Little and often makes much: identifying the time-reinforced toxicity of pesticides and their impacts on bees

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Abstract

Bees provide important pollination services for crops and wild flowers, estimated to be valued at £120 billion to the global economy. However, declining bee populations have put these services in jeopardy. Pesticides are widely blamed, at least in part, for declines in both wild and managed bee species. Bees are exposed to dietary residues of pesticides when foraging on the nectar and pollen of treated bee-attractive crops. However, these residues are generally found at such low levels that it would not be feasible for a bee to ingest an acute lethal dose. Pesticides which exhibit time-reinforced toxicity could cause mortality to bees over an extended exposure period, though, as the damage they cause can increase exponentially over time. Currently, there is no test for time-reinforced toxicity included in bee risk assessments of pesticides. The overall aims of this thesis were to identify pesticides that exhibit time-reinforced toxicity and determine their effects on a range of demographically important sublethal endpoints in bees.

Using a bioassay based on Haber's Law, I identified fipronil as a pesticide exhibiting time-reinforced toxicity (TRT) in both the honey bee (*Apis mellifera*) and bumble bee (*Bombus terrestris*), from four widely-used candidate pesticides. Fipronil at field-relevant levels was found to significantly reduce the longevity and feeding of individual worker bumble bees and those in microcolonies. This nutrient limitation was postulated to be the cause of reduced fecundity of bumble bee microcolonies exposed to dietary fipronil at concentrations of 1 part per billion and less. The toxic effect of fipronil was dramatically increased when microcolonies were placed outside to forage for food, an effect documented by several other studies, and potentially due to an increase in metabolic rate from the need to fly. However, these effects were not

observed in queenright *Bombus terrestris* colonies in the field. This disparity in effects may have been due to problems with exposure to fipronil rather than any possible resilience of colonies.

The thesis findings highlight the need for time-reinforced toxicity testing in bees to be integrated into current risk assessment protocols for pesticides. My work in this thesis has provided validation for the use of the TRT bioassay in future risk assessments of pesticides. Current-use pesticides that exhibit TRT, in this case fipronil, pose a serious threat to both wild and managed bees, impacting on demographically important endpoints including feeding and reproduction. Further research, continuing on from the work in this thesis, is needed to ascertain the impacts of TRT pesticides at both colony and population levels. Determining the mechanisms of TRT pesticides will also be key to protecting bees from the danger they pose.

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List of abbreviations and uncommon units

µg L^{-1}	Micrograms per litre
Ach	Acetylcholine
AIC	Akaike's information criterion
ANOVA	Analysis of variance
BCF	Bioconcentration factor
CNS	Central nervous system
DDT	Dichloro-diphenyl-trichloroethane
DDE	Dichloro-diphenyl-dichloroethylene
df	Degrees of freedom
DNA	Deoxyribonucleic acid
EBI	Ergosterol-biosynthesis-inhibiting
EC_{50}	Median effective concentration
edf	Effective degrees of freedom
EFSA	European Food Safety Authority
EU	European Union
FERA	Food and Environment Research Council
GABA	Gamma-aminobutyric acid
GAM	General additive model
GCMS	Gas chromatography mass spectrometry
GLM	General linear model
GLMM	Generalised linear mixed model
h	Hour
ha	Hectare
K_{ow}	Octanol-water coefficient
L_{50}	Median lethality
LC_{50}	Median lethal concentration

LCMS	Liquid chromatography mass spectrometry
LD_{50}	Median lethal dose
LEAF	Linking Environment And Farming
LOD	Limit of detection
log	Logarithm
LOQ	Limit of quantification
LT_{50}	Median lethal time
min	Minute
m/z	Mass-to-charge ratio
Na	Sodium
nAChR	Nicotinic acetylcholine receptor
NERC	Natural Environment Research Council
NOEL	No observed effect limit
OECD	Organisation for Economic Co-operation and Development
ppb	Parts per billion
PPPs	Plant protection products
Qual	Qualitative ion
rpm	Revolutions per minute
SIM	Selected-ion monitoring
TER	Toxicity exposure ratio
TRT	Time-reinforced toxicity
v/v	Volume/volume percent
w/v	Weight/volume percent

Author’s declaration

Unless otherwise stated, the author was responsible for all data collection and analysis. The use of the first person plural (i.e. ‘we’ as opposed to ‘I’) reflects the contributions of my supervisors in providing advice in experimental design, data analysis and interpretation, and of the many students and staff at the University in providing laboratory and field support during data collection.

Chapter One:

General introduction

1.1 Toxicity and bioaccumulation

1.1.1 Toxicity, toxicodynamics and toxicokinetics

The toxicity of a chemical is determined by its toxicokinetics and toxicodynamics (Rozman and Doull, 2000). Toxicokinetics determines the delivery of molecules of a toxicant which are able to reach receptors on the target tissue of a living organism (**Figure 1.1**). This encompasses toxicant uptake, transport, metabolism, sequestration and excretion. While toxicodynamics determines the number of receptors that are able to bind with the toxicant molecules that reach them, involving binding, interaction with the target molecule and the generation of injury. Toxicity occurs when the level of injury caused by a toxicant exceeds the capacity of an organism to repair and adapt, or when repair mechanisms and adaptation become dysfunctional (Klaassen, 2013). Bioaccumulative toxicants are resistant to detoxification and elimination processes, allowing more molecules to build up within an organism's tissues and potentially leading to greater injury over time (Franke et al., 1994).

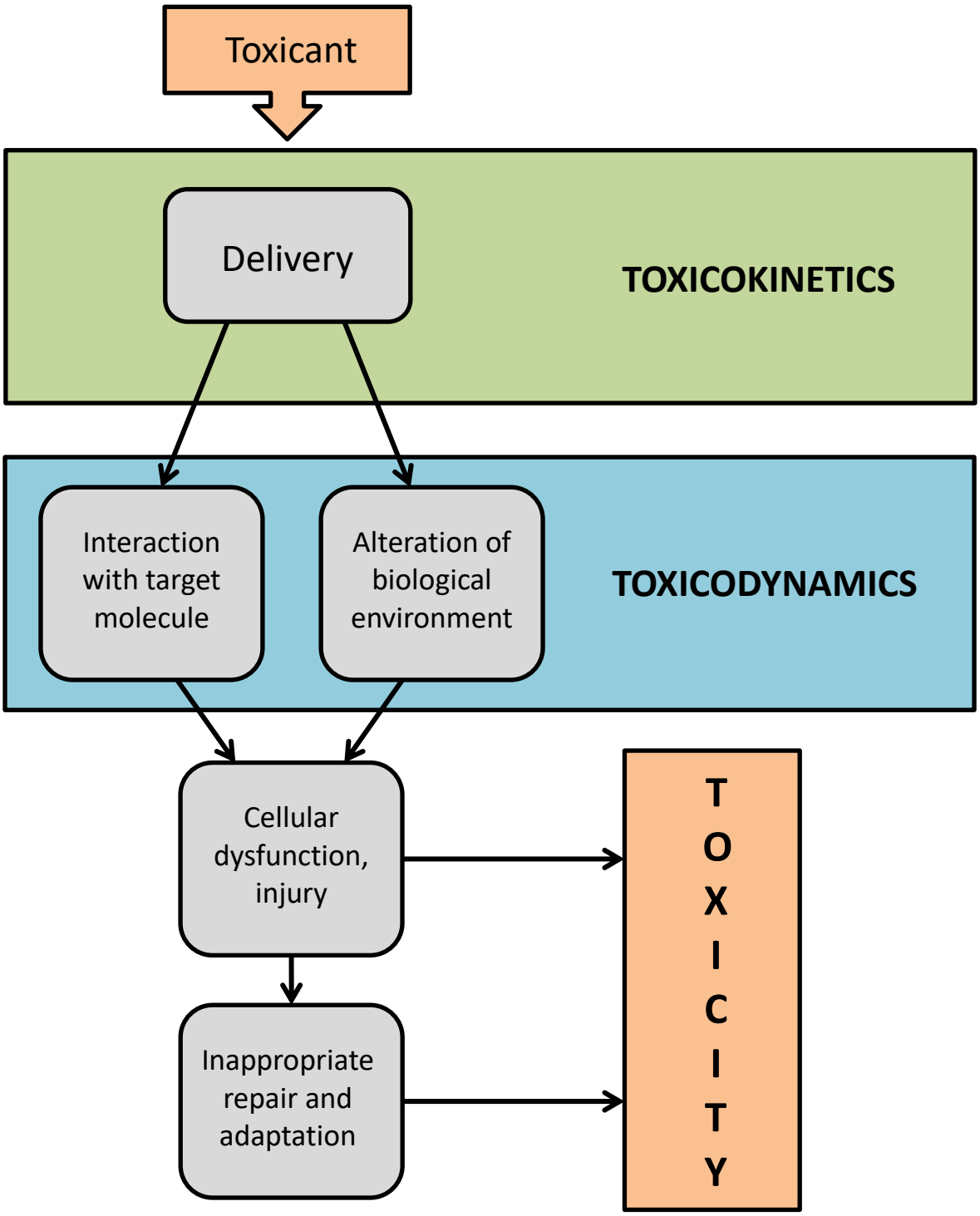

Figure 1.1 Possible stages in the development of toxicity after exposure to a toxicant. Figure adapted from (Klaassen, 2013).

1.1.2 Bioaccumulative pesticides

Historically, bioaccumulative pesticides have been the cause of large-scale damage to ecosystems. The organochlorine dichloro-diphenyl-trichloroethane (DDT) is the most well-known example of a bioaccumulative pesticide. Used as a broad-spectrum insecticide to control both human and crop pests, DDT was both highly persistent in the environment and also accumulated in the fatty tissue of exposed organisms. DDT, and its persistent metabolite(s) DDEs, are now believed to be present in every living organism (Turusov et al., 2002). This high level of bioaccumulation has led to the biomagnification of DDT through trophic levels, leading to toxic levels in some primary predators (Turusov et al., 2002). DDT accumulates in all tissues of living organisms but predominantly in fat, resulting in high storage levels (Smith, 1991). This property leads to high accumulation within fat-rich reproductive tissues and can cause complications in the reproduction of a range of species (Burdick et al., 1964, Wurster Jr. and Wingate, 1968). Though not necessarily causing fatalities to adult predatory birds, DDT exposure brought several species close to population collapse as it caused thinning of egg shells leading to reproductive failure (Grier, 1982, Turusov et al., 2002). Due to these negative impacts on wildlife, DDT was banned for use in many countries during the 1970s, though it is still used in some countries for control of vector-borne disease in humans (Turusov et al., 2002). Other organochlorines, endrin and methoxychlor, also accumulate in aquatic invertebrates, affecting locomotory activity and coordination, and increasing mortality (Anderson and DeFoe, 1980). These examples highlight the damage that can be caused by bioaccumulative toxicants in the environment. However, there is another mechanism that creates a potential threat to

organisms under prolonged exposure to environmental toxicants that bioaccumulate, which is time-reinforced toxicity (TRT).

1.2 What is time-reinforced toxicity (TRT)?

Prolonged exposure to a toxicant that exhibits TRT causes an increasing level of injury over an extended exposure period, which may lead to toxic effects emerging much earlier than expected given the impacts of an acute exposure. Specifically, TRT is manifested when for a given level of exposure, the effects of prolonged exposure are disproportionate to the effects of an acute exposure. Although the effects arising from TRT are most easily linked to bioaccumulative toxicants, bioaccumulation and time-reinforced toxicity are not necessarily linked. These linkages (**Table 1.1**) will now be reviewed.

		Time-reinforced toxicity	
Bioaccumulation		**N**	**Y**
	N		**X**
	Y		**X**

Table 1.1 Relationship between bioaccumulation and time-reinforced toxicity of a toxicant within a living organism.

1.2.1 Bioaccumulative – No, TRT – No

A non-bioaccumulative toxicant both binds reversibly to its target site and is susceptible to catabolic breakdown and elimination; during a sustained dietary exposure, the continuous and opposing actions of ingestion and elimination will establish the toxicant at a 'steady state' concentration inside the organism instead of accumulating. Consequently, the daily rate of injury is constant and the accumulated total injury is proportional to the duration of the exposure. This proportionality means that toxicological experiments on such a system will find that halving the dosage rate doubles the duration of the exposure that is required to achieve a given level of injury or effect (Rozman, 2000).

1.2.2 Bioaccumulation – Yes, TRT – No

A toxicant may bioaccumulate at its target site, irreversibly binding to target molecules, which is the case for the carcinogen Butter Yellow when binding to DNA (Warwick and Roberts, 1967). However, if each toxicant molecule only causes a single unit of injury when it binds to the target site then the accumulated total injury is still proportional to the exposure time. Therefore the toxicant will not exhibit TRT as this would require each bound toxicant molecule to induce a unit of damage for each unit of time.

Alternatively, a toxicant may bioaccumulate in non-target tissues which will not lead to as great a level of injury and therefore not generate TRT. For example, DDT bioaccumulates predominantly in fatty tissues (Smith, 1991) where it can disrupt reproduction but is generally stored until fat is metabolised and so does not cause TRT. Some organisms, such as earthworms, are able to sequester bioaccumulative toxicants away from target receptors, rendering them relatively harmless (Vijver et al., 2004).

1.2.3 Bioaccumulative – No, TRT – Yes

Potentially a non-bioaccumulative toxicant could still exhibit TRT if the injury caused when its molecules bind to the target site leads to the formation of a persistent lesion (**Figure 1.2 (2)**), which itself proliferates and thereby causes further injury over time, independent of toxicant exposure.

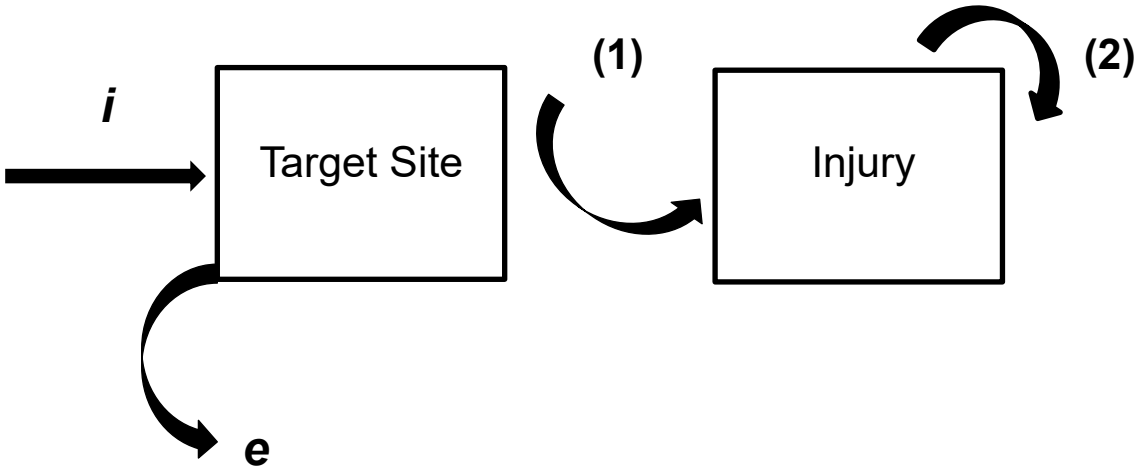

Figure 1.2 Possible pathway of a toxicant entering a living organism. The toxicant enters the organism (intake, ***i***) and binds to its target site. Some or all of the toxicant may be eliminated (***e***) from the organism before or after this occurs. Binding of the toxicant to its specific binding site can lead to the formation of injury (**1**) which may in turn lead to further injury (**2**), independent of future intake, by means of a persistent and proliferating lesion.

1.2.4 Bioaccumulative – Yes, TRT – Yes

If a toxicant bioaccumulates at its target site, resisting detoxification and elimination processes, the organism's internal concentration rises as intake proceeds during the exposure. Consequently, the rate of injury increases

during the exposure and the accumulated total injury is not proportional to exposure time but instead increases exponentially. This is the case with TRT pesticides, which over prolonged exposures can produce injury from exposure to even small residue levels as they build up to lethal levels. Therefore TRT toxicants have the potential to cause much greater injury than may be predicted from the exposure concentration, and therefore they pose a greater risk to exposed organisms. These potential impacts highlight the need to identify TRT toxicants before they are released into the environment.

1.3 How to identify TRT

1.3.1 Bioaccumulative pesticides

The ability of a toxicant to accumulate within an exposed organism can be estimated by its bioconcentration factor (BCF), calculated from its K_{ow} value (octanol-water partition coefficient) (Lu et al., 2000), which reflects its ability to accumulate in fat within organisms. For example, *a* chemical is considered to have a high bioconcentration potential if log $K_{ow} \geq 5$ or BCF ≥5000 (Dimitrov et al., 2003, Rodan et al., 1999); this is true of DDT, which has a log $K_{ow} = 5.98$ and BCF = 61,600 to 84,500 (Kenaga, 1980). However the K_{ow} value is not an infallible determinant of bioaccumulation (Franke et al., 1994) as the details of the chemical structure of the toxicant (Dimitrov et al., 2003) and the detoxification processes of the organism also govern accumulation in organisms (Baussant et al., 2001, Sundt et al., 2009). A high K_{ow} value can give a false positive result for toxicants that are broken down harmlessly by the action of internal detoxification processes. Conversely, a false negative may be given if detoxification enzymes activate or boost toxicity and accumulation of chemicals

by converting them to more toxic metabolites. For example, thiamethoxam (a neonicotinoid with log K_{ow} = - 0.13) is metabolised to clothianidin (a neonicotinoid with log K_{ow} = 0.90) (Sigma Aldrich, 2016) which exhibits much greater toxicity to insects. The K_{ow} method also only predicts accumulation within fatty tissues and is not able to predict bioaccumulation or time-reinforced toxicity by irreversible binding at the binding sit of the toxicant or the formation of a persistent and proliferating lesion.

1.3.2 Tissue residue assays

Residue assays can also be used to determine the bioaccumulation of toxicants within the tissues of living organisms. The residues of toxicants and their metabolites in tissues can be quantified using chemical analysis techniques such as liquid and gas chromatography – mass spectrometry (LC and GCMS). However, there are limitations with these techniques. Residue analysis can be both expensive and time-consuming, and it also requires knowledge of the metabolism of the toxicant to ensure all harmful metabolites are also detected and the correct tissues are tested. This information is available for relatively few contaminants in most wildlife species. Also, depending on the limits of detection (LOD) and quantification (LOQ), it may not be possible to detect potentially harmful residues within tissues. These methods are also limited to detecting the bioaccumulation of toxicants which does not necessarily translate to time-reinforced toxicity because bioaccumulation is not sufficient to cause TRT (see **Table 1.1**). Therefore another method that examines the toxic effects themselves is needed to identify TRT toxicants.

1.3.3 Haber's Law and the Druckrey-Küpfmüller equation

A method suggested by Henk Tennekes (Tennekes, 2010) uses the Druckrey-Küpfmüller equation, based on Haber's Law, as a biological assay to identify TRT toxicants. Haber's Law is a 'constant product' rule that models a non-bioaccumulative toxicant, which quickly achieves a steady state within an organism over a continuous exposure (Rozman, 2000). As such, the daily rate of injury is constant and the accumulated total injury increases proportionally with the exposure duration. Therefore halving the exposure concentration will double the duration of exposure that is required to cause a given level of injury or effect. This relationship is expressed as:

$$C \times t = k \qquad \text{Eq. 1}$$

Where C is the toxicant concentration, t is the exposure duration and k is a certain level of injury or effect, such as the time to 50% mortality (LT_{50}). Haber's Law is often used to set exposure guidelines for toxicants, calculating acceptable daily intakes for long-term exposures where only acute exposure studies are available (Gaylor, 2000).

However, toxicants which conform to Haber's Law do not show time-reinforced toxicity. The Druckrey-Küpfmüller equation is a modified version of Haber's Law (Eq. 1) which allows for toxicants which do not follow Haber's Law to be modelled (Rozman, 2000):

$$C \times t^{b} = k \qquad \text{Eq. 2}$$

The exponent, b, modifies the effect of exposure time on the given level of injury or effect (k). For a toxicant which conforms to Haber's Law $b = 1$, but a toxicant which exhibits time-reinforced toxicity will have a value of $b > 1$.

To identify toxicants which exhibit TRT it is possible to evaluate *b* by using data from 'time-to-effect' experiments that quantify the duration of exposure that cause a specific level of injury in experimental organisms in various dosage groups. From this data, the *t*-vs-*C* relationship (Eq. 2) can be derived and its slope determined on logarithmic axes to estimate parameter *b* (**Figure 1.3**), because:

$$\log (C) = - b [\log (t)] + \log (k) \qquad \text{Eq. 3}$$

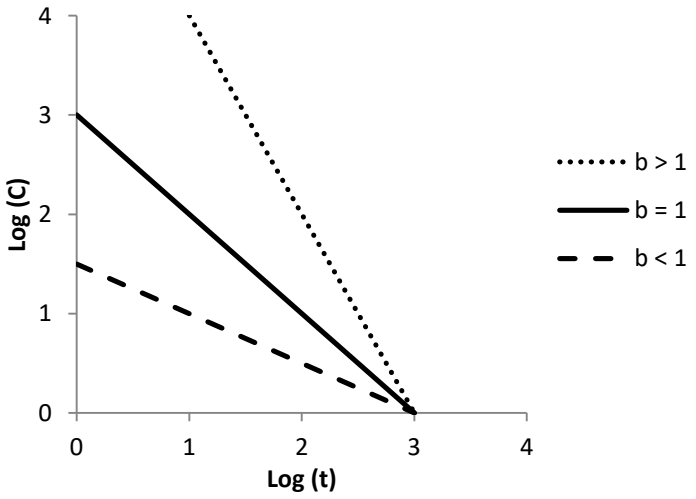

Figure 1.3 Logarithmic relationship of *t*-vs-*C* for values of parameter *b* from the Druckrey-Küpfmüller equation.

As this method is based on biological endpoints it would be able to identify TRT toxicants whether they act by bioaccumulation with the organism or by inducing a proliferating lesion. It will also take account of any TRT due to toxic metabolites from the exposure toxicant. Therefore using the Druckrey-Küpfmüller equation to test for time-reinforced toxicity would be the most

thorough and straightforward method of identification. In the case of farmland bees, the Druckrey- Küpfmüller equation has been suggested for use to identify agricultural insecticides that exhibit TRT (Tennekes, 2010), however this is not currently included in pesticides risk assessment protocols.

1.4 Farmland bees and the potential impact of TRT

Farmland bees can be split into several key groups: social or solitary, managed or wild. Social bees include both honey bee and bumble bee species, which form colonies consisting of a single queen and female workers. Workers leave the colony to forage, bringing back food for both adult bees and brood. All bee species exhibit haplodiploid sex-determination, by which unfertilised eggs develop into males while those that are fertilised become females (Cook, 1993). Therefore, unmated female workers are capable of producing male offspring.

All bees are phytophagous, feeding throughout their lives principally on nectar and pollen (Goulson, 2003). Due to their phytophagous diet, both wild and managed bees provide valuable pollination services to crops and wild flowers. Bees are vital for the production of 35% of global food crops (Klein et al., 2007) and have been estimated to be worth approximately £120 billion worldwide (Gallai et al., 2009). Honey bees unlike both bumble bees and solitary bees are a managed species, providing honey as well as pollination.

1.4.1 Honey bees

Honey bee colonies generally consist of one queen and female workers, which can number into the tens of thousands (Seeley, 1995). Only the queen reproduces, halting the development of workers' ovaries with the use of pheromones (Butler, 1959).

Colonies persist over winter, requiring the storage of large quantities of food in the form of pollen and honey. To enable workers to gather enough food, honey bees send out foragers to find floral resources. When foragers locate an area of forage they return to the hive to recruit other foragers by carrying out what is known as a ‘waggle dance’. This communicates several pieces of information to other foragers in the hive; the size of resource found, the distance from the hive and the direction in relation to the sun, thus allowing for more efficient foraging (Seeley, 1995).

Honey bee workers exhibit polyethism, by which they are split into age-related castes which determine their role in the colony. Younger workers generally act as nurse or hive bees that maintain the hive, clean it and also care for brood (eggs and larvae). As workers become older they transition to become foragers (Seeley, 1995, Winston, 1991). Workers are uniform in size, unlike those of *Bombus* species.

1.4.2 Bumble bees

There are approximately 250 species of bumble bees worldwide, 24 of which are found in the UK (Williams, 1994). The largest and commonest species of UK bumble bee is the buff-tailed bumble bee, *Bombus terrestris*. Bumble bee species can be divided into two main groups; short-tongued and long-tongued. Short-tongued species are generalist foragers, feeding on a wide range of flowers, whereas long-tongued species are more specialised foragers, focusing on deep perennial flowers. The majority of UK bumble bees facing declines are long-tongued species (Goulson et al., 2005). One of the main reasons for declines in these species is believed to be changes in land use resulting in the loss of available forage. For example, due to changes in agricultural land

management many clover fields, which are important sources of forage for long-tongued bees, have been lost (Goulson et al., 2008).

Bumble bee colonies consist of a queen and female workers. The number of workers in a colony varies greatly over the growth season and also amongst species (Goulson, 2003, Goulson et al., 2002). *Bombus* species generally have an annual life cycle (**Figure 1.4**) (Goulson, 2003, Prŷs-Jones and Corbet, 2011). New queens emerge in late winter or spring (dependent on species) and establish new colonies in trees, on or underground (including *Bombus terrestris*). Males and new queens are produced late in the summer, they mate and the fertilised new queens overwinter underground until the spring. Males, workers and existing queens perish before winter (Goulson, 2003). Some species (including *Bombus terrestris*), during favourable conditions, are capable of completing two life-cycles within one year with no overwintering of new queens (Stelzer et al., 2010).

Bumble bees can vary greatly in size within a species, with queens the largest caste. Queens and workers are structurally identical in all other aspects of the external morphology; however queens carry much larger fat deposits in their abdomens than workers, making them much heavier for their size (Cumber, 1949). Body size also varies greatly within the worker caste, with body mass of *B. terrestris* workers varying eight-fold from 0.05 to 0.40g (Goulson, 2003). Bumble bee workers do exhibit some age-based polyethism as young workers only carry out nest-based tasks and are more likely to become foragers as they age. However, workers exhibit greater behavioural plasticity than honey bees, as they are able to switch between tasks in response to changing colony requirements (Brian, 1952, Free, 1955).

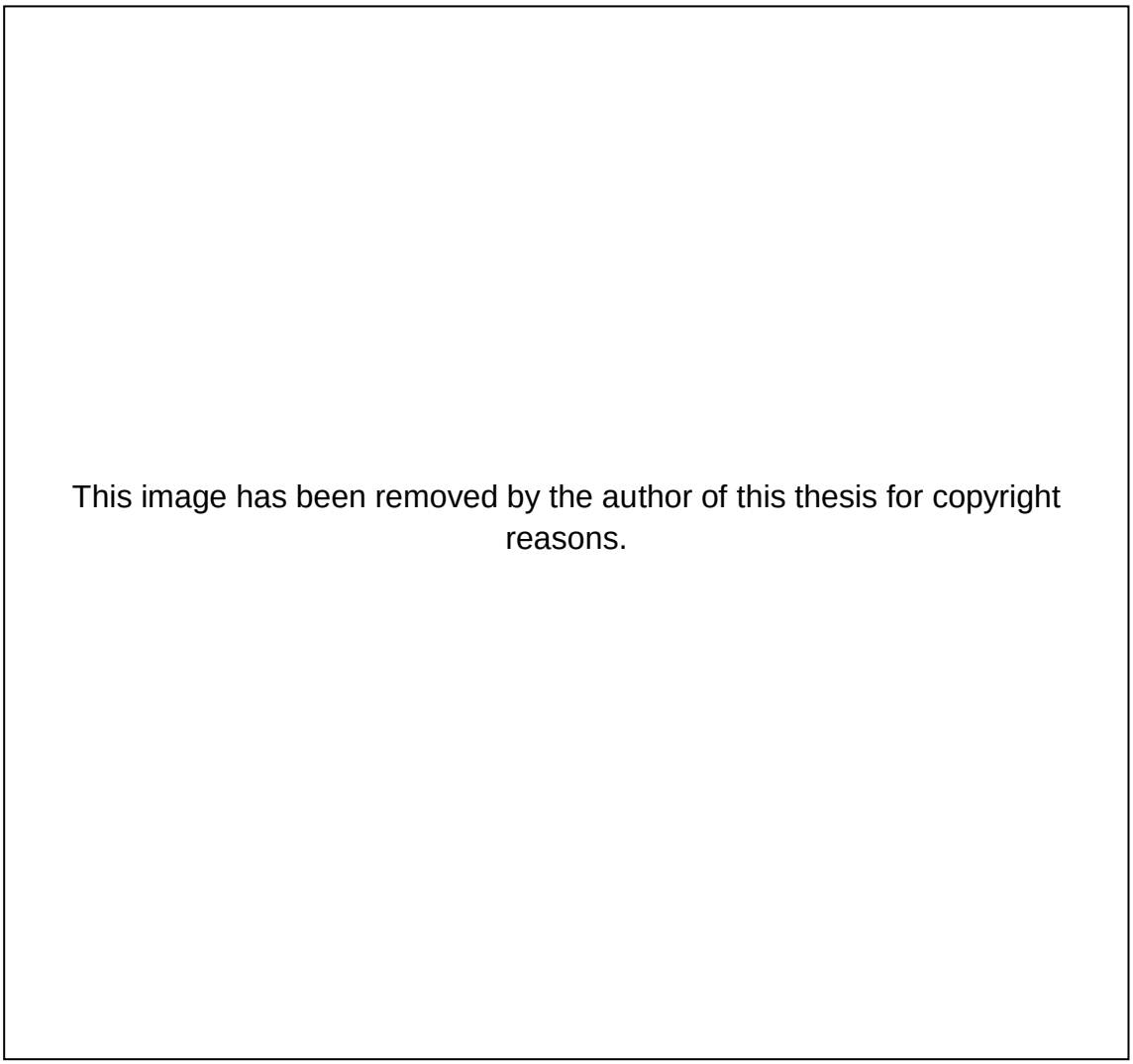

Figure 1.4 Summary of the bumble bee life-cycle, from Prŷs-Jones and Corbet (2011). Bumble bee queens emerge in spring to found new colonies, which continue to grow throughout summer until reproductives (new queens and males) are produced in late summer/early autumn when mating takes place. Newly fertilised queens then overwinter underground while the rest of the colony dies. Some species, during favourable conditions, are capable of completing two life-cycles within one year with no overwintering of new queens (Stelzer et al., 2010).

1.4.3 Solitary bees

There are 250 species of solitary bee in Great Britain and Ireland (Falk, 2015), however they are the least studied group of bees. Solitary bees are a large

group of species that exhibit nesting behaviour that can be categorised as dispersed or solitary. Nest entrances can be far apart or communal, where nest entrances are concentrated into aggregations. Solitary bees do not have worker castes as all females are capable of reproduction and nest individually, provisioning for offspring themselves. Females generally mate in spring, before constructing an individual burrow in the ground, within plants or wood, depending on the species. They will then construct a series of walled off cells, each containing an egg and provisioned with pollen and usually nectar. The eggs hatch and the larvae develop over the summer and autumn months before overwintering either as pupae or in adult form (Linsley, 1958).

1.4.3 The use of bees as study species

The honey bee *Apis mellifera* (**Figure 1.5**) has been the model species used to determine the risk to bees from pesticides for decades (EPPO, 2010). This is due to its economic importance as a pollinator (Klein et al., 2007) and its long history of domestication.

Due to differences in life history between honey bees and bumble bees, the threat posed by pesticide exposure may be very different. Therefore it is also important to investigate pesticide effects on bumble bees (Thompson and Hunt, 1999), especially as wild bees provide indispensable pollination services (Garibaldi et al., 2013). A limited number of bumble bee species have been successfully commercially bred, reducing the number of species that it is possible to utilise for pesticide toxicity testing. The most commonly-used bumble bee species in regulatory testing and research is *Bombus terrestris* (**Figure 1.5**), as it is easily maintained and bred. However, as the largest and commonest of UK bumble bee species, and also a short-tongued generalist

forager, it may not be the ideal model for the *Bombus* family as it may underestimate pesticide effects on less robust species.

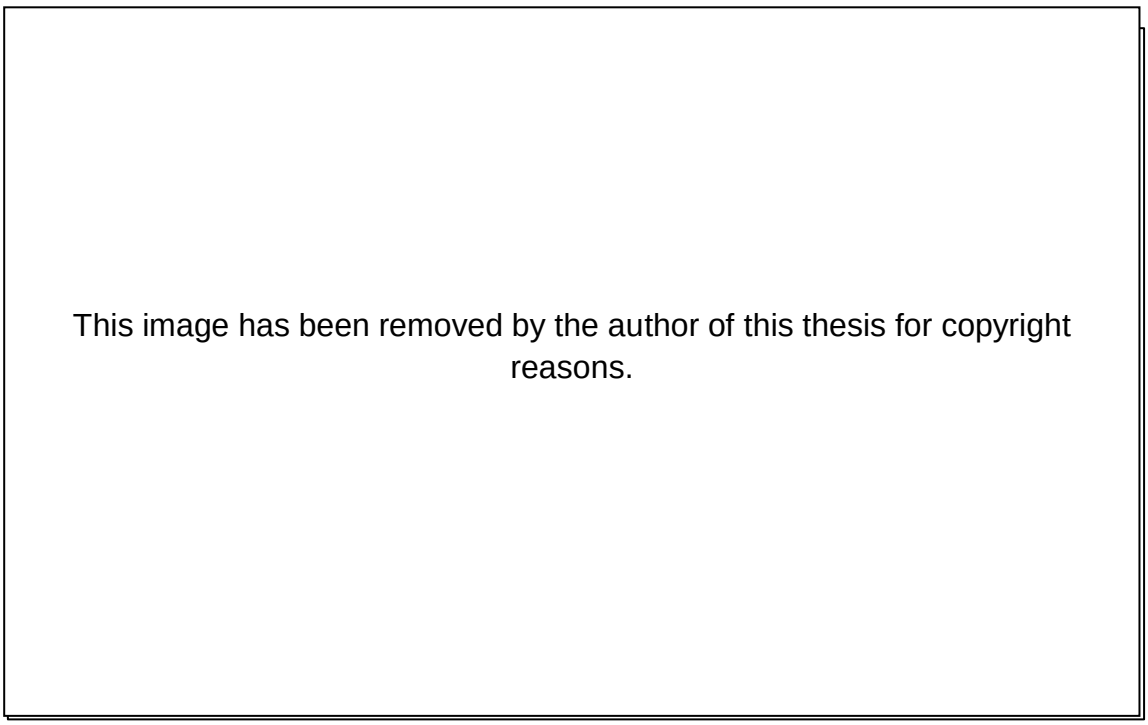

Figure 1.5 Worker bees of the species *Apis mellifera* (A) and *Bombus terrestris* (B). Image from www.beeloved.co.uk.

Pesticide research and risk assessments on bees are carried out on individuals up to whole colonies (EFSA, 2013b). The use of individual bees allows for greater replication and is more practical under laboratory conditions, however it does not allow for social interactions or investigations into the reproductive effects of pesticides. For *Bombus* species a standardised test involving queenless microcolonies can be used to investigate pesticide effects on reproduction and behaviour. Microcolonies generally consist of nests of 3-5 workers; one of which will become dominant in the absence of a queen and begin to lay unfertilised eggs. The advantages of using this method as opposed to queenright (queen is present) colonies are that it is lower cost, more

standardised, easy to use and allows for greater replication and therefore statistical power (Blacquière et al., 2012).

However, for field-realistic scenarios, such as those required in higher-tier risk assessments after initial toxicity studies (EPPO, 2010), it is necessary to use queenright colonies for both *Apis* and *Bombus* species.

1.4.5 Current concerns

There is concern over widespread declines of both managed and wild bee populations and multiple causal factors have been implicated. Disease, parasites, land use and management practices are all thought to play a part in bee declines, however, pesticide use is perceived to be a major contributor (Goulson et al., 2015). Due to the pollination services that bees provide population declines could have severe impacts on food security. Foraging on treated mass-flowering crops can lead to the exposure of bees to potentially harmful pesticides, either by ingestion of residues present in the nectar and pollen or by direct contact with sprayed vegetation (Chauzat et al., 2011, Mullin et al., 2010, Thompson et al., 2013). The impacts on bees from direct contact pesticide exposure are already well understood, however, the dietary exposure of bees to pesticides remains to be fully investigated. Pesticide residues found in nectar and pollen are generally far below a lethal concentration for bees, however they may cause toxic effects if the pesticides exhibit time-reinforced toxicity.

1.5 Insect neuroreceptors

A neuroreceptor can be a membrane receptor protein that is activated by a neurotransmitter, or a voltage-gated ion channel, present on the post-synaptic

membrane. Neuroreceptors are found at nerve synapses (**Figure 1.6**) and are essential for nerve transmission. Synapses are the junctions between two nerve cells, across which an impulse is transmitted. This occurs either by electrical coupling of ion movements through voltage-gated channels or by the release of neurotransmitters from the presynaptic membrane, which then bind to postsynaptic neuroreceptor proteins (Stewart et al., 2014).

Figure 1.6 Basic structure of a nerve synapse. Nerve impulses are transmitted across the synaptic cleft via ion exchange and the release of neurotransmitters from the presynaptic neuron. Voltage-gate ion channels and neuroreceptors within the postsynaptic membrane bind with their respective ligands, triggering changes to the postsynaptic neuron and continuing the nerve impulse. Image from www.ibguides.com.

