Developing a New Testing Paradigm for Risk-Assessment of Bee-Pesticide Interactions – Quantifying the Pace of Neonicotinoid Toxicokinetics

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature: ……..

Abstract

Neonicotinoid pesticides, which are used to protect crops from certain pests, have been correlated with the decline of non-target insect species, including bumblebees. However, despite a myriad of studies into the interaction and impact of neonicotinoids, uncertainty remains as to the risks these xenobiotics pose to bees. In particular, the question of bioaccumulation, defined here as how long neonicotinoids persist in the body (i.e. fast or slow toxicokinetics) has not yet been determined for neonicotinoids and bumblebee species. Moreover, while the implications of bioaccumulation on non-target species are severe, regulatory standards continue to rely on acute paradigm testing (e.g. 48-hour LC_{50}s or NOECs) that may inherently fail to capture bioaccumulation.

First, I reviewed the literature on the pace of toxicokinetics for neonicotinoids, found in studies on enzymatic metabolism and receptor site bonding of these substances, which are the main pathways for clearance of xenobiotics. The literature supports that neonicotinoids have face-paced toxicokinetics and are unlikely to bioaccumulate in bees. I further reviewed current regulatory practices (LC_{50}s and NOECs), and how a proxy for bioaccumulation can be derived using dose-dependence studies analysed with Haber's Law.

Next, I conducted laboratory experiments examining the usefulness of Haber's Law for quantifying bioaccumulation using the neonicotinoids imidacloprid and thiamethoxam, and the known bioaccumulative phenylpyrazole, fipronil, as a positive control. Here, not only did I corroborate the literature review findings that neonicotinoids likely have face-paced toxicokinetics, I found evidence that fipronil has bioaccumulative properties, which underscores the usefulness of Haber's Law in regulatory testing for bioaccumulation.

Finally, I used 96-hour pulse-exposures to assess a proxy for toxicokinetic pace. Bees with pulsed exposures should have less injury than constant exposures if pesticides are easily cleared. Again, thiamethoxam and fipronil showed signs of differing toxicokinetic pace. These quantifiers could be used to fill a regulatory gap for bioaccumulation addressing toxicokinetic pace.

List of Contents

Abstract 2

List of Contents 3

List of Figures 6

List of Equations 8

Definitions 9

Introduction: The Pace of Neonicotinoid Toxicokinetics 10

Chapter 1: Bioaccumulative Toxicity and Regulation of Neonicotinoids: A Review 13

1.1 Abstract 13

1.2 Introduction: Bioaccumulative Toxicity 14

1.3 The What and Why of Neonicotinoids 17

1.3.1 Crop Protection 17

1.3.2 Neurotoxic Pesticides 18

1.4 Environmental Concern Over Pesticide Usage 20

1.4.1 Environmental Contamination 20

1.4.2 Non-target Species 21

1.4.3 Bioaccumulation 23

1.5 Examining Bioaccumulation of Neonicotinoids 24

1.5.1 Enzymatic Metabolism 24

1.5.2 Receptor Site and Binding Reversibility 26

1.5.3 Evidence from Neonicotinoid-Bee Studies 30

1.6 A Note on Secondary Metabolites and Sub-lethal Effects 32

1.6.1 Secondary Effects of Enzyme Metabolites 32

1.6.2 Sub-lethal Effects of Neonicotinoids 34

1.7 The Status of Bioaccumulative Toxicity (TRT) in Current Pesticide Regulation 37

1.7.1 $LC_{50}S$ 38

1.7.2 NOECs 39

1.8 The Chronic Paradigm - Using Haber's Law to Quantify Potential for Bioaccumulative Toxicity 41

1.8.1 Haber's Law 41

1.8.2 Ingestion:Longevity Relationship **46**
1.9 Conclusion **47**
1.10 References **49**
Chapter 2: Quantifying the Pace of Toxicokinetics in Bumblebees using Haber's Law **67**
2.1 Abstract **67**
2.2 Introduction **68**
2.3 Methods **75**
2.3.1 Bee Husbandry and Acclimation **75**
2.3.2 Preparation of Doses **76**
2.3.2.1 Imidacloprid **76**
2.3.2.2 Thiamethoxam **76**
2.3.2.3 Fipronil **77**
2.3.3 LCMS Verification of Doses **77**
2.3.4 Experimental Exposures and Statistical Analysis of Mortality and Feeding **79**
2.4 Results **81**
2.4.1 Validation of Doses **81**
2.4.2 Dose-Dependence **82**
2.4.3 TRT Analysis, LCc_{50}s, and NOECs **84**
2.5 Discussion **88**
2.5.1 Relative Toxicity of Three Pesticides **88**
2.5.2 Toxicokinetics of Three Focal Pesticides **89**
2.5.3 Implications for Assessing the Risk to Bees from Trace Residues **92**
2.5.4 Potential Utility of Haber's Law in Regulatory Testing of Agrochemicals **92**
2.6 Supplementary Information **95**
2.7 References **98**
Chapter 3: Pulse Exposures Help Assess the Pace of Pesticide Toxicokinetics in Bumblebees **105**
3.1 Abstract **105**
3.2 Introduction **106**

3.3 Methods **109**
3.3.1 Provenance and Husbandry of Bees **109**
3.3.2 Treatments **109**
3.3.3 Preparation of Doses **110**
3.3.3.1 Thiamethoxam **110**
3.3.3.2 Fipronil **110**
3.3.4 Experimental Exposures and Statistical Analysis **111**
3.4 Results **112**
3.4.1 Differential Toxicokinetic Pace: Survivorship and Consumption **112**
3.4.2 Differential Toxicokinetic Pace: Lifetime AI Ingestion **114**
3.5 Discussion **116**
3.5.1 Toxicokinetic Inferences **116**
3.5.2 Correspondence of Various Toxicokinetic Bioassays **118**
3.5.3 Threats Posed to Bee Health by Focal Insecticides **119**
3.5.4 Potential Regulatory Development to Include Bioassays for TRT **120**
3.6 References **122**
Concluding Discussion **130**
4.1 Fast Toxicokinetics of Neonicotinoids and the Chronic Paradigm **130**
4.2 References **135**
Acknowledgements **137**

List of Figures

1.1	Summary and timeline of neurotoxic pesticides	**19**
1.2	(a) Summary of Sublethal endpoint papers by study type	**36**
	(b) Summary of Sublethal endpoint papers by endpoint	
	(c) Summary of whether studies found significant sublethal impact by neonicotinoids, and if in FRR	
	(d) Summary of endpoints found impacted in FRR	
1.3	(a) example: *C vs t* with Haber's exponent = 1	**43**
	(b) example *C vs t* with Haber's exponent = 2	
1.4	Example: *C vs t* with safety and intercept line	**45**
1.5	Example: ingestion:longevity relationship	**47**
2.1	Example: *C vs t* with hockey stick, safety and NOEC line	**81**
2.2	Scatter: relative longevity (to controls) for averaged lifespan	**83**
2.3	Scatter: Haber's *C vs t* for all doses with safety lines	**86**
2.4	(a) scatter: Haber's *C vs t* for all toxic bees at toxic doses	**87**
	(b) scatter: ingestion of AI vs. lifespan for all toxic bees	
SI 2.1	Scatter: mean lifespan per bee per day per dose, Tukey	**95**
SI 2.2	Scatter: mean consumption per bee per day per dose, Tukey	**96**
SI 2.3	(a) line: survivorship % dose-dependence curves	**97**
	(b) line: average consumption dose-dependence curves	
3.1	(a) line: survivorship % over time per treatment	**112**
	(b) line: mean daily consumption per treatment over time	
3.2	(a) histogram: mean lifespan by treatment	**113**
	(b) histogram: mean lifetime syrup consumption by treatment	

3.3 (a) line: mean daily AI ingestion per dosed treatment over time **115**

(b) scatter: lifetime AI ingestion per dosed treatment by lifespan

List of Equations

Eq. 1	$Ct^b = k$	**41, 71**
Eq. 2	$\log(C) = -b[\log(t)] + \log(k)$	**43, 72, 79**

Definitions

Bioaccumulation	Here defined as a substance with slow toxicokinetics
Fast toxicokinetics	A substance that is easily cleared from the body, and harm rendered is anticipated by ingestion
Plant Protection Products (PPPs)	Substances used by agricultural endeavors to shield crops from injury, including pesticides used to ward off pests
Slow toxicokinetics	A substance that persists in the body longer than predicted, thereby having longer to cause harm. Also known as bioaccumulative
Steady state (toxic load)	A state where the injury caused by a substance is proportional to injury expected from ingestion, occurring when a substance is readily cleared, i.e. has fast toxicokinetics
Time-Reinforced Toxicity (TRT)	Bioaccumulation whereby time is the amplifying factor
Toxicant	Synthetic toxin, such as the neurotoxic pesticides neonicotinoids or pyrethroids
Toxic load	The cumulative “injury” caused by ingested toxic substances. Injury occurs even if substances are easily cleared
Toxicokinetics	What happens to an ingested substance in the body – i.e. how is it metabolized or stored
Xenobiotic	A substance foreign to the body or environment

Introduction: The Pace of Neonicotinoid Toxicokinetics

Neonicotinoid pesticides, used by farmers to stave off crop losses by some pests, have been implicated as a cause of the global decline of certain domestic and wild pollinators, notably the honeybee, *Apis mellifera* (Potts et al., 2010; Goulson, 2013; Sánchez-Bayo et al., 2016). One facet of neonicotinoid exposure that remains debated is whether localized interactions between bees and pesticides can be recovered from, i.e. do neonicotinoids bioaccumulate?

Bioaccumulation is defined here as occurring when a substance has slow toxicokinetics. Where a toxic substance persists in the body, it has longer to cause harm. Pesticides with fast toxicokinetics are cleared (i.e. metabolized or released from their binding site) and would be unlikely to pose an unanticipated threat to non-target species (Cresswell, 2016). A pesticide with slow toxicokinetics may manifest symptoms even at trace dietary levels of the active ingredient.

While pesticides with slow toxicokinetics pose a potentially severe threat to non-target species (Borgå *et al.*, 2004), a metric for explicitly determining slow-toxicokinetic bioaccumulation has not yet been incorporated into pesticide regulation (Blacquière & van der Steen, 2017), leaving the market open to harmful substances, which could pose similar ecological risks as the now banned fipronil (Holder et al., 2018). In this thesis, I employed two existing tests for bioaccumulative toxicity, and develop a third.

The first method proposed to quantify the propensity for bioaccumulation is Haber's Law (Gaylor, 2000; Rozman, 2000; Holder et al., 2018). Haber's Law states that changes in dose and time-to-effect should act proportionally in the absence of bioaccumulation, and circumstances where time-to-effect is not predicted by changes in dose would indicate a substance with slow toxicokinetics (Witschi, 1999). Simple dose-dependence studies analyzed using Haber's Law may provide an inexpensive measure of bioaccumulation for regulatory purposes (Holder et al., 2018).

A second method is to measure the total active ingredient ingested compared to lifespan (the ingestion:longevity relationship), which can also be a proxy quantifier of slow or fast toxicokinetics when experiments cover a range of doses (Holder et al., 2018). If a pesticide exhibits fast-paced toxicokinetics and is easily cleared by the body, then lifespan should be predicted by the total amount of pesticide ingested, i.e. have a nonsignificant correlation, because the pesticide is quickly leaving the bee's system and injury would be consistent with units of active ingredient ingested (Holder et al., 2018). However, if lifespan is shorter than predicted by dose, i.e. a negative correlation between lifetime ingestion of pesticide and longevity, it would indicate that less pesticide than predicted was needed to shorten lifespan, as would be expected of substances with slow toxicokinetics (Holder et al., 2018). Thus, the ingestion:longevity relationship provides an additional proxy for toxicokinetic pace.

In addition, I propose to use a novel third method: pulse-exposure experiments, which use alternating periods of exposure to dosed and clean feeder syrup as a

method for determining the pace of toxicokinetics. Substances with slow toxicokinetics would not benefit from depuration in the intervals between pulsed periods of clean-syrup exposure, as they persist in the body even if the test subject is not actively ingesting the pesticide, whereas substances with fast toxicokinetics would be cleared in the rest periods of the pulse when longevity is used as the test endpoint. These differences in toxicokinetics would manifest as differences in longevity amongst exposed bees.

A pesticide that has corroborating quantifiers for all three metrics - Haber's Law, ingestion:longevity correlation and pulse-exposure experiments provides evidence of the relative toxicokinetic pace of that pesticide, which allows confident inferences into the likelihood for the substance to bioaccumulate. Thus, these frameworks could be useful tools in the regulatory testing of pesticides.

In summary, this thesis will examine the issue of bumblebee recovery from neonicotinoid exposure through (i) a literature review assessing the toxicokinetics of neonicotinoids, why slow-toxicokinetic bioaccumulation it is important to test for, and how such tests may be conducted; (ii) an experimental paper aimed at testing neonicotinoid toxicokinetics in bumblebees using the Haber's Law and ingestion:longevity methods; and (iii) an experimental paper aimed at examining toxicokinetics through pulse-exposures, which is also a more field-realistic uptake and recovery scenario.

Chapter 1: Bioaccumulative Toxicity and Regulation of Neonicotinoids: A Review

1.1 Abstract

Neonicotinoids are a class of pesticides that are used worldwide to prevent damage to crops from biting or sucking pests, but that have recently come into focus as a potential source for decline in non-target pollinating species, such as bees. While many studies have been conducted to assess the degree of impact neonicotinoids have on non-target species, a spectrum of results has created uncertainty as to the interaction between these xenobiotics with bees. One main source of debate is whether these pesticides can be quickly cleared by bee species (i.e. fast toxicokinetics) when ingested or if they instead persist in bee bodies (slow toxicokinetics), which could result in a stronger degree of harm than anticipated by exposure through bioaccumulative toxicity. Pesticides exhibiting bioaccumulative toxicity warrant strict regulation, however current regulatory testing methods may not identify slow-toxicokinetic bioaccumulation. A review of the literature relating to toxicokinetics of neonicotinoids and the evidence of their interactions within bee species reveals a likelihood that neonicotinoids have fast toxicokinetics in bee species and are unlikely to exhibit bioaccumulative toxicity. However, the need to easily identify and quantify the pace of toxicokinetics and the potential for bioaccumulative toxicity remains a gap in regulatory procedure, which continues to rely on acute-paradigm testing (i.e. 48-hour LC_{50} and NOECs). This gap may be resolved with the use of chronic paradigm testing, such as dose-dependence Haber's Law studies, explained here and implemented in a further chapter.

1.2 Introduction: Bioaccumulative Toxicity

Neonicotinoids are neurotoxic pesticides that are used globally to fight off crop damage due to sucking and biting pest insects (Oerke, 2006; Cooper and Dobson, 2007). However, neonicotinoids have been implicated in the widespread decline in non-target insects, including charismatic and utilitarian bee species (Goulson, 2013; Blacquière and van der Steen, 2017). The threat to bee species is compounded by the potential for these substances to bioaccumulate, which not only remains contentiously debated in the scientific literature on neonicotinoids (Cresswell *et al.*, 2014; Rondeau *et al.*, 2015), but also has yet to be accounted for in regulatory laboratory testing (Holder *et al.,* 2018).

Bioaccumulation occurs when only some or none of the ingested toxic substance is cleared (i.e. digested, excreted or released from the binding site) by an organism before the next uptake (Walker *et al.*, 2012). Here I define 'bioaccumulation' as being produced by a substance that has 'slow toxicokinetics', i.e. it persists in the body longer than normal, thereby having more time to impart harm (Walker *et al.*, 2012; Holder *et al.*, 2018). To best understand the threat posed by slow-toxicokinetic bioaccumulation, consider an adult human consuming a sip of alcohol daily for their entire life. Typically, the individual exposures are eliminated and leave little long-lasting effects, despite the cumulative 'lifetime' exposure being extremely high. However, consider if alcohol was not cleared between uptakes. A single dose of alcohol equivalent to the cumulative exposure of sips over several weeks would be enough to kill an adult human, while the accumulated ingestion would result in death unanticipated by

the intake of small sips. This accumulation, however, would be missed by an acute testing window (as in the 48-hours often used for regulatory testing), as the effects of sustained ingestion of small daily sips of alcohol are unlikely to manifest in such a small timespan (Walker *et al.*, 2012). The same principles apply to pesticide exposure. Trace exposures of pesticides that are cleared due to fast toxicokinetics likely do not pose the same threat as bioaccumulating pesticides with slow toxicokinetics (Holder *et al*., 2018). However, the current paradigm of acute testing is liable to miss the severe effects of bioaccumulating substances. Only chronic testing (ideally for the lifespan of the organism) could reliably catch chronic bioaccumulation effects (Cresswell 2018).

