The effects of understudied agrochemicals and parasites on bumble bee (*Bombus terrestris*) health:

How co-formulants, glyphosate and *Crithidia bombi* affect a wild pollinator species.

Thesis submitted for the degree of Doctor of Philosophy (PhD)

Royal Holloway University of London 22/09/2021

Edward A. Straw

Declaration of Authorship: I Edward A. Straw hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed: Edward A. Straw

Date: 22/09/2021

A bumble bee foraging at Savill Gardens, Windsor Great Park.

Acknowledgements

I would like to thank these wonderful people for supporting me throughout my PhD, without their guidance, help and friendship I surely would not have made it.

I could not start any other way than by thanking my supervisor, Mark Brown, for mentoring me through these last three years, his patience and guidance has been invaluable, and it is through his mentorship that I have developed as a scientist, communicator and academic.

The extended Poshbee team, spread out across Europe have offered me their insight, feedback and ingenuity throughout the years. At annual meetings, during visits and over sporadic zoom calls their expert perspectives contributed greatly to the science presented here. I am grateful to Poshbee itself, for funding both my research and trips to the continent for conferences. Poshbee, of course, would not function without Gail Turney keeping it ticking, many thanks to her for making my time as part of a multinational European grant as easy as it could have ever been. At Royal Holloway the academic oversight of Elli Leadbeater and Rudy Riesch has kept me on the academic straight and narrow, for which I am deeply grateful. I would like to thank Edward Carpentier for his questionable decision to work with me as an intern, undergraduate and master's student, he has been a good friend and colleague throughout.

I would particularly like to thank Alberto Linguadoca, who joined the Brown Lab at the same time as me, for his tireless help throughout. Many experiments in this thesis would not have seen the light of day without Alberto's deep knowledge of pesticides, and I have drawn much of the understanding of ecotoxicology and the regulatory system portrayed in this thesis from him. He also once stopped a bee that was about to sting me in the groin by flicking it onto the floor, for this I am particularly grateful.

There have been too many colleagues who have helped me to list their contributions individually, but Lewis Armstrong, Owen Vaughan, Linzi Thompson, Robin Mesnage, Hannah Wolmuth-Gordon, Morgan Morrison, Sara Hellström, Margret Jürison, Arran Folley, Peter Ian

Bee acknowledgements

Over the years I took to naming bee colonies, initially this started for convenience when trying to find a specific colony, but it was continued throughout my three years because it annoyed my supervisor, Mark.

While I would prefer to thank each bee I worked with individually, I am limited by the word count of a Royal Holloway thesis. I do hope they will forgive me for thanking them for their service, and sacrifice, by the colony instead.

Reet, Mirikel, Marcus, Elisabeth, Kersti, Una, Lotte, Helen, Paul Rudd, Elaine, Mjölnir, Chad Hogan, Optimus Prime, Disease, Famine, War, Conquest, Orvil, Nigel, Boris, Theresa, Bruce, Tony, Natasha, Clint, Wanda, Jarvis, Steve, Scott, Carol, Shuri, Peter, Gamora, Mos, Roy, Jen, Douglas, 1, 2, 3, 4, 5, 6, 7, Saxton Hale, Victoria, Bercow, Abe Lincoln, Lee Harvey, Tony, Gordon, Alistair, Scout, Soldier, Pyro, Heavy, Engineer, Demoman, Medic, Sniper, Spy, Merasmus, Bumble McBumbleFace, HamBee, Willow, Bank, Fernando, Mao, Lenin, Stalin, Geralt, Yennifer, Sanders, Warren, Biden, Roku, Caorunn, Bombay Sapphire, London Dry, Adin, Yessica, Jon, Trevor, Stephen, Ginsburg, Scalia, Sokka, Aang, Katara, Sunflower, Tulip, Zagreus, Skelly, Billy, Tommy, Omniman, Invincible, Cornwall, Dorset, Somerset, Devon, Aberdeenshire, Bedfordshire, Tyrone, Warwickshire, Anglesey, Breconshire, Caernarvonshire, Denbighshire, Eilean Siar, Falkirk, Wiltshire, Surrey, Antrim, Buckinghamshire, Cheshire, Durham, Kent, Moray, Norfolk, Stirling, Angus, Cambridgeshire, Essex, Fife, Glamorgan, Hampshire, Lancashire, Merseyside, Armagh, Cardiganshire, Flintshire, Hertfordshire, Inverclyde, Leicestershire, Pembrokeshire, Shropshire, Carmarthenshire, Derbyshire, Gloucestershire, Highland, Lincolnshire, Monmouthshire, Northumberland, Oxfordshire, Clackmannanshire, Fermanagh, Merionethshire, Nottinghamshire, Radnorshire, Staffordshire, Worcestershire, Kerry, Carlow, Donegal, Galway, Kildare, Laois, Mayo, Offaly, Roscommon, Cork, Dublin, Kilkenny, Limerick, Monaghan, Sligo, Tipperary, and Wexford.

I would note that those three bees who stung me are specifically excluded from the above acknowledgements.

Abstract

In the wild, bees face a number of distinct threats to their continued existence. This is of concern because bees play an essential role as pollinators in both agriculture specifically, and more broadly in non-agricultural ecosystems. To humans, bees are particularly important for pollinating nutritionally rich foods like fruits. The threats facing bees are multifactorial with habitat destruction/fragmentation, agricultural intensification, monoculture, climate change and pesticides all cited as drivers of their declines.

The work presented in this thesis focusses on elucidating the role of pesticides in bee declines by using laboratory testing to measure the effects of pesticide exposure on the buff-tailed bumble bee, *Bombus terrestris*. The usage and history of pesticides is detailed in the introductory chapter, as well as the effects of parasites on bee health. I have focussed on lesser studied agrochemicals like herbicides, fungicides, adjuvants and co-formulants because their effects on bees are poorly understood.

Chapters 2 and 4 present novel experimental research finding severe mortality effects of co-formulants present in herbicides and a fungicide respectively. In both instances the mortality and sublethal effects observed were not detected by regulators, highlighting that regulatory testing poorly characterises the impacts of co-formulants on bees. These experiments both challenge the notion that co-formulants are toxicologically 'inert'. Stemming from these results, several recommendations for policy makers are made on how to regulate co-formulants better.

In Chapter 5 of my thesis, I present a systematic review summarising the current state of knowledge on the effects of co-formulants or adjuvants on bees. The review finds several key knowledge gaps, particularly highlighting a lack of understanding of what real world exposure to co-formulants and adjuvants looks like.

Much like the field of co-formulant and adjuvant research, how multiple concurrent stressors affect bees' health is often overlooked, despite considerable exposure to multiple stressors in the wild. To address this, I undertook a series of experiments exposing bumble bees to two

very common stressors, the world's most used pesticide active ingredient, glyphosate, and a highly prevalent trypanosome parasite, *Crithidia bombi*. No effects of glyphosate on any metric recorded were found, and, contrary to prior research, no effect of *C. bombi* either. Further, no interaction between stressors was found.

The overarching results are synthesised in the concluding chapter, alongside a series of recommendations on how to better protect and study pollinators. Overall, the most compelling result was that co-formulants and adjuvants are a potential threat to bees. However, the research presented here alone is insufficient to conclusively determine if they are damaging as used in the field. Instead, this research presents progress towards determining the real-world impacts of co-formulants and adjuvants, and points to where future research should be allocated.

Table of contents

Table of figures

Chapter 4 Co-formulant in a commercial fungicide product causes lethal and sub-lethal effects in bumble bees

Chapter 5 'Inert' Ingredients Are Understudied, Potentially Dangerous to Bees and Deserve

Table of tables

Chapter 5 'Inert' Ingredients Are Understudied, Potentially Dangerous to Bees and Deserve

Table 1. The hazard, exposure and risk insecticides and 'inert' ingredients pose to bees.

Supplementary Table 1. The PRISMA reporting checklist.

Chapter 1

Introduction

1.1 Overview

Over the last century the limited evidence available suggests a global decline in pollinator richness and reductions in the ranges of pollinating species (Potts et al., 2010, Bartomeus et al., 2013, Nieto et al., 2014, Potts et al., 2016). There has been a particular focus in research on declines in bees, with evidence suggesting that habitat destruction/fragmentation (Garibaldi et al., 2014, Ollerton et al., 2014, Hemberger, Crossley and Gratton, 2021), agriculture (Kennedy et al., 2013, Hemberger, Crossley and Gratton, 2021) and climate change (Kerr et al., 2015) are drivers of the declines. These declines in bee populations are alarming given that bees are responsible for boosting crop yields in a range of crops, and several species depend entirely on them (Klein et al., 2007, Woodcock et al., 2019).

One aspect of agriculture, pesticides, has captured considerable attention for their potential threat to bee health. In both the public and the academic spheres the word 'pesticide' has often been treated as synonymous with 'insecticide', although since the 2010s more research has begun to focus on other classes like herbicides and fungicides (Cullen et al., 2019), as well as more obscure ingredients and products like co-formulants and adjuvants (Mullin, 2015, Mullin et al., 2015). Beyond the substances themselves the regulatory process that allows them to be used is under sustained criticism for failing to appropriately measure impacts on bee health (Straub, Strobl and Neumann, 2020) and for failing to protect the huge diversity of bees (and other pollinators) by focusing too heavily on European honey bees (*Apis mellifera*)(da Costa Domingies et al., 2020, Straub, Strobl and Neumann, 2020).

Non-chemical stressors like parasites can also harm bee health. While many parasites will have existed alongside their host bee species for evolutionary timescales, anthropogenically driven cross species transmission or spillover may be making parasitism worse for wild bees. These novel diseases and increased transmission are placing an additional burden on bee health that is contributing to the strain on their populations. When bees are diseased, they can be more susceptible to other stressors, like pesticides (Bryden et al., 2013). This means the cost of pesticides is best measured as a combination of their individual impacts, and the cost of their synergistic interaction with other pre-existing natural stressors. An increasing number of studies are now documenting high levels of synergy between separate stressors,

demonstrating that the pressures on bee health are multifaceted, complex and interactive (Siviter et al., 2021).

In this introduction I will cover the diversity and usage of pesticides in modern agriculture, explaining why they are used across the globe. I will then move to discuss pollinators and their importance both to ecosystems and agriculture, then proceed into a discussion of the effects of pesticides on pollinators. I will cover the regulatory systems in place to protect pollinators from pesticides, as well as the evidence we have for the impacts of pesticides on pollinators. I will then move to the topic of parasites afflicting pollinating species, specifically bees, before concluding in a discussion on the combined impacts of pesticides and parasites on pollinators.

In the thesis I will be exploring the individual impacts of pesticides on one pollinator species, *Bombus terrestris*. Specifically, I will study the impacts of lesser studied pesticide components like co-formulants. I will present a systematic review summarising the literature on co-formulants and adjuvants, that will contextualise the findings of the experimental studies. Two experimental studies on this topic will be presented, one on a glyphosate-based herbicide causing mortality after contact exposure, and another on a fungicide causing mortality after oral exposure. I will also look at how pesticides interact with parasites to impact bee health and fitness with a series of experiments on glyphosate and *Crithidia bombi*, a common trypanosome parasite of *B. terrestris*. Finally, I will conclude by summarising my findings more broadly.

1.2 Pesticides, their usage, uses, and diversity

The rapidly growing human population (FAOSTAT, 2021) combined with its increasingly resource intensive diet is putting biodiversity across the planet under strain (Wilson, 2002). The area of cultivated land has increased 450% over the last 300 years (Goldewijk, 2001), and farming land has also become increasingly intensively managed (Matson et al., 1997). As part of this intensive management chemical inputs, like fertiliser and pesticides, are used abundantly. These inputs increase yields, meaning less land needs to be used (Seufert, Ramankutty and Foley, 2012). However, they can also make farmland, and surrounding areas, less hospitable to wildlife through processes like eutrophication (Glibert et al., 2005).

Pesticides are among the only substances with toxic properties that we deliberately release into the environment (Cox and Surgan, 2006). While they have always had their detractors, it was not until Rachel Carson's seminal 1962 book Silent Spring, that the alarm over their impact on ecosystems was raised with a broad audience. Since 1962 the use, regulation, and research of pesticides has developed significantly, and particular focus has been given to how they impact pollinators.

The usage of pesticides

The diverse uses of pesticides, and the unique properties each class of pesticide possess, make quantifying pesticides usage difficult. A number of different metrics can be used, often with conflicting results. Weight of active ingredient and total area sprayed are the most common, each coming with a trade-off as to how explanatory they are. Toxic load is another measurement intended to standardise usage by potency of applied substance, although this measurement itself is flawed. Below I will discuss the statistics associated with each measure, as well as assessing their usefulness.

Usage by weight

Pesticide usage by weight is a complex story with different nations taking different paths. Global pesticide usage by weight has increased by ~60% from 1990 to 2017 (FAOSTAT, 2021), slightly outpacing human population growth at ~40% from 1990 to 2017 (FAOSTAT, 2021).

This increase in pesticide use by weight globally masks the progress in decreasing pesticide use made by some developed countries. For example, between 1990 and 2016 the United Kingdom (UK) cut pesticide usage by weight by 50% (FERA, 2021). However, economic development alone is not sufficient to predict pesticide use trends as even neighbouring developed nations can exhibit markedly different trajectories; with France decreasing total pesticide usage while neighbouring Germany and Spain continue to increase (FAOSTAT, 2021). Any decrease in use by developed nations has been insufficient to outweigh the rise in use in developing nations, with China alone using a million tonnes more active ingredient in 2017 than it did in 1990.

The weight of pesticides alone does not truly represent the level of potency or toxicity they possess. Insecticides are more potent gram for gram than most herbicides, so are applied in lower amounts. Because of this, the weight measurement overrepresents non-potent substances, which can mask the changes in usage of potent substances. Furthermore, pesticides are typically measured by active ingredient weight, not by weight of the whole pesticide product, meaning the application of co-formulants or adjuvants is not included in this measure. Both area of application and toxic load attempt to address these problems and are covered below.

Usage by area

Pesticide use can be measured by area of land treated, which counts each individual spray as equivalent regardless of the toxicity or class of pesticides being used. Confusion can occur as this measures does not necessarily line up with weight applied. As previously discussed, the UK reduced total weight applied by ~50% from 1990 to 2016, which means that less active ingredient will be introduced into the environment (FERA, 2021). At the same time as this reduction in weight, the total area sprayed rose (FERA, 2021). This means that farmers are using more frequent, but less concentrated sprays. Unfortunately, data reporting for area applied is highly variable globally, with major variation in definitions meaning no global statistics are readily available. This measure does not account for changes in the concentration of applications. Additionally, products containing multiple active ingredients are double counted in this measure, potentially obscuring or compounding upward trends in

usage by area, a problem occurring in Goulson, Thompson and Croombs, (2018). The measure that purports to account for these issues is toxic load.

Usage by toxic load

To account for the difference in potency between pesticides it is possible to adjust the weight of pesticide applied by the toxicity of the substance. This is commonly done by dividing weight by the LD_{50} (weight dose at which 50% of individuals die within a set timeframe) value for a single species. This makes the measure of toxic load specific for each species, limiting the interpretation of it, particularly as not all species have LD_{50}s derived. With bees for example, honey bees are commonly used as the reference point for all bees (Goulson, Thompson and Croombs, 2018) because they are the only species with a sufficient number of substances for which the LD_{50} has been derived. This measure, while imperfect, does reveal that toxic load can increase sharply while other measures like weight applied decline (Goulson, Thompson and Croombs, 2018), an effect here caused in the UK by the proliferation in usage of potent insecticides, neonicotinoids, which have since been banned (European Commission (EC), 2018a-c).

The principal issue with toxic load is that LD_{50}s are a measure of objective hazard not of risk. This means the measure does not incorporate any assessment of the exposure a species faces. European Union (EU) legislation explicitly ties application of mitigation measures to measurements of objective hazard like LD_{50}s, that leads to species experiencing less exposure to more hazardous substances (EC, 2009, European Food Safety Authority (EFSA), 2012, 2013). In this way toxic load overestimates the impact of highly potent pesticides on species.

While this brief survey demonstrates that the use of pesticides over time has developed, so too have pesticides themselves.

Innovation in pesticides

The substances farmers use have developed over time thanks to constant innovation from agrochemical companies. All chemical classes have progressed through several generations of chemical groups since their inception, albeit with faster development of insecticides than

any other group (Russell, 2005, Sparks, 2013). The history and development of specific pesticide classes are discussed in depth later. This level of investment in pesticide research reflects the economic incentive to develop new substances to mitigate the damage pests cause. The evolution of resistance has also reduced the efficacy of substances (Sparks, 2013), making novel chemical groups without cross resistance highly valuable. There has also been pressure on agrochemical companies to develop pesticides with lower environmental toxicities (Clark, 2001). This pressure comes both from the public perception of a chemical's toxicity and regulatory bans that force companies to pivot to new chemical groups (Clark, 2001). The discussion of pesticides largely focusses on active ingredients, which are the chemicals that confer pesticidal activity, but all pesticide application in the field is through formulated products that contain other chemicals, called co-formulants. Recent advances in pesticide technology have increasingly come from improving the efficacy of existing substances, rather than from bringing new active ingredients to market, which is a heavily regulated process (Hazen, 2000, Russel, 2005, Sparks, 2013). This can be achieved through the combination of active ingredients, developments of new co-formulants, increasing the diversity of product range for the same active ingredient (Mesnage, Benbrook and Antoniou, 2019), and finally by advancements in adjuvant technology (products without active ingredients added to tank mixtures to complement the action of a pesticide formulation)(Hazen, 2000). Crucially this largely avoids regulatory and academic scrutiny making it much cheaper and less risky than searching for novel substances. This is because the data requirements are less stringent for registering a new product than a new formulation (EC, 2013). Different classes of pesticide are used and developed in very different ways (Russel, 2005). As such each major pesticide class is discussed individually below.

Different classes of pesticides

Insecticides

As the most prominent class of pesticide, historically insecticides are also the most heavily debated, although in recent years the herbicide glyphosate has become particularly contentious. Insect pests not only cause direct damage to crops when they feed on them, but they also act as major disease vectors (Purcell and Almeida, 2005). Because crop diseases like

Beet Yellow Virus cannot be directly treated, control of their insect vectors like aphids is the only effective measure (Walsh et al., 1989). Insecticides are applied in a range of manners, from foliar sprays to soil drenches to seed coatings, each having unique ecotoxicological consequences. Insecticides only represent ≈2% of the weight of active ingredients applied, and ≈6% of the usage by area (FERA, 2021). However, because insecticides have such exceptionally high potency they still represent a potential threat on the same scale as herbicides or fungicides, both of which are used more abundantly (Goulson, Thompson and Croombs, 2018). Since DDT, and its analogs, were discovered in 1939, a range of distinct insecticide groups have been brought to market including, the organophosphates, carbamates, pyrethroids and neonicotinoids. Each of these new chemical groups represents innovation, with increasingly high standards being applied regarding risk to human and wildlife health. Insecticide development has led to products that are more targeted against insects, and less toxic to vertebrates (Sparks, 2013), which reduces the impact of them on farmers and consumers, but also protects vertebrate wildlife. At the same time as selectivity has increased, the potency has risen, with several order of magnitude difference between application rates of early insecticides like DDT versus more modern substances like carboxamides (Sparks, 2013). Driving the proliferation in insecticide development is the rapid development of insect resistance to insecticides, with more resistant target species than for herbicides or fungicides (Sparks and Nauen, 2015). This is perhaps driven by the rapid evolutionary capacity of insects, with high fecundity and short generation times (Pélissié et al., 2018).

Most insecticides function as neuro-disruptors, interfering with the nervous system of insects causing paralysis then death (Matsuda et al., 2001). This typically interferes with the function of synapses between nerves, for instance by binding to the signalling molecule (nicotinic acetylcholine) receptor, causing it to repeatedly fire (Matsuda et al., 2001). This leads to the insect becoming unable to control its body and eventually die. Because the nervous system, and receptors like the nicotinic acetylcholine receptor (nAChR), in insects are relatively evolutionarily conserved (Cha and Lee, 2015), this typically means that insecticides have low specificity to their intended target species. Not all insecticide are neuro-disruptors, however,

with some, like toxins derived from *Bacillus thuringiensis*, causing perforations in insect larval guts after ingestion leading to death (Heckel, 2020).

The ecologically devastating effects of the early modern insecticide DDT, and its bioaccumulation in humans, brought a spotlight to insecticides as potential threats to human health. This in part explains the much tighter restrictions applied to insecticides over other pesticide classes. Because insecticides are targeted to kill insects, and have generally low target specificity, their impacts on non-target insects, specifically pollinating insects like bees, has been the source of much recent research (Lundin et al., 2015). The neonicotinoids, a class of insecticides brought to market in the 1990s (Jeschke et al., 2010) have been a particular source of contention between scientists, farmers and regulators, with three major neonicotinoids temporarily (EC, 2013), then permanently, banned for outdoor used in the EU for their apparent detriment to bee health (EC, 2018a-c).

The toxicity of neonicotinoids, both ecologically and culturally, further prompted agrochemical companies to look for alternatives to replace them. Two key discoveries were made with Dow Agrochemicals' sulfoxaflor and Bayer's flupyradifurone. Both of these new substances were given unique chemical classifications, the sulfoximines (Sparks et al., 2013) and the butenolides (Nauen et al., 2015) respectively. This was determined by the Insecticide Resistance Action Committee (IRAC), a group created by the agrochemical industry. This distinction is hotly contested with non-governmental organisations (NGOs) like the Pesticide Action Network (PAN) arguing that they should form a subgroup of neonicotinoids. PAN contest that their unique classification is unscientific, even by IRAC's own guidelines, and is designed to distance these new products from the neonicotinoids (PAN, 2016). It is worth noting that neonicotinoids, sulfoximines and butenolides have very similar chemical structures and share the very same mode of action (nAChR antagonist). Despite this debate their classification is largely superfluous, as regulators do not consider this in decision making processes. Evidence for this can be seen from the European Commission's choice to ban just three of the many neonicotinoids on the market.

Both sulfoxaflor and flupyradifurone have become subject to intensive research focus as they are viewed as successors to the neonicotinoids (Brown et al., 2016, Siviter and Muth, 2020).

Questions have been raised over whether the same regulatory systems that authorised neonicotinoids, only later to ban them after a torrent of academic research, are sufficiently equipped to make an informed judgement on the safety of their successors (Siviter, Brown and Leadbeater, 2018). Perhaps as a result of the association with the banned neonicotinoids, the research indicating they are detrimental to bees, or more likely, a range of market forces including the high cost of the products, neither sulfoxaflor nor flupyradifurone have seen high uptake by farmers (CDPR, 2020). Because of the considerable lag between pesticides being used in the field, and reliable statistics on this being published, there is currently no reliable international data on the uptake of sulfoxaflor and flupyradifurone (Edward A. Straw, Personal Observation). The only data I am aware of is for California and reports a trivial 0.000007% market share (CDPR, 2021). Not every regulatory territory in the EU has even allowed these new insecticides to their national markets, with the UK (while still in the union) limiting sulfoxaflor's use to indoors, and the Irish regulators placing heavy restrictions on what crop types it can be used on. With neonicotinoids banned and their replacements not seeing high uptake there is a diverse array of insecticides used in agriculture today.

The diversity of insecticidal compounds farmers choose to control insect pest populations is driven by a range of factors such as specificity of insecticide to target pest species, cost of treatment, compatibility of mitigation measure and target pest species chemical resistance. Official data are not yet available on the diversity of insecticidal compound usage following the 2013 EU neonicotinoids ban (EC, 2013, 2018a-c). Official data are predicted to show an increased market share for older compounds such as pyrethroids (Ceuppens et al., 2015). While the insecticide market has shifted greatly in recent years, the market for herbicides has been relatively steady since the late 1970s.

Herbicides

Herbicides are principally used to control the growth of weeds in agriculture, although they are also used in amenity and garden settings (Duke, 2018). Weeds compete with crops for light and nutrient, and some, like *Striga* species, directly parasitise crops (Khan et al., 2002). In fertile soils, which are kept well-watered and repeatedly topped up with nutrients, weeds can proliferate if left unchecked, causing severe damage to crops and yields (Beckie, Flower

and Ashworth, 2020). Some traditional methods of weed control like stubble burning or tilling have serious environmental impacts from greenhouse gas release and soil erosion respectively, further incentivising chemical approaches (Beckie, Flower and Ashworth, 2020). While other methods for control of weeds do exist, such as mechanical weeding, none alone has the convenience or efficacy of herbicides (Beckie, Flower and Ashworth, 2020).

The modes of action of herbicides are much more varied than those of insecticides. Glyphosate, for example, blocks amino acid synthesis along the shikimate pathway (Duke, 2018), glufosinate inhibits glutamine synthetase in leaves (Takano and Dayan, 2020), and dicamba mimics the action of auxin causing abnormal growth that ultimately results in death (Hartzler, 2017). Because of this variety in their mode of action, and the wide diversity of plants considered as weeds, they can sometimes have sufficient specificity to allow application onto a crop and still provide knockdown of the intermingled weeds (Hartzler, 2017). For herbicides, the development and uptake of sophisticated biotechnology like herbicide resistant crops has facilitated a prolific increase in herbicide use in some territories (Benbrook, 2016), with an accompanying global increase in usage.

While considerable research is devoted to the development of herbicide biotechnology, the ecological costs of herbicides are less well studied (Cullen et al., 2019). This is jointly because their mode of action is targeted at plants and because they are of lower potency, so they are inherently less hazardous to consumers and animals than insecticides. Perhaps in part because of this, herbicide products have a much slower turnover rate than insecticides (Duke, 2008, Sparks et al., 2013). A single substance, glyphosate, has dominated the market from its development in the early 1970s, through to the present day (Benbrook, 2016). Only 50 years after its market entry have opposition groups succeeded in achieving major bans, such as in Mexico (Alcántara-de la Cruz et al., 2021). Major opposition is building to the pervasive use of herbicides, with opposition citing ecological concerns and consumer welfare, as well as growing worries regarding safety for farm workers (Alcántara-de la Cruz et al., 2021). This is exemplified in the debate surrounding glyphosate, with glyphosate being used prolifically in agriculture and also sold in supermarkets for untrained consumers to use. The huge extent of human and environmental exposure to glyphosate is unarguable. There is however legitimate debate among experts regarding the safety of glyphosate to consumers, with differing

international bodies listing it as either likely carcinogenic (International Agency for Research on Cancer) or not (European Commission, Environmental Protection Agency (USA)).

The cost of developing new pesticides has risen considerably since the 1970s, caused by increase testing requirements for human and environmental safety, as well as the depletion of test substances which can be seen in the drastically increasing number of substances screened per product brought to market (Sparks, 2013). This is a major problem for herbicides, as while development has happened since glyphosate was brought to market, the alternative substances lack appeal, with glufosinate having its approval revoked in 2018 for human toxicity concerns (EC, 2020) and dicamba being potentially linked to various human cancers (McDuffie et al., 2001). Without a breakthrough new chemical coming to market, farming practices will need to change if glyphosate, the 'once in a century herbicide' (as Duke and Powles, 2008 named it) is banned.

Fungicides

Fungal diseases, like rusts, smuts, and bunts, can be exceptionally damaging to crop production (Bebber and Gurr, 2015). As a result, fungal diseases are a serious threats to global food security (Bebber and Gurr, 2015); the loss of yield from rice blast (*Magnaporthe oryzae*) alone is estimated to be enough to feed 60 million people (Nalley et al., 2016). Thus, effective control measures are highly sought-after tools for alleviating poverty and starvation. Alongside crop management and breeding developments, fungicides are among the most powerful tools used to protect crops against disease. Fungicides function by inhibiting, or outright killing fungal pathogens in plants. Prior to the rapid post war development of fungicides, a range of substances were used for their fungicidal action, ranging from copper sulphate to arsenic (Russell, 2005). Modern fungicides are highly diversified in their modes of action, with Quinone outside Inhibitors (QoI) disrupting cytochrome function, and thus ATP production (Fernández-Ortuño et al., 2010) while Sterol Biosynthesis-Inhibitor (SBI) fungicides target ergosterol biosynthesis pathways, inhibiting cell wall production (Russel, 2005).

As fungicide development proliferated in the 60s and 70s, so did fungal disease resistance to them (Russell, 2005), leading to today's current tight usage regulations. To prevent the development of resistance in the UK it is now required that fungicide applications must use at least two active ingredients with distinct modes of action (Russel, 2005). There are also limits on the number of sprays per year designed to limit the scope for resistance outbreaks. These regulations inadvertently protect wildlife by limiting the number (and rate) of applications per year, but also guarantee that wildlife exposure to fungicide formulations will always be to multiple sets of chemicals which could act synergistically. The impacts of multiple stressors on human and wildlife health are not yet fully understood, and some combinations have been found to increase toxicity by several orders of magnitude (Pilling and Jepson, 1993).

Co-formulants and adjuvants

Co-formulants are a very broad category of chemical, which encompasses any ingredient in a formulation other than the active ingredient. These substances are added to formulations to help the active ingredient function or improve the stability/longevity of the product. Common categories of co-formulants are surfactants that help the pesticide droplet coat a leaf evenly, wetting agents that help the pesticide droplet stay on the leaf, and solvents that are used to dissolve the active ingredient (Mesnage and Antoniou, 2018). Adjuvants are separate products which can be added to the mixture of pesticides prior to spraying (Hazen, 2000). These modify the function of the spray to allow tailoring of the spray to the crop, conditions and desired effect. Adjuvants are typically surfactants and wetting agents, although other groups exist like crop oil concentrates, which help dissolve the waxy cuticle of leaves, dyes, which help visualise the path of spraying and water softeners which help negate the effects of hard water. The appropriate use of co-formulants and adjuvants can help achieve the same level of pest control with lower application of active ingredient which reduces environmental exposure to pesticides.

Co-formulants and adjuvants are often grouped together as 'inert' ingredients, a phrase avoided here because of the un-substantiated implication that the substances are

toxicologically benign. It is, however, appropriate to group co-formulants and adjuvants together because there is considerable overlap in the chemicals used in both categories.

Co-formulants are only tested by regulators as part of formulations, with the exception of specific research in response to academic work that has identified a potential threat to farmers and consumers, such as with Tallow Amine (EC, 2016, EFSA, 2016). When a new formulation is being brought to market it will be compared against current formulations using a simple and limited set of toxicity testing (Chemical Regulation Division, 2021). Some formulations can avoid this testing if it can be argued they are sufficiently non-distinct from pre-approved formulations (Chemical Regulation Division, 2021). This means that most co-formulants only undergo highly limited toxicity testing, if any at all, as part of formulations. Adjuvants are not tested toxicologically at all and are only tested by regulators to check that they do not cause the active ingredients of co-applied formulations to exceed maximum residue limits. As such the toxicity of co-formulants and adjuvants to humans and wildlife is very poorly understood. One group of species the effects of pesticides are particularly of concern to is pollinators, because of the vital role they play in ecosystems and agricultural production.

1.3 Pollinators

While a wide range of taxa pollinate flowers, the majority of crop pollination is carried out by bees. Around 35% of global crop production depends on animal pollination (Klein et al., 2007), predominantly by bees. Beyond just the total yield of food bees contribute to, bees are also essential pollinators for many fruit species (Klein, Steffan-Dewenter and Tscharntke, 2014), meaning that without them producing enough nutritious food for the human population would be difficult.

There is a wide diversity of bees, with an estimated 20,000 species globally (Potts et al., 2010), and nearly 2,000 species of native bee within Europe alone (Nieto et al., 2014). Unfortunately, the pressures on bee populations, from habitat destruction/fragmentation (Fuller, 1987, Garibaldi et al., 2014, Ollerton et al., 2014, Hemberger, Crossley and Gratton, 2021), agriculture (Kennedy et al., 2013, Hemberger, Crossley and Gratton, 2021), climate change (Kerr et al., 2015), parasites (Goulson et al., 2015) and pesticides (Rundlöf et al., 2015, Woodcock et al., 2016, McArt et al., 2017) combined are causing widespread declines in bee populations (Biesmeijer et al., 2006, Potts et al., 2010, Cameron et al., 2011, Carvalheiro et al., 2013).

Within the EU 37% of bee species with known trends are undergoing population declines (Nieto et al., 2014). Worse still we have insufficient data to map the population trends of 79% of all EU bee species (Nieto et al., 2014). These declines have been both in species richness and species range (Bartomeus et al., 2013), with rare species increasingly confined to small, fragmented habitats. In the UK 25 bee species have gone extinct, with around 275 remaining (Falk and Lewington, 2015). These extinction events are more notable than the declines in numbers or range because of their permanence. Some of the available evidence for north west Europe indicates a levelling off of bee declines since 1990 (Carvalheiro et al., 2013) which could be attributable to measures designed to protect bees, but could equally represent the initial loss of just the most vulnerable species (Ollerton et al., 2014). Extinction events and species range contractions lead to reduced bee diversity, this can be especially bad for pollination as Klein, Steffan-Dewenter and Tscharntke (2014) found that bee species diversity, not abundance, explained pollination success. Many farmers use honey bees to bolster

pollination (Aizen and Harder, 2009), most notably seen in the annual migration of 81% of United States (US) hives to California for the almond blossom (Goodrich, Williams and Goodhue, 2019). While honey bees are useful in crop production, they cannot substitute entirely for wild unmanaged bee species (Garibaldi et al., 2013). There is not strong evidence that yields at a global scale are currently being limited by pollination availability. However, with globally observed declines in pollinators (Potts et al., 2010), agricultural demand for pollination outstripping managed honey bee stock growth (Aizen and Harder, 2009) and increases in pollinator dependent crops (Aizen et al., 2008), whether yields will grow to become limited remains a concern.

To track a change in bee populations, long term data is required to provide a comparison point for the modern data. Such data are most readily available in Europe, and Britain in particular because of the tradition of natural history recordings (Skovgaard, 1936, Dupont et al., 2011). Elsewhere in the world where systematic and long-term data were either not collected or recorded, understanding the long-term trends in bee populations is very difficult (Grixti et al., 2009). There is nonetheless limited data for bees globally, with declines observed in, for example, North America (Grixti et al., 2009, Cameron et al., 2011, Bartomeus et al., 2013), South America (Schmid-Hempel et al., 2014), and East Asia (Inoue, Yokoyama and Washitani, 2008). Similarly, not all taxa have an equal depth of data recording. Large and charismatic taxa like bumble bees are more noticeable and often easier to identify than smaller solitary species, leading to more records of their distribution and abundance (Nieto et al., 2014).

Of the commonly attributed causes of bee declines, habitat destruction/fragmentation, agriculture, climate change, parasites and pesticides, it is only pesticides on which serious action can be taken without substantial realignment of human society. Any change to land usage to benefit pollinators would require restoration of wild and semi-wild habitats, displacing the current use type, or improvements to the existing land use which come with the trade-offs of cost and space use. As land availability is limited, large scale changes to use would require intensification of activities in remaining sites, which is associated with pressures on bee health (Le Féon et al., 2010). Some changes to agriculture to promote pollinator health have been adopted, including measures like wildflower strips which increase

pollinator diversity and abundance (Buhk et al., 2018). However, the scope for these initiatives to protect pollinators is limited, and larger scale changes like reducing the prevalence of monocultural planting would also reduce farming efficiency. Addressing climate change, while imperative, is unlikely to be motivated by its impacts on pollinators. Biological control measures can be adopted, as seen in Australia, to reduce transmission of parasites and diseases to native species, including pollinating species. However, many parasites have existed in pollinator populations for evolutionary timescales, or already have been introduced to new habitats, meaning the scope for reducing parasite load in wild populations of animals is very limited. As such pesticides, perhaps unfairly, bear the brunt of campaigning and research into their effects on bees. The effects of pesticides on bees are nonetheless substantial, with correlational studies finding relationships between increased usage of several classes of pesticides and declines in bee diversity (Ollerton et al., 2014, Woodcock et al., 2016, McArt et al., 2017, Holder et al., 2018), and several experimental studies confirming a causal link (Rundlöf et al., 2015, Tsvetkov et al., 2017).

Bumble bees

Bumble bees, which collectively form the genus *Bombus*, are highly valuable as wild pollinators that can be reared artificially (Velthuis and van Doorn, 2006), making them a valuable asset for scientific research.

Bumble bees are highly efficient pollinators, who utilise buzz pollination to extract pollen from flowers (King and Buchmann, 2003, De Luca et al., 2013). Buzz pollination entails the bee vibrating rapidly to shake pollen off of the floral reproductive parts. Alongside buzz pollination, some bumble bees have longer probosces than honey bees which allow them to pollinate important species like the nitrifying cover crop red clover (Arretz and Macfarlane, 1986). These pollination characteristics of bumble bees led them to be deliberately introduced into South America and New Zealand (Hopkins, 1914 (cited in Velthuis and van Doorn, 2006), Arretz and Macfarlane, 1986). These introductions may have benefitted crop pollination but have had devastating consequences on local bee populations (Schmid-Hempel et al., 2014).

While some European honey bee, *Apis mellifera*, colonies are wild, most are domesticated and cared for by beekeepers, and thus not considered wild bees (Nieto et al., 2014). Whereas outdoor bumble bee populations are wild and undomesticated. Multiple bumble bee species, namely *Bombus terrestris* and *Bombus impatiens*, can be reared artificially, allowing them to be used in greenhouse pollination to bolster crop yields (Velthuis and van Doorn, 2006). This means that some bumble bee species can be both artificially reared and have a stable wild population. This presents a useful opportunity for researchers, as colonies of bumble bees can be readily sourced for laboratory testing, with the results being directly applicable to a wild species. This is useful because wild bees may be impacted by stressors very differently to domesticated species because no human interventions are applied to aid them (Heard et al., 2017). As such, having model species like the European *B. terrestris* and North American *B. impatiens* aids research greatly. While some solitary bee species like *Osmia bicornis* can be reared artificially (Gruber et al., 2011), and are used under semi-domestication for crop pollination, their non-social life history makes them much less suitable for laboratory testing.

1.4 The effects of pesticides on pollinators.

Regulation of pesticides to protect pollinators

All regulatory systems have the explicit aims of protecting consumers and wildlife while balancing the need to protect food production and farmers (EPA, 1996, EC, 2009, EFSA, 2012). Reflecting differences in the value ascribed to each of these aims, the regulation of pesticides is a contentious issue. NGOs like PAN and Buglife push for further restrictions, and industry bodies like Croplife or farming organisations like the NFU push to reverse pre-existing restrictions. Reflecting differences in the success of relative groups' advocacy, regulations vary drastically across the globe (Handford, Elliott and Campbell, 2015). While strict regulatory systems, as found in the EU and the State of California, have applied ever tighter controls and restrictions on usage, others like the US federal government have sharply rolled back restrictions in recent years (Handford, Elliott and Campbell, 2015). In much of the developing world pesticide restrictions are much more relaxed, with more active ingredients approved for use, lower standards on farmer and consumer exposure levels and weaker protections for wildlife in place (Orozco et al., 2009). Considerable variation exists globally, with a patchwork of restrictions as different deliberative bodies reach conflicting conclusions, such as Mexico's federal ban on glyphosate for human health reasons (Alcántara-de la Cruz et al., 2021), something the EU has not done. This thesis will focus on pesticides as used in the EU and in the UK. At the time of writing the UK still maintained regulatory alignment with the EU regarding pesticides and as such no differentiation will be made between the two in this thesis.

European legislation on pesticides, with specific reference to their effects on bees, is comprised of three main documents, Regulation 1107/2009 (EC, 2009) and two EFSA guidance documents (EFSA, 2012, 2013). Below I discuss the principal features of this regulatory regime, which are set out in the aforementioned three main documents.

The EU relies on the European Food Safety Authority (EFSA) to synthesise and co-ordinate research into pesticides. EFSA receives submission dossiers from agrochemical companies which they review and then use to produce detailed opinion documents which are passed

onto the European Commission. The European Commission makes the ultimate judgement regarding the approval of pesticides at an EU level, although member states can opt to not authorise usage of any approved substances they choose. For an active ingredient to be registered, EFSA require a specific suite of research to be carried out such that they have the requisite information to make an informed judgement.

EU regulatory testing is divided by species group and tier. Because different species have drastically different exposure routes and ecotoxicological profiles, testing on bees (and other non-target arthropods) is segmented from mammalian testing, with toxicity in one group not triggering additional testing in another. For each group, testing starts at the lower tier with simple, small scale and typically lab-based experiments to broadly characterise the toxicity of the active ingredient. If the active ingredient surpasses a pre-determined toxicity threshold then it is entered into higher tier testing. Higher tier testing is designed to provide a much deeper insight and characterisation of a substances toxicity which can be used to inform how it can be used safely. The tiered testing structure minimises costs and is designed to reduce the number of tests performed. Reducing the number of tests reduces the number of individual animals tested upon, which helps prevent unnecessary suffering. To supplement this very structured and rigid testing scheme academic literature is also considered by EFSA. Panels of experts review and assess the quality of published literature, which then contribute to the final assessment. The use of non-standardised testing procedures in academic testing, and the pesticide doses chosen, often lead to literature being harshly criticised and discounted by these expert panels.

When a substance is found to pose a risk above specified thresholds, EFSA and the European Commission can modify the requirements on its usage to mitigate the risks it poses. These are typically warnings in the product label/environmental safety information sheet, requirements to wear personal protective equipment while spraying, or tight stipulations on how/when it can be used relative to the crop stage. Authorisation typically last 10 years, but this can be shorter for substances where there is uncertainty regarding how safe they are to use. At renewal if sufficient evidence has accrued over the previous registration period substances can be denied re-authorisation or additional stipulations can also be attached such as limitations to the types of crops they can be used on.

Research into the effects of pesticides on bees

Research conducted by regulators

As new chemicals have been brought to market, academic and regulatorily mandated research has sought to understand the impacts they have on bees, both wild and domesticated. The guidelines for toxicity testing are developed by the ICPPR (International Commission for Plant-Pollinator Relationships) which feeds into the Organisation for Economic Co-operation and Development (OECD).

The amount of testing required by regulatory bodies has grown over time (EFSA, 2013); this was partially driven by critiques of the system by academic researchers. Several principal critiques that have, and are, levelled at regulatory testing are:

- That it is focussed too heavily on honey bees, and not the other 1,964 bee species native to the EU (Nieto et al., 2014).

- That it is focussed too heavily on workers, rather than males/queens or other life stages

- That it fails to consider fitness as the ultimate metric of bee health (Straub, Strobl and Neumann, 2020).

- That it studies pesticides in isolation, thus failing to account for the multiple concurrent stressors which bees can face in the field (Pilling and Jepson, 1993, Topping et al., 2021.)

In response to these critiques the range of OECD testing guidelines has expanded over time to cover a wider array of scenarios. In 1998, protocols 213 and 214 were released detailing acute oral and contact tests for honey bees; these were the first OECD protocols for bees. 15 years later in 2013 protocol 237 was released detailing acute exposure of honey bee larvae, this expanded the scope of testing by incorporating multiple life stages. In 2017 the scope was again expanded to encompass chronic exposure of honey bees, which was recognised as a distinct threat to bees, with protocol 245. Also in 2017, protocols 246 and 247 were released detailing tests for acute contact and oral exposure of bumble bees (specifically designed for *B. terrestris* and *B. impatiens*), which for the first time expanded the range of bee species upon which regulatory testing could be performed. The ICPPR is currently developing a chronic exposure test for bumble bees to allow a second species of bee to have chronic

toxicity data entered into regulatory consideration, as well as a range of protocols for solitary bees.

Exposure of bees to pesticides

Bees are exposed to pesticides through a range of mechanisms, with pertinent differences between species, castes and life history stage, as well as application mechanism. Exposure can occur when a substance bees interact with is contaminated with pesticide, such as nectar, pollen, water, guttation fluid or soil. Alternatively, bees themselves can be exposed directly to the pesticide through contact with the tank mixture, most likely through contact exposure during spraying.

To quantify the level of exposure bees receive through contaminated substances we can directly sample those substances for pesticides before bees interact with them, for example: nectar and pollen from flowers (Fine et al., 2017), water (Tooby et al., 1981), guttation fluid (Hoffman and Castle, 2012) and soil (Krupke et al., 2012). This gives us data applicable to all bee species but is less relevant than measuring substances collected by bees, because how bees interact with the substance may skew the results, such as by selectively avoiding contaminated areas (Paul, 2019). Thus, it is often preferable to measure pesticide residues in the substances after bees have interacted with them, by collecting samples from within the nest or from bees returning to the nest.

Nectar is thought to be one of the primary sources of pesticide exposure in bees as it is their sole source of energy. To collect nectar from honey bee workers, they can be caught as they return to the colony and then their nectar crops extracted to collect the fluid within (Schatz and Wallner, 2009). Pollen can be collected using a pollen trap on the entrance to a colony, which functions by knocking pollen off of bee's corbicula (Thompson et al., 2014- honey bees, Judd et al., 2020 - Bumble bees). Pollen from solitary bee nests could be extracted although to the authors knowledge this has not yet been attempted, representing a large knowledge gap. Because many bee species store nectar and pollen, substances can accumulate in the nest over time. Thus, it is important to also sample residue levels from within a nest. Samples

can be taken from inside bee nests of honey, pollen, bee-bread and beeswax (species dependent) for analysis (Chen and Mullin, 2013). This can be done in nest without an experimental application of pesticide nearby, which is used to measure background pesticide levels in an area (Rennich et al., 2014). Alternatively, residues can be measured with experimental application of pesticide which can be used to understand how one specific pesticide impacts a colony in the days after an application (Thompson et al., 2014).

Chronic and acute exposure

In the field bees are exposed to a range of doses of pesticides through their lives. The duration and level of the exposure dictates whether it is defined as chronic or acute exposure. Chronic exposure refers to persistent exposure to a pesticide, and this can occur in any substances the bees regularly interact with. Further, it can occur both inside the nest from the build-up of residues in food reserves and nest materials, or outside the nest from contaminated resources.

Acute exposure is the interaction with a single instance of a pesticide. While acute exposure can occur at any dose, it is only commonly studied with high doses associated with little or no time between crop treatment and the bees interaction with it. Quantifying acute exposure is more difficult than chronic exposure. To use the pesticide concentration of returning nectar at the first time point after spraying is fraught, because exposure to a high enough acute dose could induce mortality, meaning that bee would not return to be measured, thus biasing the measure towards lower concentrations. Additionally, many academic semi-field trials either spray at night (Artz and Pitt-Singer, 2015) or enclose the bees during spraying (Tamburini et al., 2021), which artificially limits exposure as no such mitigation measures are used in farming, despite this being against best practice. Field residue studies could be specifically designed to measure acute exposure by spraying during foraging, and having a short period between spraying and first sampling event.

How measurement of exposure to pesticides informs study design

To mimic chronic exposure in a laboratory, either sucrose or pollen can be spiked with pesticide and bees then allowed to consume it for an extended period. The choice of concentration can be informed using background residues which are typically lower on average but more persistent, or from trials using experimental application of the pesticide, which are higher but less persistent.

To mimic acute exposure bees are treated with a single dose of pesticide either orally in sucrose or by spraying or pipetting of a pesticide directly onto them. The concentrations used can be informed by semi-field, studies, but as previously noted the methods used are not tailored to derive acute exposure. It is common to use the concentration of the tank mixture (Chapter 2) to mimic direct exposure to the spray, although this is only appropriate for contact exposure for substances without mitigation measures such as herbicides, fungicides and adjuvants. Use of the tank concentration for acute oral exposure can be fraught as it assumes bees will directly consume tank mixture, rather than tank mixture diluted with plant nectar, guttation fluid or standing water.

The effects of pesticides on bees

In this section I will review the various ways that different major classes of pesticide have been found to affect bee health.

The effects of insecticides on bees

A huge variety of different insecticides have been subject to research attention, but by far the best studied group is the neonicotinoids. The neonicotinoids are seen as a success story for academic research (in the EU at least), where the breadth and depth of the research conducted culminated in the de-authorisation of the three worst offending neonicotinoids for outdoor use. Here I shall briefly review some of the evidence for the detrimental effects of insecticides on bee health, focussing on the neonicotinoids.

As potent insecticides all three major neonicotinoids, imidacloprid, clothianidin and thiamethoxam, had LD_{50} values well below (here, lower means more lethal) the regulatory

threshold value required for additional toxicity testing. As such they were entered into higher tier testing where, after additional lab and semi-field testing, they were approved for widespread use. Their approval was contingent on a number of mitigation measures that are typically applied to insecticides. These included restrictions on the timing of application relative to crop bloom, restrictions on the rate and frequency of applications, restrictions to prevent application during bee foraging activity and the recommendation to use herbicides in the lead up to the insecticide spray to remove any weeds whose flowers may become contaminated with the insecticide.

Despite these mitigation measures there was considerable evidence that neonicotinoids were making their way into pollen and nectar, as well as bee matrices like honey and wax (reviewed in Blacquière et al., (2012)). While with any product directly applied to a crop you would expect some level of contamination of nectar and pollen, and thus commensurate contamination in bee matrices, the mitigation measures implemented are designed to prevent this contamination reaching high enough levels to negatively impact bees. While not all studies found negative effects, and much debate continues to this day over what field realistic exposure is, there is substantial evidence for detrimental effects of the neonicotinoids on bee health at a range of doses. Effects on reproduction, the ultimate currency of fitness, were observed in honey bees (Decourtye et al., 2005, Gregorc and Ellis, 2011, reviewed in Blacquière et al., (2012)), solitary bees (Abbott et al., 2008) and bumble bees (Tasei et al., 2000). In addition to directly affecting fitness, corollaries of health like learning (Piiroinen and Goulson, 2016) and homing ability were found to be impaired by neonicotinoid exposure (Henry et al., 2012, Schneider et al., 2012, Feltham, Park and Goulson, 2014, Gill and Raine, 2014, Stanley, Smith and Raine, 2015).

Not all research found negative effects, with differences in methodologies, and importantly different choices of dosing regimes, resulting in a plethora of studies that found no effect of neonicotinoids on mortality or sub-lethal traits for honey bees (e.g., Schmuck et al., 2003, Pilling et al., 2013) or bumble bees (e.g., Tasei, Ripault and Rivault, 2001, Morandin and Winston, 2003, Franklin, Winston and Morandin, 2004, Laycock et al., 2014).

Despite the conflicting literature, the strength of academic evidence was sufficient in 2013 for the European Commission to put a moratorium on the outdoor use of imidacloprid, clothianidin and thiamethoxam (EC, 2013). However, the extent of the opposition and disagreement was reflected in that only a moratorium was imposed, not an outright ban. Additional research from 2013 to 2018 continued to support the assertion that environmentally relevant concentrations of neonicotinoids were of detriment to bees. With key pieces of evidence, like Rundlöf et al., (2015), which found negative effects of neonicotinoids on bumble and solitary bee populations using a range of fitness-based metrics in full field conditions, and other studies (Woodcock et al., 2016, Tsvetkov et al., 2017) accumulating, the moratorium was extended until 2018, when it was converted into a full ban (EC, 2018a-c). Despite the ban in the EU, neonicotinoids are still the most widely used insecticides globally (FAOSTAT, 2021).