Neurotoxic insecticides have been designed to bind to insect neuroreceptors and voltage-gated ion channels, altering their action. These insecticides can act competitively or non-competitively with natural neurotransmitters to bind to

neuroreceptors. Neurotoxic insecticides can act as agonists or antagonists of insect neurons. Here, I will describe the structure and function of three key neuroreceptors of the insect nerve system and how neurotoxic insecticides target them.

1.5.1 Voltage-gated sodium channels

Voltage-gated sodium channels are activated by membrane depolarisations, leading to the opening of the channel and allowing an influx of sodium (Na^+) ions, allowing the continuation of neurotransmission. The *'para'* voltage-gated sodium channel of the insect nervous system is both structurally and functionally homologous with the α-subunit of mammalian sodium channels (**Figure 1.7**). The aqueous pore of insect sodium channels consists of four homologous domains, known as the α-subunit. Each domain is made up of six transmembrane helices (S1-S6). The S5 and S6 units combine to form the central ion-conducting pore while the S1-S4 units make up the voltage-sensitive part of the channel. P-loops between units S5 and S6 form the ion-selective filter at the extracellular end of the channel. Insect sodium channels have been shown to be binding sites for a range of potent neurotoxins (Davies et al., 2007).

Pyrethrins, pyrethroids and organochlorines, including DDT, bind to insect sodium channels and act generally as agonists, leading to hyperexcitation of neurons and eventual paralysis (Davies, 2007, Zlotkin, 1999). Some insects have evolved resistance to these insecticides by modifications to the sodium channel protein (Davies et al., 2007).

Figure 1.7 Transmembrane structure of the voltage-gated sodium channel (Davies et al., 2007). The four homologous domains (comprising the α-subunit and each consisting of six transmembrane helices) link to form a central aqueous pore (PD), lined by the S5 and S6 helices. The P-loops which link together these helices form a narrow ion-selective filter at the extracellular end of the pore. The S1-4 helices form four independent voltage sensing domains (VSD), which are responsible for the voltage sensitivity of the channel. It is thought that the voltage-dependence of channel activation is derived from the movement of the four positively charged S4 helices.

1.5.2 γ-aminobutyric acid receptors

GABA (γ-aminobutyric acid) receptors are ligand-gated chloride channels embedded in the postsynaptic membrane, which when bound to the neurotransmitter GABA inhibit neuronal activity by stopping the flow of chloride (Cl^-) ions (Bloomquist, 1996, Hosie et al., 1997). At least two distinct classes of GABA receptors have been identified, $GABA_A$ and $GABA_B$ (Olsen and DeLorey, 1999). The $GABA_A$ receptor is the target for insecticides including fipronil (Ratra et al., 2001).

The $GABA_A$ receptor consists of five subunits that form a chloride ion (Cl^-) conducting channel, known as a channel pore. These five subunits originate from seven subunit families (α, β, γ, δ, ε, θ, π), various combinations of which can form the receptor. The neurotransmitter GABA binds at the interface of the α and β subunits, shutting the ion channel (Jacob et al., 2008).

Figure 1.8 Structure of the $GABA_A$ receptor (Jacob et al., 2008). The γ-aminobutyric acid (GABA) type A receptor consists of five subunits from seven subunit families (α, β, γ, δ, ε, θ, π), which combine to form a chloride (Cl^-) permeable channel. Binding of GABA occurs at the interface of the α and β subunits, while benzodiazepines (BZ), for example, are able to bind at the interface of α and γ subunits (Jacob et al., 2008).

Various neurotoxins are able to bind to GABA receptors. Fipronil, a neurotoxic insecticide acts as a non-competitive antagonist of GABA receptors, blocking

the binding of other ligands. It is thought to bind as an allosteric modulator, potentially binding at the base of the transmembrane bundle rather than in the pore, and interrupting the channel gating mechanism rather than directly blocking the pore (Law and Lightstone, 2008).

Mutations leading to conformational changes of subunits in the insect GABA-receptor have been shown to confer resistance against GABA-targeting insecticides including fipronil and dieldrin (Li et al., 2006).

1.5.3 Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) are transmembrane ion channels found bridging the postsynaptic membrane. They are found throughout the insect central nervous system (CNS). They are activated by an increase in the concentration of acetylcholine (ACh), the neurotransmitter that binds to them, within the synaptic cleft. nAChRs consist of five subunits arranged into an aqueous pore that allows the influx of cations (Na^+, K^+, Ca^{2+}) into the postsynaptic neuron.

The composition of these subunits determines the functional and pharmacological properties of the receptor (Jones and Sattelle, 2010).

nAChRs are involved in fast excitatory synaptic transmission and are the targets of several groups of insecticides, including the neonicotinoids (Matsuda et al., 2001).

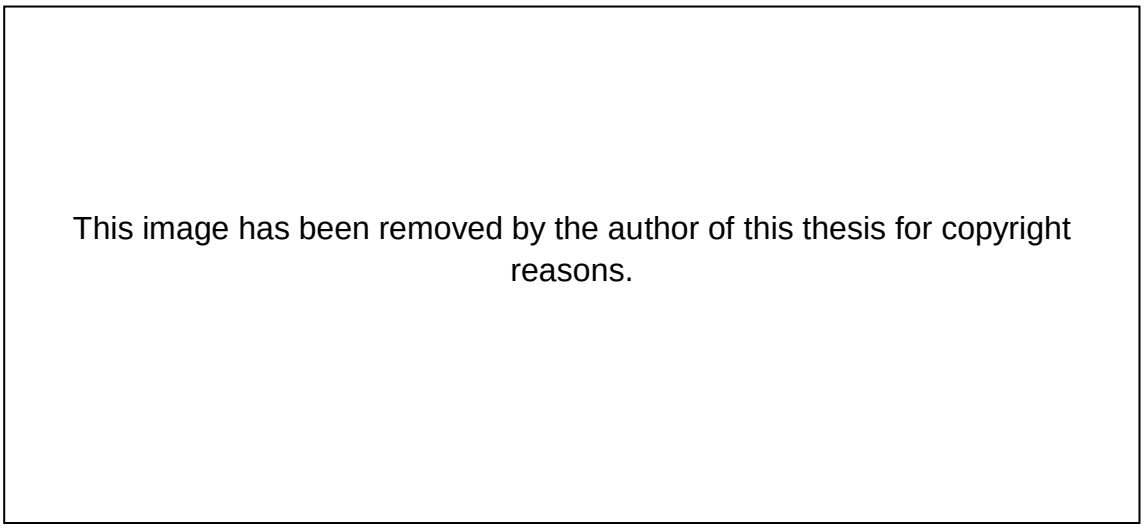

Figure 1.9 Transmembrane structure and binding sites of the nicotinic acetylcholine receptor (nAChR) (Lodish et al., 2007). nAChRs consist of five subunits which form an aqueous pore through which cations flow into the postsynaptic neuron, allowing for fast synaptic transmission. The α-helix of the second membrane-spanning segment (M2) forms the gate of the closed channel, which opens once ACh is bound to the receptor (Miyazawa et al., 2003).

1.6 Modern agrochemicals

Older generation pesticides, such as organochlorines (OCs) and organophosphates (OPs), have since been replaced for common use in agriculture by the pyrethroids and new-generation pesticides, including the neonicotinoids and fipronil (Casida, 2012). These newer pesticide classes act by a wide range of mechanisms. Though generally neurotoxic, they bind to different receptors to the OCs and OPs and elicit varying effects to exposed organisms. This is due to differences in their chemical structures. 'New generation' pesticides have been designed to be more potent so that less mass is used, and also less environmentally persistent to avoid negative impacts on non-target organisms (Jeschke and Nauen, 2008, Casida, 2012). Improved

insect binding site specificity (Yamamoto et al., 1995, Tomizawa and Casida, 2003) and systemic application methods, whereby pesticides are applied as seed dressings and then absorbed into the growing plant, have also helped to reduce the risk of pesticides to non-target organisms (Jeschke and Nauen, 2008, Casida, 2012).

1.5.1 Pyrethroids

Synthetic pyrethroid insecticides have been developed from the natural pyrethrins which occur in the flowers of chrysanthemums (*Chrysanthemum cinerariaefolium*). The early pyrethrins were unstable and expensive to produce, therefore changes to the chemicals structures were made to improve stability and increase insecticidal potency, which resulted in the synthetic pyrethroids used today. Pyrethrins are made up of an acid moiety, an alcohol moiety and an ester linkage (**Figure 1.10**). It was modifications to both moieties which lead to the development of the synthetic pyrethroids between 1924 and 1970 (Katsuda, 1999, Davies et al., 2007). Pyrethroids are applied as foliar sprays to a wide variety of crops and are still the most commonly used insecticides for crops in the UK (FERA, 2014).

The pyrethroids can be classified as either Type I or Type II, depending on whether or not they contain the α-cyano group (Casida et al., 1983). Type I pyrethroids, such as permethrin, elicit a knockdown effect in insects, whereby exposed organisms are temporarily paralysed, leaving them at risk of predation or death from exposure. Pyrethroid molecules are present as both *cis* and *trans* isomers, with *cis* isomers generally exhibiting higher toxicity, though this also depends on the alcohol moiety present (Casida et al., 1983, Davies et al., 2007). They act on both the peripheral and central nervous system of insects by

binding to sodium (Na) voltage-gated channels on sensory and motor axons, thereby slowing down the decay of action potentials and causing hyperexcitation (Vais et al., 2001, Davies et al., 2007). Na channels are closed at resting membrane potential but a small number are opened by a depolarisation of the membrane leading to a transient Na current across the membrane. Only a small proportion of Na channels need to be modified by pyrethroids to cause repetitive firing of the nerve cell and eventual death of the exposed insect (Davies et al., 2007).

Pyrethroids are classified as either highly toxic (LD_{50} of 0.1 – 10 µg a.i. bee^{-1}) or extremely toxic (LD_{50} < 0.1 µg a.i. bee^{-1}) to honey bees (Maund et al., 2012). In contact exposures they can be repellent to bees under laboratory conditions (Rieth and Levin, 1988), but these repellent effects were not observed in several field studies (Delabie et al., 1985, Karise et al., 2007) suggesting that bees may still be exposed to these chemicals.

The pyrethroids have various harmful effects on bees. In bumble bees, topical exposure to the pyrethroid lambda-cyhalothrin has been shown to cause increased worker mortality in exposed bumble bee colonies, however this did not translate to a reduction in colony growth (Gill et al., 2012). A reduction in bumble bee worker body size has also been noted, though again there were no other effects on colony performance (Baron et al., 2014). However oral exposure to lambda-cyhalothrin resulted in increased worker mortality and reduced syrup consumption and brood production, with greater effects in free-flying bumble bees (Ceuppens et al., 2015).

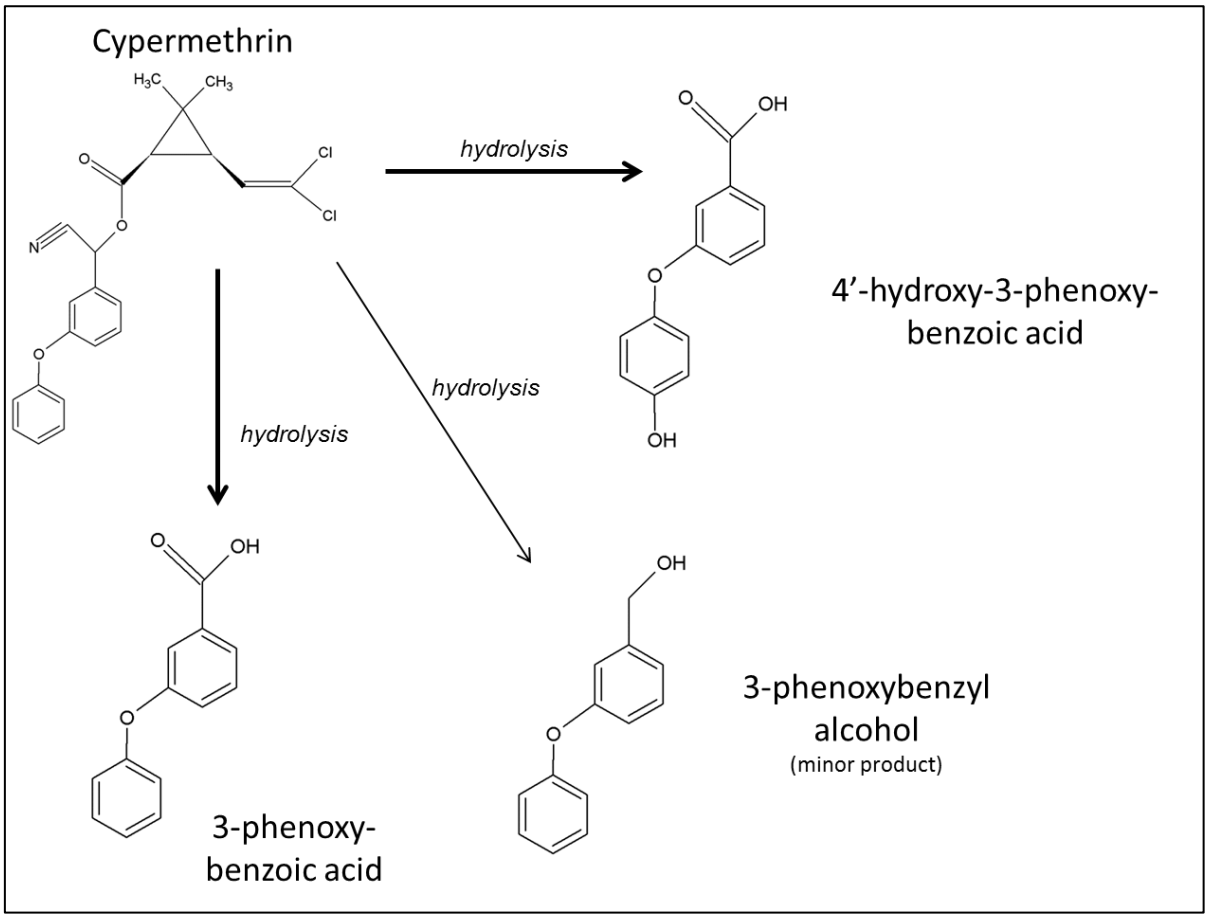

Figure 1.10 Metabolic pathways of cypermethrin in insects. Cypermethrin is readily hydrolysed via the ester linkage to non-toxic metabolites.

Exposure of honey bees to tau-fluvinate, an acaricide, can negatively impact on a range of behaviours, including learning, memory and locomotion (Frost et al., 2013, Teeters et al., 2012). The pyrethroids, cypermethrin and deltamethrin, are both widely-used on bee-attractive crops (FERA, 2014). Honey bee colonies exposed to low-level dietary cypermethrin exhibited increased in-hive mortality, queen supersedure (where the current queen is replaced by a newly-hatched queen from the same colony) and reduced brood area (Bendahou et al., 1999). Bumble bee mortalities in the United Kingdom have also been linked to cypermethrin exposure (Thompson, 2001). Deltamethrin has been shown to be

detrimental to honey bee learning performance, foraging, feeding and thermoregulation (Decourtye et al., 2004, Decourtye et al., 2005, Ramirez-Romero et al., 2005, Vandame and Belzunces, 1998). Few studies have investigated its impacts on bumble bees, though Tasei et al. (1994) found that deltamethrin exposure reduced syrup consumption, but with no impact on reproduction.

Although many sublethal effects of pyrethroids have been documented, in the majority of studies exposure doses eliciting a response were many times greater than those found in the environment, suggesting that bees may be able to detoxify lower doses without harm. Pyrethroids have been shown to be readily metabolised in the bee gut via cytochrome P450 monooxygenases and glutathione-S-transferase, producing non-toxic metabolites (**Figure 1.10**) (Fragoso et al., 2003, Johnson et al., 2006, Little et al., 1989).

Evidence of rapid metabolism of pyrethroid insecticides by bees indicates that they are unlikely to bioaccumulate within exposed individuals. There is also no evidence of pyrethroids causing proliferating lesions within organisms, therefore it is unlikely that the pyrethroids will exhibit time-reinforced toxicity to bees.

Since the pyrethroids were introduced to the market, several other groups of 'new generation' insecticides have been developed for use on crops with the aim of reducing harm to non-target organisms.

1.5.2 Phenylpyrazoles

Fipronil is the sole commercially distributed insecticide in a group of fungicides known as phenylpyrazoles. It is a non-competitive antagonist of insect γ-aminobutyric acid (GABA)-gated type A chlorine channels on post-synaptic membranes, and is thought to act as an allosteric modulator, blocking the

binding of other ligands (Law and Lightstone, 2008). Fipronil contains a unique trifluoromethylsulfinyl moiety which is responsible for its selective, highly insecticidal activity (Hainzl and Casida, 1996) and this forms the majority of hydrogen bonds in these interactions with $GABA_A$ receptors (Ci et al., 2007). The phenyl group of fipronil also forms strong hydrophobic and hydrophilic interactions with subunits of the GABA receptor (Ci et al., 2007). GABA receptors are found on the membranes of muscle cells and are important for locomotor and flight activity (Usherwood and Grundfest, 1965). The sulfone derivative of fipronil (**Figure 1.11**), a metabolite produced in insects, also has high neuroactivity at GABA-receptors, is more persistent and is less selective in its action, indicating that it may be a major contributor to fipronil toxicity (Caboni et al., 2003, Cole et al., 1993, Reynaud et al., 2012). Fipronil desulfinyl, the main photoproduct of fipronil, is also highly toxic to insects (Hainzl and Casida, 1996). Fipronil is used to control a wide range of sap-sucking insects on a variety of crops, though it is mainly used on sunflowers (*Helianthus anuus*). Fipronil is applied systemically as a seed treatment to protect the plant from germination onwards. It is taken up by the growing plant and distributed throughout its tissues, whereby some reaches the nectar and pollen. Residues of fipronil in crops and honey bee colony matrices have been found at levels generally in the range of 0 – 5 parts per billion (ppb) (Chauzat et al., 2011, Mullin et al., 2010).

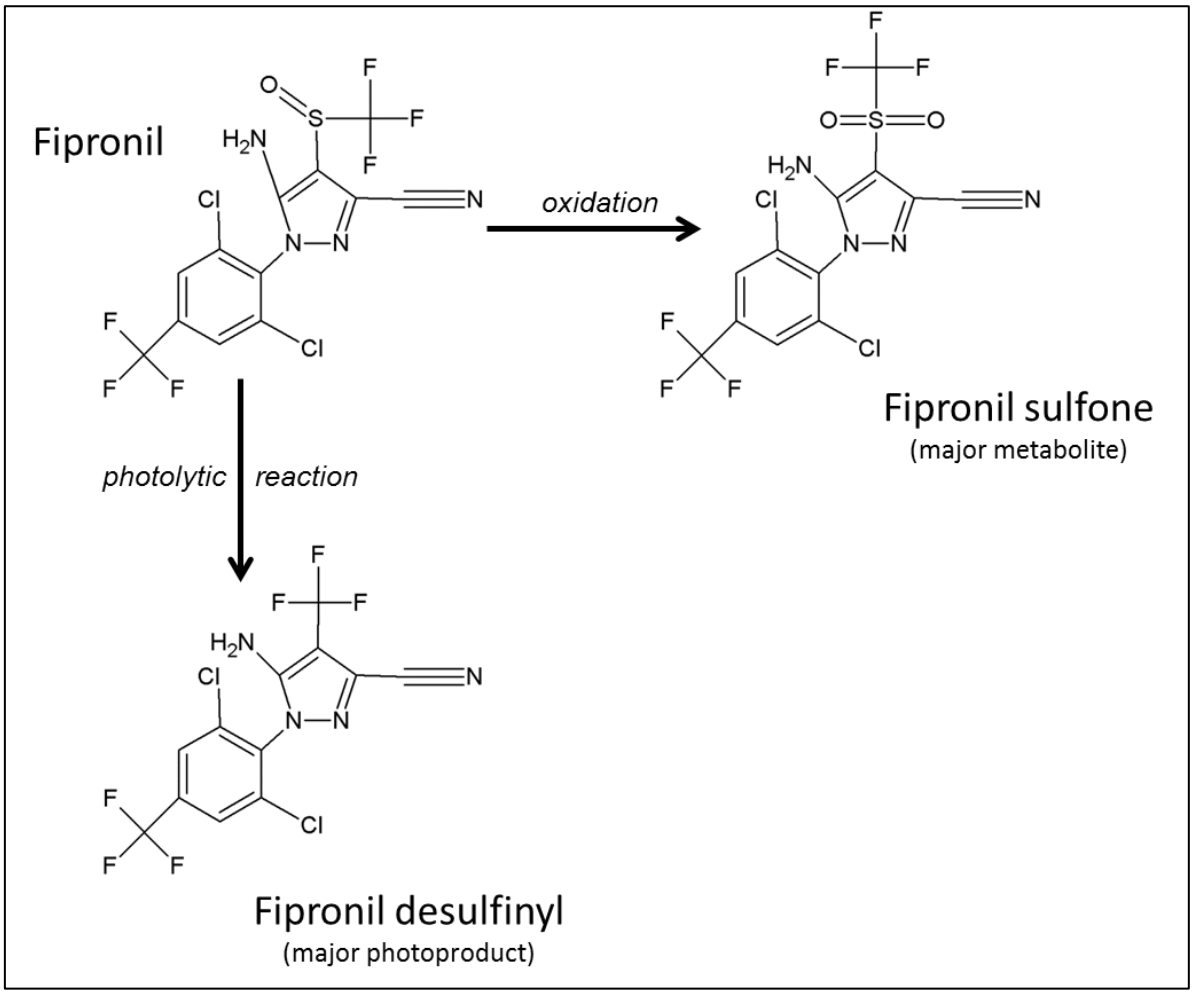

Figure 1.11 Metabolic pathways of fipronil in insects and on plants. Fipronil is metabolised to the equally toxic fipronil sulfone via oxidation. On plants fipronil undergoes a photolytic reaction to another toxic metabolite, fipronil desulfinyl.

Fipronil is classed as highly toxic to bees (EFSA, 2006), however there is limited knowledge of its sublethal effects, with no studies focusing on wild bee species. Fipronil has been shown to negatively impact honey bee behaviour, reducing foraging activity and impairing memory and learning (Aliouane et al., 2009, Colin et al., 2004, Decourtye et al., 2011, El Hassani et al., 2005). Fipronil can also inhibit honey bee mitochondrial activity and has been shown to

increase mortality in *Nosema ceranae*-infected bees (Nicodemo et al., 2014, Vidau et al., 2011).

Fipronil has been assessed as a high acute risk to honey bees when used as a maize seed dressing, due to dust drift, however the risk posed to bees from dietary residues in nectar and pollen is unknown (EFSA, 2012, EFSA, 2013a). These unresolved concerns led the European Commission to impose a provisional ban on the use of fipronil on bee-attractive crops (European Commission, 2013). There is also evidence that fipronil may bioaccumulate within bees (DEFRA, 2016) and other organisms (Cravedi et al., 2013, Reynaud et al., 2012). Due to this evidence of bioaccumulation, it may be predicted that fipronil, or its toxic sulfone derivative, will exhibit time-reinforced toxicity to bees.

1.5.3 Neonicotinoids

The neonicotinoids are a family of new-generation systemic insecticides which act with high specificity at insect nicotinic acetylcholine receptors (nAChRs) on post-synaptic membranes in the central nervous system (CNS), causing paralysis and death (Matsuda et al., 2001). They are applied as seed dressings or foliar sprays to protect crops against a broad spectrum of insect pests (Jeschke and Nauen, 2008). Due to the highly specific mode of action of the neonicotinoids there is no cross-resistance with other pesticide groups, therefore they have begun to replace previously used pesticides (Jeschke and Nauen, 2008). There are several examples of cross-resistance occuring between pesticide groups due to similarities in target sites or detoxification processes. These include cross-resistance to pyrethroids and both organochlorine and organophosphate pesticides (Brengues et al., 2003, Rodríguez et al., 2002). Precursor chemicals to the neonicotinoids with more

limited insecticidal action and stability were developed in the 1970s and 80s before the development of the first neonicotinoid, imidacloprid, which was introduced to the market in 1991 (Jeschke and Nauen, 2008).

There are now seven neonicotinoids commercially available: imidacloprid, thiamethoxam, clothianidin, acetamiprid, thiacloprid, dinotefuran and nitenpyram. These can be split into two main groups, the nitro- and cyano-substituted neonicotinoids, based on their chemical structure at the pharmacophore, which is essential for insecticidal activity. This divide also impacts on the potency of these chemicals, with nitro-substituted neonicotinoids (imidacloprid, thiamethoxam and clothianidin) exhibiting much greater toxicity to honey bees than those of the cyano-substitutes group (Iwasa et al., 2004). This difference can be ascribed to the fast metabolism of the cyano-substituted neonicotinoids (Brunet et al., 2005, Suchail et al., 2004a, b) and different nAChR subtypes that the compounds affect (Jones et al., 2006). There are two main nAChR subtypes, made up of imidacloprid-sensitive nAChRs to which imidacloprid readily binds and imidacloprid-insensitive nAChRs which do not interact with imidacloprid. Clothianidin is more toxic to insects than imidacloprid, which may be due to its action as an agonist of both nAChR subtypes (Thany, 2009). The nitrogen atom present in both pharmacophores, which is partially positively charged, is believed to contribute to the interaction of these compounds with insect nAChRs by mimicking the positively charged ammonium of acetylcholine (ACh) (Matsuda et al., 2005). Neonicotinoids are thought to bind reversibly to insect nAChRs via electrostatic forces and hydrogen bonding (Jeschke and Nauen, 2008).

The most toxic of the nitro-substituted neonicotinoids are imidacloprid, thiamethoxam and clothianidin. All are applied systemically to crops, including sunflower and oilseed rape, as seed dressings which permeate the growing plant's tissues. Residues of neonicotinoids can be found in the nectar and pollen of treated flowering crops, with concentrations in pollen generally ranging from 1 to 11 parts per billion (ppb) (Blacquière et al., 2012). A study by EFSA (2012) found the less toxic cyano-substituted neonicotinoids at concentrations up to 86 and 114 ppb in nectar and pollen, respectively, from bee-attractive crops. However, imidacloprid was only found in pollen at 2 ppb. Studies have found residues in honey bee colony matrices (including bee-collected pollen, honey bees, honey and bees wax) at concentrations ranging from 0.05 to 912 ppb, however in most instances the residues found were less than 3 ppb (Blacquière et al., 2012). It has been hypothesised that bees may avoid contaminated nectar and pollen when foraging, however the neonicotinoids have been shown to actually be attractive to bees, potentially increasing their dietary exposure (Kessler et al. 2015).

Cytochrome P450 monooxygenases (Puinean et al., 2010) and aldehyde oxidase (Shi et al., 2009) present in the gut have been shown to be important in neonicotinoid metabolism in insects (Casida, 2011). Thiamethoxam is a poor agonist of nAChRs which instead acts as a pro-insecticide that is metabolised to clothianidin, a super-agonist of insect nAChRs (**Figure 1.12**) (Casida, 2011, Ihara et al., 2004, Nauen et al., 2003). Imidacloprid is rapidly metabolised within hours in honey bee bodies (Suchail et al., 2004a, b) and is also quickly cleared from within bumble bees (Cresswell et al., 2013) (**Figure 1.12**).

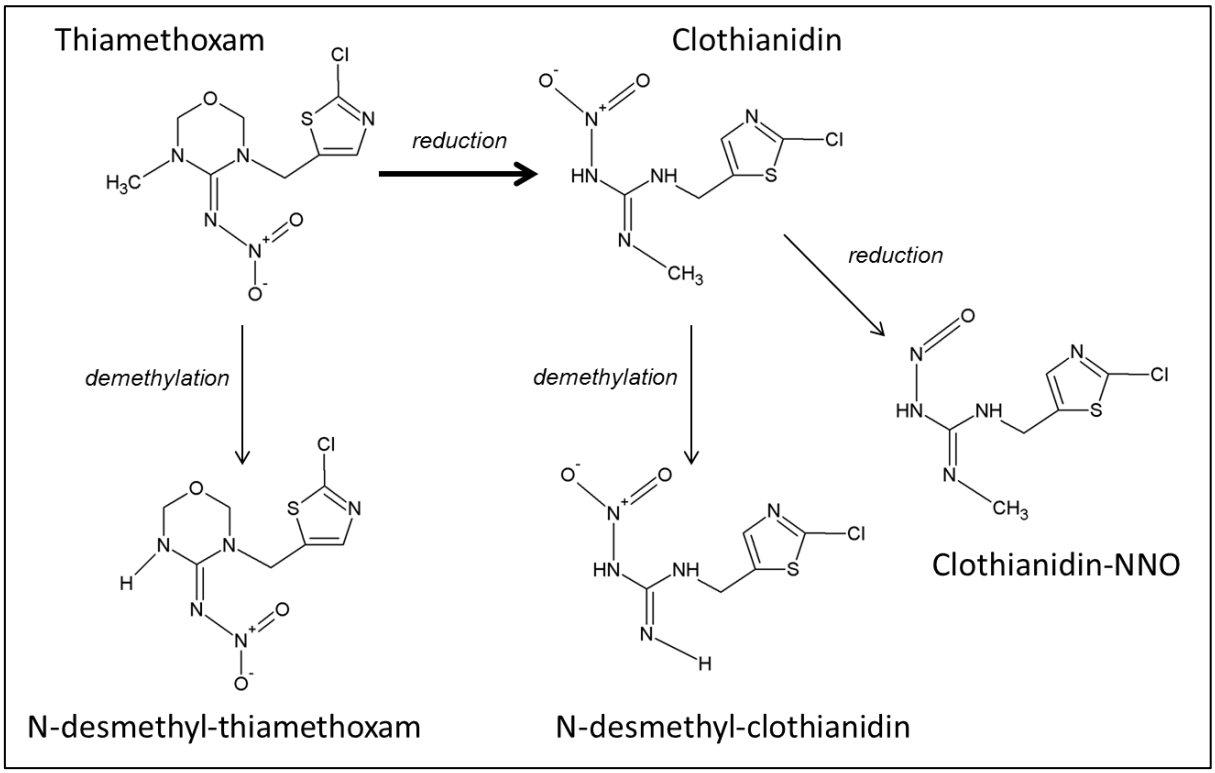

Figure 1.12 Metabolic pathways of thiamethoxam (and clothianidin) in insects. Thiamethoxam is activated by rapid metabolism to clothianidin, with the N-desmethyl-thiamethoxam also being produced as a minor product. Clothianidin itself is metabolised via demethylation to N-desmethyl-clothianidin and by reduction to clothianidin-NNO, both of which exhibit low insecticidal activity (Casida, 2011).

Neonicotinoid metabolites have also been shown to contribute to toxicity in bees (**Figure 1.12** and **1.13**) (Decourtye et al., 2003, Nauen et al., 2001, Nauen et al., 2003, Suchail et al., 2001), with the exception of acetamiprid (Iwasa et al., 2004).

The neonicotinoids, especially imidacloprid, have been widely studied with regards to their effects on bees. In a search of the literature, I identified fifty-seven studies that have reported sublethal effects of neonicotinoids since 2000 (**Table 1.2**). The majority of these studies have focused on the impact of the

neonicotinoids on learning and foraging behaviour as well as reproduction (**Figure 1.14**). Though a meta-analysis by Cresswell (2011) found that field-realistic imidacloprid residues would not be lethal to honey bees, it concluded that these residues could lead to a reduction in honey bee performance of between 6 and 20%. Imidacloprid has been shown to negatively impact both honey bees and bumble bees, causing the wide range of sublethal effects shown in **Figure 1.14**.

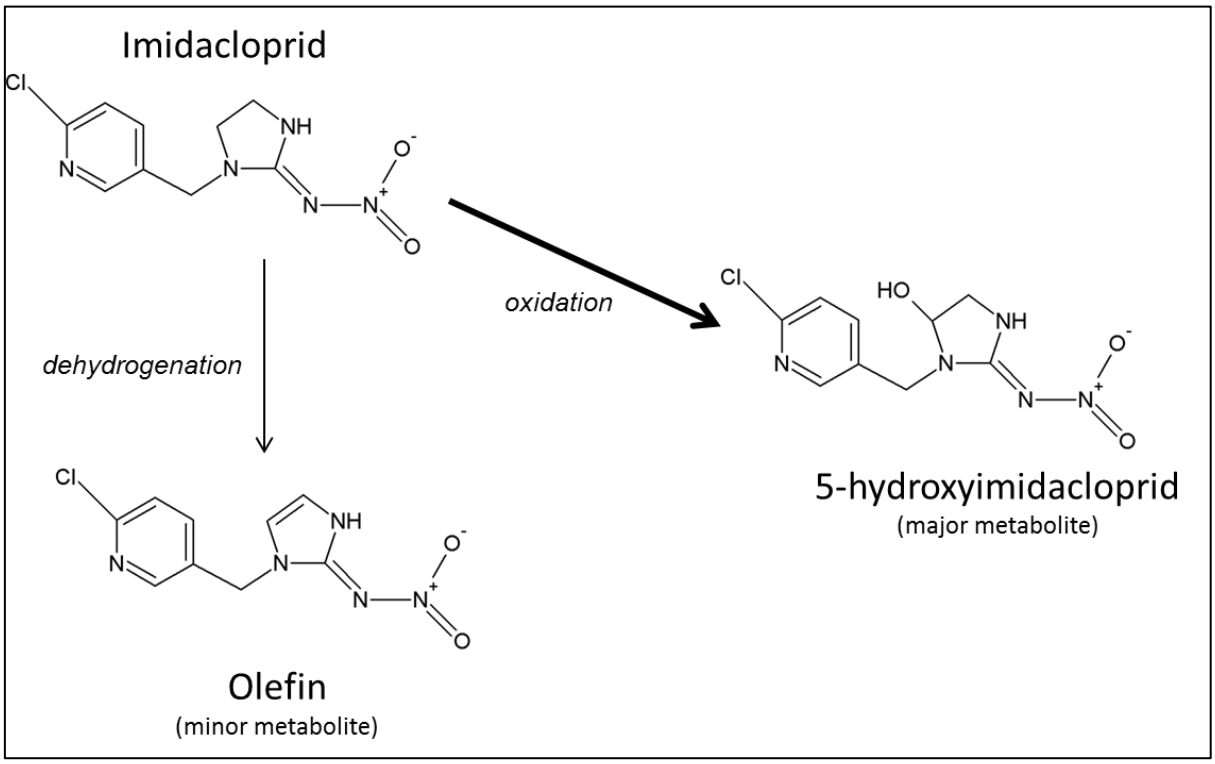

Figure 1.13 Metabolic pathways of imidacloprid in insects. Imidacloprid is mainly metabolised via oxidation to 5-hydroxyimidacloprid, which exhibits reduced insecticidal toxicity. The minor metabolite of olefin is produced via dehydrogenation (Casida, 2011).

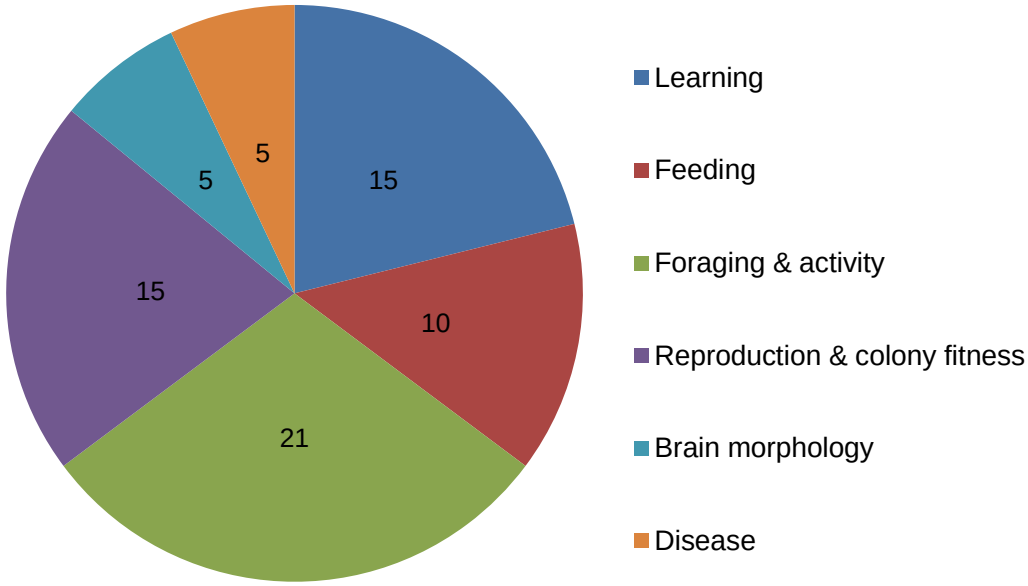

Figure 1.14 Sublethal effects of neonicotinoids on bees studied since 2000. The number of studies (N = 57 **out of a total of 88)** that have reported particular sublethal neonicotinoid effects on honey bees and bumble bees between 2000 and July 2016. **Only 65% of studies investigating sublethal endpoints found significant effects.**

These effects include reductions in bumble bee fecundity at concentrations as low as 1 ppb (Laycock et al., 2012, Tasei et al., 2000). While thaimethoxam and clothianidin have been shown to affect learning, feeding, foraging and reproduction in both honey bees and bumble bees (**Table 1.2**).