However, it is important to distinguish between injury and bioaccumulation, as they are not equal. Injury may occur without being indicative of bioaccumulation, i.e. loss of lifespan from pesticide exposure does not mean the pesticide is bioaccumulative, only that the pesticide imparted enough cumulative injury to be lethal. With bioaccumulative substances, the manifestation of cumulative injury (i.e. loss of lifespan) occurs faster than anticipated by exposure (Walker *et al.,* 2012). The cumulative injury to an organism incurred by lifetime (or experimental) exposure is known as ‘toxic load’ (Walker *et al*., 2012). Toxic load accounts for the total amount of harm rendered from pesticide uptake, even with clearance, as some injury or effect is anticipated from pesticide exposure even if the pesticide is being cleared – just as continued ingestion of alcohol still damages the liver, even as the alcohol is fully excreted from the system. Thus, sustained pesticide exposure can still render injury without bioaccumulating in the system of the organism; when exposure to injury and ability to recover from the injury (i.e.

liver detoxification from small doses of alcohol), are balanced, this is known as ‘steady-state’ (Walker *et al*., 2012). In steady state, the amount ingested is cleared before the next uptake, causing equilibrium of in-body concentration and thus injury commensurate to dose ingested (Walker *et al*., 2012). However, without full clearance before the next uptake, it would be expected to see toxicity effects magnify over time and so become increasingly disproportionate to the individual amounts consumed with continued exposure, a bioaccumulative characteristic also known as Time Reinforced Toxicity, TRT (Holder *et al*., 2018). With TRT, length of exposure becomes critical, as time is a factor amplifying the rate of toxic injury (Holder *et al*., 2018). It is therefore imperative that pesticides that may exhibit TRT are properly regulated to prevent unexpected and undue harm from realistically sustained exposure, which can only be soundly assessed by chronic testing schemes (Cresswell 2018).

To help answer the debated question of toxicokinetics in neonicotinoids, and how they could be tested for, this review aims to: (i) assess what neonicotinoids are and why they are used; (ii) evaluate the environmental threats neonicotinoids may pose, particularly in regard to bioaccumulation in non-target bee species; (iii) review what toxicokinetic evidence exists to inform whether or not neonicotinoids are expected to bioaccumulate; (iv) assess how pesticides are currently regulated; and (v) develop quantifiable metrics for bioaccumulation that could be determined experimentally through chronic testing paradigms.

1.3 The What and Why of Neonicotinoids

1.3.1 Crop Protection

Neurotoxic pesticides are part of the Plant Protection Product (PPPs) toolkit used by farmers to stave off losses as a result of pests, particularly for food crops, where high quality produce and year-round supply, even from seasonal crops, are often expected (Oerke, 2006; Cooper and Dobson, 2007). Pest and pathogens are responsible for roughly 20% of losses to global crops even with the use of pesticides (Oerke, 2006). With a growing human population, the intensification of biofuels, and increasing desertification, enhancing crop yields has become an increasing focus in agricultural industries (Lobell, Cassman and Field, 2009). Furthermore, loss of crop quantity or quality directly impacts the financial security of farmers and others dependent on income from the agriculture industry (Cooper and Dobson, 2007; Cerda *et al.*, 2017). Likewise, pests that damage one harvest of crops often linger and damage subsequent harvests (Cerda *et al.*, 2017). However, it must be addressed that use of pesticides to maintain or enhance yields are only one facet of the global agricultural trade. Food loss and waste due both to producers and consumers (Xue *et al*., 2017), as well as inefficiency and inequality in supply chains (Papargyropoulou *et al.,* 2014), accounts for increased demands for food production that could be reduced by redistribution of resources (Kummu *et al.,* 2012). Moreover, current arable land is able to generate higher yields due to better land management, and new land is continually being cleared for food production (Edgerton, 2009), efforts with their own host of environmental implications (Tilman *et al.,* 2011). Even dietary preferences influence agricultural demands, as increased meat consumption in recent years has accounted for higher need for feed crops (Edgerton, 2009).

While a worldwide overhaul of how food is grown and distributed would help alleviate the pressures that have led to pesticide application, the use of modern pesticides assists farmers in maintaining their livelihoods and meeting yield demands in the current agricultural regime. Most importantly, the positive effects on yield, disease regulation and pest control of pesticides must be weighed against the environmental and human health concerns, including intoxication of non-target organisms (Cooper and Dobson, 2007), that accompany their use.

1.3.2 Neurotoxic Pesticides

In pest insects, exposure to neurotoxic pesticides affects pathways that conduct nerve impulses, leading to paralysis and death (Christen and Fent, 2017). One of the most widely discussed neurotoxic pesticides is imidacloprid (Cresswell, 2011; Goulson, 2013) , which is a neonicotinoid pesticide (Matsuda *et al*., 2001). Neonicotinoids are toxicants (synthetic toxins), artificial analogs of the nicotine in tobacco, *Nicotiana tabacum* (Walker *et al.*, 2012). Imidacloprid, introduced in 1991, was the first neonicotinoid, followed by others, including thiamethoxam, that are particularly effective against Hemiptera, notably planthoppers and aphids (Nauen and Denholm, 2005). Applied as seed coatings, they are systemically distributed to component parts of the plant, including nectar and pollen (Jeschke and Nauen, 2008). Neonicotinoids are chemical mimics of the messenger compound acetylcholinesterase at nicotinic receptors, and their presence leads to incessant, unregulated synapse signaling (Walker *et al.*, 2012). Neonicotinoids come in two classes, divided by their possession of either a nitro group or cyano group that confers a negative charge used in their synaptic disruption (Tomizawa and Casida, 2005).

Other neurotoxic pesticide classes include pyrethroids and phenylparazoles. Pyrethroid pesticides are toxicant analogs of pyrethrin in chrysanthemums, *Chrysanthemum cinerariaefolium* (Walker *et al.*, 2012). Pyrethroids are applied as sprays , and pest management is achieved through contact with residues on plant surfaces (Knowles, 2007). Pyrethroids block sodium channels in insect nervous systems, which also overstimulates nerve impulses (Walker *et al.*, 2012). Phenylparazole pesticides are synthetic systemic pesticides that attack ɣ - aminobutyric acid (GABA) receptors, which control the flow of chlorine ions that temper nerve impulses (Walker et al., 2012). By blocking receptors and reducing ion flow, phenylparazoles also overexcite neurons, leading to paralysis and death (Walker et al., 2012).

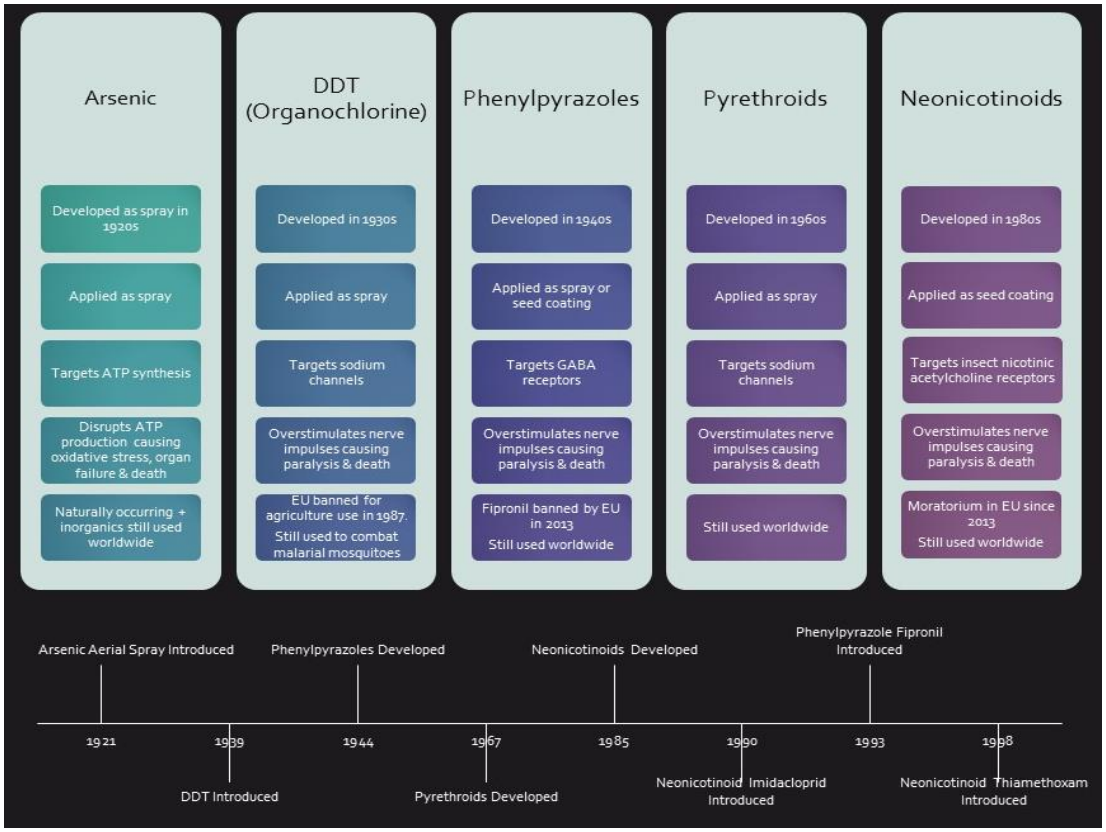

Figure 1.1: *Timeline summary of neurotoxic neonicotinoids, pyrethroids, phenylpyrazoles and DDT, as well as aerially sprayed arsenic.*

1.4 Environmental Concerns Over Pesticide Usage

1.4.1 Environmental Contamination

Despite the potential utility of pesticides in crop management, the widespread environmental ramifications associated with pesticides cannot be disregarded. These span soil and water contamination and injury to various taxa of non-target organisms, including crop pollinators (Goulson, 2013). From this it is evident that a clear concern of widespread pesticide use is the level of selectivity. Chemicals such as arsenic are highly effective lethal agents, but with no selectivity for their target species they kill without restraint and can levy unsustainable costs, even on humans (Cresswell, 2016). Low selectivity pesticides are biochemical generalists (biocides), and can poison many non-target species, including humans, even at small doses (Cresswell, 2016), which necessitates the use of chemicals with higher selectivity for their targets. Another issue is containment. Similar to low-selectivity pesticides, those applied topically, such as sprays or powders, have a higher likelihood of non-target contamination, such as of runoff into nearby water sources and soil (Knowles, 2008). The development of systemic pesticides, somewhat hydrophobic chemicals that are applied by coating seeds to be eventually incorporated into the plant itself, was intended to moderate the risk of pollution by lowering pesticide application rates (Knowles, 2008), although studies report as much as 98% of pesticide coatings still diffuse into the environment, rather than being taken up by the plant (Van der Sluijs *et al.*, 2013). Greater selectivity by targeting facets of biology more unique to the plants and pests will be the key to generating safer and more effective pesticides in the future.

In order to address problems of selectivity and containment, newer classes of pesticides, such as the neurotoxic neonicotinoids, are seed coated and adapted to be more selective for invertebrate receptors (Tomizawa and Casida, 2003). Despite these modifications, environmental concerns and threats have not been fully alleviated (Goulson, 2013; Van der Sluijs *et al.*, 2013; Vanbergen and Garratt, 2013). This is partly due to the emergence of resistance to neurotoxic pesticides, which in turn lowers the efficacy and can promote increased use of the chemicals (Pimentel *et al.*, 1992). The financial and social damage of pest resistance has sparked a variety of research into the mechanisms behind it, including genetic, biochemical and physiological aspects, in order to devise effective new pest interventions and maintain crop yields (Matsuda *et al.*, 2009). Such research also provides important mechanistic insights into the nature of the pesticides and how organisms cope with and overcome exposure to these chemicals (Nauen and Denholm, 2005). Thus far, pesticide resistance is most often linked to adaptations that render the biochemical aspects of the pesticide less active, such as mutations in the receptor sites they bind to, or else physiological adaptations that better fit the individuals to digest and excrete the pesticides without suffering higher mortality rates (Cresswell, 2016). These latter adaptations are amplifications of natural detoxification pathways, thus indicating possible avenues for non-target species to maintain resilience despite exposure to pesticides (Cresswell *et al.*, 2014; Cresswell, 2016).

1.4.2 Non-target Species

Further concern with current neurotoxic pesticides comes from potential effects

on non-target invertebrates. The most notable of these non-target species are bees, which in fact provide a valuable portion of pollination for global agriculture (Van der Sluijs *et al.*, 2013). The reported decline of certain bee populations, notably domestic honeybee and certain bumblebee populations in Europe and North America (Williams and Osbourne, 2009; Potts et al., 2010) has galvanized research into the interactions of various bee species with these chemicals, most particularly the widely applied neonicotinoid pesticides (Cresswell, 2011; Goulson, 2013; Van der Sluijs *et al.*, 2013). Yet scientific studies have not been able to conclusively determine whether or not neonicotinoids are the main cause of falling bee populations, or if they even render small-scale harm, with ardent positions on both ends of the spectrum (Laycock and Cresswell, 2013; Cresswell *et al.*, 2014; Rondeau *et al.*, 2015). These debates concern not only mortality (Cresswell, 2011), but also a variety of sub-lethal effects, including impacts on reproduction, cognition, foraging, olfaction, locomotion, metabolism, immunity and population dynamics, amongst other things (Alkassab and Kirchner, 2017). In fact, the state of pollinator decline has also been questioned as studies such as Aizen & Harder (2009) reported that domestic honeybee populations have in actually increased in recent years. However, the agricultural demand for pollination has reportedly increased more rapidly in the same time span, leaving the glut in pollination resources that originally spurred research into pollinator decline (Aizen and Holder, 2009). Arguably, the only thing made clear by the plethora of bee-neonicotinoid studies is that the exact severity of the interaction remains unclear, particularly at the trace levels of neonicotinoid exposure expected under field-realistic conditions.

1.4.3 Bioaccumulation

One major point of debate is whether or not neonicotinoids are irreversibly bioaccumulative in non-target bee species (Cresswell *et al.*, 2014; Rondeau *et al.*, 2015). Recalling the alcohol analogy, bioaccumulation occurs when the ingested pesticide cannot be cleared before the next uptake, resulting in a build-up within the organism (Walker *et al.*, 2012). A pesticide that does not accumulate in non-target species at field-realistic, trace-exposure levels, and could be recovered from when exposure is terminated (from organismal excretion or storage) would therefore pose only a transient threat. However, a bioaccumulating pesticide could render severe harm with sustained low-dose exposure to trace residues, causing eventual severe intoxication, the mechanism known as TRT. Chronic exposures to a TRT-inducing substance for sufficient periods of time would inherently lead to increased harm or mortality, necessitating particular caution for regulatory approval of bioaccumulative pesticides, even in those present at only trace doses but over large periods of time.

The regulation of toxicants capable of generating TRT is particularly daunting, as it requires testing of extended exposures (chronic paradigm) to detect, and most guidelines are based on short exposures, the acute paradigm; i.e. the minimum levels of chemicals to induce effect (No Observable Effect Concentration, NOEC) or to reduce longevity (Lethal Concentration for 50% to Die, LC_{50}) in a set time frame, normally 48-hours (Cresswell, 2018). This results in the possibility of pesticides being applied to crops that are safe for non-target species at acute trace levels, but that can accumulate to dangerous quantities in realistically sustained exposures, as seen with the now banned fipronil (Holder *et al.*, 2018).

Knowing whether a pesticide can be naturally detoxified by non-target bee species, or whether the pesticide is bioaccumulative, is essential for proper regulation, and yet no consensus has been reached as to the capacity for TRT in neonicotinoids.