The chemicals purported to be the successors to the neonicotinoids in the EU, sulfoxaflor and flupyradifurone (Brown et al., 2016, Siviter and Muth, 2020), have been found to have much the same effect on bees as their predecessors (Siviter and Muth, 2020). Notably, sulfoxaflor was shown to have a negative impact on the production of sexuals in *B. terrestris* under field conditions using a highly conservative dose of 5ppb (Siviter, Brown and Leadbeater, 2016). In contrast, there is limited evidence that these new chemicals do not cause the same detriment to learning (Siviter et al., 2019) as the neonicotinoids did (Siviter et al., 2018). However, the range of other metrics where they have been shown to be of detriment to bees (reviewed in Siviter and Muth, 2020) has led to questions about whether their approval demonstrates that regulatory systems have failed to learn the lessons of the neonicotinoids (Siviter, Brown and Leadbeater, 2016).

The effects of herbicides on bees

Because herbicide toxicity is aimed at plants, not insects, herbicide active ingredients are typically several orders of magnitude less hazardous to animals than insecticides. The routes through which herbicides do cause toxicity to insects are not fully understood, and the depth of knowledge is dependent on the active ingredient in question. A recent systematic review by Cullen et al., (2019) found just 29 studies on the effects of herbicides on bees, and while

this estimate is probably lower than the actual number, this still represents a paucity of data. Glyphosate, the most common herbicide (and pesticide generally), was subject to just 11 publications (Cullen et al., 2019). The effects of glyphosate on honey bee cognition (reviewed in Farina et al., 2019) include impaired olfactory learning (Herbert et al., 2014), reduced navigation capacity (Balbuena et al., 2015) and reduced sucrose responsivity (Herbert et al., 2014). Glyphosate can additionally perturb larval development, causing delays to moulting and decreased larval weight (Vázquez et al., 2018). The second most studied herbicide according to Cullen et al., (2019) is atrazine, with just six publications.

The mechanisms by which herbicides affect bee health are poorly understood. This is because bees often do not possess the pathways that herbicides target, for instance glyphosate inhibits the shikimate synthesis pathway, but bees themselves lack this metabolic mechanism, meaning they should theoretically be unaffected by glyphosate. Yet numerous studies have found impacts of glyphosate on various aspects of bee health (reviewed in Farina et al., 2014). To explain this, we need to consider other aspects of bee physiology, namely their microbiome. Several studies in recent years have found glyphosate to be capable of altering the microbiome composition of honey bee (Dai et al., 2018, Motta, Raymann and Moran, 2018, Blot et al., 2019, Motta and Moran, 2020, Motta et al., 2020). It is possible that effects of herbicides on bees are mediated through the microbiome, as many bacteria do have pathways susceptible to herbicide modes of action (Motta, Raymann and Moran, 2018). Beyond the microbiome though, how herbicides affect bees is poorly understood. One explanation is that herbicide active ingredients impact more pathways than previously thought. Herbicide development is centred around impacts on plants, and herbicide toxicological testing primarily looks at humans. Because insects have physiological distinctions and a large evolutionary separation from both plants and humans, herbicides may have uncharacterised impacts on pathways not shared between insects and plants/humans. One example of this is that glyphosate inhibits melanisation in several insect species, with a mechanism unlikely to involve the microbiome (Smith et al., 2021).

The effects of fungicides on bees

Fungicides have been more extensively researched than herbicides, with 79 publications reported in Cullen et al., (2019). This research has covered a range of fungicides, with no single substance dominating the field, and only six different substances having double digit publication numbers. However, unlike herbicides, which are often tested in isolation, fungicides are commonly considered as threats only from their interaction with other stressors, most frequently insecticides. Some publications fail to consider fungicides alone as potential threats to bees to the extent that they are only included in treatment groups in combination with insecticide, not alone. Part of the reason for this is the synergism between SBI fungicides and insecticides. For example, the neonicotinoid thiacloprid when applied in isolation to honey bees causes no significant mortality at a 2μg dose, and neither does the azole SBI fungicide tebuconazole at a 3μg dose, but their combination at the same doses caused 70% mortality (Schmuck, Stadler and Schmidt, 2003). However, and beyond their interaction with insecticides, fungicides can also cause mortality in bees without additional stressors (Fisher et al., 2017, Simon-Delso et al., 2018), albeit typically at higher than field realistic chronic exposure levels. At more realistic exposure levels fungicide have been found to affect a range of sublethal traits like flight performance (Liao et al., 2019), colony development (Bernauer et al., 2015), larval development (Mussen, Lopez and Peng, 2004) and nest recognition in solitary bees (Artz and Pitt-Singer, 2015). The diversity of fungicides, bees high levels of exposure to them, and their individual and synergistic effects on bees make them attractive substances to study.

Similar to herbicides, some fungicides attack pathways that are not typically present in bees. It has been suggested that these fungicides could impact symbiotic fungal species in the microbiome, which may have knock-on effects on bees (Yoder et al., 2013). However, some fungicides attack pathways that bees do possess, such as sterol biosynthesis inhibiting (SBI) fungicides like propiconazole and diniconazole (sometimes called demethylation inhibitor (DMI) or ergosterol-biosynthesis-inhibiting (EBI) fungicides, but SBI is the industry standard mode of action name as per the Fungicide Resistance Action Committee, with other names typically relating to more specific sub-categories of modes of action). In insects these SBI fungicides can inhibit P450 function, which is a vital detoxification pathway (Brattsten, Berger and Dungan, 1994). This alone damages bee health but is most noticeable in combination with other stressors where it leaves insects unable to detoxify other pesticides (Pilling and

Jepson, 1993, Iwasa et al., 2004). The hazard ratio (a measure of mortality relative to the control) for a pyrethroid pesticide (lambda-cyhalothrin) increases 16-fold with co-exposure to the SBI propiconazole (Pilling and Jepson, 1993).

The effects of formulations, co-formulants and adjuvants on bees

Just as most experimental studies focus on insecticides, most studies use only the active ingredient, rather than the product actually used in agriculture which is called a formulation (reviewed by Cullen et al., 2019 for herbicides and fungicides). Using the active ingredient alone has the benefit of allowing for the effect to be directly attributed to the active ingredient, making interpretation of the results and comparison between studies easier. Regulatory testing is also focussed predominantly on the effects of active ingredients in isolation (EFSA, 2013). Formulations are regulated at the member state level, although the level of scrutiny applied is lesser than that applied to the active ingredient (EC, 2013). Further, there is variation between nations as to the level of transparency over the regulation of formulation, with Germany releasing all reports and the UK releasing none.

Formulation composition is designed to enhance efficiency of the product against the target organisms. However, there is growing evidence that this also increases toxicity to non-target organisms (Nagy et al., 2021), like bees (Mullin, 2015, Mullin et al., 2015). For instance, Chinese regulatory data reveals the fungicide tebuconazole is 26,000x more toxic to honey bees in formulation than as an active ingredient (Zhao et al., 2011 cited in Mullin, (2015)). A review of academic literature on non-target organisms found that 24 out of 36 studies comparing active ingredient to formulations found the formulation to be more toxic (Nagy et al., 2019). This increase in toxicity could be explained by a synergy between the active ingredient and co-formulants, or the co-formulants alone (Mesnage and Antoniou, 2018).

Co-formulants is a very broad category of chemical, so the dearth of research into them leaves large knowledge gaps to be filled. Previous work on formulations and bees has identified increases in mortality not explained by the active ingredient (Abrahams et al., 2018), which is likely explained by the co-formulant surfactant. While only a limited number of studies have been performed on co-formulants or adjuvants, several have identified substances as

hazardous to bee health (Moffett and Morton, 1973, 1975, Goodwin and McBrydie, 2000, Ciarlo et al., 2012, Fine, Cox-Foster and Mullin, 2017). Co-formulants are not tested for in regulatory or academic residue monitoring programs or studies. This may be because their chemical compositions would require distinct testing methodologies (Chen and Mullin, 2013, Chen and Mullin, 2014). Worryingly, when tested for Chen and Mullin (2013, 2014) found surfactant residues above the limit of detection in 100% of beehive samples taken, demonstrating how pervasive these understudied chemicals are. Given pesticide application in agriculture exclusively uses formulations, and that there can be a large disconnect between the toxicity of an active ingredient and the formulation, the large body of research on active ingredients may be failing to accurately represent the threat wild bees face.

Because co-formulants and adjuvants are both diverse and understudied there is little that can be said of broad general mechanisms by which they impact bees. Some notable mechanisms are listed below. When exposed to surfactants via spraying bees can become coated in the low surface tension substances (Goodwin and McBrydie, 2000), which it is hypothesised may block their spiracles (breathing holes) or block the tracheal system after penetrating through the spiracles (Stevens, 1993). Surfactants also reduce the surface tension of standing water, which is hypothesised to lead to bees falling in and then being unable to escape (Moffett and Morton, 1973). Some solvents, like N-Methyl-2-Pyrrolidone have been tested on bees (Zhu et al., 2014, Fine and Mullin, 2017, Fine et al., 2017, Chen et al., 2019), finding mortality to larvae at high chronic doses, but no mechanistic understanding of these effects has been resolved.

1.5 Bee parasites

Bees play host to a range of parasites from viruses (e.g., Deformed Wing Virus (DWV)), to microsporidia (*Nosema spp.*) to trypanosomes (e.g., *Crithidia spp.*), among many others. Despite bees having co-existed with parasites for millions of years, parasites are still often cited as a potential driver of bee declines (Goulson et al., 2015). While bees and parasites have co-existed for millennia, anthropogenic pressures have caused parasites to become more harmful to bee health than in the past. This has been caused principally by the unintentional export of parasites between species, the conditions in which managed bees are kept which act to promote parasite transmission, and the spillover of parasites from managed bees to wild bees (Meeus et al., 2011, Graystock et al., 2013, Fürst et al., 2014, Hicks, Pilgrim and Marshall, 2018).

The cross-species transmission of *Varroa destructor* (henceforth referred to as Varroa) from *Apis cerana* to *A. mellifera*, and subsequent global spread has caused severe distress to *A. mellifera* colonies and beekeepers alike (Kraus and Page, 1995, Lin et al., 2018). In its original host (*A. ceranae*) Varroa is not benign, but it has a much lower impact than in *A. mellifera* colonies (Lin et al., 2018). This is because *A. ceranae* has evolved a series of defensive hygienic measures to control it. *A. mellifera* lacks these traits and thus suffers highly consequential parasitism (Page et al., 2016). Varroa, and other parasites, are spread between honey bee colonies by workers moving between densely stocked colonies, or through transmission outside the hive, such as through shared floral resources (Peck, Smith and Seeley, 2016). Varroa alone damages honey bee colonies (Kraus and Page, 1995), but it also acts as a vector of viral pathogens like DWV (Martin et al., 2012). While Varroa itself is *Apis* specific, the viruses it vectors are not (Fürst et al., 2014). Thus, the spread of Varroa has also likely worsened the parasitism of wild bees due to increased viral spillover from honey bees (Fürst et al., 2014). The human management of honey bees, which supplements and sustains heavily parasitised colonies could further worsen parasite spillover by allowing colonies to survive with levels of parasitism that could otherwise kill the colony.

While some parasites, anthropogenically introduced or exacerbated, are potentially contributing novel pressures to wild bee health, there are also parasites that have persisted

in bee populations for evolutionary timescales. While these parasites are not benign, as they do reduce colony fitness, they are however not themselves the cause of recent and ongoing bee declines. It is through their additive or synergistic interaction with other stressors, such as pesticides, that they add pressure to bee population health. So, while these established parasites alone are not the cause of bee declines, they are relevant factors in bee declines. As such it is worth considering their effects on bee health in isolation, prior to moving onto their interaction with other stressors. Here I will focus on a parasite prevalent in bumble bee populations, *C. bombi*.

Crithidia bombi

In bumble bees, *Crithidia bombi* is one of the best studied parasites, with its effects on *B. terrestris* being well characterised. One reason to focus on *C. bombi*'s effects on bumble bees is its prevalence in wild populations, with up to 82% infection rates found in some surveys (Gillespie, 2010). This high level of infection is not found in all studies, with large variation seen between years, study sites and species (e.g., Shykoff and Schmid-Hempel, 1991, Korner and Schmid-Hempel, 2005, Rutrecht and Brown, 2008, Gillespie, 2010, Jones and Brown, 2014, Hicks et al., 2018).

C. bombi interacts with the host's gut microbiome, with the abundance/presence of specific bacterial groups like *Apibacter, Lactobacillus* Firm-5 and *Gilliamella* being linked to increased resistance (Koch and Schmid-Hempel, 2011, Mockler et al., 2018). Beyond the gut microbiome both host and parasite genotype are important in determining the level of infection (Schmid-Hempel et al., 1999, Baer and Schmid-Hempel, 2003, Yourth and Schmid-Hempel, 2006, Barribeau and Schmid-Hempel, 2013).

An intestinal parasite, *C. bombi* is expelled in faeces which can then infect other workers, queens, and males (Schmid-Hempel et al., 1999). Shared floral resources lead to horizontal transmission of *C. bombi* between bumble bees (Durrer and Schmid-Hempel, 1994), potentially in nectar or through flower surfaces (Cisarovsky and Schmid-Hempel, 2014, Adler et al., 2018, Figueroa et al., 2019). Honey bees, while not able to be infected by *C. bombi,* have also been found to be able to act as vectors of it (Ruiz-González and Brown, 2006).

Within a colony larvae may act as hubs of transmission, facilitating the spread of *C. bombi* (Folly et al., 2017).

Interestingly, the buff tailed bumble bee, *B. terrestris*, a host of *C. bombi*, has recently become invasive in South America. This likely occurred after escaping after being used for supplementary crop pollination (Colla et al., 2006, Meeus et al., 2011). While impossible to know the exact source, it is highly likely that the introduction of *B. terrestris* precipitated the introduction of *C. bombi*, which was previously not present in South America (Schimd-Hempel et al., 2014, Plischuk et al., 2017). One survey found 95% prevalence of *C. bombi* in a South American native species, *Bombus atratus* (Gamboa et al., 2015). Here we see a species that is a wild bee in Europe now acting as an invasive species and disease vector, and its established parasite now acting as an emerging threat to South American bee health.

The effects of *C. bombi* on healthy, unstressed bees fitness are limited, but when infected alongside additional stressors it can have a severe impact. In otherwise unstressed *B. terrestris* workers no effect of *C. bombi* on longevity was observed (Fauser-Misslin et al., 2014). However, survival does not equate to fitness, particularly for *B. terrestris* workers, which is a social species. By measuring colony production of sexuals, a partial measure of fitness can be derived. Fauser-Misslin et al., (2014) found no effect of *C. bombi*, on sexual production, however, Yourth, Brown and Schmid-Hempel (2008) found a reduction in male production. Another partial measure of fitness is queen hibernation success, as when a queen dies prior to founding a colony her fitness becomes zero (bar any benefit she may have provided her relatives). Because *B. terrestris* are an annual social species, their entire population (bar rare overwintering colonies) narrows down to just the queens hibernating overwinter. This represents a vulnerable stage, both because of the narrowing of the population but also the harsh conditions of winter on a hibernating bee, as such *C. bombi* has been found to cause higher levels of weight loss during hibernation (Baron et al., 2017) and to reduce survival over hibernation (Fauser et al., 2017). Yet hibernation survival and success are not sufficient measures of fitness, in fact the best estimate of a parasite's impact on host fitness comes from a whole-lifecycle assessment which accounts for the most vulnerable stages. Brown, Schmid-Hempel and Schmid-Hempel (2003) did just this and found a 40% reduction in overall colony fitness, indicating that by accounting for the most vulnerable

stages, the impact of *C. bombi* can be very severe. In the wild bees are not exposed to just one stressor at a time, instead facing varying degrees of stress from a range of sources.

It has been found that alongside additional naturally occurring stressors, like nutritional deprivation, *C. bombi* can cause up to 50% higher rates of worker mortality (Brown, Loosli and Schmid-Hempel, 2000). Wild caught workers with *C. bombi* were found to have less developed ovaries than those uninfected (Shykoff and Schmid-Hempel, 1991), but this cannot be attributed to the effect of *C. bombi* alone, as it is not known if these wild workers were under additional stressors which may have acted additively or synergistically with the parasite. The effects of parasites on bee health in isolation are relatively well resolved, however the effect of parasites on bee health when those bees are also exposed to pesticides is poorly understood.

1.6 The interaction of pesticides and parasites on bee health

Pesticides are novel stressors to bee health, while established parasites have been stressors for millions of years. The true characterisation of a novel stressor's detriment to bees is both the damage it causes in isolation, and the additional damage it causes through interactions with existing stressors (Martin et al., 2012). While some pesticides in isolation, such as glyphosate, have not been shown to cause significant damage to bee health, it is possible that their interaction with established bee parasites could cause such damage (Motta, Raymann and Moran, 2018). Despite this, most studies look at the effects of pesticides in isolation, which ignores the multifactorial nature of bee declines (Alaux et al., 2010).

Combinations of neonicotinoids and parasites can inflict mortality costs, where either stressor alone would not (Fauser-Misslin et al., 2014, Doublet et al., 2015). Further, mortality inducing neonicotinoid doses are more lethal to parasite infected honey bees (Alaux et al., 2010, Vidau et al., 2011, Retschnig, Neumann and Williams, 2014). Beyond mortality, Pettis et al., (2012) found honey bees chronically exposed to a neonicotinoid through pollen had higher *Nosema* spp*.* parasite loads, although Retschnig, Neumann and Williams (2014) found another neonicotinoid provided chronically via spiked sucrose to reduce *Nosema* load. Wu et al. (2012) found honey bees exposed to higher pesticide loads during development were more susceptible to *Nosema ceranae*. While neonicotinoids and *Nosema* may interact to reduce honey bee health in some metrics, they do not act additively or synergistically in all metrics. For instance, metrics of bee health more nuanced than survival, like olfactory learning, have been studied in bumble bees and honey bees, with the effect of the combined stressors being no worse than either stressor alone (Piiroinen and Goulson, 2015, Piiroinen et al., 2015). Overall, this body of research demonstrates that studying pesticides in isolation does not reveal the full extent of their impact.

However, not all combinations of insecticide and parasite interact. For example, *C. bombi* and two neonicotinoids do not interact to reduce hibernation success in bumble bees, despite both causing detriment in isolation (Baron et al., 2017, Fauser et al., 2017). Additionally, a

pyrethroid pesticide had no impact on *C. bombi* susceptibility or intensity in bumble bees (Baron, Raine and Brown, 2014). Novel insecticides like sulfoxaflor, brought in to replace the now banned neonicotinoids, have not yet been tested alongside parasites, so their effects are unknown.

Few studies have linked herbicides and parasites in bees. Yet, recent advances in the understanding of glyphosate's effects on the gut microbiome of honey bees have developed understanding in this area. In honey bees, glyphosate has been repeatedly found to perturb the host microbiome (Dai et al., 2018, Motta, Raymann and Moran, 2018, Blot et al., 2019, Motta and Moran, 2020, Motta et al., 2020). Alone this has not been observed to cause mortality or affect other fitness corollaries. However, in bees infected with a representative dose of a typically low impact parasite, *Serratia marcescens*, this perturbation of the microbiome was associated with a significant increase in mortality (Motta, Raymann and Moran, 2018). The effect appears to be parasite species specific as no effect was observed with *Nosema ceranae* infection (Blot et al., 2019).

Similarly, little research effort has been devoted to the combined effects of fungicides and parasites, with a handful of correlational studies (Mullin et al., 2010, Pettis et al., 2013, McArt et al., 2017) finding that fungicide residues levels in honey bee colonies are strongly correlated with parasite load and colony disorders (Simon-Delso et al., 2014). Interestingly, to my knowledge no published studies explicitly testing the impacts of fungicides and parasites on bee health using an experimental methodology exist. Degrandi-Hoffman et al. (2015) found that honey bees exposed to a formulation containing the active ingredients boscalid and pyraclostrobin (Pristine) had higher viral titers than control bees for a range of damaging viruses. However, the lack of a parasite free control treatments in this experiment precludes interpretation of the effect of the fungicide-parasite interaction. Similarly, Gregorc et al. (2012) did test two fungicides (myclobutanil and chlorothalonil) on infected bees, but also lacks uninfected controls.

Co-formulants and adjuvants interaction with parasites has been subject to almost no study with just a singular publication on the topic. The sole publication, Fine et al. (2017), exposed honey bee larvae to a mixture of viruses in combination with the adjuvant Sylgard 309 (the

main component of which is organosilicone surfactants). The study found that both stressors alone reduced larval survival, and their interaction synergistically reduced it much further. Additionally, the adjuvant caused a significant increase in failed moulting attempts by larvae. Herbicides, fungicides and co-formulants/adjuvants are very heavily used pesticide groups (Mullin et al., 2015, FERA, 2021), combined accounting for the majority of all pesticides as applied by weight or area (FERA, 2021). They are used globally and typically with no mitigation measures in place to protect bees. Parasites are near ubiquitous in bee populations, with prevalence levels for individual parasites often topping half the population (e.g., Shykoff and Schmid-Hempel, 1991, Korner and Schmid-Hempel, 2005, Rutrecht and Brown, 2008, Gillespie 2010, Jones and Brown, 2014, Hicks et al., 2018). As such the almost entire lack of research into how these pesticides interact with parasites in bees is alarming.

1.7 Conclusion

In the wild, bees face a number of separate threats to their continued existence. These threats are often highly damaging alone, and even more so when in combination. One such threat, pesticides, as detailed above, is an incredibly diverse category. In the UK alone there are 276 active ingredients, 2892 pesticide products and 294 adjuvant product (Health and Safety Executive UK, 2021a-b), each used in different circumstances and each potentially interacting with other stressors like parasites in a unique manner.

The systems we use to regulate pesticides fails to adequately characterise this complexity. First, a failure to test for sublethal effects ignores the damage to populations, when even small reductions in reproductive output can have large population level effects (Bryden et al., 2013). Second, by ignoring interacting stressors, the additional damage parasites inflict upon pesticide exposed hosts is missed. Third, an explicit focus on active ingredients has led to a lack of testing for co-formulants and adjuvants which are abundantly used in agriculture (Mesnage and Antoniou, 2018).

Current testing regimes have adequately, albeit belatedly, identified and restricted use of several bee harming insecticides (EC, 2013, 2018a-c). However, their overreliance on lethality as a measure of damage ignores the complexity of the potential threat that herbicides, fungicides, co-formulants and adjuvants pose to bee health. Furthermore, the focus of testing on honey bees means that regulation largely lacks relevance to wild, non-domesticated bee populations. Consequently, in my thesis I will aim to elucidate the interaction between pesticides and parasites, looking at both lethal and sublethal effects, and focus on the effects of understudied pesticides group on the bee species *B. terrestris*. In doing so I hope to draw attention to the broad range of potential threats pollinators face, and how regulators can adapt policy to protect them.

Overview of the data chapters in my thesis

In the first data chapter, Chapter 2, of my thesis I cover the impacts of glyphosate-based herbicides on bumble bees, with a particular emphasis on the co-formulants in the mixtures. There is emerging evidence that surfactants, which are common co-formulants and common adjuvant components, may be lethal to bees when sprayed onto them. Work from the early 2000s found that honey bees sprayed with surfactant adjuvants had high levels of mortality, even when the adjuvants were at, or below, recommended dilution levels (Goodwin and McBrydie, 2000). This work was followed up on in 2018 with the application of a glyphosate-based herbicide (Sunphosate SL) to flowers on which honey bees and stingless bees were allowed to forage. Significant levels of mortality were again observed at recommended concentrations (Abraham et al., 2018). These findings combined point to surfactant co-formulants as the cause of the mortality. This conclusion was further strengthened with the publication of Motta et al. (2020) which found that Roundup ProMax caused mortality when directly applied to honey bees, but that equivalent doses of glyphosate did not. This is the topic of Chapter 2, asking the question 'What is the effect of glyphosate-based herbicides on bumble bees, and what components of the formulation are responsible'. Beyond the effects of the formulation as a whole, the impacts of glyphosate on bumble bee health deserves study, as it is the most widely used pesticide compound globally (Duke, 2018).

In Chapter 3 I present experimental work studying the combined impacts of glyphosate and *C. bombi*. These two stressors are among the most common stressors bumble bees in the wild will face. Glyphosate is the world's most used pesticide, by weight or area, and it has no mitigation measures in place to prevent bees from being exposed to it. With prevalence's of up to 82% within its native range during peak forager season (Gillespie, 2010), *C. bombi* is among the most common parasites in wild *B. terrestris* populations. While neither stressor alone has been found to cause mortality in otherwise unstressed bees, it is possible that their interaction could cause mortality. Further, glyphosate is known to perturb the honey bee gut microbiome (Dai et al., 2018, Motta, Raymann and Moran, 2018, Blot et al., 2019, Motta and Moran, 2020, Motta et al., 2020), which is similar but not identical in composition to the bumble bee microbiome. The host microbiome is known to interact with *C. bombi*, with the prevalence of certain bacterial groups conferring a degree of protection from the parasite. It

is thus possible that there would be an effect, either positive or negative, on the intensity of *C. bombi* in bees co-exposed to glyphosate. If the intensity increased, the fitness consequences of *C. bombi* would be expected to be exacerbated. While glyphosate may be the world's most used pesticide, other substances like fungicides are also abundantly used, and because they are applied to crops, not weeds, they may expose bees to even higher levels of pesticides.

Chapter 4 of my thesis will look at the impacts of a fungicide on bumble bee health, utilising a regulatory protocol adapted to provide additional information about bee health. The fungicide in question is Amistar, the flagship product of the fungicide group the strobulins, and once (and perhaps still, although data are lacking) the most used fungicide globally (Bartlett et al., 2002). This testing looked to disentangle the impacts of the fungicide formulation as a whole from each of its constituent components. To do so each individual co-formulant was tested at an equivalent level to that contained within the formulation. To assess the ability of regulatory testing to detect toxicity from substances, change in weight pre- to post-exposure was measured, along with sucrose consumption. This enabled the analysis of sublethal metrics, such that the test was not solely reliant on mortality as the only metric. The toxicity observed was attributable to a co-formulant, not the active ingredient, and this has profound implications for the active ingredient only approach to toxicity testing in academia and regulation.

The role of co-formulants and adjuvants in causing toxicity to bees is poorly understood. Some reviews have covered their impacts, but typically with a focus on how they affect the toxicity of the active ingredient (Mullin et al., 2015, Mullin, 2015). In the final data chapter, Chapter 5, of my thesis, I present a systematic review covering all the literature that tests the effects of a co-formulant or adjuvant on bees. By collating all this literature broad trends and knowledge gaps were identified. The fields of herbicide, fungicide and co-formulant/adjuvant research on bees are nascent and still small, the research contained in this thesis will hopefully help expand upon the current literature, filling in some key knowledge gaps, as well as providing direction for future research.

1.8 General methods

Crithidia bombi purification protocol

At least one *Crithidia bombi* infected colony was maintained year-round to ensure consistent access to *C. bombi*. Methodology was modified from Cole (1970). 40-60 workers were removed from a previously infected colony and moved to specimen tubes; they were then induced to defecate by physical mild agitation. Their faeces, which contains high levels of *C. bombi* (Schmid-Hempel and Schmid-Hempel, 1993) were collected in 10μL microcapillary tubes (Blaubrand, Germany) and transferred to a 1.5mL Eppendorf tube, referred to as tube 1.

1ml of 0.8% Ringer (Sigma-Aldrich, Germany) solution was added to tube 1, to dilute the faeces in a liquid with a similar osmotic balance, before being pipetted to mix. This solution was centrifuged at 800rpm for 2 minutes at 21˚C, and then the supernatant was transferred to a new tube, tube 2. This was repeated 8 times, and each time the supernatant from the most downstream tube was moved into a new tube. The pellet left in the most downstream tube was then diluted with the supernatant from the tube directly upstream of it. The supernatant from tube 1 would be moved to tube 2 each time, and as such an additional 1ml of 0.8% Ringer solution was added each round. This is a process called triangulation, whereby particles heavier than *C. bombi* cells are retained in the initial tubes, and particles lighter than *C. bombi* are moved into the later tubes. This leads to the middle tubes having very high concentrations of *C. bombi* and little else (Cole, 1970), effectively purifying it.

After the final transfer of supernatant tubes 4, 5 and 6 were centrifuged at 8000rpm for 1 minutes at 21˚C to concentrate the *C. bombi* cells in a pellet at the base of the tube. The supernatant was then removed and discarded, leaving only the concentrated *C. bombi* pellet. 100μL of 0.8% Ringer solution was then added to tube 4 and then transferred to tube 5 then 6, being pipetted to mix each time to produce a highly concentrated stock solution.

The 100μL of solution in tube 6 was vortexed for 1 minute to thoroughly mix, then 5μL was pipetted onto a haemocytometer slide and the number of *C. bombi* cells in the fixed volume

of the slide was counted to determine the concentration of the *C. bombi* solution that had been produced. This process provided a concentrated *C. bombi* stock solution, relatively free from impurities that could impact the bees set to be infected with it.

For a typical 10,000 cell dose per bee, 20µL of a 500 cell per µL solution was made by diluting the *C. bombi* stock solution in distilled water before vortexing for 1 minute, this was then diluted with 20µL of 50% w/w sucrose. This treatment solution was then again vortexed for 1 minute and maintained at 4˚C. Prior to use the solution was allowed to warm to room temperature, which is thought to improve the consumption rate. The sham inoculum used for each bee was a 20µL distilled sucrose 20µL 50% w/w sucrose and was made at the same time as the treatment solution.

Chapter 2

Roundup Causes High Levels of Mortality Following Contact Exposure in Bumble Bees

Abstract

Pollinators underpin global food production, but they are suffering significant declines across the world. Pesticides are thought to be important drivers of these declines. Herbicides are the most widely applied type of pesticides and are broadly considered 'bee safe' by regulatory bodies who explicitly allow their application directly onto foraging bees. I aimed to test the mortality effects of spraying the world's most popular herbicide brand (Roundup) directly onto bumble bees *B. terrestris audax*. I used three Roundup products, the consumer products Roundup Ready-To-Use and Roundup No Glyphosate, the agricultural product Roundup ProActive, as well as another herbicide with the same active ingredient (glyphosate), Weedol. Label recommended pesticide concentrations were applied to the bees using a Roundup Ready-To-Use spray bottle. Bees exhibited 94% mortality with Roundup Ready-To-Use and 30% mortality with Roundup ProActive, over 24 hr. Weedol did not cause significant mortality, demonstrating that the active ingredient, glyphosate, is not the cause of the mortality. The 96% mortality caused by Roundup No Glyphosate supports this conclusion. Dose-dependent mortality caused by Roundup Ready-To-Use, further confirms its acute toxicity. Roundup products caused comprehensive matting of bee body hair, suggesting that surfactants, or other co-formulants in the Roundup products, may cause death by incapacitating the gas exchange system. These mortality results demonstrate that Roundup products pose a significant hazard to bees, in both agricultural and urban systems, and that exposure of bees to them should be limited. Surfactants, or other co-formulants, in herbicides and other pesticides may contribute to global bee declines. I recommend that, as a precautionary measure until co-formulant identities are made public, label guidelines for all pesticides be altered to explicitly prohibit application to plants when bees are likely to be foraging on them. As current regulatory topical exposure toxicity testing inadequately assesses toxicity of herbicide products, I call for pesticide companies to release the full list of ingredients for each pesticide formulation, as lack of access to this information hampers research to determine safe exposure levels for beneficial insects in agro-ecosystems.

2.1 Introduction

Bees provide the crucial ecosystem service of pollination (Potts et al., 2016), but are under threat, with 37% of EU bee species with known trends exhibiting population declines (Nieto et al., 2014). One apparent cause of these declines is pesticides (Rundlöf et al., 2015, Woodcock et al., 2016, McArt et al., 2017). Pesticide usage is pervasive, with 4.1 billion kilograms of active ingredient applied globally in 2017, nearly double the amount used in 1990 (FAOSTAT, 2021). Pesticides have received significant attention from the public and policymakers due to their apparent detriment to non-target organisms, such as pollinators, but this attention has largely focused on insecticides. A recent systematic review found that only 29 studies had tested the effects of herbicides on bees (Cullen et al., 2019). Additionally, research into herbicides relative to insecticides is disproportionate to their usage, with, for example, 24 times more herbicide applied in the UK than insecticide in 2018 (FERA, 2021).

For most classes of pest, pesticide usage varies by crop and region, with a range of active ingredients being employed (Garthwaite et al., 2016a-b). However, herbicides are unique in that one substance, glyphosate, is applied at a far greater rate than any alternative (FERA, 2021). In 2014, 826 million kilograms of glyphosate were applied globally (Benbrook, 2016), accounting for around 20% of all pesticide application (Benbrook, 2016, FAOSTAT, 2021). Glyphosate (applied in products called glyphosate-based herbicides (GBHs)) has a favourable toxicity profile as a broad-spectrum herbicide, being the only herbicide to target the shikimate pathway (Duke, 2018). Its low toxicity to the majority of non-target organisms (EFSA, 2015a), has led to most regulatory regimes placing minimal restrictions on its application (Beckie et al., 2020). Bee exposure to glyphosate is poorly characterised, although it is known to be extensive, with surveys finding that 59% of honey samples had glyphosate present above the limit of detection, with a mean of 64 ppb (Rubio et al., 2014).

High acute doses (oral and contact) of glyphosate, applied as the active ingredient (glyphosate) alone, or in a single representative formulation (MON 52276 commercially called Roundup Bioflow in Italian markets (EFSA, 2015b, Mesnage et al., 2021), do not cause mortality in honeybee workers (EFSA, 2015b). Consequently, it has passed lower tier testing in the US and EU, facilitating its approval in both territories. However, GBHs contain additional

components, called co-formulants, that can have serious, but systematically underestimated risks (Cox and Surgan, 2006, Mullin et al., 2016, Mesnage and Antoniou, 2018).

Co-formulants are chemical additives that increase the efficiency of the active ingredient (Hazen, 2000). Without co-formulants, pesticide formulations would be much less effective (Hazen, 2000), and more active ingredient would need to be applied, potentially leading to more environmental damage. Most co-formulants are considered 'inert' by regulatory bodies, and thus are not subject to equivalent testing to active ingredients. Consequently, there are no requirements to test their toxicity to bees (EC, 2009), meaning that potentially toxic substances are used abundantly (Cox and Surgan, 2006, Mullin, 2015, Mullin et al., 2015). As they are not tested for in food or environmental residue monitoring programmes (Mesnage et al., 2019), our understanding of their prevalence and environmental fate is highly limited. Bee exposure to these co-formulants is likely commensurate to that of active ingredients but is poorly studied.

While our understanding of co-formulant exposure is limited, studies of hazard (i.e., the damage they cause) are more informative. Nagy et al. (2019) reported that 24 of 36 studies showed formulations to be more toxic in non-target organisms than active ingredients alone. In human cell lines and rats, Roundup products specifically were more toxic than the active ingredient alone in five of six studies, with just one study finding equivalent toxicity (Nagy et al., 2019). While only one formulation per active ingredient is typically submitted to the full range of toxicity tests in the EU (EFSA, 2015a), dozens of formulations per active ingredient are produced, each with a unique composition posing unique hazards to non-target organisms (Mesnage et al., 2019). For glyphosate in the UK there are 284 distinct consumer or agricultural formulations (Health and Safety Executive UK, 2021b), making it the most formulation diverse active ingredient in the UK. Co-formulants present in Roundup have been found to have sub-lethal effects in human cell lines (Mesnage et al., 2013, Defarge et al., 2016), demonstrating that they present a relevant hazard to health, although almost nothing is known of their effects on bees (Mullin, 2015, Mullin et al., 2015). One class of co-formulants, surfactants (**surf**ace **act**ing age**nt**), were found in 100% of American honey, pollen and beeswax samples (*n* = 27; Chen and Mullin, 2014), demonstrating their pervasiveness.

Surfactants in herbicides like Roundup spread the sprayed droplets out over target leaves, increasing glyphosate absorption and toxicity. Surfactants are major co-formulants in Roundup products, typically accounting for 15% of the concentrated weight (Mesnage et al., 2019). Surfactants are environmental pollutants that have been shown to have a range of negative impacts on honey bees (Moffett and Morton, 1973, 1975, Goodwin and McBrydie, 2000, Ciarlo et al., 2012, Fine et al., 2017) and solitary bees (Artz and Pitts-Singer, 2015).

In agriculture, direct spraying of insecticides onto bees, or bee attractive flowers, is banned as part of their mitigation strategy (EFSA, 2013) in order to prevent bees contacting the pesticide as it is being sprayed, or the residues on flowers after it is sprayed. No such restrictions apply for herbicides, with the Environmental Information Sheet for Roundup ProActive stating "Roundup ProActive is of low toxicity to honeybees; there is no requirement to avoid application of the product when bees are foraging on flowering weeds in treated crops" (Roundup ProActive Environmental Information Sheet). Consequently, with both glyphosate and the co-formulants/surfactants in GBHs being considered safe by regulators (EFSA, 2015a), there should not be lethal effects from GBHs when used following label guidelines. Abraham et al. (2018) however, found significant mortality through indirect exposure to a GBH, Sunphosate 360 SL (Zhejiang Xinan Chemical Industrial Group, Zhe-jiang, China), which is a generic GBH available in Ghana. The study found that honeybees, *A. mellifera,* and stingless bees, *Hypotrigona ruspolii,* exposed to the formulation via a branch of a flowering tree, *Senna siamea,* that had previously been sprayed with Sunphosate 360 SL suffered 28% and 23% mortality respectively, which was significantly higher than the 4% and 6% mortality for the water control. As glyphosate does not cause such mortality via contact or oral exposure (EFSA, 2015b), the mortality seen in this experiment is likely to be driven by co-formulants.

Risk assessment of the threat a pesticide poses to bees relies on the Risk = Hazard × Exposure model, where Hazard is a measure of toxicity, and Exposure is a measure of environmental contact. GBHs are currently believed to combine low to no hazard and high exposure, because they can be directly applied to bees, making them low to intermediate risk. Here I test how hazardous a range of GBHs, including Roundup products are to bumble bees. I use a study design that can distinguish between the effects of co-formulants and the active ingredient, to

allow us to test how these factors affect mortality. I predict that the GBHs will cause moderate

mortality with direct exposure, in line with Abraham et al. (2018).

2.2 Materials and methods

Ten commercial bumble bee, *B. terrestris audax,* colonies were used in the experiments (Agralan). On arrival 10 workers per colony were removed and their faeces screened for micro-parasites. No infections were detected, and all colonies were thus retained in the experiment.

In all experiments over 50 bees were exposed per treatment (excluding the control treatment in Experiment 4) in groups of five or six, as detailed in Table S2. Bees were sprayed in groups for efficiency and because an even coating could still be achieved with this number of bees in a box. For each experiment multiple source colonies were used to account for inter-colony variation, allocating them evenly across treatments. Workers were moved from source colonies into clear acrylic boxes (6.7 × 12.7 × 4.9 cm), with a plastic mesh grate bottom (6.7 × 7.3 cm). Within each box, bees were only taken from one source colony and were left to acclimatise for 10 min prior to exposure.

A mortality check was carried out prior to exposure. Mortality was defined as any moribund bee being entirely unresponsive to physical agitation with a pair of forceps. Following this, the acrylic box was sprayed in a X shape from corner to corner with two squeezes of the trigger of a Fast Action Roundup Ready-To-Use bottle (Roundup Ready-To-Use; total exposure = 1.327 ± 0.005 ml SE); the spray came out as a cone of droplets which ensured consistent and even coverage across the whole box. This amount was chosen to ensure the bees were evenly coated while keeping control mortality <10%, pilot work found this methodology to deliver the treatment evenly to all bees sprayed when visually assessed. Roundup Ready-To-Use and Roundup No Glyphosate are sold in these spray bottles, and Weedol in a similar bottle. Bees were sprayed under red light to prevent flying, I did not attempt to influence their behaviour beyond this, and they were exhibiting normal resting behaviour when sprayed. This methodology is not designed to replicate field realistic exposure (spraying conditions or label recommended application rates), it is instead designed to assess the lethality (hazard) the herbicide products pose to bumble bees. One investigator performed the spraying and mortality checks. A series of practice sprays were performed to ensure consistency. Mortality was recorded immediately after spraying, and at 10, 20 and 30 min.

After 30 min a source of sucrose (50% w/w) and small portion of pollen (1-2 g) was added. At 24 hr post-exposure mortality was recorded for a final time. Boxes that flooded due to sugar water spillage between 30 min and 24-hr observations were excluded (n = 2, both in Experiment 2, Control), as were individual bees who drowned themselves in the sucrose gravity feeder (n = 1, Experiment 5, Control).

We used a total of four herbicide products across my experiments. Fast Action Roundup Ready-To-Use (MAPP 14481; henceforth referred to as Roundup Ready-To-Use), Roundup Speed Ultra (MAPP 18692; henceforth referred to as Roundup No Glyphosate; both Scotts Miracle-Gro Company, Surrey, UK under licence from Monsanto, Cambridge, UK), and Weedol Gun! Rootkill Plus (MAPP 14554; henceforth referred to as Weedol, Scotts Miracle-Gro Company, Surrey, UK) are all consumer products that can be bought in supermarkets. Consumer products require no licence or training in the UK and are intended for garden use. Roundup ProActive (MAPP 17380, Monsanto, Cambridge, UK) can be bought online without a licence in the UK, but a licence is required to spray the substance in agriculture or horticulture (Roundup ProActive Label, 2019). All products were purchased in 2019 online or in person in the UK (full details of all products used are provided in Table S1). Table 1 shows the glyphosate and other active ingredient concentrations, as reported on the product labels, and the dilutions for the test solutions used across experiments. For pre-mixed consumer products, I used the concentration as sold, or diluted it further as in Experiments 2 and 3. For the agricultural product Roundup ProActive I used field realistic concentrations of the treatment solutions, with the product diluted as directed on the label to produce a concentration equivalent to that used in agricultural spraying. This is distinct from the rate of application, which is the amount of substance applied per area, typically expressed as active ingredient g/ha or L/ha of a pesticide mixture. I did not attempt to replicate field realistic application rates for the agricultural product Roundup ProActive for the following reasons. While we know the application rates for this product based on ground surface area (from 1 to 6 L/ha of formulation, 0.6%–33% product concentration and 10-400 L/ha of mixed solution), the exposure, or application rate on bees will be a function of the height from which the product is sprayed, the height of either crop or weed flowers and the height at which bees are present when the product is applied (which may be either the same as the flowers, or above or below this if bees are flying between flowers). As each of these factors will vary both

within crops, and from crop to crop, and as the only one for which good data exist are crop height, it is currently impossible to extrapolate from surface area application rate to bee exposure. Similarly, in the absence of label guidance on application rates for consumer products, I cannot compare my exposure to usage in gardens. Fundamentally, my experiment was designed to enable the detection of hazardous effects from substances previously reported to be non-hazardous. More complex designs using field realistic apparatus and application rates could determine the risk these substances pose.

Controls throughout were pure distilled water and were sprayed from an identical Roundup Ready-To-Use bottle at room temperature. Both the Weedol and Roundup products tested (Experiments 1 and 2) contain glyphosate at equivalent concentrations. Because Weedol is likely to have a different co-formulant composition to the Roundup products it served as a glyphosate control. A series of five independent experiments were conducted to answer the following questions:

Experiment 1: Are the impacts of consumer and agricultural Roundup products comparable? Bumble bees in three treatment groups were sprayed with either the consumer product Roundup Ready-To-Use (at its pre-mixed concentration), the agricultural product Roundup ProActive at the highest label recommended concentration of 6.25%, which covers a range of applications, or the water control.

Experiment 2: Does mortality still occur with a 1:1 dilution of consumer Roundup? Bumble bees in two treatment groups were sprayed with either the consumer product (Roundup Ready-To-Use) diluted 1:1 with pure distilled water, or the water control.

Experiment 3: Does mortality still occur with a 1:3 dilution of consumer Roundup? Bumble bees in two treatment groups were sprayed with either the consumer product (Roundup Ready-To-Use) diluted 1:3 with pure distilled water, or the water control.

Experiment 4: Does an alternative GBH (Weedol) cause mortality? Bumble bees in two treatment groups were sprayed with either the generic consumer product GBH Weedol at its pre-mixed concentration, or the water control.

Experiment 5: Does the Roundup formulation without glyphosate cause mortality? Bumble bees in two treatment groups were sprayed with either the consumer product (and GBH alternative) Roundup No Glyphosate at its pre-mixed concentration, or the water control.

All statistical analyses were carried out in 'R' programming software version 3.6.2 (R Core Team, 2019). Plots were produced using the package 'ggplot2' version 3.2.1 (Wickham, 2016) and 'survminer' version 0.4.6 (Kassambara et al., 2019). Mixed effects Cox proportional hazards models were used to analyse mortality, utilising 'survival' version 3.1-8 (Therneau, 2020a), 'coxme' version 2.2-16 (Therneau, 2020b) and 'MuMIn' version 1.43.17 for model averaging (Bartoń, 2020). AIC model simplification was used, with 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% support was reached. Parameter estimates and 95% confidence intervals are reported. The full model used was (Survival ~ Treatment + Colony of Origin + (1|Box ID)). There was no correlation between variables. For comparisons between Roundup Ready-To-Use concentrations in Experiments 2 and 3 Colony of Origin was not included as a variable, as it correlated with Treatment owing to different colonies being used for each experiment. Consequently, the final model was (Survival ~ Treatment + (1|Box ID)). Model parameters, AIC weights and final models are presented in Table S3. Proportionality of hazards was checked for each experiment to validate the Cox proportional hazards assumption, where this was violated (Experiments 2-5) a Chi-squared test of Independence was used with the model (Survival ~ Treatment).

Table 1. The concentrations of the products used, based on the amount of water added to dilute them to, or below, label concentrations, and respective glyphosate concentrations. Concentrations of other active ingredients present in formulations given in parentheses.

Experiment	Treatment	Product Concentration Used (%)	Glyphosate Concentration g/L
All	Control	0	0.0
1	Roundup Ready-To-Use	100	7.2
1	Roundup ProActive	6.25	22.5
2	Roundup Ready-To-Use 50%	50	3.6
3	Roundup Ready-To-Use 25%	25	1.8
4	Weedol	100	7.2 (0.02g/L pyraflufen-ethyl)
5	Roundup No Glyphosate	100	0.0 (60g/L acetic acid)

2.3 Results

Experiment 1: Comparing the impacts of consumer and agricultural Roundup products

There was a significant difference in mortality between both Roundup products (Ready-To-Use and ProActive) and the control (Cox proportional hazards model: parameter estimate (PE) = 5.17, 95% CI [3.52 to 6.82], and PE = 2.18, 95% CI [0.52 to 3.84] respectively), with 94% and 30% mortality respectively compared to 4% mortality in the control treatment (Figure 1). There was also a significant difference between Roundup Ready-To-Use and Roundup ProActive (Cox proportional hazards model: (PE) = 2.95, 95% CI [1.93 to 3.96]), with the Roundup Ready-To-Use causing faster and higher mortality. Of the Roundup Ready-To-Use treated bees, 38% died immediately after exposure compared to just 7% of Roundup ProActive and 0% of control bees. *Ad hoc* behavioural observations also noted bees in all Roundup treatments spent considerable time self-grooming after exposure. This may have been in response to, and potentially exacerbated, the matting of bee body hair that can be seen in Figure 4.
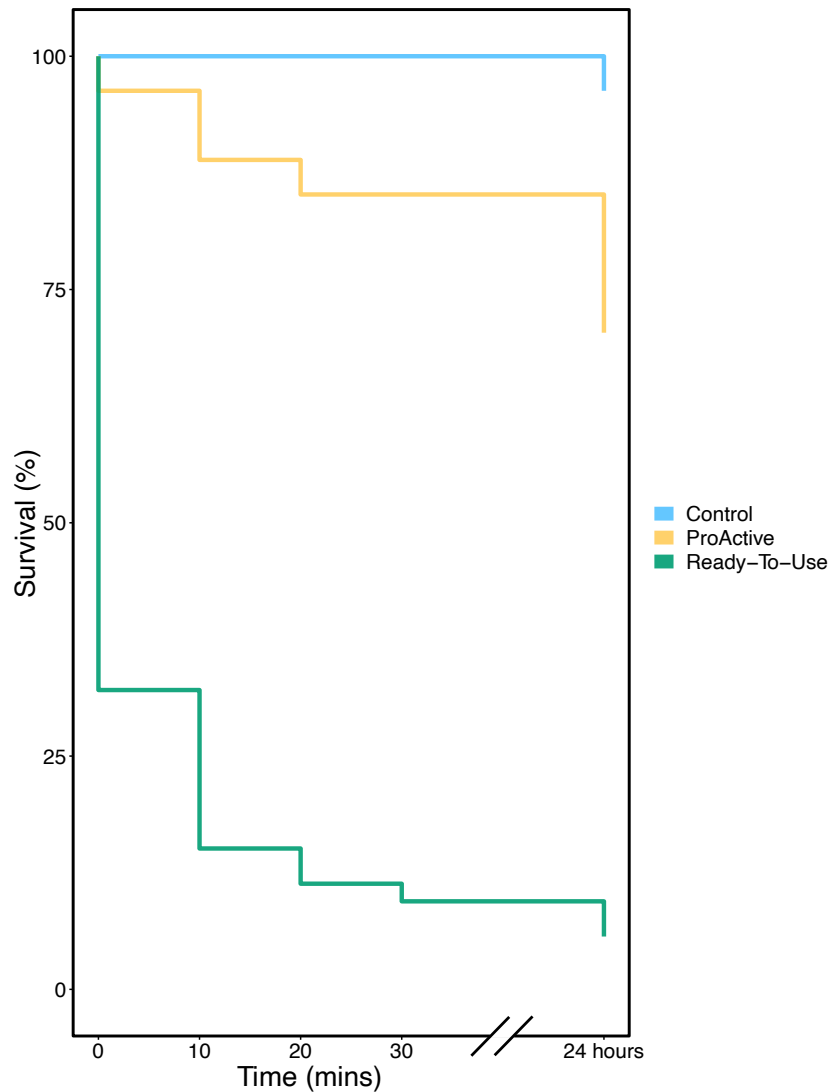
Figure 1. Experiment 1: Comparing the impacts of consumer and agricultural Roundup products against the control, demonstrating high mortality with the Ready-To-Use treatment and intermediate mortality with the ProActive treatment.

Experiment 2: Does mortality still occur with a 1:1 dilution of consumer Roundup?

The half strength Roundup Ready-To-Use solution significantly increased mortality (Chi-squared test of Independence: χ^2 = 78.26, p < 0.001), with 98% mortality respectively compared to 3% mortality in the control treatment (Figure S1).

Experiment 3: Does mortality still occur with a 1:3 dilution of consumer Roundup?