Since 2000, the number of studies investigating and reporting sublethal effects of neonicotinoids on bees have steadily increased, peaking in 2015 (**Figure 1.15**). While the majority of these studies include field-realistic exposure concentrations, the majority of sublethal effects reported occurred at higher concentrations above the field-realistic range. Nevertheless, neonicotinoids

have been shown to cause a wide range of harmful effects in bees, which could potentially impact on colony survival and population growth.

It is not thought that exposure to environmental neonicotinoids alone could be the cause of honey bee declines, however important knowledge is lacking to be sure of this (Cresswell et al., 2012a). Effects on the sustainability of wild bees are likely, but uncertain (Rundlof et al., 2015).

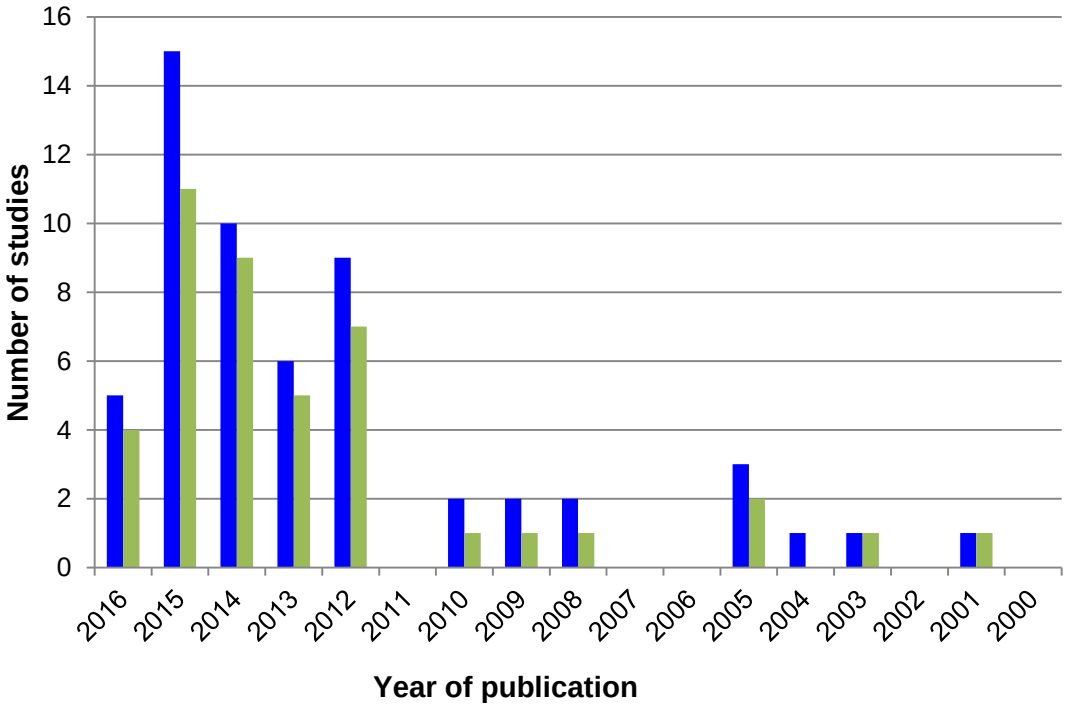

Figure 1.15 The number of studies reporting sublethal effects of neonicotinoids on bees per year since 2000. The relationship between the year of publication (*x*-axis; year of study publication from 2000 to July 2016) and the number of publications (*y*-axis; number of publications per year) that report sublethal effects of neonicotinoids on bees (shown in blue; N = 57) and the number of these publications which have included field-realistic exposure concentrations (shown in green; N = 43).

It has been hypothesised that neonicotinoid insecticides may bind irreversibly to bee nAChRs (Tennekes, 2010), however there is no impirical evidence that they can bioaccumulate within bees. Therefore, it is predicted that the neonicotinoids will not exhibit time-reinforced toxicity to bees.

1.7 Limitations of current risk assessment of agrochemicals

Current laboratory protocols for estimating the risk posed by Plant Protection Products (PPPs) to bees still rely on short-term toxicity experiments, mostly conducted on honey bees as a sentinel species for all other bee species. However, there is evidence of variation in sensitivity to pesticides between different bee species (Arena and Sgolastra, 2014) and, specifically, that honey bees may be the least sensitive among farmland bees. Also, field studies have shown that pesticides can have negative effects on wild bee species while no effect is seen in honey bees (Rundlof et al., 2015). Therefore current risk assessment protocols could be missing the potentially harmful effects of pesticide exposure on wild bees. Certainly, carrying out toxicity experiments over short time periods (less than 10 days) will miss any time-reinforced toxic effects that a pesticide may cause and therefore underestimate the potential risk in agricultural conditions where prolonged exposures are routine. As bees are likely to be exposed to small residues of pesticides from treated crops over blooming periods of several weeks, they are particularly susceptible to TRT pesticides; especially as current risk assessment is unable to identify them.

1.8 Toxicological studies of bees & insecticides

Many studies have investigated the impacts of insecticides on bee behaviour and health. In order to gain an overview of the areas on which this body of research focuses, here I present an assessment of all identified studies conducted since 2000 that investigate the effects of insecticides on bees (**Figure 1.16**). This review consisted of 196 relevant studies and clearly shows that the majority of studies focus on honey bees and neonicotinoid insecticides, primarily imidacloprid. However, imidacloprid is no longer widely-used and recent research has indicated that wild bees may be suffering greater declines and may be more sensitive to pesticides than managed bees (Rundlof et al., 2015, Arena and Sgolastra, 2014). Many neonicotinoid studies also only investigate the effects of doses above the field-realistic range of < 10 parts per billion (30 out of 93) and are therefore limited in their environmental relevance. Of those studies that do include field-realistic doses, 54% do not test a range of doses.

Residue levels of pesticides in crops are variable and the extent to which bees will be exposed to them is not easily predicted, especially as some pesticides can be attractive or repellent to bees (Kessler et al., 2015, Thompson and Wilkins, 2003). Consequently, it is necessary to test the effects of pesticide exposure over a range of doses to determine the dose-response relationship from which effects of actual crop residues can be predicted. To identify pesticides which exhibit time-reinforced toxicity in bees at field-realistic doses an exposure duration of at least 10 days is required. Of 82 subchronic exposure studies, 54 studies had an exposure duration of > 10 days, however none investigated TRT. Therefore, TRT pesticides are not being taken into consideration and thus may be going undetected.

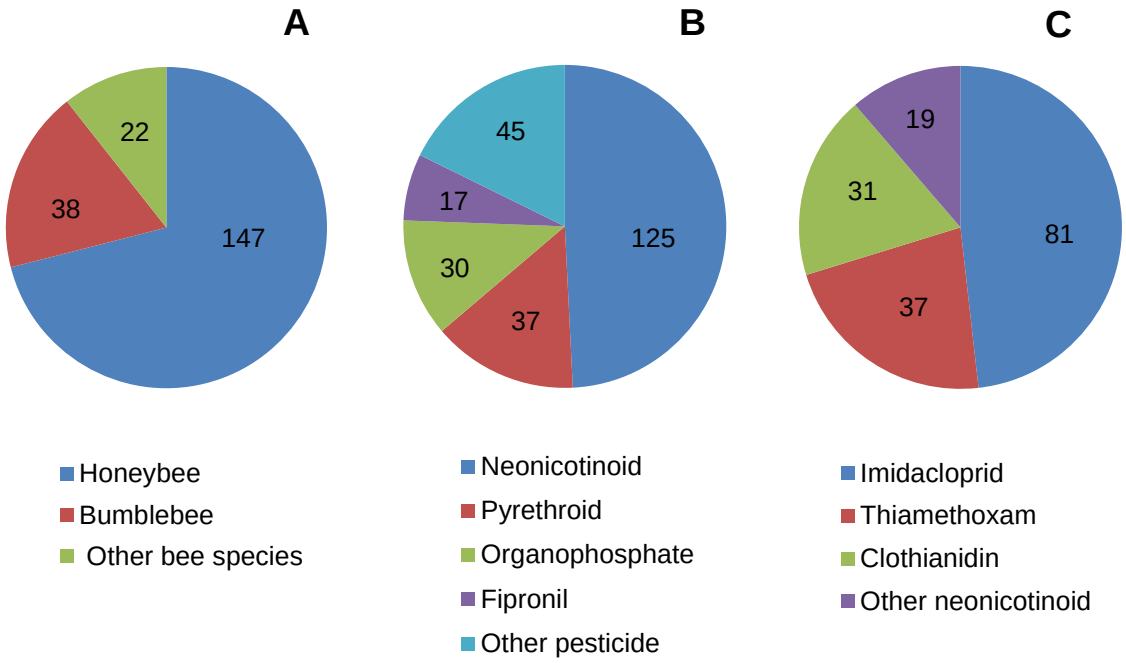

Figure 1.16 Results from the assessment of studies investigating the effects of insecticides on bees between 2000 and July 2016. The number of studies which looked at a particular bee species (**A**, n = 196), the numbers of pesticide studies focusing on honey bees or bumble bees (**B**, n = 180), and the number of neonicotinoid studies which investigated a particular insecticide within this family (**C**, n = 125). See **Appendix 1** for full list of references used. Numbers shown indicate the number of studies which include this factor.

1.8 Objectives of thesis

The objectives of this thesis are:

- To develop a simple bioassay for TRT and employ it to identify pesticides which exhibit time-reinforced toxicity in honey bees and bumble bees.

- To investigate the effects of a TRT pesticide on demographically-relevant sublethal endpoints of bumble bees, both under laboratory conditions and in the field.

To achieve these objectives I firstly utilised a simple bioassay based on Haber's Law which had previously been used to predict long-term exposure effects in humans. Using this bioassay I tested a selection of commonly-used pesticides from different chemical families on both honey bees and bumble bees (Chapter 2). Specifically, I tested the predictions developed in this chapter that: fipronil would exhibit TRT in bees while the neonicotinoid pesticides would not. My results identified fipronil as the only test pesticide that exhibits TRT in honey bees.

I then hypothesised that the TRT exhibited by fipronil would have greater impacts on colony growth than non-TRT pesticides. Using a honey bee demographic model of colony growth developed by Khoury *et al.* (2011) I determined that fipronil has the potential to cause colony collapse from exposure to environmentally-realistic dietary residues (Chapter 2).

Secondly, I investigated the effects of the same test pesticides in bumble bees to determine whether the impacts of TRT are the same across bee species, and therefore, I tested whether that the honey bee is an adequate sentinel species for risk assessment. I hypothesised that similar results would be seen when testing these pesticides for TRT in bumble bees as honey bees. Using the same bioassay based on Haber's Law I found that the neonicotinoid thiamethoxam and the pyrethroid cypermethrin did not exhibit TRT in bumble bees (Chapter 3), while fipronil acted as a TRT pesticide (Chapter 4), mirroring my results for honey bees (Chapter 2).

To determine the impacts of fipronil and non-TRT pesticides on demographically-relevant endpoints in the bumble bee I carried out a series of experiments, focusing mainly on fecundity. I hypothesised that fipronil would have significant impacts on fecundity at field-realistic exposures due to its time-reinforced toxicity, while non-TRT pesticides would not reduce fecundity at these exposure levels. I found that neither clothianidin (the main metabolite of thiamethoxam) nor cypermethrin reduced fecundity at field-realistic doses (Chapter 3). However, fipronil reduced fecundity and even halted oviposition completely, raising concerns for wild populations. When microcolonies were placed outside to forage freely exposed bees were lost or died almost immediately, presumably from a greater toxic effect from fipronil due to the increased metabolic rate required for flight (Chapter 4).

To further investigate the impacts of this dramatic mortality brought on by increased activity, I investigated the effects of fipronil exposure on queenright bumble bee colonies placed in the field. I hypothesised that fipronil exposure would impact on foraging and colony growth, potentially leading to colony failure. Unfortunately, due to technical problems with delivering the fipronil exposure, it was not possible to determine the effects of exposure on colony success. However, I was still able to investigate the variations between colonies for various endpoints including colony growth, worker size and queen production (Chapter 5).

Chapter One: Tables

Table 1.2 Overview of literature from 2016 to 2000, reporting the sublethal effects to bees of subchronic neonicotinoid exposure.

Neonicotinoid	Bee species	Pesticide (ppb)	Including field realistic range	**Sublethal effect**						**Reference**
				Learning & memory	Feeding	Foraging & activity	Reproduction & colony fitness	Brain morphology	Disease	
I / C / O	HB	1-2000 ug/L	Y	**-**	**-**	**-**	**-**	**-**	**X**	Brandt et al. (2016)
T	HB	1, 10, 100	Y	**-**	**X**	**-**	**-**	**-**	**-**	Demares et al. (2016)
C	HB / BB	4	Y	**X**	**-**	**-**	**-**	**-**	**-**	Piiroinen and Goulson (2016)
O	HB	4500-5000	N	**X**	**-**	**X**	**-**	**-**	**-**	Tison et al. (2016)
T	HB	TC	Y	**-**	**-**	**-**	**-**	**-**	**X**	Alburaki et al. (2015)
I	HB	5-100	Y	**-**	**-**	**-**	**X**	**-**	**-**	Dively et al. (2015)
I	HB	83, 166	N	**X**	**X**	**-**	**-**	**-**	**-**	Gonalons et al. (2015
I / T	HB	TC	Y	**-**	**-**	**X**	**-**	**-**	**-**	Henry et al. (2015)
I	HB	255	N	**-**	**-**	**X**	**-**	**-**	**-**	Karahan et al. (2015)

Neonicotinoid	**Bee species**	**Pesticide (ppb)**	**Including field realistic range**	Sublethal effect						Reference
				Learning & memory	Feeding	Foraging & activity	Reproduction & colony fitness	Brain morphology	Disease	
I / T / C	HB / BB	0.2-292	Y	**-**	**X**	**-**	**-**	**-**	**-**	Kessler et al. (2015)
I / C	BB	2.1	Y	**-**	**-**	**-**	**-**	**X**	**-**	Moffat et al. (2015)
T	BB	2.4-10	Y	**X**	**-**	**-**	**-**	**-**	**-**	Stanley et al. (2015a)
T	BB	2.4-10	Y	**-**	**-**	**X**	**-**	**-**	**-**	Stanley et al. (2015b)
I	HB	8.9-88.7	Y	**X**	**-**	**-**	**-**	**-**	**-**	Tan et al. (2015)
T	HB	1430	N	**-**	**-**	**-**	**-**	**X**	**-**	Tavares et al. (2015)
I / T / C	BB	1-100	Y	**-**	**X**	**-**	**-**	**-**	**-**	Thompson et al. (2015)
T / C	HB	1, 4	Y	**-**	**-**	**-**	**X**	**-**	**-**	Williams et al. (2015)
I / T	HB	0.025-2.5	Y	**X**	**-**	**-**	**-**	**-**	**-**	Wright et al. (2015)
I	HB	0.13-1.15	Y	**-**	**-**	**-**	**-**	**X**	**-**	Wu et al. (2015)
I	HB	20.8	N	**X**	**-**	**-**	**-**	**-**	**-**	Zhang et al. (2015)
I	HB	1.3-2	Y	**-**	**-**	**-**	**-**	**-**	**X**	Aufauvre et al. (2014)

Neonicotinoid	Bee species	Pesticide (ppb)	Including field realistic range	**Sublethal effect**						**Reference**
				Learning & memory	Feeding	Foraging & activity	Reproduction & colony fitness	Brain morphology	Disease	
T / C	BB	1.5, 4	Y	-	-	-	**X**	-	-	Fausser-Misslin et al. (2014)
I	BB	0.7, 6	Y	-	-	**X**	-	-	-	Feltham et al. (2014)
I	BB	10	Y	-	-	**X**	-	-	-	Gill and Raine (2014)
T	BB	0.06-98	Y	-	**X**	-	**X**	-	-	Laycock et al. (2014)
O	HB	30000-60000	N	-	-	-	-	-	**X**	Retschnig et al. (2014)
T / C	HB	2-5	Y	-	-	**X**	**X**	-	-	Sandrock et al. (2014)
I / C	BB	10-100	Y	-	-	**X**	**X**	-	-	Scholer et al. (2014)
I	HB	10-40	Y	**X**	-	**X**	-	-	-	Tan et al. (2014)
I / T / C / O	HB	2.5-2.9	Y	-	-	**X**	-	-	-	Williamson et al. (2014)
T	BB	1, 10	Y	-	**X**	-	**X**	-	-	Elston et al. (2013)
I	HB	2-3	Y	-	-	-	-	**X**	-	Hatjina et al. (2013)
C	BB	TC	Y	-	-	-	**X**	-	-	Larson et al. (2013)

Neonicotinoid	Bee species	Pesticide (ppb)	Including field realistic range	**Sublethal effect**						**Reference**
				Learning & memory	Feeding	Foraging & activity	Reproduction & colony fitness	Brain morphology	Disease	
I	BB	0.06-98	Y	**-**	**-**	**-**	**X**	**-**	**-**	Laycock et al. (2013)
I	HB	256	N	**X**	**-**	**-**	**-**	**-**	**-**	Williamson et al. (2013a)
I	HB	2.5-255	Y	**X**	**-**	**-**	**-**	**-**	**-**	Williamson et al. (2013b)
I	HB / BB	0.06-98	Y	**-**	**X**	**-**	**-**	**-**	**-**	Cresswell et al. (2012b)
I	HB	24, 241	N	**-**	**X**	**X**	**-**	**-**	**-**	Eiri et al. (2012)
I	BB	10	Y	**-**	**-**	**X**	**X**	**-**	**-**	Gill et al. (2012)
T	HB	38.5	N	**-**	**-**	**X**	**-**	**-**	**-**	Henry et al. (2012)
I	BB	0.06-98	Y	**-**	**-**	**-**	**X**	**-**	**-**	Laycock et al. (2012)
I	HB	5, 20	Y	**-**	**-**	**-**	**-**	**-**	**X**	Pettis et al. (2012)
I / C	HB	5-600	Y	**-**	**-**	**X**	**-**	**-**	**-**	Schneider et al. (2012)
I	HB	0.05-500	Y	**-**	**-**	**X**	**-**	**-**	**-**	Teeters et al. (2012)
I	BB	0.7, 6	Y	**-**	**-**	**-**	**X**	**-**	**-**	Whitehorn et al. (2012)

Neonicotinoid	Bee species	Pesticide (ppb)	Including field realistic range	**Sublethal effect**						**Reference**
				Learning & memory	Feeding	Foraging & activity	Reproduction & colony fitness	Brain morphology	Disease	
I	HB	48	N	**X**	**-**	**-**	**-**	**-**	**-**	Han et al. (2010)
I / T / O	BB	10-200000	Y	**-**	**-**	**X**	**-**	**-**	**-**	Mommaerts et al. (2010)
T / O	HB	23	N	**X**	**-**	**-**	**-**	**-**	**-**	Aliouane et al. (2009)
I	HB	0.5	Y	**-**	**-**	**-**	**-**	**X**	**-**	Skerl et al. (2009)
T / O	HB	10-100000	Y	**X**	**-**	**-**	**-**	**-**	**-**	El Hassani et al. (2008)
I	HB	30-4615	N	**-**	**-**	**X**	**-**	**-**	**-**	Yang et al. (2008)
T	BB	TC	Y	**-**	**X**	**X**	**X**	**-**	**-**	Alarcon et al. (2005)
I	HB	0.5-5	Y	**-**	**-**	**X**	**X**	**-**	**-**	Faucon et al. (2005)
I	HB	48	N	**-**	**X**	**X**	**-**	**-**	**-**	Ramirez-Romero et al. (2005)
I	HB	24	N	**X**	**-**	**X**	**-**	**-**	**-**	Decourtye et al. (2004)
I	HB	1.5-96	Y	**X**	**-**	**-**	**-**	**-**	**-**	Decourtye et al. (2003)

Neonicotinoid	Bee species	Pesticide (ppb)	Including field realistic range	**Sublethal effect**						**Reference**
				Learning & memory	Feeding	Foraging & activity	Reproduction & colony fitness	Brain morphology	Disease	
I	BB	TC	Y	**-**	**-**	**-**	**X**	**-**	**-**	Tasei et al. (2001)

Neonicotinoid: (I) imidacloprid, (T) thiamethoxam, (C) clothianidin, (O) other neonicotinoid

Bee species: (HB) honey bee, (BB) bumble bee

Pesticide: (TC) treated crop (recommended application rate)

Chapter One: Appendix 1. References from Figure 1

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Chapter Two:

Bioaccumulation of fipronil pesticide residues from pollen and nectar can cause rapid colony collapse in the honey bee *Apis mellifera*

Bioaccumulation of fipronil pesticide residues from pollen and nectar can cause rapid colony collapse in the honey bee *Apis mellifera*

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Abstract

Mass mortalities of honey bees occurred at widespread localities in France during the 1990s (Maxim and van der Sluijs, 2013) and were principally ascribed to the neonicotinoid insecticide, imidacloprid. Residues of imidacloprid were present in the nectar and pollen of treated sunflower fields, which the bees presumably collected and consumed. The actual cause of the deaths, however, has not been explained fully (Aubert et al., 2006) because dietary imidacloprid at environmentally realistic levels does not kill honey bees (Cresswell, 2011). Here we show that another insecticide used on sunflowers in France (and more widely) in the 1990s, fipronil, can bioaccumulate in individual honey bees and that the resulting time-reinforced increase in mortality rate during sustained dietary intake has the capacity to cause rapid colony collapse for environmentally realistic exposures. We used nonconformity with Haber's Law to test for time-reinforced toxicity among honey bees exposed to each of four dietary insecticides: two that were used widely in the 1990s, imidacloprid and fipronil; and two in more recent use, thiamethoxam (neonicotinoid) and cypermethrin (pyrethroid). Fipronil alone produced time-reinforcement and we confirmed its bioaccumulation by quantifying bodily residues in individual bees with mass spectrometry. When we incorporated the observed effects of realistic dietary exposures into a demographic model (Khoury et al., 2011) of honey bee colony growth, fipronil alone caused rapid colony collapse and the other pesticides tested had minimal effects. Our results identify agrochemical fipronil as among the principle suspects in causing historical instances of mass mortality in honey bees. More generally, our study highlights the importance of evaluating the potential impact of prolonged exposures on bees by testing for

time-reinforced toxicity during the regulatory approval of pesticides for use in agriculture.

2.1 Introduction

Conspicuous mass mortalities of honey bees were observed in France between 1994 and 1998 (Maxim and van der Sluijs, 2013). The onset of this phenomenon coincided with the introduction of two new-to-market systemic insecticides, imidacloprid (released in 1994) and fipronil (1993) (Tomlin, 2009), which were widely used on sunflower (*Helianthus anuus*) crops (Aubert et al., 2006). Despite being used across similar acreages (FERA, 2014), it was generally believed that the mass mortalities were caused principally by imidacloprid (Aubert et al., 2006). Imidacloprid is a neonicotinoid insecticide that disrupts the insect nervous system by acting on nicotinic acetylcholine receptors (nAChRs) (Matsuda et al., 2001) and fipronil is a phenylpyrazole insecticide that acts on the γ-aminobutyric acid (GABA) type A receptor (Cole et al., 1993, Ratra et al., 2001). Applied as seed dressings, these systemic insecticides are taken up by the growing plant and distributed throughout its tissues, including the flowers (Johnson et al., 2010). Consequently, honey bees are exposed to low-level dietary residues when feeding on nectar and pollen from systemically treated bee-attractive crops (Chauzat et al., 2011). Ongoing concerns over bee health have recently led the European Union to impose a provisional ban on the use of neonicotinoids and fipronil on bee-attractive crops (European Commission, 2013), although derogations in the United Kingdom have allowed farmers in limited areas to use neonicotinoids (including a current market product, thiamethoxam) on oilseed rape (*Brassica napus*) (Eisenstein, 2015). Meanwhile, other farmers are instead using non-systemic pyrethroid foliar sprays with active ingredients such as cypermethrin, which acts on the

sodium channels in the post-synaptic membrane of insect nerve cells (Davies et al., 2007). Our aim was to determine the capacity of these agricultural insecticides to cause mass mortality in honey bees during prolonged exposures to environmentally realistic dietary concentrations and we therefore investigated the toxicity of two historical compounds, imidacloprid and fipronil, and compared them with two in current use, thiamethoxam and cypermethrin.

All four compounds are potent insecticides, but the concentrations of their residues in nectar and pollen are typically too low to make it feasible for a honey bee to ingest an acute lethal dose. For example, imidacloprid has been detected in pollen at approximately five parts per billion (ppb) by mass so that the acute lethal dose (from 4 to > 81 ng per bee) (Blacquiere et al., 2012) is many times larger than the daily intake by an adult honey bee eating pollen (< 1 ng d^{-1}) (Henry et al., 2012a). However, the lifespan of adult bees (Seeley, 1995) and the blooming period of mass-flowering crops like sunflower (Arias and Rieseberg, 1994) and oilseed rape (Hoyle et al., 2007) both extend over several weeks, so that the exposure of individuals is not acute, but sustained. Consequently, even an insecticide that is present at trace dietary levels eventually may become lethal if it bioaccumulates in an individual bee, which has been termed ‘time-reinforced toxicity’ (TRT) (Tennekes and Sanchez-Bayo, 2011). We therefore sought the signature of TRT among bees exposed to the four target compounds by testing for conformity to Haber’s law as follows.

When a toxicant binds reversibly to its target site and is substantially susceptible to catabolic breakdown and elimination, then during a sustained dietary exposure the continuous and opposing actions of ingestion and elimination will establish a ‘steady state’ concentration inside the organism. If

the daily rate of injury resulting from this steady state is constant, a simple pharmacokinetic compartment model of toxic load (**Supplementary Figure 2.1**) predicts that the accumulated total injury is proportional to the duration of the exposure (**Supplementary Figure 2.2**). This proportionality under steady state conditions means that toxicological experiments on such a system will find that halving the dosage rate doubles the duration of the exposure that is required to achieve a given level of injury or effect (**Supplementary Figure 2.3**). Toxicants with these properties will produce the specified injury from any exposure whose dose-duration combination conforms to a 'constant product' rule known as Haber's Law (Rozman, 2000):

$$C t^{b} = k \qquad \text{Eq. 1}$$

where C denotes the concentration of the toxicant in the diet, t denotes the duration of the exposure and the exponent takes the value b =1, which reflects the proportionality relationship. If instead the organism's internal concentration at the target site rises as intake proceeds during the exposure because the toxicant bioaccumulates, the rate of injury increases with time and so the accumulated total injury is not proportional to exposure time but instead increases quasi-exponentially as a power function (**Supplementary Figure 2.4**). Since the rate of injury accelerates over time towards the level required to produce a given effects, it exhibits TRT. In this case, the exponent in Eq 1 takes the value $b > 1$. In toxicological systems where $b > 1$, halving the dosage will require less than double the duration of the exposure to achieve the given injury because time reinforces toxicity. In the pharmacokinetic model of an idealised bioaccumulative toxicant, the exponent in Eq. 1 takes the value $b = 2$ (**Supplementary Figure 2.5**).

When the development of a specified injury across a range of different dose-duration combinations is best described by setting $b > 1$ in Eq. 1, we have detected TRT. Consequently, it is straightforward to test for TRT by evaluating b using data from a series of 'time-to-effect' experiments that quantify the exposure durations required to produce a specified level of injury in experimental subjects under various doses. After conducting exposures at various doses, a suitable test for TRT involves fitting the t-vs.-C relationship and determining its slope on logarithmic axes, which estimates parameter b (Eq 1) because:

$$\log(C) = -b[\log(t)] + \log(k)$$

We screened all four candidate pesticides for time-reinforced toxicity during dietary exposures and investigated the bioaccumulative potential of any that exhibited TRT. We also evaluated the impacts of each of the four test pesticides on honey bee colony growth by incorporating their observed dose-appropriate effects on foraging and hive bees into a demographic model (Khoury et al., 2011). Within a colony, female honey bees are separated into several castes; queens and workers. Workers are also subdivided to perform age-related tasks, with younger workers staying within the hive to care for brood and maintain the nest while older workers take on foraging roles (Seeley, 1995, Winston, 1991). Due to potentially sustained exposures, it would be possible for not only foragers to be exposed to a TRT pesticide but also those that worker within the hive. Therefore, as both worker roles are integral to colony function, losses to both are included in the demographic model.

We expect imidacloprid, thiamethoxam and cypermethrin to have toxicological effects on honey bees that conform to Haber's Law ($b = 1$) because they bind

reversibly to their target sites and they are readily metabolised. For example, ingested imidacloprid has a biological half-life of approximately five hours in honey bees (Cresswell et al., 2013), its metabolites are rapidly eliminated and it binds reversibly to target receptors (nAChRs) in the insect nervous system (Jeschke and Nauen, 2008) , from which it is displaced competitively by the native neurotransmitter. In contrast, fipronil and its toxic metabolite, fipronil sulfone, are non-competitive allosteric modulators of the GABA-gated chlorine channels in the insect synapse (Law and Lightstone, 2008) and the interaction between these toxicants and their target sites is poorly reversible (Cole et al., 1993).

2.2 Materials and methods

2.2.1 Time-reinforced toxicity bioassay

2.2.1.1 Preparation of chemicals

Imidacloprid was obtained as a solution in acetonitrile (analytical standard, PESTANAL®, Sigma Aldrich Co. LLC; product code: 46341). A vacuum concentrator (ScanSpeed MaxiVac Beta; LaboGene ApS, Lynge, Denmark) was used to remove the acetonitrile and the imidacloprid was resuspended in deionised water to form a stock solution of 10 mg L^{-1}. Thiamethoxam, fipronil and cypermethrin (analytical standards, PESTANAL®, Sigma Aldrich Co. LLC; product codes: 37924, 46451, 36128, respectively) were suspended in water (thiamethoxam) and acetone (fipronil and cypermethrin) to form stock solutions (10 mg L^{-1}, 10 mg L^{-1} and 400 mg L^{-1}, respectively) before being combined with 50% w/v aqueous sugar solution (Attraker: 1.27 kg L^{-1} fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs,

Netherlands). Doses of cypermethrin contained a maximum of 0.95% acetone v/v, reducing with cypermethrin concentration (control doses contained 0.95% acetone v/v). Doses of fipronil contained a maximum of 1.25% acetone v/v, reducing with fipronil concentration (control doses contained 1.25% acetone v/v).

2.2.1.2 Bees and pesticide diets

Adult worker honey bees (*Apis mellifera*) of various ages were obtained from domesticated colonies (Devon, UK). Newly-eclosed bees were not used as we wanted to determine the effects of pesticide on a demographically representative sample of adults, which is a more environmentally realistic scenario. Honey bees were caged in groups of 10 (cage dimensions: approx. 0.10m diameter x 0.04m height) in plastic containers, with 7 cages per dose. Bees were maintained in a semi-controlled environment (temperature between 21.2 and 27.8 °C; relative humidity between 20 and 56%; 12:12 hours of light:darkness) and were fed *ad libitum* on syrup containing either imidacloprid (dosages: 0.00, 8.00, 20.00, 50.00, 125.00, 187.50, 250.00, 500.00, 1000.00 or 2000.00 µg L^{-1}), thiamethoxam (0.00, 8.00, 20.00, 50.00, 125.00, 218.75 or 312.50 µg L^{-1}), fipronil (0.00, 3.20, 8.00, 20.00, 50.00, 87.50 or 125.00 µg L^{-1}) or cypermethrin (0.00, 0.78, 1.95, 4.88, 12.21, 21.36, 30.52, 41.99, 53.46 or 64.94 mg L^{-1}) . Each cage received one of the dosages, whose range spanned and exceeded the environmentally realistic concentrations. Environmentally realistic concentrations of these pesticides are here defined as < 10 ppb (imidacloprid and thiamethoxam), < 10 ppb (fipronil) and < 100 ppb (cypermethrin) (Chauzat et al., 2011, EFSA, 2012, Mullin et al., 2010).

Bees were monitored daily for mortality and syrup consumption was measured daily by weighing syrup feeders for the first 10 days of treatment, and every 2-3 days thereafter. The LC_{50} (48h) was estimated for each dosage. Further details of the methods and results of short-term toxicity, longevity and syrup consumption can be found in *Supplementary Materials*.

2.2.1.3 Statistical analysis

All statistical analyses were carried out using R version 3.0.2 (R Core Team, 2013). The power law relationship between dietary concentration of pesticide and LT_{50} was log-transformed and the slope of the relationship (parameter *b*) was determined by regression. Using the Akaike Information Criterion (AIC), the fit of both linear and non-linear regression models were compared to ensure that the appropriate regression was applied to the power law relationship, as described in Xiao *et al.* (2011). For all test pesticides, linear regression was shown to be the most appropriate for use. For each pesticide, data points were excluded from the regression analysis if the observed LT_{50} fell within the 95% confidence interval of the undosed controls, which we calculated as the control's mean $LT_{50} \pm 1.96$ S.D. Note that we were evaluating whether the LT_{50} of an individual dosed cage could be reasonably attributed to senescence, hence the confidence interval is calculated using the S.D. and not the S.E. Further details of the analysis of short-term toxicity, longevity and syrup consumption data can be found in *Supplementary Materials*.

2.2.2 Honey bee demographic model

To evaluate the impact of dietary pesticides on honey bee colonies, we simulated the population dynamics of a control (unexposed) colony using a published demographic model (Khoury et al., 2011) and then perturbed the vital

rates of population growth according to the effects that we had quantified experimentally. The previous application of the model to a toxicological perturbation (Henry et al., 2012a) had investigated only the loss of intoxicated foragers through homing failure, but we sought to explore the case where hive bees also experience an elevated rate of mortality by feeding on stored honey that contains a dietary pesticide. We therefore modified the original model (**Supplementary Figure 2.6**). The population dynamics of the control colony were described using previously determined parameter values (L = 2000, alpha = 0.25, theta = 0.75 (Khoury et al., 2011); M_B = 0.154 (Henry et al., 2012a); w = 22000 (Henry et al., 2012b) so that the colony's population of bees increased by approximately 25% over 30 days from an initial size of 18000 (13500 hive bees, 4500 foragers), which simulates the rates of development typical in France coincident with the blooming of sunflower and oilseed rape.

We simulated the colony's exposure to four dietary pesticides by perturbing the baseline mortality rate, M_{base}, according to our experimental observations as follows. We calculated a mean daily mortality rate among honey bees in the control treatment and in each concentration of the laboratory pesticide exposures (see *Supplementary Materials* for longevity data). Using these values, we fitted a least-squares linear relationship between the dietary concentration of pesticide (*dose*) and the total daily mortality rate, denoted M_{total} (Fipronil: M_{total} = 0.0056*dose* + 0.1833; r-squared > 0.99; Imidacloprid: M_{total} = 0.0006*dose* + 0.0659, r-squared = 0.65; Thiamethoxam: M_{total} = 0.0011*dose* + 0.1721, r-squared > 0.99; Cypermethrin: $M_{total} = 7x10^{-7}dose$ + 0.1468, r-squared = 0.28; **Supplementary Figure 2.7**). Using these fitted dose-response (i.e. M_{total} *vs*. *dose*) relationships, we estimated the pesticide-independent mortality

rate, denoted M_{base}, from the intercept of the linear regression (i.e. the rate at zero-dose)

Using the values of M_{total} and M_{base} obtained above, we then estimated the daily mortality rate due to each dietary pesticide, denoted $M_{pesticide}$ as follows. We first assume that pesticide-induced mortality applies to individual bees that survive the baseline mortality rate. I.e.

$$M_{total} = M_{base} + (1 - M_{base})\, M_{pesticide} \qquad \text{Eq M2}$$

Using this assumption, we obtain:

$$M_{pesticide} = (M_{total} - M_{base})/(1 - M_{base}) \qquad \text{Eq M3}$$

We then solve Eq M3 for $M_{pesticide}$ after using the fitted dose-response (M_{total} *vs*. *dose*) relationships obtained previously to estimate M_{total} at the environmentally realistic dietary concentration of nectar (imidacloprid, fipronil and thiamethoxam: 5ppb; cypermethrin: 100 ppb). Solving Eq M3 yielded four pesticide-specific values of $M_{pesticide}$ as follows: fipronil $M_{pesticide}$ = 0.0342, imidacloprid, 0.0003, thiamethoxam, 0.0066, cypermethrin, 0.0001. Our values of $M_{pesticide}$ based on residues measured in nectar are likely to be conservative because honey bees concentrate the solutes in collected nectar before adding it to their food stores as honey.

For predicting the number of dead bees found outside a hive, we assume that 2.5% of natural mortalities in control colonies occur at the hive whereas under fipronil exposure all mortalities occur at the hive. For comparison, the overall mortalities under the various exposures are displayed in **Supplementary Figure 2.8**.

2.2.3 Honey bee internal residue analysis

2.2.3.1 Preparation of chemicals

Fipronil was obtained as a powder (analytical standard, PESTANAL®, Sigma Aldrich Co. LLC; product code: 46451) and was suspended in acetone to form a stock solution of 2.9 µg ml^{-1}, before being mixed with 50% w/v aqueous sugar solution (Attraker: 1.27 kg L^{-1} fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) to produce the final dose used.