1.5 Examining Bioaccumulation of Neonicotinoids

An examination of pesticide clearance mechanisms may help shed light on whether slow or fast toxicokinetics are anticipated in bees exposed to neonicotinoids. Pesticide detoxification is most often attributed to enzymatic metabolism of the toxicant that allows for excretion, although release of the pesticide from the receptor site due to weak bonds or low affinity is also a requirement for successful clearance (Bass *et al.*, 2015; Cresswell, 2016).

1.5.1 Enzymatic Metabolism

Living organisms are typically equipped with natural detoxifying systems that combat the uptake of dietary xenobiotics, such as pesticides. In resilient individuals, sustained uptake will lead inevitably to safe storage or excretion of metabolites, with a varied array of enzymatic metabolic mechanisms in place to reduce the pesticide to an excretable or storable form (Walker *et al.*, 2012). The cytochrome P450s (CYPs) family of enzymes are found in all kingdoms of life, and are vital to a variety of internal systems, including detoxification of foreign substances (Olsen, Oostenbrink and Jørgensen, 2015). CYPs are monooxygenases with heme groups, which they employ in oxidative reactions to render xenobiotics, including neurotoxic pesticides, less toxic, more soluble and easier to store or excrete (Walker *et al.*, 2012; Olsen, Oostenbrink and

Jørgensen, 2015). CYPs are considered one of the major enzymatic detoxifying systems for coping with pesticide uptake (Rauch and Nauen, 2003), and their induction/overexpression is most often the cause of pesticide resistance (Nebert and Russell, 2002; Iwasa *et al.*, 2004; Zhang *et al.*, 2016).

In silico docking studies adapted from pharmacology are computer-based 3-D models of proteins which help identify target sites of pesticides, aiding the understanding of their toxicity, or potential sites whose modification can confer resistance (Roncaglioni *et al.*, 2013; Liu *et al.*, 2015). Such studies conducted on CYPs show the proteins have six active sites, which provide places for pesticide docking, and sources for regulating gene expression (Liu *et al.*, 2015). Gene assays and synergistic studies with known substances that inhibit CYPs, such as piperonyl butoxide (PBO), are used in conjunction with pesticides to help determine whether CYPs are involved, or if an overexpression of detoxifying enzymes already exists (Iwasa *et al.*, 2004; Liu *et al.*, 2015).

It has long been established that bees employ CYPs in detoxification. A 1992 study showed CYPs induction in honey bees, *Apis mellifera,* within 48-hours of exposure to xenobiotics, with a peak monooxygenase activity after nine days of continued dosing (Kezić, Lucić and Sulimanović, 1992). Such insights are especially relevant to low-dose chronic exposures characteristic of pesticide applications (Cresswell, 2016), and may explain post-exposure recovery (I.e. recuperation) (Laycock and Cresswell, 2013). CYPs are particularly critical to honey bee detoxification of pyrethroids, and are likely responsible for the inconsistent levels of toxicity between types of pyrethroids (Johnson *et al.*, 2006).

A study of P450-inhibitors and neonicotinoids, including imidacloprid, shows P450s are an important mechanism for neonicotinoid detoxification in honey bees, reducing their susceptibility to them (Iwasa *et al.*, 2004). However, the role of CYPs in imidacloprid detoxification is still under scrutiny and some suggest detoxification may be linked to other mechanisms of detoxification and clearance (Liu *et al.*, 2005). Regardless, the overall implication from these findings are that bees likely engage metabolic enzymes to guard against neonicotinoid uptake, which likely contributes to the reports of clearance by both proxy (i.e. brood production) and body residue measurements of neonicotinoids in honeybees (Cresswell *et al.*, 2014) and bumblebees (Laycock and Cresswell, 2013; Thompson *et al.*, 2015) within 24-hours for *Apis mellifera,* and 48-hours for *Bombus terrestris.* Mutations and overexpression of these enzymes seen in other insect species as a result of sustained pesticide exposure might further allow bee species to maintain resilience in the face of continued applications of neonicotinoids (Kezić, Lucić and Sulimanović, 1992; Liu *et al.*, 2011, 2015; Schmehl *et al.*, 2014).

1.5.2 Receptor Site and Binding Reversibility

With advancements in receptor site modeling and *in silico* docking studies, where and how neonicotinoids interact with their target sites has become clearer (Matsuda *et al.*, 2009), and also how these sites may confer resistance (Shimomura *et al.*, 2006). Neonicotinoids bind as ligands to the nicotinic acetylcholine receptors (nAChRs), which are found in invertebrate nervous systems and vertebrate neuromuscular junctions (Matsuda *et al.*, 2001). Insect nAChRs are varied, extensive, and despite massive strides in the field, their

function and design are not fully understood (Tomizawa and Casida, 2005), though it is known that neonicotinoids bind with their electronegative end (from their nitro or cyano group) to selectively depolarize and block nerve synapse transmission in nAChRs (Matsuda *et al.*, 2001; Tomizawa and Casida, 2005). The main points of interest of receptor sites concerning potential bioaccumulation at the target site are affinity of neonicotinoids to their binding site and the strength of the bonds between neonicotinoids and their target receptors.

Neonicotinoids have been found to bind with high affinity to invertebrate nAChRs, as demonstrated by competitive binding or displacement studies using α-bungarotoxin, α-BTX (Tomizawa and Casida, 2005). α-BTX is derived from snake (*Bungarus multicinctus)* venom, and has a high selectivity, affinity and saturation of acetylcholine receptors (Freeman, Schmidt and Oswald, 1980), so it is often used as an aid for studying the relative binding ability of other acetylcholine agonists. These studies reveal the complex relationship between binding affinity and toxicity. For instance, one α-BTX study shows honey bees and houseflies have the same receptor affinity for neonicotinoids, despite having drastically different degree of susceptibility to impact, with honeybees having lower susceptibility to the neonicotinoid acetamiprid (Iwasa *et al.*, 2004). The difference in toxic effects between the organisms, despite equal affinity for the binding site, may relate to differences in metabolism, or could point to differences in the receptors themselves. NAChRs provide many, varied binding sites for neonicotinoids within an insects nervous systems, from cognition centers to controls of muscular movements, so different pesticides may confer different modes (or observable levels) of toxicity by targeting different receptor sites

(Cresswell, 2016). Even toxicants in the same family can have different modes of action, as demonstrated by imidacloprid sharing a binding site with α-BTX that thiamethoxam shows low affinity for (Wiesner and Kayser, 2000). This suggests that either thiamethoxam is a low affinity binder to nAChRs (which given the reported potency of thiamethoxam and the known action of neonicotinoids is unlikely (Maienfisch *et al*., 2001)), or it has a different array of isoreceptor binding sites (suggested to be neuromuscular junctions) than imidacloprid, whose various receptors are concentrated in olfactory, learning and memory centers (Buckingham *et al.*, 1997; Wiesner and Kayser, 2000; Williamson, Willis and Wright, 2014). Ultimately what this tells us is that future studies are needed to identify the scope and nature of the diverse neonicotinoid binding sites.

Potentially more important in conferring toxicity than affinity for target sites is the strength of the bonds between pesticides and their targeted receptors. Substances such as α-BTX , which attach to receptors with strong covalent bonds, will likely persist in the system even if exposure is an isolated incident, as the ligand bond is unlikely to be disrupted (Robinson et al., 1975). Substances that bind to ligand receptors with strong covalent bonds are known as irreversible binders (Robinson et al., 1975). Continued exposure to a chemical that irreversibly binds would eventually block enough receptors to cause death. Reversible bonds, however, are likely to occur with weak bonds, such as hydrogen, van der Waals interactions or ion bonds, that allow relatively easy detachment of the chemical ligand from its receptor (Takahashi, 2011). In fact, the reversibility of ligand-binding between acetylcholine and its receptor is essential for the proper functioning of nAChRs. Evidence suggests that

neonicotinoids mimic these natural ligands by attaching to nAChRs with weak hydrogen or electrostatic bonds (Kezić, Lucić and Sulimanović, 1992; Bao *et al.*, 2016), a mechanism that allows for recovery of receptors after a cessation of exposure (Cresswell *et al.*, 2014). Modern risk assessments acknowledge that neonicotinoids are reversible binders (Brandt *et al.*, 2016). Equally enlightening are studies that show reversibility of sub-lethal effects in honey bee (Cresswell *et al.*, 2014) and bumblebee species (Laycock and Cresswell, 2013) exposed to imidacloprid. However, having binding reversibility does not prevent toxicity, or even death, it merely provides evidence that recovery from sub-lethal exposure may be feasible and provides a necessary requirement for 'steady-state' toxicokinetics.

Creating or enhancing reversibility of ligand-receptor binding is a source of emerging pest resistance. Point mutations alter binding affinities to either prevent or weaken bonds between the neuroreceptor and the pesticides, reducing their toxicity and allowing recovery from their uptake (Cresswell *et al.*, 2014; Cresswell, 2016). In fact, nAChR mutations in the cotton aphid, *Aphis gossypii* (Kim *et al.*, 2015; Chen *et al.*, 2016), *N. lugens* (Liu *et al.*, 2005, 2006), the peach potato aphid, *Myzus persicae* (Beckingham *et al.*, 2013), *Drosophila melanogaster* and *Leptinotarsa decemlineata* (Matsuda *et al.*, 2009) have all been linked to neonicotinoid resistance. This means new pesticides will need to be developed as increasing resistance weakens the efficacy of neonicotinoids (Krupke *et al.*, 2017). Fortunately, honeybees have been found to have one of the most diverse arrays of nAChRs receptor subunits in any known insect species, providing opportunity to devise effective neurotoxic pesticides with low selectivity for certain

bee species, particularly honeybees (Jones *et al.*, 2006). The diversity of nAChR receptors also hints at the likelihood that future mutations in this vast web of nAChRs have or will result in lowered binding affinity of neonicotinoids in bee species in a process of evolutionary adaptation.

1.5.3 Evidence from Neonicotinoid-Bee Studies

Despite evidence from CYP-450 metabolism and weak ligand bonding of neonicotinoids indicating reversibility, there is still no consensus on whether bioaccumulation and TRT occur in neonicotinoids, especially imidacloprid (Cresswell *et al.*, 2014; Sánchez-Bayo, Belzunces and Bonmatin, 2017). Cresswell et al. (2014) did a pulsed-exposure experiment, showing bumblebees given high doses of imidacloprid (98 ppb, compared to a field realistic range of <10 ppb (Cresswell, 2011)) could recover if exposure was terminated, negating the idea that irreversible binding could be the basis of TRT in exposure to imidacloprid (Cresswell *et al.*, 2014). Sanchez-Bayo et al. (2017) alternatively found evidence of imidacloprid bioaccumulating, however, they only continually exposed winter honey bees to 98 ppb (Sánchez-Bayo, Belzunces and Bonmatin, 2017). This study cited TRT occurred on the basis that imidacloprid was present in the fat residues of exposed bees (Sánchez-Bayo, Belzunces and Bonmatin, 2017). Fat residues (a classic site for bioaccumulation in lipophilic toxicants) in wild bees have also been said to contain neonicotinoids, although the exact chemical components of the residues were undetermined (Feng *et al.*, 2016). However, the paucity of evidence linking increased bee mortality with field-realistic (i.e. low-dose, sustained) exposures to neonicotinoids further support neonicotinoids as non-bioaccumulating in bee species (Tasei, Lerin and Ripault,

2000; Schmuck *et al.*, 2001; Schmuck, 2004; Faucon *et al.*, 2005; Alkassab and Kirchner, 2017), or at least as bioaccumulating at insignificant rates. While contentious, it is nevertheless plausible that field realistic neonicotinoid doses (0.7 -10 ug/L for imidacloprid (Cresswell, 2011)) may be low enough for some of many bee species to detoxify without directly incurring mortality. However, lack of reduced longevity does not negate the potential existence of sub-lethal effects from neonicotinoid exposure on other endpoints, or even the possibility of a low level of accumulation.

Some disparity between lifetime consumption and anticipated effects have been the source of doubt in pesticide ecotoxicology studies. However, if the study species has an inherently short lifespan, and if a pesticide does not bioaccumulate, then it would be logical that exposure times, restricted by lifespan, are too limited for pesticide ingestion to manifest as observable or significant effect. In the wild, the lifespan of a foraging honeybee is less than seven days (Khoury, Myerscough and Barron, 2011), and bumblebees forage for 20 days (Doums *et al.*, 2002). Small exposures in such short timescales are likely to have negligible effect, if the pesticide is being cleared between exposures and the organism does not live long enough for sub-lethal effects to become severe, even if they still impact fitness, i.e. there is non-zero toxic load ‘injury’. Recalling the human-alcohol analogy, it is probable that bees chronically exposed to realistically low-level doses of non-bioaccumulative neonicotinoids would not exhibit increased mortality, given that clearance is continuous (i.e. steady-state in-body concentrations).

1.6 A Note on Secondary Metabolites and Sub-lethal Effects

Even if neonicotinoids may not cause bioaccumulative toxicity, ingestion of the xenobiotics could still damage non-target species. The detoxification process itself may render harm, either because the metabolic degradation of pesticides requires an alteration of their chemical structure in order to store or excrete them, creating mobile intermediaries which may come with their own damaging properties, or because of the energetic costs of detoxification. Moreover, the pesticides may not lead directly to death, but they can manifest a multitude of sub-lethal effects that can impair fitness, and therefore they should be briefly addressed.

1.6.1 Secondary Effects of Enzyme Metabolites

While detoxification may offset uptakes, it may come at a cost. The cost is both energetic, in terms of resources expended to induce enzymes and to store/excrete, and from the by-products of these transformations themselves (Walker et al., 2012). Most simply, in order to store/excrete a pesticide, the toxicant's molecular makeup is altered, sometimes creating by-products or intermediates that do more damage than the original xenobiotic (Walker et al., 2012). For instance, the pathway used by CYPs to reduce the pesticide to an iron-oxo (or similar) compound may actually create intermediaries which are more toxic than the pesticide itself, known as 'metabolic activation' (Simon-Delso *et al.*, 2015). One concern is the oxidated by-products produced when metabolizing xenobiotics, which are linked to mutations and cancer (Walker et al., 2012; Simon-Delso *et al*., 2015).

Hydrophobicity, used to describe molecules that repel water, is related to membrane penetrability, and therefore toxic activity, regardless of binding affinity (Yamamoto *et al.*, 1998). Hydrophobic molecules less than 800 MW (Molecular weight) are better adept at infiltrating lipid layers, such as membranes, often leading to higher activity of the pesticide (Yamamoto *et al.*, 1998; Walker *et al.*, 2012). Hydrophobicity is in fact postulated to be more important than binding affinity in regards to pesticide toxicity (Yamamoto *et al.*, 1998). Those metabolites, which are intentionally reduced in order to make them more fat-soluble for storage are likely to have increased hydrophobicity, and thus more potential toxicity. However, most detoxification activity involves oxidation of xenobiotics.

The metabolites that result from CYPs digestion of cyano-neonicotinoids appear to be relatively inert to honeybees, whereas metabolites of the more commercially relevant nitro-substituted neonicotinoids, such as imidacloprid and thiamethoxam, have been said to produce damaging hydroxyl metabolites (Iwasa *et al.*, 2004). Imidacloprid was reported as three orders of magnitude more lethal than acetamiprid and thiacloprid, which are both cyano-substituted neonicotinoids, despite the fact that both groups of neonicotinoids have the same binding affinity for receptor sites (Iwasa *et al.*, 2004). This suggests the metabolites produced during honeybee digestion of neonicotinoids could propagate toxicity of the substance. In fact, metabolic products of imidacloprid have been found in higher residues than imidacloprid itself in Canadian bees, and the oxidative reductions have been linked to heightened toxicity (Codling *et al.*, 2016). However, even highly toxic metabolites are themselves only intermediates

in a breakdown pathway, which means that metabolic activation may be followed by metabolic inactivation eventually (Walker et al., 2012).