The quarter strength Roundup Ready-To-Use solution also produced significantly higher mortality than the control (Chi-squared test of Independence: χ^2 = 47.16, p < 0.001), with 78% mortality as opposed to 8% mortality in the control treatment (Figure S2). However, the mortality was less than either half or full strength (98% and 94% respectively; Figure 1; Figures S1 and S2). Furthermore, the mortality was delayed with only 10% of bumble bees dying within 30 min.

There was a significant difference between full-strength and both half and quarter-strength Roundup Ready-To-Use solutions in their effects on mortality (Cox proportional hazards model: (PE) = 1.23, 95% CI [0.766-1.70], and 2.33, 95% CI [1.54-3.20] respectively), with the highest and fastest mortality in the whole strength treatment, followed by the half strength.

Experiment 4: Does an alternative GBH (Weedol) cause mortality?

Weedol did not cause a significant difference in mortality relative to the control. (Chi-squared test of Independence: χ^2 = 0.00, p = 0.983), with 4% and 6% mortality respectively (Figure 2).
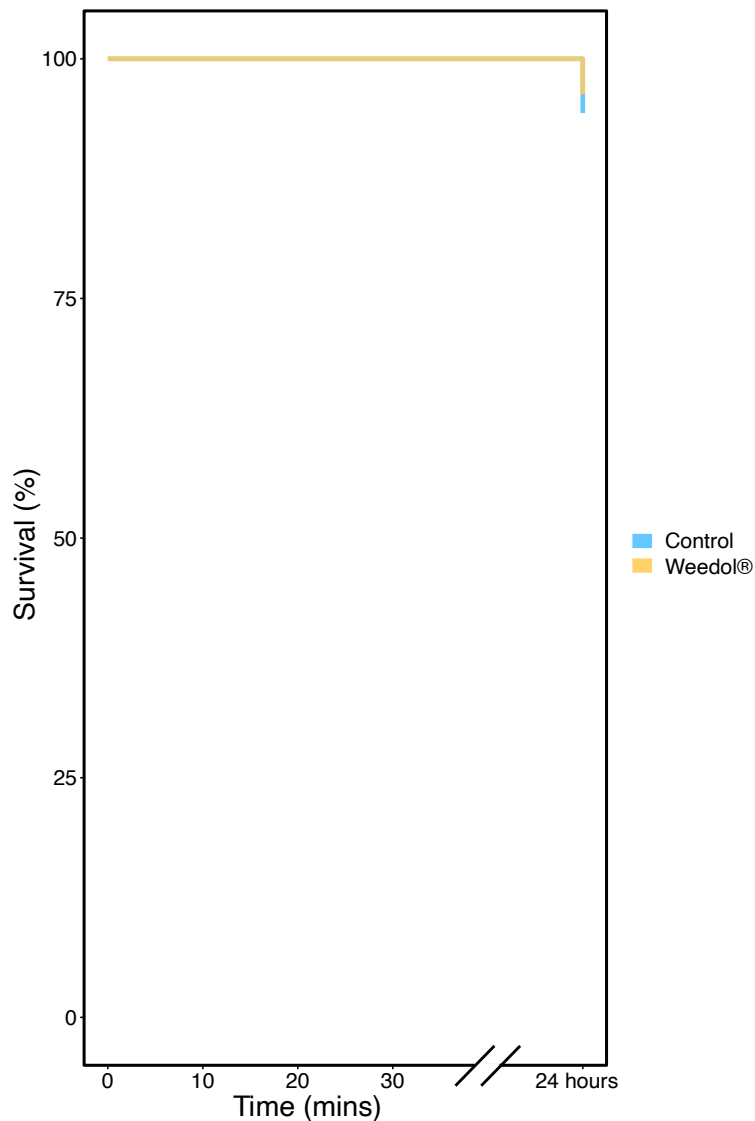
Figure 2. Experiment 4: Consumer product, and GBH alternative, Weedol does not cause mortality relative to the control.

Experiment 5: Does the roundup formulation without glyphosate cause mortality?

Roundup No Glyphosate produced significantly higher mortality than the control (Chi-squared test of Independence: χ^2 = 87.51, p < 0.001), with 96% mortality respectively compared to 0% mortality in the control treatment (Figure 3).
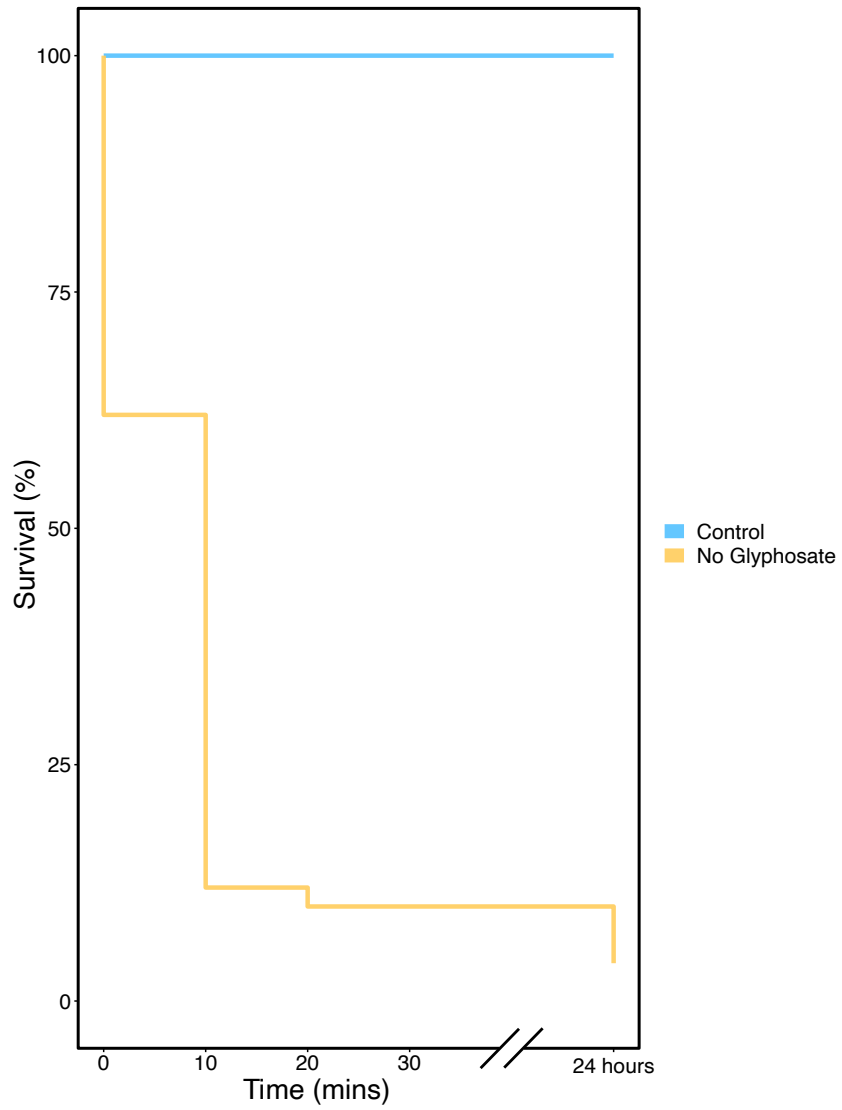
Figure 3. Experiment 5: The consumer product, and alternative to GBHs, Roundup No Glyphosate causes high mortality.

2.4 Discussion

My results are the first to show that contact exposure to either consumer or agricultural Roundup products at label recommended concentrations can cause high levels of mortality in bumble bees. The consumer product Roundup Ready-To-Use caused 94% mortality at the pre-mixed concentration, and still caused significant mortality at a quarter strength. The agricultural product Roundup ProActive also caused significant mortality, although over a longer time period. Interestingly, Roundup No Glyphosate caused 96% mortality while the generic GBH Weedol did not significantly increase mortality. Together, this demonstrates that the co-formulants in these Roundup products, not the active ingredient glyphosate, are driving mortality. I suggest that the mechanism driving this mortality may be surfactants in the formulations blocking the tracheal system of the bees, which is essential for gas exchange. Given the hazard demonstrated here with all tested Roundup products, and the extensive exposure of bees to such GBHs world-wide, GBHs may pose a high risk to bees, and thus may be an as yet unidentified driver of the bee declines that are occurring around the globe.
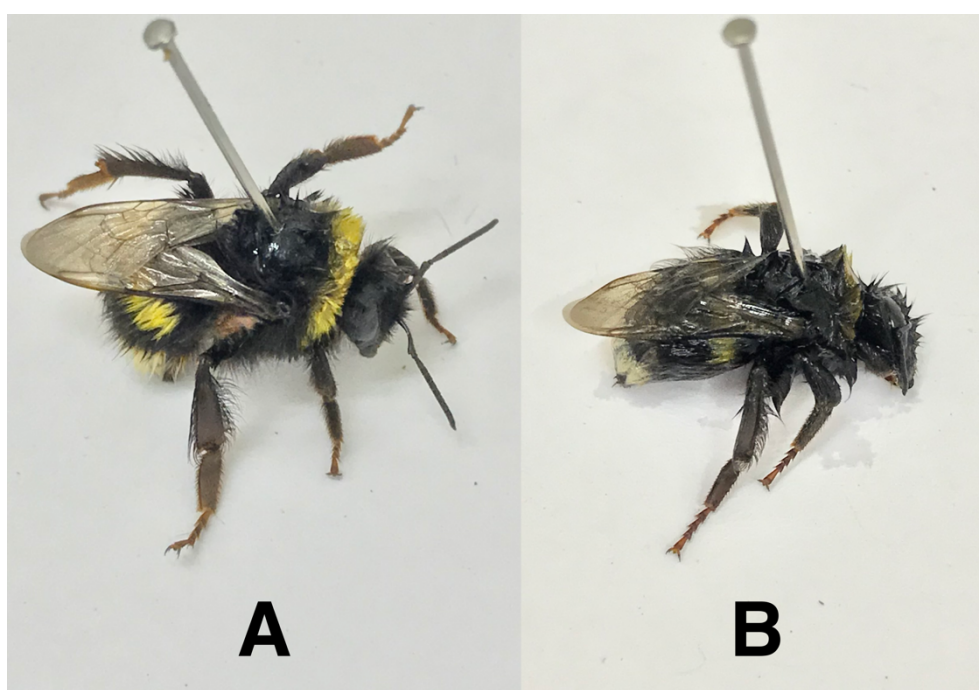
Figure 4. (A) Control and (B) Roundup Ready-To-Use full concentration bumble bees sprayed and photographed within five minutes. Matting of the hairs over the bee's whole body can be seen in B.

At a quarter strength, the consumer product Roundup Ready-To-Use still caused 78% mortality, demonstrating that the formulation is sufficiently toxic to cause mortality despite being 75% water. The dose dependency shown in my experiments confirms the products' toxicity and aids our understanding of how to use them safely. At a quarter strength the mortality seen is equivalent to the double strength Sunphosate 360 SL used in Abraham et al. (2018), suggesting that Roundup Ready-To-Use would also cause indirect contact mortality as even exposure to a severely reduced concentration caused high mortality with direct application. While consumer herbicides are unlikely to be applied directly to bees, they are likely to be applied to bee-attractive weeds which could drive mortality, with the Roundup Ready-To-Use label even advising "Treat established perennial weeds at the start of flowering to give best results" (Roundup Ready-To-Use Label, 2019). Consequently, label restrictions should explicitly caution against application to flowering plants. While the agricultural product Roundup ProActive requires a licence to spray, and has clear label instructions, the product label of Roundup Ready-To-Use has no guidance pertaining to bees. A first step should be to amend household product labels to reflect the hazard posed to bees. Finally, whether consumers need access to potent pesticides, especially when nearly half of consumers either do not follow or take no notice of label recommendations (Grey et al., 2005), requires revisiting by policymakers; consumer pesticide products should not be overlooked in policy initiatives to reduce pesticide use.

The consumer product Roundup Ready-To-Use caused more and faster mortality than the agricultural product Roundup ProActive, but the latter still caused 30% mortality over 24 hr. The Material Safety Data Sheet (MSDS) for Roundup ProActive lists Nitroryl (CAS no. 226563-63-9) and Alkylpolyglycoside (CAS no. 68515-73-1) as ingredients (Roundup ProActive MSDS, 2020), possibly acting as a surfactants (US Patent 20100113274A1, 2010, US Patent 5266690A, 1993), although I do not know what, or if, other surfactants are in the formulation. If these substances are driving the mortality in the Roundup ProActive treatment, this would be concerning as they are common in recently introduced products (Mesnage et al., 2019). I would suggest that the topical toxicity of these substances be assessed by regulatory agencies, to allow judgement to be made on their safety for inclusion in products bees are

exposed to. This Roundup ProActive driven mortality is in contrast to the guidance in the product's UK Environmental Information Sheet stating, "Roundup ProActive is of low toxicity to honeybees; there is no requirement to avoid application of the product when bees are foraging on flowering weeds in treated crops" (Roundup ProActive Environmental Information Sheet). This means that on-label guidance explicitly allows application directly onto bees, along with spraying onto flowering weeds, which are frequently visited by bees (Wood et al., 2019). This means that the exposure bees will face is incredibly high, with no attempt being made to mitigate their exposure. Furthermore, in the United States, Roundup products can be directly applied to genetically modified glyphosate resistant (Roundup Ready) crops, in order to knockdown weeds growing among the crop (Roundup Ready Plus Information Sheet). For Roundup Ready Soybeans this includes allowing application to the crop during flowering (Roundup Ready Plus Information Sheet). As soybean flowers are an attractive floral resource for bees (EFSA, 2013), this will lead to direct exposure of bees to Roundup products, which I have shown can drive significant mortality. Exposure through such herbicide tolerant crops is likely to be significantly higher than through flowering weeds, with herbicide tolerant soybeans covering 84.5 million hectares globally in 2014 (James, 2014 cited in Benbrook, 2016). Agricultural labels should preclude application to flowering plants or bees to reduce exposure.

Previous studies have examined the contact toxicity of surfactant adjuvants and Roundup products. Results vary for studies testing similar surfactant spray adjuvants, with Goodwin and McBrydie (2000) finding 100% mortality below label recommended concentrations, while Donovan and Elliott (2001) found no mortality even in their highest treatments. This is likely explained by the different methodologies, with the former using a Potter spray tower which is close to field realistic spray conditions and the latter using pipette application using OECD 214 (OECD, 1998). Following OECD 214, 1–2 µl of a solution is pipetted onto the backs of anaesthetised bees and then mortality assessed for 48 hr (OECD, 1998). This protocol is appropriate to assess the toxicity of active ingredients, particularly potent insecticides, but inappropriate for assessing the toxicity of more dilute surfactant solutions. Due to EU law protecting co-formulant composition (EC, 2009), I do not know if the components of the adjuvants used in either study are present in any of the formulations tested here.

This study diverges from the previously described results of Abraham et al. (2018) by using direct application onto bees, rather than indirect exposure (spraying flowers for the bees to then visit). I also used bumble bees, not honeybees or stingless bees, and still found high mortality suggesting the effects of GBH formulations on bees is widespread. The results presented here expand our understanding of how GBH formulations can cause mortality through contact exposure by isolating the co-formulants as driving the mortality and suggesting a mechanism behind the mortality. Recent work suggests similar mortality impacts in honey bees using a different Roundup formulation (Motta et al., 2020).

The only regulatory studies of contact mortality with GBHs have used honey bees and the protocol OECD 214 (see above, OECD, 1998). This protocol does not accurately assess contact toxicity for formulations like Roundup products, which can be sprayed directly onto bees. Regulatory testing should assess the contact toxicity of all formulations prior to approval/renewal using more field realistic methodologies than OECD 214, incorporating label recommended spraying apparatus and concentrations.

My results clearly show that Weedol does not produce higher mortality than the water control, and together with results from regulatory assessments (EFSA, 2015b), this confirms that the mortality seen in my experiments is not driven by glyphosate. This is supported by the findings of Motta et al. (2020), who found spraying honeybees with glyphosate did not cause mortality. Furthermore, Roundup No Glyphosate caused 96% mortality, which demonstrates that the co-formulants in Roundup products are toxic, and that the mortality I see does not derive from an interaction between co-formulants and glyphosate. This is encouraging, as it indicates the mortality could be eliminated entirely with a change to the co-formulants, without affecting the active ingredient content. The contrast between Weedol and Roundup products, which both use glyphosate as their active ingredient, demonstrates that co-formulants and formulations as well as active ingredients should be tested and regulated individually. This is especially true as active ingredient registrations have been greatly outstripped by novel formulation production, as pesticide manufacturers improve the efficiency of their products through changes to their co-formulants (Green and Beestman, 2007). That two of the three GBH's tested here produced significant mortality is concerning given that there are 281 other GBH's currently licenced for use in the UK.

The three Roundup substances tested produced significant mortality, which shows that the current regulatory testing for contact toxicity is inadequate to detect mortality effects. While the testing performed here was not agriculturally field realistic, it highlights that these products pose a legitimate hazard that requires risk assessment through field realistic testing. These results contradict the regulatory assessment that GBHs are entirely bee-safe and do not require mitigation measures. Finally, for each active ingredient only a single representative formulation is mandated for testing at an EU level (EFSA, 2013). The only contact toxicity testing on bees with whole formulations presented in the EFSA, 2015 renewal assessment report is on the original version of Roundup (MON 2139) in 1972 and the representative formulation Roundup Bioflow (MON 52276), which lacks the alkylamine ethoxylates common in other GBH's, instead using a quarternary ammonium compound (EFSA, 2015b).

While I have not explicitly tested the mechanism through which this mortality is generated, I suggest that the surfactants in the formulations are interfering with the action of the spiracles, or tracheal system more broadly. Insects conduct gas exchange through the tracheal system, with spiracles (surface holes on the thorax and abdomen) enabling airflow into the tracheal system, and the tracheae carrying air to tissues and cells where gas exchange occurs (Bailey, 1954). My observations show that the Roundup products are spreading the formulation over the surface of the bumble bees, possibly limiting gas exchange. This spread may have been exacerbated by the self-grooming behaviour observed in the Roundup treatments, and future research should formally assess this. This could be through a range of mechanisms, either by matting hairs down over the spiracles and physically smothering them, by blocking narrow sections in the respiratory system, or by coating the surface of the whole system in a non-permeable lining (see Figure 4 and Figure S3). Stevens (1993) noted that insect spiracles are similar in size to plant stomata, which GBHs are designed to penetrate, and suggested therefore that the surfactants allow water penetration into the tracheal system, causing drowning. It is unlikely that the immediate mortality seen most prominently in the standard strength Roundup Ready-To-Use treatment is caused by oral ingestion as even high doses of potent insecticides require several hours to produce mortality (Edward Straw, pers. obs.). I do not know if the mechanism driving the 38% immediate mortality in the

Roundup Ready-To-Use treatment is the same mechanism driving the further 56% mortality in the 30 min to 24-hr timeframe. Surfactant driven mortality in honeybees, which typically act as a sentinel for all beneficial insects, is unlikely to have been detected by beekeepers as the knockdown of bees is so fast they are unlikely to return to the hive before dying; this would mean the only symptom beekeepers would see is a reduced worker population (Goodwin and McBrydie, 2000).

Further work is required to elucidate the mechanism by which these products produce mortality. However, a significant difficulty in isolating this mechanism is that formulation composition is protected under EU law (EC, 2009), preventing researchers from knowing the identity and concentration of the surfactants involved, or what other co-formulant groups are present (Cox and Surgan, 2006). This severely impedes our ability to understand what mechanism(s) is/are at play and hinders academic testing of relevant ecological pollutants. If the MSDS that accompanies a product included a list of all the components, then each component could be tested individually to isolate the compounds (or interaction of compounds) causing the observed mortality. I suggest that the necessity to properly test pesticide effects on wildlife outweighs company rights to withhold proprietary information.

2.5 Acknowledgements

2.6 Data availability statement

Data available from the Dryad Digital Repository https://doi.org/ 10.5061/dryad.80gb5mkqn

Chapter 3

No evidence of effects or interaction between the widely used herbicide, glyphosate, and a common parasite in bumble bees

Abstract

Glyphosate is the world's most used pesticide and it is used without the mitigation measures that could reduce the exposure of pollinators to it. However, studies are starting to suggest negative impacts of this pesticide on bees, an essential group of pollinators. Accordingly, whether glyphosate, alone or alongside other stressors, is detrimental to bee health is a vital question. Bees are suffering declines across the globe, and pesticides, including glyphosate, have been suggested as being factors in these declines. Here I test, across a range of experimental paradigms, whether glyphosate impacts a wild bumble bee species, *B. terrestris*. In addition, I build upon existing work with honey bees testing glyphosate-parasite interactions by conducting fully crossed experiments with glyphosate and a common bumble bee trypanosome gut parasite, *Crithidia bombi*. I utilised regulatory acute toxicity testing protocols, modified to allow for exposure to multiple stressors. These protocols are expanded upon to test for effects on long term survival (20 days). Microcolony testing, using unmated workers, was employed to measure the impacts of either stressor on a proxy of reproductive success. This microcolony testing was conducted with both acute and chronic exposure to cover a range of exposure scenarios. I found no effects of acute or chronic exposure to glyphosate, over a range of timespans post-exposure, on mortality or a range of sublethal metrics. I also found no interaction between glyphosate and *C. bombi* in any metric, although there was conflicting evidence of increased parasite intensity after an acute exposure to glyphosate. In contrast to published literature, I found no direct impacts of this parasite on bee health. My testing focussed on mortality and worker reproduction, so impacts of either or both of these stressors on other sublethal metrics could still exist. My results expand the current knowledge on glyphosate by testing a previously untested species, *B. terrestris*, using acute exposure, and by incorporating a parasite never before tested alongside glyphosate. In conclusion my results find that glyphosate, as an active ingredient, is unlikely to be harmful to bumble bees either alone, or alongside *C. bombi*.

3.1 Introduction

Glyphosate is the world's most used pesticide (Duke and Powles, 2008, Benbrook, 2016). It is a herbicide used to suppress weeds in agricultural and amenity settings (Duke and Powles, 2008, Duke, 2018). Glyphosate helps reduce the need for tilling and mechanical weeding, which helps protect against soil erosion and boosts farmers yields and profits (Becki, Flower and Ashworth, 2020). Bees are exposed to glyphosate frequently in nature through spraying of weeds, contamination of water, and application onto glyphosate resistant flowering crops (Odemer et al., 2020, Chapter 2). Glyphosate-based herbicide products typically do not carry any mitigation measures aimed at reducing bees exposure to them. Research into herbicides, and glyphosate specifically, has grown considerably in recent years, with just 15 papers found in a systematic review of literature up to 2018 (Cullen et al., 2019), the first of which was published in 2011, while five were published in the final year searched. Several more publications have emerged since then (e.g., Motta et al., 2020, Motta et al., 2020, Odemer et al., 2020). To date most of these studies have used honey bees, *A. mellifera*, with only a few testing the impacts on other bee species (Ruiz-Toledo and Sánchez-Guillén, 2014, Abraham et al., 2018, Seide et al., 2018). Glyphosate has also undergone regulatory testing for governmental authorities worldwide to determine its effects on bees (EFSA, 2015, Duke 2018). Glyphosate is currently approved in all major territories (Duke, 2018), and where it is not approved (Mexico, for example) this is for human health reasons, not bee health reasons (Alcántara-de la Cruz et al., 2021).

In the EU and US pesticide regulation uses a tiered approach, with initial toxicity testing focussing solely on mortality (lower tier), and, if toxicity thresholds are met in the lower tier tests, then more complex experiments are conducted (higher tier)(EFSA, 2013, EPA, 2014). In the EU specifically this initial testing comprises two tests, acute oral exposure and acute contact exposure, both performed with the pure active ingredient and the representative formulation (EFSA, 2013). It has been suggested that this mortality-focussed approach is inadequate to properly assess the toxicity of a substance, and that there should be a move towards a fitness-based approach that also considers sublethal and reproductive effects at lower tiers (Straub, Strobl and Neumann, 2020). In the EU, glyphosate did not meet the toxicity thresholds required to trigger higher tier testing, so was approved for use with only

very minimal bee testing (EFSA, 2015). Alongside regulatory testing a number of academic experiments have found that oral exposure to glyphosate does not cause mortality in adult bees (Herbert et al., 2014, Goñalons and Farina, 2018, Motta, Raymann and Moran, 2019, Blot et al., 2019, Faita et al., 2020, Almasri et al., 2021), although there is mixed evidence, with Almasri et al. (2020) and Motta and Moran (2020) finding mortality at doses considerably lower than doses found to be non-lethal in other work.

While there is little strong evidence that glyphosate causes mortality in adult bees, it has been found to cause a range of sublethal effects in honey bees (reviewed in Farina et al., 2019). Chronic exposure to field realistic doses has been found to impair learning (Herbert et al., 2014, Balbuena et al., 2015) and increase the length of time taken to return to a colony (Balbuena et al., 2015). Chronic exposure has also been linked to larval mortality, reduced body mass, and a reduction in successful moulting (Vazquez et al., 2018), although the evidence here is mixed with conflicting results across years and colonies. At a molecular level, glyphosate has been found to impair antioxidant and acetylcholinesterase production (Boily et al., 2013, Helmer et al., 2015). While these results are limited in their scope, and derive only from honey bees, they represent clear evidence that the herbicide glyphosate can be biologically active in bees, and that examining the mortality effects of glyphosate in isolation are insufficient to understand its impacts on bees.

Motta, Raymann and Moran (2018) and Motta et al. (2020) found that in honey bees chronic exposure to glyphosate does not typically cause significant mortality, but that glyphosate can synergise with parasites to cause mortality. Exposure to the opportunistic parasite *Serratia marcescens* caused some mortality, around 20-30% more than the control, while glyphosate caused no more mortality than the control. However, when both stressors were applied simultaneously the mortality increased by almost 80% compared to the control. This result was replicated with a glyphosate-based formulation, Roundup ProMAX, in Motta et al. (2020), showing that the formulation also causes the synergism. Glyphosate induced a knockdown of protective gut bacteria that allowed the parasite to be more deadly, thus explaining how an otherwise non-lethal pesticide synergises to cause substantial mortality. This result highlights the importance of testing multiple stressors on bees, as even individually non-lethal pesticides can cause considerable synergism alongside common parasites.

In addition to this synergism, there is mixed evidence for the interaction between another bee parasite group, *Nosema spp.,* and glyphosate in honey bees. Both Blot et al. (2019) and Faita et al. (2020) found no effect of chronic exposure to glyphosate on mortality and a significant effect of *Nosema spp.*. However, only Faita et al. (2020) observed a significant interaction between the two stressors, with a 17% increase in mortality compared to the *Nosema spp.* alone. This difference may be attributable to the use of a formulation by Faita et al. (2020), rather than just the active ingredient used by Blot et al. (2020), or the mix of *Nosema apis* and *Nosema ceranae* used by Faita et al. (2020), rather than just *N. ceranae* used by Blot et al. (2020). In fact, the use of a formulation in Faita et al. (2020) does prevent the effect observed being attributable to glyphosate as the other ingredients may have driven the effect.

The studies described above focus on honey bees and common pathogens. Here I extend this approach to bumble bees (*Bombus* spp.) and their common trypanosome gut parasite *C. bombi*, which has been found at prevalence's of up to 82% in the wild (Gillespie, 2010), although this level of infection is not found in all studies, with large variation between years, sites and species (Shykoff and Schmid-Hempel, 1991, Korner and Schmid-Hempel, 2005, Rutrecht and Brown, 2008, Gillespie, 2010, Jones and Brown, 2014, Hicks et al., 2018). It is likely that *C. bombi* is less damaging of a parasite to *B. terrestris* than either *Nosema spp.* or *S. marcescens* are to honey bees, with no individual effect on mortality in otherwise unstressed bees (Brown, Loosli and Schmid-Hempel, 2000, Fauser-Misslin et al., 2014, Baron, Raine and Brown, 2014). At the colony level, uncontrolled or post-founding infections have no impact on growth or production of sexuals (Shykoff and Schmid-Hempel, 1991, Fauser-Misslin et al., 2014). In contrast, when experimentally infected bees are starved, worker mortality rates increase by 50% (Brown, Loosli and Schmid-Hempel, 2000), and when infections are experimentally controlled and occur before the stressful hibernation period, the parasite has dramatic negative impacts of up to 40% on host fitness (Brown, Schmid-Hempel and Schmid-Hempel, 2003, Yourth, Brown and Schmid-Hempel, 2008). Thus, this parasite is most likely to have impacts on bumble bees when combined with other stressors.

Finally, *C. bombi* infection is strongly related to the host gut microbiome, with specific bacterial groups like *Apibacter, Lactobacillus* Firm-5 and *Gilliamella* conferring increased resistance in *B. terrestris* (Koch and Schmid-Hempel, 2011, Mockler et al., 2018). Interestingly, a range of studies have found an effect of glyphosate on the honey bee microbiome (Dai et al., 2018, Motta, Raymann and Moran, 2018, Motta and Moran, 2020, Blot et al., 2019, Motta et al., 2020), consistently finding that it changes the microbiome composition. This suggests that, despite differences between *A. mellifera* and *B. terrestris* and their microbiomes, glyphosate might impact *C. bombi* indirectly through modifications of the gut microbiome.

In this study, I test whether glyphosate has direct impacts on worker mortality or reproduction, whether it interacts with *C. bombi* to impact these metrics of bee health, and whether infected bumble bees that are exposed to glyphosate, either acutely or chronically, will have increased *C. bombi* intensities.

3.2 Materials and methods

General

B. terrestris audax colonies were ordered from Agralan Ltd, Swindon, UK. Colonies were maintained on *ad libitum* sucrose and honey bee collected pollen from Thorne, Windsor, UK and Agralan Ltd, Swindon, UK respectively. On arrival, 10 workers per colony were removed and their faeces screened for micro-parasites (Rutrecht and Brown, 2008). No infections were detected, and all colonies were thus retained in the experiment. The number of bees or microcolonies included in each treatment group is presented in Tables S1-5. Pesticides were applied as pure active ingredient, glyphosate (Sigma-Aldrich) CAS-no: 1071-83-6 and dimethoate (Sigma-Aldrich) CAS-no: 60-51-5.

Modified ecotoxicological protocol OECD 247: general methods

OECD 247 (OECD, 2017) is an internationally agreed upon protocol for testing the toxicity effects of acute exposure to an oral solution in bumble bees (*Bombus spp*.). The protocol only allows for a single exposure phase, so modifications based on Siviter, Matthews and Brown (In Submission) were used to include an additional parasite exposure phase.

Worker bees were housed in Nicot cages a day in advance of parasite exposure, and then rank allocated 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. Syringes with 50% (w/w) sucrose were added to the Nicot cages for sustenance. The tip of the syringe was clipped off to allow access to the sucrose.

The subsequent day, following the OECD 247 protocol (OECD, 2017), I exposed bees in the parasite treatments to an inoculum containing 10,000 cells of *C. bombi*. The parasite inoculum was prepared by removing 40 worker bees from a *C. bombi* infected colony and inducing them to defecate. The faeces were then purified following Cole (1970). Purified *C. bombi* solution

was then diluted in distilled water and mixed 1:1 with 50% (w/w) sucrose to produce the test solution with 10,000 cells in 40µL of inoculum. A control solution of 1:1 distilled water and 50% (w/w) sucrose was also produced. Pilot work had demonstrated that this method leads to very high infection rates (>95%). At dissection any bees with a parasite intensity of 0 cells per µL were deemed to have a failed infection, and were excluded from the experiment. A further single worker with an intensity of 100 cells per µl, which is more likely to have resulted from contamination of the slide than an infection, was also excluded

Sucrose syringes were removed for 2-4 hours prior to exposure to the inoculum, starving the bees. Then 40µL of solution was pipetted into a fresh syringe and this was added to each cage. The bees were left to feed on the inoculum for a further four hours, at which point the syringe was removed and consumption visually verified. Bees that did not consume >80% of the solution were excluded from the experiment. Bees were returned to *ad libitum* sucrose with a syringe of 50% (w/w) sucrose and had a small ball of pollen added (~1g).

Bees were left for 7 days for the parasite infection to develop, at which point they entered the pesticide exposure phase. Here the above steps for parasite exposure were repeated, but with pesticide-laced treatment solutions replacing the parasite treatment solutions. The treatment doses used in all acute exposure experiment are listed in Table 1. below.

Table 1. Showing the doses of parasite or pesticide given to each worker in a given treatment.

Control	C. bombi only	Positive control
	10,000 cells per worker	4µg dimethoate per worker
Glyphosate only	Glyphosate and C. bombi	
200µg glyphosate per worker	10,000 cells per worker 200µg glyphosate per worker	

After exposure to the pesticide, mortality was recorded at four hours, 24 hours and 48 hours. Mortality was defined as a lack of response to physical agitation. Dead bees were discarded as their corpses degrade too quickly to be dissected.

Any bees who survived the full 48 hours were weighed, then transferred to a 2mL Eppendorf tube and frozen at -80C° for later dissection. Bees in the *C. bombi* or Glyphosate + *C. bombi* treatment groups were later dissected. Bees were removed from the freezer and placed on ice. The abdomen was cut off and was pinned to a black wax plate. The abdomen was cut open on one side, and pinned open. 100μL of 0.8% Ringers solution was pipetted directly onto the gut to prevent desiccation and another 100μL onto the wax to the side of the body. The honey crop was cut, and the gut transferred to the droplet on the wax. The ileum was isolated and cut at both ends, with care to remove any Malpighian tubules and tracheal tissue. The ileum was moved to a 1.5ml Eppendorf with 100μL of 0.8% Ringers solution and ground using a pestle for five seconds in a set pattern of movements. The ground gut was then vortexed for a single second and 10μL pipetted onto a Neubauer haemocytometer slide and the *C. bombi* concentration counted. All endpoints are presented as mean ± one standard deviation.

Experiment one: modified ecotoxicological protocol OECD 247: small scale

In this initial exploratory experiment only the *C. bombi* only and Glyphosate + *C. bombi* treatments were included. While bees were evenly allocated to treatments by colony of origin, colony origin was not tracked through the experiment and as such this is not accounted for in the statistics. Due to non-feeder events and deaths prior to the glyphosate exposure stage the final treatment groups may have had an uneven allocation of colony of origin, although this is unlikely due to the initial even distribution and low occurrence of such events. Sucrose consumption was not measured.

Experiment two: modified ecotoxicological protocol OECD 247: full scale

This experiment was a full-scale repetition of experiment one, with all treatment groups included. The Modified Ecotoxicological Protocol OECD 247 protocol described above was followed with a single major deviation, in that haemolymph samples were taken from all bees at the end of the experiment. The haemolymph was analysed as part of a different project.

This manipulation did not affect the mortality metric as mortality was recorded prior to the manipulation. Further, it would not affect the parasite intensity measure as there is no by treatment differences, and the timescale of the extraction is too short to influence *C. bombi* levels. This experiment was conducted in two batches with just a single day stagger between them.

Experiment three: modified ecotoxicological protocol OECD 247: long term

To test for longer term effects a version of the Modified Ecotoxicological Protocol OECD 247 protocol described above was performed, with the only deviation being that bees were maintained for 20 days post exposure rather than 48 hours. Mortality checks were made daily and pollen balls renewed weekly.

Experiment four: microcolony exposure- acute exposure

To test for effects on reproduction a microcolony experiment was performed. Bees were moved into microcolony boxes (clear acrylic boxes (6.7x12.7x4.9cm), with a plastic mesh grate bottom (6.7x7.3cm)) a day prior to parasite exposure. Initially 8 workers per microcolony box were added.

Pathogen inoculation and glyphosate exposure followed the Modified Ecotoxicological Protocol OECD 247, with bees being moved into Nicot cages for this exposure. Between treatments bumble bees were maintained in microcolony boxes.

Due to time constraints only bumble bees receiving a treatment were moved to Nicot cages and exposed. Bees in the control treatment were never moved to Nicot cages, bees in the *C. bombi* only treatment and the glyphosate only treatment were moved to Nicot cages just once, and those in the Glyphosate + *C. bombi* treatment were moved to Nicot cages twice. This had the potential to cause a by treatment effect as being moved to a Nicot cage is a potentially stressful experience. However, the day prior to the *C. bombi* exposure day all bees were manipulated as they were moved from their source colony to a microcolony box.

Similarly, on the glyphosate exposure day all bees not moved into Nicot cages were manipulated as they were moved into a fresh microcolony box. As such it is only the marginal additional level of stress from the time in the Nicot cages that could produce a by treatment effect. Bees in the Nicot cages were also kept in their microcolony box adjacent to nest-mates to reduce stress.

Non-feeders were excluded from the experiment at each of the exposure steps, which alongside mortality led to slightly lower worker numbers in the micro-colonies (Glyphosate: 6.9 ± 1.2, *C. bombi*: 6.7 ± 1.1, Glyphosate + *C. bombi*: 6.4 ± 1.1 (SD)), versus the control (7.8 ± 0.4 (SD)). Workers who died ($n = 4$) or escaped ($n = 5$) during the experiment were recorded, but not replaced. This was accounted for in the analysis, however, with reproductive output expressed per worker present at end of experiment. Given that worker reproduction is highly dependent on the laying individual (Blacquière et al., 2012), this should robustly account for differing worker numbers.

After glyphosate exposure bumble bees were moved to a fresh microcolony box to reset their reproductive efforts, and then provided *ad libitum* sucrose and pollen for 14 days. 14 days is shorter than the time required for a bee to develop from egg to eclosion, so all adults at the end of the experiment were those initially added.

On day 14, adult bumble bees were counted and frozen for later dissection to quantify pathogen intensity, the total number of eggs and larvae number were counted, and total larval weight measured. Larval weight was chosen as the best measurement of reproductive success as it reflects output better than larval number. By using weight, the greater investment required to rear a L4 larvae, versus a L1 larvae, is reflected, whereas number of larvae would not reflect this investment disparity. As such larval weight per worker was chosen as the quantitative metric used for analysis.

Experiment five: microcolony exposure- chronic exposure

This protocol is derived from the OECD 245 honey bee chronic oral toxicity test, with modification to account for the different test species.

Workers used in the experiment were age controlled, to achieve this 8 workers were taken from a source colony, tagged and moved into a microcolony box. Pupae and enclosed larvae from the same colony were added, with the 8 tagged workers acting as nurses for them. Newly emerged workers were identified by their lack of a tag, and 10 days after the start of emergence they were moved to Nicot cages for parasite inoculation. This inoculation followed the Modified Ecotoxicological Protocol OECD 247, with parasite treatment groups detailed in Table 1. After excluding non-feeders, bees were then allocated to microcolonies in groups of six based on treatment, with all workers within a microcolony originating from the same source colony. Because the allocation to microcolonies occurred after non-feeders were excluded there is no by treatment exclusion effect. By selecting newly emerged workers over a 10-day period, workers were age controlled to be within 10 days of one another. Workers were left on *ad libitum* sucrose and pollen for a week while the parasite developed. After seven days the workers were moved to a fresh microcolony to reset their reproductive effort.

Data from Thompson et al. (2014) were used to inform the chronic exposure scenario. Thompson et al. (2014) measured glyphosate concentration in returning nectar and pollen from honey bees foraging on *Phacelia tanacetifolia* sprayed with a glyphosate-based herbicide formulation (MON 52276) according to full label restrictions. Using WebPlotDigitiser (Rohatgi, 2020), the values from Thompson et al. (2014)'s graphs were extracted. An inverse relationship model was used to model the declining residue concentration: $Glyphosate\ Concentration = Intercept + \frac{Constant}{Time}$. As the data from Thompson et al. (2014) has missing days and no data after 7 days, missing data were either interpolated or extrapolated. These modelled concentrations of returning nectar and pollen were then used to generate an exposure regime. Sucrose was fed to the bees *ad libitum* and was spiked with pesticides in concentrations shown in Figure 1. In all treatments 50% w/w sucrose was changed daily, and the previous day's consumption was recorded. The glyphosate concentration provided decreased over time with the modelled values, see Figure 8. Degradation of the glyphosate will have occurred in the sucrose; however, this is largely insignificant given glyphosate's long half-life of 47-267 days (as measured in seawater) (Mercurio et al., 2014). 5g of pollen was provided and in glyphosate treatments this was

spiked with an average concentration of glyphosate over the 10 days exposure (110mg/kg). This was done as changing pollen daily was not feasible, and 5g was used as this amount was rarely wholly consumed by a group of workers in 14 days. In the positive control, the dimethoate concentration was maintained at a constant 1mg/L, and pollen was not spiked in this treatment. Following OECD 245 for honey bees (OECD, 2017a), exposure ended on day 10, and all bumble bees were fed unspiked sucrose for another four days. On day 14 bumble bees were frozen and reproductive output measured, as described above. Mortality was recorded daily.

As the dataset used to calculate my chronic exposure regime was from a semi-field exposure studied conducted in honey bees (Thompson et al., 2014), the use of these data for *B. terrestris* may be unrealistic. There are no comparable data from honey bees and bumble bees to be able to see if the same spraying regime leads to similar returning nectar concentrations. However, as the only available dataset it is the best choice to inform the chronic exposure regime.
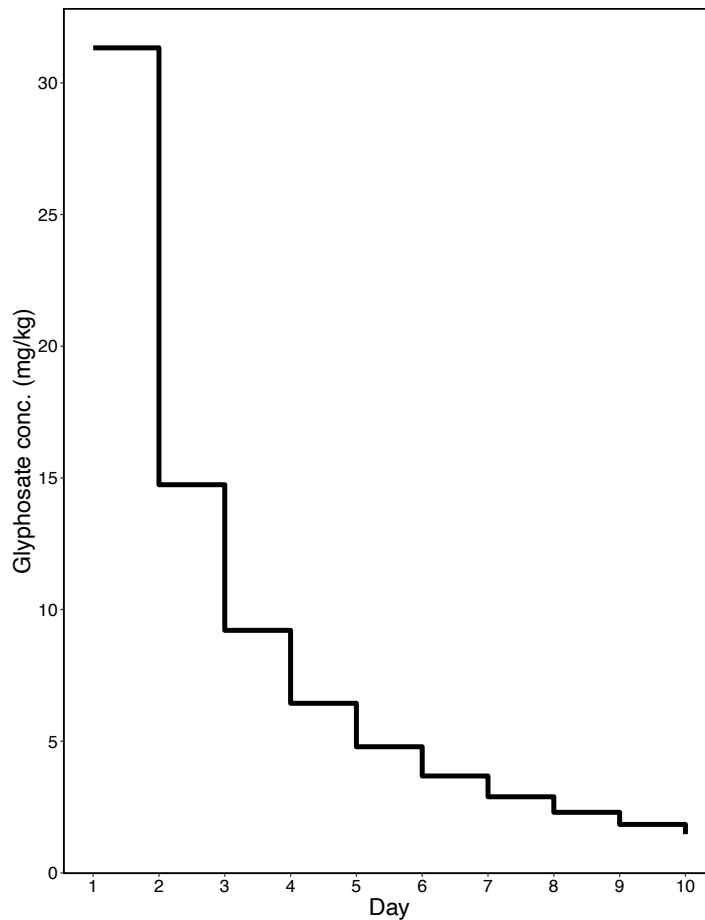
Figure 1. Showing a stepwise chronic exposure profile generated from Thompson et al. (2014). With glyphosate concentration (in mg/kg) presented on the Y axis and time in days on the X axis.

Statistical testing

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 and 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. 'lme4' version 1.1-23 was used for Linear Mixed Effects models (Bates et al., 2020) and

'coxme' version 2.2-16 was used for Mixed Effects Cox Proportional Hazards models (Therneau, 2020). Confidence intervals not crossing zero indicate a significant effect, so a confidence interval of -1.00 to 1.00 would not be significant, but a confidence interval of -2.00 to -1.00 would be. Model assumptions were checked graphically and using statistical testing, including using 'e1071' version 1.7-4 (Mayer et al., 2021). Model parameters, AIC weights and final models are presented in Tables S6-11.

Experiment one: modified ecotoxicological protocol OECD 247: small scale

Parasite intensity: Data were found to be non-normal using a Shapiro-Wilks test, so a Kruskal Wallis test was used with the model (Parasite Intensity ~ Treatment).

Mortality: Due to an absence of mortality in the experiment no statistical testing was conducted.

Experiment two: modified ecotoxicological protocol OECD 247: full scale

Parasite intensity: Data were found to be non-normal using a Shapiro-Wilks test, so a Kruskal Wallis test was used with the model (Parasite Intensity ~ Treatment).

Mortality: Due to an absence of mortality in the experiment, except in the positive control where all bees died, no statistical testing was conducted.

Experiment three: modified ecotoxicological protocol OECD 247: long term

Mortality: A Cox Proportional Hazards model was used to analyse the mortality data. Due to the near complete mortality in the positive control treatment, this treatment was excluded from the mortality analysis as it violates the proportionality of hazards assumption. The full model used was (Mortality ~ Treatment + Body Weight + (1|Colony)). Proportionality of hazards was checked graphically.

Experiments four and five: microcolony exposure- acute exposure and chronic exposure

Reproduction: Larval weight, adjusted to the number of workers present at the end of the experiment, was found to be non-normal using a Shapiro-Wilks test. It was accordingly square root transformed, and confirmed to be normal using a further Shapiro-Wilks test. The full model used was (Larval Weight per Worker ~ Treatment + Body Weight of Initial Workers + Number of Workers Alive at the End of the Experiment + (1|Colony)).

Parasite intensity: A Linear Mixed Effect model was used to analyse the parasite intensity data. The full model used was (Parasite Intensity ~ Treatment + (1|Micro Colony ID) + (1|Colony)).

Acute exposure only

Mortality: Mortality was too low to allow a Linear model, Linear Mixed Effects models, or Chi-Square test. Accordingly, a Fishers Exact test was used with the model (Survival ~ Treatment).

Chronic exposure only

Sucrose/Glyphosate consumption: A Linear Mixed Effect model was used to analyse the Sucrose Consumption data. The full model used was (Sucrose Consumption ~ Treatment * Time + Weight of Bees at Start of Exposure + (1|Micro Colony ID) + (1|Colony)).

Mortality: Mortality was too low to allow a Linear model, Linear Mixed Effects models, or Chi-Square test. Accordingly, a Fishers Exact test was used with the model (Survival ~ Treatment).

3.3 Results

Experiment one: modified ecotoxicological protocol OECD 247: small scale

Parasite intensity

The Glyphosate + *C. bombi* treatment had a significantly higher parasite intensity than the *C. bombi* only treatment (Kruskal-Wallis $X^2(1)= 7.885$, $p = 0.005$). Glyphosate + *C. bombi* treated bees (n = 21) had an average parasite intensity of 14,519 ± 10,462 (SD) cells per μL compared to 6,946 ± 5,682 cells per μL in the *C. bombi* only treatment (n = 23) (Figure 2).

Figure 2. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.

Mortality

No mortality was observed in either the *C. bombi*, or the Glyphosate + *C. bombi* treatment.

Experiment two: modified ecotoxicological protocol OECD 247: full scale

Parasite intensity

In contrast to the first experiment, Glyphosate + *C. bombi* did not have a significantly different parasite intensity to the *C. bombi* only treatment (Kruskal-Wallis $X^2(1)= 0.428$, $p = 0.513$). Glyphosate + *C. bombi* treated bees ($n = 34$) had an average parasite intensity of 24,124 ± 14,664 cells per μL, compared to the 20,756 ± 14,473 cells per μL in the *C. bombi* only treatment ($n = 32$) (see Figure 3). Neither body weight or batch had a significant effect on parasite intensity (Linear Mixed Effect model: parameter estimate (PE) = 66,940.7, 95% CI [-19,878.3 to 152,664.5] and (PE) = 897.3, 95% CI [-6,843.0 to 8,512.8] respectively).
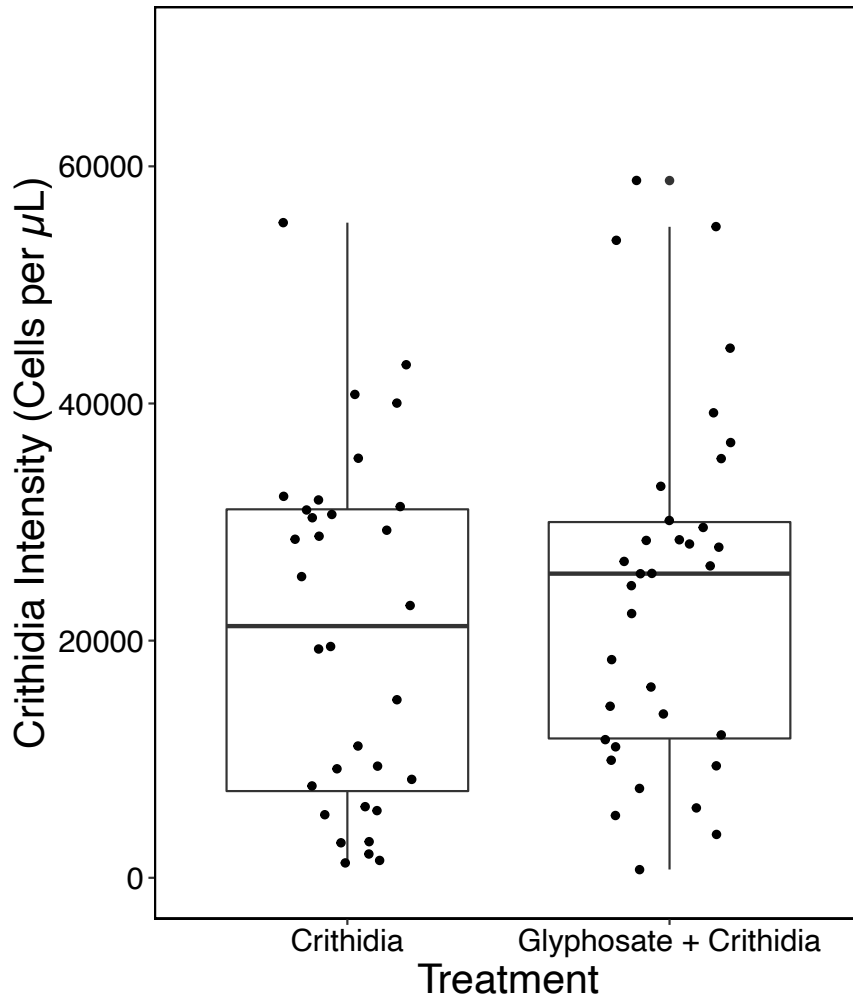
Figure 3. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.

Mortality

No mortality was observed in any treatment bar the positive control, where all bees died within 24 hours.

Experiment three: modified ecotoxicological protocol OECD 247: long term

Mortality

All bees in the positive control treatment, bar one, died within two days, while all other treatments experienced mortality over the 20-day period.