2.2.3.2 Bees and pesticide diets

These methods were based on OECD Test Guideline No. 213 Honey bee acute oral toxicity test (OECD, 1998). Adult worker honey bees of variable age were collected from domesticated colonies in Devon, UK. Honey bees were starved 1.5 – 2 hours prior to dosing and were then briefly chilled to inactivity before being placed into cages in batches of 10 (round, plastic containers; cage dimensions: approx. 0.10m diameter x 0.04m height). Bees were maintained under controlled laboratory conditions (temperature 25 °C; relative humidity 40 %, 12:12 hours light:darkness) and each cage of 10 bees was fed 200 µL of either control syrup or syrup containing fipronil at a concentration of 145 µg L^{-1}. This dose was chosen to cause negligible mortality by the end of the experiment but was also still high enough to produce quantifiable residues. After this initial dose had been consumed (time 0), bees were provided with control syrup for the remaining duration. Cages were sampled at the time points of 0, 0.5, 1, 1.5, 2, 4 and 6 days post-dose and frozen at -20 °C. Control honey bees (no fipronil exposure) were collected after 0, 1, 2 and 4 days post syrup ingestion. Replication of each treatment (dosed, control) was $n = 2$ or $n = 3$ cages per time point (**Supplementary Figure 2.9**).

2.2.3.3 Residue analysis

Residues of fipronil and its main toxic metabolite fipronil sulfone in treated bees were measured with gas chromatography mass spectrometry (GC-MS). Bees fed only control syrup were also analysed for residues of fipronil sulfone.

To each sample (approximately 10 bees) 10 ml each of water and acetonitrile were added and this was then homogenised (Ultra Turrax homogeniser T25, IKA Labortechnik, Staufen, Germany) at 9000 rpm for 2 minutes. Each sample was then shaken with 4 g of $MgSO_4$ (anhydrous) and 2 g of sodium chloride for 1 minute before being centrifuged (Allegra X-15R, Beckman Coulter (UK) Ltd, High Wicombe, UK) at 3500 rpm for 2 minutes. The supernatant was collected and a 1 ml aliquot was evaporated to dryness at 45 °C using a TurboVap LV Concentrator (Biotage Ltd, Uppsala, Sweden). The sample was then re-dissolved in 1 ml of ethyl acetate with the aid of ultra-sonication.

GC-MS analysis was conducted using an Agilent 7890B gas chromatograph and an Agilent 5977A mass spectrometer, fitted with an Agilent DB-5ms, 0.25 mm x 30 m x 0.25 µm film thickness column and operated in selected-ion monitoring mode (SIM) (**Table 2.**1) with helium carrier gas. Instrument conditions included splitless injection, 250 °C inlet, 300 °C transfer line; oven temperature programme: 70 °C for 0.50 minutes, 20 °C/min to 150 °C, 5 °C/min to 300 °C, then isothermal for 15 minutes. The limit of detection (LOD) for fipronil was 0.005 ng/bee and for fipronil sulfone was 0.01 ng/bee.

2.3 Results and Discussion

Our analyses of the log(*t*)-*vs.*-log(*C*) relationships among the four insecticides revealed that fipronil alone showed evidence of time-reinforced mortality

(fipronil: b = 2.2 ± 0.08; imidacloprid: 0.1 ± 0.32; thiamethoxam: 0.8 ± 0.11; cypermethrin: 0.3 ± 0.11; **Figure 2.1**). In our experiments, the bees had experienced a sustained exposure because they fed continuously until death, albeit at dose-dependent rates (**Supplementary Figure 2.10**). The exponent of the constant-product law (Eq. 2) fitted for fipronil closely approximates the theoretical value for a bioaccumulative toxicant (b = 2). Using GC-MS analysis, we established that the sulfone produced from a single fipronil-laced meal persisted undiminished in honey bees for at least six days (**Figure 2.2**), a result also found by a recent study, carried out over a 48 hour period (DEFRA, 2016). Consequently, fipronil sulfone is very likely to be highly bioaccumulative under sustained dietary intake. Taken together, these findings suggest that bioaccumulation is the cause of the time-reinforced toxicity observed in our experiments. The pharmacokinetics of fipronil in honey bees contrast with those of imidacloprid in dietary exposures. As a parent molecule, imidacloprid is rapidly metabolised with a biological half-life of approximately 5 hours and following a single imidacloprid-laced meal its known toxic metabolites account for less than 5% of the ingested mass after 48 hours (Suchail et al., 2004), whereas we found that fipronil sulfone accounted for almost all of the ingested fipronil even after six days. The fitted exponents of the constant-product law fitted for imidacloprid and cypermethrin were substantially below one, indicating that sustained low-level doses were disproportionately ineffective compared to acute higher doses.

For each pesticides, we used the mortality data from the time-reinforcement bioassay to calculate the daily mortality rate of honey bees exposed to field-realistic dietary residues, and added this rate to a honey bee demographic model (Khoury et al., 2011) to determine its effects on honey bee colony growth

(see Methods, **Supplementary Figure 2.6**). Unlike previous studies (Henry et al., 2012a, Khoury et al., 2011), we applied the insecticide-related mortality rate to both foragers and nurse bees in the simulated colony because all adult bees in a colony feed on stored forage. When the effect of each insecticide was included separately in the model, fipronil alone caused mass mortality. Specifically, the simulation predicts the death of approximately 1000 bees per day during the first week of exposure (**Supplementary Figure 2.8**), which accounts for the 'tapis d'abeilles mortes' (carpet of dead bees) in front of each colony that characterised the affected French apiaries during the 1990s (de Villiers, 2004). If the exposure was prolonged, the effect of fipronil causes colony failure within 40 days (**Figure 2.3**). There was virtually no effect of imidacloprid or cypermethrin exposure on colony growth and although exposure to thiamethoxam residues reduced the rate of colony growth, worker numbers still increased over time (**Supplementary Figures 2.8 and 2.11**).

Of the candidate compounds examined, only fipronil was predicted to cause colony collapse in honey bees at environmentally realistic residue levels. We therefore postulate that fipronil, not imidacloprid, was the cause of mass mortalities of honey bees that were associated with agricultural sunflower in France during the 1990s. Of course, some recent mass mortalities of honey bees, such as the 2008 instance in Baden-Würtemberg, Germany (Nuyttens et al., 2013) were instead caused by clouds of insecticidal neonicotinoid dust that were released as treated maize seeds were planted by pneumatic drilling machinery. Dust emission cannot account for the mass mortalities that coincided with the mid-summer bloom of French sunflower crops, however, because agricultural sowing (including maize) occurs weeks earlier in the year. Despite the ongoing ban in Europe, contamination of floral forage by fipronil

continues to be an occasional cause of mass mortalities, such as the 2014 event that involved 172 hives across 23 apiaries in the Canton of Bern, Switzerland (Seiler et al., 2014). In this instance, investigators suspected that fipronil residues were present as an accidental trace contaminant in an imported batch of an approved fungicide that had been used to treat fruit trees. This incident supports our hypothesis that fipronil is capable of causing major impacts on honey bees. Furthermore, our analysis has examined in detail only a lethal endpoint, but fipronil-induced TRT may also detrimentally affect sublethal endpoints in individual honey bees, such as foraging intensity and homing success (Decourtye et al., 2011), which also could contribute to colony collapse.

Our findings highlight the need to identify agrochemicals that cause time-reinforced toxicity, because this property enables trace contamination to become disproportionately harmful by sustained exposure. Previously, regulatory procedures for the risk assessment of plant protection products in the European Union have relied on short-term toxicity laboratory tests on honey bees (so called 'first tier' tests), which do not take account of the possible harm that results from TRT during realistic sustained exposures. The octanol-water coefficient (K_{ow}) has been used conventionally to predict the ability of a compound to accumulate within organisms, with a log value > 5 suggesting high bioaccumulative potential (Rodan et al., 1999). However, the K_{ow} value for fipronil (log K_{ow} = 3.75 (Bonmatin et al., 2014) fails to predict that it causes TRT. Therefore, newly formulated draft guidelines issued by the European Food Safety Authority (EFSA) for risk assessment in bees require both longer conventional laboratory exposures (10 days) in first tier procedures that could reveal TRT and a new experimental protocol aimed specifically at evaluating

conformity with Haber's Law (EFSA, 2013). By explicitly including an evaluation of TRT due to dietary exposure, future risk assessments will enable regulatory testing to better protect farmland bees and the valuable ecosystem services that they deliver by pollinating crops and wild flowers.

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Author Contributions As first author I co-designed the study, carried out the lab work and statistical analysis, and co-wrote the manuscript; Ainsley Jones carried out the GC-MS analysis; Charles Tyler helped in the methods and co-wrote the manuscript; James Cresswell conceived the study, co-designed the study and co-wrote the manuscript.

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Chapter Two: Figures

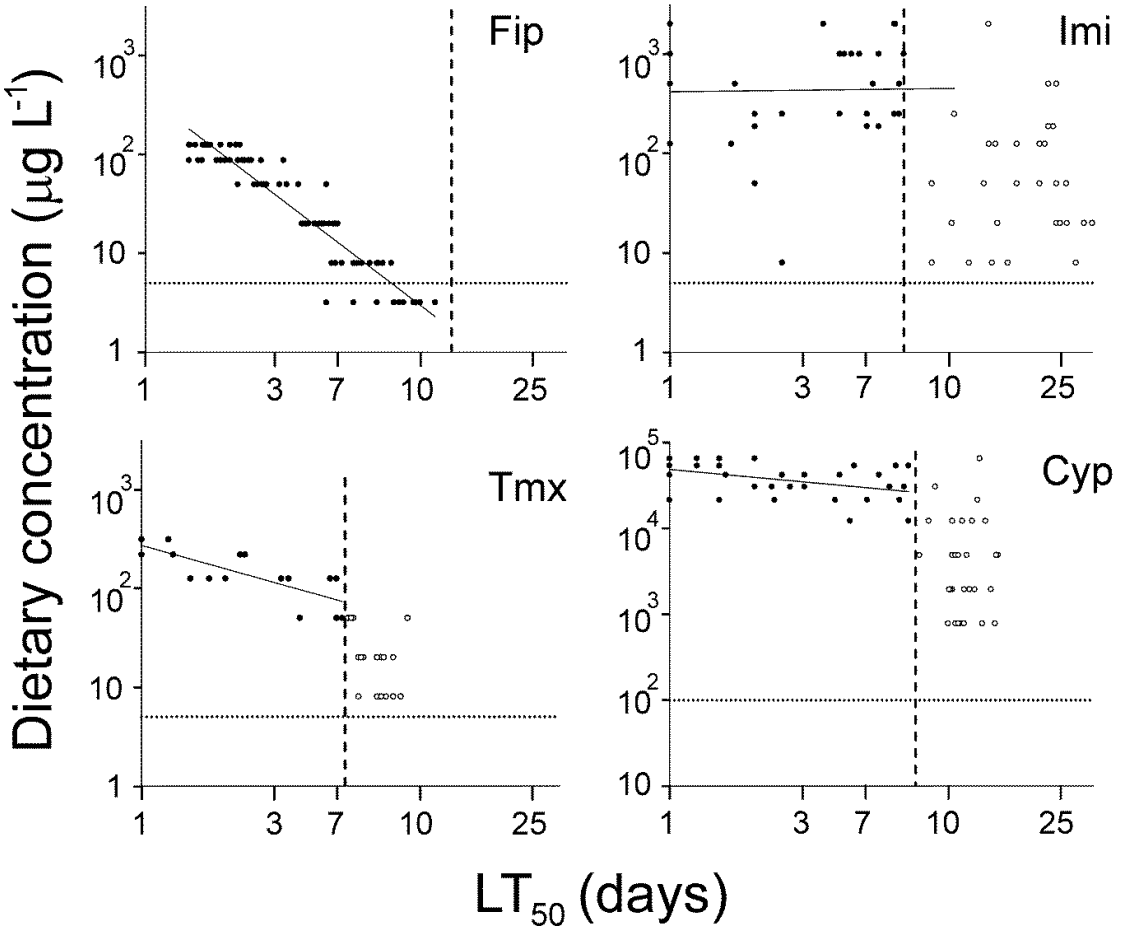


Figure 2.1 Four pesticides (Fip = fipronil; Imi = imidacloprid; Tmx = thiamethoxam; Cyp = cypermethrin) evaluated for time-reinforced toxicity. Solid lines indicate best-fit relationships between dose (*y*-axis: dietary concentration in µg L^{-1}) and time-to-effect (*x*-axis: time to 50% mortality among exposed subjects, or LT_{50}). The vertical dashed line indicates the upper limit on LT_{50} imposed by senescence (see Methods); the horizontal dotted line indicates the environmentally realistic residue concentrations used in the honey bee demographic model. Filled symbols indicate data points used to fit the *t-vs.-C* relationship; open symbols indicate excluded data where observed longevity was attributed to senescence.

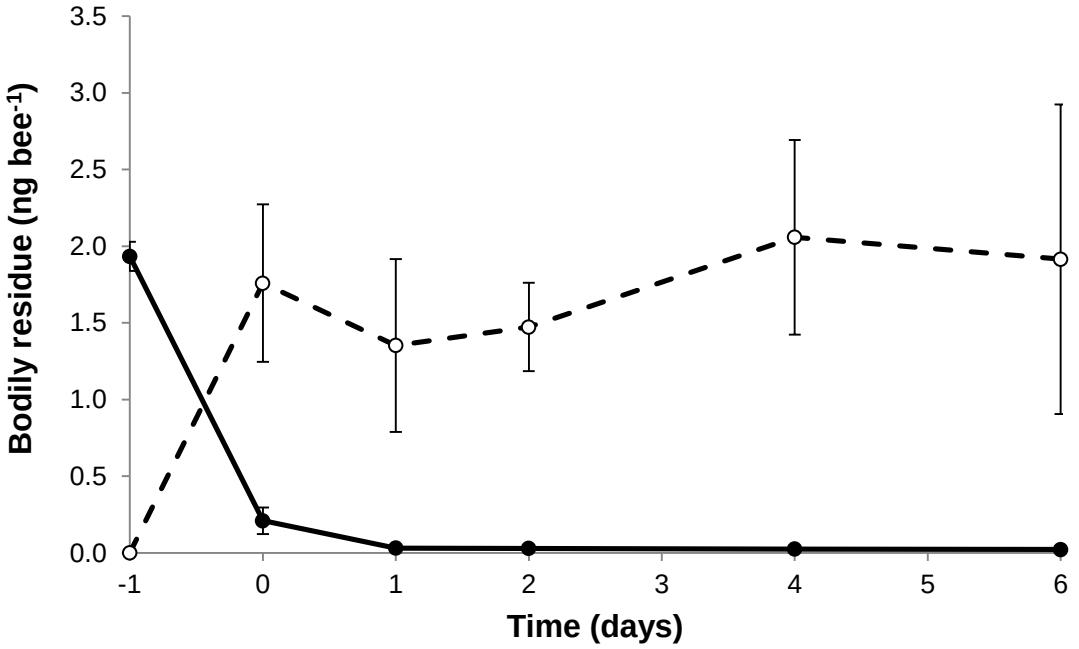


Figure 2.2 Time-course of whole-body residues of fipronil (solid line) and its sulfone metabolite (dashed line) in honey bees after a single fipronil-laced meal. Residues (*y*-axis: ng of compound bee^{-1}) were measured at intervals over a six day period (*x*-axis: time in days) after a single acute dietary exposure to syrup containing fipronil at 145 µg L^{-1}. The initial fipronil content of bees was estimated from their syrup consumption and is indicated for ease of inspection at $x = -1$. Similarly, the initial bodily residue of the sulfone metabolite is assumed to be zero. Symbols indicate sample means and error bars denote ± 1 SE. Fipronil concentrations in control (undosed) samples were less than 0.02 ng bee^{-1} and fipronil sulfone concentrations were less than 0.11 ng bee^{-1}. Mean residues are connected for ease of inspection only.

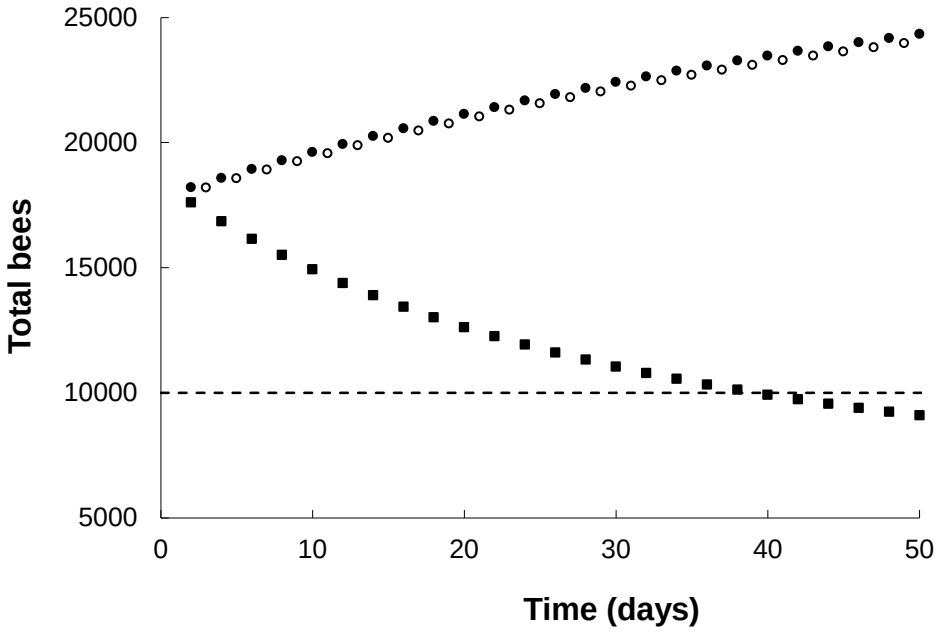
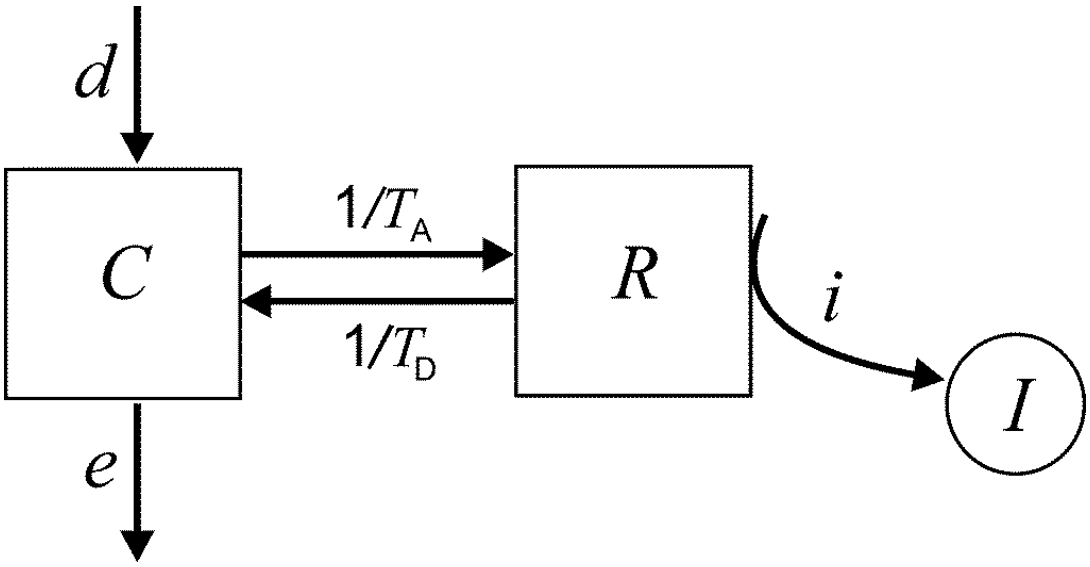
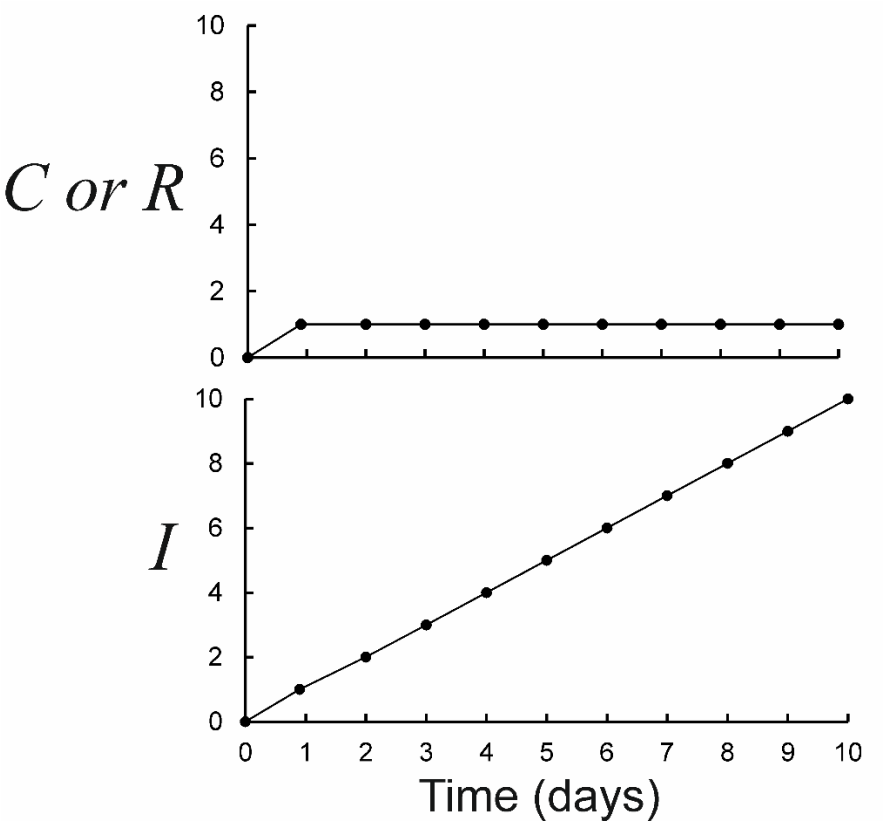


Figure 2.3 Population dynamics in simulated honey bee colonies during exposures to fipronil and imidacloprid. Model predictions of colony size (*y*-axis: number of adult workers) over a seven week period (*x*-axis: time in days) under control conditions (symbol: filled circles) and during environmentally realistic dietary exposures to imidacloprid (open circles) or fipronil (filled squares). The dashed line indicates the presumed minimum for colony survival.

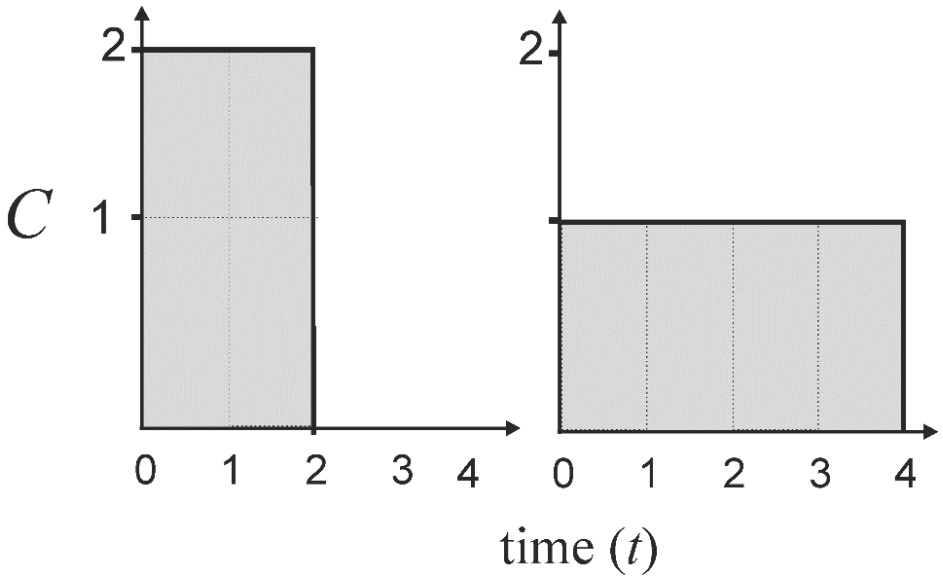
Chapter Two: Supplementary Figures



Supplementary Figure 2.1 Compartment model of pharmacokinetics during dietary exposure to a toxicant. Assume that the pharmacokinetics of the toxicant in an animal's body are governed by this simple compartment model. The animal ingests the toxicant at a dose rate of d ng d^{-1} and assume that the animal's detoxification enzyme system has surplus capacity, which means that the rate of the detoxification is proportional to the internal concentration of the toxicant, C. Hence, the toxicant is detoxified metabolically (or otherwise eliminated from the animal's body) with first order dynamics at a rate of eC ng d^{-1}. Let R denote the concentration of target receptors bound by the toxicant and assume that the formation of the toxicant-receptor complex is governed by coefficients of association and dissociation, denoted T_A and T_D respectively so that the rate at which the toxicant binds to receptors is R/T_A., etc. Assume that the animal incurs irreversible injury at a daily rate Ri. The total injury incurred by the organism is denoted by circular box I (the circle is used to distinguish a box that accumulates an effect from one that accumulates a mass) and the oblique arrow into the circular box indicates transfer of influence, not mass.



Supplementary Figure 2.2 Pharmacokinetics of a non-bioaccumulative toxicant. When the toxicant binds reversibly to its target site and is substantially susceptible to catabolic breakdown and elimination, then during a sustained dietary exposure the continuous and opposing actions of ingestion and elimination will establish a 'steady state' concentration inside the organism and C is constant. Since R is proportional to C, R is also constant over time and injury accrues at a constant rate. Hence, $I \propto t$ and $kI = Ct$, where t denotes the duration of the exposure. In this hypothetical discrete-time example: $d = e = 1$; $T_A = T_D = 1$.



Supplementary Figure 2.3 Toxic load in a non-bioaccumulative toxicant and Haber's Law. The total injury across the exposure, or toxic load, is proportional to the area under the curve (AUC) of the plot of *C* over time, which can be visualized as a rectangular geometry with area *C*× *t* (grey fill). Consider two groups of animals that feed separately on diets whose toxicant concentrations differ by a factor of α (i.e $d_1 = d_2 / \alpha$); in this hypothetical example, α = 2. If the feeding rates on the diets are equal and the animals on the more toxic diet have an internal concentration of toxicant C_1, the internal concentration of toxicant of those that feed on the less toxic diet is C_1/α. Assume that animals feeding on the more toxic diet reach a given level of injury (toxic load) in t_1 days and those in the less toxic diet reach the same level in t_2 days (in this hypothetical example, t_1 = 2 days). Since the AUCs have rectangular geometry, then for both groups to experience the same injury, those on the less toxic diet must be exposed for $t_2 = \alpha t_1$ days (i.e. t_2 = 4 days). Formally, we can write:

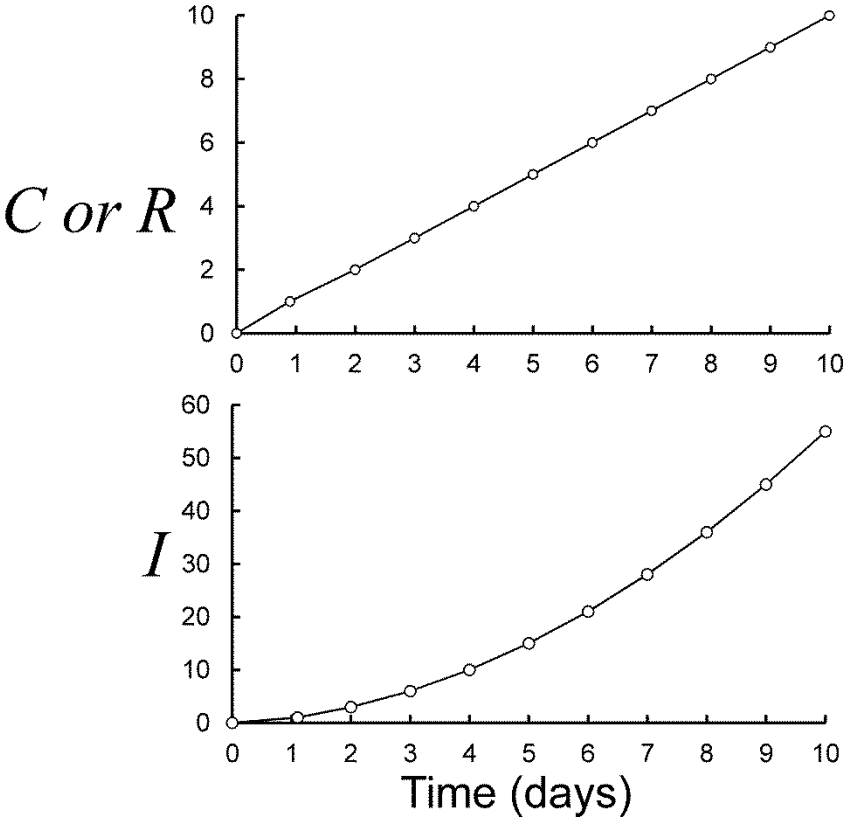
$$C_1 t_1 = \frac{C_1}{\alpha} \times t_2 = \frac{C_1}{\alpha} \times \alpha t_1 \qquad \text{Eq. S1}$$

Simplification of Eq ED1 and generalisation for all conforming *C* and *t* combinations yields $Ct = k$. Hence, subjects exposed to perfectly non-bioaccumulative toxicants in appropriate 'time-to-effect' experiments will exhibit outcomes that conform to a

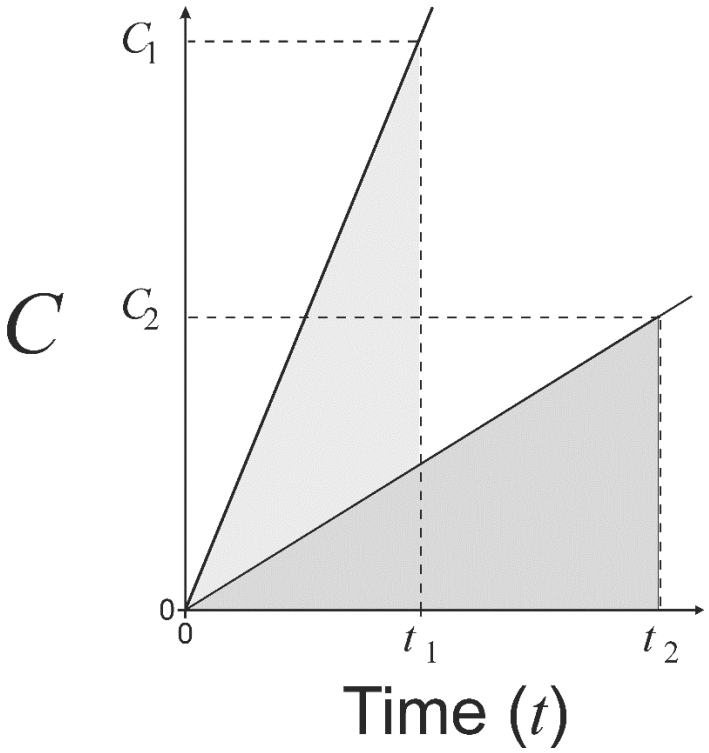
constant-product law of $C^b t = k$ where b =1, which is Haber's Law. Taking logarithms of both sides of $Ct = k$ and rearranging yields:

$$\log(C) = -1\log(T) + \log(k) \qquad \text{Eq. S2}$$

Therefore, a non-bioaccumulative toxicant delivered in a time-to-effect experiment will produce a *C-vs.-t* relationship with a slope of -1 on log-log axes.



Supplementary Figure 2.4 Pharmacokinetics of a bioaccumulative toxicant. When the toxicant is not susceptible to catabolic breakdown and elimination, then during a sustained dietary exposure continuous ingestion will cause an accumulation of toxicant inside the organism and *C* increases over time. Since *R* is proportional to *C*, *R* also increases over time and injury accrues at an increasing rate as exposure progresses, which is 'time reinforcement'. In this hypothetical discrete-time example: $d = 1$; $e = 0$; $T_A = T_D = 1$.



Supplementary Figure 2.5 Toxic load in a bioaccumulative toxicant. Given constant ingestion of a bioaccumulative toxicant, let the internal concentration at time *t* be given by:

$$C = \beta t \qquad \text{Eq. S3}$$

The total injury across the exposure, or toxic load, is proportional to the area under the curve (AUC) of the plot of *C* over time, which can be visualized as a triangular geometry with area 0.5*t* × *C* (i.e. half base × height). As before, consider two groups of animals that feed separately on diets whose toxicant concentrations differ by a factor of α. If the feeding rates on the diets are equal, the animals on the more toxic diet have an internal concentration of toxicant $C_1 = \beta t_1$ and those on the less toxic diet have $C_2 = (\beta/\alpha)t_2$. Since the AUCs have triangular geometry, then for both groups to experience the same injury we require:

$$0.5t_1 \times \beta t_1 = 0.5t_2 \times \frac{\beta}{\alpha} t_2 \qquad \text{Eq. S4}$$

Simplification yields:

$$t_1^2 = \frac{t_2^2}{\alpha}$$ Eq. S5

Multiplying both side by C_1 yields:

$$C_1 t_1^2 = \frac{C_1}{\alpha} t_2^2$$ Eq. S6

Recall that the internal concentrations differ by a factor of α, so that we can write:

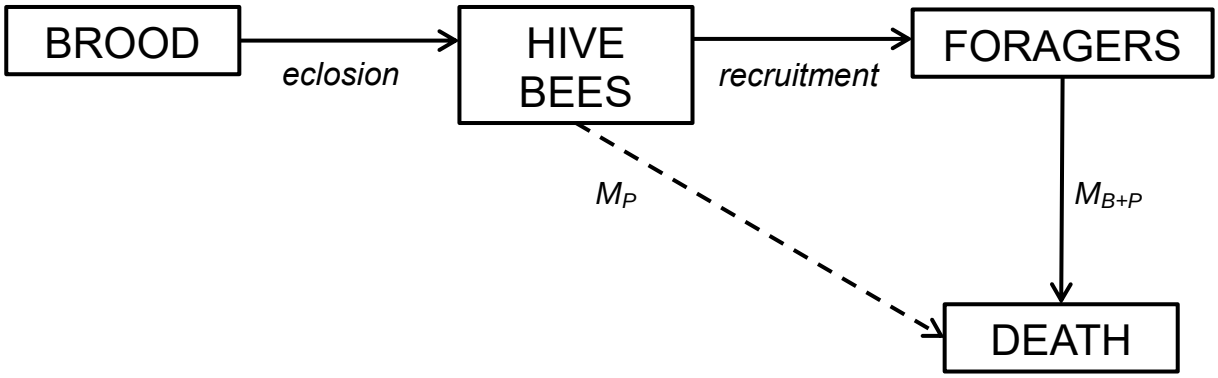
$$C_1 t_1^2 = C_2 t_2^2$$ Eq. S7

Generalisation for all conforming *C* and *t* combinations yields $Ct^2 = k$. Hence, subjects exposed to perfectly bioaccumulative toxicants in appropriate 'time-to-effect' experiments will exhibit outcomes that conform to a constant-product law of $Ct^b = k$ where b =2.