1.6.2 Sub-lethal Effects of Neonicotinoids

In the case of bees and neonicotinoids, evidence does exist showing continued uptake may confer detriments to fitness without directly enhancing mortality, known as sub-lethal effects (Alkassab and Kirchner, 2017). Wild and managed bees require high-functioning cognitive abilities in order to best forage and survive in complex or changing landscapes, and brain function is highly vulnerable to disruption and thus sub-lethal effects of neurotoxic pesticides are possible, and their implications could be critical (Klein *et al.*, 2017). Sub-lethal effects are manifestations of toxic 'injury'.

Sub-lethal effects are varied and wide-ranging, and may include impacts on cognition (Tison, 2016; Alkassab and Kirchner, 2017), foraging (Colin *et al.*, 2004; Schneider *et al.*, 2012; Karahan *et al.*, 2015), immunity (Brandt *et al.*, 2016; Coulon *et al.*, 2017), colony dynamics (Gill, Ramos-Rodriguez and Raine, 2012; Larson, Redmond and Potter, 2013; Feltham, Park and Goulson, 2014; Sandrock *et al.*, 2014; Rondeau *et al.*, 2015), longevity (Suchail, Guez and Belzunces, 2001), fecundity (Laycock *et al.*, 2012; Elston, Thompson and Walters, 2013), locomotion (Lambin *et al.*, 2001; Teeters *et al.*, 2012; Williamson, Willis and Wright, 2014) and olfaction (Han *et al.*, 2010; Tan *et al.*, 2015; Peng and Yang, 2016), which have all been linked neonicotinoids, although with many caveats related to exposure, administration, timing, and doses (Alkassab and Kirchner,

2017). In fact, of the studies examined, compelling results in field-realistic doses of neonicotinoids were most often concerned with cognition (Tison, 2016) or olfaction/foraging (Karahan *et al*., 2015; Fig. 1.2), and as imidacloprid in particular is known to target cognition receptors (Buckingham *et al.*, 1997; Wiesner and Kayser, 2000; Williamson, Willis and Wright, 2014), it would be unsurprisingly to find that chronic exposure to neonicotinoids had impact on such endpoints. Of note were studies which found effects at field-realistic doses only when neonicotinoids interacted with another stressor, i.e. Nosema (Alaux *et al.*, 2009), or Chronic bee paralysis virus (Schurr *et al*., 2017), as co-exposure with neonicotinoids appears to cause additive effects on both longevity and sub-lethal endpoints in these studies. Given that wild (or free-foraging domestic) bees are likely to be exposed to a variety of environmental contaminants and stressors, the negative synergistic capacity of neonicotinoids may help explain declines not accounted for by field realistic doses of these pesticides alone. Therefore, the many studies that show no effects on neonicotinoids for these same endpoints (Fig. 1.2), namely: foraging (Karahan *et al.*, 2015; Thompson *et al.*, 2016), immunity (Alaux *et al.*, 2010), colony dynamics (Faucon *et al.*, 2005; Cutler and Scott-Dupree, 2007; Franklin, Winston and Morandin, 2009; Morandin and Winston, 2009; Pohorecka *et al.*, 2012; Pilling *et al.*, 2013), longevity (Tasei, Lerin and Ripault, 2000; Schmuck *et al.*, 2001; Schmuck, 2004; Faucon *et al.*, 2005; Cutler and Scott-Dupree, 2007; Aliouane *et al.*, 2009), fecundity (Cutler and Scott-Dupree, 2007; Pohorecka *et al.*, 2012; Laycock and Cresswell, 2013; Laycock *et al.*, 2014), locomotion (El Hassani *et al.*, 2008; Aliouane *et al.*, 2009), and olfaction (Bortolotti *et al.*, 2003; Williamson, Baker and Wright, 2013; Alkassab and Kirchner, 2016), help provide evidence that neonicotinoids alone

are not likely the sole generator of widespread pollinator declines, but could be a contributing factor along with a host of other stressors (Brown and Paxton, 2009), such as disease (Blanken, van Langevelde, and van Dooremalen, 2015), climate change (Parmesan *et al*., 1999; Kerr *et al.*, 2015), other agrochemicals such as fungicides (Vanbergen and Initiative, 2013; Kiljaneck *et al.,* 2017), or habitat loss (Potts *et al*., 2010)

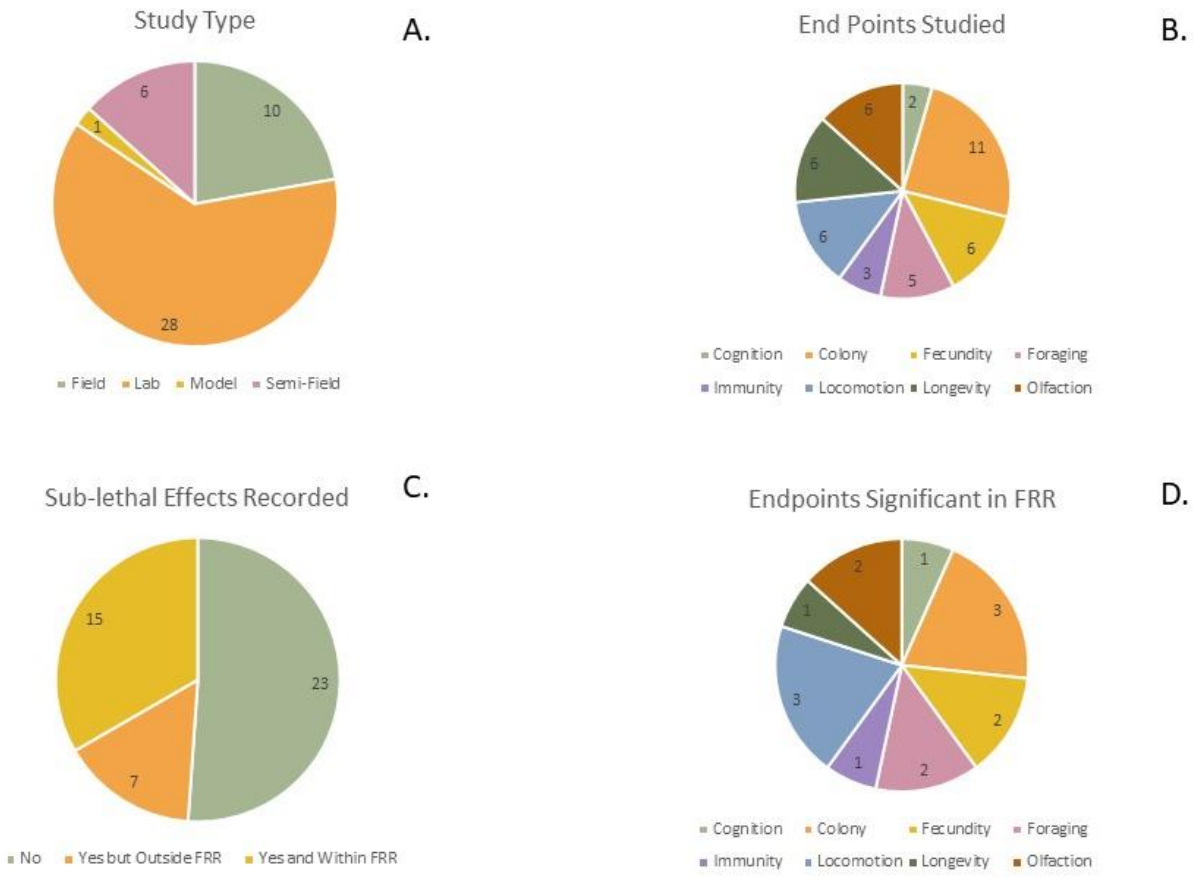

Figure 1.2: *Summary of papers examining sub-lethal effects of neonicotinoids based on (a) type of study (N=45). (b) endpoint examined (N=45), (c) whether the study found sub-lethal effects were induced by neonicotinoid exposure and if so, whether the sub-lethal effects were recorded in the field-realistic range (FRR, N=45), and (d) endpoint studied for experiments where sub-lethal effects were recorded in the FRR (N=15).*

This brief review represents a subset of the extensive studies and the potential myriad of sub-lethal effects that have been examined for bees exposed to neonicotinoids. The main lesson gained is that no inevitable sub-lethal effects attributable to field realistic doses of neonicotinoids has yet to be determined, but sustained exposures under environmentally realistic conditions may be rendering harm to bees that is not currently accounted for by current acute testing regimes and should continue to be examined, especially in conjunction with other stressors.

1.7 The Status of Bioaccumulative Toxicity (TRT) in Current Pesticide Regulation

While bioaccumulative toxicity is a challenging and critical problem for pesticide regulation, it has been largely ignored in regulatory standards (EFSA, 2012b). In fact, uncertainty over the environmental effects of neonicotinoids, including their potential to accumulate, has led to a provisionary ban of usage in the EU, although it is worth noting that some initial results of the ban appear to indicate neonicotinoids were not the likely cause of pollinator mortality (Blacquière and van der Steen, 2017). Despite the controversy of modern pesticides arising mainly from their association with the decline of non-target species and contamination of the environment (Goulson, 2013; Van der Sluijs *et al.*, 2013; Vanbergen and Initiative, 2013), insufficient industry standards for assessing pesticide safety are also blamed for neglecting the potency of unsafe chemicals in the past (Holder *et al.*, 2018). Most safe pestcide doses are determined by 48 or 72-hour laboratory studies that search for the single-exposure concentration

(dose) that kills 50% of exposed subjects in that time – or the LC_{50} (LD_{50}), coupled with a statistically determined no-effect concentration, NOEC (Walker *et al.*, 2012). While 48-hour LC_{50} studies provide a quick assessment of the relative lethal toxicity of a substance, they have three weaknesses, as follows. First, they do not reflect environmentally relevant exposures, as in the field, pesticides are administered at chronic low doses. Second, they do not address time reinforced toxicity (TRT). Third**,** they therefore do not produce biologically relevant NOECs. To adequately assess pesticide safety, a measure of LC_{50} must be supplemented with a a quantifiable representation of TRT, and a reliable, biologically based NOEC, which I propose deriving from Haber's Law exponent.

1.7.1 LC_{50}s

LC_{50}s are used to summarize dose-response curves of mortality of an organism in reaction to a toxic substance (Chapman *et al.*, 1996). These metrics provide a simple way to rank the relative lethality of substances, with low values corresponding to highly toxic substances. While a useful tool for quick reference of relative toxicity, LC_{50}s may not be the best way to summarise dose-response data.

The first failing of LC_{50}s is that they are bound to the laboratory conditions used to calculate them (Cresswell 2018). LC_{50}s inherently represent only acute, high-concentration exposures of pesticides in controlled conditions. Yet, barring accidental spills, pesticides used under good agricultural practice are dessiminated at low concentrations (Devillers and Pham-Delègue, 2003) and realistic exposures are sustained over many days. The disparity between

laboratory findings and environmental realism must be bridged in order to adequately regulate pesticides and protect bee health.

One such factor lacking in LC_{50}s is their inability to indicate TRT (or bioaccumulative toxicity), which arises as the concentration of a substance in the organism increases with time. In non-bioaccumulative pesticides, each uptake is cleared either by excretion or reversibility of receptor binding sites before the next uptake, creating a continual clean slate (Liu *et al.*, 2006). With TRT, these detoxification mechanisms fail to clear any or all of the toxicant, leading to rising incidence of toxic effects (Walker *et al.*, 2012). As such, even 72-hour studies do not represent realistic exposure times/lifespans in many species, including bees - with foraging worker honey bees living on average seven days (Khoury, Myerscough and Barron, 2011), and foraging bumblebees as long as 20 days (Doums *et al.*, 2002). When determined under the acute-exposure paradigm, 48-hour LC_{50}s may neglect TRT by simply not running long enough to enable expression of maximum toxicity in a bioaccumulative substance. Such flaws could be responsible for substances, such as organochlorines (Chopra, Sharma and Chamoli, 2011) and fipronil (Holder *et al.*, 2018), being approved for use commercially despite their capacity to generate TRT.

1.7.2 NOECs

NOECs are statistical extrapolations of the dose-response curves, used to find the highest dose that does not have a significant increase in mortality from the control (Chapman *et al.*, 1996). As such, NOECs are subject to variance due to differences in replication, computer software, statistical tests chosen, alpha

levels, laboratory protocols, and controls (Chapman *et al.*, 1996; Chapman, Caldwell and Chapman, 1996). They also note that if statistical tests are low powered, this leaves little protection from drawing the false conclusion that a particular dose of pesticide is safe when it actually is not (Chapman *et al.*, 1996). The short time span of the acute paradigm and its inherent failure to address TRT makes it likely that LC_{50}s derived in this way also misrepresent safe doses derived from so-called 'safety factors' such as 10% LC_{50} (NOECs). Indeed, for these reasons Chapman et al. (1996) warned against the use of NOECs in regulation over 20 years ago (Chapman, Caldwell and Chapman, 1996). Thus, regulators are potentially prone to wrongly deem a pesticide safe than shut down production on a harmless substance. This is particularly worrying when NOECs are used as protection thresholds (Cresswell, no date).

Another concern is that NOEC values increase (i.e. overrepresent safety) as the accuracy of an experiment decreases, meaning less rigorously determined NOECs deem higher (potentially unsafe) doses to be 'safe' (Chapman *et al.*, 1996). Furthermore, as they are subject to statistical sensitivity, and fail unless the dose-response relationship is monotonic, NOECs cannot provide a reasonable measure of no-effect (Chapman *et al.*, 1996; Chapman, Caldwell and Chapman, 1996). In fact, NOECs can in some cases be 10-30% more toxic than 'no effect' (Warne and van Dam, 2008). Yet some regulators still seek a NOEC in order to determine reasonable pesticide applications. The concept of a NOEC may not be the issue, but rather the unsound way in which its value is determined (Warne and van Dam, 2008). A NOEC grounded in biology, rather than in shaky statistics could help resolve regulatory inconsistencies. Below I will outline a new

way to estimate a NOEC using a basis in Haber's Law, which will be implemented in Chapter 2.

1.8 The Chronic Paradigm - Using Haber's Law to Quantify Potential for Bioaccumulative Toxicity

1.8.1 Haber's Law

While a knowledge of the toxicokinetics of pesticides is useful in understanding bioaccumulation (EFSA, 2012b), metabolic and receptor-site systems are complex, subject to evolutionary change, poorly understood and difficult to map. Haber's Law, however, is tailor-made to aid in the determination of TRT and a biologically relevant NOEC (Gaylor, 2000; Rozman, 2000; Holder *et al.*, 2018). Haber's Law is a simple equation that has been successfully used in inhalation toxicity and aquatic toxicology since its inception over 100 years ago (Witschi, 1999; Rozman, 2000). The simplest, conventional form of Haber's Law states that if a chemical concentration is doubled, the time for 50% to die (or mean death) should halve (Rowland, Benet and Graham, 1973), and can be calculated as:

$$Ct^b = k$$

Eq. 1

With *C* representing concentration, *t* exposure time, and *b* the Haber's exponent. The conventional, monotonic form of Haber's law occurs when *b*=1, whereby changes in exposure and consumption are inversely proportional in order to maintain an equivalent toxic effect. In fact, Haber's Law can be considered a

family of curves generated from the range of doses that vary by the value of *b*, and thus the family of curves can be used to analyze the degree to which time-to-effect is amplified by changes in dose, *C*, and thereby the degree to which a substance is likely to bioaccumulate (see Eq. 2).

Haber's Law is the ideal equation for assessing safety of substances that are applied at consistent rates (Gaylor, 2000) and concentrations (Witschi, 1999). Used to maintain agricultural yields and liveliehoods, seed-coating pesticides (such as neonicotinoids) are model candidates for Haber's Law experiments when they are disseminated at relatively consistent exposure concentrations through direct ingestion of constituent plant parts (pollen, nectar, leaves, etc), which creates sustained exposure of consistent dietary concentrations.

In order to estimate the Haber exponent, a series of 'time-to-effect' experiments should be conducted over a serial dilution of concentrations. Experiments that run for the lifespan of the study species (i.e. the chronic paradigm) generate a dose-response curve that easily reveals if changes in 'time-to-effect' (i.e. mortality) are commensurate with changes in doses, as anticipated by Haber's Law. If mortality occurs faster at lower doses than expected by Haber's idealized exponent, it indicates TRT by bioaccumulation (i.e. $b>1$). If the dilution series incorporates sufficient doses, an LC_{50} and NOEC can also be calculated using these same series of experimental exposures.