C. bombi only, Glyphosate only, and Glyphosate + *C. bombi* did not have significantly different mortality compared to the negative control (Cox proportional hazards mixed effects model: parameter estimate (PE) = 0.728, 95% CI [-0.81 to 0.96], (PE) = 1.27, 95% CI [-0.92 to
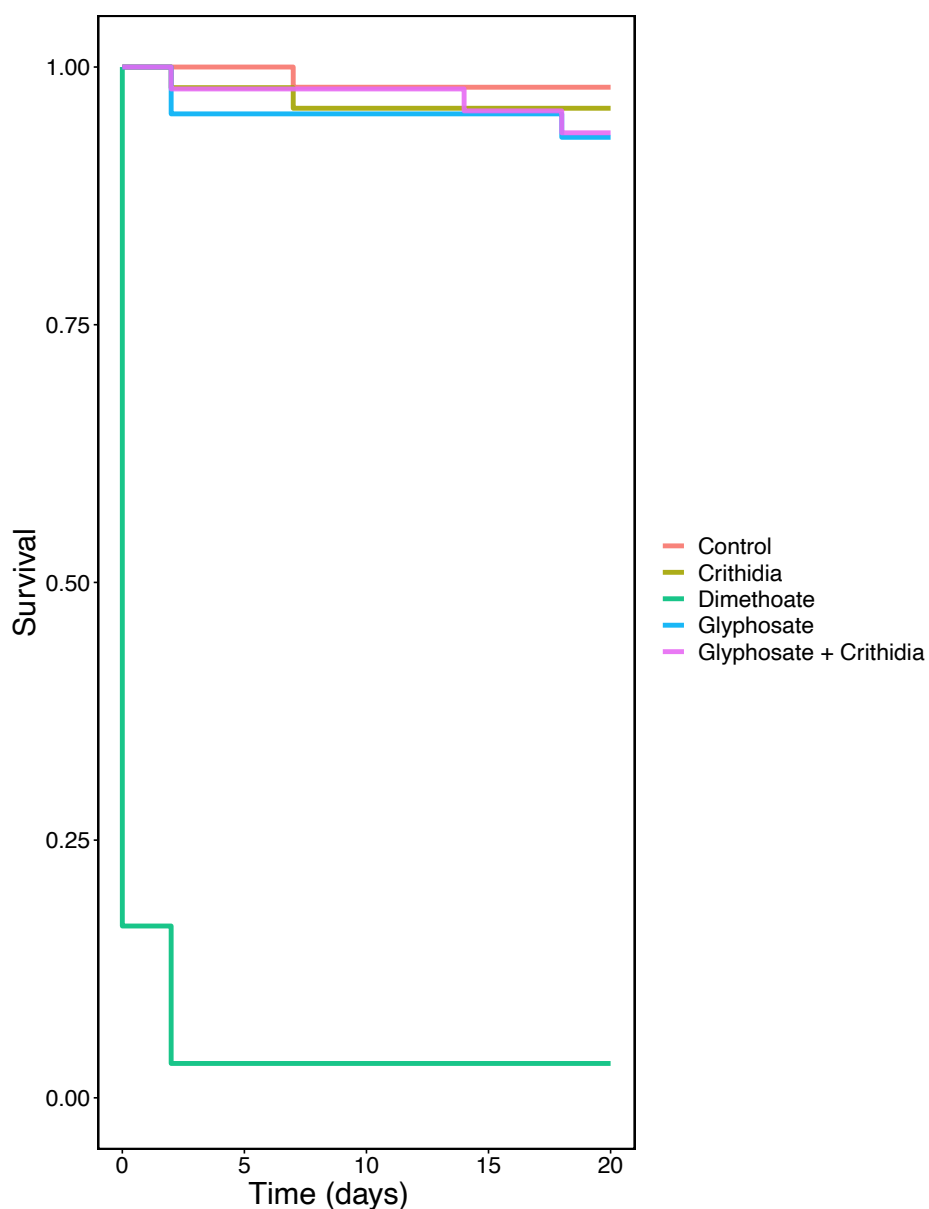
1.18], and PE = 1.19, 95% CI [-0.89 to 1.14], respectively). *C. bombi* only, Glyphosate only, and Glyphosate + *C. bombi* had 4%, 7% and 6% mortality respectively, while the control had 2% mortality (see Figure 4), a real terms difference of one to two bees.

Figure 4. A Kaplan-Meier plot showing the survival over time by treatment.

Experiments four: microcolony exposure- acute exposure

Reproduction

There was no significant difference in reproductive output between treatments. While the mean larval weight per worker (±SD and number of microcolonies) varied between treatments (0.510 ± 0.224g, *n* = 8 in the control, 0.458 ± 0.349g, *n* = 11 in the *C. bombi* only treatment, 0.405 ± 0.141g, *n* = 9 in the Glyphosate only treatment and 0.339 ± 0.224g, *n* = 10 in the Glyphosate + *C. bombi* treatment (see Figure 5)), a null model, which contained the response variable, the co-variate of initial worker weight and the random colony variable, but not the treatment variable, was the best supported model with ≥95% AIC support. This model found a significant effect of Original Weight of Nurse Workers on reproductive output (Linear mixed effects model (LMER) =0.26, 95% CI [0.14 to 0.37]), with heavier workers being more successful at rearing offspring.
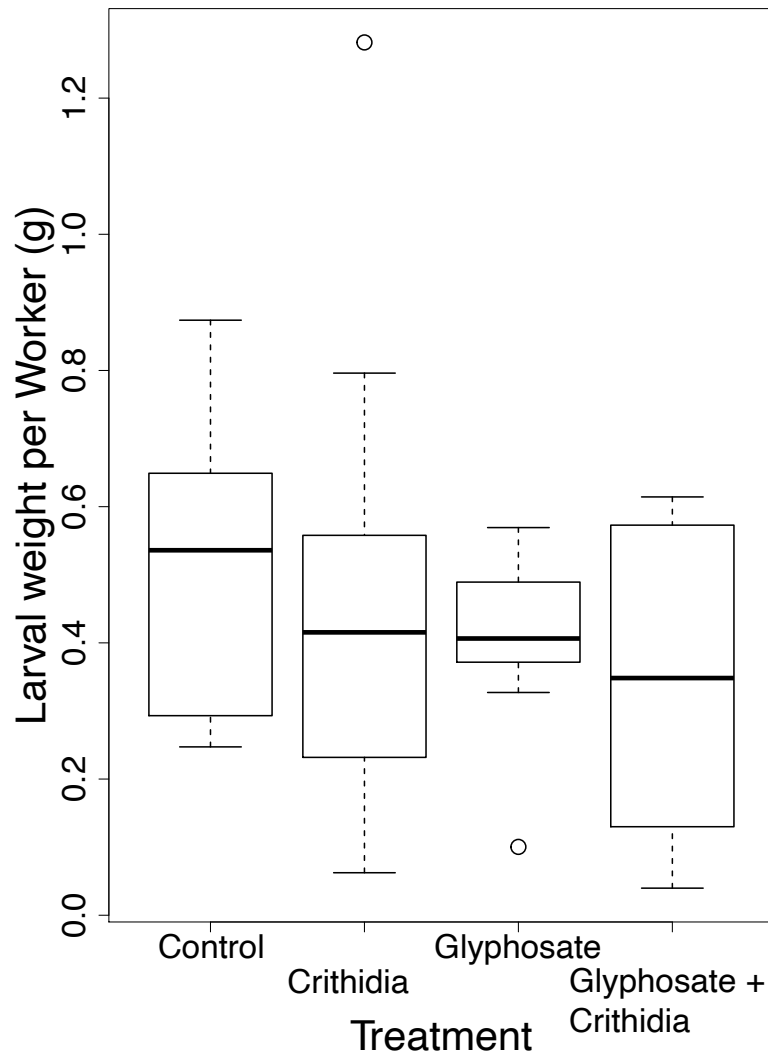
Figure 5. A boxplot showing the larval weight per microcolony standardised by the number of workers, presented by treatment.

Parasite intensity

Glyphosate + *C. bombi* exposed bees did not have a significantly different parasite intensity to the *C. bombi* only treatment (Linear Mixed Effect model: parameter estimate (PE) = -314.6, 95% CI [-2,865.81 to 2,236.55]). Glyphosate + *C. bombi* treated bees (*n* = 64) had an average parasite intensity of 18,362 ± 7,704 cells per μL, compared to the 18,635 ± 5,884 cells per μL in the *C. bombi* only treatment (*n* = 74) (see Figure 6).
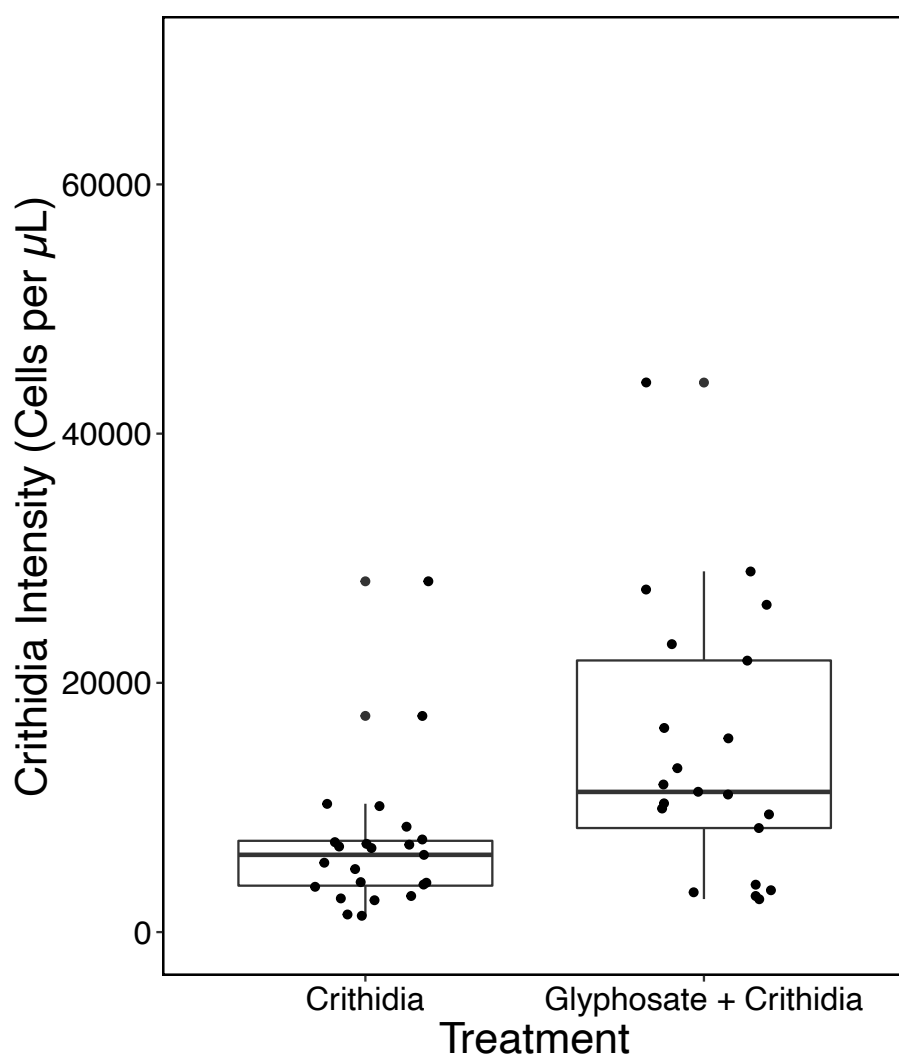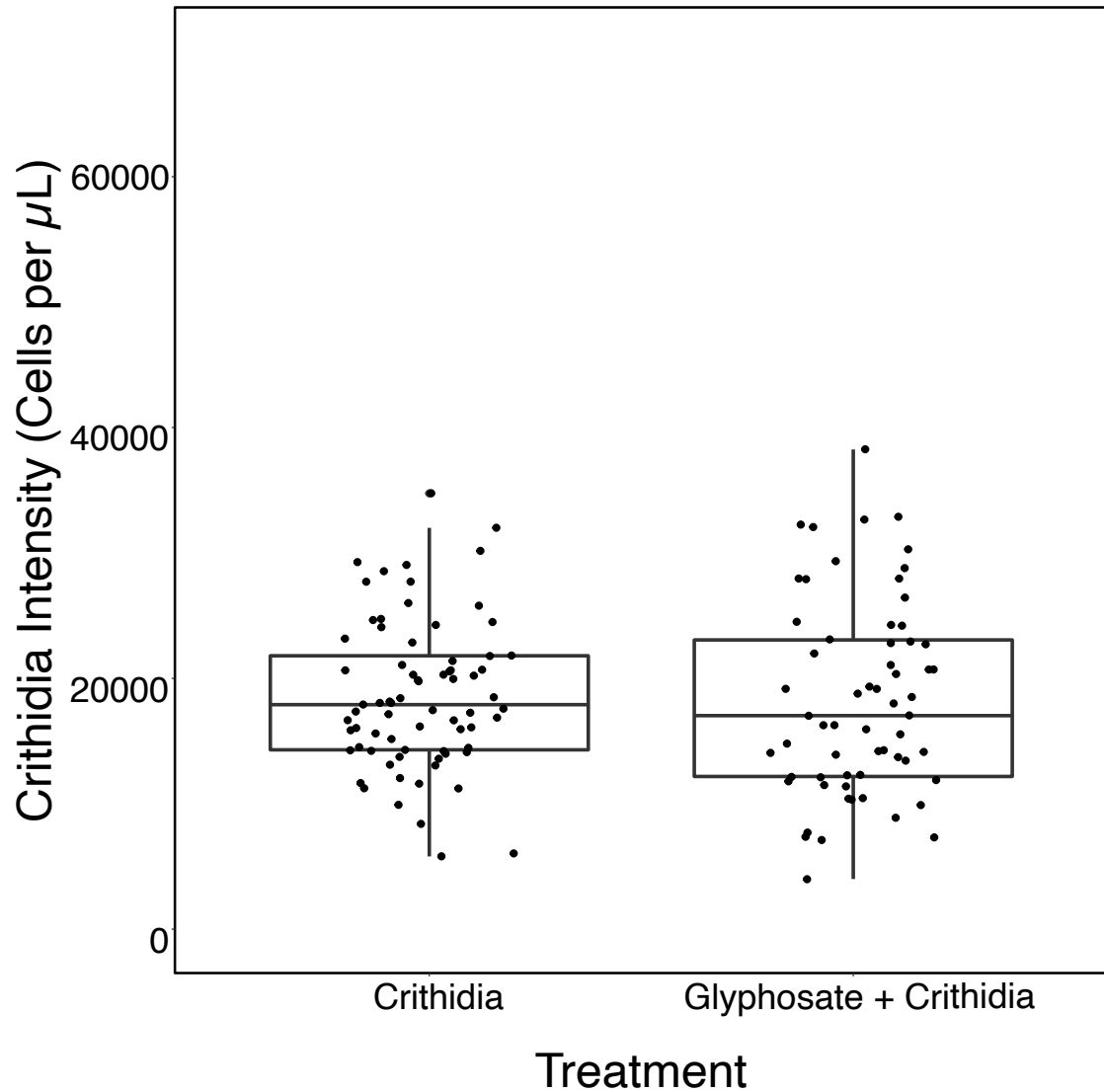
Figure 6. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.

Mortality

There was no significant difference in mortality by treatment (Fisher Exact test (two sided) *p*= 0.679). *C. bombi* only, Glyphosate only and Glyphosate + *C. bombi* had 1%, 0% and 3% mortality respectively, while the control had 2% mortality, a real terms difference of one bee.

Experiments five: microcolony exposure- chronic exposure

Reproduction

There was no significant difference in reproductive output between treatments. The mean larval weight per worker (±SD and number of microcolonies) varied between treatments, with 0.106 ± 0.077g, n = 8 in the control, 0.053 ± 0.054g, n = 8 in the *C. bombi* only treatment, 0.143 ± 0.139g, n = 8 in the Glyphosate only treatment and 0.124 ± 0.103g, n = 8 in the Glyphosate + *C. bombi* treatment (see Figure 7). The model average with a cumulative ≥95% AIC support did not include the treatment term. The two models included were both null models, one with the co-variate of initial worker weight and random colony variable, and the second with just the random colony variable. This model found no significant effect of Original Weight of Nurse Workers on reproductive output (Linear mixed effects model (LMER) =0.20, 95% CI [-0.15 to 0.27]).
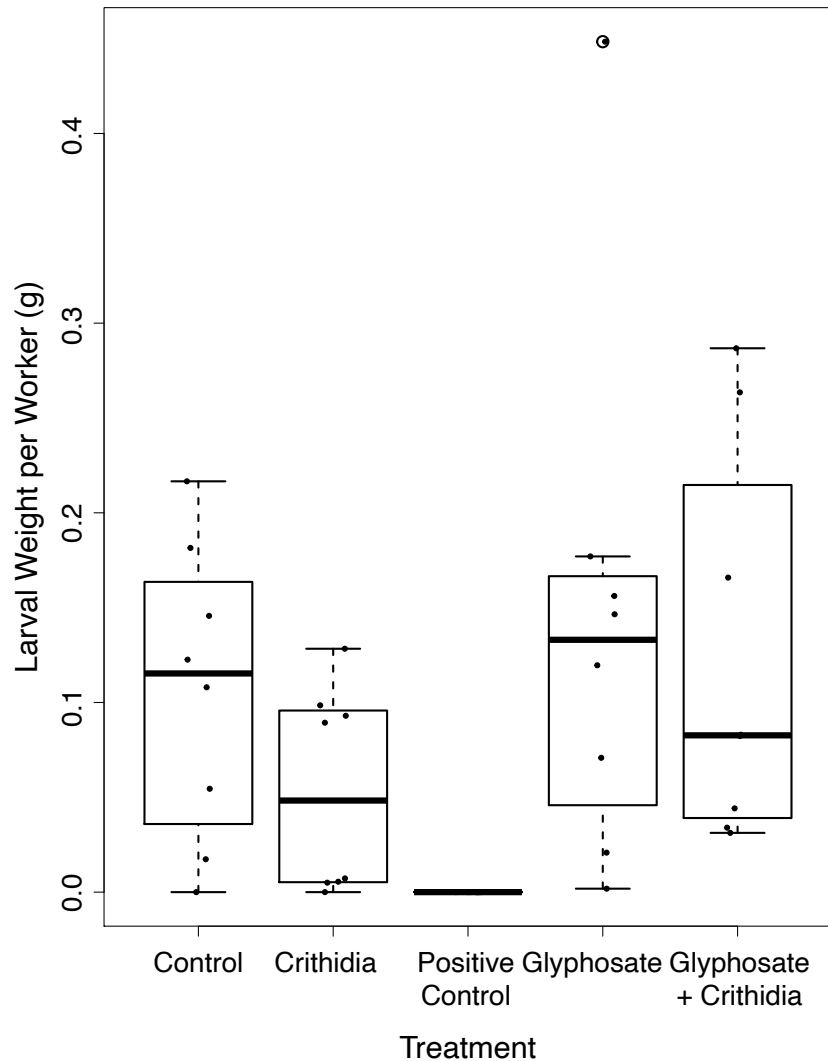
Figure 7. A boxplot showing the larval weight per microcolony standardised by the number of workers, presented by treatment with overlaid jittered data points. All bees in the positive control died, accordingly they produced no larvae.

Sucrose consumption

Over the 10-day exposure period the average consumption of sucrose per worker was 5.890 ± 0.676mL in the control, 5.880 ± 0.865mL in the *C. bombi* only treatment, 5.947 ± 0.875mL in the Glyphosate only treatment, and 6.271 ± 0.746mL in the Glyphosate + *C. bombi* treatment.

The model average that contained models with a cumulative ≥95% AIC support did not include the Treatment term. As such Treatment had no effect on sucrose consumption. The weight of the bees at the start of exposure also did not affect sucrose consumption, (Linear Mixed Effect model: parameter estimate (PE) = 0.062, 95% CI [-0.052 to 0.069]).

Over the 10-day exposure period the average consumption of glyphosate per worker was 38.7 ± 5.4μg in the Glyphosate only treatment, and 41.4 ± 4.3μg in the Glyphosate + *C. bombi* treatment. The majority of this consumption was in the initial few days, as the concentration decreased markedly over time. Figure 8 shows the sharp decline in glyphosate consumption over time.
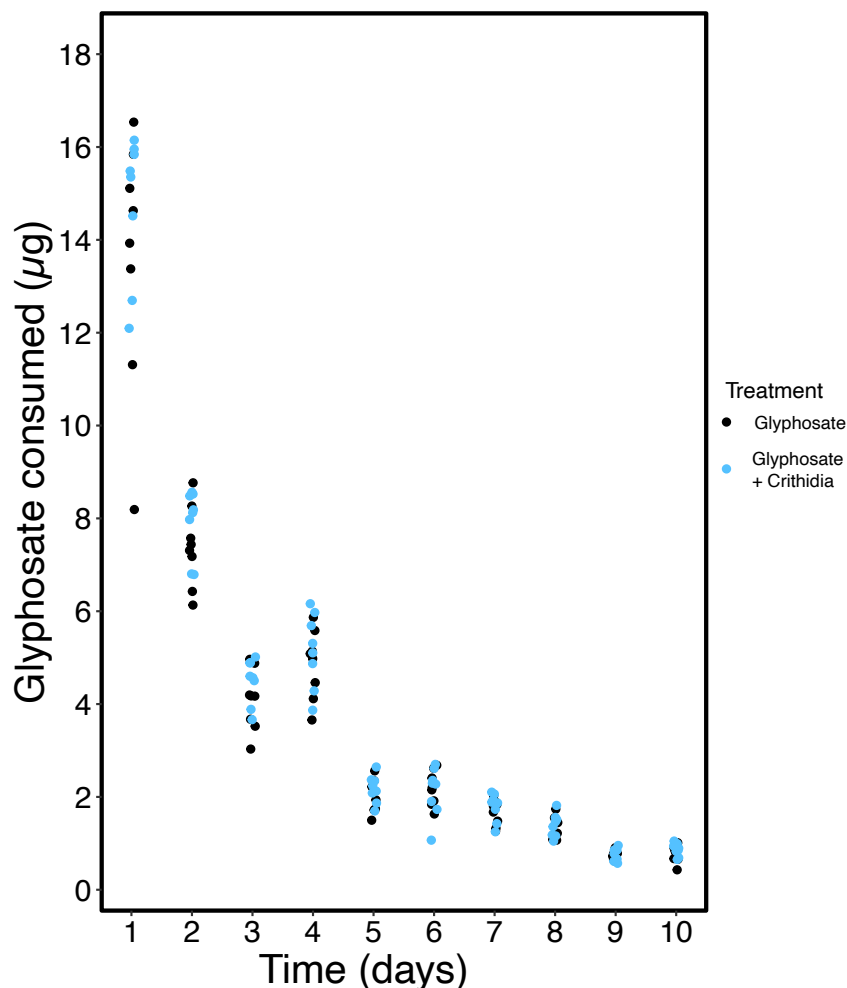
Figure 8. A scatter plot showing the daily consumption of the active ingredient glyphosate over time, presented by treatment. Data points have been horizontally jittered for clarity. Bees in the Control and *C. bombi* only treatments had glyphosate exposures of zero, and have been omitted from the graph.

Parasite intensity

Glyphosate + *C. bombi* did not have a significantly different parasite intensity to the *C. bombi* only treatment (Linear Mixed Effect model: parameter estimate (PE) = 1649.0, 95% CI [-3251.24 to 6529.72]). Glyphosate + *C. bombi* treated bees (*n* = 42) had an average parasite intensity of 20,562 ± 7065 cells per µL compared to 18,759 ± 9403 cells per µL for the *C. bombi* only treatment (*n* = 44) (see Figure 9).
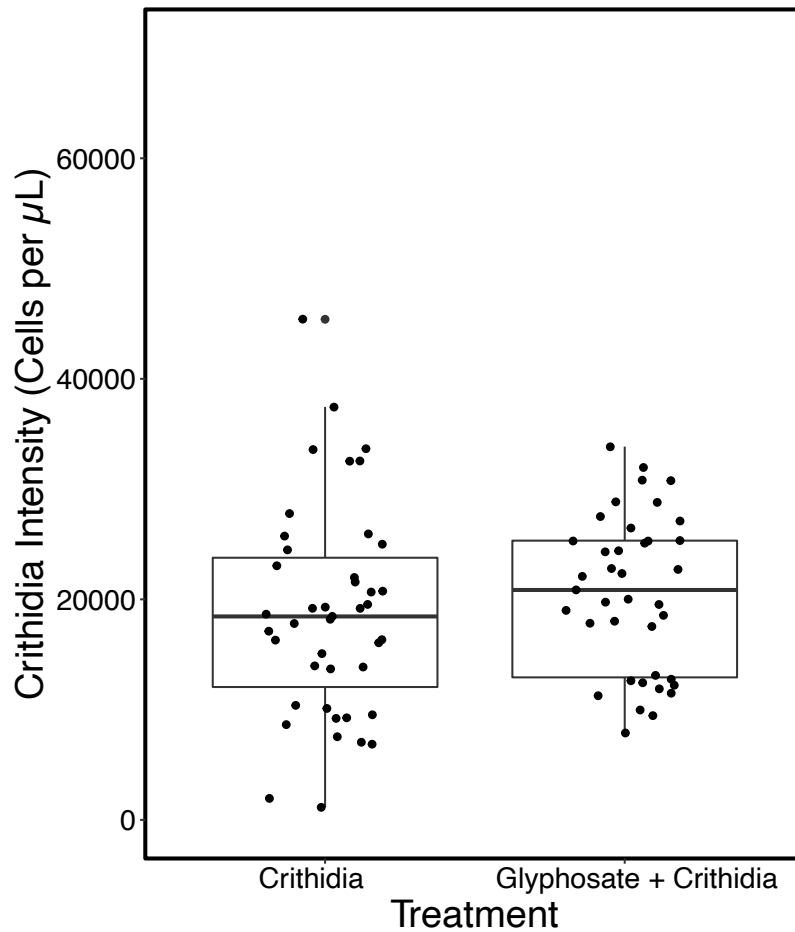
Figure 9. A boxplot with overlaid jittered data points showing the parasite intensity by treatment.

Mortality

All bees in the positive control died. There was no significant difference in mortality between the remaining treatments (Fisher Exact test (two sided) p= 0.903). *C. bombi* only, Glyphosate only and Glyphosate + *C. bombi* had 0%, 2% and 2% mortality respectively, while the control had 4% mortality, a real terms difference of one to two bees.

3.4 Discussion

Through a series of experiments, I show no robust evidence for the effects of either glyphosate, *C. bombi*, or their combination, on mortality or a range of sublethal effects in bumble bees. Acute exposure to either stressor or their combination over a range of timescales representing the majority of a bee's lifespan did not cause mortality, nor did chronic exposure over a 10-day period. While an initial experiment found an acute dose of 200μg of glyphosate caused a considerable increase in the intensity of the parasite *C. bombi*, this effect was not seen in any of the follow up experiments. I found no evidence to suggest glyphosate affects reproduction among workers, and, contrary to predictions from previous studies (Shykoff and Schmid-Hempel, 1991, Brown, Loosli and Schmid-Hempel, 2000), no evidence that *C. bombi* does either.

Mortality

The most basic metric of bee health is mortality. A dead bee can contribute nothing further to its fitness, as it is unable to contribute to the provisioning of brood or production of sexuals. Most regulatory systems use mortality as the initial metric to assess toxicity (EFSA, 2012, 2013, EPA, 2014). In the EU, lower tier testing considers just acute contact and oral toxicity in honey bees and bumble bees workers (including OECD 247 studies), and then additionally in just honey bees; chronic oral worker toxicity and acute larvae oral toxicity. Although the addition of bumble bee data has not yet been fully implemented (EFSA, 2015). In the case of glyphosate, the LD_{50}s derived were found to be above the threshold value of 200μg active ingredient per bee (or equivalent highest possible tested dose)(EFSA, 2015), although this was only done with honey bees, as bumble bee data are not due to be submitted until the 2025 EU renewal of glyphosate. As such, glyphosate was not entered into higher tier testing, meaning that from a regulatory testing standpoint only short-term mortality was considered (EFSA, 2015). This was used to justify the current lack of any mitigation measures for exposure of bees to glyphosate or glyphosate-based herbicides.

The data presented here supports the regulatory conclusion that glyphosate does not cause mortality in the short term (EFSA, 2015). These data also expand the species upon which I have evidence of the mortality effects of glyphosate, with the addition of a bumble bee to the previously studied honey bee. My results show no mortality over a range of exposures and time periods from 2-20 days, going well beyond the two-day test regulators will conduct on bumble bees using OECD 247. Additionally, there were no mortality effects from the interaction between glyphosate, with either acute or chronic exposure, and *C. bombi* in worker bumble bees. It is important to clarify that my experiments used glyphosate as an active ingredient, not as a formulation.

Several experiments have tested glyphosate-based herbicide formulations, as opposed to the active ingredient glyphosate, on honey bees (Abraham et al., 2018, Faita et al., 2020, Odemer et al., 2020 and Motta et al., 2020) and non-*Apis* bees (Ruiz-Toledo and Sánchez-Guillén, 2014, Abraham et al., 2018, Seide et al., 2018, Chapter 2). However, co-formulants in glyphosate-based herbicides can have significant effects on toxicity (Motta et al., 2020, Chapter 2), making these studies difficult to interpret from the perspective of the active ingredient. Consequently, the following discussion of existing academic literature will be limited to experiments that solely test the active ingredient glyphosate.

In line with my results, the academic literature has largely found no evidence for effects of glyphosate on adult honey bee worker survival. Over a range of concentrations up to 210mg/kg, and across a range of timelines, no significant mortality has been observed in multiple studies (Herbert et al., 2014, Goñalons and Farina, 2018, Blot et al., 2019, Motta, Raymann and Moran, 2019). Yet, despite these results, Almasri et al. (2020) found that just 0.00083mg/kg, a concentration approximately two million times lower than 210mg/kg, significantly reduced survival over 20 days. It is not clear from Almasri et al.'s (2020) methods if the solvent dimethyl sulfoxide was present in the control treatment, which could potentially have confounded the results. Interestingly, a follow up study by the same authors, Almasri et al. (2021), failed to replicate this result using the same concentration. Further, Motta and Moran, (2020) found that concentrations as low as 9.625mg/kg caused significant mortality over 20-40 days. However, neither Almasri et al. (2020) or Motta and Moran (2020) report screening their honey bees for parasites prior to the trial, and so synergistic effects cannot be

ruled out. While a recent meta-analysis of the mortality effects of glyphosate on bees suggested a significant effect of glyphosate on mortality (Battisti et al., 2021), the methods used heavily predisposed the results to confirm the mortality hypothesis (Straw, 2021), In addition, errors in the data extraction process and analysis mean that the conclusions drawn in this meta-analysis lack support (Straw, 2021).

Interestingly, work on honey bees has not been limited to adult workers, honey bee larvae have also been tested. In honey bees, evidence for mortality in larvae is heavily mixed. Tomé et al. (2020) found that six days of exposure to 0.054mg/kg, but not 0.0008mg/kg, caused significant mortality at 18 days after treatment started, although the authors note that the 16% mortality is 'considered incidental because [their methodology] accepts up to 30% control mortality'. Vazquez et al. (2018) had very mixed results over five days exposure, with their highest treatment group 5mg/kg causing significant mortality in one colony, but no change in four colonies, and significantly reduced mortality in one colony. Dai et al. (2018) found that over 21 days exposure to 4mg/kg or 20mg/kg caused significant mortality, but that 0.8mg/kg did not.

These mixed results, for both adults and larvae, heavily indicate strong colony effects, or that some bees were infected with a parasite, like *Serratia marcescens*, which synergises with glyphosate to cause mortality (Motta, Raymann and Moran, 2018, Motta et al., 2020). Notably, none of these studies, in adults or larvae, explicitly reported screening their bees for signs of disease. Thompson et al. (2014) used verifiably healthy bees making it the most robust study to date. They found that over 15 days of exposure neither 75mg/L, 150mg/L or 301 mg/L caused any larval mortality. This highlights the importance of screening bees for diseases prior to experiments, as well as the need for more work to understand the effects of pesticides on parasite exposed bees. Odemer et al. (2020) also found no evidence of mortality in a range of experiments (adults and larvae) using parasite-free honey bees, but, as noted above, these results are not directly comparable because of the use of a glyphosate-based formulation (although glyphosate co-formulants are typically linked to increased, not reduced, toxicity (Mesnage, Bernay and Séralini, 2012, Nagy et al., 2021)).

The larval mortality literature relates to the experiments presented here because the larvae in the chronic exposure experiment will also have been fed glyphosate by the nurse workers. However, in my experimental paradigm the peak exposure for micro-colonies would have occurred while new offspring were still in the egg stage. My results did not explicitly consider larval mortality, but no effect was seen on larval number or weight (consistent with Thompson et al. (2014)), which indicates that if any mortality occurred it was below the level required to reduce reproductive success. Further experiments, where peak exposure occurs at the larval feeding stage, are required to understand whether results from honey bee larvae extrapolate to bumble bee larvae. Larvae were not the primary subjects of this study, adult workers were, and as such the evidence collected on their mortality is more substantial.

In the short term (two days) and long term (20 days) after exposure to a relatively high acute dose of glyphosate, no mortality was seen in individually housed bees in three separate experiments (Modified Ecotoxicological Protocol OECD 247: Small Scale, Full Scale and Long-Term Mortality). As 20 days is representative of a considerable proportion of a bumble bee worker's lifespan (Brian, 1952, Rodd, Plowright and Owen, 1980, Goldblatt and Fell, 1987), this indicates that there is no delayed mortality response and no meaningful shortening of longevity. All the academic studies cited above have used chronic exposure to glyphosate, not acute exposure. As such, there is presently no non-regulatory data on acute exposure to glyphosate in any bee species, nor any data on glyphosate exposure in bumble bees, so my results represent a substantive contribution to the understanding of glyphosate's effects on bee mortality.

In the microcolony experiments no significant mortality was seen with either adult workers acutely exposed, or age controlled young adult workers with chronic exposure. This demonstrates that even while the bees are housed collectively under more natural conditions, and exerting themselves rearing young, any potential stress was insufficient to cause mortality. The finding of no mortality with a fully field realistic chronic exposure regime in parasite free bumble bees supports the evidence that chronic glyphosate exposure is non-lethal to healthy worker bees (Herbert et al., 2014, Goñalons and Farina, 2018, Blot et al., 2019, Motta, Raymann and Moran, 2019). The lack of increased mortality alongside *C. bombi* infection also aids our understanding of which parasites can synergise with glyphosate to

cause mortality in bees. Mortality, however, is not the only metric of bee health, and other sublethal metrics like parasite intensity are important to consider for a more complete picture of bee health.

Parasite intensity

The initial experiment found a 109% increase in *C. bombi* intensity. As a preliminary experiment the methods were less robust than later experiments, with a smaller sample size and no tracking of colony of origin or body weight through the experiment. However, the balanced experimental design accounts for this variation and as such it is unlikely to be confounded. Further the sample size of *C. bombi* n = 21 and Glyphosate + *C. bombi* n = 23 is appropriately powered (Logan, Ruis-González and Brown, 2005).

The follow up experiment to this, found a 16% increase in *C. bombi* intensity, although this effect was not statistically significant. In this trial the sample size was larger, and the co-variates of colony of origin and body weight were tracked throughout.

These opposing results can be explained in several ways. Principally either of the two experiment could have delivered a false positive or a false negative result, which is the simplest solution, and there is no evidence to confirm or contradict this. Alternatively, it is possible that some of the other variables in the experiment such as the parasite, the colonies used, or other unknown effects are acting individually or in combination to alter the parasite intensity.

As with all bumble bee toxicity testing the colonies used differed between experiments. Because of this, there could be a parasite by host genotype interaction (Baer and Schmid-Hempel, 2003), or a parasite by host microbiome interaction (Koch and Schmid-Hempel, 2011, Mockler et al., 2018) as has been observed in experiments previously. However, I believe that this is unlikely as in each experiment three or more colonies were used to account for inter-colony variation, these were evenly distributed to treatment groups, and colonies were sourced from the same supplier.

More interestingly, the two experiments differed heavily in the average parasite intensity in the *C. bombi* only treatments. While the first experiment had a parasite intensity of 6,946 ± 5,682 cells per µL (SD), the follow up experiment had a mean parasite intensity of 20,756 ± 14,473 cells per µL, which is considerably higher. It is possible that this increase in parasite intensity reached a plateau, meaning any increase in parasite intensity caused by glyphosate could no longer occur, as there was no further scope for intensity to rise. To assess the evidence for this hypothesis we can look to prior *C. bombi* literature.

The majority of the literature on *C. bombi* in *B. terrestris* uses faecal counts, which are not directly comparable to homogenised gut counts. Further, there are currently no comparable data on peak parasite intensity using homogenised gut counts. As such it is not possible to know if the levels seen in my second experiment do represent a plateau. However, my methods were based on unpublished work (Siviter, Matthews and Brown, In Submission), that found a mean parasite intensity of 1,849 ± 1,966 cells per µL, comparable to levels in my first experiment, but more than ten times lower than intensity levels in my second experiment. This is indicative that a plateau may have been reached. Similarly, in my microcolony experiments, which took place in between the two experiments on individual bumble bees, a high parasite intensity (Acute: 18,635 ± 5,884 cells per µL and Chronic: 18,759 ± 9403 cells per µL) was recorded.

Faecal parasite counts from Logan, Ruiz-González and Brown, (2005) found parasite intensity to rise to a peak at around 13 days post inoculation. So, at 9 days post-exposure the parasite intensity should not have plateaued. However, in all experiments after my first small scale experiment, the parasite intensities at either 9 days or 21 days were in the 20,000 cells per µL range. This again supports the plateau hypothesis because there is a consistent and high parasite intensity across a range of experiments and conditions.

If the *C. bombi* intensity had reached a plateau, that such high parasite intensities do not cause any measurable impacts on the other metrics recorded under the conditions tested here does indicate that even if glyphosate does increase parasite intensity, this is not likely to lead to any reduction in fitness. As such any effect that might exist is unlikely to be environmentally relevant or robust.

A final explanation for these conflicting results may come from the parasite source used. The *C. bombi* used in the experiments was from the same original source, wild caught infected *B. terrestris* spring queens. Faeces collected from infected queens were used to infect a commercial colony which were kept as a parasite source. As each commercial colony neared the end of its lifespan, faeces was collected from workers in it and used to infect a new commercial colony. Theoretically, within a year the serial passage of the parasite could lead to selection for higher infection levels, and if this were the case it could explain my experimental results. However, previous work with *C. bombi* suggests that the opposite occurs, with serial passage within a colony reducing infectivity to non-colony members (Yourth and Schmid-Hempel, 2006), which would result in lower prevalence and intensity of infections in my experimental paradigm, a pattern I did not see. Consequently, it seems unlikely that an increase in transmissibility or growth in *C. bombi* across the course of experiments can explain my results. While parasite intensity is an important factor in bee health, reproductive success is much more important to a bee's fitness.

Reproduction

Reproductive success is the ultimate metric of bee health, directly representing bee fitness. Drone production by unmated workers in a microcolony set up is designed to function as a proxy of this, and itself does not directly represent a field realistic measure of whole colony sexual production. There is even some evidence that microcolonies can give contradictory results to queenright laboratory or full field experiments (Oystaeyen et al., 2020). As such my results should be interpreted with caution and are not a field realistic measure of reproductive success.

No significant effect on reproduction was found in any experiment, despite at times large differences between treatments (up to a 33.5% difference in reproductive success versus the control), which is potentially indicative of power limitation. Indeed, it is possible that both microcolony experiments were power limited, with ~10 microcolonies per treatment (a total of 38 and 36 microcolonies in each experiment). This is less than other microcolony

experiments like Oystaeyen et al. (2020) which used 20 per treatment, and Siviter et al. (2019) which used 30 per treatment. The power limitation hypothesis is supported by the lack of a significant effect of *C. bombi* on reproductive success in both experiments, which contrasts with a range of published literature (Shykoff and Schmid-Hempel, 1991, Brown, Schmid-Hempel and Schmid-Hempel, 2003, Yourth, Brown and Schmid-Hempel, 2008). Interestingly, while not significant, *C. bombi* reduced reproductive success by 10.2% and 50.0% in the Acute and Chronic experiments respectively. This is a similar scale of reduction to previously published data (Brown, Schmid-Hempel and Schmid-Hempel, 2003). The data presented here also indicate that acute exposure to glyphosate is more likely to impact reproductive success than chronic exposure, with a 20.6% decline in reproductive success after acute exposure, versus a 34.9% increase after chronic exposure. Overall, I would suggest that this evidence be used to guide future studies, conducted ideally in field conditions with larger sample sizes to provide more high quality and definitive evidence for any potential effects.

There was a considerably lower reproductive output overall in the Chronic experiment than in the Acute exposure experiment. This is likely because the workers in the Chronic exposure experiment were age controlled, and thus likely to be much younger on average. This could have led to a delay in ovary development retarding reproductive output. In the Chronic exposure experiment, sucrose consumption was also tracked to allow for the total glyphosate exposure to be measured.

Sucrose consumption

Sucrose consumption can be an indicator of bee health (Chapter 4). While in isolation this metric has no clear relation to fitness, the ultimate measure of bee health, it can be useful in indicating that a bee is acting abnormally. In the case of exposure to the co-formulant alcohol ethoxylates, reduced sucrose consumption went hand in hand with weight loss and gut melanisation (Chapter 4). Further, sucrose consumption could be a corollary of pollination services, as bees with lower appetites might forage less, although in social bees nectar foraging is a response to both individual and colony-level nectar needs (Hendriksma, Toth and

Shafir, 2019). Under chronic exposure, no treatment affected sucrose consumption, indicating that glyphosate did not significantly affect the bees dietary consumption.

Under microcolony conditions worker bees consumed an average of 38.7 or 41.4μg of glyphosate (Glyphosate and Glyphosate + *C. bombi* treatments respectively) under a field realistic, degrading concentration exposure regime. This can be used to inform future research as to the cumulative exposure bees would experience in the wild. The majority of this glyphosate was consumed within the first few days of exposure, with the rapidly declining residues causing the consumption from day five onwards to contribute little to overall exposure. Consequently, future studies could truncate the glyphosate exposure to five days with little reduction in exposure. However, it is also worth noting that there is no limit on the number of sprays of a glyphosate-based herbicide per year, or a mandated time gap between them (Roundup ProActive Label), so repeat exposure could occur. As such, the 38.7 or 41.4μg dose does not necessarily represent the total dose a bee could be exposed to over their lifetime.

The stepwise degradation method of exposure, as developed for bees in Linguadoca et al. (2021), is the most field realistic existing method of simulating real pesticide exposure in a laboratory setting. By mimicking the degradation of the substance the exposure profile is accurately portrayed, whereas a flat exposure, even using a time-weighted average dose, would lack the nuance of the initial peak followed by a lengthy tail. As such, the lack of mortality resulting from this chronic exposure can be seen as a very rigorous result, representing the best approximation of the effects of a field realistic exposure possible.

The research presented here principally used acute oral exposure to 200μg of glyphosate as an active ingredient. None of the research into the effects of glyphosate on the honey bee microbiome has used acute exposure, instead using chronic exposure at a range of concentrations from 0.8mg/kg (Dai et al., 2018) to 210mg/kg (Blot et al., 2019). It is possible that sustained exposure to glyphosate is more impactful than a single more concentrated instance of exposure because the gut microbial community is not afforded opportunity to recover. Alternatively, exposure to the considerably higher acute concentration may also have a more severe impact, potentially acting to cull sensitive species and strains. Given that

bees are exposed to both acute and chronic exposure to glyphosate in the wild, if future research considered acute exposure my understanding of how glyphosate affects bee health would be more complete.

How the acute exposure to 200μg of glyphosate used in this study relates to in-field exposure is unknown. There is no data, even from honey bees, to be able to accurately predict acute exposure to herbicides that lack any mitigation measures. Given that flowering weeds can be sprayed while bees are foraging on them, and glyphosate is typically sprayed in very concentrated sprays (compared with insecticides), for a bee to consume 200μg in a short period of time immediately after a spray application is not implausible, although lower doses are more likely. More work on acute exposure of bees to agrochemicals without bee specific mitigation measures is needed to inform future research. However, with no effects on a range of metrics seen at this potentially high-end dose, it is likely that more field realistic acute exposures would also not have an effect on bumble bees.

3.5 Conclusion

As the world's most used pesticide (Duke and Powles, 2008, Benbrook, 2016), the application of glyphosate is a hotly debated topic, largely due to its human carcinogenicity (Alcántara-de la Cruz et al., 2021), but increasingly regarding its potential toxicity to bees (Cullen et al., 2019). Given its wide usage, the implications for changing its regulatory status would substantively reshape conventional farming practices (Beckie, Flower and Ashworth, 2020), and thus need to be made using robust and environmentally sound science. As such it is imperative that evidence for or against its impacts on bees is of the highest of standards.

With that in mind, the findings presented here provide robust evidence that oral exposure to the active ingredient glyphosate does not induce mortality in the bumble bee *B. terrestris*. I report mixed evidence for the effect of glyphosate on *C. bombi* parasite intensity, with insufficient evidence to describe the effect as environmentally robust. While future research could elucidate the impacts of glyphosate on *C. bombi* intensity, as I found no effects in any metric of their combination, research efforts are best focussed on other pesticide-parasite combinations. Further I report no effects of glyphosate, *C. bombi* or their combination on worker reproductive output, but this conclusion is potentially limited by the power of the study. My results thus do not indicate any requirement to change the regulatory status of the active ingredient glyphosate as it pertains to bumble bees. As glyphosate has been found to impact honey bees as measured by a range of sublethal metrics (Boily et al., 2013, Herbert et al., 2014, Balbuena et al., 2015, Helmer et al., 2015, Vazquez et al., 2018), further research using wild bee species and sublethal metrics would help resolve whether this widely used chemical is safe for bees.

3.6 Acknowledgements

3.7 Data availability statement

Data available from the Zendono http://doi.org/10.5281/zenodo.5235407

Chapter 4

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

Abstract

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 been overlooked as potential threats. By testing an acute oral dose of the fungicide product Amistar, and equivalent doses of each individual co-formulant, I was able to measure the toxicity of the formulation and identify the ingredient responsible. I found that a co-formulant, alcohol ethoxylates, caused a range of damage to bumble bee health. Exposure to alcohol ethoxylates caused 30% mortality and a range of sublethal effects. Alcohol ethoxylates treated bees consumed half as much sucrose as negative control bees over the course of the experiment and lost weight. Alcohol ethoxylates treated bees had significant melanisation of their midguts, evidence of gut damage. I suggest that this gut damage explains the reduction in appetite, weight loss and mortality, with bees dying from energy depletion. My results demonstrate that sublethal impacts of pesticide formulations need to be considered during regulatory consideration, and that co-formulants can be more toxic than active ingredients.

4.1 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 and Antoniou, 2018). However, the toxicological effects of co-formulants have been consistently underestimated for bees and other non-target organisms, including humans (Cox and Surgan, 2006, Mullin 2015, Mullin et al., 2015, Mesnage and 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, because they lack mitigation measures to protect bees from exposure.

Fungicides are very widely used agrochemicals, with almost half a million metric tonnes applied globally in 2014 (FAOSTAT, 2021), 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, 2020b). 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 and 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 (NMP) and dimethyl sulfoxide (DMSO) have been tested on honeybees ((Zhu et al., 2014, Fine and Mullin, 2017, Fine et al., 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)(see Table 1). Of these, C16-18 alcohols ethoxylated (CAS-no 68439-49-6), which are part of the chemical group alcohol ethoxylates, constitute 10-20% of the formulation and 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., 2018a). Alcohol ethoxylates are currently being used to replace alkylphenol ethoxylates as surfactant co-formulants because they can synergise with the active ingredient to a greater extent (Li et al., 2018a).

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-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. The experiment was specifically tailored to identify any potential toxicity of co-formulants or co-formulant mixtures. I 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 my 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 I predicted that the Amistar treatment would cause significant mortality and gut damage. I hypothesised that bees would compensate for reduced gut function by over-consuming sucrose and that the reduced gut function would cause weight loss.

4.2 Materials and methods

We used Amistar, a broad-spectrum fungicide, purchased online through Agrigem Ltd (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 Table S3. This list may not be comprehensive as European law (EC, 2009) explicitly protects whole formulation composition as proprietary knowledge, so I 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, 2006). From the difference between the appearance of the co-formulant mixture and Amistar it is likely that there are other ingredients not listed, see Figure S1. 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 I trialled, and therefore azoxystrobin was not included as an individual treatment, nor in the co-formulant mixture. Regulatory testing of azoxystrobin on honey bees did not detect any lethal effects (EFSA, 2010).

Table 1. Doses of chemicals used in each treatment group. Each co-formulant dose is proportionate to its concentration in the formulation. Calculations, per bee, 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) per bee
Negative control	Water	0.0
Positive control	Dimethoate	4
Alcohol ethoxylates	C16-18 alcohols, ethoxylated	160
Naphthalenesulfonic acid	Naphthalenesulfonic acid, dimethyl-, 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 *B. terrestris audax* were used in the experiments (Agralan, Wiltshire, UK). On arrival, 10 workers per colony were removed and their faeces visually screened for micro-parasites (Rutrecht and 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, (OECD, 2017), used by industry and regulators, where deviations from OECD 247 served to increase the richness of data captured. Below I give a brief summary of the method used, and list all deviations from OECD 247, for the base method in full detail see OECD 247.

We housed and weighed worker bees in individual Nicot cages a day in advance of their chemical exposure, and then rank allocated them to treatments based on their weight, with

an even distribution of source colonies by treatment. Bees outside the range of 0.1g-0.4g were not used. Number of bees exposed can be seen in Table S1. Prior to, and after exposure, bees had access to *ad libitum* 45% w/w sucrose solution (Thorne, Windsor, UK). Bees were kept at 25 ± 2°C and 50 ± 10% relative humidity, under red light or darkness.

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, per bee. 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. Bees who did not consume the droplet were excluded from the entire experiment. While the proportions of bees who did not feed on the droplet did significantly differ between treatments (Fishers Exact Test, p=<0.001), with more non-feeders in treatment groups containing alcohol ethoxylates, there is no reason to believe this would impact the results of the study.

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. Consumption was first checked at 2 hours, and again at 4 hours. The syringe was checked visually to ensure the bees consumed the whole droplet. The syringe of each bee who had consumed the droplet was then filled with 45% w/w sucrose solution, without any pesticide. 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. As soon as they were found dead, any bees who died were weighed then transferred to a 2mL Eppendorf tube and frozen at -80C°, as were any bees who survived the full 120 hours.

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.

While dissecting the guts of bees exposed to Amistar during pilot work, gut melanisation was observed, which led to the formal quantification of gut melanisation in this experiment. As a proxy for the level of damage to the gut I used the presence of melanisation (dark brown patches and striations) on the midgut, which are not seen in healthy bees (EA Straw and MJF Brown pers. obs., also see Results). 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 mm^2. 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. The use of Fiji to analyse insect gut lesions is common in the literature (e.g., da Costa Domingues et al., 2020, Smith et al., 2020).