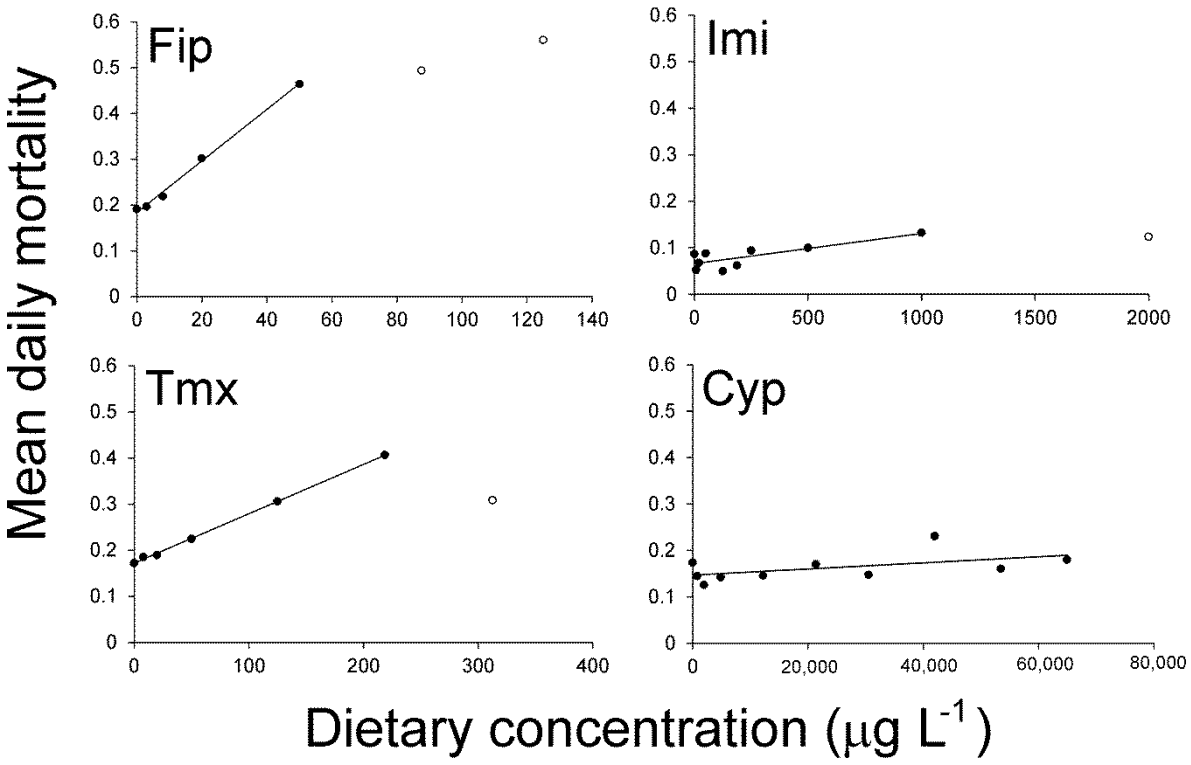
Taking logarithms of both sides of $Ct^2 = k$ and rearranging yields:

$$\log(C) = -2\log(T) + \log(k)$$ Eq. S8

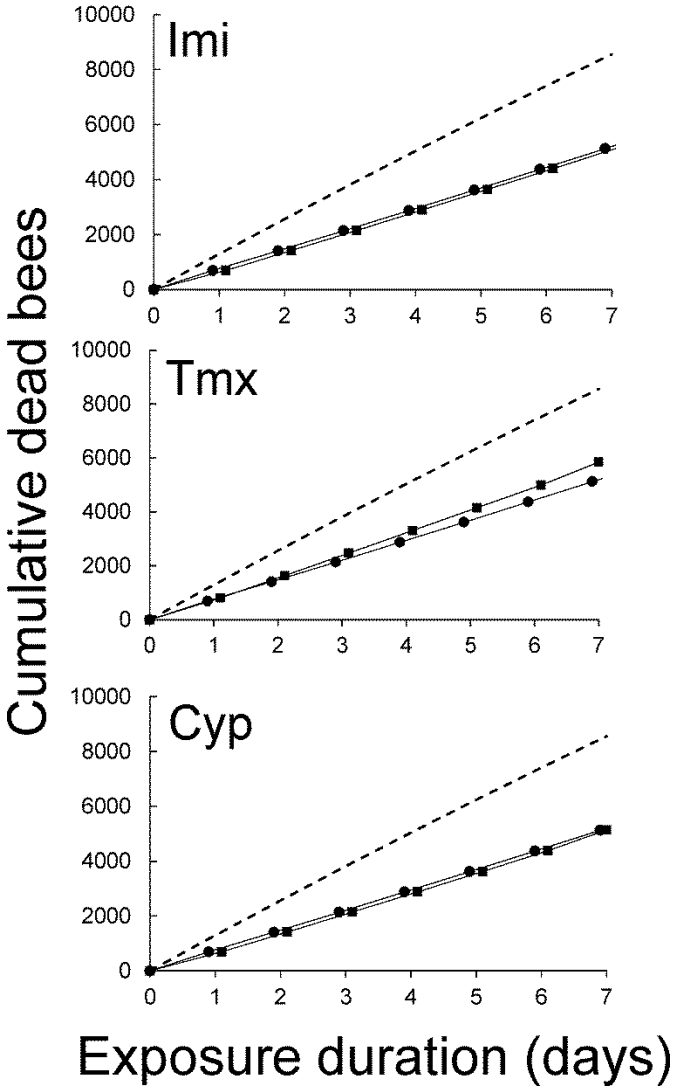
Therefore, a bioaccumulative toxicant delivered in a time-to-effect experiment will produce a *C-vs.-t* relationship with a slope of -2 on log-log axes.



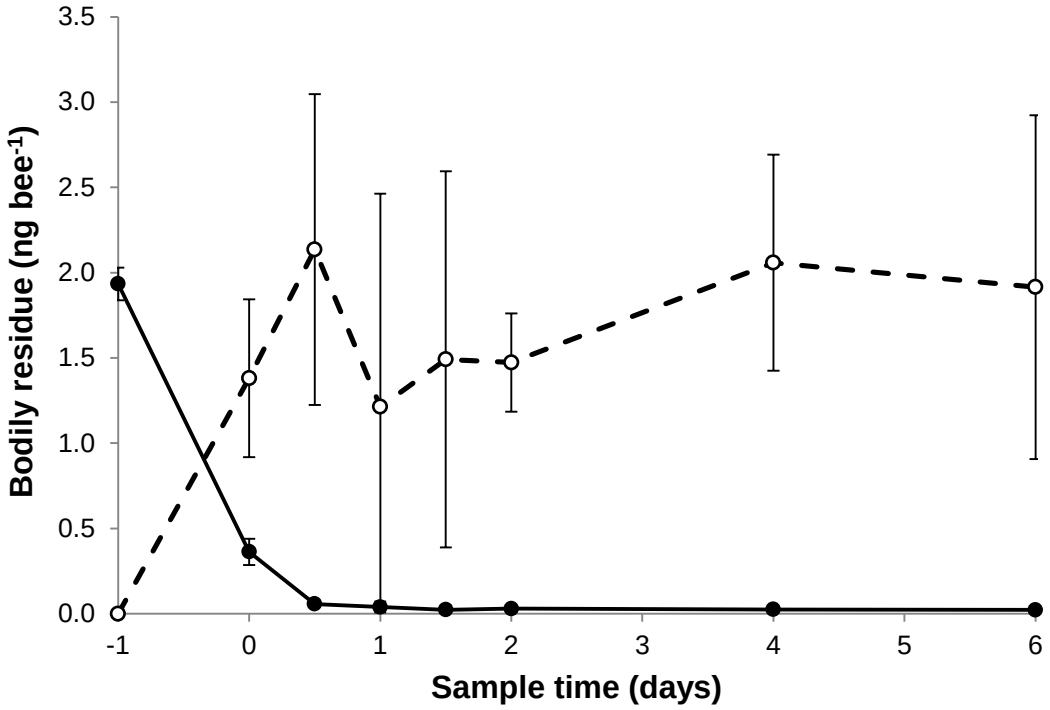
Supplementary Figure 2.6 Schematic diagram of a demographic honey bee colony model, based on Khoury et al. (2011). The number of hive bees is determined by the number of brood and the rate of eclosion, given as $L\left(\frac{N}{w+N}\right)$ where *L* is the queen's laying rate, *N* is the total number of bees in the hive and *w* determines the rate at which the rate of eclosion approaches *L* as *N* increases. The number of hive bees recruited as foragers is given by $H\left[\alpha-\sigma\left(\frac{F}{N}\right)\right]$, where α is the maximum rate of recruitment, σ is the rate of recruitment of foragers back to hive bees, *H* is the number of hive bees and *F* is the number of foragers present in the colony. Foragers die at a rate, M_{B+P}, that compounds the baseline rate, M_{base}, and the rate due to pesticide exposure, $M_{pesticide}$ (see Eq M2). Hive bees die only when exposed to pesticides, at a rate of $M_P = M_{pesticide}$. Values used in the model were; L = 2000, N_0 = 18000, H_0 = 13500, F_0 = 4500, α = 0.25, σ = 0.75 (Khoury et al., 2011); M_B = 0.154 (Henry et al., 2012a); w = 22000 (Henry et al., 2012b). Values of $M_{pesticide}$ for each pesticide were determined from experimental toxicity data (Eq M3).



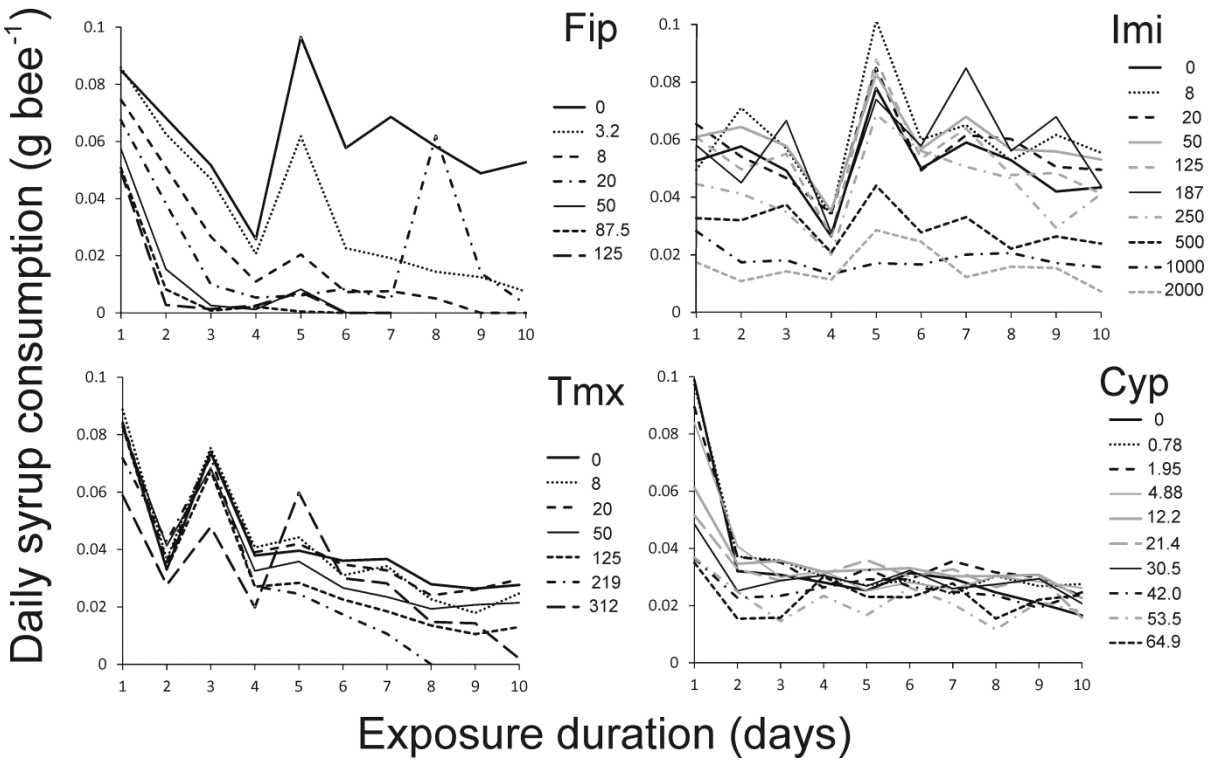
Supplementary Figure 2.7 Relationships between dose and mean daily mortality rate of honey bee workers. For each pesticide (Fip = fipronil, Imi = imidacloprid, Tmx = thiamethoxam, Cyp = cypermethrin), the four panels each show the mean daily mortality rate of honey bee workers (*y*-axis: mean daily mortality rate) exposed to various dietary concentrations of the pesticide (*x*-axis; toxicant concentration in dietary syrup in µg L^{-1}. Each solid line indicates the fitted linear regression used to estimate the mortality rate at an environmentally relevant exposure. (Fip: $r^2 > 0.99$; Imi: $r^2 = 0.65$; Tmx: $r^2 > 0.99$; Cyp: $r^2 = 0.28$). Open symbols indicate data points not included in the regression analysis.



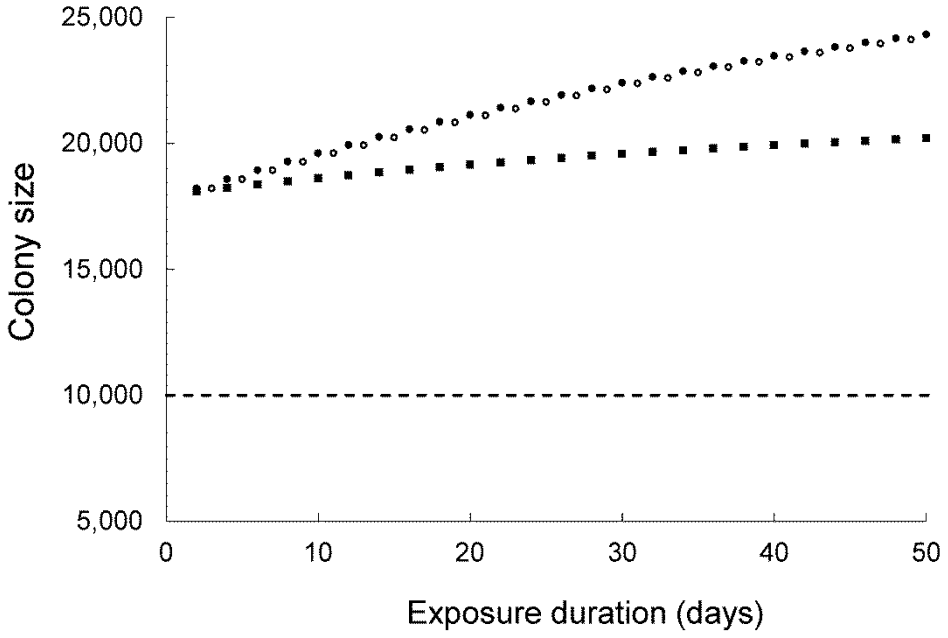
Supplementary Figure 2.8 Comparison of predicted cumulative mortality during exposures to fipronil, imidacloprid, thiamethoxam or cypermethrin. Upper panel (Imi): Cumulative mortality (*y*-axis: total number of dead adult workers) over one week (*x*-axis: days) for a control colony (filled circles) vs. a colony exposed to either dietary imidacloprid (square symbols) or fipronil (dashed line). Lower panels the same except for (Tmx), where square symbols denote a colony exposed to dietary thiamethoxam and (Cyp) cypermethrin. All panels depict outputs of the demographic model[18] with toxicant-specific mortality parameters.



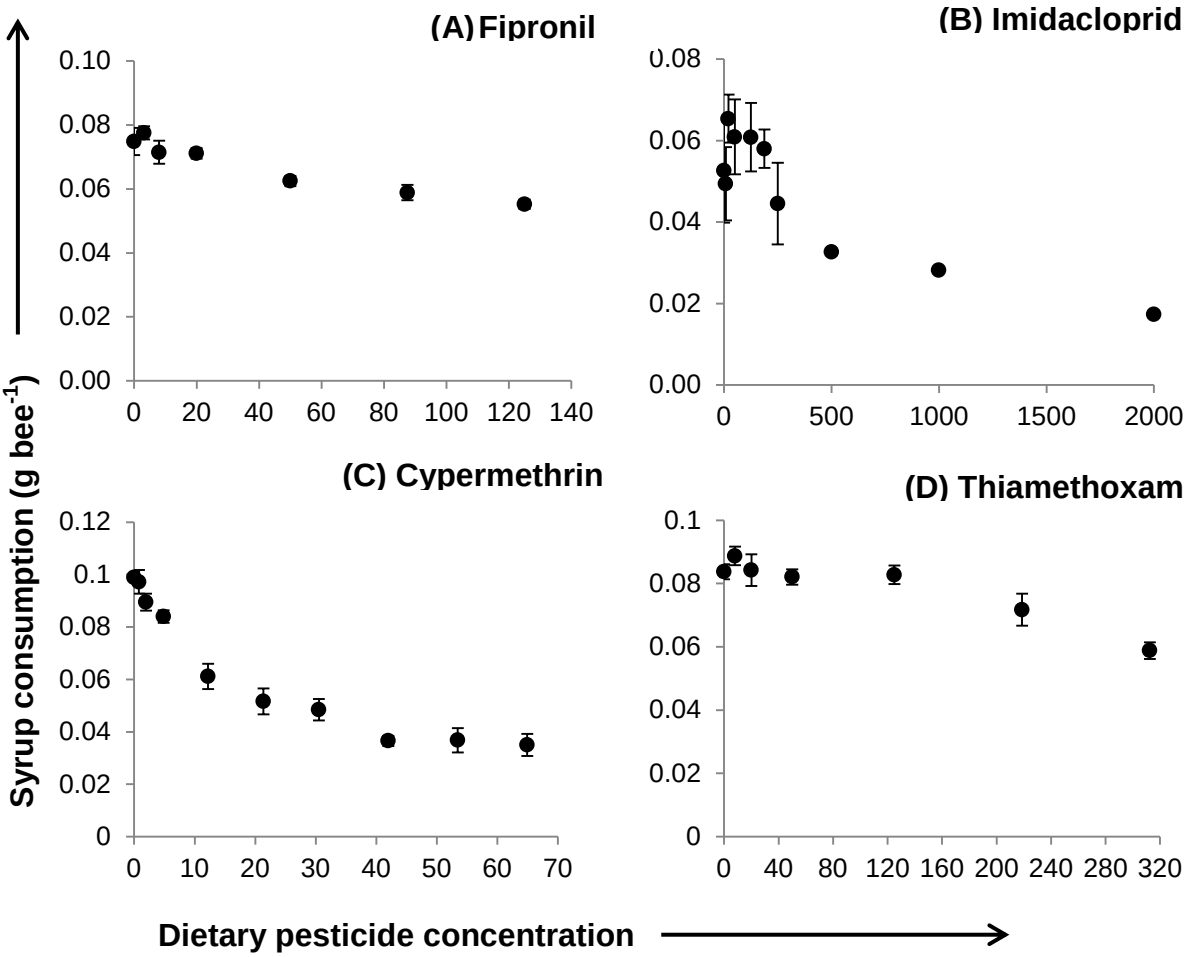
Supplementary Figure 2.9 Residue analysis over time of honey bees exposed to fipronil; including all time points. Residue level detected (*y*-axis; mean residue level of toxicant within individual honey bee workers in nanograms (ng)) of fipronil (filled symbols; N = 3, each consisting of 10 bees) and its toxic metabolite fipronil sulfone (open symbols; N = 3, each consisting of 10 bees) within individuals honey bee workers after fipronil exposure over time (*x*-axis; sample time in days after acute exposure to dietary fipronil at 145 µg L^{-1}). Data from the consumption of fipronil diet was used to estimate residue levels of fipronil at time point -1. Time point 0 was sampled immediately after the acute fipronil dose had been ingested. Error bars denote ± 1 SEM. Note some error bars are obscured by data points. Fipronil concentrations in control (undosed) samples were less than 0.02 ng bee^{-1} and fipronil sulfone concentrations were less than 0.11 ng bee^{-1}. Mean residues are connected for ease of inspection only.



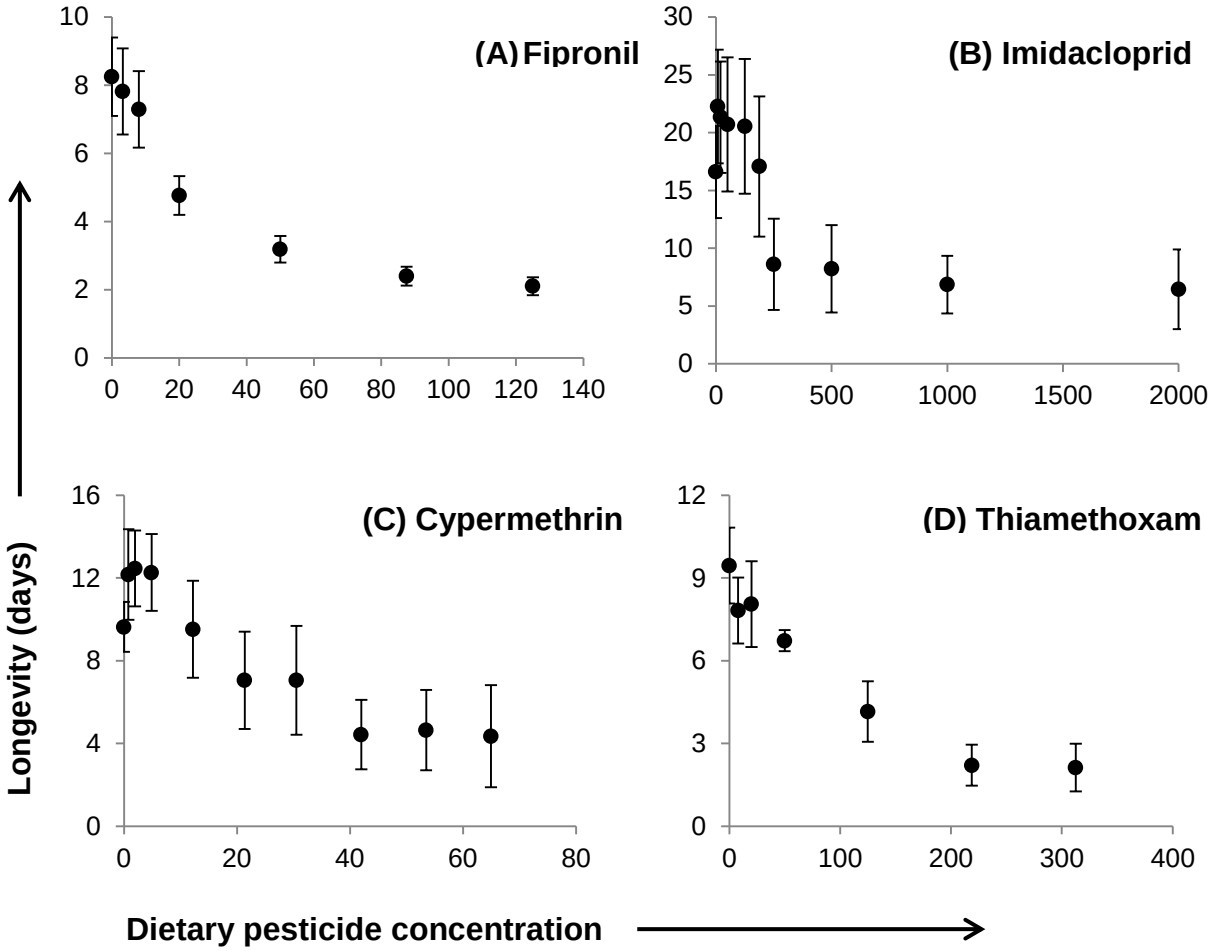
Supplementary Figure 2.10 Effects of four pesticides on honey bee syrup consumption over time. Relationship between syrup consumption of honey bees at various pesticide doses (*y*-axis: mean daily syrup consumption per bees in g) and exposure time (*x*-axis: duration of exposure in days) for honey bees exposed to: (Fip) fipronil (0 to 125 µg L^{-1}, N = 7); (Imi) imidacloprid (0 to 250 µg L^{-1}, N = 7); (Tmx) thiamethoxam (0 to 312.5 µg L^{-1}, N = 7); and (Cyp) cypermethrin (0 to 64.94 mg L^{-1}, N = 7).



Supplementary Figure 2.11 Impacts of exposure to thiamethoxam and cypermethrin on honey bee colony growth. Demographic model simulations of colony growth (*y*-axis: number of adult workers) over time (*x*-axis: duration of exposure in days) when workers are exposed to field realistic residues of thiamethoxam (5ppb; square data points), cypermethrin (100 ppb; open circle data points, offset for inspection from control by one day), and without pesticide exposure (closed circle data points). The dashed line indicates the minimum threshold for colony survival. Data points of control and cypermethrin-exposed colonies have been slightly shifted in the *x*-plane for ease of inspection.



Supplementary Figure 2.12 Effects of four pesticides on honey bee syrup consumption on day 1 of exposure. Relationship between syrup consumption (*y*-axis: mean syrup consumption of individual worker bees in g) and concentration of dietary pesticide (*x*-axis; concentration of pesticide in syrup in µg L^{-1}; cypermethrin concentration in mg L^{-1}) for honey bees exposed to **(A)** fipronil (day 1 consumption; 0 to 125 µg L^{-1}, N = 10); **(B)** imidacloprid (day 1-6 consumption; 0 to 250 µg L^{-1}, N = 10); **(C)** cypermethrin (day 1 consumption; 0 to 64.94 mg L^{-1}, N = 10); **(D)** thiamethoxam (day 1 consumption; 0 to 312.5 µg L^{-1}, N = 10). Error bars indicate 1 standard error (SE). Note some error bars are obscured by data points.



Supplementary Figure 2.13 Longevity of honey bees exposed to four pesticides. Longevity (*y*-axis: mean longevity of individual worker bees in days after initial exposure) of individual worker honey bees after exposure (*x*-axis; concentration of pesticide in syrup in µg L^{-1}; cypermethrin concentration in mg L^{-1}) to **(A)** fipronil (0 to 125 µg L^{-1}, N = 10); **(B)** imidacloprid (0 to 2000 µg L^{-1}, N = 10); **(C)** cypermethrin (0 to 64.94 mg L^{-1}, N = 10); **(D)** thiamethoxam (0 to 312.5 µg L^{-1}, N = 10). Error bars indicate 1 standard error (SE). Note some error bars are obscured by data points.

Chapter Two: Tables

	Target		**Qual1**		**Qual2**	
Analyte	**m/z**	**dwell**	**m/z**	**dwell**	**m/z**	**dwell**
Fipronil	367	160	351	140	369	140
Fipronil sulfone	383	40	385	40	255	40

Table 2.1 Selected-ion monitoring (SIM) parameters used for GC-MS analysis.

Chapter Two: Supplementary information

Justification for the use of 5ppb

Residues of neonicotinoids applied as seed treatments (imidacloprid, thiamethoxam and clothianidin) are typically found in the pollen and nectar of treated bee-attractive crops in the range of <1 to 10 parts per billion (ppb) (Cresswell, 2011, EFSA, 2012). Therefore we chose the mid-range value of 5 ppb to determine the pesticide mortality rate applied to a demographic model (Khoury et al., 2011). For fipronil, however, little data is available as to the levels of the residues in the pollen and nectar of crops. However, the application rates (50 – 75 g ha^{-1}) of fipronil and imidacloprid (Pisa et al., 2015) are very similar and so are the levels of their residues in honey bees and bee-collected pollen (Chauzat et al., 2011). On this basis, we argue that bees are probably exposed to a similar degree to both of these pesticides and therefore the value of 5 ppb measured in pollen and nectar for imidacloprid is a plausible estimate for fipronil residues.

Justification for exclusion of data points in fipronil mortality assessment for demographic model

The average daily mortality rate due to dietary fipronil at 5 ppb was estimated using only data points in the range of 5 ppb, because the dose-response relationship was linear in this range. Two data points measured at high doses (> 80 ppb) were excluded, because the dose-response relationship saturated at these highest doses (**Supplementary Figure 2.7**).

Syrup consumption

Syrup consumption of control and pesticide exposed bees was monitored to determine whether observed toxic effects could be linked to starvation.

Variation in syrup consumption of bees relating to dose was tested by one-way analysis of variance (ANOVA), with dose as a categorical variable. Significant differences between dosed groups were identified with Tukey tests and pair-wise comparisons were carried out for significant groups by further ANOVA tests. Where necessary, the response variable was log-transformed to conform to test assumptions.

Syrup consumption on Day 1 of exposure varied significantly with dose concentration for all pesticides (Fipronil: $F_{6,39} = 11.33$, Imidacloprid: $F_{9,57} = 4.58$, Thiamethoxam: $F_{13,35} = 10.05$, Cypermethrin: $F_{9,58} = 53.20$; $P < 0.001$ in all cases; **Supplementary Figure 2.12**), however even bees exposed to the highest doses ate at least a third of that of the controls, indicating that starvation was not a factor in observed toxicity. Fipronil reduced the syrup consumption of honey bees at dose concentrations ≥ 50 µg L^{-1} compared with the control ($F_{1,24} = 26.12$, $P < 0.001$). For cypermethrin, honey bee syrup consumption was reduced at concentrations ≥ 1.95 mg L^{-1} compared to the control ($F_{1,66} = 255.96$, $P < 0.001$).

Short-term toxicity

Percentage mortality (48h) data were transformed using logit analysis and the LC_{50} (48h) calculated as described by Crawley (2007). LC_{50} 48-hour values for honey bees exposed to thiamethoxam, fipronil and cypermethrin were calculated, with standard error, as 196.04 ± 8.85 µg L^{-1} , 83.81 ± 3.30 µg L^{-1} and

49.44 ± 2.13 mg L^{-1}, respectively. It was not possible to determine the LC_{50} 48-hour value for imidacloprid due to low mortality at all doses.

Longevity

Longevity data was analysed using GLMMs with a gamma error structure, including “dose” as a categorical fixed variable and “box” as a categorical random variable. The best fit of the models used was determined by comparison of Akaike’s Information Criterion (AIC) values. Significance of dose effects was determined by comparison with a GLMM omitting “dose”. Individual dose effects were assessed by Tukey pair-wise comparisons.

Longevity was reduced by higher doses for all pesticides (Fipronil: x^2 = 139.84, df = 6, Imidacloprid: x^2 = 59.94, df = 9, Thiamethoxam: x^2 = 113.33, df = 6, Cypermethrin: x^2 = 88.84, df = 9, P < 0.001 in all cases; **Supplementary Figure 2.13**). Only exposure to imidacloprid at 2000 µg L^{-1} significantly reduced longevity compared with the control (P < 0.05). Fipronil caused reductions in the longevity of honey bees at dose concentrations ≥ 20 µg L^{-1} compared with the control (P < 0.001). For thiamethoxam, longevity was reduced at dose concentrations ≥ 50 µg L^{-1} compared to the control (P < 0.005 (50 µg L^{-1}), P < 0.001 (>50 µg L^{-1})). For cypermethrin, honey bee longevity was reduced at dose concentrations ≥ 41.99 mg L^{-1} compared with the control group (P < 0.001).

Honey bee internal residue analysis

Fipronil residues in treated bees peaked directly after exposure (day 0 = 0.363 ng bee^{-1}, S.E. ± 0.077) before rapidly falling to 0.040 ng bee^{-1} (S.E. ± 0.032) within 24 hours of fipronil exposure. During this period fipronil sulfone residues were found to increase, indicating rapid metabolism (**Supplementary Figure**

2.9). Fipronil sulfone residue levels are maintained over the 6 day sample period indicating a bioaccumulative toxicant which bees are unable to metabolise or eliminate. Fipronil concentrations in control (undosed) samples were less than 0.02 ng bee^{-1} and fipronil sulfone concentrations were less than 0.11 ng bee^{-1}.

Data from half-day time points were combined with the previous whole-day measures (e.g. day 0.5 with day 0) to give an average residue level across each 24 hour time period (Figure 2). **Supplementary Figure 2.9** presents all original time points tested.

Supplementary References

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Chapter Three:

Effects of two widely-used pesticides, clothianidin and cypermethrin, on worker reproduction and trophic conversion efficiencies in bumble bee microcolonies

Effects of two widely-used pesticides, clothianidin and cypermethrin, on worker reproduction and trophic conversion efficiencies in *Bombus terrestris* microcolonies

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Keywords: bumble bee, microcolonies, clothianidin, cypermethrin, reproduction, conversion efficiency

Abstract

There are concerns over continuing declines in wild bee populations and the role of agrochemical pesticide use as a potential cause. Bees are exposed to pesticide residues when foraging on the nectar and pollen of treated mass-flowering crops. Although these residues are generally far below the lethal level, they may have sublethal detrimental effects on bumble bees. Clothianidin and cypermethrin are two commonly used pesticides from different chemical classes that appear as residues in pollen and nectar. To assess the risk posed by these residues to wild bees it is important to establish the dose-dependence of their impacts and also whether they exert time-reinforced toxicity. We therefore exposed *Bombus terrestris* microcolonies to dietary residues of clothianidin or cypermethrin and evaluated the dose-dependence of various sublethal effects, including fecundity and trophic conversion efficiency. We analysed the experimental outcomes using a bioassay based on Haber's Law to determine whether either pesticide showed time-reinforced toxicity during prolonged exposure.

At field-realistic doses, neither cypermethrin nor clothianidin affected brood production, syrup and pollen consumption or individual longevity in bumble bee microcolonies. Neither compound generated patterns of mortality consistent with time-dependent mortality. In control microcolonies, conversion efficiency of diet to reproductive output varied between 9 and 27 %. The conversion efficiency of microcolonies was independent of cypermethrin exposure; however clothianidin at the highest doses caused a significant reduction. Our results indicate that low level dietary exposures to clothianidin and cypermethrin residues in crops are unlikely to harm bumble bee reproductive success in the wild. Our results also have implications for risk assessment generally, indicating

the importance of testing for dose-dependence of demographically relevant endpoints at realistically prolonged exposures.

3.1 Introduction

Bumble bees provide valuable ecosystem services, pollinating both crops and wild plant species (Williams and Osborne, 2009). However, there is widespread concern that these services are under threat based on evidence that several bumble bee species are in decline (Cameron et al., 2011, Williams and Osborne, 2009, Biesmeijer et al., 2006). There are a range of factors that may be driving these declines, including habitat loss and emerging diseases, but for farmland bees much of the focus has been on pesticide exposure (Vanbergen and Insect Pollinators Initiative, 2013). A wide range of agrochemical pesticides are currently used to protect crops from pest insects, including the neonicotinoids and pyrethroids. These groups of pesticides were applied to approximately 1.4 million ha and 4.9 million ha of crops in 2013, respectively, making them two of the most widely used pesticide families in the UK (FERA, 2014).

The regulation of agrochemical use has been largely successful in safeguarding bees against mass mortalities and the pesticide residues found in the nectar and pollen of treated mass-flowering crops are often at low, non-lethal levels. However, research into the toxicological impacts of crop residues on wild farmland bees is still limited because most studies either focus on surveys of residue levels in honey bee-collected pollen or other honey bee colony matrices (Chauzat et al., 2011, Mullin et al., 2010). To ensure that the effects of environmentally realistic residue concentrations are known it is necessary to

evaluate the dose-dependency of a range of relevant toxicological endpoints. In particular, to be able to safeguard wild bee populations it is necessary to determine the effects of pesticides on demographically-relevant, sublethal endpoints rather than focussing simply on lethality to individuals.

Pesticides from the neonicotinoid and pyrethroid families have already been shown to cause various sublethal effects in bees. For example, the neonicotinoid, clothianidin, a super-agonist of insect nicotinic acetylcholine receptors (nAChRs), negatively affects motor function and foraging behaviour and decreases the immune response of exposed honey bees at sublethal levels (Di Prisco et al., 2013, Williamson et al., 2014, Schneider et al., 2012). Cypermethrin, a pyrethroid that modulates sodium channels on insect postsynaptic membranes, has mixed effects at sublethal endpoints. Decourtye et al. (2005) found no effect on honey bee olfactory learning of cypermethrin up to 782 µg L^{-1}, while a study by Bendahou et al. (1999) reported that a chronic exposure of 12.5 µg L^{-1} increased queen supersedure, brood abortion and ultimately failure of honey bee colonies. However, there is limited knowledge of the effects of these pesticides on wild bee species in demographically important endpoints relating to colony performance and reproduction. We therefore set out to investigate the dose-dependency of a demographically relevant endpoint that is incompletely studied, namely fecundity.

Reduced fecundity in the foundress queen could be particularly detrimental to bumble bees for two reasons due to their annual life cycle. First, with only new queens surviving over winter (Goulson, 2003), a reduction in the number of new queens (gynes) produced is likely to constrain the number of new colonies founded the following year. Second, reduced foundress fecundity will reduce the

number of adult workers produced by a colony. Queen production in bumble bee colonies is related to colony size, with typically only the largest colonies producing reproductives, so a reduction in workers produced is likely to reduce colony success (Owen et al., 1980, Müller and Schmid-Hempel, 1992). Fewer worker bees could also lead to reduced brood care and foraging, further impacting on reproductive success. Fecundity is very sensitive to dietary pesticide exposure in bees. For example, when delivered orally to laboratory colonies of bumble bees, the neonicotinoid imidacloprid gave an EC_{50} value of just one part per billion (ppb), the lowest demonstrated to date (Laycock et al., 2012).

To fully exploit studies aiming to model the relationship between the availability of floral resources and bumble bee abundance (Crone and Williams, 2016), it is necessary to know the trophic conversion efficiency of bumble bees. Based on floral resources alone, it is then possible to determine the optimum environment required to conserve bumble bee populations. The efficiency with which bumble bees are able to convert dietary inputs of pollen and nectar into the reproductive outputs of brood and nest structure is vitally important to reproduction, though at present this trophic conversion efficiency is not known. If exposure to a pesticide reduced this conversion efficiency it could have important detrimental effects on bumble bee reproductive success. Pollen is an important protein component of the larval diet and is also the main source of lipids for adult bees (Roulston and Cane, 2000), which are then incorporated into wax production. Nectar is the main carbohydrate source for both adults and larvae (Brodschneider and Crailsheim, 2010) and is used both for respiration and also wax production (Rortais et al., 2005). By measuring the food consumption and reproductive outputs of laboratory colonies, we determined the efficiency of

bumble bees in converting dietary inputs (nectar and pollen) into reproductive outputs (brood and wax structures) and we also investigated the impacts of dietary pesticide exposure on this process.

When carrying out risk assessments on the effects of agrochemicals on bees, it is important to test whether pesticides exhibit time-reinforced toxicity (TRT), which can lead to enhanced risk from prolonged pesticide exposure. TRT toxicants cause disproportionately strong toxic effects over prolonged exposures than would be predicted by shorter, acute exposures. Therefore, pesticides with TRT could cause much greater harm to bees than predicted by the conventional short-term exposure experiments which are currently performed for risk assessments in Europe and North America. To determine whether TRT was a factor for toxicity in our experiments, we investigated whether clothianidin or cypermethrin exhibited time-reinforced toxicity in bumble bees.