Most simply, the Haber's exponent can be estimated from the relationship between the log value of pesticide concentration plotted against a log value of time-to-effect at each dose, which yields a straight-line response curve:

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

Eq. 2

The Haber's exponent (*b*), represented here as the slope of the log:log curve, reveals if a pesticide exhibits TRT. A *b* of |1|, the idealized Haber's exponent, indicates a substance where dose and time-to-effect are proportional, which is consistent with steady-state toxicokinetics (elimination balances ingestion), and indicates that the chemical does not produce TRT (Fig. 1.3a). A *b* of |2| would represent an ideal bioaccumulating substance (Holder *et al.*, 2018), where time-to-effect is clearly disproportionate to dose (elimination is slower than ingestion; Fig 1.3b). In fact, the exponent b>1 is the quantifiable amplifier of time represented in the concept of TRT, accounting for why a substance with b=1 does not exhibit TRT.

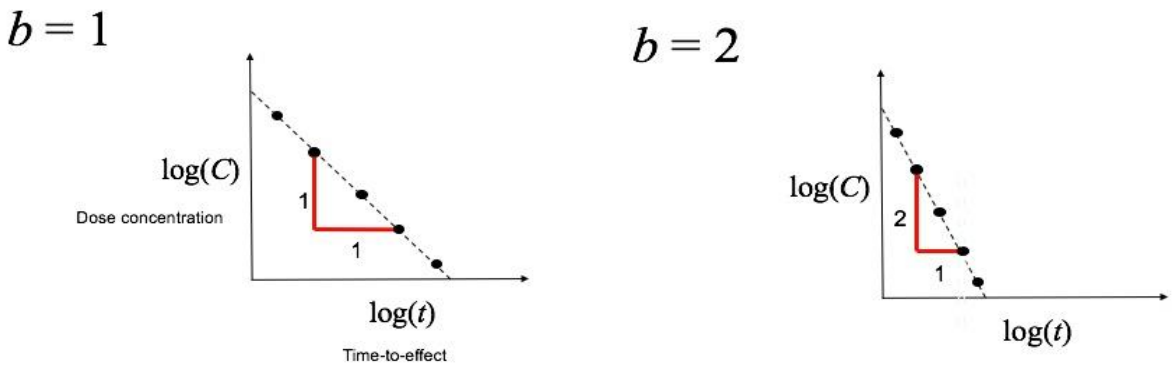

*Figure **1.3**: (a) Model of a no TRT Haber's Law dose-response with a slope of |1| based on log(pesticide consumption) against log(time-to-effect). (b) Model of TRT Haber's Law dose-response with a slope of |2| based on log(pesticide consumption) against log(time-to-effect).*

In the case of pesticides that do not exhibit TRT, safe doses can additionally be determined (Fig 1.4). Doses that do not lower longevity compared to controls are considered safe doses – points that are easily mapped when the experiments run until all subjects die. Thus, a biological NOEC can be calculated by the intersection of the Haber's regression line with a vertical line through the controls, which here I term the 'safety line' (Fig 1.4). A relatively non-toxic pesticide will exhibit a distinctive pattern, where multiple safe doses have similar longevity to controls and 'safe' concentrations form a vertical line, constrained by inherent lifespan of the study species (Fig 1.4). Alternatively, in highly toxic pesticides, even the smallest doses fail to match the longevity of the controls and are likely to appear as a straightforward linear trend that intercepts the x-axis at the control longevity because of the dose-dependent reductions in longevity across the exposure range.

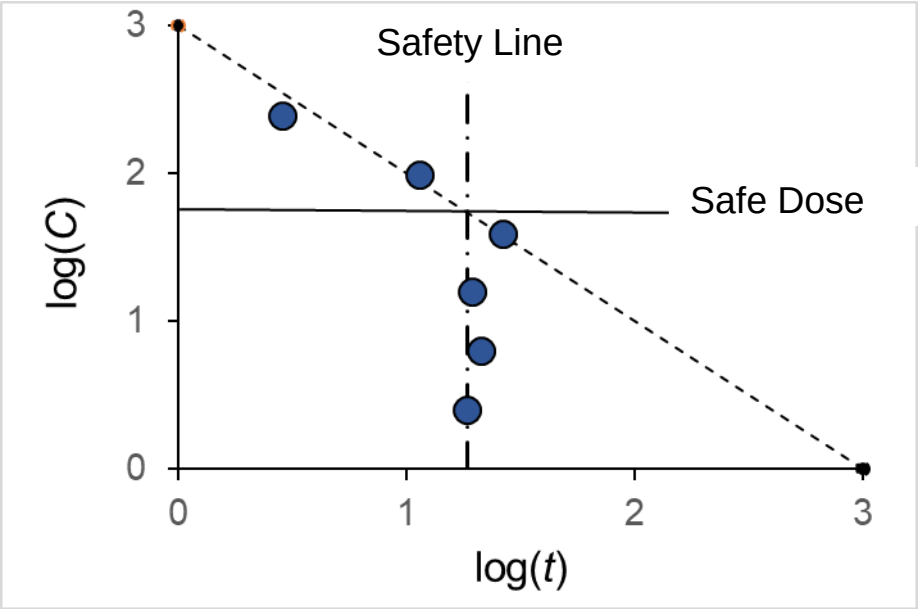

*Figure **1.4**: Example of b |1| pesticide with safe dose. Vertical dotted line represents natural senescence, determined by mean mortality of control study subjects. Points to the left of the vertical dotted 'safety' line represent doses that kill subjects faster than they would die naturally, while doses to the right represent doses that do not alter longevity of study subjects. The solid line represents the safe dose determined by the dose-response regression's interception with the line of control death. The characteristic shape of non-toxic pesticides is a vertical line of safe doses with unsafe doses acting as the regressing curve.*

The use of Haber's Law is not constrained to mortality, pesticides or bees, but could be applied to any substance or study subject, as long as the time-to-effect end point is discrete (0 or 1), and the dose-dependence experiments can be run for the duration for time-to-effect to occur. However, time-to-effect endpoints with a discrete 0,1 occurrence are limited. As Haber's Law is proposed here as an additional tool for laboratory determinations of pesticide safety, which are currently limited to acute paradigm LC testing, it requires uncontaminated laboratory environments to function properly, making it unsuitable for study

subjects with long lifespans or complex needs (i.e. humans). It also makes it unsuitable for field studies, or even studies with confounding variables, such as co-exposure studies. Simply put, Haber's Law is a simplistic equation and therefore is limited to simplistic experiments. While these attributes of simplicity make such studies inexpensive, definitive and easy to run and replicate, they also invariably limit Haber's Law ability to make statements about field realistic scenarios or the state of wild bees. It must also be stressed that Haber's Law can only provide a proxy for toxicokinetic pace or bioaccumulation, not definitive clearance/accumulation rates. More sophisticated studies utilizing body-residue analyses would be needed to determine with more certainty the bioaccumulative propensity of substances or the rates in which they are cleared from subject's systems.

1.8.2 Ingestion:Longevity Relationship

A second metric for TRT lies in the relationship between total mass of ingested pesticide and longevity, which can also be used to validate Haber's Law findings. Pesticides exhibiting TRT should have a negative correlation between pesticide mass and longevity, as bioaccumulative toxicants are not cleared before the next ingestion, and therefore the level of internal exposure is the critical factor to determining mortality (Holder *et al.*, 2018), because pesticides with slow toxicokinetics have longer 'in body' residence, which affords them greater opportunity to cause harm (Fig. 1.5). Hence, each unit of a bioaccumulative toxicant causes more harm through the greater length of time in the subject body. Therefore, long-lived subjects at lower doses require less of the toxicant to kill them (TRT, b>1, Fig. 1.5). Alternatively, a nonsignificant or positive relationship

between ingested pesticide mass and longevity is indicative of quickly cleared toxicants, evidenced by lifetime ingestion not affecting longevity. This means either that lifetime ingestion of toxicant is diminishing lifespan at an expected rate (Clearance, b=1, no correlation, Fig. 1.5), or even that subjects are exceeding expected lifespans based on lifetime consumption (Low-Dose Inefficacy, positive correlation. Fig. 1.5). This could only occur with 'fast toxicokinetics' characteristic of non-bioaccumulative substances, whereby toxicants are cleared before rendering unanticipated injury. Thus, analyzing the slope of the ingestion:longevity relationship is a corroborating test for TRT.

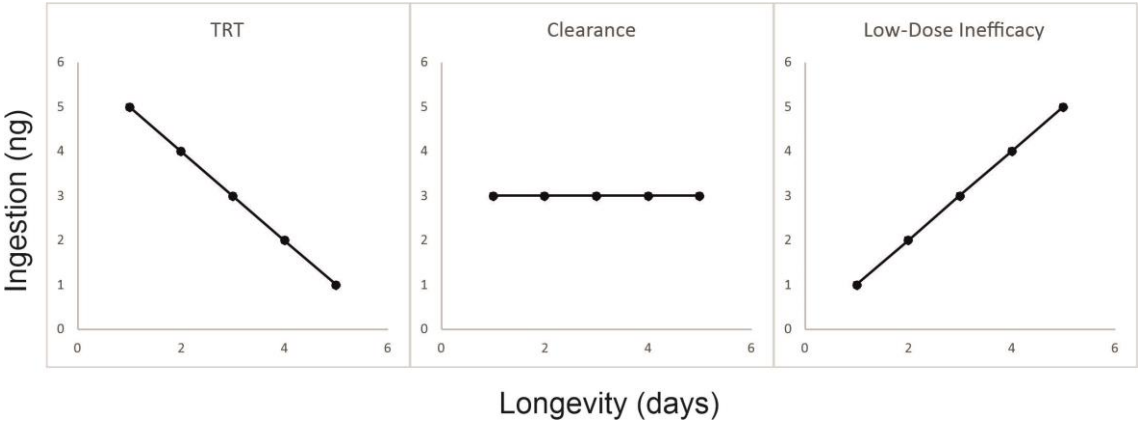

*Figure **1.5**: Example of ingestion:longevity relationship curves, showing TRT (left), with a negative slope of lifespan per ng of active ingredient ingested (b>1), clearance (middle) with no slope (b=1), and inefficacy (right, b<1), whereby the ingestion appears to have a positive effect on longevity, indicating that the pesticide is likely not having a negative effect on lifespan.*

1.9 Conclusion

The use of neonicotinoids has sparked a heated debate into their toxicity in bees, particularly whether or not they are reversible. Resilience of exposed insects is linked to reversibility of ligand-receptor binding and to robust enzymatic metabolism and weak target site affinity or bonding strength. The evidence here

suggests that bees are capable of metabolic degradation of neonicotinoids, and, while they may have high affinity for target receptors, neonicotinoids employ weak bonds that are easily detached, making them unlikely to bioaccumulate in bee species.

While the evidence suggests that neonicotinoids are not expected to bioaccumulate, a quantifiable metric of TRT should be incorporated into regulatory studies in order to best safeguard non-target species and should be employed to ensure neonicotinoids do not bioaccumulate in bee species. One such method is the application of Haber's Law to chronic dose-dependence studies, which is an inexpensive and simple solution to assessing bioaccumulation of pesticides. The slope of the ingestion:longevity relationship is a second revealing index for detecting TRT. The aim of the next chapter is to deploy these two indices (Haber's analysis and ingestion:longevity relationship) in a real chronic exposure of bumblebees, which I conducted in the laboratory of the University of Exeter.

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Chapter 2: Quantifying the Pace of Toxicokinetics in Bumblebees using Haber's Law

2.1 Abstract

Declines in wild pollinators, including key bumblebee species, have spurred concerns that pesticides, namely neonicotinoids, may generate bioaccumulative toxicity (also known as Time Reinforced Toxicity, TRT) due to slow toxicokinetics within bee systems. However, the continued dependency on acute-paradigm testing (i.e. 48-hour LC_{50}s) represents a gap in laboratory assessments, whereby pesticides capable of generating TRT due to persistence in bee bodies could be missed, or their safety overestimated. The application of the Haber's Law proportionality equation to dose-dependence studies represents a simple method for quantifying the potential for bioaccumulative toxicity (represented by the Haber's slope *b*, where *b* approaching 2 indicates TRT), a chronic paradigm test that allows inferences to be made about the pace of toxicokinetics of pesticides within study subjects, which I demonstrated here in bumblebees (*Bombus terrestris*) with two controversial neonicotinoids, imidacloprid and thiamethoxam, and the phenylpyrazole fipronil. To corroborate the Haber's findings, I also analyzed the relationship between lifetime ingestion of active ingredient and longevity, where negative relationships similarly indicate TRT. Both neonicotinoids showed no indication of TRT, with Haber's exponents of approximately 1 (imidacloprid: *b*=1.0; thiamethoxam: *b*=1.2), and no correlation between lifespan and active ingredient ingestion, while fipronil (known to generate TRT) had a Haber's exponent of 1.8 and a significantly negative correlation between

total active ingredient ingested and lifespan. Not only do our results help clarify that neonicotinoids are unlikely to generate TRT, indicative of fast toxicokinetics within bumblebee bodies, but they also demonstrate the usefulness of Haber's Law and the ingestion:longevity relationship in chronic paradigm testing for bioaccumulative toxicity and establishing the pace of pesticide toxicokinetics.

2.2 Introduction

Bumblebees are part of a contingent of wild pollinating insects that contribute to crop production in the multibillion dollar-per-year agricultural industries of Europe and North America (Van der Sluijs *et al.*, 2013; Vanbergen and Garratt, 2013). Recent declines in some populations of wild bees (Colla and Packer, 2008; Brown and Paxton, 2009) have stirred a flurry of scientific activity aimed at understanding the threats to bee health and sustaining effective pollinating services (Potts *et al.*, 2010). The neurotoxic insecticides used in intensive agriculture as plant protection products (PPPs) have been implicated as one potential threat to bee health. These PPPs include neonicotinoids, such as imidacloprid and thiamethoxam (Alkassab and Kirchner, 2017) and phenylparazoles, like fipronil (Pisa *et al.*, 2015).

Neurotoxic insecticides are applied to disrupt normal nerve action in pest insects leading to their death (Walker *et al.*, 2012). Imidacloprid, thiamethoxam and fipronil are often applied as a seed coating and subsequently become systemic in the maturing plant (Simon-Delso *et al.*, 2015). Consequently, insecticide residues are ingested by insects as they

feed on the stems, leaves, nectar or pollen of treated plants (Thompson, 2001; Walker *et al.*, 2012; Pisa *et al.*, 2015). The specific modes of neurotoxic action vary among the classes of chemical toxicant: neonicotinoids inhibit acetylcholine breakdown (Tomizawa and Casida, 2003); and phenylparazoles like fipronil bind to ɣ-aminobutyric acid (GABA) receptors (Gunasekara *et al.*, 2007). The high neurotoxicity of these agrochemicals has been suggested as a cause of population declines in wild bees, including bumblebees (Goulson, 2013), but the magnitude their effects is unclear (Cresswell, 2011; Alkassab and Kirchner, 2017). For example, in crops where systemic PPPs are used under good agricultural practice, flower-visiting bees should encounter only trace residues of the PPPs in the nectar and pollen that is their principal diet. For neonicotinoids, nectar residues are expected to be between 1 and 11 ppb (Cresswell 2016). However, even trace dietary residues may eventually cause severe harm if they build up to injurious levels in the insect body during sustained exposures because they resist metabolic degradation and/or elimination (Borgå *et al.*, 2004). Toxic substances that accumulate in an organism above levels expected from dietary concentrations are said to bioaccumulate (Walker *et al.*, 2012) or to have slow toxicokinetics. Where clearance is poor or failing, the toxicant builds up and thereby causes symptoms to intensify over time. In contrast, during dietary exposure to a non-bioaccumulative toxicant with fast toxicokinetics, each uptake would be rapidly cleared by either excretion or metabolic detoxification and so toxicodynamic equilibrium is established (so-called ‘steady-state’), and symptoms should not intensify over time provided that the toxicant binds reversibly to its target receptor site (Liu *et al.*, 2006). Clearly, the harmfulness of trace dietary

residues depends in part on the degree to which their effects intensify over time. This intensification is termed Time-Reinforced Toxicity (TRT) (Walker *et al.*, 2012) and establishing the degree to which a PPP produces TRT could be an important consideration in assessing its risk to non-target organisms, such as wild bees. Fundamentally, pesticides with slow toxicokinetics could generate TRT.