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 and 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, so a 95% confidence interval of -1.00 to 1.00 would not be significant, but a 95% confidence interval of -2.00 to -1.00 would be. Model parameters, AIC weights and final models are presented in Tables S4-7. Where a bee lacked a response variable result due to mortality or experimental error it was excluded from that particular analysis, see Table S1 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. Cox proportional hazards models were used to analyse mortality, utilising 'survival' version 3.1-8 (Therneau, 2020). The full model for mortality used was (Mortality ~ Treatment + Bee Weight+ 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. Generalised linear models were used to analyse sucrose consumption of bees who survived the full 120 hours. Generalised linear models were used to analyse gut melanisation and weight change on all bees. The full models used was (Metric ~ Treatment +

Bee Weight + Colony of Origin), with the metric being either Sucrose Consumption, Weight Change or Gut Melanisation. Model assumptions were checked graphically and met.

4.3 Results

Mortality

All bees in the positive control treatment died within four hours (n=34), no bees in either the negative control (n=35) or the benzisothiazol treatment (n=36) died over the period of 120 hours, and only one bee died in the naphthalenesulfonic acid treatment (n=33).

There was significantly higher mortality in all treatments containing alcohol ethoxylates, compared to the negative control. Amistar (n=31), co-formulant mixture (n=25) and alcohol ethoxylates (n=30) all had significantly higher mortality than the negative control (Cox proportional hazards model: parameter estimate (PE) = 2.16, 95% CI [0.07 to 4.26], (PE) = 2.57, 95% CI [0.65 to 4.83], and PE = 2.28, 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). Bees whose initial weight was heavier were significantly less likely to die than lighter bees, and bees from one colony were slightly less likely to die also (see Supplementary Results).
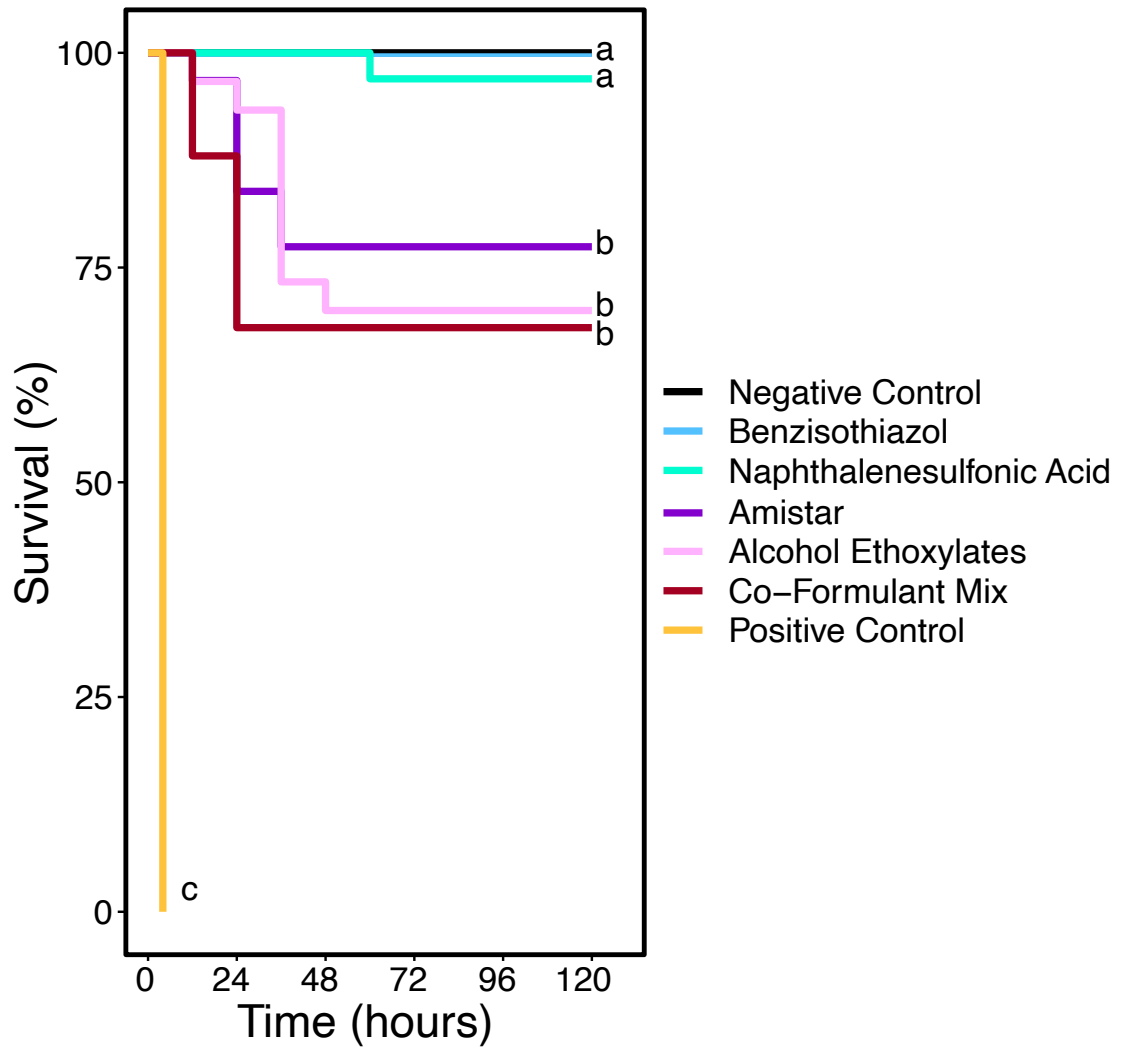
Figure 1. A Kaplan-Meier plot showing survival against time; colour coded by treatment. The negative control and benzisothiazol line is split to allow both to be visible, as both treatments had 0% mortality. Different letters indicate statistically significant differences.

Sucrose consumption

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

Amistar (n=24), co-formulant mixture (n=17) and alcohol ethoxylates (n=21) all had significantly lower consumption than the negative control (n=35) (Generalised linear 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. Bees in neither benzisothiazol (n=36) nor naphthalenesulfonic acid (n=32) had significantly different consumption versus the negative control (see Supplementary Results). Bees whose initial weight was heavier drank significantly more sucrose than lighter bees, and bees from one colony were significantly more likely to drink slightly less (see Supplementary Results).
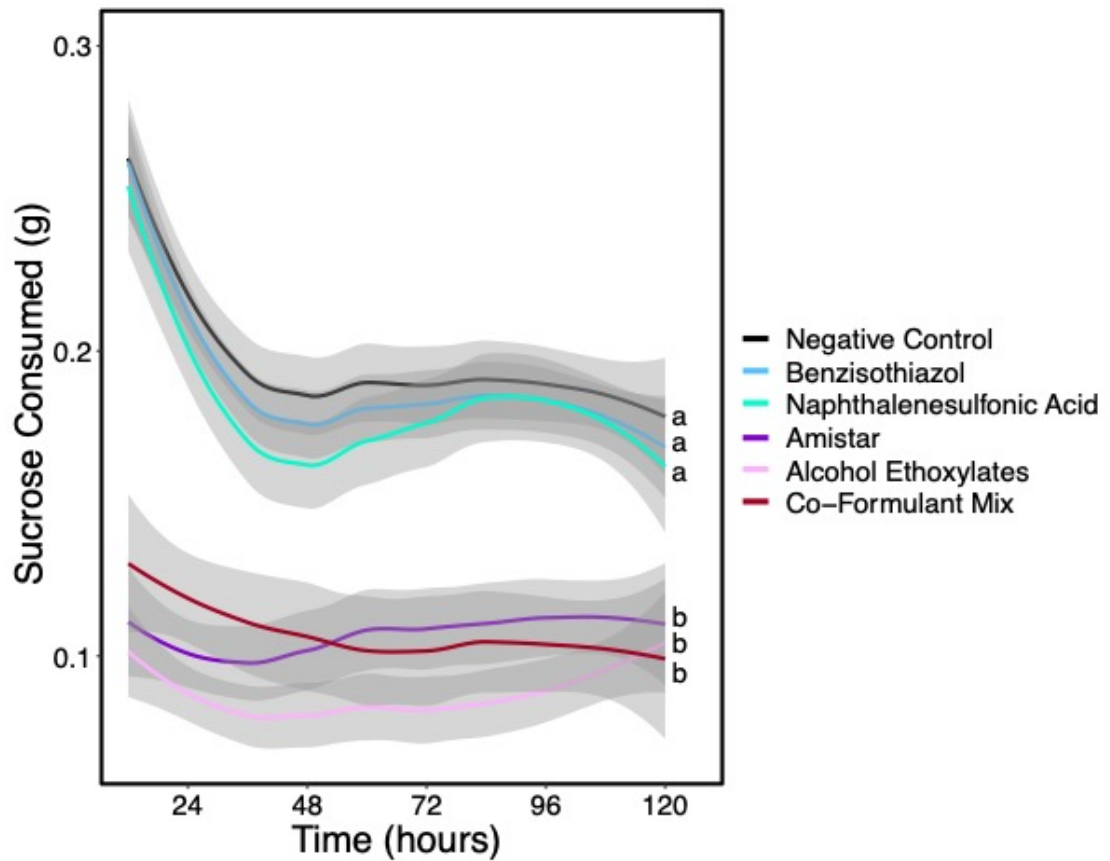
Figure 2. A time series plot showing sucrose consumption over a 120-hour period; colour coded by treatment. Average consumption and 95% confidence intervals are shown. Sucrose consumption data collected every 12 hours has been LOESS smoothed. Y axis scale refers to sucrose consumption per bee over a 12-hour period. Different letters indicate statistically significant differences.

Weight Change

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

Amistar (n=31), co-formulant mixture (n=25) and alcohol ethoxylates (n=30) all had a significantly different weight change compared to the negative control (n=35) (Generalised linear 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. Bees in neither benzisothiazol (n=36) nor naphthalenesulfonic acid (n=33) had significantly different consumption versus the negative control (see Supplementary Results). There was no effect of initial weight or colony on weight change (see Supplementary Results).
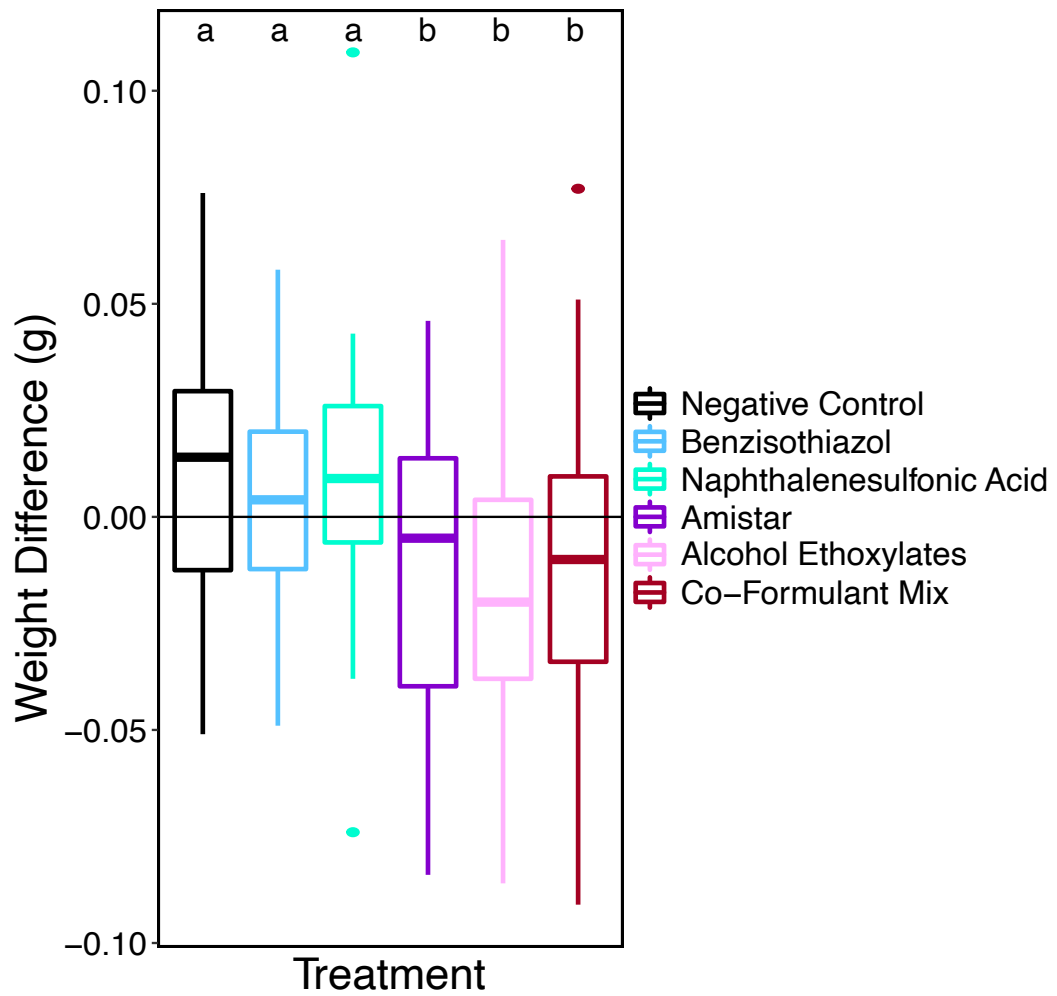
Figure 3. A boxplot showing change in weight over the 120-hour period, or until death colour coded by treatment. Boxes represent the Inter-Quartile Range (IQR), with the bold horizontal line the median value. The whiskers represent the furthest datapoint within 1.5 times the IQR and points beyond this are plotted as outliers. Different letters indicate statistically significant differences.

Area of Gut Melanisation

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

Amistar (n=30), co-formulant mixture (n=23) and alcohol ethoxylates (n=29) all had significantly more melanisation than the negative control (n=35) (Generalised linear model: parameter estimate (PE) = 0.67, 95% CI [0.22 to 1.12], (PE) = 1.13, 95% CI [0.64 to 1.62], and PE = 0.61, 95% CI [0.16 to 1.07], respectively). Amistar, co-formulant mixture and alcohol ethoxylates treated bees had an average melanised area of $0.925mm^2$, $1.350mm^2$ and $0.850mm^2$ respectively, compared to the $0.230mm^2$ 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. Bees in neither benzisothiazol (n=36) nor naphthalenesulfonic acid (n=33) had significantly different consumption versus the negative control (see Supplementary Results). There was no effect of initial weight or colony on gut melanisation (see Supplementary Results).
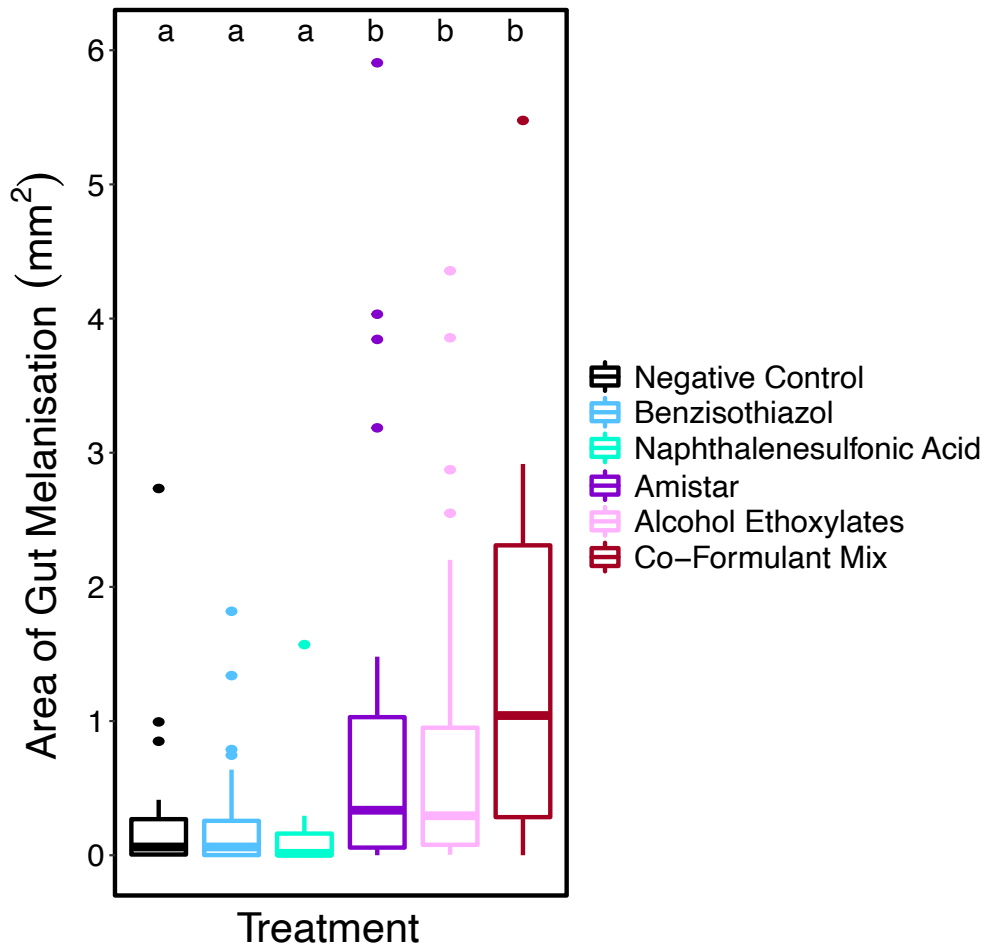
Figure 4. A boxplot showing area of gut melanisation, colour coded by treatment. Boxes represent the IQR, with the bold horizontal line the median value. The whiskers represent the furthest datapoint within 1.5 times the IQR and points beyond this are plotted as outliers. Different letters indicate statistically significant differences.
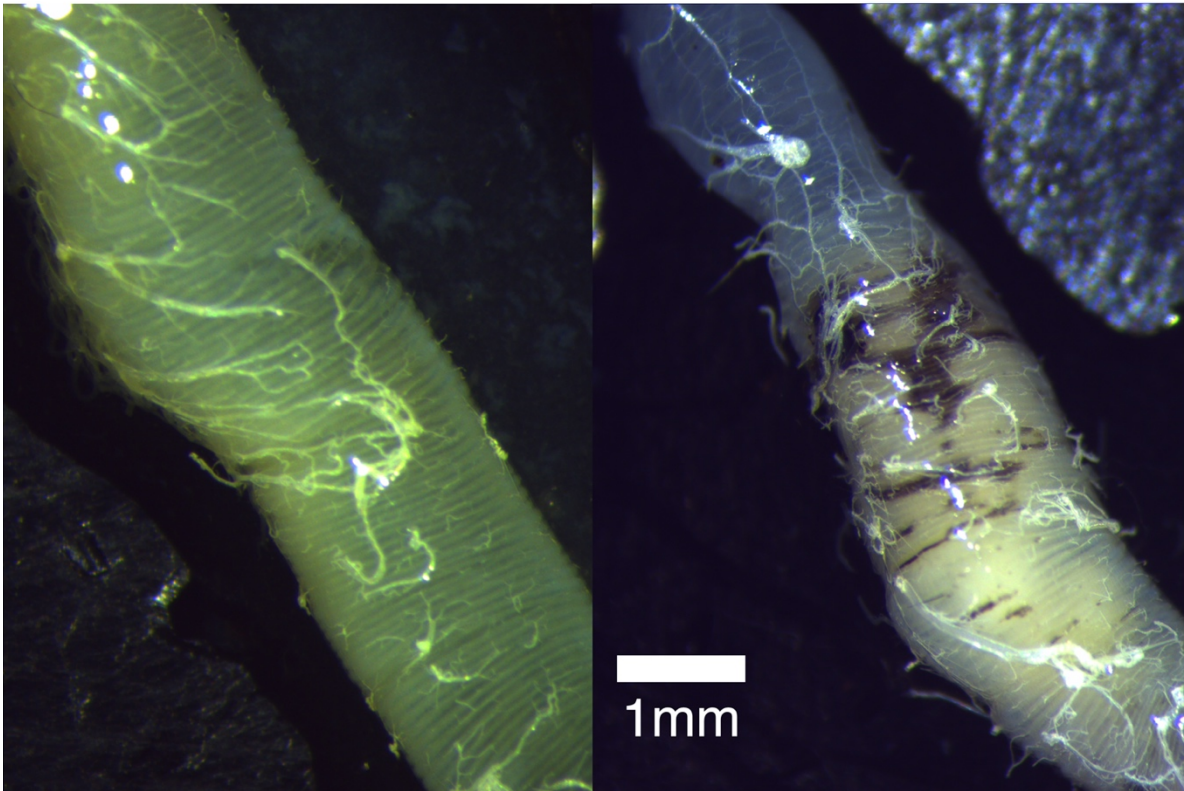
Figure 5. (Left) Bumble bee midgut in the negative control treatment. (Right) Bumble bee midgut in the co-formulant mixture treatment, which contains alcohol ethoxylates. The dark brown patches are areas of melanisation, indicative of damage to the gut. Both bees survived the full 120 hours.

4.4 Discussion

Here I 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 tested, 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.

The mortality in the Amistar treatment, and treatments containing alcohol ethoxylates reached 32% at its highest, which is substantial given that bees are likely to have a high level of exposure to Amistar and alcohol ethoxylates. The mechanism by which the alcohol ethoxylates cause mortality has not been explicitly isolated, but my results suggest two potential, possibly related, causes. I recorded a 302% increase in the melanised area of bee midguts in the alcohol ethoxylates treatment. A similar effect was observed in *Melipona scutellaris* exposed to the pure fungicide active ingredient pyraclostrobin alongside a similar reduction in survival (da Costa Domingues et al., 2020). I 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. Figure S3 shows a plot comparing sugar consumption against gut melanisation, with increasing gut melanisation correlated to reduced sugar consumption in the Amistar, co-formulant mixture and alcohol ethoxylates treatments. Consequently, I 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 treatment lost 8.4% of their original weight, in stark contrast to the negative

control where bees gained 4.8% over the five-day period. This indicates the alcohol ethoxylate treated bees are expending more energy than they are consuming, and thus exhibiting a negative energy balance. This weight loss, while considerable as a percentage of the bee's total body mass, is also similar in scale to the weight of the sucrose bees consume in one sitting (EA Straw pers. obs.), for which rigorous data do not exist. As such it is possible that a portion of the weight loss is attributable to the reduced sucrose consumption of the bees, meaning they would have less sucrose in their guts at the time of weighing. Sucrose consumption does not, however, explain the failure of alcohol ethoxylate treated bees to gain weight, which was observed in the control treatment. 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 and 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 subsequently have effects on ecosystem services. In my experiment, bumble bee appetite was reduced immediately after ingesting a single dose of alcohol ethoxylates or Amistar. This effect persisted for five days after exposure, indicating a persistent change in consumption behaviour. While nectar-foraging in bumble bees 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 I have found map onto foraging and pollination are clearly needed. Importantly, the reduction in appetite recorded in my 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. I suggest that a simple modification to the regulatory protocol OECD 247 would be to weigh the sucrose syringes provided to the bees after the pesticide exposure and again at the end

of the trials to calculate sucrose consumption, which would allow measurement of this sublethal effect with minimal additional workload. This is only two additional measurements using kit already used in the experiment, as the standard OECD 247 protocol requires the dose consumption be validated by weighting the syringe after pesticide exposure

My 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 I used the upper end of the range. The co-formulant mixture treatment in all metrics was statistically indistinguishable from the alcohol ethoxylates treatment, showing that the toxicity of alcohol ethoxylates is not a result of synergism with other co-formulants.

We believe that the implications of my results are not limited to a laboratory setting and a single species, as other published and unpublished research supports my findings. Semi-field flight cage experiments, where Amistar was applied to a crop, found effects on full bumble bee colonies (*B. terrestris*). Amistar caused a reduction in average bee weight and a reduction in foraging activity, as my results predict (Tamburini et al., 2021a and Wintermantel et al. In Submission). This demonstrates that the effects observed in my laboratory testing scale up to effects at a field realistic level. Additionally, in honeybees (*A. mellifera*) Amistar has been found to cause mortality in laboratory experiments at a range of doses (Medrzycki, Di Prisco and Costa, In Preparation, Tamburini et al., 2021b), demonstrating the mortality effect found in my 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).

To measure the exposure of bees to agrochemicals, 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 and Antoniou, 2018). 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 crops, such as 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). In addition, I 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). 89 adjuvant products licenced in the UK containing alkoxylated alcohols as the primary ingredient (Health and Safety Executive UK, 2020a). To my knowledge, these adjuvants have never been toxicity tested on bees and have no bee exposure mitigation measures in place whatsoever.

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, 2020b), 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 (Chapter 2). Some progress has been made in Europe, with the recent legislation explicitly recognising the potential harm co-formulants could pose to the environment, but this has yet to lead to regulatory change (EC, 2021).

To complement measures to promote academic research, moving regulatory research beyond its mortality and active ingredient-centric approach to toxicity testing would better reflect the risks pesticides, as used in the field, pose. 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 could undergo a much more rigorous set of lower tier testing or be automatically entered for higher tier testing.

In the face of declining bee populations I advocate that a precautionary approach minimising the exposure of bees to potential stressors, where possible, would be prudent. The current legislation allowing application of pesticides 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 (Mullin, 2015, Chapter 2). The wealth of untested and undisclosed co-formulants used abundantly in agriculture is a serious and pressing concern for the health of pollinators worldwide.

4.5 Acknowledgements

2.6 Data availability statement

We intend to use Dryad Digital Repository. Until then all data will be available upon request.

Chapter 5

'Inert' Ingredients Are Understudied, Potentially Dangerous to Bees and Deserve More Research Attention

Abstract

Agrochemical formulations are composed of two broad groups of chemicals: active ingredients, which confer pest control action, and 'inert' ingredients, which facilitate the action of the active ingredient. Most research into the effects of agrochemicals focusses on the effects of active ingredients. This reflects the assumption, engrained in legislation, that 'inert' ingredients are non-toxic. A review of relevant research shows that for bees – a significant focus of research into agrochemical impacts, due to their essential role as pollinators – this assumption is both without empirical foundation and likely to be untrue. After conducting a systematic literature search, I found just 16 studies that tested the effects of 'inert' ingredients on bee health. In these studies, 'inert' ingredients were found to cause mortality in bees through multiple exposure routes. 'Inert' ingredients also acted synergistically with other stressors, and caused sublethal and colony level effects. In addition, the lack of research on 'inert' ingredient effects on bees is compounded by a lack of diversity in study organism used, with only two studies assessing effects on non-*Apis* bees. I argue that 'inert' ingredients have distinct, and poorly understood, ecological persistency profiles and toxicities, making research into their individual effects necessary. I highlight the near total lack of mitigation in place to protect bees from 'inert' ingredients, and argue that research efforts should be redistributed to address the knowledge gap identified here. Specifically, I call for a new focus on testing the wealth of understudied 'inert' substances, and a shift away from testing well understood chemicals like neonicotinoids. If so-called 'inert' ingredients are, in fact, detrimental to bee health, their potential role in widespread bee declines needs urgent assessment.

5.1 Introduction

Ecosystem services provided by pollinators contribute $235-577 billion to the global economy each year, with bees providing the majority of pollination (Potts et al., 2016). However, declines in bees have been identified, with, for example, 37% of European bee species with known population trends being in decline (Nieto et al., 2014). These declines pose a significant threat to the economic value bees provide (Potts et al., 2016). While numerous factors are likely contributing to these declines, one factor that has been repeatedly implicated is the widespread use of pesticides, with experimental, correlational, and modelling work at a range of scales confirming this link (Rundlöf et al., 2015, Woodcock et al., 2016, McArt et al., 2017, Tsvetkov et al., 2017). However, pesticides are not applied alone, but rather are used within complex formulations. Each formulation includes both the active ingredient itself, and co-formulants that facilitate the action of the active ingredient (Hazen, 2000). When applied to crops, such formulations are often further accompanied by separate products called adjuvants that complement the action of the pesticide. Both co-formulants and adjuvants play a range of roles, including as surfactants that help active ingredients penetrate leaves, emulsifiers that help products stay thoroughly mixed, and solvents that help to dissolve the active ingredient. These substances are referred to as 'inert' ingredients, because they are not intended to have direct pest control action.

There are no comprehensive figures for global 'inert' use, as California is the only regulatory zone to accurately record their application (Mullin et al., 2016), but they are known to be heavily used across the globe. According to the US federal Environmental Protection Agency there are around 4,000 'inert' ingredients in use in the US (Weinhold, 2010). No equivalent data are available for the EU, but there are 294 separate adjuvant products and 2892 separate pesticide products registered for use in the UK alone (Health and Safety Executive UK, 2020a-b). As all active ingredients are applied as part of formulations, all formulations contain co-formulants, and formulations are commonly sprayed in a tank mix containing an adjuvant product, we can surmise that the quantity of 'inert' ingredient application is commensurate to, or likely even exceeds, that of active ingredients. Further, no mitigation measures are attached to adjuvants, meaning they can often be sprayed onto crops while bees forage on them. Co-formulants typically only have mitigation measures carried over from the active

ingredient, not measures tailor to their specific toxicity. Thus, the exposure of bees to them, though currently unquantified, is likely to be considerable.

While regulatory bodies require active ingredients to undergo a suite of toxicity testing on bees (e.g., EPA, 1996, EC, 2009, EFSA, 2012, 2013), no parallel testing is required for individual 'inert' ingredients (EPA, 1996, EC, 2009), despite evidence of potential toxicity (Cox and Surgan, 2006, Mesnage and Antoniou, 2018). Instead, in the EU there is toxicity testing of a single commercial product per active ingredient, called the 'representative formulation' (EC, 2013), while in the US only the toxicity of the active ingredient is considered (EPA, 1996, Mullin et al., 2015). In the EU, at the national level, all other formulations with the same active ingredient, of which there can be hundreds (Health and Safety Executive UK, 2020b), need individual approval. Which additional formulations trigger testing is determined by the similarity of their composition relative to already tested substances (Chemical Regulation Division, 2021). If their toxicity to bees can be predicted based on existing data from formulations with a similar composition, then no additional testing is required. Formulations for which toxicity cannot be reliably predicted are not submitted to the full suite of ecotoxicological testing, but instead benchmarked against existing products using mortality at a single dose to demonstrate equivalent toxicity (Chemical Regulation Division, 2021).

Current regulatory regimes are insufficient to protect bees for three main reasons. Firstly, the adjuvants that are added to these formulations via tank mixes undergo no bee toxicity testing at all (EPA, 1996, EC, 2009), meaning that there is no regulatory data confirming their safety to bees. An otherwise safe formulation could become toxic to bees if the adjuvant added is toxic to bees (Moffet, Morton and MacDonald, 1972). Secondly, extensive data, including that collected by regulators, has demonstrated high variation in the toxicity of formulations with the same active ingredient to bees (Mullin, 2015, Chapter 2). Finally, regulatory testing regimes are tailored to detect toxicity from potent insecticides capable of causing short term mortality at low doses, not from 'inert' ingredients which may have more subtle, but still pertinent, sublethal effects at higher doses. This could mean their toxicity is underestimated by regulatory testing.

Current understanding of the effects of 'inert' ingredients is almost exclusively centred around how they impact the toxicity of active ingredients (Mullin et al., 2015, Nagy et al., 2021). Here, I focus on the individual impacts of 'inert' ingredients, rather than how they impact active ingredient toxicity, which while relevant is outside the scope of this review. It is important that we understand the effects of 'inert' ingredients in isolation because the ecological fate of each ingredient is unlikely to be uniform across the formulation (Katagi, 2008).

Importantly, the development process of active ingredients makes them less likely to be ecologically persistent than 'inert' ingredients. In the development of active ingredients, specific attention is paid to their environmental persistence. Regulations like Maximum Residue Limits (MRLs) that are aimed at capping consumer exposure incentivise agrochemical companies to produce active ingredients that readily degrade. There are no MRLs for 'inert' ingredients (EC, 2009), and as such no pressure to produce fast-decaying substances. For example, the pyrethroid insecticides cypermethrin, permethrin, and deltamethrin all have half-lives in pond water of <1 day (Tooby et al., 1981, Crossland, Shires and Bennet, 1982, Rawn et al., 1982). In contrast, the surfactant adjuvant Multi-Film X-77, which can be applied as part of the same tank mix as pyrethroids, can repel honeybee visitation from a pond for six months after an initial spiking of 500ppm (Moffett and Morton, 1973, 1975). This concentration of Multi-Film X-77 also causes honeybees to drown at high rates for 60 days after application (Moffett and Morton, 1973). In this scenario the pyrethroid active ingredient has degraded well below the limit of detection whilst the 'inert' adjuvant is still causing significant mortality for months afterwards. While not all active ingredients degrade as fast as pyrethroids, and not all 'inert' ingredients are likely to be as persistent as surfactants, this illustrates that assuming that all ingredients in a formulation will behave in a uniform manner once in the environment is unlikely to be true.

One of the reasons that there is a paucity of data on the environmental fate or toxicity of 'inert' ingredients' is that, under EU law, only co-formulants with specific human hazard statements attached need to be reported as ingredients (EC, 2006). EU laws are nonetheless among the most stringent in the world, with comparable documents from the US having even less information. The identity and concentration of other ingredients are explicitly protected

under EU law as proprietary information (EC, 2009). Maintaining the identity of 'inert' ingredients as trade secrets severely impedes researchers' capacity to understand how they spread in, and affect, nature (Chen, Fine and Mullin, 2018, Chapter 2).

The limitations of current regulatory testing regimes are illustrated by the fate of the three neonicotinoid insecticides (imidacloprid, thiamethoxam and clothianidin) for which authorization for outdoor use was revoked in the EU in 2013 (EC, 2013). These substances had undergone, and passed, full ecotoxicological testing (including assessment of risks to bees) but were nonetheless later shown through academic research to cause serious detriment to bees and bee populations, mediated through sublethal effects that the regulatory process failed to detect (Rundlöf et al., 2015, Sgolastra et al., 2020). Just as the limited scope of the regulatory system failed to detect the risk that these neonicotinoids posed to bees, 'inert' ingredients too could be severely damaging to bees without triggering concern during the regulatory process. Consequently, academic research has a significant role to play in assessing the exposure, hazards, and risks associated with 'inert' ingredients within pesticide formulations.

Existing academic research on 'inert' ingredients has focussed on surfactants (most commonly as adjuvants) and solvents (most commonly as co-formulants). Surfactants (derived from **surf**ace **act**ive **ag**ent) are among the most common adjuvant type (Health and Safety Executive UK, 2020b). They function by reducing surface tension, enabling the spray to spread out over the surface of the leaf, increasing contact area and active ingredient uptake by the plant (Stevens, 1993). Solvents are co-formulants that allow an active ingredient to be dissolved at a higher concentration than if it were dissolved in water (Hazen, 2000). Because formulations are sold as concentrated stocks, this makes formulations cheaper to produce, distribute, and store. Given that many active ingredients are poorly soluble in water, solvents are likely very common co-formulants. Crop oil concentrates are a much less frequently studied type of 'inert'. They are typically petroleum-based spray adjuvants used to reduce droplet evaporation, and aid degradation of the wax surface on a leaf, to aid active ingredient penetration. The substances described above are used widely in agriculture, and their impacts on bee health is not well understood. As such I use a systematic review approach to

comprehensively summarise what is known about the effects of such 'inert' ingredients on bees.

5.2 Methods

Web of Science Core Collection and Google Scholar searches were undertaken based on the methods used by Cullen et al. (2019), using the PRISMA framework (Moher et al., 2009), and combined with forward and backwards citation tracing to ensure that all relevant literature was captured, although I acknowledge that using only the English language potentially excludes relevant literature. Peer reviewed studies were included in the review if they presented experimental research testing at least one treatment of an agricultural co-formulant or adjuvant, with an appropriate control, or measured residues of an agricultural co-formulant or adjuvant in bees, honey, wax, or bee-collected nectar or pollen. A condition of inclusion was that the respective studies' authors must have noted that the co-formulant or adjuvant tested was agriculturally relevant. Studies measuring residues of 'inert' ingredients in flowers or non-bee-collected pollen or nectar are excluded from the review (not included in the results) as they have not interacted with a bee, but are nevertheless discussed for comprehensiveness.

Literature was initially characterised by title, with titles lacking relevance to agriculture, bees or pesticides being excluded, then by abstract, with abstracts lacking relevance to the search criteria removed. Finally, remaining literature was read in full and the full exclusion criteria were applied. Throughout the search ambiguous studies were retained to the next stage. Studies not accessible online were excluded.

Because the word adjuvant is used to refer to co-formulants by some authors I define it here as meaning a separate product used as a tank additive (Hazen, 2000). I focused on 'inert' ingredients, so adjuvants with the intended purpose of specific pesticidal action (regardless of organic/regulatory status) were excluded. For instance, neem oil adjuvants that are marketed as insecticidal would be excluded. Synergist co-formulants were also excluded as they are not intended to be biologically inert. Because solvents are often used in studies testing active ingredients, they were only included in the review if the study explicitly mentioned the solvent as being used in agricultural products; I imposed this restriction because the solvents commonly used by researchers are rarely the same substances used by the agrochemical industry.

The Web of Science Core Collection search was conducted in November 2020 using the following terms Topic, Title and Abstract Search = (((adjuvant* OR coformulant* OR co-formulant* OR *formulant* OR inert) OR (penetra* OR "odour mask*" OR stabiliz* OR stabilis* OR preservative* OR surfactant* OR emulsifier* OR diluent* OR propellant* OR anti-foaming OR antifoaming OR solvent* OR carrier*)) AND (*bee OR *bees)))). The Abstract search did not use wildcards before words because this functionality was not supported. A supplementary Google Scholar search was made to ensure all literature was captured with the terms ("bee" OR "bees") AND ("adjuvant" OR "coformulant" OR "co-formulant" OR "formulant"), with the first 200 studies searched in May 2020. Forward citation tracing was performed with Google Scholar in May 2020, as well as reverse citation tracing using the studies reference list. More comprehensive exclusion criteria, methodology and results are available in the Supplementary Materials.

Following Haddaway et al. (2020), the 'Critical Friend' approach was adopted, with Linzi J. Thompson (a co-author), joining the project after the initial literature characterisation was conducted. Linzi J. Thompson provided critical feedback on the systematic review methods and execution, while blinded to the results.

5.3 Results and Discussion

A total of 16 studies (from 1973 to 2019) fulfilled the inclusion criteria, comprising 13 experimental studies, two residue analysis studies, and one experimental and residue analysis study (Figure 1). There was a mixture of methodological approaches, with 9 laboratory, three semi-field, and four field studies. However, diversity among study organisms was severely limited, with 14 studies testing honeybees, and just two studies on a species other than *A. mellifera* (specifically, the solitary bees *Osmia lignaria* and *Megachile rotundata*). This demonstrates the lack of knowledge about how these widely applied substances could impact any of the other approximately 20,000 bee species (Potts et al., 2010).

Most studies (n = 11) tested surfactants, while some tested solvents (n = 4) and only one tested crop oil concentrates, stickers or wetting agents (n = 1). The life history stage studied varied, with adults being the most commonly studied stage (n = 12), followed by larvae (n = 5), and then pupae (n = 1) and eggs (n = 1). Nearly all studies focused on mortality (n = 12), while reproduction and food consumption were the second most studied metrics (n = 4), followed by nesting behaviour (n = 3). Among the studies measuring 'inert' ingredient residues, two focussed on surfactants, and one on solvents. In total 48 substances or products have been experimentally tested in the academic literature, and just 8 have been tested in more than one study, indicating a lack of a depth of study for those tested. For further analysis of the studies included in this study, see the Supplementary Material. While the frequency of studies has increased in recent years (Figure 1) this is more likely to represent an increase in studies in general (Bornmann and Mutz, 2015), rather than increased interest in 'inert' ingredients. It is also worth noting that 7 of the studies dating from post-2010 are from one network of authors.
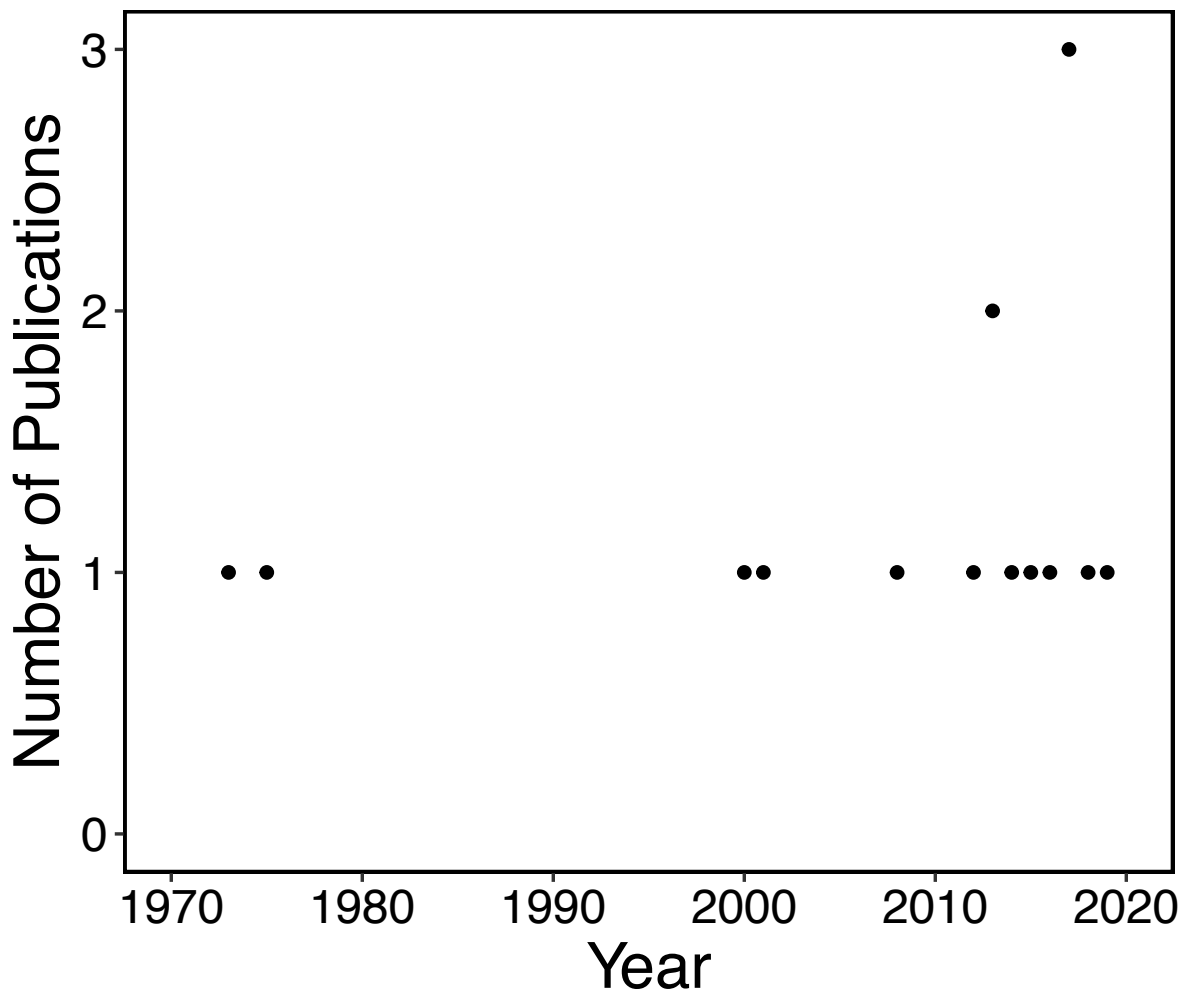
Figure 1. The number of studies testing co-formulants/adjuvants on bees that fulfil the inclusion criteria of this systematic review plotted against their year of publication.

The risk an agrochemical poses to bees is a combination of the exposure bees face and the likely consequences if exposed (hazard). Below, the research identified in this systematic review is divided into residue studies, which quantify exposure, and experimental studies, which quantify hazard.

Residue studies

Because the ecological persistency of 'inert' ingredients in nature is poorly understood we do not know to what extent exposure occurs. To address this question, it is possible to measure 'inert' ingredient residues in bee matrices, such as honey, pollen, nectar, wax, and bees

themselves. The limited evidence available has typically identified wax as a major substrate for residue accumulation (Chen and Mullin, 2013, 2014). Two studies have looked at various surfactants, and one at the solvent NMP.

Chen and Mullin (2013) developed a methodology for detecting trisiloxane surfactants in honeybee matrices, using the QuEChERS technique. Trisiloxane surfactants are common surfactant co-formulants in the organosilicone group and are included in spray adjuvants like Silwet L-77 and Dyne-Amic. They can be used with a range of pesticide classes, and on a range of crops. Chen and Mullin (2013) sampled honey, pollen and wax samples from 7 US states, and while there were no positive detections in honey, 60% of pollen and all wax samples had positive detections (maximum concentrations 39ppb and 390ppb respectively). The same authors later tested for nonylphenol ethoxylate and octylphenol ethoxylate surfactants in the same matrices (Chen and Mullin, 2014). Again, honey was the least contaminated (46 ± 26ppb, mean ± standard deviation), followed by pollen (429 ± 203ppb) and wax (1051 ± 2897ppb). Octylphenol ethoxylate surfactants were less prevalent and presented at lower average concentrations. Chen and Mullin (2015) used LC-MS chromatography and identified trisiloxane surfactants in almond flowers, as well as the chemical compositions of several surfactant adjuvants. However, this study is not included in the systematic review results because the matrix analysed was not collected by bees. These studies demonstrate that bees are exposed to surfactants at non-negligible concentrations, however, whether these concentrations have a meaningful toxic impact is unknown, particularly as the experimental literature covered below typically uses much higher concentrations.

NMP is a solvent co-formulant often used in insecticide formulations (Fine and Mullin, 2017). Experimentally exposed honey bee larvae were less capable of metabolising NMP residues than workers (Fine and Mullin, 2017). While another residue analysis study, Fine et al. (2017), was excluded from this systematic review (because the matrices studied were not collected by bees), its results are still of interest as it is the only study in which an 'inert' ingredient was purposefully applied to a crop to enable the explicit measurement of residues in pollen or other bee relevant matrices (Fine et al., 2017). Following manufacturer's instructions an insecticide formulation (Rimon 0.83EC), containing 40-50% NMP, was applied to apple trees either at the bud stage or while flowering. When sprayed at bud, a high of 22,000ppb (17,150

± 4,390ppb) in pollen was detected 12 hours after application, while direct application to the flowers found a high of 234,600ppb in pollen 2.5 hours after application (Fine et al., 2017). These residue levels were very high (58x higher) compared to the active ingredients novaluron, although the methodology was not directly comparable between substances.

The lack of exposure studies I identify here has important implications for experimental tests of hazard. For active ingredients, exposure regimes are typically designed with reference to the results of semi-field studies where the pesticide is deliberately applied to a crop. Pollen and nectar brought back to the nest by foraging honeybees is collected and measured over time. Using these data, chronic exposure scenarios can be constructed that assess the potential effects on individuals or colonies of bees foraging on a recently sprayed crop. Without similar experiments for a range of 'inert' ingredients, it is not possible to inform experimental exposure regimes with real world data. If regulatory bodies were to mandate residue analysis for all agrochemicals, including 'inert' ingredients, we would have a better understanding of the complex exposure bees face to a diverse range of chemicals. The kind of well-funded and systematic approach to residue monitoring required is something only a regulatorily mandated process can offer. Without this, academic researchers will not be able to properly assess whether their exposure regimes are field-realistic, which could lead to over- or under-estimates of the risks that 'inert' ingredients pose to bees.

Experimental studies

Given the general lack of exposure and residue studies we identify above, the only reference point we can use for exposure regimes in experimental studies is likely to be the in-tank mix concentration, which is the concentration of the 'inert' ingredient in the solution as sprayed. For co-formulants this is not always known because their concentration and identity are not required to be publicly disclosed (EC, 2009). For adjuvants, most labels mandate a maximum concentration of 1% (10,000ppm). This means that without bioaccumulation we would expect around 10,000ppm (1%) to be the very upper end of field realistic exposure, which is equivalent to feeding directly on in-tank mix. While this may be appropriate for acute exposure (see Ciarlo et al., (2012)), it is likely to vastly overestimate field realistic chronic

exposure. As such, the studies detailed below use a range of exposure values that may or may not be field realistic. Hence, they elucidate the relative hazard the substances pose, but cannot yet fully document risk. While little is known about the ecological persistencies of 'inert' ingredients' and how they map to the ecotoxicological risk posed to non-target organisms like bees, we have known for nearly a century that surfactants have strong insecticidal action.

Soaps, which are surfactants, have been recognised as posing risks to insects as far back as 1931. "Insecticidal soaps are the oldest of recognized insect destroyers. Almost any form of soap, if used in a strong enough mixture, will kill soft-bodied insects" (Sanderson and Pearis, 1931, cited in Wolfenbarger (1957)). The mechanism through which surfactants cause mortality in insects is unresolved, although Stevens (1993) notes that insect spiracles are similar in size to plant stomata, which surfactants are designed to penetrate. Thus, surfactants may inadvertently block the breathing apparatus of the insects and cause them to drown (Chapter 2).

Adjuvants have been tested since the 1970s (Moffett and Morton, 1973, 1975), and these studies found significant effects of surfactant adjuvants on honeybee drowning events when added to the bees' water supplies, and commensurate repellence from the spiked water. Moffett and Morton (1975) then expanded upon the repellence seen in the prior study, finding that adjuvants could repel honeybee visitation to water sources for up to six months but did not deter visitation to sprayed flowers. A lack of deterrence to sprayed flowers means bees will not avoid contaminated flowers, and as such will be exposed to higher levels of surfactants. These types of studies have not been repeated since, meaning we do not know if the new generations of 'inert' ingredients could similarly be causing honeybee drownings and repellence.

Exposure to adjuvants is not limited to contamination of water sources, as farmers spray adjuvants in a range of situations, and labels do not include any guidance for reducing bees' exposure. As such, label guidance allows for direct overspray of bees, which could cause mortality through contact exposure. Contact exposure occurs when a bee is exposed to spray droplets of a pesticide, or when it lands on a recently sprayed surface such as a flower or leaf.

In experimental studies, this is often simulated by either using a spraying apparatus to mimic direct overspray of bees, or by pipetting 2µL of the pesticide onto the dorsal side of the thorax/abdomen of anesthetized bees (OECD 214 (OECD, 1998)). Using a Potter spray tower, which replicates recommended spraying apparatus, two surfactant adjuvants, Pulse and Boost, were found to cause 100% mortality in honeybees at 40-50% of the label recommended concentration (Goodwin and McBrydie, 2000). The use of a Potter spray tower and use of label recommended concentrations makes this study reasonably representative of in-field application, although the application rate (L/ha) used is likely an overestimate of realistic application, because they used an equivalent rate of 2,000L/ha for most experiments which is an unrealistically high application rate in nearly all setting. This suggests that these substances are highly likely to be driving mortality in the field given that bee exposure to surfactant adjuvants is high. However, as only two studies have measured surfactant exposure, our understanding of how bees are exposed to surfactants is highly limited.