In order to test for TRT, we used a bioassay based on Haber's Law. Haber's Law assumes that the toxicant is in a toxicokinetic steady state within the organism. Internal concentration is not easy to determine, but to investigate Haber's Law it is sufficient to assume that each different dose produces an internal concentration of the toxicant that is equal (or at least proportional) to the exposure concentration (C). The injury sustained from the exposure is the product of the interaction between the toxicant and its target site. Haber's Law assumes that the number of target sites is in excess, therefore the reaction rate will be proportional to C, and so the total injury incurred over the exposure duration (t) is given by Ct (Rozman, 2000). If the median tolerance to injury of a

bee population is *k*, then the exposure required to reach LD_{50} (lethal dose causing 50% mortality) is given by

$$C t^b = k \qquad \text{Eq. 1}$$

and Haber's Law applies when exponent *b* = 1. If instead the toxicant bioaccumulates at the target tissue, the internal concentration of the organism rises over the exposure duration and the accumulated total injury increases exponentially with time. In this case, the exponent in Eq 1 takes the value *b* > 1. When the progression of injury across a range of different exposures is described by *b* > 1 in Eq. 1, we have detected time-reinforced toxicity. Consequently, it is straightforward to test for time-reinforced toxicity using data from 'time-to-effect' experiments that quantify the exposure time required to produce a specified level of injury in experimental subjects. After conducting exposures at various dietary concentrations, a suitable test involves deriving the *t-vs.-C* relationship and determining its slope on logarithmic axes, which estimates parameter *b* (Eq 1) because:

$$\log(C) = -b[\log(t)] + \log(k) \qquad \text{Eq. 2}$$

We studied the effects of environmentally realistic exposures of two widely-used neurotoxic pesticides, with different mechanisms of toxicity, on reproduction and trophic conversion efficiency in *Bombus terrestris* queenless microcolonies. Clothianidin, a neonicotinoid, and cypermethrin, a pyrethroid, have both been found at sublethal concentrations in pollen and honey bee colonies (Mullin et al., 2010, Chauzat et al., 2011). Clothianidin has also been placed under restriction for use on bee-attractive crops by the European Union, along with thiamethoxam and imidacloprid (European Commission, 2013), due to concerns

for their negative impacts to bee health (EFSA, 2013a, EFSA, 2013b, EFSA, 2013c).

Queenless microcolonies, generally consisting of 3-5 workers bumble bees, can be used to study the effects of dietary pesticides on *Bombus terrestris* reproduction, under controlled laboratory conditions (Mommaerts et al., 2010, Laycock et al., 2012). When workers are kept in the absence of the queen, one or two will develop their ovaries over several days and lay unfertilised eggs which are capable of developing into drones, while the other workers care for the brood (Tasei et al., 2000). Several microcolony studies have investigated the effects of neonicotinoids on worker bumble bee reproduction. While thiamethoxam, the metabolic precursor of clothianidin, was shown to have no effect on reproduction at field realistic exposure concentrations (Laycock et al., 2014), imidacloprid at a concentration of 1 µg L^{-1} reduced brood production by a third (Laycock et al., 2012). The impacts and dose-dependence of clothianidin and cypermethrin have not previously been studied. In summary, the objectives of this investigation were: (1) to evaluate clothianidin and cypermethrin for time-reinforced toxicity in bumble bees; (2) to determine the effects of these insecticides on fecundity and trophic conversion efficiency of worker bumble bees in microcolonies.

3.2 Materials and methods

3.2.1 Microcolonies and dosing regime

Adult worker bumble bees (*Bombus terrestris* audax) were obtained from 2 commercial colonies (Biobest colony; Agralan Ltd., Swindon, U.K.) per experiment (6 colonies in total). Fifty queenless microcolonies (per experiment) consisting of 4 worker bumble bees were established in softwood boxes (120 x 120 x 45 mm) by randomly allocating workers from the same queenright colony (100 from each colony). Each microcolony was provided with 2 syrup feeders and maintained under conditions of: 22 - 27 °C, 30 - 55 % relative humidity, in darkness, except during data collection. All microcolonies were fed *ad libitum* on control syrup (Attraker: 1.27 kg L^{-1} fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) for 24 hours to acclimatise to experimental conditions. Any bees that died during this period were replaced with workers from their source colony. Following acclimatisation microcolonies were fed *ad libitum* on control syrup or syrup dosed with one of 9 pesticide concentrations spanning the field realistic range, for 28 days.

Clothianidin and cypermethrin (PESTANAL®, Sigma Aldrich Co. LLC; product codes: 37924 and 36128, respectively) were dissolved in water (clothianidin) and acetone (cypermethrin) to form stock solutions (10 mg L^{-1} and 1000 mg L^{-1}, respectively) before being combined with control syrup (manufacturer). Pesticide doses were as follows; clothianidin: 0.50, 1.28, 3.20, 5.60, 8.00, 14.00, 20.00, 30.00 and 40.00 µg L^{-1}, cypermethrin: 8.00, 20.00, 35.00, 50.00, 125.00, 312.50, 781.25, 1953.00 and 4882.80 µg L^{-1}. Field realistic concentrations of clothianidin and cypermethrin are here defined respectively as < 10ppb and < 40 ppb. Each microcolony was also provided with an undosed

pollen ball (pollen pellets obtained from Koppert, ground and mixed with water, mean mass = 4.767 g, S.E ± 0.071 g) for the 28 day exposure period. Pollen balls were weighed before and after they were placed in microcolonies while syrup feeders were weighed and replenished daily. Mean daily *per capita* consumption of pollen and syrup was then calculated. Feeding data was corrected for evaporation from both pollen and syrup using unoccupied microcolony domiciles. Where syrup was collected and stored in honey pots its mass at the end of the exposure was measured and subtracted from syrup consumption. Microcolonies were monitored daily for worker mortality and appearance of honey pots and wax-covered egg cells that indicated oviposition. Microcolonies were freeze-killed after 28 days of exposure and all eggs and larvae were collected from the nest, counted and weighed. Brood number was used as a measure of reproductive success.

3.2.2 Conversion efficiency

Trophic conversion efficiency is defined here as the rate by which an individual bumble bee worker is able to convert the mass of pollen and syrup (a proxy for nectar) that it ingests into 'reproductive' mass made up of both brood (eggs and larvae) and wax, which is used to build nest structures for brood rearing. Calculating this efficiency gives a percentage rate of conversion which can aid in determining the floral resources required for bumble bee colony success. If exposure to a pesticide lead solely to a reduction in pollen and syrup ingestion, we would not expect to find any changes to conversion efficiency. However, if conversion efficiency was reduced by pesticide exposure, this would suggest some other mechanism of toxicity at work or a cost from detoxification processes. Brood and wax nest structures were collected from freeze-killed microcolonies and the dry mass of each was calculated. The dry mass of pollen

ingested over the 28-day exposure period was also calculated. Samples were placed in a drying oven and heated at 65°C for a period of 3 days. Samples were weighed daily to ensure that they were completely dry.

3.2.3 Time-reinforced toxicity testing using Haber's Law

Cypermethrin and thiamethoxam, the parent compound of clothianidin, were tested for time-reinforced toxicity in bumble bees (*Bombus terrestris* audax) using a bioassay based on an analysis of mortality patterns using Haber's Law.

Cypermethrin and thiamethoxam (analytical standards, PESTANAL®, Sigma Aldrich Co. LLC; product codes: 36128 and 37924, respectively) were suspended in acetone (cypermethrin) and water (thiamethoxam) to form stock solutions before being combined with control syrup.

Bumble bees (Bombus terrestris audax) were obtained as commercial colonies (Biobest colony; Agralan Ltd., Swindon, U.K.). Workers were collected in equal number from 2 separate colonies (for each pesticide tested) and were caged individually (cage dimensions: 0.07 m x 0.07 m x 0.035 m) in wooden cages with the two largest faces covered with fine plastic mesh, and 10 individuals per dose. Caged bumble bees were maintained in a controlled laboratory environment (temperature between 22 and 26 °C; relative humidity between 30 and 54%; 12:12 hours of light:darkness) and were fed ad libitum on syrup containing cypermethrin (at 0.00, 0.13, 0.31, 0.78, 1.95, 4.88, 6.71, 8.54, 10.38, 12.21 mg L-1) or thiamethoxam (at 0.00, 3.20, 8.00, 20.00, 50.00, 125.00 172.25, 219.00, 265.75, 312.5 µg L-1. The LT50 (time to 50% mortality) for each dose group was recorded for bees exposed to both thiamethoxam and cypermethrin.

3.2.4 Statistical analysis

We determined the dose-dependence of brood number, consumption of syrup and pollen and trophic conversion efficiency (cypermethrin only) using Generalised Additive Modelling (GAM) to include differences between original colonies. The smoothed independent variable in each model was *concentration* (dietary concentration of clothianidin or cypermethrin in µg L^{-1}) and *colony* (the original colony that the bees were sourced from) was treated as a categorical independent variable. The interaction between these variables was included in the initial models prior to simplification, which was carried out using a backwards step-wise approach. The best model fit was determined by comparison of Akaike's Information Criterion (AIC). Where a linear response was indicated by GAM a Generalised Linear Model was used instead and simplified as stated previously. To determine the effects of dietary clothianidin on trophic conversion efficiency we used one-way ANOVA (Analysis of Variance). To calculate the probability that all zero-values for conversion efficiency were found in the highest two doses we used hypergeometric distribution. Analysis of variation in survival was carried out using Cox's proportional hazards analysis, treating *dose* and *box number* as independent variables, with *survival* treated as a censored dependent variable. Simplification of the survival model was carried out by backwards step-wise analysis. Time-reinforced toxicity was tested for using linear regression to determine the value of *b*. All statistical analyses were conducted in R version 3.0.2 (R Core Team, 2013).

3.3 Results

3.3.1 Syrup and pollen consumption

Syrup and pollen consumption per capita were both significantly reduced by clothianidin exposure (GLM, df = 49, P < 0.001, **Figure 3.1 A & C**). Total ingestion of clothianidin increased with dose (ANOVA, F9,40 = 17.02, P < 0.001) until a decrease in syrup consumption constrains it at the highest doses (**Figure 3.2 A**). In contrast, exposure to cypermethrin had no effect on syrup consumption (GLM, df = 49, P = 0.95; **Figure 3.1 B**), with total intake of cypermethrin increasing with dose (ANOVA, F9,40 = 32.15, P < 0.001; **Figure 3.2 B**), though pollen consumption declined with dose (GLM, df = 49, P < 0.05; **Figure 3.1 D**).

3.3.2 Fecundity

Brood production (number of laid eggs and larvae) showed a significant dose-dependent reduction after dietary exposure to clothianidin (GAM; edf = 1.925, χ^2 = 37.26, P < 0.001; **Figure 3.3 A**), though not in the field-realistic concentration range. There was no effect of cypermethrin exposure on brood number (GAM; edf = 1.408, χ^2 = 4.267, P > 0.08; **Figure 3.3 B**).

3.3.3 Trophic conversion efficiency

The average trophic conversion efficiencies of microcolonies exposed to clothianidin (excluding the highest two doses) and cypermethrin were 15.7% (± 1.5 S.E.) and 8.9 % (± 0.7 S.E.), respectively. However, exposure to clothianidin significantly reduced the conversion efficiency of microcolonies (ANOVA; $F_{9,40}$ = 4.803, P < 0.001). We calculated the likelihood that all 8 zero-conversion efficiency microcolonies were found in the highest two clothianidin doses using the hypergeometric distribution:

$$\mathrm{P\ (k\ in\ n\ draws)} = \frac{\binom{K}{k}\binom{N-K}{n-k}}{\binom{N}{n}}$$

where N = 50 objects, K = 8 possible successes, n = 10 draws, and k = 8 picked successes. We found P = 8.38 x 10^{-8} and therefore highly significant (**Figure 3.3 C**). However, no effect of clothianidin was observed at doses within the field realistic range (≤ 10 ppb). Conversion efficiency was not significantly affected by dietary cypermethrin exposure (GAM; edf = 1.879, F = 0.956, P > 0.05, **Figure 3.3 D**).

3.3.4 Time-reinforced toxicity

Cypermethrin and thiamethoxam both acted without time-reinforcement (linear regression; *b* = 0.27 ± 0.051 and *b* = 0.31 ± 0.181, respectively; **Figure 3.4 A & B**). A pesticide that exhibits TRT would be expected to have a value of b > 1, the fact that the values for cypermethrin and thiamethoxam are far below this threshold indicates that these pesticides may be readily detoxified before reaching their target tissues. This is also indicated by senescence, rather than pesticide toxicity, being the main cause of bee mortality in lower doses.

3.3.5 Longevity

Longevity was significantly reduced with increasing concentrations of both clothianidin and cypermethrin (Cox's analysis: *clothianidin*, *z* = 8.220, P < 0.001; *cypermethrin*, *z* = 3.710, P < 0.001; **Figure 3.5 A & B**), however this was again only observed at the highest pesticide doses and not within the field realistic range of either pesticide.

3.4 Discussion

The acute oral lethal dose causing 50% mortality (LD_{50}) of clothianidin is 3.79 ng honey bee^{-1} (European Commission, 2005). However, all the bees that we exposed to field realistic doses ingested more than the LD50 of clothianidin, with bees at the top end of the range (8 µg L^{-1}) ingesting 82.16 ng each on average (SEM ± 4.74 ng), more than 20 times greater than the LD_{50}. This was also the case for cypermethrin, which has an oral LD_{50} of 15 ng bee^{-1} (FAO, 2007). As with clothianidin, all bees exposed to field realistic dietary residues ingested a greater amount of cypermethrin than the LD_{50}, with bees at the high end of the field realistic range (50 µg L^{-1}) again ingesting more than 20 times the LD_{50} (mean = 329 ng/bee; SEM ± 33.45 ng). The high amounts of these pesticides ingested were not only non-lethal but even had no effect (within the field realistic range) on fecundity, a known sensitive endpoint (Laycock et al., 2012).

The lack of toxic effects observed in these microcolonies can potentially be explained by the lack of time-reinforced toxicity exhibited by cypermethrin and thiamethoxam (the precursor to clothianidin) in bumble bees (**Figure 3.3**). Both insecticides induced disproportionately low mortality for the concentrations ingested. This may be due to these pesticides being quickly detoxified and so not accumulating over time within bees. These pesticides elicit a lesser effect than predicted by Haber's Law, which implies that they are rapidly metabolised or eliminated before injury is sustained. Cypermethrin is known to be readily detoxified via mono-oxygenases and glutathione-S-transferase which are present in the bee gut (Little et al., 1989, Fragoso et al., 2003). Thiamethoxam, and so clothianidin, is metabolised by cytochrome P450s and aldehyde oxidase enzymes to less-toxic metabolites (Casida, 2011, Honda et al., 2006).

Imidacloprid, another neonicotinoid pesticide, has also been shown to be quickly eliminated by bees (Cresswell et al., 2013).

Neither clothianidin nor cypermethrin caused any observed sublethal effects at concentrations within the field realistic range (< 10ppb and < 100 ppb, respectively). Comparisons of the sensitivity of toxicity endpoints in bees have shown that reproduction is a more sensitive measure of toxicity than other endpoints, such as feeding or mortality (Laycock et al., 2012). Therefore, a lack of toxic effect on reproduction suggests that there may also be no effect on other sublethal endpoints. Our results are consistent with those of several other studies. Schneider et al. (2012) found that exposure to clothianidin at < 0.5 ng bee^{-1} had no effect on honey bee foraging ability, while no long term effects were observed in honey bee colonies foraging on clothianidin-treated oilseed rape (Cutler and Scott-Dupree, 2007). A study by Franklin et al. (2004) found no effect of clothianidin exposure at either 6 or 36 ppb on the health or foraging ability of *Bombus impatiens* colonies maintained under laboratory conditions. However, in our study bumble bee reproduction did show some dose-dependence as brood number was significantly reduced or completely absent at the highest doses of clothianidin, though these were far in excess of field realistic residues. The reduction in reproduction observed in these microcolonies was mirrored by a reduction in both syrup and pollen consumption. These results suggest that oviposition may have been impacted by reduced feeding, as pollen consumption is necessary for egg production and larval growth (Plowright and Pendrel, 1977). The observed reduction in reproduction could also be due to disruption of ovary development by exposure to the pesticides, however this was not studied. In a previous study, microcolonies exposed to thiamethoxam were also unaffected at field realistic

concentrations (Laycock et al., 2014) however brood production was reduced by a third in microcolonies exposed to just 1 µg L^{-1} of imidacloprid (Laycock et al., 2012). The disparity between these results from pesticides within the same chemical family and with similar short-term toxicity to bees highlights the importance of testing all pesticides individually. Our results suggest that there is not necessarily a close resemblance among members of the neonicotinoid family when considering dose-dependence on bumble bees in the environmentally relevant range. Cypermethrin had no effect on the endpoints tested, highlighting its relatively low toxicity to bees.

From our results and other studies, there is good evidence of high rates of detoxification within bumble bees, which probably allow them to cope with some farmland dietary pesticide residues. However, this high rate of detoxification is vulnerable to potential interactions, including synergies, with other agrochemicals that act as detoxification enzyme inhibitors. The pyrethroids have been shown to be synergised by certain fungicides which inhibit cytochrome P450s. For example, there is evidence of synergy between neonicotinoid insecticides and ergosterol biosynthesis inhibiting (EBI) fungicides (Thompson et al 2014). Deltamethrin is synergised by prochloraz, a widely-used imidazole fungicide applied as a foliar spray (Colin & Belzunces 2006). Some fungicides have also been shown to reduce the repellency of pyrethroids, therefore increasing the risk of exposure (Thompson & Wilkins 2003).

Other studies have, however, found effects of various insecticides on *Bombus terrestris* reproduction and colony success. Field-realistic concentrations of imidacloprid (< 10 ppb) were found to significantly reduce colony growth and lead to an 85% reduction in the number of new queens produced by colonies

under field conditions (Whitehorn et al., 2012), which could be due reduced foraging efficiency of workers (Feltham et al., 2014). While Gill et al. (2012) found that combined exposure to a neonicotinoid (imidacloprid) and a pyrethroid (λ-cyhalothrin) under semi-field conditions impaired foraging behaviour and increased worker mortality, which also lead to reduced brood production and colony success. Exposure to field-realistic concentrations of the neonicotinoid thiamethoxam also impaired foraging behaviour, leading to reduced pollination services (Stanley et al., 2015). No effects on colony health were observed when bumble bee colonies foraged on neonicotinoid-treated oilseed rape crops (Thompson et al., 2013). However, Rundlof et al. (2015) found that exposure to oilseed rape seed-treated with clothianidin and β-cyfluthrin (pyrethroid) reduced both bumble bee colony growth and reproductive success under field conditions. This indicates that a combined pesticide exposure may pose more of a threat to bumble bees than exposure to a single pesticide.

The efficiency of bumble bees in converting food (nectar and pollen) into reproductive output has not been reported previously. Here we found bees had a mean conversion efficiency of approximately 15 %. Conversion efficiency is vital to reproductive success and a reduction in efficiency could impact on nest building and brood production. We found that there was no dose-dependency of conversion efficiency to clothianidin or cypermethrin exposure in the environmentally relevant range. This information is important for studies aiming to model bumble bee colony success in agricultural landscapes, such as that by (Crone and Williams, 2016), as it can be used to determine the required pollen and nectar per unit area for colonies to succeed. Based on a combined average conversion efficiency of 12.3%, we calculate that 1 kg of sugar / ha /

month will lead to bumble bee abundance of 3796 bees/ha, or 15 colonies (assuming 250 bees / colony/ month)

Conclusions

Our results do not suggest that clothianidin and cypermethrin pose a threat to bees when exposed to field realistic doses as assessed in a laboratory environment. These results indicate that these pesticides may not impact on the reproduction of wild bees, although this does not rule out other modes of harm to which bees are prone outside the laboratory, such as effects on homing behaviour, or other routes of exposure that might deliver higher concentrations than we studied here, such as by direct contact. These pesticides may also have effects on other endpoints, such as foraging and navigation, that were not investigated in our study, and which could also affect colony success. There is also the potential for synergism between these pesticides and P450 inhibitors. Therefore, careful consideration is still needed for their continued use.

This microcolony method would be a useful addition to risk assessment protocols of pesticides to bees as these currently focus on more short-term exposures and lethal endpoints. The test for TRT also uses simple toxicological data which can be produced from existing risk assessment protocols. It is important to investigate the dose-dependence of a range of endpoints so that effects at different exposure levels can be determined, as the current understanding of the level to which bees are exposed to pesticides is limited. Testing should be as field relevant as possible, involving field realistic dosing and demographically relevant endpoints.

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Author Contributions As first author I carried out the lab work and statistical analysis, co-designed the study and co-wrote the manuscript; Charles Tyler helped in the methods and co-wrote the manuscript; James Cresswell co-designed the study and co-wrote the manuscript.

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Chapter Three: Figures

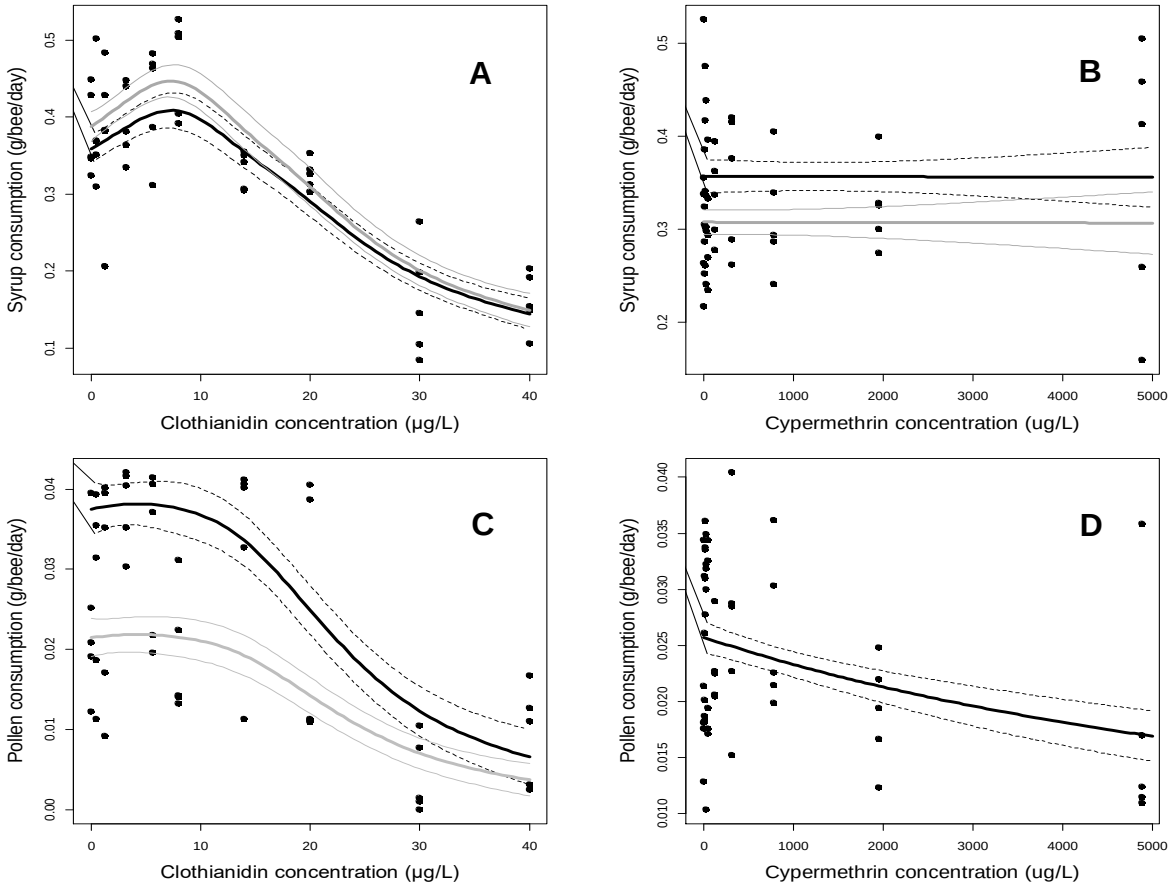

Figure 3.1 Syrup and pollen consumption of *Bombus terrestris* workers in microcolonies exposed to pesticides for 28 days. The relationship (modelled with GAMs) between: syrup consumption (*y*-axis; mean daily *per capita* consumption of syrup in g per bee) and dietary pesticide concentration (*x*-axis; concentration of pesticide in syrup in µg L^{-1}) of bumble bee workers exposed to (**A**) clothianidin (0 to 40 µg L^{-1}, N = 5) and (**B**) cypermethrin (0 to 4882.8 µg L^{-1}, N = 5); and pollen consumption (*y*-axis; daily *per capita* consumption of undosed pollen in g per bee) of bumble bee workers exposed to either (**C**) clothianidin (0 to 40 µg L^{-1}, N = 5) or (**D**) cypermethrin (0 to 4882.8 µg L^{-1}, N = 5); over a 28-day exposure period. Black line indicates the modelled response of microcolonies originating from colony A and the grey line from colony B. The 95% confidence intervals are shown.

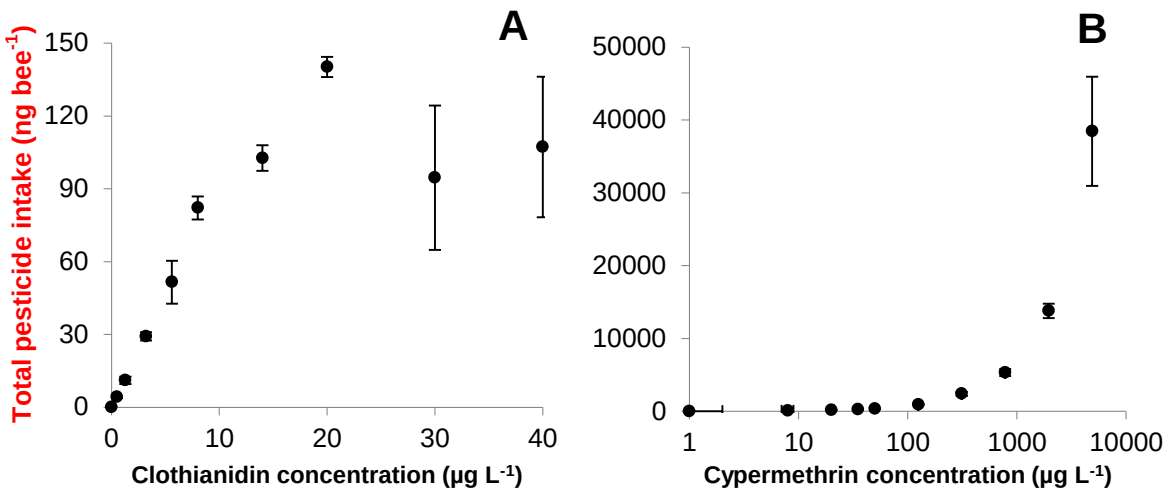

Figure 3.2 Total pesticide intake of bumble bees exposed to clothianidin and cypermethrin over a 28-day period. The relationship between dietary concentration (*x*-axis; concentration of dietary pesticide in syrup in µg L^{-1}) and **total pesticide load** (*y*-axis; mean total amount of pesticide ingested per bee in ng) of worker bumble bees exposed to **(A)** clothianidin (0 to 40 µg L^{-1}, N = 5) and **(B)** cypermethrin (0.0 to 4882.8 µg L^{-1}, N = 5) **over a 28-day period.** Error bars denoted S.E. and may be obscured by some data points.

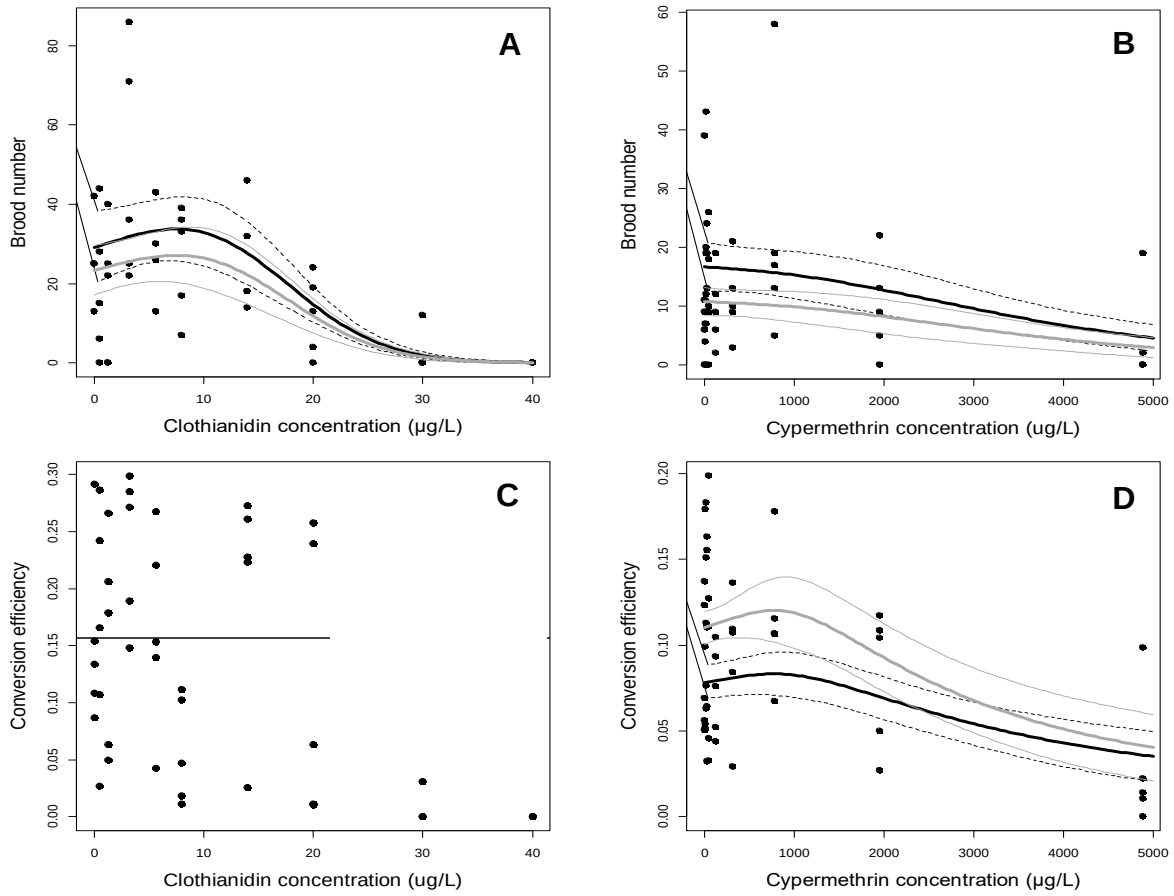

Figure 3.3 Brood production and conversion efficiency of *Bombus terrestris* workers in microcolonies exposed to pesticides for 28 days. Generalised additive models of the relationship between: brood number (*y*-axis; number of eggs and larvae produced per microcolony) and dietary pesticide concentration (*x*-axis; concentration of pesticide in syrup in µg L^{-1}) of bumble bee workers exposed to (**A**) clothianidin (0.0 to 40.0 µg L^{-1}, N = 5) and (**B**) cypermethrin (0.0 to 4882.8 µg L^{-1}, N = 5); conversion efficiency (*y*-axis; conversion efficiency of the dry weight of dietary inputs (pollen and syrup) consumed into reproductive output (dry weight of brood and wax produced) per microcolony) and dietary concentration (*x*-axis; concentration of pesticide in syrup in µg L^{-1}) of bumble bee workers exposed to (**C**) clothianidin (0.0 to 40.0 µg L^{-1}, N = 5), horizontal line indicates mean conversion efficiency of microcolonies (excluding highest two doses) and (**D**) cypermethrin (0.0 to 4882.8 µg L^{-1}, N = 5). Black line indicates the modelled response of microcolonies originating from colony A and the grey line from colony B. The 95% confidence intervals are shown.

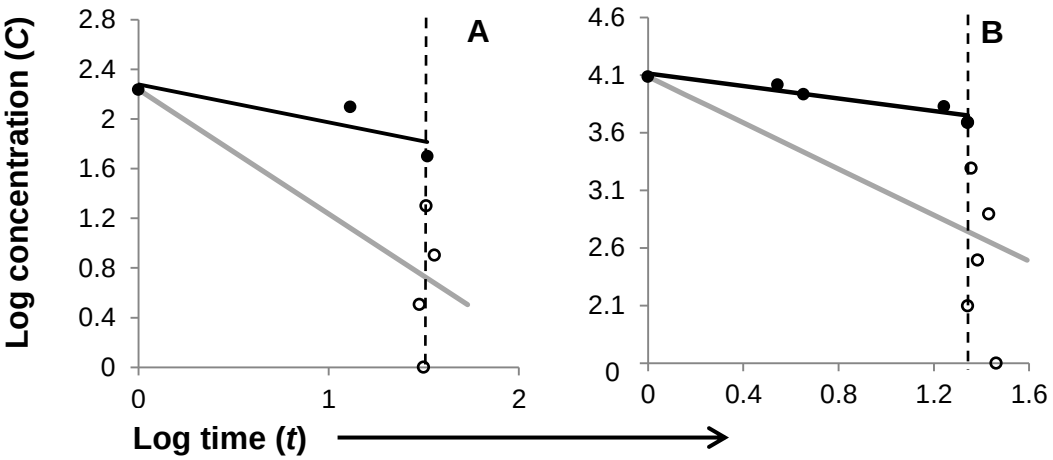

Figure 3.4 Two pesticides, thiamethoxam and cypermethrin, screened for time-reinforced toxicity using a bioassay based on Haber's Law. Logarithmic relationship between dietary concentration (*y*-axis; log 10 transformation of concentration of test pesticide in syrup in µg L^{-1}) and time to 50% mortality (LT_{50}) (*x*-axis; log 10 transformation of time to 50% mortality (LT_{50}) for each treatment group) for bumble bee workers exposed to **(A)** thiamethoxam (0.00 to 312.5 µg L^{-1}, N = 10) and **(B)** cypermethrin (0.00 to 12.21 mg L^{-1}, N = 10). Only concentrations shown to have a toxic effect have been included in the linear regression calculation (filled symbols). Open symbols indicate excluded data where observed longevity was attributed to senescence. The dashed line indicates the point at which mortality is due to senescence. The grey lines indicate the expected results of a Haberian steady-state relationship, where $b = 1$.

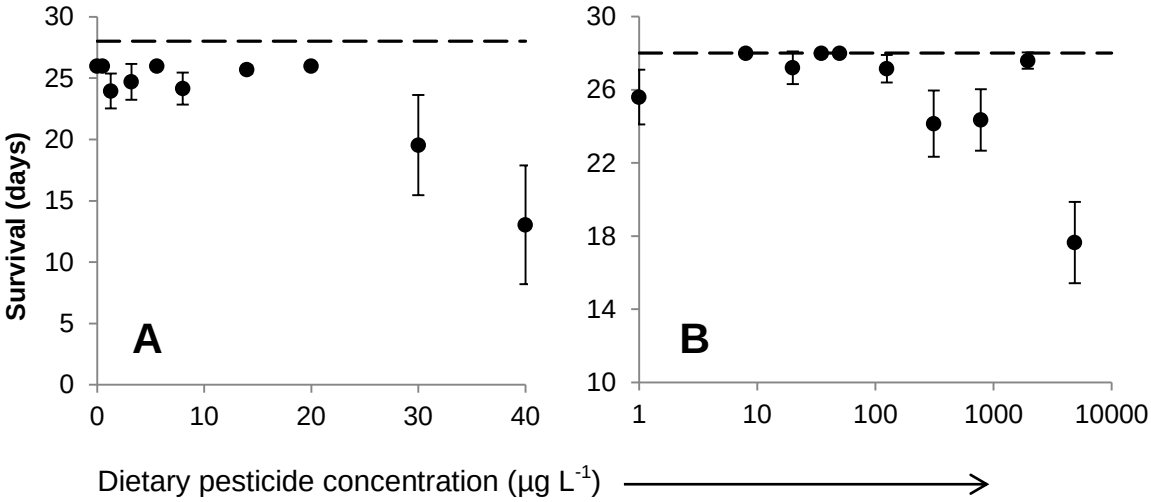

Figure 3.5 Effect of clothianidin and cypermethrin exposure on bumble bee worker survival. The relationship between dietary concentration (*x*-axis; concentration of dietary pesticide in syrup in µg L^{-1}) and survival (y-axis; mean survival in days; maximum = 28 days) of worker bumble bees exposed to **(A)** clothianidin (0 to 40 µg L^{-1}, N = 5) and **(B)** cypermethrin (0.0 to 4882.8 µg L^{-1}, N = 5). Dashed lines indicate the maximum survival time (28 days). Error bars denoted S.E. and may be obscured by some data points.

Chapter Four:

Effects of fipronil, a persistent pesticide, on bumble bees: comparative sensitivity of enclosed and free-flying test paradigms

Effects of fipronil, a persistent pesticide, on bumble bees: comparative sensitivity of enclosed and free-flying test paradigms

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Abstract

Fipronil is a widely-used pesticide that is currently restricted from use on bee-attractive crops in the European Union due to concerns about its effects on bee health (EFSA, 2013f). It is found in very low levels as residues in the nectar and pollen of treated mass-flowering crops on which bees feed (Chauzat et al., 2011, Mullin et al., 2010). These residues could not cause bee mortality unless fipronil exhibits time-reinforced toxicity, leading to increasing internal residues (and associated injury) with time. It is not known whether fipronil exhibits time-reinforced toxicity in bumble bees and effects on reproduction and colony growth have not previously been studied. We therefore used a bioassay based on Haber's Law to determine whether fipronil exhibits time-reinforced toxicity in individual *Bombus terrestris* workers. We also exposed two groups of microcolonies to dietary fipronil and evaluated the dose-dependence of a range of endpoints, including mortality, reproduction and feeding. The first microcolony group were maintained under enclosed laboratory conditions while the second group were free-flying in semi-field conditions.