Government regulation of agrochemicals involves a difficult conundrum because the yield of certain entomophilous crops (oilseeds, sunflowers, etc.) in intensive agriculture relies both on pesticidal insecticides and the services of (non-target) insect pollinators. Currently, the development of the regulation of agrochemicals for better protection of bee health is an ongoing endeavor (Blacquière and van der Steen, 2017). PPPs exhibiting TRT should be of high concern, but TRT is not yet widely targeted in regulatory risk assessments that aim to protect bee health (Anatra-Cordone and Durkin, 2005). Until recently, one of the mainstays of laboratory testing in regulatory risk assessment has been the 48-hour dosing study, or the so-called 'acute paradigm' exposure, which is used to determine two cardinal comparative indicators: (a) the LC_{50} (LD_{50}), or the lethal concentration (dose) for 50% of the exposed subjects to die; and (b) the NOEC (no observable effect concentration) (Cresswell, no date; Gaines, 1969). Of course, these acute exposures will not reveal TRT, which manifests increasingly over time, and a 48-hour study does not usually represent the environmentally realistic duration of exposure for many pollinator species. For example, worker bumblebees live on average 20 days after the onset of foraging (Doums *et al.*, 2002) and may therefore forage on

sunflower or oilseed crops for several weeks. However, new test guidelines are requiring sustained exposures (10-day) under a so-called 'chronic' exposure paradigm (Potts *et al.*, 2017). Potentially, the results of these experiments could be used to evaluate a PPP's potential for generating TRT. Therefore, in order to investigate the potential efficacy of testing paradigms suited to the detection of TRT, I conducted sustained exposures of bumblebees to dietary insecticides. I used the results of these laboratory experiments to test for TRT using Haber's Law. In so doing, I illustrate how deviations from Haber's Law can be used to generate a quantifiable metric for bioaccumulative toxicity in pesticide studies.

The conventional variant of Haber's Law states that the 'time-to-effect' on any particular endpoint (e.g. fatality) is inversely proportional to the concentration of the exposure to the toxicant. If *C* denotes the concentration of a toxicant, *t* is exposure time then the general form of Haber's Law is given by:

$$C t^b = k$$

Eq. 1

In the conventional variant *b*=1 and, for example, doubling the concentration of the exposure will halve the time required to reach a specified end point (e.g. for 50% of exposed bees to die) (Rowland, Benet and Graham, 1973). The appearance of concentration-vs-time relationships in which the exponent of Haber's Law takes the value b=1 are consistent with non-bioaccumulative toxicants that have rapid clearance and fast toxicokinetics. The appearance

of concentration-vs-time relationships in which the exponent of Haber's Law takes the value b=2 are consistent with bioaccumulative toxicants, slow toxicokinetics, and the presence of TRT. If mortality occurs with a TRT profile within the lifespan of the study organism, it indicates potential bioaccumulation and a need to revisit regulation, using equation 1.

It is straightforward to estimate the Haber exponent from conventional 'time-to-effect' experiments by logarithmic transformation of the concentration-vs-time relationships as follows:

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

Eq. 2

Hence, in order to investigate time reinforced toxicity, the Haber exponent can be estimated by regression analysis following logarithmic transformation of Eq. 1.

A second metric for assessing TRT lies in the relationship between the mass of ingested toxicant that precedes fatality and longevity, which can be used to corroborate the inferences made from Haber's Law. When experimental exposures are conducted across a range of doses, a toxicokinetic inference can be made by comparing the ingested mass that precedes fatality across the different doses. A toxicant with fast toxicokinetics (a short in-body residence) will cause the same injury per unit ingested irrespective of dose because each unit has approximately the same in-body residence time, which

means that each bee must ingest the same total mass of toxicant to cause its fatality irrespective of the duration of the exposure. In contrast, a toxicant with slow toxicokinetics is retained in the bee's body, which means that each ingested unit has longer to cause injury in the longer-lived bees feeding on lower doses, so these bees need to consume less of the toxicant in total to be killed. Hence, TRT produces a negative correlation between longevity and the total mass of toxicant ingested by an individual before death (Holder *et al.*, 2018).

In order to assess the risk to bee health posed by a PPP, regulators might use the NOEC directly as a protection threshold to safeguard a focal non-target species from obvious toxic effects. Where the test endpoint is fatality, the NOEC restricts permissible exposures to levels that do not increase the death rate above normal background levels. The NOEC is determined by experimental exposures and is taken to be the lowest of the tested doses in which the measured response of the exposed subjects is not statistically different from the response of undosed controls. As the conventional NOEC is designated to be the smallest dose that causes a response different to the control given the size of the experiment, specifically, the value of this NOEC has no intrinsic biological basis, but instead changes with the power of the experimental design. However, a NOEC can also be determined using the Haber regression line. Extrapolation of the *C*-vs.-*t* relationship on log-log scales yields an estimate of the NOEC because the intercept between the *C*-vs.-*t* relationship and the response of control undosed subjects is the concentration at which dose-dependent toxicity begins (Cresswell, 2018).

Doses that do not reduce longevity compared to controls are considered ‘safe doses’ – points that are easily quantified when the experiments run until all specimens die. Thus, a biologically relevant and parametric NOEC can be calculated by the intersection of the Haber’s regression line with a vertical line through the controls, the ‘safety line’. Specifically, the intercept between the log(*C*)-vs.- log(*t*) regression and the lower confidence interval on the responses of the control population is, in theory, the lowest toxic dose.

Here, I report the results of a series of dose-dependence studies examining mortality in bumblebees (*Bombus terrestris*) due to exposures to three neurotoxic pesticides - imidacloprid, thiamethoxam and fipronil - to model the application of Haber’s Law as a way to examine TRT. Previous studies show thiamethoxam has little indication of bioaccumulation (Tox Services, 2015), while fipronil is regarded as highly toxic and bioaccumulative, especially in aquatic systems (Gunasekara *et al.*, 2007), and may be the culprit in the 1990’s ‘carpet of bees’ mass mortality of bees in France (Cresswell, 2016; Holder *et al.*, 2018). Alternatively, imidacloprid’s lethality and potential for bioaccumulation is still debated in bee toxicology (Cresswell *et al.*, 2014; Sánchez-Bayo, Belzunces and Bonmatin, 2017), prompting much of the research and regulatory discourse into neonicotinoids. The debate is encapsulated by the disparity in LD_{50} measurements published for imidacloprid, which range from 3 - 280 ng/bee for 48-hour LD_{50} (Anatra-Cordone and Durkin, 2005), which is very large when compared to the realistic exposure which range from 1 - 11 ppb (Cresswell, 2016). I believe the use of

Haber's Law to assess TRT may help identify, and better regulate, the effects of pesticides on non-target species.

In summary, the aims of this study are: (i) to characterize the time profiles of dose-dependent toxicity in bees exposed to three pesticides; (ii) to evaluate thereby whether any of the focal pesticides generates TRT in the mortality endpoint; and (iii) to assess the relevance of TRT for future regulatory use.

2.3 Methods

2.3.1 Bee Husbandry and Acclimation

I used adult workers taken from commercial colonies of *B. terrestris* (either Biobest, Westerlo, Belgium or Koppert B.V., Berkel en Rodenrijs, the Netherlands). For each toxicant that I investigated, the experiment involved 80 individuals (40 bees collected from each of two colonies). Bees were placed individually in softwood boxes (0.07 x 0.07 x 0.035 m) fitted with mesh sides and given access to syrup feeders (punctured micro-centrifuge tubes containing 1.27 Kg/L fructose/glucose/saccharose solution; Attracker, Koppert B.V.). The caged bees were kept in a semi-controlled environment (approximately 25 °C, 45% relative humidity). The bees were fed *ad libitum* undosed syrup for 72 hours prior to experimental dosing for acclimation. In each experiment, the two colonies were equally represented in each dose treatments and bees were randomly allocated otherwise. The exposure lasted for the lifespan of the individual. Bees that died during the acclimation days were replaced with a worker from the corresponding original colony.

2.3.2 Preparation of Doses

2.3.2.1 Imidacloprid

A primary stock solution of imidacloprid (Pestanal 37894; Sigma-Aldrich, Gillingham, UK) was produced by dissolving powdered toxicant in 20% acetone solution (10 mL acetone + 40 mL deionized water; 47.5 mg in 50 mL liquid). The primary stock was further diluted in deionized water to produce a concentrated stock of the highest dose (195,300 µg L^{-1}), from which I produced 40% serially diluted experimental stocks (100x concentrated). Finally, I produced the following various experimental doses by adding 1 mL of stock to 99 mL feeder syrup to produce the series of dosed syrups as follows: 1953, 781, 312.5, 125, 50, 20, 8 µg L^{-1}.

Controls were given feeders with 0.2 mL acetone and 0.8 mL of deionized water per 99 mL of clean syrup.

2.3.2.2 Thiamethoxam

A primary stock of thiamethoxam (Pestanal 37924; Sigma-Aldrich, Gillingham, UK) was produced by dissolving powdered toxicant in deionized water (10.1 mg in 50 mL water). The primary stock was further diluted in deionized water to produce a concentrated stock of the highest dose (31,250 µg L^{-1}) from which I produced experimental stocks by 40% serial dilution (100x concentrated), finally adding 1 mL aliquots to 99 mL of feeder syrup to produce the final experimental doses: 312.5, 125, 50, 20, 5, 3.2 µg L^{-1}. The same protocol was used to produce the following doses for a second experiment covering doses 1953, 781, 312.5, 125, 50, 20 µg L^{-1}.

Controls were fed syrup composed of 1 mL deionized water per 99 mL of clean syrup.

2.3.2.3 Fipronil

Given the hydrophobic nature of fipronil, I produced a primary stock solution by dissolving powdered toxicant (Pestanal 46451.; Sigma-Aldrich, Gillingham, UK) initially in acetone (20.2 mg in 50 mL acetone). The primary stock was then diluted in acetone in 40% serial dilution (100x concentrated) before adding 1 mL aliquots to 99 mL of feeder syrup to produce the following doses: 125, 50, 20, 5, 3.2, 1.28 µg L^{-1}.

Controls were fed syrup composed of 1 mL acetone per 99 mL of undosed syrup.

2.3.3 LCMS Verification of Doses

Quantitative analysis of toxicant concentrations was performed using an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. 10 µl of each standard concentration was injected onto an Eclipse Plus C_{18} reverse phase analytical column (3.5 µm, 2.1 x 150 mm) (Agilent Technologies, Palo Alto, USA). Analysis was conducted in positive ion mode, and all solvents were LC-MS grade. Mobile phase A comprised 2 % acetonitrile, 98% water, 0.1 % Formic Acid, and mobile phase B was 95 % acetonitrile, 5 % water and 0.1 % formic acid. The following gradient was used:

0 min – 0% B; 1 min – 70 % B; 10 min – 80 % B; 10.2 min – 100 % B; 12 min – 100 % B; 13 min – 0 % B, followed by 4 min re-equilibration time (post time). The flow rate for the first minute and post time was 0.3 ml min^{-1}, which was then ramped up to 0.45 ml min^{-1} between 1 and 12 min. The QQQ source conditions for electrospray ionisation were as follows: gas temperature was 350 °C with a drying gas flow rate of 11 l min^{-1} and a nebuliser pressure of 35 psig. The capillary voltage was 4 kV. The transition m/z of each toxicant (labelled and non-labelled) was predetermined prior to the run using Mass Hunter optimizer software (version B.08.00; manufacturer) to optimise the fragmentation conditions for each of the standards.

For each toxicant, nominal concentrations were tested using one of two methods. For imidacloprid and thiamethoxam, toxicant levels in the experimental syrups were quantified by spiking them with an appropriate labelled standard: imidacloprid was spiked with 100 µg L^{-1} d_4-imidacloprid (Pestanal 34170; Sigma-Aldrich, Gillingham, UK); thiamethoxam was spiked with 10 µg L^{-1} d_3-thiamethoxam (Pestanal 38176; Sigma-Aldrich, Gillingham, UK). This use of labelled standards negates any differences in instrument sensitivity over time. For fipronil, a similar quantification with spiked standards, using ^{13}C-fipronil (79157; Sigma-Aldrich, Gillingham, UK) revealed that the two-month delay before LCMS analysis had produced a substantial reduction in estimated concentration (c. 40%), probably because the hydrophobic toxicant had attached to the interior surfaces of the plastic container. The syrup preparation was therefore repeated by the same

protocol as used for the experimental syrups and compared these nominal levels against a calibration curve of the non-labelled toxicant.

2.3.4 Experimental Exposures and Statistical Analysis of Mortality and Feeding

During each exposure, I monitored bees daily to record mortality and measured syrup consumption gravimetrically every 48 hours. Fresh batches of dosed feeder syrup were produced from stock solutions every 10 days. In order to investigate dose-dependent toxicity, I analysed variation among exposed bees in longevity and syrup consumption as follows.

In order to investigate time reinforced toxicity, I determined the value of the Haber exponent, *b,* by regression analysis (see Eq. 2)

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

Eq. 2

Therefore, regression analysis of the log(*C*):log(*t*) relationship was used to evaluate the Haber's exponent. It is essential that a regression test for TRT is performed only on mortality data that describes toxic effects because the effects of old age also intensify with time, which means that senescence can introduce time reinforcement into mortality data. To exclude mortality due to senescence, I identified dosed bees whose death could not be reasonably ascribed to natural causes as those whose longevity was shorter than the

lower confidence interval on the longevity of the control (undosed) bees (the confidence interval was based on the sampling variation among individual bees, hence the standard deviation was used to defined the interval rather than the standard error). An observation of longevity significantly shorter than among controls (i.e. observed longevity < control mean - 1.96 SD) in a dosed bee was considered a dose-dependent death.

I conducted a second test for TRT by analyzing the slope of the ingestion:longevity relationship. I conducted this analysis using only dosed bees whose longevity was significantly shorter than the controls, i.e. whose deaths could be reasonably attributed to the toxicant. Where dose-response relationships in longevity were not linearly monotonic, I analysed variation among doses with one-way Analysis of Variance (ANOVA). I used Spearman rank correlations to test the significance of ingestion:longevity relationships.

48-hour LC_{50}s were estimated through the Haber's regression equation, using the log(2) as a value for *t.* NOECs were analysed through the intersection of the control *t* with the Haber's regression equation, using the log(Control *t*) as the estimate of the intersection between the regression and safety lines (Fig. 2.1).

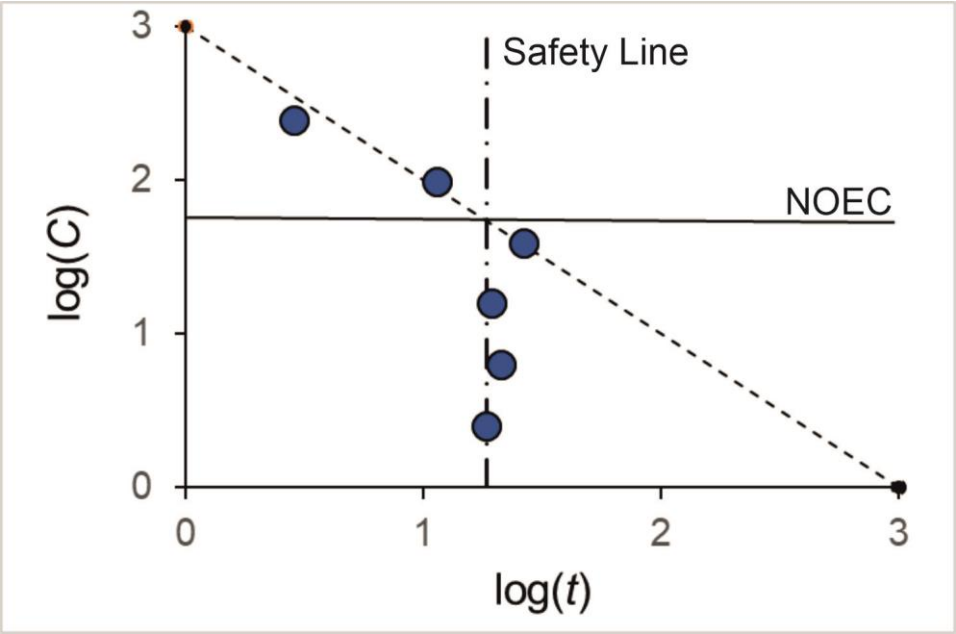


*Figure **2.1**: example of "hockey stick" C vs t graph and the resulting NOEC of a relatively non-toxic pesticide, where b=1. The dotted vertical line represents natural senescence, determined by lower confidence interval on mean mortality of control subjects. Points to the left of the dotted vertical 'safety' line represent doses that kill subjects faster than they would die naturally, while doses to the right represent doses that do not alter longevity of subjects. The solid horizontal line represents the safe dose determined by the dose-response curve's interception with the line of senescence, and this intersection represents a NOEC. This "hockey-stick" appearance is the characteristic shape of non-TRT pesticides.*

2.4 Results

2.4.1 Validation of Doses

Nominal doses of the neonicotinoids corresponded closely to their analytically determined values as they were fitted with y=mx + b models which show observed relationships are close approximations of idealized values when m approaches 1 as observed here: imidacloprid, *analytic*=1.07*nominal* - 7.4, R^2 > 0.99; thiamethoxam, *analytic*=1.04*nominal* + 3.6; R^2 > 0.99.