When testing the toxicity of surfactants as adjuvants, the methodology chosen is likely to influence the size of the observed effect. The standard contact toxicity test for honeybees, OECD 214, has been used to determine the toxicity (hazard) of both Silwet L-77 and Triton X-100, with LD_{50}s of 357µg and 1436µg respectively (Chen et al., 2019). This can be used to inform risk management strategies by allowing comparison of the toxicity with other substances. Donovan and Elliott, (2001) used OECD 214 to test the toxicity of several adjuvants, mostly surfactants, on honeybees and found no significant mortality from any substance. However, the dosing regime lacked the range needed to detect lethal effects and is insufficient to justify the conclusion that the substances tested were 'non-toxic to honey bees'. In a separate study, Sims and Appel (2007) sprayed a single acute dose of alcohol ethoxylates onto honey bees, *A. mellifera*, causing 100% mortality. While some alcohol ethoxylates are used in agricultural formulations, the authors made no mention of the substances tested being agriculturally relevant and the product tested was a cleaning product, Tomadol 23-1. It is for these reasons that the study is excluded from the conclusions of this systematic review.

Chronic oral toxicity of surfactants has been tested on honeybees in two studies. Moffett and Morton (1973) found two out of 7 adjuvants/surfactant co-formulants caused mortality at the

very high exposure level of 1,000ppm in nectar over 60 days (nearly equivalent to drinking in-tank mix for the entire honeybee worker lifespan). At 10 and 100ppm, no significant difference was detected from the control even over the full 60-day exposure period. In contrast, Chen, Fine and Mullin (2018) found that three trisiloxane surfactants at 100ppm reduced survival over an 8- or 10-day period. There was a clear effect of the class of surfactant, with trisiloxane surfactants causing >90% mortality relative to the control, while alkylphenol polyethoxylates and fatty amine polyethoxylates surfactants caused less than 20%. These results indicate that the hazard surfactants pose could be mitigated by redesigning formulations/adjuvants to choose the safer options. A dose-dependent relationship between the surfactant adjuvant Dyne-Amic and honey bee larval mortality was observed in Kordecki and Johnson (2019), however the study lacked a control and so was excluded from the systematic review.

The effects of pesticides are not limited to mortality, and a vast body of research now documents the importance of sub-lethal impacts of agrochemicals for social bees (Straub, Strobl and Neumann, 2020). For example, impairment of learning ability may impact upon foraging efficiency (Raine and Chittka, 2008), which may then impact colony reproductive success. 10 studies have measured sublethal effects of 'inert' ingredients on bees, providing more information on their effects on fitness. Ciarlo et al. (2012) tested acute 20µg doses of several adjuvant products individually on honeybee learning using the proboscis extension reflex methodology. In the field, a honeybee feeding for just two seconds on sprayed tank mixture (which can be sprayed onto flowering crops or weeds) would imbibe a 20µg dose of the surfactant adjuvants tested (Ciarlo et al., 2012). All 20µg doses of surfactant adjuvants impaired learning, but crop oil concentrates did not, suggesting that the different classes of 'inert' ingredient are toxicologically distinct.

Another important sublethal effect in social bees is queen rearing success, with reduced queen production being likely to reduce colony fitness. However, in the only study so far to examine this question, Johnson and Percel (2013) found no effect of the surfactant adjuvant Break-Thru, at 200ppm in pollen, on several metrics of honey bee queen rearing success.

While the studies described above have looked at 'inert' ingredients individually, pressures on bee health are multifactorial (Vanbergen and the Insect Pollinators Initiative, 2013, Goulson et al., 2015, Main et al., 2020, Siviter et al., 2021), with novel stressors like agrochemicals adding to pre-existing stressors like parasites. Consequently, we may only be able to appreciate the impact pesticides have when we understand how they interact with other stressors. In only one study has the interaction between an 'inert' ingredient and a stressor other than another agrochemical been tested. In a fully crossed experimental design, Fine, Cox-Foster and Mullin (2017) spiked honeybee larval diets with 10ppm of the surfactant adjuvant Sylgard 309 and a representative dose of a mixed virus inoculum. The surfactant adjuvant was found to increase black queen cell viral titre significantly, demonstrating an interaction between the stressors. Both stressors alone reduced larval survival, causing failed moults, melanisation and other developmental abnormalities. When combined the stressors acted synergistically, causing more larval mortality than the additive impacts of either stressors relative to the control.

The systematic review conducted returned no studies on bumble bees (*Bombus spp.*), which is alarming given their agricultural and ecological importance (Potts et al., 2016). Only two studies have tested the effects of 'inert' ingredients on bee species other than honeybees, both of which focussed upon solitary species. Ladurner et al. (2008) tested the effects of the surfactant adjuvant Dyne-Amic on *Osmia lignaria* nesting behaviour and reproduction and reported no lethal or behavioural effects of Dyne-Amic. In contrast, Artz and Pitts-Singer (2015) tested the effects of the surfactant adjuvant N-90 on both *O. lignaria* and *Megachile rotunda* when sprayed on *Phacelia tanacetifolia* and *Sinapis alba* at label-recommended rates. In flight cages with the sprayed crops, nest recognition ability in both species was significantly impaired by N-90. While no mortality was found, these results are likely to be conservative, as the N-90 spray was applied at night when bees were not foraging, whereas label guidance for N-90 is unlikely to mandate night application, and so realistic field usage may result in direct contact with the spray, rather than residues that may have dried by the time bees become active.

Changing the time of application is one approach to reducing bees exposure, another is to apply a substance that will repel bees from visiting recently sprayed crops. Mayer (1997)

sprayed trees with methyl salicate to test if it could repel bees from visiting trees recently sprayed with insecticides. They found methyl salicate to be unsuccessful at repelling honey bees for a meaningful time. How this fits within the context of the results and definitions of 'adjuvant' and 'inert' given here is ambiguous, and as such has been excluded from the results, but is nonetheless mentioned here for comprehensiveness.

Beyond the research into surfactant adjuvants listed above (and Ciarlo et al. (2012), which tested the effects of crop oil concentrates on honey bee learning ability) the only other groups of 'inert' ingredients tested have been the two solvents NMP and DMSO. These solvents are alternatives to one another with one producer of DMSO advertising it as safer and less toxic than NMP (Gaylord Chemical Information Sheet). Both NMP and DMSO are widely used solvents in agricultural formulations (Zhu et al., 2014, Gaylord Chemical Information Sheet).

All work on oral exposure to NMP has used a chronic feeding regime whereby NMP was administered through sucrose, while the residue work has measured NMP in pollen, and as such it is difficult to assess the field realism of the exposure regimes in the experimental work. As no residue analyses of field realistic NMP nectar concentrations are currently available, a wide range of concentrations (0.537- 10,000ppm) have been used in the exposure regimes in experimental work. The first study to assess NMP toxicity to honey bee larvae was Zhu et al. (2014), which found 50% mortality within 12 hours at 10,000ppm; however, in the absence of a control, these results cannot be interpreted (and as such this study is excluded from the systematic review results). When repeated, in a study by Fine et al. (2017), 100ppm of NMP caused significant larval mortality compared to the control, although mortality did not reach 50% over the 20-day trial period. In contrast, adult honeybees only experienced significant mortality at doses as high as 5,000ppm treatment (Fine and Mullin, 2017), which is unlikely to be a field realistic chronic exposure. This suggests that larvae are more susceptible to NMP than adults. The effects of chronic exposure to 500ppm NMP for 7-10 days on honeybee colony health was also investigated by Fine et al. (2017). This dose is above the 100ppm that is known to cause larval mortality, but below the 5,000ppm that causes adult mortality. In this study, NMP inhibited colony weight gain and emerging forager counts, which is most likely to be caused by larval mortality and knock-on effects on colony foraging.

To investigate whether higher impacts of NMP on larvae were a function of differential detoxification, Fine and Mullin (2017) fed honeybee workers and larvae 200ppm NMP for six days and quantified residues of the NMP and its metabolites from the adults and larvae. They found that larvae were less able to detoxify the NMP, and this may explain the higher sensitivity of larvae to NMP. Using OECD 214, NMP was found to have an acute contact LD_{50} greater than 2,000µg per honeybee (Chen et al., 2019). This finding suggests NMP is of negligible toxicity when applied via acute contact.

DMSO has received less attention than NMP, with only two studies assessing its toxicity to bees. Moffett and Morton (1973) found that DMSO produced no significant lethal effects in honeybees with chronic exposure of 1,000ppm for 60 days. Milchreit et al. (2016) found mixed effects of chronic oral exposure (500ppm) on honeybee brood development, with no detriment to fitness clearly demonstrated. Together, these results support the producer's assertion that this substance is less toxic than its alternative NMP (Gaylord Chemical Information Sheet). If this is substantiated in directly comparable trials, DMSO could be used to replace NMP as a solvent to reduce the toxicity of pesticide formulations to bees.

A call to reprioritise research into 'inert' ingredients

Research into the effects of pesticides on bees is disproportionately focussed on active ingredients, with 'inert' ingredients receiving significantly less attention. This is most clearly visible when considering the number of studies focussing on them relative to the best studied pesticide class, insecticides. For example, a single active ingredient, the neonicotinoid imidacloprid, was the subject of 168 studies as of 2015 (Lundin et al., 2015). This dwarfs the literature on 'inert' ingredients, with the systematic review here finding just 16 studies up to 2021. The allocation of research is partially explained by the intended purpose of insecticides- to kill insects. However, as I detail above, despite 'inert' ingredients not being designed to kill insects, they can have unintended consequences on bee health.

If bee ecotoxicological research is an applied science with the aim of understanding the risks pesticides could pose to bees, the optimal allocation of research effort to substances should

match the potential risk each substance poses. This risk is a combination of the hazard posed to bees and the likelihood of exposure. The hazard is likely greatest with insecticides. However, exposure is likely to be greatest with 'inert' ingredients that are used in far higher quantities (Mullin et al., 2015), with little in the way of exposure mitigation. The current allocation of research effort has focussed very strongly on the hazard posed by insecticides, without recognising that 'inert' ingredients have vastly higher exposure levels. This means that the allocation of research is primarily based on hazard, not risk as it should be.

	Hazard	Exposure	Risk
Insecticide	High	Low- Stringent mitigation measures	Intermediate
'Inert' ingredients	Poorly characterised but non-negligible	Very high- Little to no mitigation measures	Intermediate, but poorly characterised

Table 1. Detailing the hazard, exposure and risk insecticides and 'inert' ingredients pose to bees. Risk = Hazard * Exposure.

As illustrated in Table 1, while the hazards and exposures of insecticides and 'inert' ingredients differ, their risk to bees could be equivalent. As such, research effort should be reallocated to inert ingredients to characterise their exposure and hazard to bees, after which the benefits of further research can be evaluated.

To be clear, research into insecticidal active ingredients is in my opinion clearly justified, but a reallocation of resources to better reflect the risks bees face in the wild would encompass 'inert' ingredients as well. Applied bee pesticide research would therefore benefit from allocating resources to agrochemicals in proportion to their potential risk to bees. This would require research into large numbers of chemicals that may have never been tested on bees before. I propose that the potential, and likely impacts of these widely applied substances on bee health represents a key knowledge gap that urgently requires research attention and funding.

5.4 Conclusion

The literature reviewed above raises a number of concerns around the impacts of 'inert' ingredients on bee health and productivity at the individual and colony levels. What little research we have on 'inert' ingredient residues in nature shows them to be widespread, and at high concentration (Chen and Mullin, 2013, 2014, Fine et al., 2017), although our understanding of what the normal concentration range of 'inert' ingredients is in agricultural systems is underdeveloped. More research into the environmental pervasiveness and persistence of 'inert' ingredients would inform future experimental research on appropriate dosing regimes and expand our understanding of the risk they pose to bees. Importantly, and in addition to this limited understanding of environmental residues, the research identified here demonstrates that 'inert' ingredients are not ecotoxicologically benign, and as such they should be subject to greater regulation.

'Inert' ingredients drive mortality through multiple exposure routes, synergise with other stressors, and cause sublethal effects. While I call on regulators to require testing of 'inert' ingredients on bees, I also caution that the current regulatory testing system is ill-equipped to test the effects of 'inert' ingredients. Current regulatory testing exclusively uses methodologies designed for neurotoxic insecticides, which may not properly characterise the risks of 'inert' ingredients to which bees face considerably higher exposure. Given that surfactants have been identified as causing both sublethal (Ciarlo et al., 2012, Artz and Pitts-Singer, 2015) and synergistic effects alongside other stressors (Fine, Cox-Foster and Mullin, 2016), a regulatory testing approach that measures sublethal effects and incorporates multiple stressors is essential.

'Inert' ingredients interact with a range of stressors, but perhaps most importantly with active ingredients. A systematic comparison of active ingredient toxicity versus whole formulation toxicity covering academic and regulatory data would give highly informative results, but is outside of the scope of this systematic review. As prior reviews have demonstrated (Mullin et al., 2015, Nagy et al., 2020), formulations are commonly more toxic to non-target organisms than active ingredients, suggesting that the term 'inert' ingredients may not be appropriate. In fact, the use of the words 'inert' or 'inactive' to describe co-formulants and adjuvants posits

that they are toxicologically benign substances. The research collated here demonstrates that this is not true for all such substances and highlights a lack of data for many more, although there is currently too little evidence to make broad conclusions about 'inert' ingredients in general, or for any bee species, particularly any species other than honey bees. As such I would suggest that the terms 'co-formulant' or 'adjuvant', where appropriate, are better descriptors of the substances because they are neutral regarding their toxicological activity.

Just as the language used to describe 'inert' ingredients does not reflect their potential toxicity, neither does the legislation regulating them. Legislation that protects formulation composition as trade secrets hampers research into the impacts of 'inert' ingredients (EC, 2009, Weinhold, 2010, Mullin et al., 2015, Chapter 2), as such publication of formulation composition would be a critical step forward for environmental risk assessment. Further, full disclosure of ingredients would improve transparency and build trust for both consumers and farmers (Mullin et al., 2015, Chapter 2).

Progress has come with the recent European Commission legislation on co-formulants (EC, 2021), where the ostensible aim is to ban co-formulants harmful to humans or the environment. However, the legislation will only effect change if the European regulatory process is adapted accordingly. EFSA have made progress in this area with proposals to regulate by product, and with explicit consideration of the co-formulants (EFSA, 2018), but this has yet to become practice. The progress in regulating co-formulants has almost exclusively been driven by human toxicity concerns, with little consideration given to pollinators and other non-target organisms (see EFSA, 2016 which considered only the human toxicity of POEA). Despite these proposals for co-formulants, adjuvants are still entirely unregulated at the European level, despite many containing the same chemicals as many co-formulants (EC, 2021).

In conclusion, evidence of 'inert' ingredients having the potential to cause mortality in bees dates back to the 1970's (Moffett and Morton, 1973), yet in the EU and US there is still no regulatorily mandated toxicity testing of 'inert' ingredients (EC, 2009). This means that the only currently available research stream is academic testing, which has produced just 16 studies to date. This represents a large gap in our understanding of pesticide ecotoxicology.

The research collated here demonstrates that 'inert' ingredients are not inert and can pose significant and pertinent risks to bee health. I call on researchers to devote more attention to 'inert' ingredients and regulators to require testing of 'inert' ingredients to ensure their safety to bees.

5.5 Acknowledgements

5.6 Data availability statement

Data is available upon request.

Chapter 6

Discussion

6.1 Summary overview

What impacts do pesticides have on wild pollinators is a contentious and important question. The field devoted to its study, pollinator ecotoxicology, is well established, with considerable research effort being devoted to it. Much of this research has been on the impacts of pesticides in isolation (Mullin et al., 2015, Cullen et al., 2018), without consideration of the broader implications of their use. Three key examples of this highlighted by my research are: firstly, research typically focusses on active ingredients, while all pesticide application in agriculture uses formulations. Secondly, most research is conducted on healthy animals, yet in the wild pollinators like bees are frequently exposed to other stressors. And finally, research is overwhelmingly directed to chemical groups that are already heavily researched by regulators (insecticides), and already have very strict mitigation measures in place, leaving other substances understudied. For the field of pollinator ecotoxicology to realise its goal of informing the protection of wild pollinators it needs to move beyond these limitations, and to look at the effects of pesticides through a wider lens.

Summary of thesis data chapters

The first data chapter in this thesis, Chapter 2 challenges the notion that studying the impacts of an active ingredient alone is enough to understand the risk it poses with real world use. Several formulations of glyphosate-based herbicides were found to be highly lethal to bees with contact exposure, yet others, with identical active ingredient compositions were not. While the responsible chemical(s) for the toxicity could not be determined due to trade secrecy regulations, co-formulants were identified as the cause of the toxicity. This research highlights that classes of pesticide other than insecticides can also pose substantial hazard to non-target organisms.

While glyphosate was found not to be responsible for the mortality observed in Chapter 2, other exposure routes were investigated to build a more complete understanding of its effects. Chapter 3 looked at the impacts of two concurrent stressors on bee health, glyphosate and *Crithidia bombi*, expanding upon prior testing that has principally looked at

stressors in isolation. Neither stressor was found to have an observable impact on bee health, alone or in combination. This indicates that while pollinators do experience multiple concurrent stressors in the field, they do not necessarily interact to the detriment of pollinator health.

Non-insecticidal pesticides are not limited to herbicides, and the impacts of fungicides on bee health was also investigated in Chapter 4. This chapter again finds toxicity of a formulation not explained by the active ingredient. A popular fungicide was found to cause a range of sublethal damage to bumble bees, with the chemical responsible being a co-formulant surfactant/emulsifier. The protocol used expanded upon an established regulatory protocol, with the inclusion of sublethal metrics to allowed for a more nuanced detection of effects.

The final data chapter, Chapter 5, summarises the state of knowledge on adjuvants and co-formulants impacts on bees, contextualising the results of chapters 2 and 4 within the wider literature. A key finding was that there are considerable knowledge gaps; research was almost exclusively limited to honey bees, and the effects of surfactant adjuvants. Further, the environmental fate of adjuvants and co-formulants was found to be incredibly poorly resolved, limiting the capacity for research to simulate field realism. This precludes the type of testing conducted in Chapter 3 which used field residue data to inform laboratory testing.

The research presented in this thesis finds major knowledge gaps in pollinator ecotoxicology's efforts to protect wild pollinators. This research goes only a small way to addressing these knowledge gaps and highlights more questions than it answers. Below I detail what can be broadly concluded from this work, what future research could be inspired by it, and what specific tangible recommendations are supported by its findings.

6.3 Key topics

How regulators and academics research the impacts of pesticides on pollinators is disconnected from the reality of how pesticides are used in the field. Below I discuss four key topics of ways in which research could better reflect real world impacts of pesticides.

Active ingredients, formulations, or 'inert' ingredients, what to test?

One large difference between pesticides as used in research, and pesticides as used in agriculture is the active ingredient versus formulation split, with the former being researched (Cullen et al., 2018) and the latter being applied. Studies comparing active ingredient toxicity to formulation toxicity have consistently found formulations to be more toxic (reviewed in Mullin, 2015 and Nagy et al., 2021). This difference is caused by the co-formulants in a formulation, either having toxicity alone (chapters 2, 4 and 5, Fine et al., 2017), or by synergising with the active ingredient (Mullin, 2015, Nagy et al., 2021). Formulations are also commonly applied alongside adjuvants, which can be toxic alone (Moffet and Morton, 1973, 1975, Goodwin and McBrydie, 2000, Mullin et al., 2015, Fine, Cox-Foster and Mullin, 2017) or in synergy with the formulation (Mullin, 2015). The most common research approach, of testing just the active ingredient, does not reflect the real toxicity of pesticides as used in agriculture, where they are sprayed alongside co-formulants and adjuvants.

The research in chapters 2, 3 and 4 demonstrates that some active ingredients can show no observable toxicity, while the 'inert' ingredients in the same formulation can be incredibly toxic (chapters 2 and 4). This is particularly notable in Chapter 2, where two formulations, with identical active ingredient compositions, but distinct co-formulant mixtures, have drastically different toxicities. This is also visible in Chapter 4, where if the experiment had considered only the active ingredient, no toxicity would have been observed, but by using the formulation, and each individual co-formulant, considerable toxicity was observed. Together, this demonstrates that an explicit focus on active ingredients alone fails to replicate the impact of realistic exposure to pesticides.

While the use of formulations does present several benefits, namely increased field realism, it also has limitations and is not applicable to all study designs. For example, a common experimental methodology, chronic oral exposure, is not suited to the use of formulations. This is because once a formulation is sprayed, each ingredient will have a different half-life and environmental fate (Katagi, 2008), meaning that persistent exposure to the formulated mixture of ingredients would be increasingly unrepresentative of real exposure as time progresses. Contrastingly, some types of testing like contact toxicity testing are well suited to using formulations because they mimic formulation exposure directly as it is sprayed. Acute exposure broadly is more suited to using formulations because it tends to replicate exposure to pesticides shortly after spraying.

While some methodologies are well suited to testing formulations, there are some broader methodological problems when testing formulations. For instance, commercial formulations are highly changeable, with products with the same name and manufacturer ID frequently having slight formulation changes, limiting replicability (UK Health and Safety Executive, 2021b). Further, access to pesticide products is limited, with many products being only sold wholesale (personal observation), further limiting replicability. Finally, testing of pure active ingredients is required for some experimental designs, such as when researchers want to explicitly isolate the cause of an effect (as in Chapter 5). So, while more research could use formulations, they are not appropriate for all testing. Pesticides, as sprayed in agriculture, often do not contain just diluted formulations, they can also include adjuvants as a cost-effective way to boost efficacy (Hazen, 2000).

Adjuvants, as Chapter 5 demonstrates, can be toxicologically hazardous, with potential to reduce honey bee learning (Ciarlo et al., 2012), increase viral titres (Fine, Cox-Foster and Mullin, 2017), cause considerable drowning events for honey bee colonies (Moffet and Morton, 1973, 1975), and cause mortality when directly sprayed onto bees (Goodwin and McBrydie, 2000). As such, to quantify the hazard pesticides cause when used in the real world, the additional cost of adjuvant toxicity must be considered (Mesnage and Antoniou, 2018). This poses several challenges, as a range of adjuvants can be used with each formulation. Unfortunately, there is no data for any territory bar the state of California on adjuvant usage

(Mullin et al., 2015), making it hard to inform treatment regimes with real world usage. Including adjuvants alongside formulations would improve the real-world applicability of contact toxicity testing, and some acute oral testing, but again is of limited value to chronic oral testing due to the different compounds having different levels of ecological persistency (Katagi, 2008). Testing of adjuvants alone can be conducted with chronic exposure, although as Chapter 5 identifies, there is little real-world data to inform these exposure regimes.

Chapter 2 could have tested Roundup formulations sprayed alongside an adjuvant, which is commonly how Roundup is sprayed in agriculture. However, because of the difficulty in purchasing adjuvants, they were not included. Because the toxicity observed in Chapter 2 is likely caused by a surfactant co-formulant, it is highly likely that had a surfactant adjuvant been added to the mix more mortality would have been observed. This is a limitation of my work, as it may have underestimated the worst case for toxicity, and this is a topic for future research to continue. While the effects of formulations is a largely unaddressed issue in the field, the interaction of pesticides with other stressors is also largely unresolved.

Testing stressors of bee health in isolation

All regulatory research, and nearly all academic research is conducted using healthy, unparasitized, well fed bees, who have never had pesticide exposure prior to the experiment (EFSA, 2012, 2013, 2021, EPA, 2014, Siviter et al., 2021). This is very intuitive for testing as it allows for any effect observed to be directly attributable to the treatment chemical. Yet it also fails to replicate the considerable exposure to stressors that bees in the wild will face (Gillespie, 2010, Vanbergen and the Insect Pollinators Initiative, 2013, Hicks et al., 2018, Main et al., 2020, EFSA, 2021, Siviter et al., 2021).

Honey bee hives used for ecotoxicology testing are given health checks to ensure parasitism is at a low level (EFSA, 2013, Odemer et al., 2021), they are deliberately shielded from nutritional deprivation, with supplementary sugar, and they are shielded as best as possible from external pesticide exposure. For bumble bees the situation is even more artificial, as bees used in testing are sourced from commercial suppliers that produce colonies largely free

from any of the aforementioned stressors (OECD, 2017). They are sheltered to the extent that the bumble bees used in most ecotoxicology testing will have never left their colony box. This lack of exposure to the stressors bees in the wild face is a real concern because these additional stressors could predispose bees to being susceptible to pesticides, and this would be missed by testing unstressed bees. As such, the testing done on healthy bees could underestimate the hazard a pesticide poses to stressed wild bees (EFSA, 2021, Siviter et al., 2021).

My testing in chapters 2 and 4 is a victim of this, looking exclusively at a single stressor at a time. Chapter 3, however, addresses this question directly by expanding the number of stressors from one to two. While two stressors does not perfectly simulate reality, it builds progress towards that goal. One critical issue with addressing this problem is that for every additional stressor added to the experiment the sample size must double (if using the fully crossed methodology required to properly interpret the results). This makes testing more than two concurrent stressors exceedingly difficult to do in well powered experiments. This is seen in Chapter 3 especially, as the sample sizes chosen were typically at the limit of what one investigator could handle in one batch, meaning that to expand the scope of the study to a third stressor would have required multiple investigators, adding complexity, or a multi-batch design, reducing the experiments power. Further, considerable expertise is required to properly simulate each additional stressor, especially with stressors of different groups, i.e., parasites, nutrition, pesticide, thus increasing the chances of poor study design. It is for these reasons that multi-stressor testing, while possible, is not conducted more frequently.

Chapter 3 tested two very common stressors bumble bees can face, the world's most widely used pesticide active ingredient, glyphosate (Duke and Powles, 2008, Duke, 2018), and a parasite with perhaps the highest prevalence in wild *B. terrestris*, *C. bombi* (Shykoff and Schmid-Hempel, 1991, Korner and Schmid-Hempel, 2005, Rutrecht and Brown, 2008, Gillespie, 2010, Jones and Brown, 2014, Hicks et al., 2018). Ultimately, the testing found no effect of either stressor alone, or any synergy between them, in a range of metrics. So, while it is true bees will be under multiple stressors simultaneously, this may not necessarily tangibly impact them for all combinations of stressors (Siviter et al., 2021).

The lack of synergism in Chapter 3 illustrates that multi-stressor interactions need to be assessed on a case-by-case basis, as confirmed by research in honey bees that showed that chronic glyphosate exposure alongside the parasite *Serratia marcescens* can synergise to cause considerable lethality (Motta, Raymann and Moran, 2018). As such, in bumble bees it is entirely possible that a more virulent parasite, like *Nosema bombi*, may have a synergistic impact with glyphosate in adult workers. It is also possible that at different lifecycle stages, particularly energetically demanding stages like queen hibernation where parasite stress can manifest (Brown, Schmid-Hempel and Schmid-Hempel, 2003, Yourth, Brown and Schmid-Hempel, 2008), stressors could be more likely to synergise.

Testing pesticides at a range of life history stages or alongside a range of parasites is relatively simple in comparison to the innumerable potential interactions between pesticides. There are 474 active ingredients registered within the EU (EC, 2021), and 2,892 distinct pesticide products registered in the UK alone (UK Health and Safety Executive, 2021b). The average American honey bee wax contains 6 detectable pesticide active ingredient residues, although there can be up to 39 (Mullin et al., 2010). So exhaustive testing of all possible combinations of pesticides, especially considering more than two concurrent stressors, is patently infeasible. It would be more feasible to test all combinations of pesticides licenced to be sprayed from the same tank mixture, which would cover contact toxicity and most acute oral toxicity, however even this would drastically inflate testing requirements. It is worth noting that current EU regulation requires any pesticide formulation with more than one active ingredient to be submitted to ecotoxicity testing as if it were a new substance, and as such this does cover the most extreme cases of multi-pesticide co-exposure (EC, 2009).

While regulation would be far more representative of reality with comprehensive multi-stressor testing, or even a highly limited version of this, it is unlikely this is the best possible allocation of resources. Regulation has yet to address much more basic questions like the impacts of adjuvants (Mullin et al., 2015, Chapter 5), the impacts of non-representative formulations (Chapter 2, Chemical Research Division, 2013), the sublethal impacts of active ingredients (Straub, Strobl and Neumann, 2020, Chapter 3), and the impacts of pesticides in general on the wide diversity of bee species (Potts et al., 2016), questions that are more feasible to answer. So given that multi-stressor testing is not possible to do comprehensively,

and not of the highest priority to perform in a limited version, other approaches to tackling multiple stressors could be explored. Incorporating conservative risk factors into pesticide regulation is one such approach.

An example of a risk factor, as proposed for use in pesticide regulation, is the risk factor to account for interspecies pesticide sensitivities. EFSA has proposed use of a 10x risk factor, whereby it assumes that honey bees (the primary species upon which pesticide testing is conducted) are 10x less susceptible to a pesticide than the most susceptible bee species (EFSA, 2012). The mitigation measures for a substance would then be designed based on the assumption that it is 10x more harmful to other species that it has to honey bees, in theory protecting all bees who with less than 10x as sensitive. This proposal has not yet been adopted.

This same approach could be applied to additional stressors. The degree of synergism of key stressors with a representative selection of pesticides could be derived, and from this a sensible risk factor derived. This would be difficult because of the case-by-case nature of multi-stressor synergies, but with a battery of tests the worst-case synergism could be found, and this value used as the risk factor in an attempt to be conservative. While this suggestion does involve considerable workload, it is eminently more feasible than systematic screening of all combinations. If regulation accounted for the worst-case impacts of multiple stressors, researchers would be able to better study the impacts of individual pesticides. This would potentially free up resources such that the hazards of understudied substances could be quantified.

Should pollinator ecotoxicology focus on insecticides?

In relation to testing on bees, as of 2015 there were 216 papers directly testing neonicotinoid insecticides, (Lundin et al., 2015), as of 2018 there were only 89 papers testing any herbicide or fungicide, and as of 2021 there were just 17 papers testing co-formulants or adjuvants (16 papers identified in chapters 4, and the unpublished research in Chapter 5). This huge disparity highlights the attention given to insecticides, and contrasts very heavily with the

usage of these substances. Globally herbicides, fungicides, adjuvants and co-formulants are used in considerably greater quantities, and over considerably greater areas, than insecticides. However, as described in the introduction, usage by weight or area does not fully capture the toxicity of a substance. The metric of toxic load approaches an assessment of risk, but falsely assumes the weight of application is a proxy for exposure, which is not true because different substances have different mitigation measures in place. When considering; usage, mitigation measures and hazard, a more complex picture of risk emerges.

Usage:

By weight or any measure of area, co-formulants and adjuvants unarguably have the highest usage, followed by herbicides and fungicides (approximately joint)(FERA, 2021, FAOSTAT, 2021), and with insecticides being least used (FERA, 2021, FAOSTAT, 2021).

Mitigation measure:

Co-formulants and adjuvants, as well as herbicides and fungicides, have no specific mitigation measures in place to protect bees from exposure (EFSA, 2012, 2013). Although, because herbicides are not applied to crops (excluding genetically modified herbicide resistant crops) bees exposure to them will be lower. In most regulatory regimes insecticides have stringent mitigation measures in place to reduce bees' exposure, and although this does not eliminate exposure entirely, it heavily limits it (EFSA, 2012, 2013).

Hazard:

Hazard is highest with insecticides; as compounds designed to kill insects their potency is obvious. Herbicides, fungicides, co-formulants and adjuvants can be hazardous to bees, although their toxicity is very poorly characterised (Cullen et al., 2019, chapters 4 and 5). Some substances that regulators would describe as 'bee-safe' can be highly hazardous, as Chapter 2 identifies, highlighting that the understanding of hazard as derived by regulatory testing is flawed.

So, bringing this all together, it could well be argued that an integrated approach, looking at risk, which is a composite of hazard and exposure (itself a composite of usage and mitigation measures), would not weight research so heavily in favour of insecticides, principally because

of the mitigation measures in place reducing bees exposure to them. However, it is far too early in the nascent fields of co-formulants and adjuvants (Chapter 5), and, to a lesser degree, herbicides and fungicides (Cullen et al., 2018) for this conclusion to be drawn. Several insecticides, like the neonicotinoids, have been demonstrated to be dangerous to pollinators, despite their mitigation measures (Rundlöf et al., 2015, Woodcock et al., 2016, McArt et al., 2017, Tsvetkov et al., 2017), leading to bans in the EU (EC, 2013). These bans were a direct result of the intensive research (EFSA, 2018, Goulson, 2018). So, while it is also possible that other pesticide products may also be dangerous, it is premature to call for a wholesale reallocation of research. A middle ground with a greater emphasis on preliminary research into these nascent fields, would allow for the relative weighting of each group's usage, exposure and hazard to be better quantified. If residue measurements of these understudied groups confirm the suspicion that the lack of mitigation measures causes massive exposure, research using the residue values derived could determine if field realistic exposure is damaging to bees. This would then allow for direct comparison to the effects of insecticides to be drawn and for the appropriate allocation of research to be decided.

6.4 Recommendations

Clear and transparent labelling of all pesticide ingredients

One key recommendation that stems primarily from Chapter 2 is that pesticide labels should list every ingredient present in them. The current system protects formulation composition as a trade secret (Weinhold, 2010), with the assumed reason for this being to promote innovation. There is merit to promoting innovation in pesticide formulations, as by changing the co-formulant composition it could be possible to achieve equivalent results with a lower dose, therefore reducing the environmental consequences (Hazen, 2000). However, the argument of protecting innovation holds little water as it assumes that the lack of labelling effectively protects the formulation composition. LC-MS and other analytical tools can be used to discern the chemical composition of a formulation (Chen and Mullin, 2015), and these tools are readily available to competing agrochemical companies. As such, if secrecy is the only barrier to protecting the composition it is unlikely to be effective. Secrecy does however function to effectively exclude academics from studying formulations, because of the costs, skills and equipment needed to analyse each individual formulation. Further, the need for academics to publicly share their results could open them up to lawsuits if they revealed proprietary information (formulation composition is explicitly protected in EU law (EC, 2009)). It is my view that the most appropriate balance between fostering innovation and promoting transparency is to rely upon patent laws. If an innovation is truly novel, then patent laws should be utilised, or expanded, to protect that development. Patenting an innovation would prevent other companies from using the design, while still allowing researchers to openly study the formulations using it. It is, or would be, a more effective tool than secrecy and would strike a better balance between corporate needs and societal values like transparency (Weinhold, 2010).

Submit adjuvants to ecotoxicology testing

Chapter 5 summarises the small amount of existing literature on the effects of adjuvants on bees. The research demonstrates that even at field realistic concentrations, adjuvants can have negative consequences on honey bees, while data is lacking for other species. Despite this hazard, adjuvants are not tested on bees whatsoever during regulation. This means that potentially harmful substances are applied broadly, with no mitigation measures, and no regulatory assessment of hazard at all. So, while regulatory testing of non-insecticidal compounds has dubious merit, discussed below, it is nonetheless better than no testing at all. As such regulators should require adjuvants to be submitted to toxicity testing equivalent to representative formulations.

Re-evaluate the applicability of current regulatory testing to non-insecticidal compounds

Regulatory testing of pesticides uses protocols designed to quantify effects of highly hazardous insecticides. Concepts like the LD_{50} work well for insecticides, as they are typically sufficiently hazardous to cause 50% mortality at low doses. This is because they are designed to target pest insects, and as such have non-target toxicity to beneficial pollinator insects. However, non-insecticidal pesticide compounds and formulations can also be dangerous to bees (Cullen et al., 2018, chapters 2, 4 and 5), without causing mortality at low doses. One example of this is found in Chapter 2, where glyphosate-based herbicides were found to be safe for contact exposure to bees by regulators using a protocol designed for insecticides (EFSA, 2015a, OECD, 1998). This protocol involved pipetting 2μL of solution on a bee's back (with up to 200μg of active ingredient dissolved within). For insecticides with neurotoxic action this is sufficient for mortality to occur, but for herbicides with mechanical action (smothering of spiracles) this quantity is insufficient. The protocol limits the upper dose at 2μL, artificially imposing a cut off for toxicity, which may be below real-world exposure levels.

To fix this issue, toxicity testing could be segregated by mode of action, to better reflect differences between groups. As the hazard of insecticidal compound's is well quantified by

existing protocols (excluding sub-lethal effects), this should not be changed. Other compounds, like herbicides, fungicides and adjuvants, should be submitted to additional testing at higher exposure levels to quantify their toxicity. Ideally, this would be conducted using different protocols like using Potter spray towers, to better simulate direct overspray, and be tailored to the substance being tested.

Because of these incongruities in the system, research should be allocated to developing novel methodologies to properly quantify the impacts of non-insecticidal compounds, which reflect the lower potencies they have, but also reflect the higher exposure levels bees face. Without proper assessment of what damage exposure without mitigation measures causes, we do not properly understand the risks non-insecticidal compounds pose to pollinators.

6.5 Conclusion

To summarise my thesis, I believe that the way we understand pesticides effects on bees is currently too limited. The assumption that only highly potent insecticides are capable of causing damage to bees is too narrow, and in direct contradiction to a growing body of evidence. Just as the European regulatory bodies incorrectly characterised the effects of the neonicotinoids, they too may be mischaracterising the toxicity of substances used in incredible abundance. The potential damage from herbicides, fungicides, co-formulants and adjuvants is a critical knowledge gap to be addressed before the role of pesticides in bee declines can be resolved.

"We bequeath to you the synthetic jungles of Hawaii and a scrubland where once thrived the prodigious Amazon forest, along with some remnants of wild environments here and there we chose not to lay waste. Your challenge is to create new kinds of plants and animals by genetic engineering and somehow fit them together into free-living artificial ecosystems. We understand that this feat may prove impossible. We are certain that for many of you even the thought of doing so will be repugnant. We wish you luck. And if you go ahead and succeed in the attempt, we regret that what you manufacture can never be as satisfying as the original creation. Accept our apologies and this audiovisual library that illustrates the wondrous world that used to be."

Edward O. Wilson, 2002- The Future of Life.

References

Abbott, V.A., Nadeau, J.L., Higo, H.A., and Winston, M.L. (2008). Lethal and sublethal effects of imidacloprid on *Osmia lignaria* and clothianidin on *Megachile rotundata* (Hymenoptera: Megachilidae*). Journal of Economic Entomology*, **101**, 784–796.

Abraham, J., Benhotons, G.S., Krampah, I., Tagba, J., Amissah, C., and Abraham, J.D. (2018). Commercially formulated glyphosate can kill non-target pollinator bees under laboratory conditions. *Entomologia Experimentalis et Applicata*, **166**, 695-702.

Adler, L.S., Michaud, K.M., Ellner, S.P., McArt, S.H., Stevenson, P.C., and Irwin, R.E. (2018). Disease where you dine: plant species and floral traits associated with pathogen transmission in bumblebees. *Ecology*, **99**, 2535–2545.

Aizen, M.A., and Harder, L.D. (2009). The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. *Current Biology*, **19**, 915–918.

Aizen, M.A., Garibaldi, L.A., Cunningham, S.A., and Klein, A.M. (2008). Long-term global trends in crop yield and production reveal no current pollination shortage but increasing pollinator dependency. *Current Biology*, **18**, 1572–1575.

Alaux, C., Brunet, J.L., Dussaubat, C., Mondet, F., Tchamitchan, S., Cousin, M., et al. (2010). Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental Microbiology*, **12**, 774–782.

Alcántara-de la Cruz, R., Cruz-Hipolito, H.E., Alfredo Domínguez-Valenzuela, J., and De Prado, R. (2021). Glyphosate ban in Mexico: potential impacts on agriculture and weed management. *Pest Management Science*, **77**, 3820-3831.

Almasri, H., Tavares, D.A., Diogon, M., Pioz, M., Alamil, M., Sené, D., et al. (2021). Physiological effects of the interaction between Nosema ceranae and sequential and overlapping exposure to glyphosate and difenoconazole in the honey bee *Apis mellifera*. *Ecotoxicology and Environmental Safety*, **217**, 112258.

Almasri, H., Tavares, D.A., Pioz, M., Sené, D., Tchamitchian, S., Cousin, et al. (2020). Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter *Apis mellifera* honey bees. *Ecotoxicology and Environmental Safety*, **203**, 111013.

Amistar Environmental Information Sheet. (2020). Available at: https://www.syngenta.co.uk/sites/g/files/zhg151/f/amistar_0.pdf?token=1511171806. Last accessed in January 2021.

Amistar Label. (2020). Available at: https://www.syngenta.co.uk/product/crop-protection/amistar-0. Last accessed in January 2021.

Amistar Material Safety Data Sheet. (2020). Available at: https://www.syngenta.co.uk/sites/g/files/zhg151/f/amistar_v18.pdf?token=15336575 64. Last accessed in January 2021.

Arretz, P.V., and Macfarlane, R.P. (1986). The introduction of *Bombus ruderatus* to Chile for red clover pollination. *Bee World*, **67**, 15–22.

Artz, D.R., and Pitts-Singer, T.L. (2015). Effects of fungicide and adjuvant sprays on nesting behavior in two managed solitary bees, *Osmia lignaria* and *Megachile rotundata*. *PLoS ONE*, **10**, e0135688.

Baer, B., and Schmid-Hempel, P. (2003). Bumblebee workers from different sire groups vary in susceptibility to parasite infection. *Ecology Letters,* **6**, 106–110.

Bailey, L. (1954). The respiratory currents in the tracheal system of the adult honey-bee. *Journal of Experimental Biology*, **31**, 589-593.

Balbuena, M.S., Tison, L., Hahn, M.L., Greggers, U., Menzel, R., and Farina, W.M. (2015). Effects of sublethal doses of glyphosate on honeybee navigation. *Journal of Experimental Biology*, **218**, 2799–2805.

Baron, G.L., Raine, N.E., and Brown, M.J.F. (2014). Impact of chronic exposure to a pyrethroid pesticide on bumblebees and interactions with a trypanosome parasite. *Journal of Applied Ecology*, **51**, 460–469.

Barribeau, S.M., and Schmid-Hempel, P. (2013). Qualitatively different immune response of the bumblebee host, *Bombus terrestris*, to infection by different genotypes of the trypanosome gut parasite, *Crithidia bombi*. *Infection, Genetics and Evolution*, **20**, 249–256.

Bartlett, D.W., Clough, J.M., Godwin, J.R., Hall, A.A., Hamer, M. and Parr-Dobrzanski, B. (2002). The strobilurin fungicides. *Pesticide Management Science*, **58**, 649-662.

Bartomeus, I., Ascher, J.S., Gibbs, J., Danforth, N., Wagner, D.L., Hedtke., et al. (2013). Historical changes in northeastern US bee pollinators related to shared ecological traits. *Proceedings of the National Academy of Sciences*, **110**, 4656–4660.

Bartoń, K. (2020). MuMIn: Multi-Model Inference. R package version 1.0.0. https://CRAN.R-project.org/package=MuMIn

Bates, D., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, **67**, 1–48.

Battisti, L., Potrich, M., Sampaio, A.R., de Castilhos Ghisi, N., Costa-Maia, M., Abati, R., et al. (2021). Is glyphosate toxic to bees? A meta- analytical review. *Science of the Total Environment*, **767**, 145397.

Bebber, D.P., and Gurr, S.J. (2015).Crop-destroying fungal and oomycete pathogens challenge food security. *Fungal Genetics and Biology*, **74**, 62-64.

Beckie. H.J., Flower, K.C., and Ashworth, M.B. (2020). Farming without glyphosate? *Plants*, **9**, 1-15.

Benbrook, C.M. (2016). Trends in glyphosate herbicide use in the United States and globally. *Environmental Science Europe*, **28**, 1-15.

Bernauer, O.M., Gaines-Day, H.R., and Steffan, S.A. (2015). Colonies of bumble bees (*Bombus impatiens*) produce fewer workers, less bee biomass, and have smaller mother queens following fungicide exposure. *Insects*, **6**, 478-488.

Blacquière, T., Smagghe, G., van Gestel, C.A.M., and Mommaerts, V. (2012). Neonicotinoids in bees: a review on concentrations, side-effects and risk assessment. *Ecotoxicology*, **21**, 973–992.

Blot, N., Veillat, L., Rouzé, R., and Delatte, H. (2019). Glyphosate, but not its metabolite AMPA, alters the honeybee gut microbiota. *PLoS One*, **14**, e0215466.

Boily, M., Sarrasin, B., DeBlois, C., Aras, P., and Chagnon, M. (2013). Acetylcholinesterase in honey bees (*Apis mellifera*) exposed to neonicotinoids, atrazine and glyphosate: laboratory and field experiments. *Environmental Science and Pollution Research*, **20**, 5603–5614.

Bornmann, L. and Mutz, R. (2015). Growth rates of modern science: a bibliometric analysis based on the number of publications and cited references. *Journal of the Association for Information Science and Technology*, **66**, 2215-2222.

Brattsten, L.B., Berger, D.A., and Dungan, L.B. (1994). In vitro inhibition of midgut microsomal P450s from *Spodoptera eridania* caterpillars by demethylation inhibitor fungicides and plant growth regulators. *Pesticide Biochemistry and Physiology*, **49**, 234–243.

Brian, A.D. (1952). Division of labour and foraging in *Bombus agrorum* Fabricius. *Journal of*

Animal Ecology, **21**, 223–240.

Brown, M.J.F., Dicks, L.V., Paxton, R.J., Baldock, K.C.R., Barrron, A.B., Chauzat, M-P., et al. (2016). A horizon scan of future threats and opportunities for pollinators and pollination. *PeerJ*, **4**, e2249.

Brown, M.J.F., Loosli, R., and Schmid-Hempel, P. (2000). Condition-dependant expression of virulence in a trypanosome infecting bumblebees. *Oikos*, **91**, 421–427.

Brown, M.J.F., Schmid-Hempel, R., and Schmid-Hempel, P. (2003). Strong context-dependent virulence in a host-parasite system: reconciling genetic evidence with theory. *Journal of Animal Ecology,* **72**, 994–1002.

Bryden, J., Gill, R.J., Mitton, R.A.A., Raine, R.E., and Jansen, V.A.A. (2013). Chronic sublethal stress causes bee colony failure. *Ecology Letters*, **16**, 1463–1469.

Buhk, C., Oppermann, R., Schanowski, A., Bleil, R., Lüdemann, J., and Maus, C. (2018). Flower strip networks offer promising long term effects on pollinator species richness in intensively cultivated agricultural areas. *BMC Ecology*, **18**, 1-13.

Cameron, S.A., Lozier, J.D., Strange, J.P., Koch, J.B., Cordes, N., Solter, L.F., et al. (2011). Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences*, **108**, 662–667.

Carvalheiro, L.G., Kunin, W.E., Aguirre-Gutiérrez, J., Ellis, W., Fox., R., Groom, Q., et al. (2013). Species richness declines and biotic homogenisation have slowed down for NW-European pollinators and plants. *Ecology Letters*, **16**, 870–78.

California Department of Pesticide Regulation. 2021. California Pesticide Sales Report Data. Available at https://www.cdpr.ca.gov/docs/mill/nopdsold.htm. Last accessed in June 2021.

Ceuppens, B., Eeraerts, M., Vleugels, T., Cnops, G., Roldan-Ruiz, I., and Smagghe, G. (2015). Effects of dietary lambda-cyhalothrin exposure on bumblebee survival, reproduction, and foraging behavior in laboratory and greenhouse. *Journal of Pest Science*, **88**, 777–783.

Cha, D.J, and Lee., S.H. (2015). Evolutionary origin and status of two insect acetylcholinesterases and their structural conservation and differentiation. *Evolution and Development*, **17**, 109–119.

Chemical Regulation Division. (2021). Formulation studies and combined risk assessment in ecotoxicology: Guidance on the need for studies and their use in risk assessment.

Available at https://www.hse.gov.uk/pesticides/resources/E/CRD-Formulation-Guidance-ecotox.pdf Last accessed in March 2020.

Chen, J., and Mullin, C. (2013). Quantitative determination of trisiloxane surfactants in beehive environments based on liquid chromatography coupled to mass spectrometry. *Environmental Science and Technology*, **47**, 9317-9323.

Chen, J., Fine, J. and Mullin, C. (2018). Are organosilicon surfactants safe for bees or humans? *Science of The Total Environment*, **612**, 415-421.

Chen, J., Mullin, C.A. (2014). Determination of nonylphenol ethoxylate and octylphenol ethoxylate surfactants in beehive samples by high performance liquid chromatography coupled to mass spectrometry. *Food Chemistry*, **158**, 473-479.

Chen, J., Mullin, C.A. (2015). Characterization of trisiloxane surfactants from agrochemical adjuvants and pollinator-related matrices using liquid chromatography coupled to mass spectrometry. *Journal of Agricultural and Food Chemistry*, **63**, 5120-5125.

Chen, L., Yan, Q., Zhang, J., Yuan, S., and Liu, X. (2019). Joint toxicity of acetamiprid and co-applied pesticide adjuvants on honeybees under semifield and laboratory conditions. *Environmental Toxicology and Chemistry*, **38**, 1940-1946.

Ciarlo, T.J., Mullin, C.A., Frazier, J.L., and Schmehl, D.R. (2012). Learning impairment in honey bees caused by agricultural spray adjuvants. *PLoS ONE*, **7**, e40848.

Cisarovsky, G., and Schmid-Hempel, P. (2014). Combining laboratory and field approaches to investigate the importance of flower nectar in the horizontal transmission of a bumblebee parasite. *Entomologia Experimentalis et Applicata*, **152**, 209–215.

Clark, T. (2000). Pesticides in Perspective. *Journal of Environmental Monitoring,* **3**, 64-69.

Cole, R.J. (1970). The application of the "triangulation" method to the purification of *Nosema* spores from insect tissues. *Journal of Invertebrate Pathology*, **15**, 193-195.

Colla, S.R., Otterstatter, M.C., Gegear, R.J., Thomson, J.D. (2006). Plight of the bumble bee: pathogen spillover from commercial to wild populations. *Biological Conservation*, **129**, 461–467.

Cox, C., and Surgan, M. (2006). Unidentified inert ingredients in pesticides: implications for human and environmental health. *Environmental Health Perspectives*, **114**, 1803-1806.

Crossland, N.O., Shires, S.W., and Bennett, D. (1982). Aquatic toxicology of cypermethrin. III. Fate and biological effects of spray drift deposits in fresh water adjacent to agricultural land. *Aquatic Toxicology,* **2**, 25

Cullen, M.G., Thompson, L.J., Carolan, J.C., Stout, J.C., and Stanley, D.A. (2019). Fungicides, herbicides and bees: a systematic review of existing research and methods. *PLoS ONE*, **14**, e0225743.

da Costa Domingues, C.E., Inoue, L.V.B., da Silva-Zacarin, E.C.M., and Malaspina, O. (2020). Fungicide pyraclostrobin affects midgut morphophysiology and reduces survival of Brazilian native stingless bee *Melipona scutellaris*. *Ecotoxicology and Environmental Safety,* **206**, 111395.

Dai, P., Yan, Z., Ma, S., Yang, Y., Wang, Q., Hou, C., et al. (2018). The herbicide glyphosate negatively affects midgut bacterial communities and survival of honey bee during larvae reared *in vitro*. *Journal of Agricultural and Food Chemistry*, **66**, 7786–93.