We found that exposure to fipronil concentrations as low as 0.5 µg L^{-1} for 28 days significantly reduced fecundity of worker bumble bees in queenless microcolonies. Microcolonies exposed to fipronil exhibited increased larval mortality, and when allowed to forage freely workers were rapidly lost or deceased. Additionally, we show that fipronil exhibits time-reinforced toxicity in bumble bees, which significantly reduces individual longevity and reproductive success in environmentally realistic exposures. These results indicate that fipronil poses a potential threat to bees exposed to residues in the environment, and previous research using sedentary bees under laboratory conditions may

be underestimating the negative impacts that this pesticide, and others, have on bees.

4.1 Introduction

Fipronil and GABA receptors

Fipronil, a phenylpyrazole insecticide, is a potent neurotoxin which acts as a non-competitive antagonist of the γ-aminobutyric acid-gated (GABA) type A receptor on insect postsynaptic membranes, as does its equally toxic and more persistent metabolite fipronil sulfone (Hainzl et al., 1998, Law and Lightstone, 2008, Ratra et al., 2001). Fipronil is oxidised to fipronil sulfone by the actions of P450 monoxygenases and esterase in insects (Hainzl et al., 1998, Tang et al., 2009). Cytochrome P450s have also been shown to be important in resistance to fipronil by other insect species, including cockroaches and house flies (Gondhalekar and Scharf, 2012, Liu and Yue, 2000). Both compounds show high specificity for insect $GABA_A$ receptors, causing increased depolarisations which can lead to paralysis and death (Cole et al., 1993). $GABA_A$ receptors are found on the membranes of muscle cells and are important for locomotor and flight activity (Usherwood and Grundfest, 1965), therefore the actions of fipronil on insect receptors could seriously impact on bee foraging ability. In agriculture, fipronil is conventionally used as a systemic insecticide, applied as a seed dressing on a range of crops including sunflower and maize (Aubert et al., 2006). It permeates the plant's tissues, protecting against biting and sucking pests. However, it can also be present in the nectar and pollen of treated flowering crops on which bees feed. Fipronil residues have been found in honey

bee colonies and collected pollen at sublethal concentrations of 0.4 to 28.5 µg L^{-1}, though most residues are ≤ 3 µg L^{-1} (Chauzat et al., 2011, Mullin et al., 2010, Stoner and Eitzer, 2013). Fipronil has been classed as highly toxic to bees (EFSA, 2006).

Concerns

There is limited knowledge of the sublethal effects of fipronil on bees, with no studies focusing on wild bee species. Honey bee studies have found that fipronil has a range of negative sublethal effects; including on behaviour, foraging activity and memory and learning (Aliouane et al., 2009, Colin et al., 2004, Decourtye et al., 2011, El Hassani et al., 2005). Fipronil has also been shown to inhibit honey bee mitochondrial activity and increase mortality in *Nosema ceranae*-infected bees (Nicodemo et al., 2014, Vidau et al., 2011).

Fipronil has been found to bioaccumulate in vertebrates, including rats and frogs (Cravedi et al., 2013, Reynaud et al., 2012), however its action in bumble bees has previously been unknown. It is thought that fipronil binds with limited reversibility to $GABA_A$ receptors as an allosteric modulator, altering the main binding site structure and so blocking the binding of other ligands (Law and Lightstone, 2008), which may explain its bioaccumulation. The interaction between fipronil (and its sulfone metabolite) and GABA receptors is also poorly reversible (Cole et al., 1993). Bioaccumulative pesticides can exhibit “time-reinforced toxicity” (Tennekes and Sanchez-Bayo, 2011), whereby the internal concentration of the pesticide increases over time so that the injury it causes increases exponentially over the exposure duration. If fipronil exhibits time-reinforced toxicity in bees it could pose a threat to bees foraging on fipronil-

treated crops, because small, sublethal residues could build up over time to cause major toxic effects.

Haber's Law bioassay

Haber's Law, a 'constant product' rule, has long been used in toxicology risk assessments to determine safe levels of human exposure to toxicants (Gaylor, 2000). Here, we use a bioassay based on a modified version of Haber's Law, known as the Druckrey-Küpfmüller equation (Tennekes, 2010) to determine whether fipronil exhibits time-reinforced toxicity in bumble bees.

Haber's Law models a non-bioaccumulative toxicant that binds reversibly to its target site and that is susceptible to metabolism and/or elimination. During a sustained dietary exposure, a 'steady state' concentration inside the organism will be established by the continuous and opposing actions of ingestion and elimination. Therefore, the daily rate of injury is constant and the accumulated total injury is proportional to the exposure duration. This proportionality means that toxicological experiments on such a system will find that halving the dosage rate doubles the duration of the exposure that is required to achieve a given level of injury or effect. Toxicants with these properties will produce the specified injurious effect from any exposure whose dosage-duration combination conforms to the 'constant product' rule known as Haber's Law (Rozman, 2000):

$$Ct^b = k \qquad \text{Eq. 1}$$

where C denotes the dietary concentration of the toxicant, t denotes the exposure duration and the exponent takes the value b =1, which reflects the

proportionality relationship. If instead the toxicant bioaccumulates, the internal concentration within an organism rises as exposure continues. Subsequently, the rate of injury increases and the accumulated total injury increases exponentially. In this case, the exponent in Eq 1 takes the value $b > 1$. Where this is the case, halving the dosage will require less than double the duration of the exposure to achieve the given injury. Therefore the value of exponent *b* will be greater than 1 for these TRT toxicants. Consequently, it is straightforward to test for TRT by evaluating *b* using data from 'time-to-effect' experiments that quantify the durations of exposure required to produce a specified level of injury in experimental subjects exposed to various dosages. TRT can be tested for by using this data to derive the *t-vs.-C* relationship and determine its slope on logarithmic axes, which estimates parameter *b* (Eq 1) because:

$$\log(C) = -b[\log(t)] + \log(k)$$

Sublethal effects

Fipronil is highly toxic to bees, with an LC_{50} of 160 ppb (parts per billion) (EFSA, 2006). Residues in nectar and pollen however are found at much lower concentrations. Consequently there have been a number of studies to determine the sublethal impacts of fipronil to bees. Fipronil has been shown to negatively affect honey bee learning and behaviour, reducing locomotory activity, olfactory learning ability and foraging efficiency (Colin et al., 2004, Decourtye et al., 2011, Aliouane et al., 2009, El Hassani et al., 2005). However, the impacts of fipronil exposure on wild bees have not been assessed. In wild populations, fecundity is a demographically important endpoint to investigate as any reduction in fecundity would directly impact successful growth and

reproduction of colonies and, consequently, the sustainability of the population.. This is especially the case for bumble bees, due to their life history. Typically only large bumble bee colonies will produce the new queens required to found new colonies the following spring (Owen et al., 1980, Müller and Schmid-Hempel, 1992). Consequently colony success hinges on the fecundity of a foundress queen, which will produce workers in the short term and eventually reproductives at the end of the season. Therefore we investigated the impacts of dietary exposure of field realistic residues of fipronil on the fecundity of bumble bees. As a laboratory proxy, we studied the fecundity of bumble bee worker microcolonies. Previous studies (Laycock et al., 2012, Laycock and Cresswell, 2013) have found close correspondence between toxic effects on fecundity in queen-right colonies vs. orphaned microcolonies.

Enclosed to free-flying

A much-debated topic in scientific research is how translatable results from enclosed laboratory-based studies are to free-flying situations. Some effects of pesticides observed under laboratory conditions are not observed when translated to field exposures (Blacquiere et al., 2012). For example, several studies have found that the negative impacts of pesticides on bumble bees were exacerbated when bees were made to fly to a feeder in a greenhouse compared to being enclosed under laboratory conditions (Mommaerts et al., 2010, Ceuppens et al., 2015). To determine whether the effects of dietary fipronil exposure on the fecundity and longevity of bumble bees are increased with activity we also exposed established microcolonies to fipronil prior to placing outside to forage freely. We compared the results with those of

microcolonies maintain under laboratory conditions where bumble bees were unable to fly.

4.2 Materials and methods

4.2.1 Preparation of chemicals

Fipronil (PESTANAL®, Sigma Aldrich Co. LLC; product code: 46451) was suspended in acetone to form a stock solutions (10^4 µg L^{-1}) before being combined with control syrup.

4.2.2 Time-reinforced toxicity testing

Bumble bees (*Bombus terrestris* audax) were obtained from commercial suppliers (Biobest colony; Agralan Ltd., Swindon, U.K.). Workers were collected in equal numbers from 2 separate colonies and were caged individually (cage dimensions: 0.07 m x 0.07 m x 0.035 m) in wooden cages with the two largest faces of the cages covered with fine plastic mesh, and with 10 individuals per dose. Caged bumble bees were maintained in a semi-controlled laboratory environment with temperature between 21.2 and 27.8 °C, relative humidity between 20 and 56%, 12:12 hours of light:darkness, and were fed *ad libitum* on syrup containing fipronil at concentrations of 0.0, 3.2, 8.0, 20.0, 87.5 or 125 µg L^{-1}, which spans the field realistic range (< 10 ppb (Mullin et al., 2010, EFSA, 2012, Chauzat et al., 2011)). Bees were monitored daily for mortality and the number of days to reach 50% mortality (LT_{50}) in each dose group was recorded.

The time-reinforced toxicity of fipronil was tested using time to 50% mortality in the dosage groups of individual bumble bees (*Bombus terrestris* audax) using a bioassay based on Haber's Law, as previously described in Chapters 2 and 3.

4.2.3 Enclosed queenless microcolonies

Adult worker bumble bees (*Bombus terrestris* audax) were obtained from 2 commercial colonies (Biobest colony; Agralan Ltd., Swindon, U.K.) per experiment (6 colonies in total). Fifty queenless microcolonies (per experiment) consisting of 4 worker bumble bees were established in softwood boxes (120 x 120 x 45 mm) by randomly allocating workers from the same queenright colony (100 from each colony). Each microcolony was provided with 2 syrup feeders and maintained under conditions of 20 - 26 °C, 30 - 54 % relative humidity and kept in darkness except during data collection. All microcolonies were fed *ad libitum* on control syrup (Attraker: 1.27 kg L^{-1} fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) for 24 hours to acclimatise to the experimental conditions. Any bees that died during this period were replaced with workers from their source colony. Following acclimatisation microcolonies were fed *ad libitum* on control syrup or syrup dosed with one of 4 pesticide concentrations for 28 days. Fipronil doses were as follows: 0.08, 0.2, 0.5 and 1.28 µg L^{-1}. Each microcolony was also provided with an undosed pollen ball (pollen pellets obtained from Koppert, ground and mixed with water, mean mass = 5.46 g, S.E ± 0.038 g) for the 28 day exposure period. Pollen balls were weighed before and after they were placed in microcolonies while syrup feeders were weighed daily to allow pollen and syrup consumption to be calculated. Feeding data was corrected for evaporation from both pollen and syrup. Where syrup was collected and stored in honey pots its mass at the end of the experiment was measured and subtracted from syrup consumption. Microcolonies were also monitored daily for worker mortality and appearance of honey pots and wax covered egg cells that indicated oviposition. Microcolonies

were freeze-killed after 28 days of exposure and all eggs and larvae were collected from the nest and weighed.

4.2.4 Queenless microcolonies under free-flying conditions

4.2.4.1 Preparation of chemicals

Fipronil stock (10^4 µg L^{-1}) was mixed with control syrup to a concentration of 125 µg L^{-1}, this mixture was then diluted further with control syrup to make final fipronil concentrations of 1.28 µg L^{-1} /1 ppb (parts per billion) and 2.56 µg L^{-1} / 2 ppb (0.025% acetone maximum).

4.2.4.2 Microcolonies

Twenty one queenless microcolonies were set up detailed above and maintained under semi-controlled conditions in darkness (except during data collection) for a period of 35 days. Each microcolony was provided with two syrup feeders and fed *ad libitum* on control syrup (Attraker: 1.27 kg L^{-1} fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) for 28 days to allow for the development of nest structures and brood. Syrup feeders were weighed daily and consumption of undosed syrup was calculated, correcting for evaporation. Each microcolony was also provided with an undosed pollen ball (pollen pellets obtained from Koppert, ground and mixed with water). After a period of 28 days microcolonies were fed *ad libitum* on control syrup (0.025% acetone) or syrup containing fipronil at a concentration of 1 or 2 ppb (7 microcolonies per dose). Microcolonies were maintained on dose in the laboratory for one week prior to being placed outside to forage freely for two weeks without further syrup provided.

4.2.5 Statistical analysis

All statistical analysis was carried out using R version 3.0.2 (R Core Team, 2013). A Generalised Linear Model (GLM) with a negative binomial error structure was used to determine the effect of fipronil on the number of brood in both enclosed and free-flying microcolonies, with *concentration* as the independent variable and *brood number* as the dependant variable. The effect of fipronil exposure on the proportion of dead larvae present in free-flying microcolonies was determined using a Generalised Linear Mixed Model (GLMM) with binomial error structure, *concentration* as the independent variable and *microcolony number* as a random factor.

4.3 Results

4.3.1 Time-reinforced toxicity

Fipronil was found to exhibit time-reinforced toxicity on longevity in individually caged bumble bees (b = 2.18 ± 0.057 S.E.; **Figure 4.1**). This b-value is very close to the value expected of an ideal bioaccumulative toxicant (b = 2), as described in Chapter 2. It was also highly toxic to bumble bees with a 48 hour LC_{50} value of 85.62 µg L^{-1} (Probit analysis, ± 12.99 S.E.).

4.3.2 Effects on enclosed queenless microcolonies

4.3.2.1 Fecundity

Exposure to dietary fipronil significantly reduced the number of brood (eggs and larvae) produced by microcolonies over the 28-day study period (GLM; df = 49, z = -4.192, P < 0.001; **Figure 4.2 A**). There was no difference among dose

groups in the number of days elapsed before initial oviposition (mean = 9.37, S.E. ± 1.04; ANOVA: $F_{4,25} = 0.41$, P = 0.80).

4.3.2.2 Longevity

Worker bumble bee longevity was significantly reduced with increasing fipronil dose (Cox's proportional hazards model: coefficient = 0.5854, *z* = 4.196, P < 0.001; **Figure 4.2 B**) with undosed bees living on average 3 days longer than those exposed to fipronil at the highest dose.

4.3.2.3 Food consumption and fipronil intake

Exposure to dietary fipronil caused a significant decrease in the *per capita* daily syrup and pollen consumption of worker bumble bees (ANOVA; *syrup*: $F_{4,39} = 18.84$, P < 0.001; *pollen*: $F_{4,44} = 6.681$, P < 0.001: **Figure 4.2 C & D**). All bees still ate, indicating that observed reductions in longevity were not due to starvation. Syrup consumption was reduced by fipronil at concentrations of 0.5 µg L^{-1} and above (Tukey's pair-wise comparison: P < 0.001), however, the amount of fipronil ingested by individual workers each day still increased significantly with dietary concentration (ANOVA, $F_{4,45} = 118.7$, P < 0.001). Pollen consumption was only reduced at 1.28 µg L^{-1} of fipronil (Tukey's pair-wise comparison: P < 0.001).

4.3.3 Queenless microcolonies under free-flying conditions

4.3.3.1 Brood production

Brood number increased with fipronil concentration (GLM; df = 20, z = 2.139, P < 0.04). However, the proportion of dead larvae present in microcolonies also significantly increased with fipronil concentration (GLMM; z = 3.668, P < 0.001, **Figure 4.3 A**), indicating that although brood production was not affected,

survival of brood was, either due to direct toxicity of fipronil in the larval diet or from poor brood care by intoxicated workers.

4.3.3.2 Worker mortality

There was no worker mortality during the 8-day dosing period under enclosed laboratory conditions. However, once microcolonies were placed outside, high mortality was immediately observed in dosed microcolonies compared to undosed microcolonies (ANOVA; $F_{2,18} = 15.47$, $P < 0.001$; **Figure 4.3 B**). This was due to the observed inability of any dosed bees to fly or return to the nest.

4.4 Discussion

Fipronil exhibits TRT in bumble bees

Fipronil has been suspected of bioaccumulative properties from several studies on a range of different organisms (Cravedi et al., 2013, Reynaud et al., 2012). Here, we have shown that fipronil exhibits time-reinforced toxicity in exposed bumble bees, leading to disproportionately high mortality given the exposure duration. Fipronil can be found in the nectar and pollen of treated crops and in bee-collected pollen at concentrations generally between 0.1 and 100 ppb, however the majority of residues detected are below 3 ppb (Bonmatin et al., 2015). The LD_{50} for honey bees exposed to fipronil is between 4 and 6.2 ng per bee in a 48 h exposure. In our TRT experiment, bees fed on field-realistic concentrations of fipronil (1.28 and 3.2 µg L^{-1}) only ingested an average of 0.70 and 1.1 ng, respectively, by the time 50% mortality (LT_{50}) occurred, even though the exposure duration was greater than 48 hours. These results highlight the relationship between the impacts of TRT pesticides over prolonged exposures.

The amounts ingested are only a small fraction of the LD_{50} and so would be assumed to be within safe limits of exposure. However, exposure to just 1 ppb (1.28 µg L^{-1}) of fipronil reduced the longevity of individual bumble bee workers by almost 10 days. The observed enhancement of the toxicological effect could be due to fipronil itself or its main metabolite, fipronil sulfone, which is equally toxic (Caboni et al., 2003). Fipronil sulfone is also more persistent at the binding site and less selective in its action, indicating that it may be a major contributor to fipronil toxicity (Hainzl et al., 1998). This property of time-reinforced toxicity raises concerns regarding the effects that fipronil may have on bees foraging for prolonged periods on small residues in treated crops. The injury caused by ingesting these residues over a prolonged exposure could lead to sublethal effects or even mortality of bees.

Laboratory microcolonies under enclosed conditions

Few studies to date have investigated the effects of field-realistic exposures of fipronil on bees and its effects on bumble bee fecundity were previously unknown. We found that fecundity was greatly reduced by field-realistic dietary concentrations of fipronil. Bumble bee workers exposed to only 1 ppb (1.28 µg L^{-1}) of fipronil did not lay eggs nor built nest structures. This could be due to fipronil stopping ovary development in workers, as has been reported for high doses of imidacloprid (Laycock et al., 2012). Fipronil may in some way be obstructing nesting behaviour by direct effects on the nervous system because fipronil is neurotoxic. Alternatively, fipronil exposure may have disrupted social interaction between worker bumble bees, which has been shown to be necessary for brood production in orphaned *B. terrestris* workers (Amsalem et al., 2009). However this was not the case for bees exposed to fipronil at

concentrations < 1 ppb, which were able to lay eggs, although fipronil did cause a reduction in final brood numbers. These reductions cannot be explained by delayed brood production as no difference was found in the timing of initial oviposition with fipronil exposure. Syrup and pollen consumption were both reduced with fipronil exposure. Thus, we speculate that nutrient limitation could have led to the observed reduction in brood at lower fipronil doses as the protein and carbohydrates these food sources provide are vital for both oviposition and larval development (Brodschneider and Crailsheim, 2010, Roulston et al., 2000, Vaudo et al., 2015). The effects of fipronil on bumble bee fecundity are similar to, though greater than, those of imidacloprid, which reduces fecundity by a third at 1 ppb (Laycock et al., 2012). However, exposure to field-realistic doses of another neonicotinoid, thiamethoxam, has no effect on bumble bee fecundity (Laycock et al., 2014). These results indicate that fipronil may pose a greater risk to exposed wild bumble bees than neonicotinoids. However, the effects of fipronil on bumble bee queens still need to be ascertained.

Semi-field microcolonies under free-flying conditions

There are a limited number of bee studies which focus on both laboratory- and field-based experiments. Though, of these studies, several have shown greater effects of dietary pesticide exposure in bees made to forage than those kept under laboratory conditions and unable to fly. Mommaerts et al. (2010) suggested that using bumble bee microcolonies without foraging activity to determine pesticide toxicity may be underestimating pesticide effects. They found that in microcolonies that allowed foraging activity bees were 3-10 times more sensitive to neonicotinoids than those without foraging. Ceuppens et al.

(2015) also found a similar effect when bumble bees were exposed to the pyrethroid, lambda-cyhalothrin.

We found that bumble bee workers in microcolonies exposed to 1 or 2 ppb fipronil were able to survive for 8 days under laboratory conditions, however when they were free to forage outside mortality was rapid. Within 1 day of free-flying nearly 20% of bees were lost from exposed microcolonies, while no losses were observed among controls in the free-flying microcolonies. Bees from treated microcolonies were observed to have limited coordination when exiting the microcolony boxes, with a high insidence of bees spinning on their backs on the ground before expiring (Philippa Holder, personal observations). The insect GABA-receptors on which fipronil acts are located on the membranes of muscle cells and are important for locomotor and flight activity in insects (Leal and Neckmeyer, 2002, Usherwood and Grundfest, 1965). Therefore, the antagonistic action of fipronil may be expected to affect locomotory activity as observed. There is great disparity in bumble bee survival post-exposure between the enclosed and free-flying microcolonies, suggesting that flight activity has a major impact on fipronil toxicity. Losses from semi-field microcolonies occurred too rapidly to be due to impaired homing ability and therefore may have been caused by an increase in toxicity of fipronil due to increased metabolic rate from flight. An increased metabolic rate required for flight may increase the activity of detoxification enzymes, such as cytochrome P450s and glutathione-S-transferases (Scharf et al., 2000), which metabolise fipronil to its main metabolite, fipronil sulfone. However fipronil sulfone is equally toxic and exhibits increased persistence toxicity (Caboni et al., 2003, Cole et al.,

1993, Reynaud et al., 2012), therefore the metabolism of fipronil will not reduce the sulfone's toxic effects.

While exposure to fipronil increased the number of brood produced by free-flying microcolonies, the proportion of larvae that were discarded (dead) also increased with fipronil concentration. Therefore, though it appears that fipronil exposure did not limit oviposition, larval survival was reduced. We speculate that increased larval mortality led to increased brood production to compensate for larval losses.

The negative effects observed from exposure to just 1ppb of dietary fipronil in laboratory microcolonies are in themsleves a cause for concern, however the increased toxicity and larval losses found under semi-field conditions indicates that fipronil exposure may have severe impacts on wild bees. The high toxicity of low-level fipronil is due to its time-reinforced toxicity over extended exposures, which may be due to its bioaccumulation (or that of its metabolite fipronil sulfone) in bees.

Conclusions

The fact that both worker survival and brood production were significantly reduced in bumble bee microcolonies at low, field-realistic exposures is of serious concern. These effects could have negative impacts on wild bumble bee populations foraging on fipronil-treated crops, with the risk of colony collapse from both increased worker losses and reduced brood. Although fipronil is currently under a temporary EU ban for use on bee-attractive crops, this ban is due to be reviewed within the next year and fipronil is also still widely used outside of the European Union. Risk assessments which do not test for TRT or

include foraging may be underestimating the impacts of pesticides on bees, potentially allowing dangerous pesticides to be used in the environment and risking the health of bee populations.

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Author Contributions As first author I carried out the majority of the lab work, all statistical analysis, co-designed the study and co-wrote the manuscript; project students Andrew Ball and Angharad Thomas carried out the fipronil microcolony study; summer intern Juliette Seneze assisted with lab work; Ian Laycock co-designed the study; Charles Tyler helped in the methods and co-wrote the manuscript; James Cresswell co-designed the study and co-wrote the manuscript.

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Chapter Four: Figures

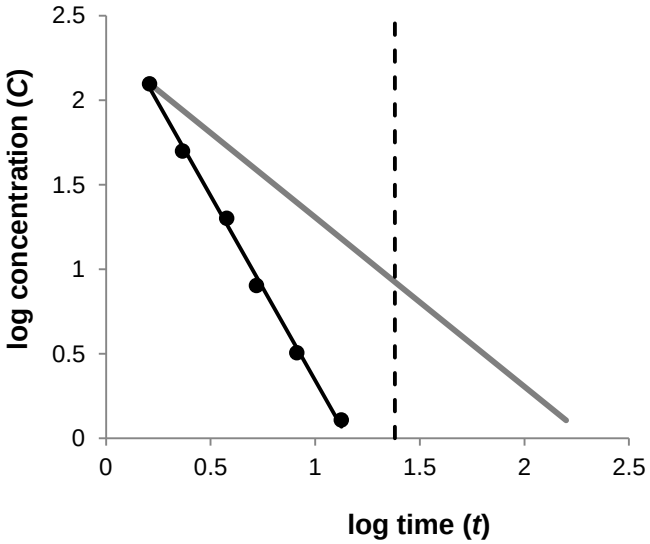

Figure 4.1 Fipronil evaluated for time-reinforced toxicity with Haber's Law. Logarithmic relationship between dietary fipronil concentration (*y*-axis; log 10 transformation of concentration of fipronil in syrup in µg L^{-1}) and time to 50% mortality among cohorts of individually-caged bumble bees (LT_{50}) (*x*-axis; log 10 transformation of time to 50% mortality (LT_{50}) for each treatment group) for bumble bee workers exposed to fipronil (0.00 to 125 µg L^{-1}, N = 10). The grey line indicates the expected results of a Haberian steady-state relationship, where *b* = 1. The dashed line indicates the point of senescence.

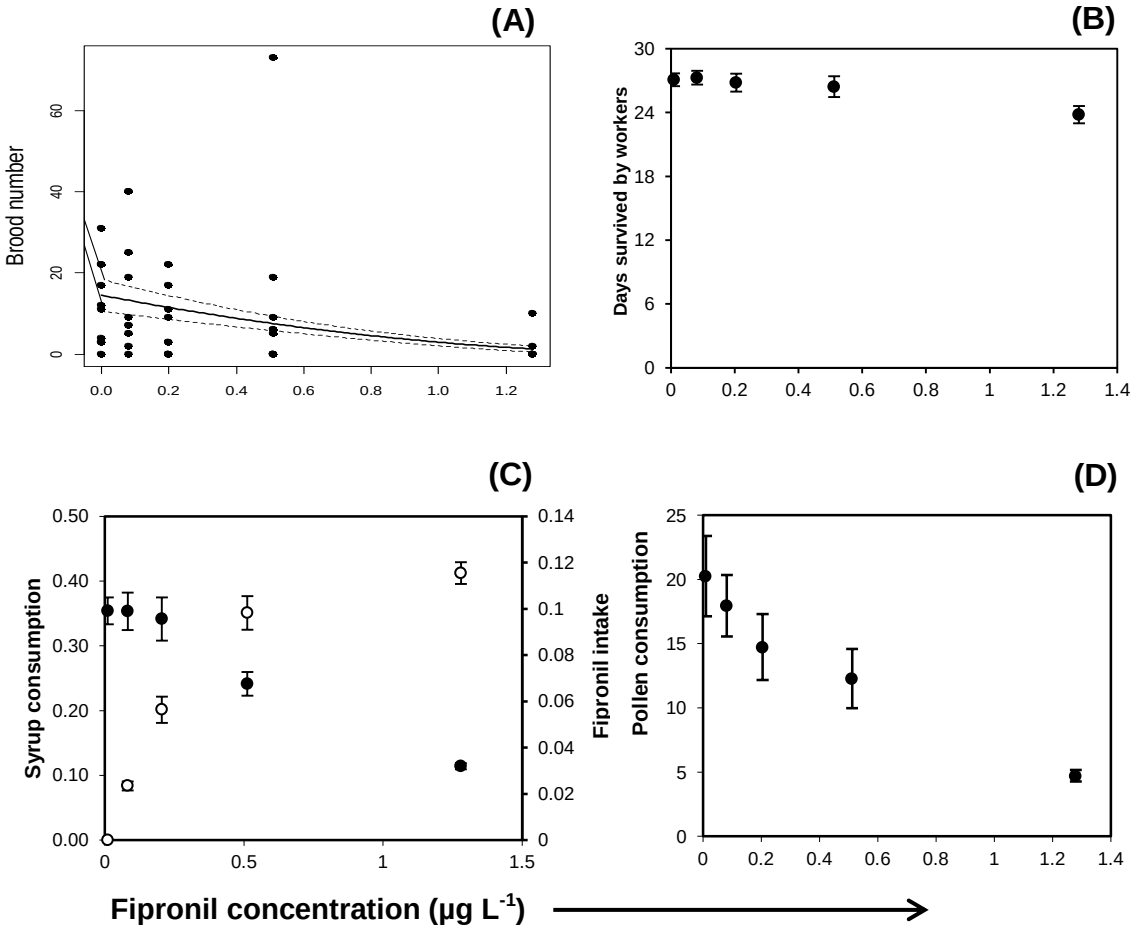

Figure 4.2 Lethal and sublethal effects in enclosed laboratory microcolonies of *Bombus terrestris* workers exposed to dietary fipronil. The effects of fipronil (*x*-axis; dietary fipronil concentration in syrup in µg L^{-1}) on **(A)** brood weight (total weight of eggs and larvae in grams (g)); **(B)** number of days survived by workers under exposure (maximum = 28 days); **(C)** daily *per capita* syrup consumption (lefthand *y*-axis; syrup consumption per bee per day in grams) and daily fipronil intake (righthand *y*-axis; amount of fipronil ingested per bee per day in ng), and **(D)** daily *per capita* consumption of undosed pollen. Control data (0 µg L^{-1}) are displayed slightly displaced for ease of inspection. Data shown are the means and error bars indicate ± 1 S.E. (replicates per dosage group; N =10 microcolonies). Note some error bars are obscured by data points.

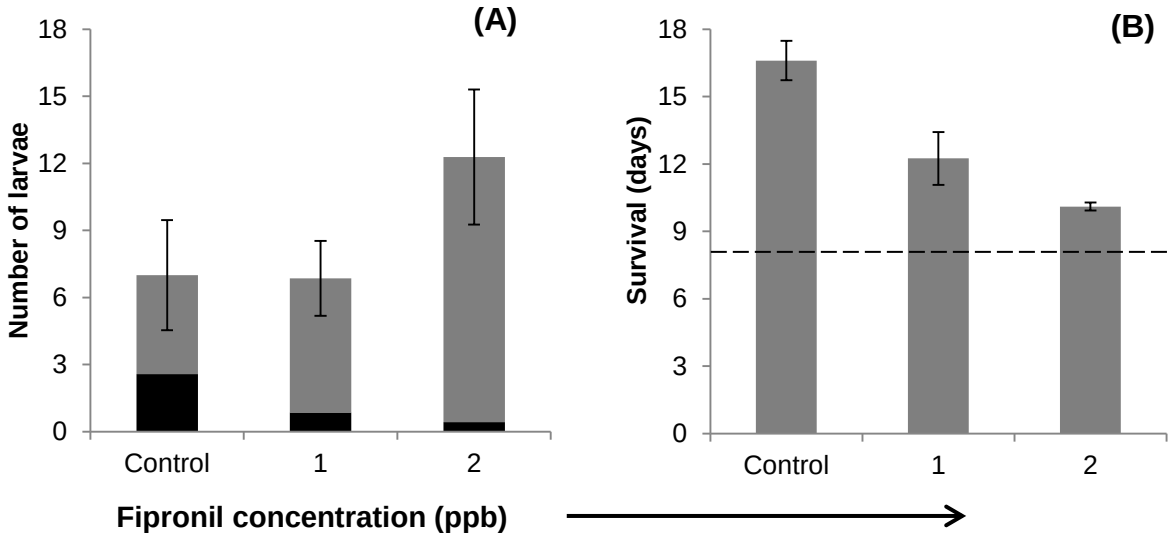

Figure 4.3 Fecundity and survival of worker bumble bees in free-flying semi-field microcolonies after exposure to field-realistic doses of fipronil. The dose-response relationship between dietary fipronil (*x*-axis; concentration of fipronil in syrup in ppb) and **(A)** the number of larvae produced (*y*-axis; number of larvae produced per microcolony, N = 21) with the number of viable larvae (black bars) and discarded larvae (grey bars) shown (S.E. bars shown are for total larvae); **(B)** survival (*y*-axis; the number of days survived after induction of fipronil exposure, N = 21), error bars indicate ± 1 S.E. and the dashed horizontal line indicates 8-day margin, after which previously enclosed microcolonies were placed outside to free-forage.

Chapter Five:

Impacts of fipronil on *Bombus terrestris* colonies in the field

Impacts of fipronil on *Bombus terrestris* colonies in the field

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Keywords: bumble bee, fipronil, reproduction, persistent, colonies, field realistic

Abstract

Fipronil is a widely-used, systemic pesticide which has been shown to adversely affect bees. Laboratory trials that used field-realistic exposures demonstrate that fipronil significantly reduces both feeding and fecundity of bumble bee workers in microcolonies. Dietary fipronil also reduces longevity, with increased mortality rates induced by foraging flights. Previous research has shown significant effects of fipronil at concentrations as low as 0.4 parts per billion. However, the effects of fipronil on the success of queenright bumble bee colonies have yet to be established. We therefore conducted a field experiment in which 30 queenright colonies of Bombus terrestris were exposed to dietary fipronil at concentrations of 1 and 2 parts per billion (ppb) for 14 days and then situated in the borders of an agricultural field.

We observed no variation among the *Bombus terrestris* colony success or queen production that could be attributed to fipronil diet. There was no significant difference in the number of new queens produced between colonies in different treatment groups, however only 14% of colonies succeeded in producing new queens. Weight increase of all colonies over six weeks was also low. It is postulated that the lack of effects may be due to incomplete exposure because of a technical flaw. Specifically, we speculate that the plastic wicks of the feeders absorbed the active ingredient, which is highly hydrophobic. Therefore, further research is needed to determine the effects of fipronil on colony success and future studies that include foraging in the field should be carried out earlier in the year to improve foraging choices.

5.1 Introduction

Fipronil

Fipronil is a widely used phenylpyrazole insecticide which was introduced to the market in the 1990s (Tomlin, 2009). Fipronil is a systemic insecticide, applied as a seed dressing, to protect a range of crops from herbivorous pests throughout growth. It is absorbed and distributed throughout plant tissues, where it can end up in pollen and nectar. Bees are exposed to small residues (generally < 3 μg L^{-1}) of fipronil when foraging on the contaminated nectar and pollen of treated mass-flowering crops, and residues have also been found within honey bee colonies (Chauzat et al., 2011, Mullin et al., 2010, Stoner and Eitzer, 2013). Fipronil is a non-competitive inhibitor of insect γ-aminobutyric acid (GABA) receptors, blocking the binding of other ligands as an allosteric modulator (Law and Lightstone, 2008). The sulfone derivative of fipronil, the main metabolite in insects, is also a potent insecticide (Caboni et al., 2003, Cole et al., 1993).

Impacts of fipronil

Fipronil is known to be highly toxic to bees and I have shown that it also exhibits time-reinforced toxicity in both honey bees and bumble bees (Chapters 2 & 4). Fipronil reduces bumble bee longevity by almost 10 days after exposure to only 1 ppb (Chapter 4). Nectar and pollen consumption are also reduced at field realistic concentrations (Chapter 4). This nutrient deficit could impact on colony functions such as foraging, brood rearing and disease resistance. However, possibly the greatest impact on colony success is reproductive failure, which could have a greater impact than loss of older bees (Decourtye and Devillers, 2010). I have shown that fecundity of bumble bee workers is greatly reduced

after exposure to fipronil concentrations as low as 0.5 ppb (Chapter 4). Bumble bee populations are particularly vulnerable to a fall in reproductive output compared to honey bees due to their annual life cycle. Bumble bee queens emerge in spring to found new colonies, which continue to grow with increasing numbers of workers throughout summer, until reproductives (new queens and males) are produced in late summer/early autumn after which mating takes place. Newly fertilised queens then overwinter underground while the rest of the colony dies (Goulson, 2003).

Therefore, for a bumble bee population to succeed its colonies must survive to produce reproductives. There is also evidence that a minimum colony size threshold exists, below which the production of new queens and males may not occur (Owen et al., 1980, Müller and Schmid-Hempel, 1992). Therefore, any loss in reproductive output of workers or reproductives could have an impact on colony success. Concerns over the impacts of fipronil exposure to bee health have led to temporary restrictions for fipronil use on bee-attractive crops within the European Union (EFSA, 2013a, EFSA, 2013b).

Colony-level field studies

Few studies have assessed the impacts of insecticides on bumble bee colonies in the field, and those that have focus on the neonicotinoids. Whitehorn et al. (2012), on which this study is modelled, found that field-realistic exposure to imidacloprid lead to reduced colony growth and an 85% reduction in the production of new queens. While, a semi-field study by Feltham et al. (2014) found that a similar exposure to imidacloprid lead to reduced foraging efficiency of workers, which may provide a causal mechanism for the reduction in colony

success observed by Whitehorn et al. (2012). However, foraging on neonicotinoid seed-treated oilseed rape had no effect on bumble bee colony health (Thompson et al., 2013). While Rundlof et al. (2015) showed that exposure to oilseed rape treated with a seed coating of clothianidin (a neonicotinoid) and β-cyfluthrin (a pyrethroid) reduced both bumble bee colony growth and reproduction. The disparity in these results illustrates the difficulties in determining colony-level effects under field conditions.

Microcolonies to colonies

Although I have previously shown that fipronil exposure negatively affects fecundity in microcolonies of bumble bee workers, there is no knowledge of the effects that fipronil may have on queenright colonies. If fipronil exposure affects queens similarly to workers it could have a severe impact on colony growth and success. We therefore exposed queenright *Bombus terrestris* colonies to dietary fipronil for 2 weeks under laboratory conditions prior to placing them in the field to investigate potential effects on fecundity and colony success.