Analysis of the original fipronil syrups produced values that were substantially lower than the nominal doses (*analytic*= 0.67 *nominal* + 2.7; $R^2 > 0.99$). The analysis was performed several months after the syrups were prepared and I speculate that the hydrophobic toxicant had accumulated on the surfaces of the plastic containers. When fresh syrups were prepared, comparison with a standard curve yielded a much closer correspondence (analytic= 0.93 *nominal* + 0.8; $R^2 > 0.9$

2.4.2 Dose-Dependence

For all three pesticides, dietary exposure of bumblebees produced dose-dependent variation in longevity (Fig. 2.2, SI Fig. 2.1) and syrup consumption (SI Fig. 2.2, SI Fig. 2.3).

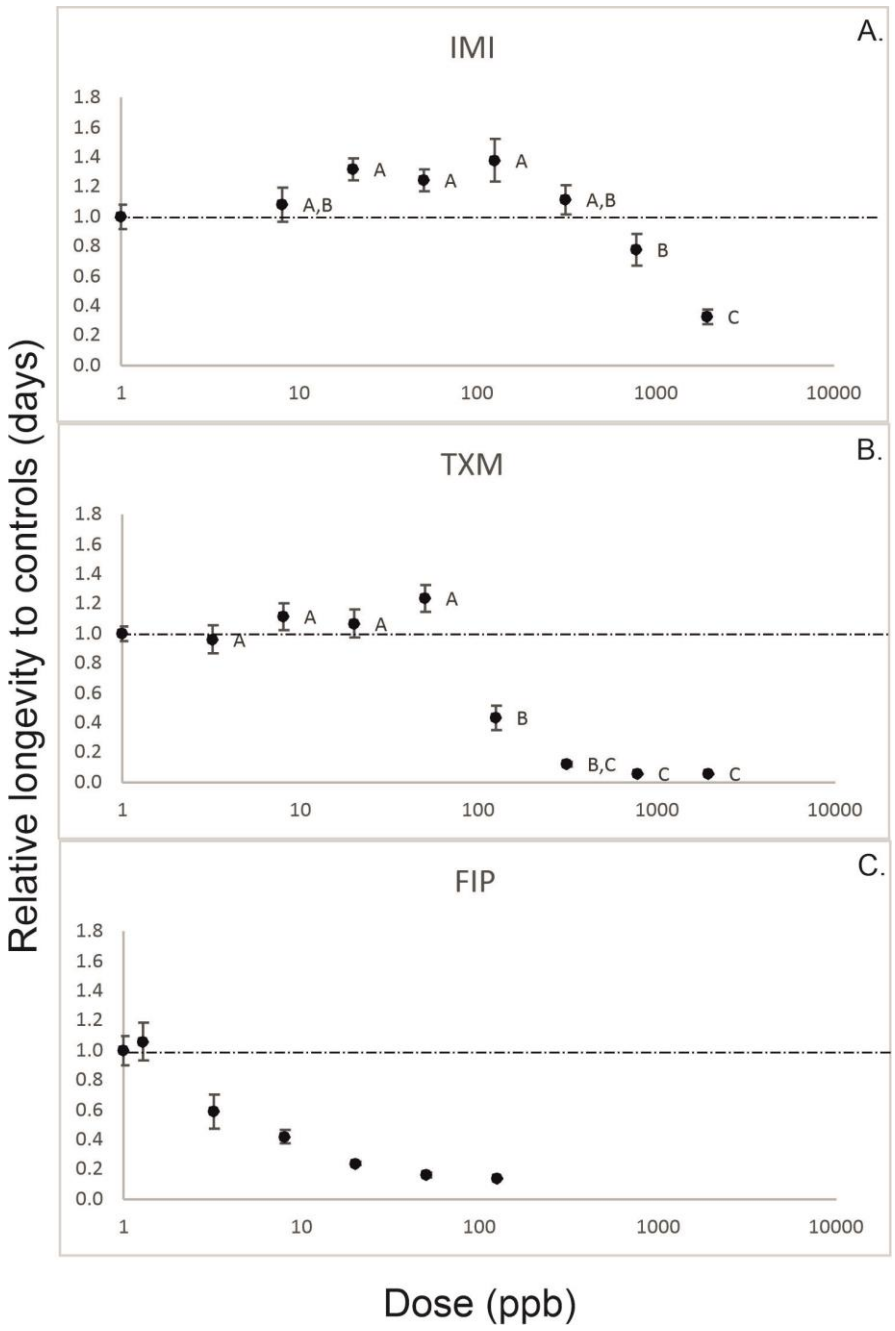


*Figure **2.2:** Relative longevity in days (to control longevity) in relation to dose (ppb), with standard error bars for (a) imidacloprid (b) thiamethoxam, and (c) fipronil. The dotted line represents the relativized control longevity. A.B,C labels reflect Tukey test groupings.*

Imidacloprid displayed the least pronounced dose-dependence effects, and even exhibited hormesis in survivorship (Fig. 2.2A), with low doses stimulating longevity, and high doses reducing lifespan (ANOVA: $F(7,75)= 12.4$, $p<0.001$; Tukey $\alpha<0.05$). Imidacloprid had a mild effect on syrup consumption, with no

consistent reduction in feeding rate (SI Fig 2.2A. ANOVA: $F(7,75)= 22.5$, $p<0.001$; Tukey $\alpha<0.05$).

Exposure to thiamethoxam only showed strong dose-dependence at doses that far exceed even worst-case scenario exposures (Fig. 2.2). Average longevity was maintained at doses up to 98 ppb (SI Fig. 2.1B; ANOVA: $F(8,151)= 40.4$, $p<0.001$; Tukey $\alpha<0.05$), while syrup consumption only consistently dropped after 248 ppb (SI Fig. 2.2B; ANOVA: $F(8,151)= 5.3$, $p<0.001$; Tukey $\alpha<0.05$).

Only fipronil reduced the longevity of bumblebees at dietary concentrations a within the field realistic range (Fig. 2.2; SI Fig. 2.1C; ANOVA: $F(6,71)= 24.9$, $p<0.001$; Tukey $\alpha<0.05$). Averaged longevity was consistently reduced by dietary fipronil as was syrup consumption (SI Fig. 2.2C; ANOVA: $F(6,71)= 6.3$, $p<0.001$; Tukey $\alpha<0.05$).

2.4.3 TRT Analysis, LC_{50}s, and NOECs

Dietary exposures to imidacloprid produced neither signature of TRT (Haber's exponent $b= 1.0 \pm 0.14$; Fig. 2.3a, Fig. 2.5a; ingestion:longevity relationship, Spearman's *rho* IMI= 0.71, P= 0.01; Fig. 2.4b). I interpret the positive correlation in the ingestion:longevity relationship to indicate that bumblebees on the lowest doses cleared imidacloprid more effectively. Even at doses as high as 1500 ppb, only 11 bees had lifespans significantly shorter than

undosed controls. The effects were so slight that I cannot estimate a NOEC (Fig 2.3a) or LC_{50} in our dose range.

The exposures of bumblebees to thiamethoxam also produced neither signature of TRT (Haber's exponent *b*= -1.2 ± 0.35; Fig 2.3b, Fig. 2.4a; ingestion:longevity relationship, Spearman's rho= -0.24, P=0.08; Fig. 2.4b). Using extrapolation of the ingestion:longevity relationship, I estimated a NOEC of 15.6 ppb (Fig. 2.3b) and a 48-hour LC_{50} of 240.7 ppb for thiamethoxam in bumblebees.

In our dietary exposures of bumblebees, only fipronil showed the signatures of TRT (Haber's exponent *b*= -1.89 ± 0.38; Figs 2.3c, Fig. 2.4a; ingestion:longevity relationship, Spearman's *rho* FIP= -0.47, P<0.01; Fig. 2.5b). I estimate a NOEC 1.3 ppb (Fig. 2.3c) and a 48-hour LC_{50} of 48.4 ppb.

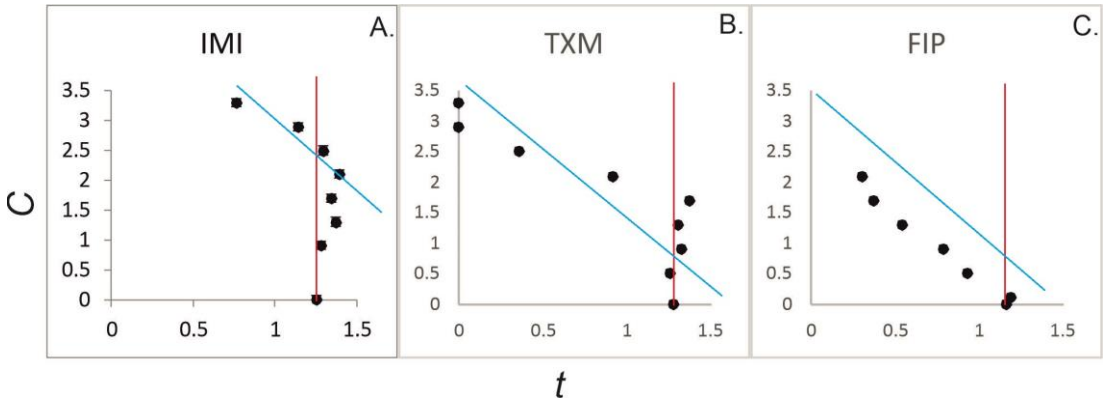


*Figure **2.3**: Haber's Law of log(mean time-to-death in days) plotted against log(concentration in ug/L) for (A) imidacloprid, (B) thiamethoxam and (C) fipronil based on dose means. Regression lines indicate an idealized -1 slope (b= |1|). Vertical lines are 'safety lines' that represent lower confidence intervals on average mortality of the controls. Doses on the right of the vertical line may be considered safe, as they do not reduce mortality compared to the controls. The intersection of the line of safety and regression line determines a NOEC.*

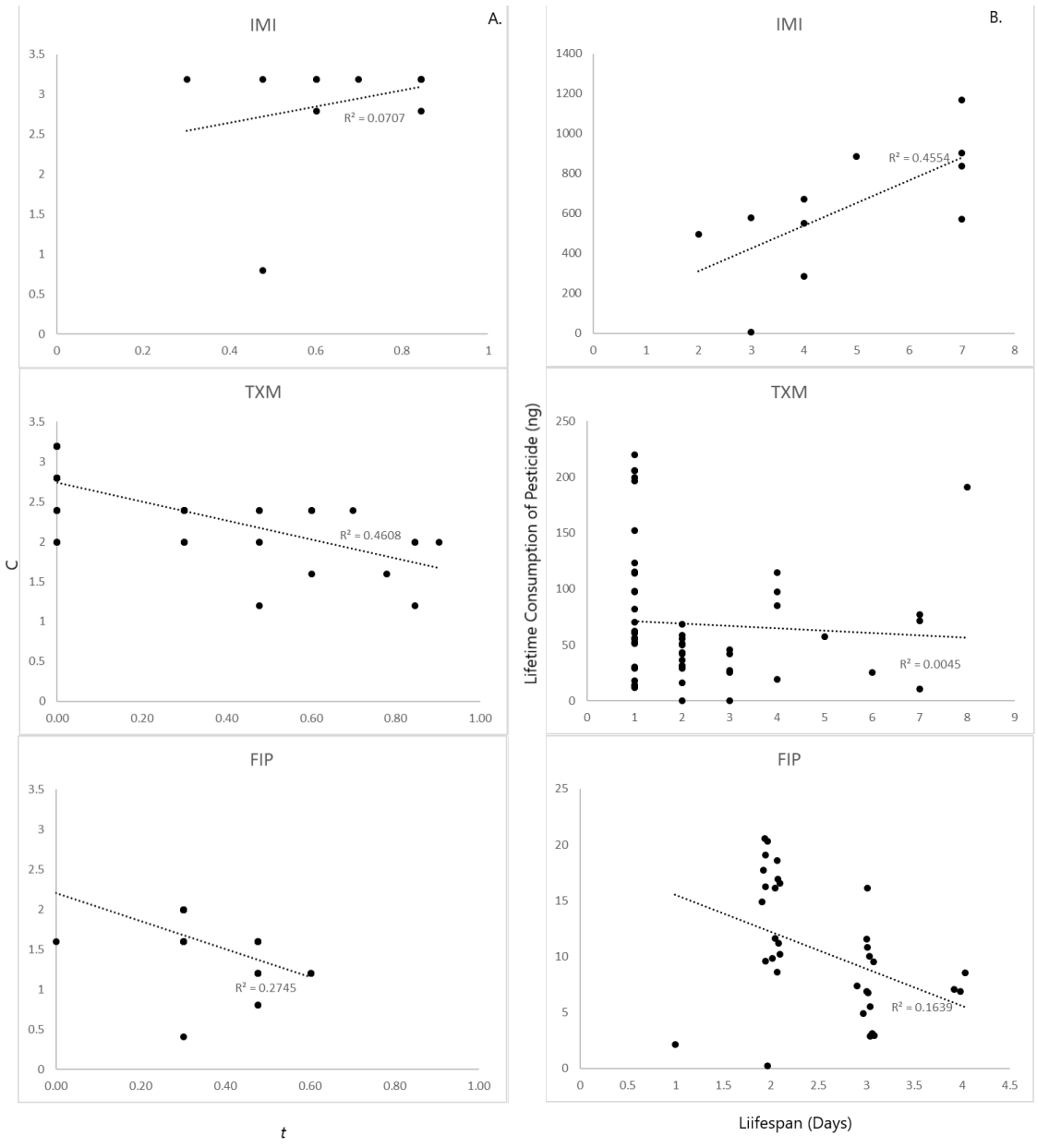


*Figure **2.4a**: Haber's exponent, C vs. t analysis for TRT. Each dot represents a bee whose longevity (in days) fell outside the 95% CI for control longevity (IMI N=11, TXM N=57, FIP N=35). C represents the log of doses in ug/L, and t represents log of time-to-death of toxic bees. The slope of the regression line represents Haber's exponent, b. **2.4b**: Assessing TRT by longevity vs. pesticide ingested relationship with regression and R^2 values. Each dot represents individual dosed bees whose longevity lay outside the 95% confidence interval of controls. FIP was jittered for clarity.*

2.5 Discussion

2.5.1 Relative Toxicity of Three Pesticides

This study revealed differential toxicity between fipronil and the neonicotinoids, whereby only fipronil reduced longevity at doses within the field-realistic range of exposure. Bees given as low as 2.5 ppb of fipronil, well within the field realistic range (Zaluski, Justulin Jr. and Orsi, 2017), lived significantly shorter lives than controls. Reduced longevity was not seen in thiamethoxam until 98.4 ppb and imidacloprid until 1538 ppb, and bees given either neonicotinoid consistently lived much longer than bees exposed to fipronil, despite being administered equal dosages. Interestingly, imidacloprid showed the least effect on either consumption or longevity, with no consistent decrease in consumption rate, and an effect in longevity only at doses nearly 150 times the concentration of those found in the field. Thiamethoxam similarly showed effects only at relatively high doses (threshold for reduced consumption: 246 ppb; threshold for reduced longevity: 98.43 ppb), with the strongest (longevity) effect at a dose nearly 10 times the highest expected field dose (Cresswell, 2016). The lack of severe effects of thiamethoxam and imidacloprid stand in stark contrast to fipronil, whose profound effects on consumption and longevity even at trace doses speaks to the strong toxicity of fipronil.