Danihlík, J., Škrabišová, M., Lenobel, R., Šebela, M., Omar, E., Petřivalský, M., et al. (2018). Does the pollen diet influence the production and expression of antimicrobial peptides in individual honey bees? *Insects*, **9**, 1-12.

De Luca, P.A., Bussière, L.F., Souto-Vilaros, D., Goulson, D., Mason, A.C., and Vallejo-Marín, M. (2013). Variability in bumblebee pollination buzzes affects the quantity of pollen released from flowers. *Oecologia*, **172**, 805-816.

Decourtye, A., Armengaud, C., Renou, M., Devillers, J., Cluzeau, S., Gauthier, M., and Pham-Delègue, M.H. (2004). Imidacloprid impairs memory and brain metabolism in the honeybee (*Apis mellifera* L.). *Pesticide Biochemistry and Physiology,* **78**, 83–92.

Defarge, N., Takács, E., Lozano, V.L., Mesnage, R., de Vendômois, J.S., Séralini, G.E., et al. (2016). Co-formulants in glyphosate-based herbicides disrupt aromatase activity in human cells below toxic levels. *International Journal of Environmental Research and Public Health*, **13**, 264.

DeGrandi-Hoffmann, G., Chen, Y., and Simonds, R. (2013). The effects of pesticides on queen rearing and virus titers in honey bees (*Apis mellifera* L.). *Insects*, **4**, 71-89.

Donovan, B.J., and Elliott, G.S. (2001). Honey bee response to high concentrations of some new spray adjuvants. *New Zealand Plant Protection*, **54**, 51-55.

Doublet, V., Labarussias, M., de Miranda, J.R., Moritz, R.F.A., and Paxton, R.J. (2015). Bees under stress: sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. *Environmental Microbiology*, **17**, 969–983.

Duke, S.O. (2018). The history and current status of glyphosate. *Pest Management Science*, **74**, 1027-1034.

Duke, S.O., and Powles, S.B. (2008). Glyphosate: a once-in-a-century herbicide. *Pest Management Science*, **64**, 319–325.

Dupont, Y.L., Damgaard, C., and Simonsen, V. (2011). Quantitative historical change in bumblebee (*Bombus* spp.) assemblages of red clover fields. *PLoS ONE*, **6**, e25172.

Durrer, S., and Schmid-Hempel, P. (1994). Shared use of flowers leads to horizontal pathogen transmission. *Proceedings of the Royal Society of London B: Biological Sciences*, **258**, 299–302.

European Food Safety Authority. (2018). Scientific risk assessment of pesticides in the European Union (EU): EFSA contribution to on-going reflections by the EC. *EFSA Journal*, **15**, 1367.

Environment Protection Agency. (1996). Federal insecticide, fungicide, and rodenticide act. 7, U.S.C §136 1996.

Environment Protection Agency. (2014). Guidance for assessing pesticide risks to bees. Office of Pesticide Programs, Washington DC.

European Commission. (2006). Regulation (EU) No 1907/2006. *Official Journal of the European Union,* **L396**, 1-849.

European Commission. (2009). Regulation (EU) No 1107/2009. *Official Journal of the European Union*, **L309**, 1-50.

European Commission. (2013). Regulation (EU) No 284/2013. *Official Journal of the European Union*, **L93**, 85-152.

European Commission. (2016). Regulation (EU) No 1313/2016. *Official Journal of the European Union*, **L208**, 1-3.

European Commission. (2018a). Regulation (EU) No 783/2018. *Official Journal of the European Union*, **L132**, 31-34.

European Commission. (2018b). Regulation (EU) No 784/2018. *Official Journal of the European Union*, **L132**, 35-39.

European Commission. (2018c). Regulation (EU) No 785/2018. *Official Journal of the European Union*, **L132**, 40-44.

European Commission. (2021). Regulation (EU) No 383/2021. *Official Journal of the European Union*, **L74**, 7-46.

European Food Safety Authority. (2010). Conclusion on the peer review of the pesticide risk assessment of the active substance azoxystrobin. *EFSA Journal*, **8**, 1-110.

European Food Safety Authority. (2012). Scientific opinion on the science behind the development of a risk assessment of Plant Protection Products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). *EFSA Journal*, **10**, 2668.

European Food Safety Authority. (2013). EFSA guidance document on the risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus spp*. and solitary bees). *EFSA Journal*, **11**, 3295.

European Food Safety Authority. (2015a). Conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. *EFSA Journal*, **13**, 4302.

European Food Safety Authority. (2015b). Appendix to the conclusion on the peer review of the pesticide risk assessment of the active substance glyphosate. *EFSA Journal*, **13**, 4302.

European Food Safety Authority. (2016). Request for the evaluation of the toxicological assessment of the co-formulant POE-tallowamine. *EFSA Journal*, **13**, 4303.

European Food Safety Authority. (2018). Evaluation of the data on clothianidin, imidacloprid and thiamethoxam for the updated risk assessment to bees for seed treatments and granules in the EU. *EFSA Supporting Publications*, **EN-1378**, 1-31.

European Food Safety Authority. (2021). A systems-based approach to the environmental risk assessment of multiple stressors in honey bees. *EFSA Journal*, **19**, 6607.

European Patent EP2004194B1. Azoxystrobin formulations. Available at: https://patents.google.com/patent/EP2004194B1/en. Last accessed in January 2021.

Faita, M.R., Cardozo, M.M., Amandio, D.T.T., Orth, A.I., and Nodari, R.O. (2020). Glyphosate-based herbicides and *Nosema sp.* microsporidia reduce honey bee (*Apis mellifera* L.) survivability under laboratory conditions. *Journal of Apicultural Research*, **59**, 332–342.

Falk, S., and Lewington, R. (2015). Field guide to the bees of Great Britain and Ireland. London: Bloomsbury Wildlife.

Farina, W.M., Balbuena, M.S., Herbert, L.T., Megoni Goñalons, C., and Vázquez, D.E. (2019). Effects of the herbicide glyphosate on honey bee sensory and cognitive abilities: individual impairments with implications for the hive. *Insects*, **354**, 1–16.

Fauser-Misslin, A., Sadd, B.M., Neumann, P., Sandrock, C. (2014). Influence of combined pesticide and parasite exposure on bumblebee colony traits in the laboratory. *Journal of Applied Ecology*, **51**, 450–459.

Fauser, A., Sandrock, C., Neumann, P., and Sadd, B.M. (2017). Neonicotinoids override a parasite exposure impact on hibernation success of a key bumblebee pollinator. *Ecological Entomology*, **42**, 306–314.

Feltham, H., Park, K., and Goulson, D. (2014). Field realistic doses of pesticide imidacloprid reduce bumblebee pollen foraging efficiency. *Ecotoxicology*, **23**, 317–323.

FERA. (2021). UK Pesticide Usage Data. Available at https://secure.fera.defra.gov.uk/pusstats/surveys/. Last accessed in June 2021.

Fernández-Ortuño, D., Torés, J.A., de Vicente, A., and Pérez-García A. (2010). The QoI fungicides, the rise and fall of a successful class of agricultural fungicides. Fungicides, Odile Carisse (Ed.), IntechOpen, London, UK.

Figueroa, L.L., Blinder, M., Grincavitch, C., Jelinek, A., Mann, E.K., Merva, L.A., et al. (2019). Bee pathogen transmission dynamics: deposition, persistence and acquisition on flowers. *Proceedings of the Royal Society B: Biological Sciences*, **286**, 20190603.

Fine, J., and Mullin, C. (2017). Metabolism of N-methyl-2-pyrrolidone in honey bee adults and larvae: exploring age related differences in toxic effects. *Environmental Science and Technology*, **51**, 11412-11422.

Fine, J.D., Cox-Foster, D.L., and Mullin, C.A. (2017). An inert pesticide adjuvant synergizes viral pathogenicity and mortality in honey bee larvae. *Scientific Reports*, **7**, 1-9.

Fine, J.D., Mullin, C.A, Frazier, M.T., and Reynolds, R.D. (2017). Field residues and effects of the insect growth regulator novaluron and its major co-formulant N-methyl-2-pyrrolidone on honey bee reproduction and development. *Journal of Economic Entomology*, **110**, 1993–2001.

Fisher II, A., Coleman, C., Hoffman, C., Fritz, B., Rangel, J. (2017). The synergistic effects of almond protection fungicides on honey bee (Hymenoptera: Apidae) forager survival. *Journal of Economic Entomology*, **110**, 1–7.

Folly, A.J., Koch, H., Stevenson, P.C., and Brown, M.J.F. (2017). Larvae act as a transient transmission hub for the prevalent bumblebee parasite *Crithidia bombi*. *Journal of Invertebrate Pathology*, **148**, 81–85.

Food and Agriculture Organisation. (2021). FAOSTAT pesticides use dataset. Available at http://www.fao.org/faostat/en/#data/RP. Version updated June 22, 2021. Last accessed in June 2021.

Franklin, M.T., Winston, M.L., Morandin, L.A. (2004). Effects of clothianidin on *Bombus impatiens* (Hymenoptera: Apidae) colony health and foraging ability. *Journal of Economic Entomology*, **97**, 369–373.

Fuller, R.M. (1987). The changing extent and conservation interest of lowland grasslands in England and Wales: a review of grassland surveys 1930-1984. *Biological Conservation*, **40**, 281–300.

Gamboa, V., Ravoet, J., Brunain, M., Smagghe, G., Meeus, I., Figueroa, J., et al. (2015). Bee pathogens found in *Bombus atratus* from Colombia: a case study. *Journal of Invertebrate Pathology*, **129**, 36–39.

Garibaldi, L.A., Carvalheiro, L.G., Leonhard, S.D., Aizen, M.A., Blaauw, B.R., Isaacs, R., et al. (2014). From research to action: enhancing crop yield through wild pollinators. *Frontiers in Ecology and the Environment*, **12,** 439–447.

Garibaldi, L.A., Steffan-Dewenter, I., Winfree, R., Aizen, M.A., Bommarco, R., Cunningham, S.A., et al. (2013). Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science*, **339**, 1608-1611.

Garthwaite D., Barker, I., Ridley, L., Mace, A., Parrish, G., MacArthur, R., and Luet, Y. (2016a). Orchards in the United Kingdom 2016. *Pesticide Usage Survey Report*, **273**, 1-68.

Garthwaite D., Barker, I., Ridley, L., Mace, A., Parrish, G., MacArthur, R., and Luet, Y. (2016b). Arable crops in the United Kingdom 2016. *Pesticide Usage Survey Report*, **284**, 1-68.

Gaylord Chemical Information Sheet. (2020). Available at https://www.gaylordchemical.com/wp-content/uploads/2015/11/NMP-vs-DMSO-brochure.pdf. Last accessed November 2020.

Gill, R.J., and Raine, N.E. (2014). Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Functional Ecology*, **28**, 1459–1471.

Gillespie, S. (2010). Factors affecting parasite prevalence among wild bumblebees. *Ecological Entomology*, **35**, 737–747.

Glibert, P.M., Seitzinger, S., Heil, C.A., Burkholder, J.M., Parrow, M.W., Codispoti, L.A., et al. (2005). The role of eutrophication in the proliferation of harmful algal blooms. New perspectives and new approaches. *Oceanography*, **18**, 198-209.

Goldblatt J.W., and Fell, R.D. (1987). Adult longevity of workers of the bumble bees *Bombus fervidus* (F.) and *Bombus pennsylvanicus* (De Geer) (Hymenoptera: Apidae). *Canadian Journal of Zoology*, **65**, 2349-2353.

Goldewijk, K.K. (2001). Estimating global land use change over the past 300 years: The HYDE Database. *Global Biogeochemical Cycles*, **15**, 417-433.

Gonalons, C.M., and Farina, W.M. (2018). Impaired associative learning after chronic exposure to pesticides in young adult honey bees. *Journal of Experimental Biology*, **221**, 176644.

Goodrich, B., Williams, J., and Goodhue, R. (2019). the great bee migration: supply analysis of honey bee colony shipments into California for almond pollination services. Invited paper presented at the 2019 Annual Meeting of the Allied Social Sciences Association. Atlanta, Georgia.

Goodwin, R.M., and McBrydie, H.M. (2000). Effects of surfactants on honey bee survival. *New Zealand Plant Protection*, **53**, 230-234.

Goulson, D. (2018). Call to restrict neonicotinoids. *Science*, **360,** 973.

Goulson, D., Nicholls, E., Botías, C., and Rotheray, E.L. (2015). Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, **347,** 1255957.

Goulson, D., Thompson, J., and Croombs, A. (2018). Rapid rise in toxic load for bees revealed by analysis of pesticide use in Great Britain. *PeerJ*, **7**, 6-13.

Green J.M., and Beestman, G.B. (2007). Recently patented and commercialized formulation and adjuvant technology. *Crop Protection*, **26**, 320–327.

Gregorc, A., and Ellis, J.D. (2011). Cell death localization in situ in laboratory reared honey bee (*Apis mellifera* L.) larvae treated with pesticides. *Pesticide Biochemistry and Physiology,* **99**, 200-207.

Gregorc, A., Evans, J.D., Scharfc, M., and Ellis, J.D. (2012). Gene expression in honey bee (*Apis mellifera*) larvae exposed to pesticides and Varroa mites (*Varroa destructor*). *Journal of Insect Physiology*, **58**, 1042–1049.

Grey, C.N.B., Nieuwenhuijsen, M.J., Golding, J., and the ALSPAC Team. (2005). The use and disposal of household pesticides. *Environmental Research*, **97**, 109-115.

Grixti, J.C., Wong, L.T., Cameron, S.A., and Favret, C. (2009). Decline of bumble bees (*Bombus*) in the North American Midwest. *Biological Conservation*, **142**, 75–84.

Gruber, B., Eckel, K., Everaars, J., and Dornmann, C. (2011). On managing the red mason bee (*Osmia bicornis*) in apple orchards. *Apidologie*, **42**, 564-576.

Haddaway, N.R., Bethel, A., Dicks, L.V., Koricheva, J., Macura, B., Petrokofsky, G., Pullin, A.S., Savilaakso, S., and Stewart G.B. (2020). Eight problems with literature reviews and how to fix them. *Nature Ecology and Evolution*, **4**, 1582-1589.

Hartzler, B. (2017). Dicamba: past, present and future. *2017 Integrated Crop Management Conference - Iowa State University*, **1**, 71-76.

Hazen, J.L. (2000). Adjuvants – terminology, classification, and chemistry. *Weed Technology*, **14**, 773–784.

Health and Safety Executive UK. (2021a). Adjuvant Database Search. Available at: https://secure.pesticides.gov.uk/adjuvants/Search.aspx Accessed in November 2020.

Health and Safety Executive UK. (2021b). Plant Protection Products with Authorisation for use in the UK Database Search. Available at: https://secure.pesticides.gov.uk/pestreg/ProdSearch.asp Accessed in November 2020.

Heard, M.A., Baas, J., Dorne, J.L., Lahive, E., Robinson, A.G., Rortais, A., et al. (2017). Comparative toxicity of pesticides and environmental contaminants in bees: are honey bees a useful proxy for wild bee species? *Science of the Total Environment*, **578**, 357–365.

Heckel, D.G. (2020). How do toxins from *Bacillus thuringiensis* kill insects? An evolutionary perspective. *Archives of Insect Biochemistry and Physiology*, **104**, 1-12.

Helmer, S.H., Kerbaol, A., Aras, P., Jumarie, C., and Boily, M. (2015). Effects of realistic doses of atrazine, metolachlor, and glyphosate on lipid peroxidation and diet-derived antioxidants in caged honey bees (*Apis mellifera*). *Environmental Science and Pollution Research*, **22**, 8010–8021.

Hendriksma, H.P., Toth, A.L., and Shafir, S. (2019). Individual and colony level foraging decisions of bumble bees and honey bees in relation to balancing of nutrient needs. *Frontiers in Ecology and Evolution*, **7**, 177.

Henry, M., Béguin, M., Requier, F., Rollin, O., Odoux, J.F. Aupinel, et al. (2012). A common pesticide decreases foraging success and survival in honey bees. *Science*, **336**, 348-350.

Herbert, L.T., Vázquez, D.E., Arenas, A., and Farina, W.M. (2014). Effects of field-realistic doses of glyphosate on honeybee appetitive behaviour. *Journal of Experimental Biology*, **217**, 3457–3464.

Hicks, B.J., Pilgrim, B.L., Perry, E., and Marshall, H.D. (2018). Observations of native bumble bees inside of commercial colonies of *Bombus impatiens* (Hymenoptera: Apidae) and the potential for pathogen spillover. *Canadian Entomologist*, **150**, 520–531.

Hoffman, E.J., and Castle, S.J. (2012). Imidacloprid in melon guttation fluid: a potential mode of exposure for pest and beneficial organisms. *Journal of Ecotoxicology*, **105**, 67-71.

Holder, P.J., Jones, A., Tyler, C.R., and Cresswell, J.E. (2018). Fipronil pesticide as a suspect in historical mass mortalities of honey bees. *PNAS*, **16**, 16–21.

Hopkins, I. (1914). History of the humble-bee in New Zealand: its introduction and results. *New Zealand Department of Agriculture and Industry Communications*, **46**, 1–29.

Inoue, M.N., Yokoyama, J., and Washitani, I. (2013). Displacement of Japanese native bumblebees by the recently introduced *Bombus terrestris* (L.) (Hymenoptera: Apidae). *Journal of Insect Conservation,* **12**, 135–146.

Iwasa, T., Motoyama, N., Ambrose, J.T., and Roe, R.M. (2004). Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protection*, **23**, 371–378.

James, C. (2014). Global status of Commercialized biotech/GM Crops: 2014. ISAAA Briefs *18*.

Jansen, V.A.A., Brown, M.J.F., and Raine, N.E. (2017). Pesticide reduces bumblebee colony initiation and increases probability of population extinction. *Nature Ecology and Evolution*, **1**, 1308-1316.

Jeschke, P., Nauen, R., Schindler, M., and Elbert, A. (2011). Overview of the status and global strategy for neonicotinoids. *Journal of Agricultural and Food Chemistry*, **59**, 2897-2908.

Johnson, R., and Percel, E. (2013). Effect of a fungicide and spray adjuvant on queen-rearing success in honey bees (Hymenoptera: Apidae). *Journal of Economic Entomology*, **106**, 1952-1957.

Jones, C.M., and Brown, M.J.F. (2014). Parasites and genetic diversity in an invasive bumble bee. *Journal of Animal Ecology*, **83**, 1428-1440.

Judd, H.J., Huntzinger, C., Ramirez, R., and Strange, J.P. (2020). A 3D printed pollen trap for bumble bee (*Bombus*) hive entrances. *Journal of Visual Experiments*, **161**, e61500.

Kassambara, A., Kosinski, M., and Biecek, P. (2019). survminer: Drawing survival curves using 'ggplot2'. R package version 0.4.6. https://CRAN.R-project.org/package=survminer

Katagi, T. (2008). Surfactant effects on environmental behavior of pesticides. In Reviews of Environmental Contamination and Toxicology. (New York, New York: Springer).

Kennedy, C.M., Lonsdorf, E., Neel, M.C., Williams, N.M., Ricketts, T.H., Winfree, R., et al. (2013). A global quantitative synthesis of local and landscape effects on wild bee pollinators in agroecosystems. *Ecology Letters*, **16**, 584–599.

Kerr, J.T., Pindar, A., Galpern, P., Packer, L., Potts, S.G., Roberts, S.M., et al. (2015). Climate change impacts on bumblebees converge across continents. *Science*, **349**, 177–80.

Khan, Z.R., Hassanali, A., Overholt, W., Khamis, T.M., Hooper, A.M., Picket, J.A. et al. (2002). Control of witchweed *Striga Hermonthica* by intercropping with *Desmodium Spp.*, and the mechanism defined as allelopathic. *Journal of Chemical Ecology*, **28**, 1871-1885.

King, M.J., and Buchmann, S.L. (2003). Floral sonication by bees: mesosomal vibration by *Bombus* and *Xylocopa*, but not *Apis* (Hymenoptera: Apidae), ejects pollen from poricidal anthers. *Journal of the Kansas Entomological Society*, **76**, 295-305.

Klein, A.M., Steffan-Dewenter, I., and Tscharntke, T. (2003). Fruit set of highland coffee increases with the diversity of pollinating bees. *Proceedings of the Royal Society of London B: Biological Sciences*, **270**, 955-961.

Klein, A.M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C. et al. (2007). Importance of pollinators in changing landscapes for world crops. *Proceedings of the Royal Society B: Biological Sciences*, **274**, 303–313.

Koch, H., and Schmid-Hempel, P. (2011). Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. *Proceedings of the National Academy of Sciences*, **108**, 19288–19292.

Koch, H., Woodward, J., Langat, M.K., Brown, M.J.F., and Stevenson, P.C. (2019). Flagellum removal by a nectar metabolite inhibits infectivity of a bumblebee parasite. *Current Biology*, **29**, 3494-3500.

Kordecki, H., and Johnson, R.M. (2019). Effects of insecticides, fungicides, and adjuvant combinations on honey bee brood development. Undergraduate Thesis, Ohio State University.

Korner, P., and Schmid-Hempel, P. (2005). Correlates of parasite load in bumblebees in an Alpine habitat. *Entomological Science,* **8**, 151–160.

Krupke, C.H., Hunt, G.J., Eitzer, B.D., Andino, G., and Given, K. (2012). Multiple routes of pesticide exposure for honey bees living near agricultural fields. *PLoS ONE*, **7**, e29268.

Ladurner, E., Bosch, J., Kemp, W., and Maini, S. (2008). Foraging and nesting behavior of *Osmia lignaria* (Hymenoptera: Megachilidae) in the presence of fungicides: Cage studies. *Journal of Economic Entomology*, **101**, 647-653.

Laycock, I., Cotterell, K.C., O'Shea-Wheller, T.A., Cresswell, J.E. (2014). Effects of the neonicotinoid pesticide thiamethoxam at field-realistic levels on microcolonies of *Bombus terrestris* worker bumble bees. *Ecotoxicology and Environmental Safety,* **100**, 153–158.

Le Féon, V., Schermann-Legionnet, A., Delettre, Y., Aviron, S., Billeter, R., Henrickx, F., et al. (2010). Intensification of agriculture, landscape composition and wild bee communities: A large scale study in four European countries. *Agriculture, Ecosystems and Environment*, **137**, 143–150.

Li, B., Pang, X., Zhang, P., Lin, J., Li, X., Liu, Y., et al. (2018b). Alcohol ethoxylates significantly synergize pesticides than alkylphenol ethoxylates considering bioactivity against three pests and joint toxicity to *Daphnia magna*. *Science of the Total Environment*, **644,** 1452–1459.

Li, B.X., Lin, J., Pang, X., Li, H., Li, X., and Liu, F. (2018a). Binary mixtures of alcohol ethoxylates, nonylphenol ethoxylates and pesticides exhibit comparative bioactivity against three pests and toxicological risks to aquatic organisms. *Chemosphere*, **204**, 44-50.

Liao, L.H., Wu, W.Y., Dad, A., and Berenbaum, M.R. (2019). Fungicide suppression of flight performance in the honeybee (*Apis mellifera*) and its amelioration by quercetin. *Proceedings of the Royal Society of London B: Biological Sciences,* **286**, 20192041.

Linguadoca, A., Rizzi C., Villa, S., and Brown, M.J.F. (2021). Sulfoxaflor and nutritional deficiency synergistically reduce survival and fecundity in bumblebees. *Science of the Total Environment*, **795**, 148680.

Logan, A., Ruiz-González, M.X., and Brown, M.J.F. (2005). The impact of host starvation on parasite development and population dynamics in an intestinal trypanosome parasite of bumble bees. *Parasitology*, **130**, 637-642.

Lundin, O., Rundlöf, M., Smith, H.G., Fries, I., and Bommarco, R. (2015). Neonicotinoid insecticides and their impacts on bees: a systematic review of research approaches and identification of knowledge gaps. *PLoS One*, **10**, e0136928.

Main, A.R., Hladik, M.L., Webb, E.B., Goyne, K.W., and Mengel, D. (2020). Beyond neonicotinoids – wild pollinators are exposed to a range of pesticides while foraging in agroecosystems. *Science of the Total Environment*, **742**, 140436.

Martin, S.J.M., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M. et al. (2012). Global honey bee viral landscape altered by a parasitic mite. *Science*, **33**, 1304–1306.

Matson, P.A., Parton, W.J, Power, A.G., and Swift, M.J. (1997). Agricultural intensification and ecosystem properties. *Science*, **227**, 504-509.

Matsuda, K., Buckingham, S.D., Kleier, D., Rauh, J.J., Grauso, M., and Sattele, D.B. (2011). Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *TRENDS in Pharmacological Sciences*, **22**, 573-580.

Mayer, D. Dimitriadou, E., Hornik, K., Weingessel, A., Leisch, F., Chang, C.C., et al. (2021). Misc Functions of the Department of Statistics, Probability Theory Group (Formerly: E1071), TU Wien. R package version 1.7-4. https://cran.r-project.org/web/packages/e1071/index.html

McArt, S.H., Urbanowicz, C., McCoshum, S., Irwin, R.E., Adler, L.S. (2017). Landscape predictors of pathogen prevalence and range contractions in US bumblebees. *Proceedings of the Royal Society of London B: Biological Sciences*, **284**, 20172181.

McDuffie, H.H., Pahwa, P., McLaughlin, J.R., Spinellie, J.J., Fincham, S., Dosman, J.A., et al. (2001). Non-Hodgkin's Lymphoma and specific pesticide exposures in men: cross-Canada study of pesticides and health. *Cancer Epidemiology, Biomarkers and Prevention*, **10**, 1155–1163.

Meeus, I., Brown, M.J.F., De Graaf, D.C., and Smagghe, G. (2011). Effects of invasive parasites on bumble bee declines. *Conservation Biology*, **25**, 662–671.

Mercurio, P., Flores, F., Mueller, J.F., Carter, S., and Negri, A.P. (2014). Glyphosate persistence in seawater. *Marine Pollution Bulletin*, **85**, 385-390.

Mesnage, R., and Antoniou, M.N. (2018). Ignoring adjuvant toxicity falsifies the safety profile of commercial pesticides. *Frontiers in Public Health*, **5**, 1-8.

Mesnage, R., Benbrook, C., and Antoniou, M.N. (2019). Insight into the confusion over surfactant co-formulants in glyphosate-based herbicides. *Food Chemistry and Toxicology*, **128**, 137-145.

Mesnage, R., Bernay, B., and Séralini, G.E. (2013). Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. *Toxicology*, **313**, 122-128.

Mesnage, R., Teixeira, M., Mandrioli, D., Falcioni, L., Ducarmon, Q.R., Zwittink, R.D., et al. (2021). Use of shotgun metagenomics and metabolomics to evaluate the impact of glyphosate or Roundup MON 52276 on the gut microbiota and serum metabolome of Sprague-Dawley rats. *Environmental Health Perspectives*, **129***, 017005.

Milchreit, K., Ruhnke, H., Wegener, J., and Bienefeld, K. (2016). Effects of an insect growth regulator and a solvent on honeybee (*Apis mellifera* L.) brood development and queen viability. *Ecotoxicology*, **25**, 530-537.

Mockler, B.K., Kwong, W.K., Moran, N.A., and Koch, H. (2018). Microbiome structure influences infection by the parasite *Crithidia bombi* in bumble bees. *Applied and Environmental Microbiology*, **83**, e02335-17.

Moffet, J.O., Morton, H.L., and MacDonald, R.H. (1972). Toxicity of some herbicidal sprays to honey bees. *Journal of Economic Entomology,* **65**, 32-36.

Moffett, J.O., and Morton, H.L. (1973). Surfactants in water drown honey bees. *Environmental Entomology*, **2**, 227-231.

Moffett, J.O., and Morton, H.L. (1975). Repellency of surfactants to honey bees. *Environmental* Entomology, **4**, 780-782.

Moher, D., Liberati, A., Tetzlaff, J., Altman, D.G., and the PRISMA Group. (2009). *Preferred reporting items for systematic reviews and meta-analyses:* The PRISMA statement. *PLoS Medicine,* **6**, e1000097.

Morandin, L.A., and Winston, M.L. (2003). Effects of novel pesticides on bumble bee (Hymenoptera: Apidae) colony health and foraging ability. *Environmental Entomology*, **32**, 555-563.

Motta, E.V.S., and Moran, N.A. (2020). Impact of glyphosate on the honey bee gut microbiota: effects of intensity, duration, and timing of exposure. *mSystems*, **5**, e00268-20.

Motta, E.V.S., Mak, M., De Jong, T.K., Powell, J.E., O'Donnell, A., Suhr, K.J., et al. (2020). Oral or topical exposure to glyphosate in herbicide formulation impacts the gut microbiota and survival rates of honey bees. *Applied Environmental Microbiology*, **86**, e01150-20.

Motta, E.V.S., Raymann, K., and Moran, N.A. (2018). Glyphosate perturbs the gut microbiota of honey bees. *Proceedings of the National Academy of Science* USA, **115**, 10305–10310.

Mullin, C.A. (2015). Effects of 'inactive' ingredients on bees. Current Opinion in Insect Science, **10**, 194-200.

Mullin, C.A., Chen, J., Fine J.D., Frazier, M.T., and Frazier, J.L. (2015). The formulation makes the honey bee poison. *Pesticide Biochemistry and Physiology*, **120**, 27-35.

Mullin, C.A., Fine J.D., Reynolds, R.D., and Frazier, M.T. (2016). Toxicological risks of agrochemical spray adjuvants: organosilicone surfactants may not be safe. *Frontiers in Public Health*, **4**, 1-8.

Mullin, C.A., Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., van Engelsdorp, D., and Pettis, J.S. (2010). High levels of miticides and agrochemicals in North American apiaries: implications for honey bee health. *PLoS ONE*, **5**, e9754.

Mussen, E.C., Lopez, J.E., Peng, C.Y.S. (2004). Effects of selected fungicides on growth and development of larval honey bees, *Apis mellifera* L. (Hymenoptera: Apidae). *Environmental Entomology*, **33**, 1151-1154.

Nagy, K., Duca, R.C., Lovas, S., Creta, M., Scheepers, P.T.J., Godderis, L., and Ádám, B. (2020). Systematic review of comparative studies assessing the toxicity of pesticide active ingredients and their product formulations. *Environmental Research*, **181**, 108926.

Nalley, L., Tsiboe, F., Durand-Morat, A., Shew, A., and Thoma, G. (2016). Economic and environmental impact of rice blast pathogen (*Magnaporthe oryzae*) alleviation in the United States. *PLoS ONE*, **11**, e0167295.

Nauen, R., Jeschke, P., Velten, R., Beck, M.E., Ebbinghaus-Kintscher, U., Thielert, W., et al. (2015). Flupyradifurone: a brief profile of a new butenolide insecticide. *Pest Management Science*, **71**, 850-862.

Nieto, A., Roberts, S.P.M., Kemp, J., Rasmont, P., Kuhlmann, M., García Criado, M., et al. (2014). European red list of bees. Luxembourg: Publication Office of the European Union.

Odemer, R., Alkassab, A.T., Bischoff, G., Frommberger, M., Wernecke, A., Wirtz, I.P., et al. (2020). Chronic high glyphosate exposure delays individual worker bee (*Apis mellifera* l.) development under field conditions. *Insects*, **11**, 1-20.

Ollerton, J., Erenler, H., Edwards, M., and Crockett, R. (2014). Extinctions of Aculeate pollinators in Britain and the role of large-scale agricultural changes. *Science*, **346**, 1360–1362.

Organisation for Economic Co-Operation and Development. (1998). Honeybees, acute contact toxicity test. *OECD Guidelines for the Testing of Chemicals,* **214**, 1–7.

Organisation for Economic Co-Operation and Development. (2017a). Honey Bee (*Apis mellifera* L.), Chronic oral toxicity test (10-day feeding). *OECD Guidelines for the Testing of Chemicals*, **245**, 1–9.

Organisation for Economic Co-Operation and Development. (2017b). Bumblebee, acute oral toxicity test. *OECD Guidelines for the Testing of Chemicals*, **247**, 1–12.

Orozco, F.A., Cole, D.C., Forbes, G., Kroschel, J., Wanigaratne, S., and Arica, D. (2009). Monitoring adherence to the international code of conduct: highly hazardous pesticides in central Andean agriculture and farmers' rights to health. *International Journal of Occupational and Environmental Health*, **15**, 255-268.

Oystaeyen, A.V., Klatt, B.K., Petit, C., Lenaerts, N., and Wäckers, F. (2020). Short-term lab assessments and microcolonies are insufficient for the risk assessment of insecticides for bees. *Chemosphere*, **273**, 128518.

Paul, N. (2019). The impacts of glyphosate on bumble bee productivity and parasite load. Master's Thesis, Queen's University Belfast.

Peck, D.T., Smith, M.L., and Seeley, T.D. (2016). *Varroa destructor* mites can nimbly climb from flowers onto foraging honey bees. *PLoS ONE*, **11**, 1–12.

Pélissié, B., Crossley, M.S., Cohen, Z.P., and Schoville, S.D. (2018). Rapid evolution in insect pests: the importance of space and time in population genomics studies. *Current Opinion in Insect Science*, **26**, 8–16.

Pesticide Action Network. (2015). Sulfoxaflor and flupyradifurone: Neonicotinoids or not? Available at https://www.buzzaboutbees.net/support-files/pannewpesticidesfactsheet.pdf. Last accessed in June 2021.

Pettis, J.S., Lichtenberg, E.M., Andree, M., Stitzinger, J., Rose, R., and VanEngelsdorp, D. (2013). Crop pollination exposes honey bees to pesticides which alters their susceptibility to the gut pathogen *Nosema ceranae*. *PLoS ONE,* **8**, e70182.

Pettis, J.S., Vanengelsdorp, D., Johnson, J., and Dively, G. (2012). Pesticide exposure in honey bees results in increased levels of the gut pathogen *Nosema*. *Naturwissenschaften*, **99**, 153–158.

Piiroinen, S., and D. Goulson. (2016). Chronic neonicotinoid pesticide exposure and parasite stress differentially affects learning in honeybees and bumblebees. *Proceedings of the Royal Society B: Biological Sciences*, **283**, 20160246.

Piiroinen, S., Botías, C., Nicholls, E., and Goulson, D. (2016). No effect of low-level chronic neonicotinoid exposure on bumblebee learning and fecundity. *PeerJ*, **4**, e1808.

Pilling, E., Campbell, P., Coulson, M., Ruddle, N., and Tornier, I. (2013). A four-year field program investigating long-term effects of repeated exposure of honey bee colonies to flowering crops treated with thiamethoxam. *PLoS ONE*, **8**, e77193.

Pilling, E.D., and Jepson, P.C. (1993). Pyrethroid insecticide in the honeybee synergism between EBI fungicides and a pyrethroid insecticide in the honeybee (*Apis mellifera*). *Pesticide Science*, **39**, 293-297.

Plischuk, S., Antúnez, K., Haramboure, M., Minardi, G.M., and Lange, C.E. (2017). Long-term prevalence of the protists *Crithidia bombi* and *Apicystis bombi* and detection of the microsporidium *Nosema bombi* in invasive bumble bees. *Environmental Microbiology Reports,* **9**, 169–173.

Potts, S.G., Biesmeijer, J.C., Kremen, C., Neumann, P., Schweiger, O., and Kunin, W.E. (2010). Global pollinator declines: trends, impacts and drivers. *Trends in Ecology and Evolution*, **25**, 345– 353.

Potts, S.G., Ngo, H.T., Biesmeijer, J.C., Breeze, T.D., Dicks, L.V., Garibaldi, L.A., et al. eds. (2016). The assessment report of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services on pollinators, pollination and food production. (Bonn, Germany: Secretariat of the Intergovernmental Science-Policy Platform on Biodiversity and Ecosystem Services).

Purcell, A.H., and Almeida, R.P.P. (2014). Insects as vectors of disease agents. Encyclopaedia of Plant and Crop Science, 1-14.

R Development Core Team. (2019). R: A language and environment for statistical computing. R Foundation for Statistical Computing. https://www.R-project.org.

Raine, N.E., and Chittka, L. (2008). The correlation of learning speed and natural foraging success in bumble-bees. *Proceedings of the Royal Society B*, **275**, 803-808.

Rawn, G.P., Webster, G.R.B., and Muir, D.C.G. (1982). Fate of permethrin in model outdoor ponds. *Journal of Environmental Science and Health, Part B*, **17**, 463.

Rennich, K., Kunkel, G., Abban, S., Fahey, R., Eversole, H., Evans, J., et al. (2013). 2013-2014 national honey bee pests and diseases survey report. USDA Report.

Retschnig, G., Neumann, P., and Williams, G.R. (2014). Thiacloprid-*Nosema ceranae* interactions in honey bees: host survivorship but not parasite reproduction is dependent on pesticide dose. *Journal of Invertebrate Pathology*, **118**, 18–19.

Rodd, F.H., Plowright, R.C., and Owen, R.E. (1980). Mortality rates of adult bumble bee workers (Hymenoptera: Apidae). *Canadian Journal of Zoology*, **58**, 1718-1721.

Rohatgi, A. (2020). WebPlotDigitizer. Version 4.4. https://automeris.io/WebPlotDigitizer

Roundup ProActive Environmental Information Sheet. (2020). Available at: https://www.monsanto-ag.co.uk/media/2029/roundup-proactive-eia.pdf. Last accessed in April 2020.

Roundup ProActive Label. (2019). Available at: https://www.monsanto-ag.co.uk/media/1950/roundup-proactive-label-november-2016.pdf. Last accessed in April 2020.

Roundup ProActive Label. (2019). Retrieved from https://www.green-tech.co.uk/media/shared/chemical-labels/Roundup%20Pro%20Active%20360%20Product%20Label-min.pdf

Roundup ProActive MSDS. (2020). Available at: https://www.monsanto-ag.co.uk/media/1947/msds-roundup-proactive-04052017.pdf. Last accessed in April 2020.

Roundup Ready Plus Information Sheet. (2020). Available at http://www.roundupreadyplus.com/resourcecenter/postemergence_herbicide_applications_in_soybean. Last accessed in April 2020.

Roundup Ready-To-Use Label. (2019). Available at: https://www.lovethegarden.com/sites/default/files/content/products/documents/pack_label/UK_017829_RUP_PL.pdf

Rubio, F., Guo, E., and Kamp, L. (2014). Survey of glyphosate residues in honey corn and soy products. *Journal of Environmental and Analytical Toxicology*, **5**, 1000249.

Ruiz-González, M.X., and Brown, M.J.F. (2006). Honey bee and bumblebee trypanosomatids: specificity and potential for transmission. *Ecological Entomology*, **31**, 616–622.

Ruiz-Toledo, J., and Sánchez-Guillén, D. (2014). Effect of the concentration of glyphosate present in bodywaters near transgenic soybean fields on the honeybee *Apis mellifera*, and the stingless bee *Tetragonisca angustula*. *Acta zoológica Mexico*, **30**, 408–413.

Rundlöf, M., Andersson, G.K., Bommarco, R., Fries, I., Hederström, V., Herbertsson, L., et al. (2015). Seed coating with a neonicotinoid insecticide negatively affects wild bees. *Nature*, **521**, 77–80.

Russel, P.E. (2015). A century of fungicide evolution. *Journal of Agricultural Science*, **143**, 11–25.

Rutrecht, S.T., and Brown M.J.F. (2009). Differential virulence in a multiple-host parasite of bumble bees: resolving the paradox of parasite survival? *Oikos*, **116**, 941-949.

Rutrecht, S.T., and Brown, M.J.F. (2008). The life-history impact and implications of multiple parasites for bumble bee queens. *International Journal for Parasitology*, **38**, 799–808.

Sanderson, E.D., and Peairs, L.M. (1931). Insect pests of farm, garden and orchard. John Wiley and Sons, New York, 2nd Ed.

Schatz, K., and Wallner, F. (2009). Pflanzenschutzmittelapplikation in blühenden raps (*Brassica napus*) und deren auswirkungen auf die rückstandssituation in honig, nektar und pollen der honigbiene (*Apis mellifera*). PhD Thesis, Universität Hohenheim.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nature Methods*, **9**, 676-682.

Schmid-Hempel, P., Puhr, K., Kruger, N., Reber, C., and Schmid-Hempel, R. (1999). Dynamic and genetic consequences of variation in horizontal transmission for a microparasitic infection. *Evolution*, **53**, 426– 434.

Schmid-Hempel, R., Eckhardt, M., Goulson, D., Heinzmann, D., Lange, C., Plischuk, S., et al. (2014). The invasion of southern South America by imported bumblebees and associated parasites. *Journal of Animal Ecology*, **83**, 823–37.

Schmid-Hempel, R., Eckhardt, M., Goulson, D., Heinzmann, D., Lange, C., Plischuk, S., et al. (2014). The invasion of southern South America by imported bumblebees and associated parasites. *Journal of Animal Ecology*, **83**, 823–837.

Schmuck, R., Stadler, T., and Werner Schmidt, H. (2003). Field relevance of a synergistic effect observed in the laboratory between an EBI fungicide and a chloronicotinyl insecticide in the honeybee (*Apis mellifera* L, Hymenoptera). *Pest Management Science,* **59**, 279–286.

Schneider, C.W., Tautz, J., Grünewald, B., Fuchs, S. (2012). RFID tracking of sublethal effects of two neonicotinoid insecticides on the foraging behavior of *Apis mellifera. PLoS ONE,* **7**, e30023.

Seide, V.E., Bernardes, R.C., Pereira, E.J.G., and Lima, M.A.P. (2018). Glyphosate is lethal and cry toxins alter the development of the stingless bee *Melipona quadrifasciata*. *Environmental Pollution*, **243**, 1854–1860.

Seufert, V., Ramankutty, N., and Foley, J.A. (2012). Comparing the yields of organic and conventional agriculture. *Nature*, **485**, 229–232.

Sgolastra, F., Kemp, W.P., Buckner, J.S., Pitts-Singer, T.L., Maini, S., and Bosch, J. (2011). The long summer: pre-wintering temperatures affect metabolic expenditure and winter survival in a solitary bee. *Journal of Insect Physiology*, **57**, 1651-1659.

Sgolastra, F., Medrzycki, P., Bortolotti, L., Maini, S., Porrini, C., Simon-Delso, N., and Bosch, J. (2020). Bees and pesticide regulation: Lessons from the neonicotinoid experience. *Biological Conversation*, **241**, 108356.

Shykoff, J.A., and Schmid-Hempel, P. (1991). Incidence and effects of four parasites in natural populations of bumble bees in Switzerland. *Apidologie*, **22**, 117–125.

Simon-Delso, N., San Martin, G., Bruneau, E., and Hautier, L. (2018). Time-to-death approach to reveal chronic and cumulative toxicity of a fungicide for honeybees not revealed with the standard ten-day test. *Scientific Reports*, **8**, 7241.

Simon-Delso, N., San Martin, G., Bruneau, E., Minsart, L.-A., Mouret, C., and Hautier, L. (2014). Honeybee colony disorder in crop areas: the role of pesticides and viruses. *PLoS One*, **9**, e103073.

Sims, S.R., and Appel, A.G. (2007). Linear alcohol ethoxylates: insecticidal and synergistic effects on German cockroaches (Blattodea: Blattellidae) and other insects. *Journal of Economic Entomology*, **100**, 871-879.

Siviter, H., and Muth, F. (2020). Do novel insecticides pose a threat to beneficial insects? *Proceedings of the Royal Society B: Biological Sciences*, **287**, 20201265.

Siviter, H., Bailes, E.J., Martin, C.D., Oliver, T.R., Koricheva, J., Leadbeater, E., et al. (2021). Agrochemicals interact synergistically to increase bee mortality. *Nature*, **596**, 389-392

Siviter, H., Brown, M.J.F., and Leadbeater, E. (2018). Sulfoxaflor exposure reduces bumblebee reproductive success. *Nature*, **561**, 109–112.

Siviter, H., Horner, J., Brown, M.J.F., and Leadbeater, E. (2020). Sulfoxaflor exposure reduces egg laying in bumblebees *Bombus terrestris*. *Journal of Applied Ecology*, **57**, 160–169.

Siviter, H., Koricheva, J., Brown, M.J.F., and Leadbeater, E. (2018). Quantifying the impact of pesticides on learning and memory in bees. *Journal of Applied Ecology*, **55**, 2812-2821.

Siviter, H., Scott, A., Pasquier, G., Pull, C.D., Brown, M.J.F., and Leadbeater, E. (2018). No evidence for negative impacts of acute sulfoxaflor exposure on bee olfactory conditioning or working memory. *PeerJ*, **7**, e7208.

Skovgaard O.S. (1936). Rødkløverens bestøvning, humlebier og humleboer. *Det Kongelige Danske Videnskabernes Selskabs Skrifter Naturvidenskabelig og Matematisk Afdeling Række*, **96**, 1– 140.

Smith, D.F.Q., Camacho, E., Thakur, R., Barron, A.J., Dong, Y., Dimopoulos, et al. (2021). Glyphosate inhibits melanization and increases susceptibility to infection in insects. *PLoS Biol*, **19**, e3001182.

Söderhäll, K., and Cernius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. *Current Opinion in Immunology*, **10**, 23-28.

Sparks, T.C. (2013). Insecticide discovery: an evaluation and analysis. *Pesticide Biochemistry and Physiology*, **107**, 8–17.

Stanley, D.A., Smith, K.E., and Raine, N.E. (2015). Bumblebee learning and memory is impaired by chronic exposure to a neonicotinoid pesticide. *Scientific Reports*, **5**, 16508.

Stevens, P.J.G. (1993). Organosilicone surfactants as adjuvants for agrochemicals. *Pesticide Science*, **38**, 103-122.

Straub, L., Strobl, V., and Neumann, P. (2020). The need for an evolutionary approach to ecotoxicology. *Nature Ecology and Evolution*, **4**, 895.

Straw, E.A. (2021). Letter to the editors regarding Battisti et al (2021): Is glyphosate toxic to bees? A meta-analytical review. *Science of the Total Environment*, **790**, 147556.

Straw, E.A., Carpentier, E.N., and Brown, M.J.F. (2021). Data from: Roundup Causes High Levels of Mortality Following Contact Exposure in Bumble Bees. Dryad Digital Repository, https://doi:10.5061/dryad.80gb5mkqn

Straw, E.A., Carpentier, E.N., and Brown, M.J.F. (2021). Roundup causes high levels of mortality following contact exposure in bumble bees. *Journal of Applied Ecology*, **58**, 1167– 1176.

Sygenta Website. (2020). Available at: https://www.syngenta.co.uk/products/search/crop-protection. Last accessed in November 2020.

Takano, H.K., and Dayan, F.E. (2020). Glufosinate-ammonium: a review of the current state of knowledge. *Pest Managament Science*, **76**, 3911–3925.

Tamburini, G., Pereira-Peixoto, M.H., Borth, J., Lotz, S., Wintermantel, D., Allan, M., et al. (2021). Fungicide and insecticide exposure adversely impacts bumblebees and pollination services under semi-field conditions. *Environment International*, **157**, 106813.

Tamburini, G., Wintermantel, D., Allan, M., Dean, R., Knauer, A., Albrecht, M., and Klein A.M. (2021). Sulfoxaflor insecticide and azoxystrobin fungicide have no major impact on honeybees in a realistic-exposure semi-field experiment. *Science of the Total Environment*, **778**, 146084.

Tasei, J.N., Lerin, J., and Ripault, G. (2000). Sublethal effects of imidacloprid on bumblebees, *Bombus terrestris* (Hymenoptera: Apidae), during a laboratory feeding test. *Pest Management Science,* **56**, 784–788.

Tasei, J.N., Ripault, G., and Rivault, E. (2001). Hazards of imidacloprid seed coating to *Bombus terrestris* (Hymenoptera: Apidea) when applied to sunflower. *Journal of Economic Entomology*, **94**, 623–627.

Therneau, T.M. (2020a). A package for survival analysis in R. Version 3.1-11. https://CRAN.R-project.org/package=survival.

Therneau, T.M. (2020b). coxme: Mixed effects Cox models. R package version 2.2-5. https://CRAN.R-project.org/package=coxme.

Thompson, H.M., Levine, S.L., Doering, J., Norman, S., Manson, P., Sutton, P., et al. (2014). Evaluating exposure and potential effects on honeybee brood (*Apis mellifera*) development using glyphosate as an example. *Integrated Environmental Assessment and Management,* **10**, 463–470.

Tomé, H.V.V., Schmehl, D.R., Wedde, A.E., Godoy, R.S.M., Ravaiano, S.V., Guedes, R.N.C., et al. (2020). Frequently encountered pesticides can cause multiple disorders in developing worker honey bees. *Environmental Pollution*, **256**, 113420.

Tooby, T.E., Thompson, A.N., Rycroft, R.J., Black, I.A., and Hewson, R.T. (1981). A pond study to investigate the effects of fish and aquatic invertebrates of deltamethrin applied directly onto water. UK MAFF Report PRD 1276.

Topping, C.J., Brown, M.J.F., Chetcuti, J., de Miranda, J.R., Nazzi, F., Neumann, P., et al. (2021). Holistic environmental risk assessment for bees. *Science,* **37**, 897.

Tsvetkov, N., Samson-Robert, O., Sood, K., Patel, H.S., Malena, A., Gajiwala, P.H., et al. (2017). Chronic exposure to neonicotinoids reduces honey bee health near corn crops. *Science*, **356**, 1395–1397.

US Patent 20100113274A1. (2010). Glyphosate formulations containing amidoalkylamine surfactants. Available at: https://patents.google.com/patent/US20100113274A1/en. Last accessed in March 2020.

US Patent US5266690A. (1993). Preparation of alkylpolyglycosides. Available at: https://patents.google.com/patent/US5266690A/en. Last accessed in August 2020.

Vanbergen, A.J., and the Insect Pollinators Initiative. (2013). Threats to an ecosystem service: pressures on pollinators. *Frontiers in Ecology and the Environment*, **11**, 251-259.

Vázquez, D.E., Ilina, N., Pagano, E.A., Zavala, J.A., and Farina, W.M. (2018). Glyphosate affects the larval development of honey bees depending on the susceptibility of colonies. *PLoS One*, **13**, 1-19.

Velthuis, H.H.W., and van Doorn, A. (2006). A century of advances in bumblebee domestication and the economic and environmental aspects of its commercialization for pollination. *Apidologie*, **37**, 421–451.

Vidau, C., Diogon, M., Aufauvre, J., Fontbonne, R., Viguè, B., Brunet., J.L., et al. (2011). Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybees previously infected by *Nosema ceranae*. *PLoS ONE*, **6**, e21550.

Vilmos, P., and Kurucz, É. (1998). Insect immunity: evolutionary roots of the mammalian innate immune system. *Immunology Letters*, **62**, 59-66.

Walsh, J.A., Perrin, R.M., Miller, A., and Laycock, D.S. (1989). Studies on beet western yellows virus in winter oilseed rape (*Brassica napus* ssp*. oleifera*) and the effect of insecticidal treatment on its spread. *Crop Protection*, **8**, 137-143.