5.2 Materials and methods

5.2.1 Preparation of fipronil

Fipronil (PESTANAL®, Sigma Aldrich Co. LLC; product code: 46451) was suspended in acetone to form a stock solution (10^4 µg L^{-1} fipronil) before being combined with diluted control syrup (90:10 syrup:water) (Attraker: 1.27 kg L^{-1} fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands).

5.2.2 Bombus terrestris colonies

30 commercial queenright *Bombus terrestris* colonies (Biobest colony; Agralan Ltd., Swindon, U.K.) containing on average 51.2 workers (range: 36 to 82; ± 2.07 S.E.) were randomly assigned to 1 of 3 treatments: control, low dose or high dose. The low dose treatment group were fed on sugar syrup containing 1ppb fipronil (0.01% acetone) and the high dose treatment group were fed on syrup containing 2ppb fipronil (0.02% acetone). The control group were fed syrup (90:10 syrup:water) containing only 0.02% acetone (equal to the high dose acetone content). Colonies were maintained under laboratory conditions as follows: temperature = 25 °C, humidity = 30%, 12:12hr light:darkness and fed *ad libitum* on their respective syrups for 14 days. Each colony was also provided with an undosed pollen ball (pollen pellets obtained from Koppert, ground and mixed with water) on day 1 and day 10 (day 1 mean mass = 14.50g, S.E. ± 0.1g; day 10 mean mass = 15.55g, S.E. ± 0.09g) of treatment. Syrup consumption per colony was monitored during the 14 day exposure. After 14 days of exposure colonies were placed in the field on 15th July where workers could forage under natural conditions for a further 4 weeks, without further fipronil exposure. Worker number per colony was recorded prior to placement in the field. The field site (approximately 8 hectares in area) was situated in an agricultural landscape which was part of a LEAF (Linking Environment and Farming) farm in Devon, UK (50°49'35.8"N 3°29'55.0"W). Colonies were randomly allocated to positions in the uncut field margin of a field bean crop which was not treated with insecticides. Colony weight was recorded initially and weekly thereafter. Once in the field colonies were weighed in the early morning before workers started to forage. Nests were weighed along with

the plastic box containing them. Colonies were brought in after 4 weeks in the field, all bees and nest structures were removed from colony boxes and stored at -20 °C. The nests of 6 randomly-selected colonies from each dose group were then dissected and the number of new queens, males, workers, pupae, larvae and eggs was recorded. The number of empty and closed pupal cells was recorded and any pupal cells with a diameter greater than 11mm we deemed to be gynes, while smaller cells were those of workers or males (Inoue et al., 2010). Larval length was also recorded. The body size of workers from all 30 colonies was recorded using the maximum thorax width (Goulson et al., 2002, Goulson, 2003).

5.2.3 Statistical analysis

All statistical analyses were carried out using R version 3.0.2 (R Core Team, 2013). Differences in worker numbers between treatment groups at various time points, the total number of cells (brood and nectar storage) and initial and final colony weight were analysed using a one-way ANOVA test. General linear mixed models (GLMM) were used to determine the effect of fipronil exposure on both worker body size and larvae length, with *dose* treated as a fixed variable and *colony* as a random variable for both. New queen production was analysed using a generalised linear model (GLM) with a negative binomial error structure, due to very few colonies producing new queens.

5.3 Results

5.3.1 Syrup consumption

Exposure to fipronil had no significant effect on bumble bee colony daily syrup consumption (ANOVA, $F_{2,26} = 0.82$, P = 0.45; **Figure 5.1**).

5.3.2 Queen production

Only 14 % of colonies succeeded in producing new queens. The number of new queens produced by colonies was not significantly affected by fipronil exposure (GLM, df = 27, P = 0.08; mean values: control = 0.6 ± 0.42 S.E., 1 ppb = 0.6 ± 0.71 S.E., 2 ppb = 0.1 ± 0.11 S.E.).

5.3.3 Colony growth and worker numbers

The initial number of bumble bee workers per colony and the initial colony weight did not vary significantly between dose groups (ANOVA; $F_{2,26} = 2.119$, P = 0.14, and $F_{2,26} = 2.11$, P = 0.14, respectively; **Figure 5.2**). At their greatest weight, in week five, colonies had increased in weight by an average of 21.85 g (S.E. ± 8.66 g), while by week six there was no increase from the colonies' initial weights (mean = - 0.05 g, S.E. ± 10.12). Exposure to dietary fipronil did not significantly affect worker numbers over the initial 2 week laboratory phase (ANOVA, $F_{2,26} = 1.77$, P = 0.19). There was still no significant difference in final worker numbers (**Figure 5.3**), or colony weight, between fipronil exposures after 4 weeks in the field (ANOVA; $F_{2,26} = 0.20$, P = 0.82, and $F_{2,26} = 0.86$, P = 0.43, respectively; **Figure 5.2**).

5.3.4 Worker body size

The average body size of bumble bee workers (thorax width = 4.63 mm ± 0.04 S.E.) was not affected by fipronil exposure (GLMM, df = 27, t = - 0.09, P = 0.93).

5.3.5 Nest measures

Colony mass (**Table 5.1**) was positively correlated with the total number of both brood and nectar cells present: *colony mass* = 0.4529 * *total cells* + 403.22, r^2 = 0.36. There was no significant difference in the total number of cells (brood and nectar storage) between colonies in different treatment groups (ANOVA, $F_{2,15}$ = 0.88, P = 0.44). The total number of brood present in each colony was not significantly affected by fipronil exposure (ANOVA, $F_{2,15}$ = 0.31, P = 0.74; **Figure 5.4**). Larvae length was also unaffected by fipronil exposure (GLMM, df = 14, t = 0.38, P = 0.71).

5.4 Discussion

Problem with exposure

Fipronil exposure had no effect on colonies, suggesting that there may have been problems with delivery of the pesticide to the bumble bee diet. Previous research has shown that fipronil at comparable concentrations causes significant reductions in feeding, fecundity and longevity, especially under field conditions, where bees are exposed to more of the rigors of the natural environment. Bees were fed using plastic wick feeders provided within the commercial bee colonies. It is possible that the wicks acted as a filter, removing fipronil from the syrup ingested. A previous study also using these feeders had successfully delivered an exposure of bees to imidacloprid (Laycock and Cresswell, 2013). In contrast to imidacloprid, fipronil is a larger, hydrophobic molecule and so it may have been unable to pass through the wick. In other areas of research covered in this thesis (Chapter 2), it has proven impossible to obtain a sound analytical assay of fipronil concentrations in test syrups. Potentially, the fipronil had come out of solution by adsorption onto the walls of plastic tubes used to store syrup samples (Ainsley Jones, personal communication).

Feeding

Fipronil has been shown to reduce both syrup and pollen consumption of bumble bees in microcolonies at dietary concentrations as low as 0.4 ppb (Chapter 4). The nutrient limitation due to fipronil exposure may have led to the observed reductions in fecundity in these microcolonies. Therefore, exposure of colonies to either 1 or 2 ppb should be expected to not only reduce feeding but

also fecundity, and consequently colony growth. However, the feeding rates of bumble bee colonies did not differ with the intended fipronil exposure (**Figure 5.2**), suggesting that fipronil may not have been properly delivered and ingested.

Colony growth

Fecundity of workers in microcolonies was further impacted when exposed to 1ppb of fipronil (Chapter 4), leading to a complete lack of nest building and oviposition. Exposure to fipronil residues at this level therefore should be detrimentally affecting bumble bee behaviour and social interactions that are necessary for proper nest construction. Exposed bumble bee colonies showed no reduction in fecundity or colony growth at either 1 or 2 ppb (**Figure 5.4**). Colony growth, measured using colony mass, was limited throughout all the groups in the present study (**Figure 5.3**). Colonies initially lost mass under laboratory conditions, which may have been due to evaporation in the relatively dry environment. Colonies did increase in mass until week five, under a combination of laboratory then field conditions, but only by an average of 4.37 g/week. In the final week colonies began to lose mass as they switched from the growth phase to producing reproductives. By week six colonies had returned, on average, to their initial mass (mass gain = - 0.049 g, ± 10.12 g S.E.). In comparison, in the study by Whitehorn et al. (2012) on which this study is modelled, control colonies gained approximately 25 g/week each over the study period. We speculate that this comparatively low mass gain by the colonies studied in this chapter may have been due to a lack of available forage. The number of colonies producing new queens was also comparatively low at only 14%. Since queen production is related to colony size (number of

workers) and usually has a threshold trigger, this lack of gynes may have been due to the limited colony growth observed or possible nutrient limitation.

Worker numbers and body size

Worker numbers throughout the six week experimental period did not vary systematically among treatment groups. In our previous study, bees in microcolonies that were dosed with the same concentrations of fipronil showed strong increases in mortality when placed in the field to forage, possibly due to an increase in metabolic rate. If this effect translated to colonies in the field a reduction in worker numbers could impact on the colony's foraging ability, as well as levels of social hygiene and thermoregulation by effects on housekeeping tasks, as house bees are recruited to make up the shortfall in forager numbers. Fipronil could also affect colony success by altering worker body size. A study by Goulson and Sparrow (2009) found that competition with honey bees led to reductions in bumble bee worker body size, presumably due to nutrient limitation from lack of available forage. Reductions in feeding caused by fipronil could lead to similar effects. Bumble bee workers naturally vary greatly in body size, with an up to 10-fold difference in body mass (Alford, 1975). It has been hypothesised that this variation is adaptive, with bees of different sizes performing different tasks, known as alloethism. There is evidence that this is the case for bumble bees, with larger bees performing foraging tasks more efficiently and smaller bees housekeeping and brood rearing (Goulson et al., 2002). Therefore, a change to size variation in workers could upset work balance and impact on normal colony function. However, we found no impact of the intended fipronil exposures on worker body size in the colonies that we studied.

Wider research

Previous studies investigating the effects of pesticides on bumble bees in field settings have had mixed results. A study by Whitehorn et al. (2012), on which our own study was modelled, found that field-realistic levels exposure to imidacloprid had dramatic effects on colony success, with an 85% decrease in queen production and reduced colony growth. Given that our own study was started at the same time of year, the observed levels of colony growth by Whitehorn et al. were far in excess of our own study. This disparity may indicate that our colonies had limited available forage and therefore reductions in colony growth due to fipronil exposure would have been much harder to identify. However, limited availability of forage could have increased colony stress levels and therefore it may be expected that pesticide effects would be more apparent. Field studies by Thompson et al. (2013) and Rundlof et al. (2015) both investigated the effects of exposure to neonicotinoid-treated oilseed rape crops on bumble bee colony success. Thompson et al. (2013) found no effect on bumble bee colonies from nearby treated crops, however replication within the experiment was low and did not allow for statistical analysis. Conversely, Rundlof et al. (2015) found that exposure to crops treated with a mixture of clothianidin (a neonicotinoid) and β-cyfluthrin (a pyrethroid) reduced both bumble bee colony growth and reproduction. The disparity in these results illustrates the difficulties in determining colony-level effects under field conditions.

To determine that pesticide exposure does not have an effect on field colonies it is necessary to include appropriate replication within the experiment to allow for sufficient statistical power to identify potential effects. Residue analysis of bees

and colony matrices is also necessary to determine whether negative results are due to a lack of pesticide exposure. From our own findings it is not possible to rule out a lack of fipronil effect as opposed to problems with fipronil delivery as residue analysis was not carried out. If our results give a true picture of fipronil effects on field colonies this raises serious questions about the validity of laboratory experiments to determine pesticide risks. From our previous laboratory and semi-field based research we predicted that fipronil would have significant effects on colony growth and success. The disparity in these results would indicate that colonies under field conditions may be more resilient to fipronil exposure than individuals and queenless microcolonies in the laboratory.

However, if our lack of observed effects were due to problems with fipronil delivery this indicates that residue analysis of exposed colonies is vital to rule out this issue as a potential cause of negative results found in other field studies.

Conclusions

Studies of bumble bee colony performance in relation to fecundity and mortality are important for modelling demographically population level effects, especially with respect to sustainability. Many laboratory studies do not generally investigate effects on queens and queenright colonies which are required to understand the impacts of pesticides on bumble bee populations (but see Laycock and Cresswell (2013)). Therefore, a greater focus needs to be given to these studies. Due to potential problems with delivering the intended exposure, it is not possible to draw any conclusions from the present experimental study regarding the effects of fipronil exposure on bumble bee colonies. These issues

also indicate the need for residue analysis of exposed colonies to rule out potential delivery problems as a cause of negative results in future studies. Due to the slow growth of our colonies and limited queen production, it can be concluded that for future colony studies colonies should be placed in the field earlier in the year to ensure ample forage. The impacts of fipronil on bumble bee colony require further research and the present study provides an informative template for the design of future experiments.

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Author Contributions As first author, I carried out the experimental work and statistical analysis, co-designed the study and co-wrote the manuscript; Charles Tyler helped in the methods and co-wrote the manuscript; James Cresswell co-designed the study and co-wrote the manuscript.

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Chapter Five: Figures

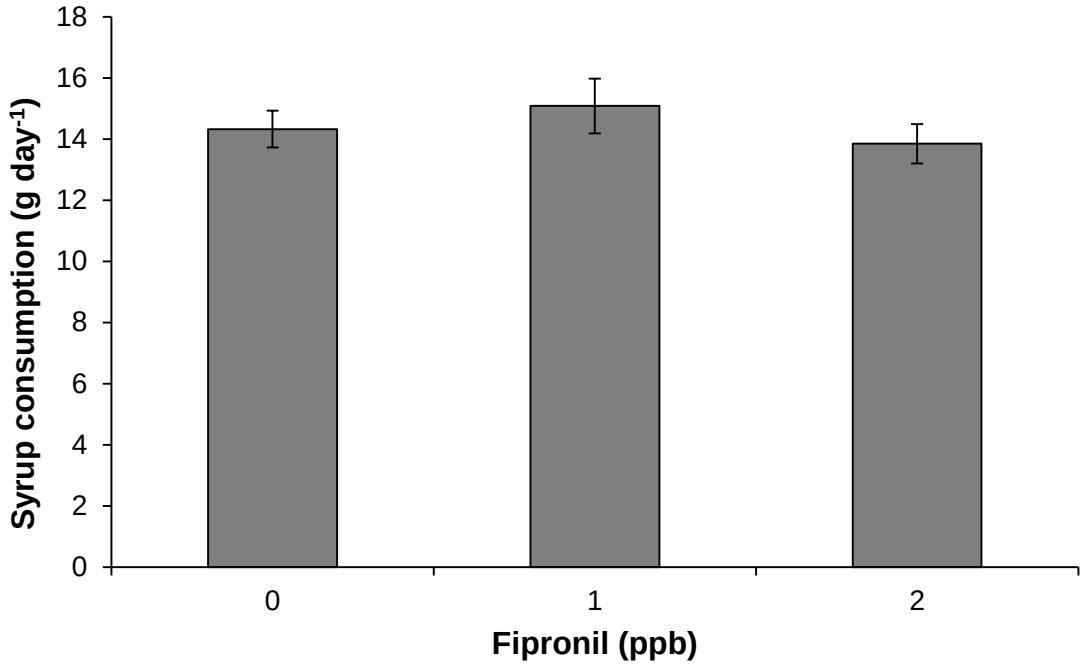

Figure 5.1 Syrup consumption of colonies exposed to fipronil under laboratory conditions. Relationship between the daily amount of syrup consumed by colonies (*y*-axis; mean daily syrup consumption per colony over the two week exposure period in grams (g) per day) and fipronil concentration (*x*-axis; dietary concentration of fipronil in syrup in ppb). Error bars indicate ± 1 S.E (n = 6).

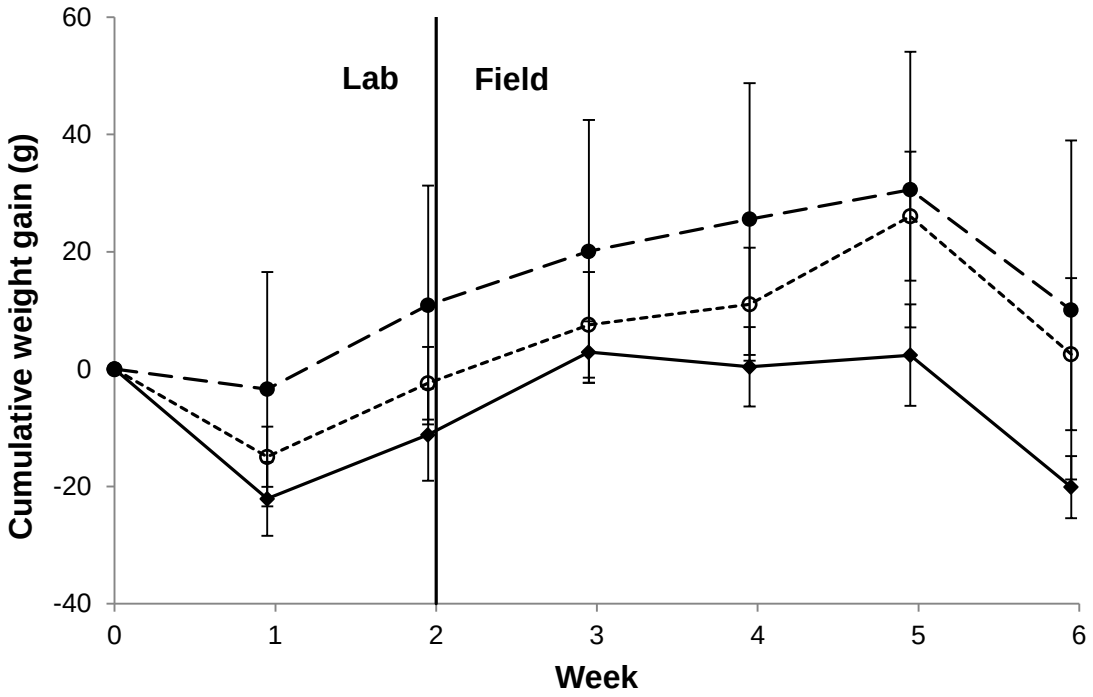

Figure 5.2 Changes in the mass of bumble bee colonies over time. Relationship between colony mass (*y*-axis; change in colony mass in grams (g)) and time (*x*-axis; time elapsed in weeks) for queenright bumble bee colonies fed on control syrup (filled diamonds) or fipronil at 1 ppb (filled circles) or 2 ppb (open circles). Data points are connected and offset for ease of inspection. Data shown are mean values and error bars indicate ± 1 S.E (n = 10).

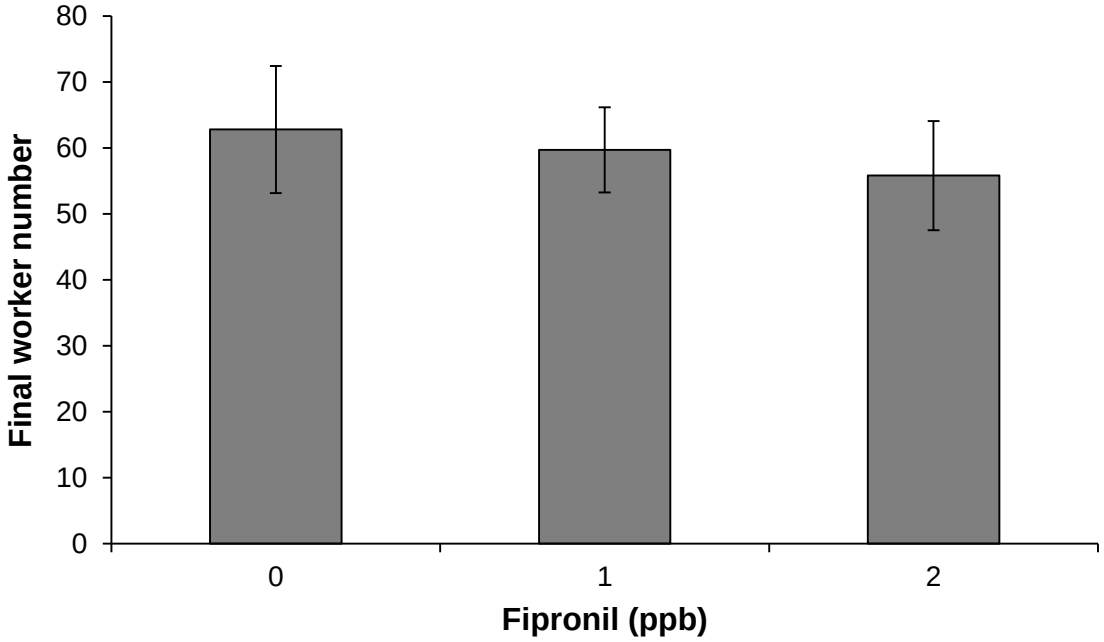

Figure 5.3 Final numbers of workers present in colonies after six weeks. Relationship between the final number of workers present in colonies (*y*-axis; mean final number of workers per colony) and dietary fipronil concentration (*x*-axis; concentration of fipronil in syrup in ppb). Error bars indicate ± 1 S.E (n = 6).

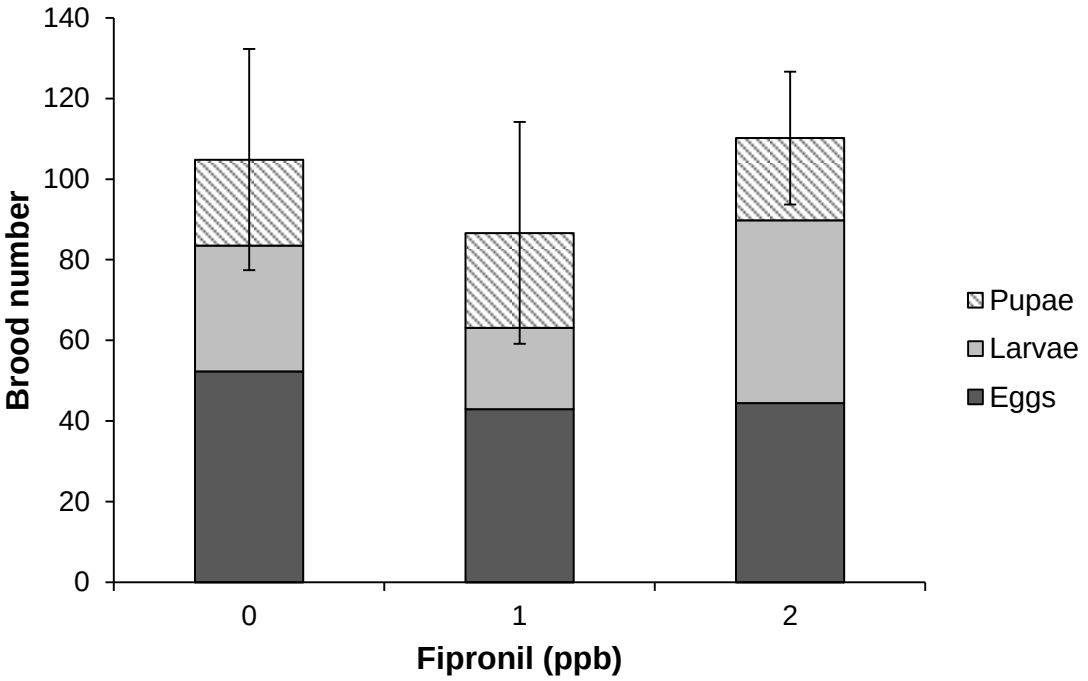

Figure 5.4 Brood production of colonies exposed to dietary fipronil. The relationship between final brood number (*y*-axis; the mean number of brood contained in each colony at week six of study) and fipronil exposure (*x*-axis; concentration of dietary fipronil in ppb). Each bar is split to show the number of eggs, larvae and pupae. Error bars indicate ± 1 S.E. of total brood (n = 6).

Chapter Five: Tables

Table 5.1 *Bombus terrestris* colony mass before and after exposure to a range of dietary fipronil concentrations.

	Initial colony mass (g)		Final colony mass (g)		Mass difference (g)	
Fipronil concentration (ppb)	**Mean**	**S.E.**	**Mean**	**S.E.**	**Mean**	**S.E.**
0	468.5	8.28	463.0	6.10	-20.1	5.31
1	449.4	24.72	466.7	16.56	10.1	28.88
2	458.5	6.48	477.8	10.96	2.5	12.94

Chapter Six:

General discussion

6.1 Overall thesis aims and objectives

The objectives of this thesis were to identify time-reinforced toxicity (TRT) pesticides and investigate their effects on demographically relevant endpoints in bees. Here I discuss my findings and their implications in relation to two important regulatory questions which are yet to be fully answered. I also suggest areas where further insight could help to finally answer these questions.

6.2 Which are the most acceptable insecticides to use on crops?

Global food security is a major concern as human population growth continues. The UK food security policy (Barclay, 2012) highlights five key challenges for future global food security:

1. Managing future supply and demand sustainably
2. Ensuring stability in food supplies
3. Ending global hunger
4. Managing the effects of the food system on climate change mitigation
5. **Maintaining biodiversity and ecosystems**

To improve crop yields to meet increasing demand, plant protection products, including pesticides, are widely used. These chemicals can have a negative impact on non-target species, affecting biodiversity and ecosystems. To help to maintain biodiversity and ecosystems, as highlighted in the UK food security policy, development on pesticides has focused on enhancing several key attributes of pesticides: efficacy, crop protection and selectivity. Here I will discuss how the research I conducted improves understanding of these

pesticide attributes and highlights the need to also consider whether pesticides exhibit time-reinforced toxicity.

Efficacy

New generation insecticides have been developed to increase insecticidal potency, reducing the time to effect and thereby improving crop protection and reducing damage to plants. Pyrethroids were formulated from natural pyrethrins, enhancing their insecticidal action and enabling them to cause a rapid 'knockdown' effect on exposed insects (Davies et al., 2007). Further research into novel insecticides led to the development of the neonicotinoids, which were designed to be more toxic and, consequently, lethal at much lower concentrations (Jeschke and Nauen, 2008). Theoretically, this property allows for much less pesticide to be used to provide the same level of protection for the crop. However, many other factors determine the efficacy of pesticides, including ingestion by the pest insect, transport to target sites and vulnerability to detoxification enzymes. I confirmed that new generation insecticides, the neonicotinoids and fipronil, were indeed far more toxic to bees than cypermethrin (a pyrethroid) (Chapter 1). The implication of this finding is that the efficacy of pesticides is increasing over time, and that these new generation pesticides could pose a greater threat to exposed bees from small residues.

Crop protection

Until recently, pesticides, such as pyrethroids, were generally applied as foliar sprays to crop plants. This application method has several potential issues for use. Firstly, spray application does not guarantee uniform coverage and some areas of the crop may be missed, resulting in a lack of protection from pests.

Secondly, foliar sprays are easily eroded by the weather. Consequently, it is necessary to repeat their application several times over the crop growth period. Thirdly, spray application results in relatively high concentration residues of the pesticide on the leaves and in the pollen and nectar of flowering crops. This poses a risk to non-target organisms that may be present on the crop. Also, non-target organisms may be directly sprayed during the application process or be exposed on nearby plants after pesticide drift, leading to an increased chance of mortality.

To reduce the problems associated with spray application, new generation pesticides have been formulated to be systemic. This property enables the pesticide to be absorbed by the plant and distributed throughout its tissues, ensuring total pesticide coverage. These pesticides are applied as seed dressings, protecting the crop from soil pests during germination, through to harvest. This also limits the amount of pesticide required to protect crops as repeated applications may not be necessary. However, because of their longevity in the plant, systemic insecticide residues are necessarily found at low concentrations in the nectar and pollen of treated flowering crops. Bees then forage on these crops, ingesting the insecticide residues. My thesis shows that some systemic residues, namely fipronil, are toxic to bees even at these low doses. The implications of these results are that, firstly, current risk assessments have failed to identify this attribute of fipronil and potentially other pesticides that have been approved for agricultural use. Secondly, the systemic nature of pesticides does not guarantee their safety for use, and thirdly, fipronil could lead to increased mortality of farmland bees from tiny residues that were previously believed to be harmless.

Selectivity

Over time pesticides have been formulated with high target specificity to reduce negative effects on non-target organisms such as birds, mammals and fish (Casida, 2012, Jeschke and Nauen, 2008, Yamamoto et al., 1995). However, non-target insects are still at risk from insecticides as it has not been possible to develop species-specific insecticides because the chemical target sites are conserved in insects. Systemic applications limit the exposure of non-target insecticides as only those feeding on the plant will be exposed. Unfortunately, as well as herbivorous insect pests this group also includes pollinators, beneficial non-target insects. There is also evidence of insecticides from seed coatings leaching into the surrounding soil, potentially harming soil biota and ecosystem services (Chagnon et al., 2015, Pisa et al., 2015). My work shows that whereas detoxification system can vary in capacity both within species, families and insect orders, there is no reason to attribute particularly strong or weak capacity to bees. Consequently, insecticides are not selectively protecting bees.

Which pesticide is most acceptable?

Fipronil and the neonicotinoids are all 'new generation' systemic insecticides, applied as seed-dressings and so minimise exposure to non-target organisms, unlike cypermethrin which is applied directly as a foliar spray. Therefore, these newer insecticides would appear to safer for use, limiting negative effects to biodiversity. The 'new generation' also exhibit increased insecticidal potency compared to the pyrethroids, improving the effectiveness of their pest control. All of the insecticides that I have investigated show selectivity for their insect

targets, though this is again improved in the newer insecticides, especially the neonicotinoids which have very limited mammalian toxicity. But they are not selectively benign towards bees. Taking these attributes into account, fipronil and the neonicotinoids appear to be more acceptable for use on crops due to improved efficacy and safety. However, my research has also highlighted that time-reinforced toxicity is another attribute that requires consideration.

Table 6.1 Desirable attributes of four insecticides applied to bee-attractive crops.

	Phenylpyrazole	**Neonicotinoid**		**Pyrethroid**
Attribute	Fipronil	Imidacloprid	Thiamethoxam	Cypermethrin
Efficacy	✓	✓	✓	
Systemic	✓	✓	✓	
Selectivity	✓	✓	✓	✓
TRT	✓	☒	☒	☒

Although it has been hypothesised that the neonicotinoids bind irreversibly to insect neurons (Tennekes and Sanchez-Bayo, 2011), and consequently bioaccumulate, neither the neonicotinoids tested nor cypermethrin exhibit TRT. Also, exposure to field-realistic concentrations of neonicotinoids and cypermethrin had no effects on a range of bumble bee endpoints tested. However, previous research has found that imidacloprid does negatively impact bumble bee fecundity at as little as 1 ppb (Laycock et al., 2012). From these

results, thiamethoxam and its metabolite clothianidin appear to pose a smaller risk to bees than the other insecticides tested (**Table 6.1**).

Fipronil has previously been widely-used on crops in Europe until a 3-year moratorium was imposed on its use on bee-attractive crops, however this temporary ban will soon expire. Fipronil is also still widely used in many countries, including the USA and throughout Asia. My research into the time-reinforced toxicity of pesticides has shown fipronil to pose a danger to bees foraging on treated flowering crops, as well as any other agricultural insecticide with this property. I have shown that a TRT pesticide can be lethal at very low doses over prolonged exposures, highlighting the danger that these pesticides pose to bees, potentially no matter how small the dose.

6.3 Which toxicity endpoints are most valuable for risk assessment?

There are two main factors involved in toxicity testing that I will discuss here: exposure time and the endpoint measured. Current risk assessment protocols focus mainly on short-term exposures of fewer than 10 days with mortality as the endpoint. Risk assessments for bees are also predominantly carried out using honey bees as a model species, however sensitivity to insecticides has been shown to vary between bee species (Arena and Sgolastra, 2014, Cresswell et al., 2012).

Tier 1 testing for sprayed pesticides involves determining the contact LD_{50} (median lethal acute dose) of a pesticide, from which a hazard ratio is calculated using the highest recommended application rate of the manufacturer:

$$\text{Hazard ratio} = \text{application rate (g ha}^{-1}\text{)} / \text{contact LD}_{50}\ (\mu\text{g bee}^{-1})$$

If the hazard ratio is less than 50 the pesticide is categorised as low risk to bees. To determine the risk posed by pesticides applied as soil or seed treatments, the oral LD_{50} of the test pesticide and the concentration of residues of this pesticide in nectar and pollen of treated crops are used to calculate a first-tier toxicity exposure ratio (TER):

$$\text{TER} = \text{oral LD}_{50}\ (\mu\text{g bee}^{-1}) / \text{plant residues (mg kg}^{-1}\text{)}$$

A TER value greater than 10 indicates that the pesticide is of low risk to bees. Conversely, if a pesticide does not meet these criteria, second-tier testing over a 10-day exposure period is conducted to calculate the 10-day NOEL (no observed effect limit) and from this a Tier 2 TER can be calculated (EPPO, 2010).

However, there are several shortcomings to these risk assessment methods. Firstly, I will address the issues surrounding the exposure time tested. My research has shown that short-term mortality testing will underestimate the toxic effects of TRT pesticides. An exposure duration of less than 10 days is too short to identify pesticides that exhibit time-reinforced toxicity, as evidenced by the approval and use of fipronil since 1994. Using a simple laboratory bioassay with a prolonged exposure time, I have shown that fipronil exhibits TRT in both honey bees and bumble bees, posing a risk to bees at field-realistic

concentrations (Chapters 2 & 4). The bioassay is feasible for use as a regulatory tool because its logistical requirements are not much greater than existing Tier 1 procedures. The importance of testing for TRT is clear because I have also linked fipronil to mass bee deaths which took place in France during the 1990s and shown it capable of causing honey bee colony collapse (Chapter 2). These findings highlight the need to increase the exposure times used for pesticide risk assessment with bees. Newly formulated draft guidelines issued by the European Food Safety Authority (EFSA) for risk assessment in bees require both longer conventional laboratory exposures (10 days) in first tier procedures that could reveal TRT and a new experimental protocol aimed specifically at evaluating conformity with Haber's Law (EFSA, 2013b).

Secondly, there are also problems with using solely lethal endpoints. Lethality has been shown to be a low sensitivity endpoint of pesticide exposure in bees, and a similarity in pesticide lethality does not guarantee similar sublethal effects. For example, the EC_{50} of dietary imidacloprid on bumble bee fecundity was found to be just 1ppb, while imidacloprid at 98 ppb resulted in the death of a single bee (Laycock et al., 2012). While the neonicotinoids imidacloprid, thiamethoxam and clothianidin have similar short-term toxicities, only imidacloprid negatively impacts reproduction and feeding at field-realistic exposures (Chapter 3) (Laycock et al., 2014, Laycock et al., 2012). I found that bumble bee fecundity had a much greater sensitivity to pesticide exposure than mortality, or even other sublethal endpoints including syrup and pollen consumption (Chapters 2 – 4). Fipronil exposure strongly reduced fecundity of bumble bee workers at dietary concentrations as low as 0.5 ppb (Chapter 4). This is a concerning result, especially as a reduction in fecundity (number of

brood) may have a greater impact on colony success than the loss of older workers (Decourtye and Devillers, 2010). These results indicate that the focus of future risk assessments needs to be on more realistic exposure durations and also on demographically relevant endpoints, such as fecundity. Risk assessments should also focus more on the results from field experiments where bees are required to forage for food. I found that fipronil toxicity was greatly increased once bees were made to fly for food, resulting in almost immediate mortality (Chapter 4). This increased toxic effect also found in bumble bees exposed to neonicotinoids and a pyrethroid (Ceuppens et al., 2015, Mommaerts et al., 2010). Higher tier testing under current protocols has always required field testing for most insecticides, so it is puzzling that fipronil was approved for use in the EU. Perhaps lack of statistical power in the protocols.

6.4 Future applications and research

Future applications

The TRT bioassay I used could be applied to screen new plant protection products in future risk assessments as well as to identify any current-used pesticides that exhibit time-reinforced toxicity. This bioassay has been included in newly formulated draft guidelines issued by the European Food Safety Authority (EFSA) for risk assessment in bees (EFSA, 2013b).

The work in this thesis could also be used to aid the design of more appropriate risk assessments of PPPs to bees, including the testing of more bee species, using prolonged exposures and including more demographically important and

sensitive endpoints, such as fecundity. My research also highlights the need to include testing in free-flying conditions where bees are exposed to the rigors that they would experience in the agricultural environment.

Future research

To fully understand the impacts of TRT pesticides on bees, as well as other species, it is necessary to conduct further research to determine the mechanisms by which pesticides exhibit TRT. To do this neurophysiological studies are needed to study the binding behaviour of these neurotoxic pesticides as well as their potential to permanently alter neurological activity within the bee brain. The impacts of these changes need to be investigated also as they could impact on bee behaviour, potentially affecting colony survivorship.

Future research into pesticides effects also needs to focus on real-world scenarios, where bees are exposed to the rigors required to maintain and grow a colony under natural conditions. Foraging, navigation and acclimating to changing temperatures are all factors which could affect the ability of bees to cope with pesticide exposure, and so lab-based studies are at best only surrogates for real-world scenarios. This needs more due consideration in future population level modelling. Further research focussing on queen-level effects of pesticide exposure is key to determining impacts on colony survivorship, as to date few studies have focussed on this aspect.

Ergokinetics, the relationship between the concentration of a toxicant at its site of action and the injury caused, is still poorly understood and warrants further research. This could improve understanding of the mechanisms by which pesticides may exhibit time-reinforced toxicity.

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