Weinhold, B. (2010). Mystery in a Bottle. *Environmental Health Perspectives*, **22**, 169-171.

Wickham, W. (2016). ggplot2: Elegant graphics for data analysis. Springer-Verlag New York. https://CRAN.R-project.org/package=ggplot2.

Wilson, E.O. (2002). *The future of life*. Knopf: New York.

Wolfenbarger, D.O. (1957). Observations of insecticidal control by surface active agents. *The Florida Entomolo*gist, **40**, 53-59.

Wood, T.J., Kaplan, I., Zhang, Y., and Szendrei, Z. (2019). Honeybee dietary neonicotinoid exposure is associated with pollen collection from agricultural weeds. *Proceedings of the Royal Society B*, **286**, 20190989.

Woodcock, B.A., Isaac, N.J.B, Bullock, J.M., Roy, D.B., Garthwaite, D.G., Crowe, A., et al. (2016). Impacts of neonicotinoid use on long-term population changes in wild bees in England. *Nature Communications*, **7**, 1–8.

Wu, J.Y., Smart, M.D., Anelli, C.M., and Sheppard, W.S. (2012). Honey bees (*Apis mellifera*) reared in brood combs containing high levels of pesticide residues exhibit increased susceptibility to *Nosema* (Microsporidia) infection. *Journal of Invertebrate Pathology*, **109**, 326–329.

Yoder, J.A., Jajack, A.J., Rosselot, A.E., Smith, T.J., Yerke, M.C., and Sammatora, D. (2013). Fungicide contamination reduces beneficial fungi in bee bread based on an area-wide field study in honey bee, *Apis mellifera*, colonies. *Journal of Toxicology and Environmental Health, Part A*, **76**, 587-600.

Yourth, C.P., and Schmid-Hempel, P. (2006). Serial passage of the parasite *Crithidia bombi* within a colony of its host, *Bombus terrestris*, reduces success in unrelated hosts. *Proceedings of the Royal Society B: Biological Sciences,* **273**, 655–659.

Yourth, C.P., Brown, M.J.F, and Schmid-Hempel, P. (2008). Effects of natal and novel *Crithidia bombi* (Trypanosomatidae) infections on *Bombus terrestris* hosts. *Insectes Sociaux*, **55**, 86– 90.

Zhao, S., Yuan, S.-K., Cai, B., Jiang H, Wang X-J, Lin R-H, et al. (2011). The acute oral toxicity of 300 formulated pesticides to *Apis mellifera*. *Agrochemicals*, **50**, 278-280.

Zhu, W., Schmehl, D., Mullin, C., and Frazier, J. (2014). Four common pesticides, their mixtures and a formulation solvent in the hive environment have high oral toxicity to honey bee larvae. *PLoS ONE*, **9**, e77547.

Appendices

Appendix 1
Roundup causes high levels of contact mortality in bumble bees

1.1 Supplementary methods

I used five or six bees per box because this number could be sprayed accurately with even application. Higher numbers led to the risk that the exposure of individual bees might be blocked by other bees, while lower numbers would have been inefficient. I did not make an assessment of the dose applied to each bee because this was determined to be impractical. Any physical manipulation of a bee causes it to buzz in response, which could dislodge moisture and thus disrupt the treatment. Further it is standard practice not to measure the dose received in spray application studies (Goodwin and McBrydie, 2000).

Supplementary Table 1. Full details for herbicides used in the experiments.

Brand Name	Given Name in Manuscript	Glyphosate Concentration Pure (g/L)	MAPP	PCS No	Lot No	Production Date	Producer	Licensed From	Purchased From
Weedol Gun! Rootkill Plus	Weedol	7.2	14554	2465	Not Listed	Not Listed	Scotts Miracle-Gro Company, Surrey UK		Homebase, Staines, UK
Fast Action Roundup Ready-To-Use	Roundup Ready-To-Use	7.2	14481	1669	C8534	05/11/2018	Scotts Miracle-Gro Company, Surrey UK	Monsanto, Cambridge UK	Homebase, Staines, UK
Roundup Speed Ultra	Roundup No Glyphosate	0	18692	6259	C8N557	31/10/2018	Scotts Miracle-Gro Company, Surrey UK	Monsanto, Cambridge UK	RHS Wisley Garden Shop, Woking, UK
Roundup ProActive	Roundup ProActive	360	17380	Not Listed	AZE090910A	10/05/2018	Monsanto, Cambridge UK		Agrigem.co.uk, Lincoln, UK

Supplementary Table 2. Treatments used in the experiments, the number of boxes sprayed per experiment, number of bees per box and total number of bees used.

Experiment	Treatment	Number of Boxes Sprayed	Boxes Contained Groups of	Total Bees
1	Control	9	6	54
	Roundup Ready-To-Use	9	6 (one group of 5)	53
	Roundup ProActive	9	6	54
2	Control	8	5	40
	Roundup Ready-To-Use 50%	10	5	50
3	Control	10	5	50
	Roundup Ready-To-Use 25%	10	5	50
4	Control	9	6	54
	Weedol	9	6	54
5	Control	10	5	50
	Roundup No Glyphosate	10	5	50

1.2 Supplementary results

Supplementary Table 3. The results of the model selection process for each analysis using the package 'MuMIn' (Bartoń, 2020). Predictors, AIC, ΔAIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented. Experiment 1: Comparing the impacts of consumer and agricultural Roundup products.

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Colony of Origin, (1|Box ID)	520.7	1.57	0.313	Yes
M1	Treatment, (1|Box ID)	519.1	0.00	0.687	Yes
M2	Colony of Origin, (1|Box ID)	539.8	20.70	0.000	No
M0	(1|Box ID)	539.0	19.92	0.000	No
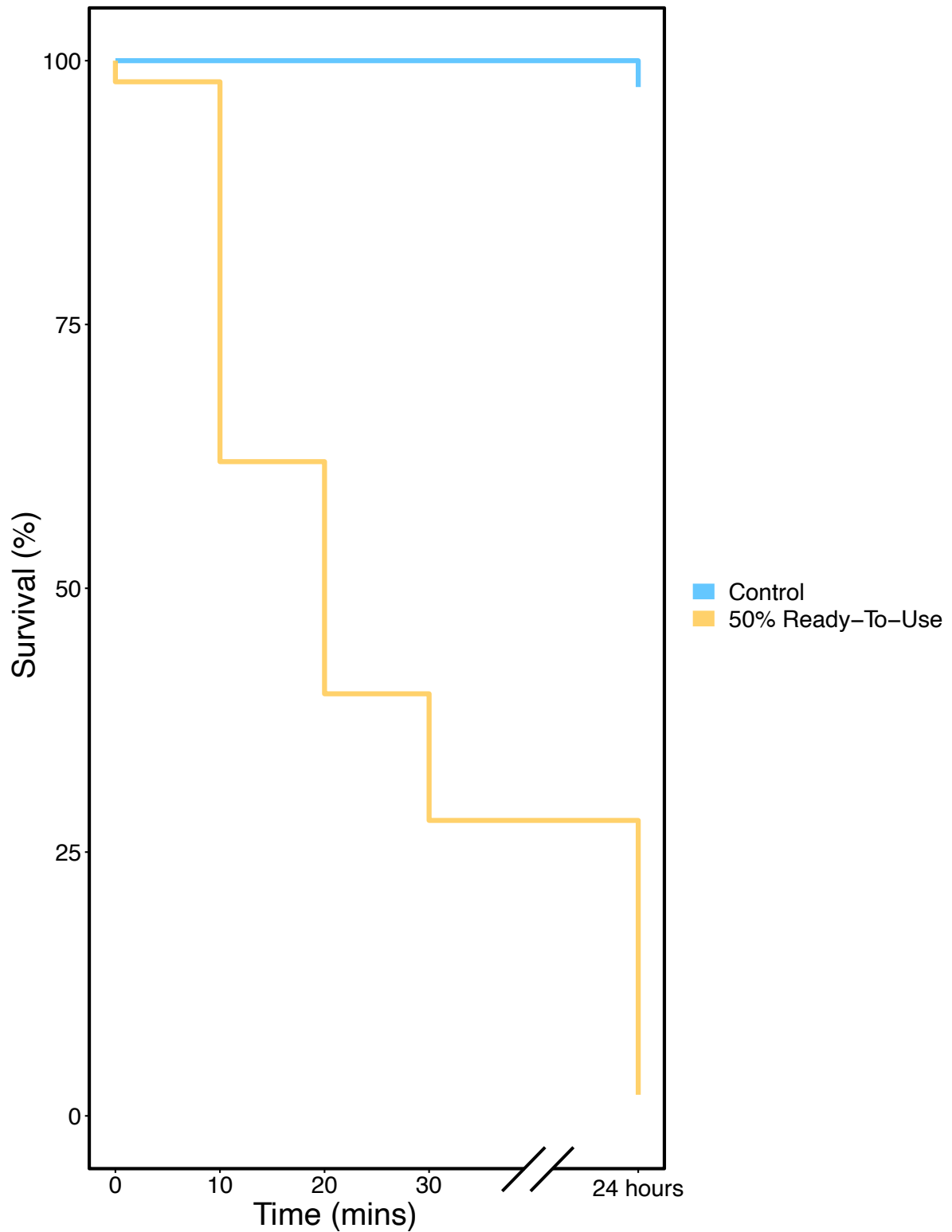
Supplementary Figure 1. Experiment 2: Does mortality still occur with a 1:1 dilution of consumer Roundup? High mortality over 24 hours can be seen, with minimal mortality in the control.
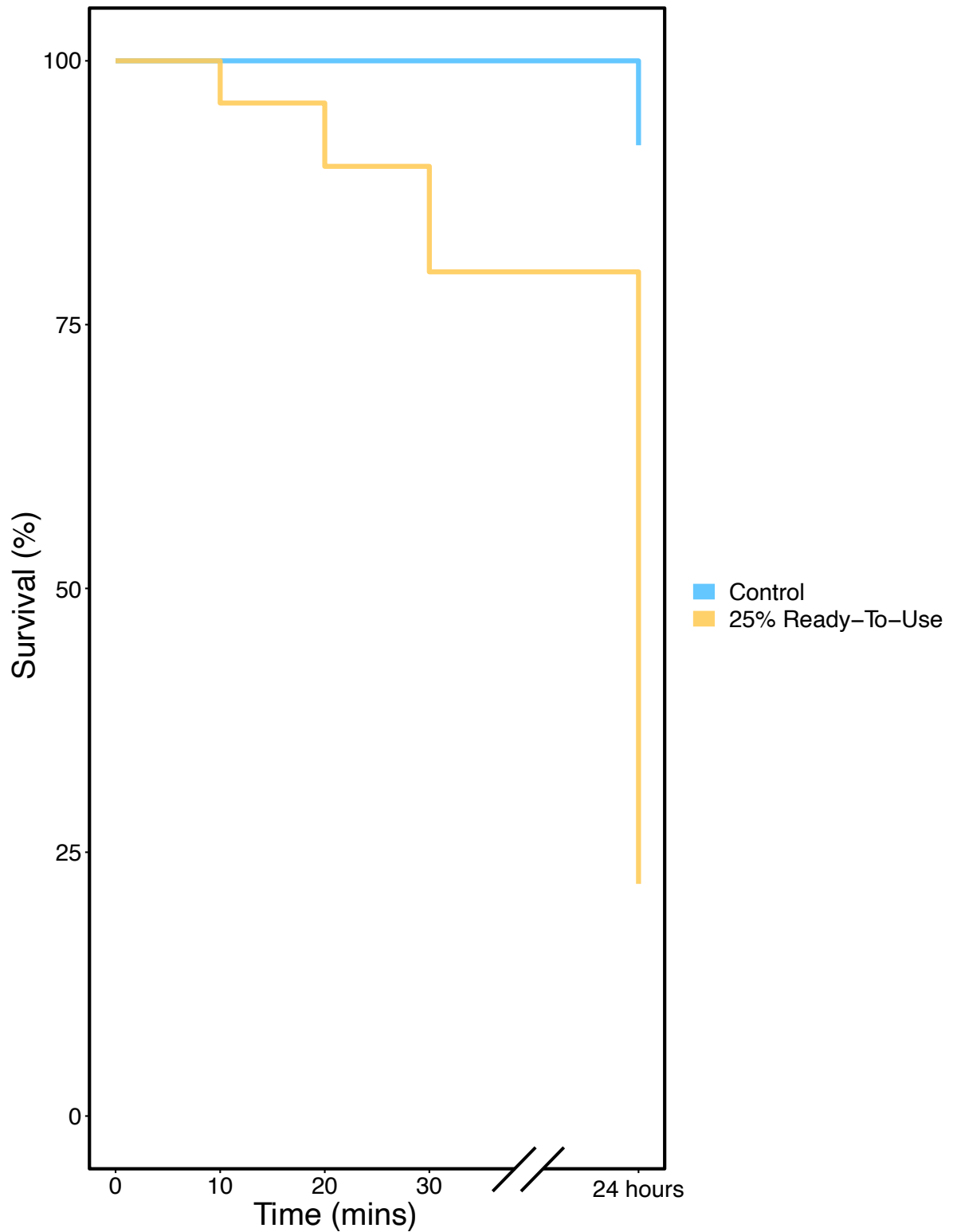
Supplementary Figure 2. Experiment 3: Does mortality still occur with a 1:3 dilution of consumer Roundup? High mortality over 24 hours can be seen, and a delayed response respective to the 50% treatment, with minimal mortality in the control.
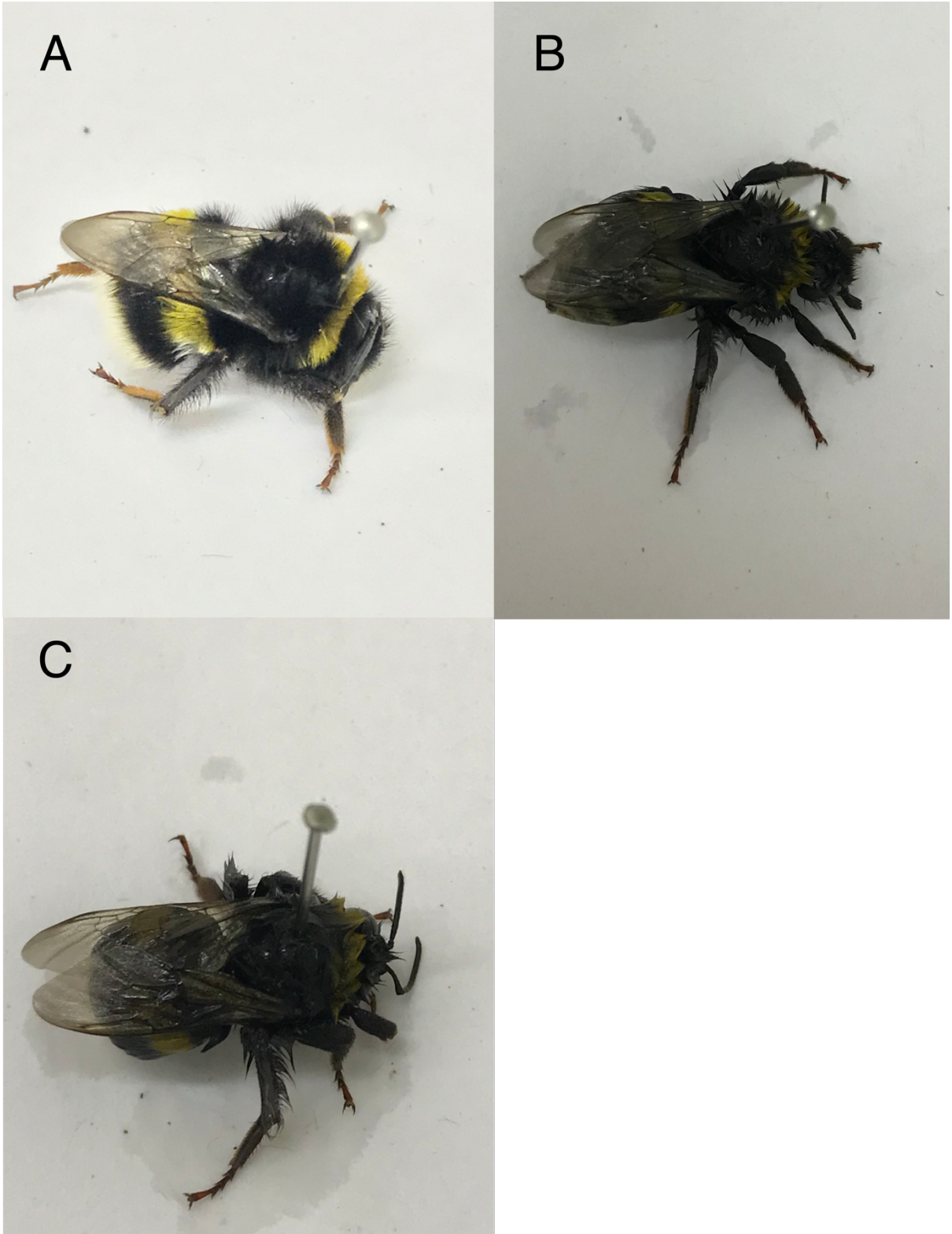
Supplementary Figure 3. (A) Weedol, (B) Roundup No Glyphosate and (C) Roundup ProActive sprayed bumble bees, photographed within 5 minutes of spraying. Matting of the hairs over the bee's whole body can be seen in B and C.

Appendix 2
No evidence of effects or interaction between the widely used herbicide, glyphosate, and a common parasite in bumble bees.

2.1 Supplementary methods

Supplementary Table 1. Experiment 1: Modified OECD 247: small scale treatment conditions and number of bees per treatment.

***Crithidia bombi* only**
10,000 *C. bombi* cells per worker
n = 23
Glyphosate and *C. bombi*
10,000 *C. bombi* cells per worker
200μg per worker
n = 21

Supplementary Table 2. Experiment 2: Modified OECD 247: full scale treatment conditions and number of bees per treatment.

Control	*C. bombi* only	Positive control
	10,000 *C. bombi* cells per worker	4µg dimethoate per worker
n = 45	*n* = 32	*n* = 36
Glyphosate only	**Glyphosate and *C. bombi***	
	10,000 *C. bombi* cells per worker	
200µg per worker	200µg per worker	
n = 40	*n* = 34	

Supplementary Table 3. Experiment 3: Modified OECD 247: Long Term Survival treatment conditions and number of bees per treatment.

Control	*C. bombi* only	Positive control
n = 51	10,000 *C. bombi* cells per worker *n* = 50	4µg dimethoate per worker *n* = 30
Glyphosate only 200µg per worker *n* = 44	**Glyphosate and *C. bombi*** 10,000 *C. bombi* cells per worker 200µg per worker *n* = 47	

Supplementary Table 4. Experiment 4: Microcolony Exposure- Acute treatment conditions and number of bees per treatment. n_s - n_e does not always equal the number of deaths, because a small number of bees escaped the cages, or did not feed on the solutions.

Control	C. bombi only
Microcolonies n_m = 8 Number of bees alive at start of experiment n_s = 64 Number of bees alive at end of experiment n_e = 62	10,000 C. bombi cells per worker Microcolonies n_m = 11 Number of bees alive at start of experiment n_s = 88 Number of bees alive at end of experiment n_e = 74
Glyphosate only	Glyphosate and C. bombi
200µg per worker Microcolonies n_m =9 Number of bees alive at start of experiment n_s = 72 Number of bees alive at end of experiment n_e = 62	10,000 cells per worker 200µg per worker Microcolonies n_m =10 Number of bees alive at start of experiment n_s = 80 Number of bees alive at end of experiment n_e = 64

Supplementary Table 5. Experiment 5: Microcolony Exposure- Chronic treatment conditions and number of bees per treatment. n_s - n_e does not always equal the number of deaths, because a small number of bees escaped the cages, or did not feed on the solutions.

Control	C. bombi only
	10,000 cells per worker
Microcolonies n_m = 8	Microcolonies n_m = 8
Number of bees alive at start of experiment n_s = 64	Number of bees alive at start of experiment n_s = 64
Number of bees alive at end of experiment n_e = 45	Number of bees alive at end of experiment n_e = 44
Glyphosate only	**Glyphosate and C. bombi**
	10,000 cells per worker
200μg per worker	200μg per worker
n = microcolonies 8	n = microcolonies 8
Number of bees alive at start of experiment n_s = 64	Number of bees alive at start of experiment n_s = 64
Number of bees alive at end of experiment n_e = 46	Number of bees alive at end of experiment n_e = 42

2.2 Supplementary results

Supplementary Table 6. Modified Ecotoxicological Protocol OECD 247: Long Term Survival, Mortality: The results of the model selection process for each analysis using the package 'MuMIn' (Bartoń, 2020). Predictors, AIC, ΔAIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Colony of Origin	100.4	6.18	0.039	No
M1	Treatment, Colony of Origin	98.6	4.34	0.098	Yes
M0	Treatment, Bee Weight, Colony of Origin	94.3	0.00	0.862	Yes

Supplementary Table 7. Experiment four: Microcolony Exposure- Acute Exposure, Reproduction. The results of the model selection process for each analysis using the package 'MuMIn' (Bartoń, 2020). Predictors, AIC, ΔAIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Bee Weight, Bees Alive at End, Colony of Origin	7.2	27.35	0.000	Yes
M1	Treatment, Bee Weight, Colony of Origin	-1.8	18.32	0.000	No
M2	Treatment, Bees Alive at End, Colony of Origin	10.3	30.39	0.000	No
M3	Treatment, Colony of Origin	2.7	22.79	0.000	No
M4	Bee Weight, Colony of Origin	-20.1	0	0.991	Yes
M5	Bees Alive at End, Colony of Origin	-5.1	14.97	0.001	No
M0	Colony of Origin	-10.5	0.63	0.008	No

Supplementary Table 8. Experiment four: Microcolony Exposure- Acute Exposure, Parasite Intensity. The results of the model selection process for each analysis using the package 'MuMIn' (Bartoń, 2020). Predictors, AIC, ΔAIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Microcolony, Colony of Origin	2742.6	2.13	0.177	Yes
M1	Treatment, Colony of Origin	2740.4	0	0.513	Yes
M2	Treatment, Microcolony	2741.4	1.01	0.309	Yes
M0a	Colony of Origin	2754.4	14.01	0.000	No
M0b	Microcolony	2755.7	15.22	0.000	No

Supplementary Table 9. Experiment four: Microcolony Exposure- Chronic Exposure, Reproduction. The results of the model selection process for each analysis using the package 'MuMIn' (Bartoń, 2020). Predictors, AIC, ΔAIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Bee Weight, Bees Alive at End, Colony of Origin	5.7	24.57	0.000	Yes
M1	Treatment, Bee Weight, Colony of Origin	-0.9	17.93	0.000	No
M2	Treatment, Bees Alive at End, Colony of Origin	3.6	22.48	0.000	No
M3	Treatment, Colony of Origin	-4.2	14.64	0.000	No
M4	Bee Weight, Colony of Origin	-17.0	1.84	0.279	Yes
M5	Bees Alive at End, Colony of Origin	-11.8	7.03	0.021	No
M0	Colony of Origin	-18.9	0.00	0.699	Yes

Supplementary Table 10. Experiment four: Microcolony Exposure- Chronic Exposure, Sucrose Consumption. The results of the model selection process for each analysis using the package 'MuMIn' (Bartoń, 2020). Predictors, AIC, ΔAIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Bee Weight, Microcolony, Colony of Origin	-224.0	63.77	0.000	No
M1	Treatment, Microcolony, Colony of Origin	-229.3	72.01	0.000	No
M2	Treatment, Colony of Origin	-215.7	22.48	0.000	No
M3	Treatment, Microcolony	-229.0	58.73	0.000	No
M0a	Bee Weight, Microcolony, Colony of Origin	-282.1	5.59	0.053	Yes
M0b	Bee Weight, Colony of Origin	-267.6	20.12	0.000	No
M0c	Bee Weight, Microcolony	-283.0	4.73	0.081	Yes

| M0d | Microcolony, Colony of Origin | -287.7 | 0.00 | 0.866 | Yes |

Supplementary Table 11. Experiment four: Microcolony Exposure- Chronic Exposure, Parasite Intensity. The results of the model selection process for each analysis using the package 'MuMIn' (Bartoń, 2020). Predictors, AIC, ΔAIC from the Best Model, AIC Weight and whether the model was included in the final parameter estimates are all presented.

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Microcolony, Colony of Origin	1683.6	1.69	0.239	Yes
M1	Treatment, Colony of Origin	1684.0	2.04	0.202	Yes
M2	Treatment, Microcolony	1681.9	0.00	0.558	Yes
M0a	Colony of Origin	1697.6	15.68	0.000	No
M0b	Microcolony	1699.3	17.36	0.000	No
M0c	Microcolony, Colony of Origin	1700.0	18.07	0.000	No

Appendix 3
Co-formulant in a commercial fungicide product causes lethal and sub-lethal effects in bumble bees

3.1 Supplementary methods

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

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

Materials

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. Ministerially Approved Pesticide Product (MAPP).

Brand Name	Azoxystrobin concentration 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

3.2 Supplementary results
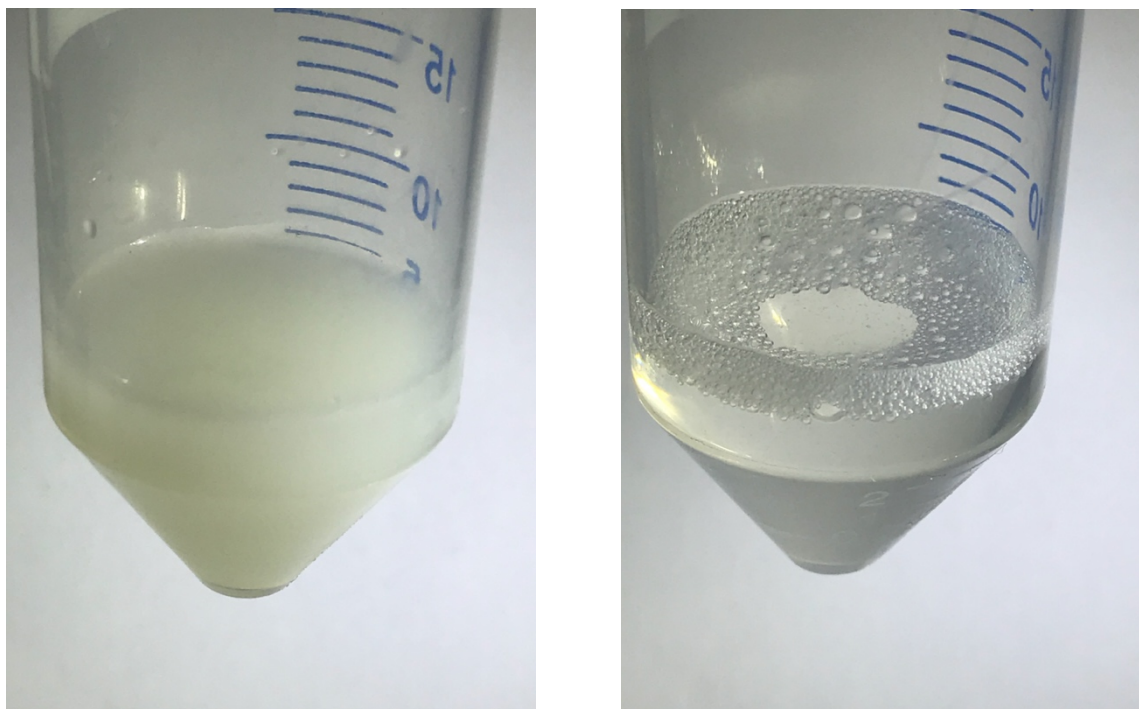
Supplementary Figure 1. (Left) Amistar treatment solution. (Right) co-formulant mixture treatment solution.

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. To dissolve azoxystrobin I trialled both acetone and water, with neither being suitable because flocculation occurred once mixed with sucrose.
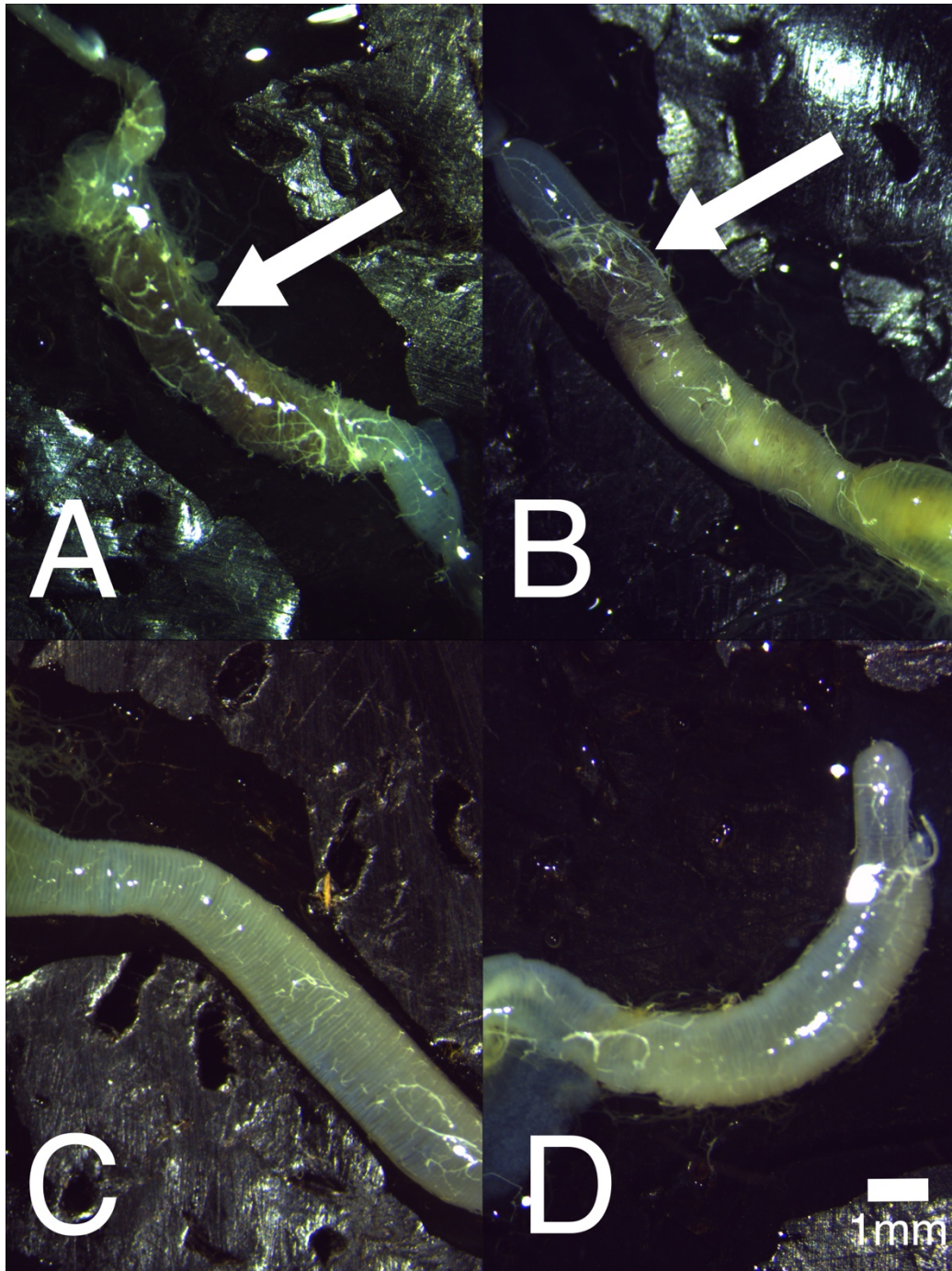
Supplementary Figure 2. Bumble bee foreguts of bees who survived the full 120 hours.

(Top Left-A) Amistar treatment. A large area of the gut is visibly darkened. (Top Right-B) alcohol ethoxylates treatment. A large area of the gut is visibly darkened, and some brown spots are visible. (Bottom left-C) Naphthalenesulfonic acid treatment. No melanisation is visible. (Bottom Right-D) Benzisothiazol treatment. No melanisation is visible. The images in supplementary Figure 2 are sample pictures, and all images are available upon request from the authors.
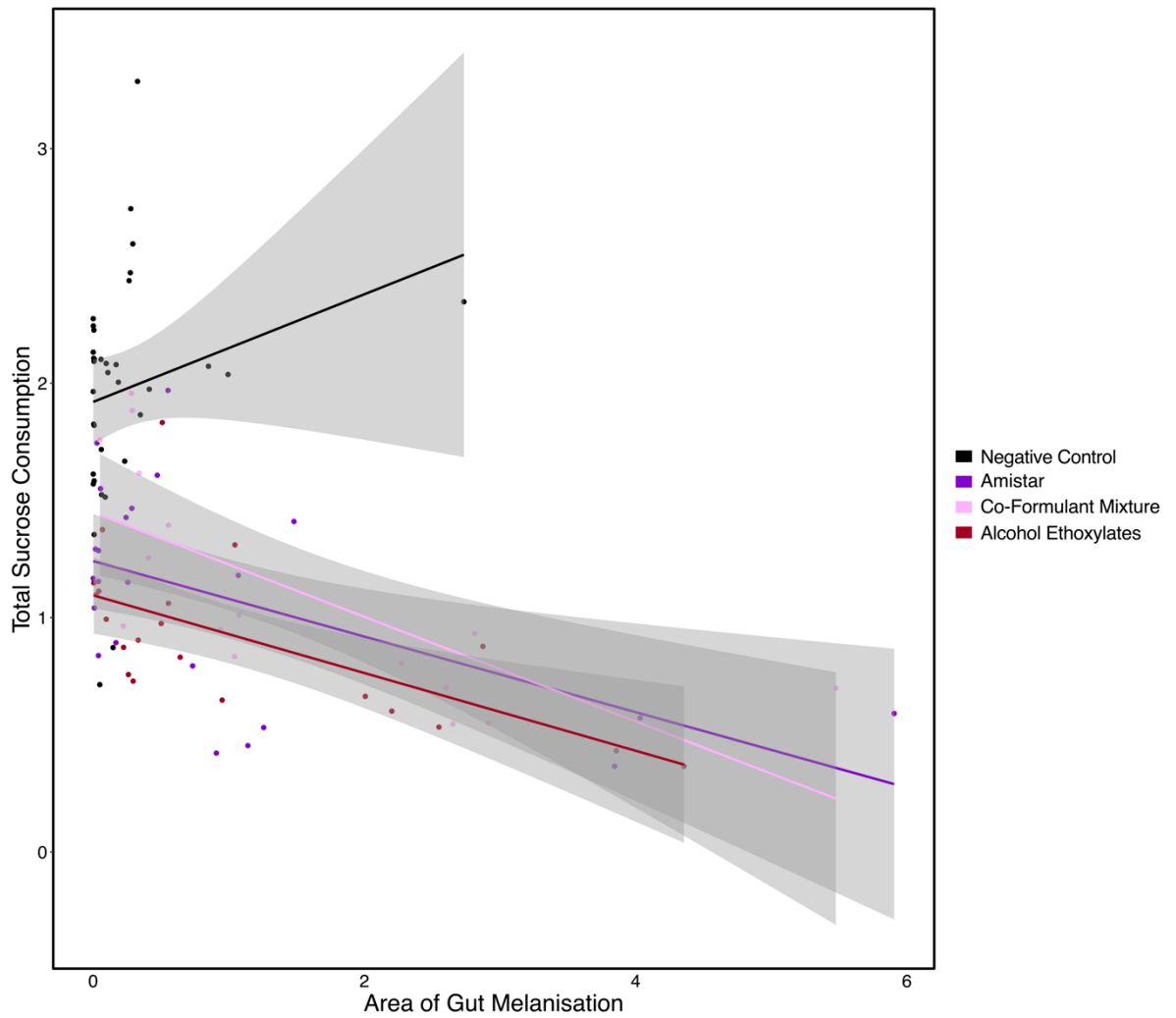
Supplementary Figure 3. Total Sucrose Consumption per bumble bee plotted against area of gut melanisation, with 95% CI. Naphthalenesulfonic acid and benzisothiazol treated bees have been omitted to aid the clarity of the graph. A correlation between increasing area of gut melanisation and reduced sucrose consumption is visible.

Mortality

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

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Bee Weight, Colony of Origin	225.1	0.00	0.352	Yes
M1	Treatment, Colony of Origin	225.7	0.59	0.262	Yes
M2	Treatment, Bee_Weight	225.7	0.58	0.263	Yes
M3	Treatment	228.2	3.09	0.075	Yes
M0a	Colony of Origin	229.7	4.60	0.011	No
M0b	Bee Weight	232.3	7.19	0.246	No
M0c	Nothing	233.5	9.38	0.011	No

The slightly lower mortality seen in the Amistar treatment versus co-formulant mixture and alcohol ethoxylates, is not statistically significant (Cox proportional hazards model: parameter estimate PE = 0.08, 95% CI [-0.48 to 0.65] and (PE) = 0.04, 95% CI [-0.38 to 0.45], respectively).

There was a small effect of bee weight at the beginning of the experiment on mortality (Cox proportional hazards model: parameter estimate (PE) = -9.31, 95% CI [-17.89 to -0.74]), with heavier bees less likely to die.

There was a significant effect of colony of origin on mortality for one of two colonies (Generalised linear model: parameter estimate (PE) = -1.73, 95% CI [-3.22 to -0.24] and PE = -0.41, 95% CI [-1.36 to 0.54]), when compared to an arbitrarily chosen reference colony.

Sucrose Consumption

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

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Bee Weight, Colony of Origin	150.4	0.00	1.000	Yes
M1	Treatment, Colony of Origin	181.2	30.80	0.000	No
M2	Treatment, Bee_Weight	180.4	30.05	0.000	No
M3	Treatment	202.0	51.62	0.000	No
M0a	Colony of Origin	296.7	145.35	0.000	No
M0b	Bee Weight	302.4	152.00	0.000	No
M0c	Nothing	307.2	156.78	0.000	No

Neither benzisothiazol nor naphthalenesulfonic acid had significantly different consumption versus the control (Generalised linear model: parameter estimate (PE) = -0.05, 95% CI [-0.22 to 0.12] and PE = -0.15, 95% CI [-0.33 to 0.02], 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 (Generalised linear

model: parameter estimate (PE) = -0.09, 95% CI [-0.34 to 0.15] and PE = -0.21, 95% CI [-0.43 to 0.02], respectively).

There was a significant effect of bee weight on sucrose consumption (Generalised linear model: parameter estimate (PE) = 3.66, 95% CI [2.44 to 4.86]), with heavier bees drinking more.

There was a significant effect of colony of origin on mortality for one of two colonies (Generalised linear model: parameter estimate (PE) = -0.42, 95% CI [-0.55 to -0.28] and PE = --0.12, 95% CI [-27 to 0.03]), when compared to an arbitrarily chosen reference colony.

Weight Change

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

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Bee Weight, Colony of Origin	-760.2	2.65	0.191	Yes
M1	Treatment, Colony of Origin	-754.2	8.70	0.009	No
M2	Treatment, Bee_Weight	-762.9	0.00	0.719	Yes
M3	Treatment	-758.5	4.37	0.191	Yes
M0a	Colony of Origin	-737.4	17.51	0.000	No
M0b	Bee Weight	-745.4	152.00	0.000	No
M0c	Nothing	-741.4	21.468	0.000	No

Neither benzisothazol nor naphthalenesulfonic acid had significantly different weight change versus the negative control (Generalised linear 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 (Generalised linear

model: parameter estimate (PE) = -0.01, 95% CI [-0.01 to 0.01] and PE = -0.01, 95% CI [-0.02 to 0.01], respectively).

There was no significant effect of bee weight on weight change (Generalised linear model: parameter estimate (PE) = -0.11, 95% CI [-0.22 to 0.00]).

There was no significant effect of colony of origin on weight change (Generalised linear model: parameter estimate (PE) = 0.00, 95% CI [-0.00 to 0.01] and PE = -0.00, 95% CI [-0.01 to 0.01], for either colony compared to an arbitrarily chosen reference colony).

Gut Melanisation

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

Model Name	Predictors	AIC	ΔAIC from best model	AIC Weight	Included in Final Model Set
FM	Treatment, Bee Weight, Colony of Origin	509.1	3.94	0.108	Yes
M1	Treatment, Colony of Origin	513.2	8.06	0.014	No
M2	Treatment, Bee_Weight	505.1	0.00	0.779	Yes
M3	Treatment	509.3	4.14	0.099	Yes
M0a	Colony of Origin	539.4	34.23	0.000	No
M0b	Bee Weight	533.5	28.38	0.000	No
M0c	Nothing	536.2	31.11	0.000	No

Neither benzisothiazol nor naphthalenesulfonic acid had significantly different melanised area versus the negative control (Generalised linear 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 $0.240mm^2$ and $0.116mm^2$ respectively, compared to the $0.230mm^2$ 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 (Generalised linear

model: parameter estimate (PE) = 0.50, 95% CI [-0.45 to 0.76] and PE = -0.04, 95% CI [-0.38 to 0.36], respectively).

There was no significant effect of bee weight on gut melanisation (Generalised linear model: parameter estimate (PE) = -3.33, 95% CI [-6.18 to 0.19]).

There was no significant effect of colony of origin on weight change (Generalised linear model: parameter estimate (PE) = -0.04, 95% CI [-0.12 to 0.11] and PE = -0.12, 95% CI [-0.15 to 0.12], for either colony compared to an arbitrarily chosen reference colony).

Appendix 4
A systematic review of the effects of 'inert' ingredients on bees

4.1 Supplementary methods

Primary Exclusion Criteria

Studies which:

Present new experimental research

>> Testing at least one treatment being an agricultural co-formulant or adjuvant (as defined by the authors), with an appropriate control.

> OR

>> Measuring residues of an agricultural co-formulant or adjuvant in bees, honey, or wax, or bee collected nectar or pollen

Literature was initially characterised by title, with titles lacking relevance to agriculture, bees or pesticides being excluded.

Literature was then characterised by abstract, with abstracts lacking relevance to the search criteria removed.

Literature was finally characterised by a full read. Here the full exclusion criteria were applied. Throughout the search ambiguous titles like '100 pesticides…' were retained to the next stage until detail could be found to exclude or include them.

Specific exclusion criteria:

Studies identified from Web of Science, Google Scholar and forward/backward tracing must be accessible with the information provided by those sources.

The novel research fitting the above criteria must be written in the English language.

Publications only accepted from peer reviewed journals.

Papers not accessible online, despite all reasonable efforts made to acquire them were excluded.

Edge case definitions:

Adjuvant definition: tank additives without purpose of specific pesticidal action (regardless of organic/regulatory status). For instance, neem oil adjuvants which are marketed as insecticidal would be excluded.

Synergists: because synergists are included in insecticide formulations for their specific toxicity/immune inhibitory function to insects, despite being non-lethal themselves, they fall outside the scope of co-formulants as defined here.

Standard solvents: Solvents are used to dissolve pure active ingredients, would only meet the criteria for a valid treatment group if the manuscript explicitly mentions that the same solvent is used in a formulations. This is because the solvents used commonly by researchers are rarely the same substances used by industry, so it cannot be assumed to be true.

Search Terms and Information

A Web of Science Core Collection advanced search was conducted on 30/04/2020 and repeated on 09/07/2021 with the following terms:

The Web of Science Core Collection search was conducted in November 2020 using the following terms Topic, Title and Abstract Search = (((adjuvant* OR coformulant* OR co-formulant* OR *formulant* OR inert) OR (penetra* OR "odour mask*" OR stabiliz* OR stabilis* OR preservative* OR surfactant* OR emulsifier* OR diluent* OR propellant* OR anti-foaming OR antifoaming OR solvent* OR carrier*)) AND (*bee OR *bees)))).

The Abstract search did not use wildcards before words because left hand truncation is not supported for this search type. A supplementary Google Scholar search was made to ensure all literature was captured with the terms ("bee" OR "bees") AND ("adjuvant" OR "coformulant" OR "co-formulant" OR "formulant"), searched on 06/05/2020. Forward citation tracing was performed with Google Scholar on 07/05/2020.

Assessment of bias

There is a time lag between publication and indexing on databases which may mean relevant literature published recently was missed. Further older literature may not have been digitised or indexed appropriately, leading to early research being missed. Exclusion of studies written in languages other than English could bias reporting toward agricultural practices in English speaking countries. This would lead to an underestimation of the published research.

There is a risk of a publication bias towards studies detecting an effect of 'inert' ingredient on bees, or of finding 'inert' ingredient residues in relevant bee matrices. There is no reason to believe this is more likely to be occurring than in any other field. Further no quantitative synthesis has been performed, so a publication bias would not affect the qualitative reporting.

Supplementary Assessment of Comprehensiveness.

As the systematic review has demonstrated there is very little research on the effects of 'inert' ingredients on bees. Because of this several publications focus primarily on whole formulations, tank mixes or active ingredients. This means that 'inert' ingredients are often secondary study subjects, which could prevent them from properly featuring in titles, abstracts and topic fields which would affect their likelihood of appearing in search. The emerging field also has yet to properly settle on definitions of adjuvant, often being used to describe a co-formulant. There is industry accepted definitions of adjuvant as reviewed in Hazen, (2000) who's adoption would promote clear discourse. Authors did not always specify if the 'inert' ingredient tested was an adjuvant or a co-formulant, or what chemical property it conferred to the tank mixture, as such these search terms may not have detected some literature. The use of the specific terms and general terms was an attempt to mitigate this. The lack of explicit specification prevented proper categorisation of some studies, and going forward studies should explicitly describe their treatments chemicals in as much detail as possible. When using products like adjuvants reporting levels varied with not all studies listing sufficient information to identify the specific product tested, this caused difficulties in categorising some substances. High reporting standards would include providing enough information for the product to be sourced independently, and with the confidence that it was the same mixture. Producer product codes are an excellent resource here. Because the

distribution of pesticides is restricted providing details of where products were purchased from will also help future work, and aid researchers entering the field. Not all authors ascribed field realism or a lack of field realism to their experimental work. This was partly caused by several studies testing a range of doses, demonstrating hazard not risk.
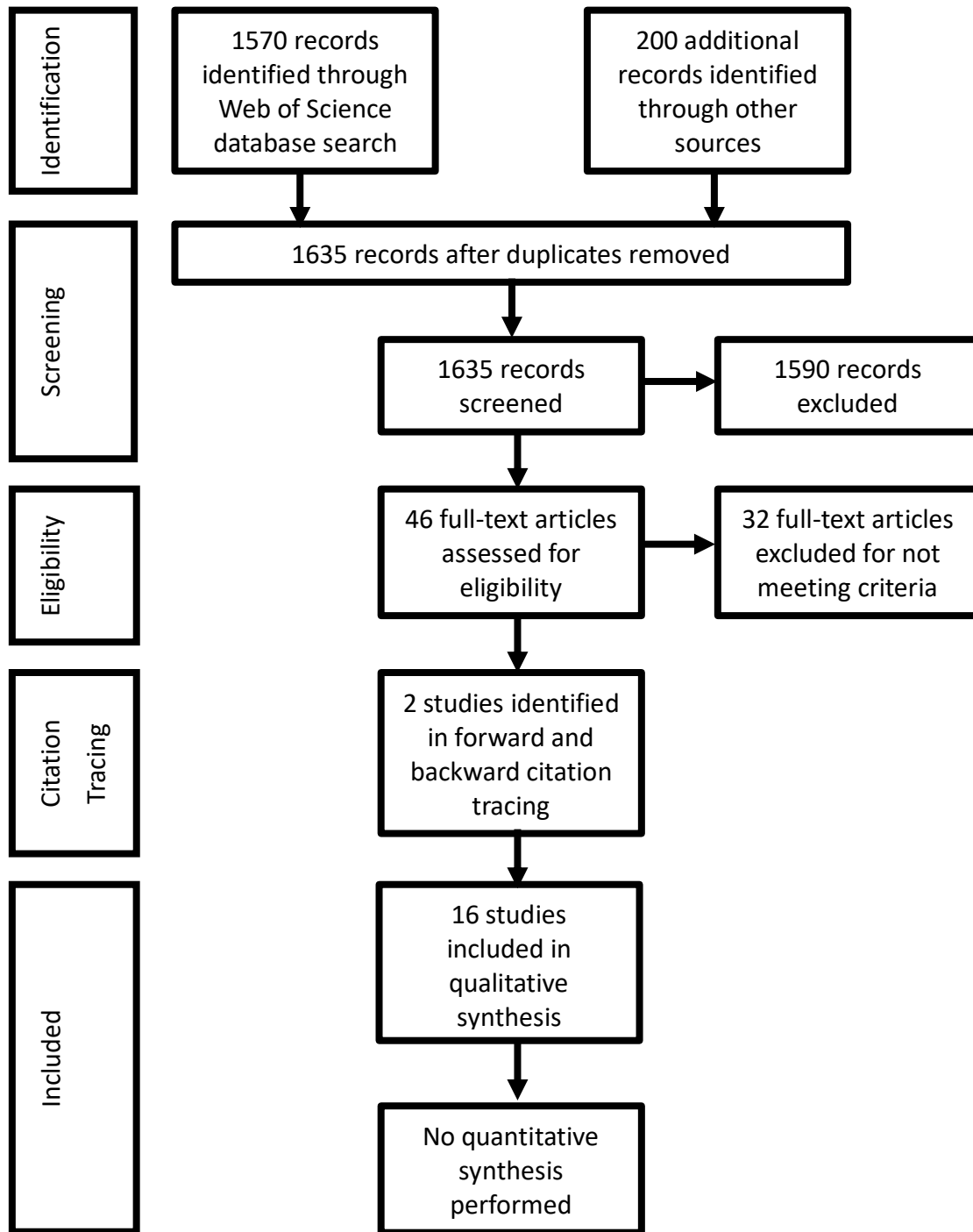
Supplementary Figure 1. A PRISMA flow diagram detailing search protocol.

Supplementary Table 1. The PRISMA reporting checklist.

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Main text
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	Main text. No systematic review registration
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	Main text
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	Supplementary Information
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	Supplementary Information. No registration
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	Supplementary Information
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	Main text

Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Main text and Supplementary Information
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	Main text and Supplementary Information
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	Supplementary Information
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	Supplementary Information
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	Supplementary Information
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	Main text
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	Main text
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	Main text
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	Supplementary Information
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	Supplementary Information

Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	No meta-analysis performed
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome-level assessment (see Item 12).	Not performed
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group and (b) effect estimates and confidence intervals, ideally with a forest plot.	Supplementary Information
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	No meta-analysis performed
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	No quantitative assessment of bias performed
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	NA
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., health care providers, users, and policy makers).	Main text
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review level (e.g., incomplete retrieval of identified research, reporting bias).	Supplementary Information
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	Main text
FUNDING			

Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	Main text

Final Acknowledgements

A bumble bee resting on a burnt log at Bagshot Heath.

To all the bees and people who made this happen, thank you.

"It's all about the greater good.

All: The greater good"