The Haber's Law analysis reinforced the relative toxicity patterns seen in the dose-dependence analysis, as fipronil, the only pesticide reporting observable effect on lifespan or consumption in the field realistic range, was also the only pesticide to show indications of bioaccumulative toxicity. Using the Haber's

exponent regression, I was also able to determine a NOEC and 48-hour LC_{50} for thiamethoxam and fipronil, which are indicative of the relative toxicities noticeable in the dose-dependence analysis, although our use of chronic exposures, rather than the acute exposures of LD/NOED studies (reported in ng/bee) makes direct comparison to previous studies difficult. Our estimation for fipronil NOEC was 1.3 ppb, a realistically low dose, indicating that bees exposed to fipronil in the field could have noticeable declines in lifespan and consumption. For thiamethoxam, I estimated a NOEC of 15.6 ppb, a dose above the upper limit of neonicotinoids expected in the field. This suggests that at current dosing rates, thiamethoxam is not likely to affect lifespan or feeding of bumblebees. The LC_{50}s also further the story of relative toxicities, as I report 240 ppb LC_{50} for thiamethoxam, and only 48 ppb for fipronil. Reports of thiamethoxam 48-hour LD_{50} ranged from 5 – 30 ng/bee, displaying the uncertainty of thiamethoxam toxicity (EFSA, 2012b). Our results depict thiamethoxam as a pesticide with low relative lethal toxicity. With fipronil, the low LC_{50} marks it as a pesticide with relatively high lethal toxicity. Given our NOEC/LC_{50} values, it is not surprising that doses as low as 0.1 ng/bee of fipronil reportedly killed all experimental honeybees within one week of exposure (Aliouane *et al.*, 2009).

2.5.2 Toxicokinetics of the Three Focal Pesticides

With both the Haber's exponent and ingestion:longevity analysis, fipronil distinguishes itself as a likely generator of bioaccumulative toxicity, with a |1.8| Haber's exponent, almost the idealized 'perfectly irreversible accumulator (*b*=2), and a significantly negative ingestion:longevity correlation. These

indicators of TRT fail to manifest for the two neonicotinoids, and imidacloprid even showed low toxicity throughout the tested range. Even at doses 25-150 times that of the field realistic imidacloprid dosages, not enough bees were dying outside the CIs of the controls for it to be considered toxic when lethality is the focal endpoint. Regardless, the perfect |1| Haber's exponent and the significantly positive correlation between ingestion and longevity suggest strongly that imidacloprid is unlikely to exhibit TRT. Moreover, thiamethoxam, a pesticide in the same class as imidacloprid, similarly established itself as relatively non-toxic and non-bioaccumulative, with a |1.2| Haber's exponent, and a non-significant relationship between pesticide ingestion and longevity.

Our inability to detect TRT in the neonicotinoids imidacloprid and thiamethoxam tallies with reports of evidence of clearance via behavioural/reproductive rate proxies and analysis of body residues in this class of pesticides in honeybees (Cresswell *et al.*, 2014) and bumblebees (Laycock and Cresswell, 2013; Thompson *et al.*, 2015), with a <24-hour clearance for *Apis mellifera,* and 48-hour clearance for *Bombus terrestris.* Similar reports have been made for amphipods (*Gammarus pulex)* exposed to aquatic imidacloprid (Nyman *et al.*, 2013). Not only is evidence of clearance a strong indicator that these neonicotinoids do not bioaccumulate, a trademark of TRT is unexpected effects on mortality, yet many studies, including ours, fail to find increased mortality at environmentally realistic chronic doses of thiamethoxam or imidacloprid (Cresswell, 2011, 2016).

Developed to mimic the natural ligand binding with acetylcholine receptors, the hydrogen or electrostatic bonds that neonicotinoids use to bind to their targets sites are inherently weak, designed to rapidly release from the site, as opposed to strong, covalent bonds associated with irreversible binders (Kezić, Lucić and Sulimanović, 1992; Bao *et al.*, 2016). The weak bonding of neonicotinoids couples with the robust enzymatic metabolism (Kezić, Lucić and Sulimanović, 1992; Iwasa *et al.*, 2004) and the complex acetylcholine receptor networks associated with bees (Jones *et al.*, 2006), which may confer adaptive mechanisms for coping with neonicotinoid exposure (Zhu *et al.*, 2008; Liu *et al.*, 2015), making it unlikely that these substances bioaccumulate.

By comparison, fipronil, known to bioaccumulate, exhibits every hallmark of TRT in our analysis. Our results are consistent with the multitude of findings of high toxicity and TRT in fipronil, which has already been banned in Europe and China (Wu *et al.*, 2015) due to its disproportionate toxicity. Fipronil has shown that pesticides that should be flagged for high toxicity are characterized by several distinguishing markers, including low dose effects on consumption and mortality, and a signature of TRT, in both a Haber's exponent approaching |2|, and a negative ingestion:longevity relationship. It is therefore plausible that fipronil ligand bonding utilizes less reversible bonds, such as covalent, to attach to target sites. The nature of its receptor-ligand interactions should be a focus for future investigations.

2.5.3 Implications for Assessing the Risk to Bees from Trace Residues

Environmentally realistic residues of neonicotinoids in the field are rarely above 11 ppb, and more often the values are much lower (Cresswell, 2016). At these levels, neither imidacloprid nor thiamethoxam exhibited observable effects on bumblebee lifespan or ingestion rates in our studies. In fact, both pesticides may stimulate extended lifespan at low doses, a known hormesis (Haddi *et al.*, 2016; Holder *et al.*, 2018), possibly due to pesticide-induced energy reallocations (Jager, Barsi and Ducrot, 2013). Moreover, the lack of TRT signatures for these pesticides, and the high NOEC/LC_{50} values for thiamethoxam suggest these neonicotinoids might be relatively non-toxic at field realistic doses. However, sub-lethal (i.e. reproduction rates) or synergistic (interaction with other substances found in the field) effects may still exist at low exposures of these neonicotinoids, but the extent of these detriments remain undetermined (Alkassab and Kirchner, 2017; Raimets *et al.*, 2018). Alternatively, the indications of TRT-generating slow toxicokinetics in fipronil that manifest as longevity and consumption reductions even at trace exposures support the widespread ban on fipronil seen across Europe and Asia (Wu *et al.*, 2015).

2.5.4 Potential Utility of Haber's Law in Regulatory Testing of Agrochemicals

The correspondence of the known toxicology of these substances with the quantifiable metrics produced by our Haber's Law studies show the potential reliability of such analysis in assessing TRT of toxic substances. Moreover,

dose-dependence studies are applicable to any discrete endpoint of time-to-effect, and therefore could possibly be expanded to explore sub-lethal effects. Most importantly, they are inexpensive, simple, and provide vital information otherwise missed by current regulatory (48-hour LC_{50}) studies.

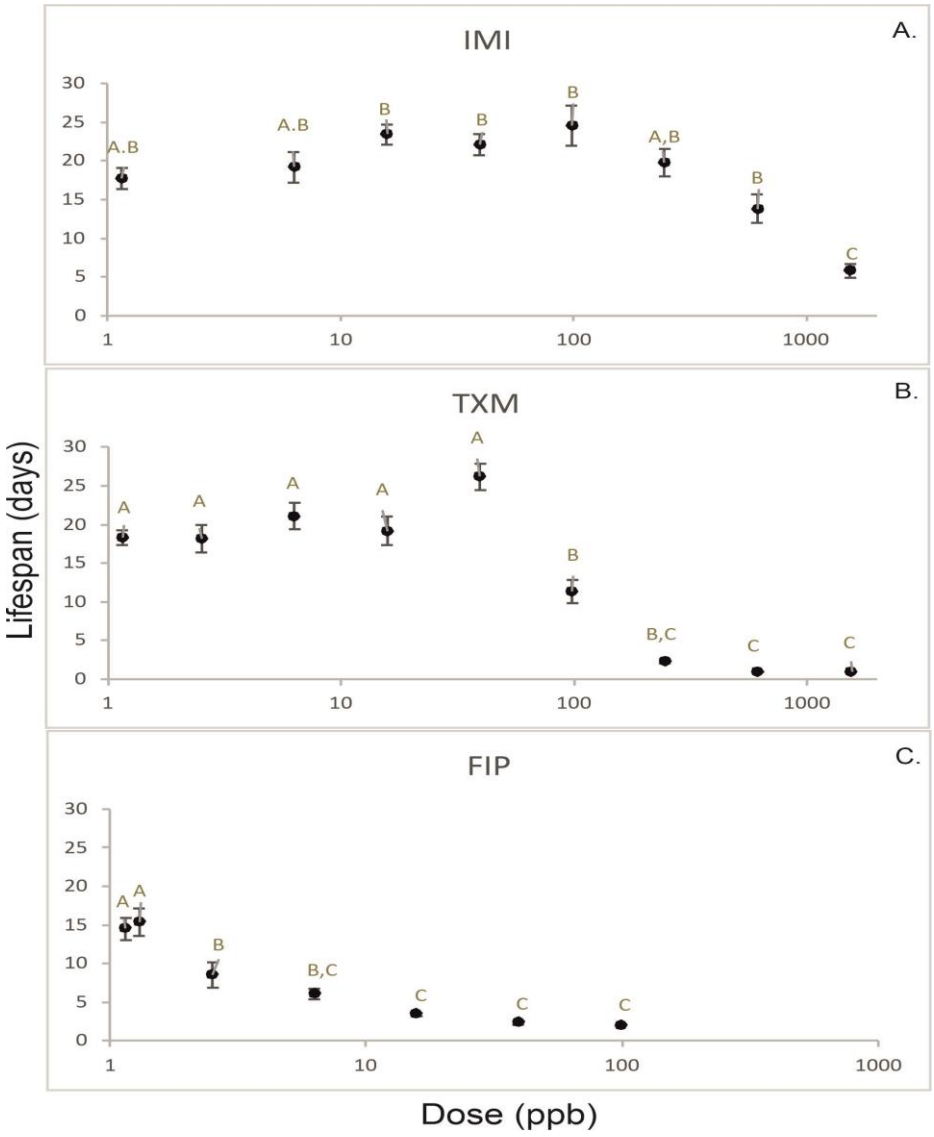
The distinctive and quantifiable pattern between toxicants with high toxicity/TRT and pesticides with low toxicity and little sign of bioaccumulation extends beyond the Haber's slope. The graphs themselves also produce unique and useful signatures on the toxicity/TRT of the test substance. A substance with fast toxicokinetics would produce small effects at low doses with similar longevity to the controls, creating a vertical line of log(*t*):log(*C*) points above the controls. The toxic doses would reduce longevity and push the curve towards the horizontal and so generate the characteristic visual pattern of an inverted 'hockey stick', with toxic doses comprising the curved blade and the non-toxic doses forming a stick (Fig. 2.1, Fig. 2.3a,b). For highly toxic/TRT substances, few or no doses reduce longevity, and the pattern would lack an inflection point (Fig. 2.3c). These distinguishing characteristics, along with the Haber's exponent and ingestion:longevity relationship, could aid regulators in readily identifying disproportionately harmful substances that current methods may not be designed to discern.

I am not the first to raise concerns about the over-reliance on LC_{50}s in pesticide regulation (Persoone and Gilette 1990, Chapman *et al.*, 1996; Halm *et al.*, 2006), or the limitations of NOECs (Chapman, Caldwell and Chapman, 1996), nor am I the first to propose the wider application of Haber's Law in

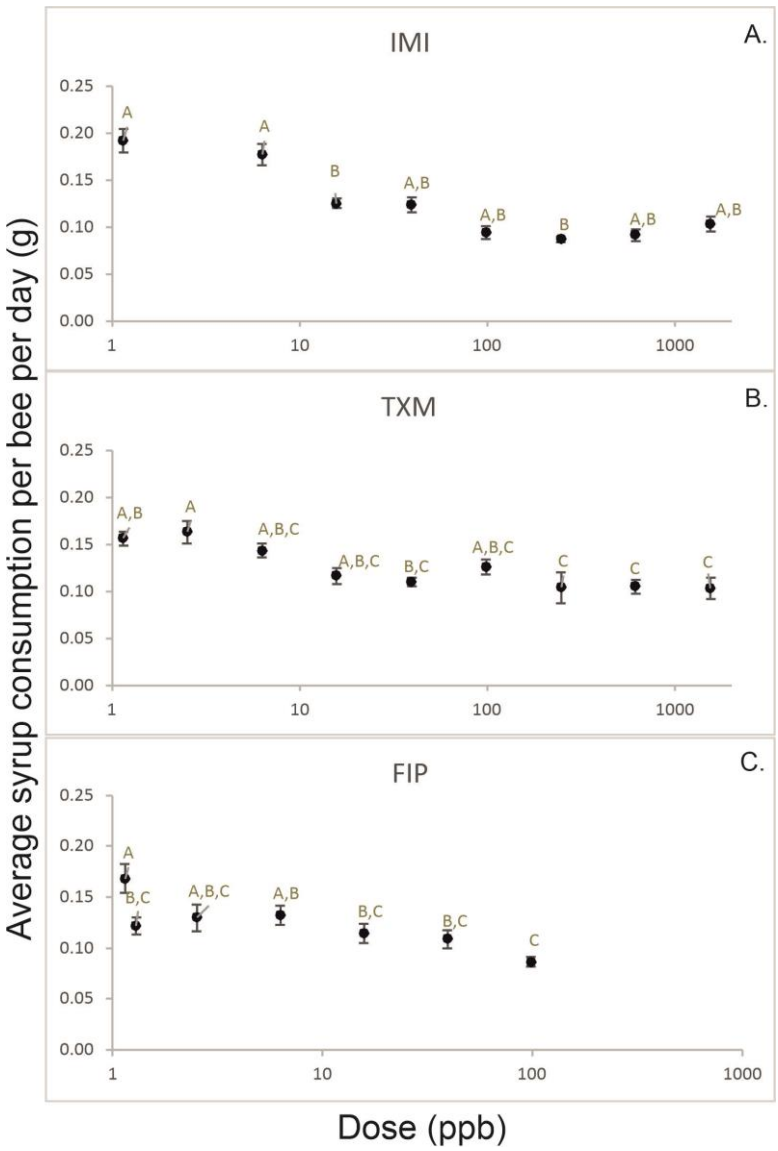
toxicology (Rozman, 2000; Schramm *et al.*, 2002). However, I propose, rather than overhauling conventional methods for Haber's equation, that LC_{50}s and NOECs represent part of a wider picture of safety that can be completed with the use of Haber's Law, whereby the three indicators of safety (NOEC, LC_{50}, and Haber's exponent) are used in conjunction to aid regulatory decisions. Haber's exponent gives a clear and biologically grounded quantifier for TRT, while appropriate doses can reveal a NOEC, and even an LC_{50}. These three 'cardinal numbers' (Cresswell, no date) together can give a better picture of pesticide toxicity than current methods.

Accurate and comprehensive metrics of pesticide toxicity may help not only to protect non-targets, including humans (Cooper and Dobson, 2007), and mitigate environmental contamination (Knowles, 2008), but also to aid the agricultural industry, which relies on pesticides to maintain yields (Oerke, 2006). Farmers' livelihoods rely on successful harvests, and toxic substances like pesticides are used by many for economic gain – not to mention to meet the global demand for agricultural products essential for economy and subsistence (Oerke, 2006; Cooper and Dobson, 2007). As such, the use of toxic interventions is likely to persist, and regulators must find reliable methods for preventing dissemination of unduly harmful chemicals into the environment. My experiments provide evidence that incorporating Haber's Law experiments into laboratory assessments of safety are one such safeguard.

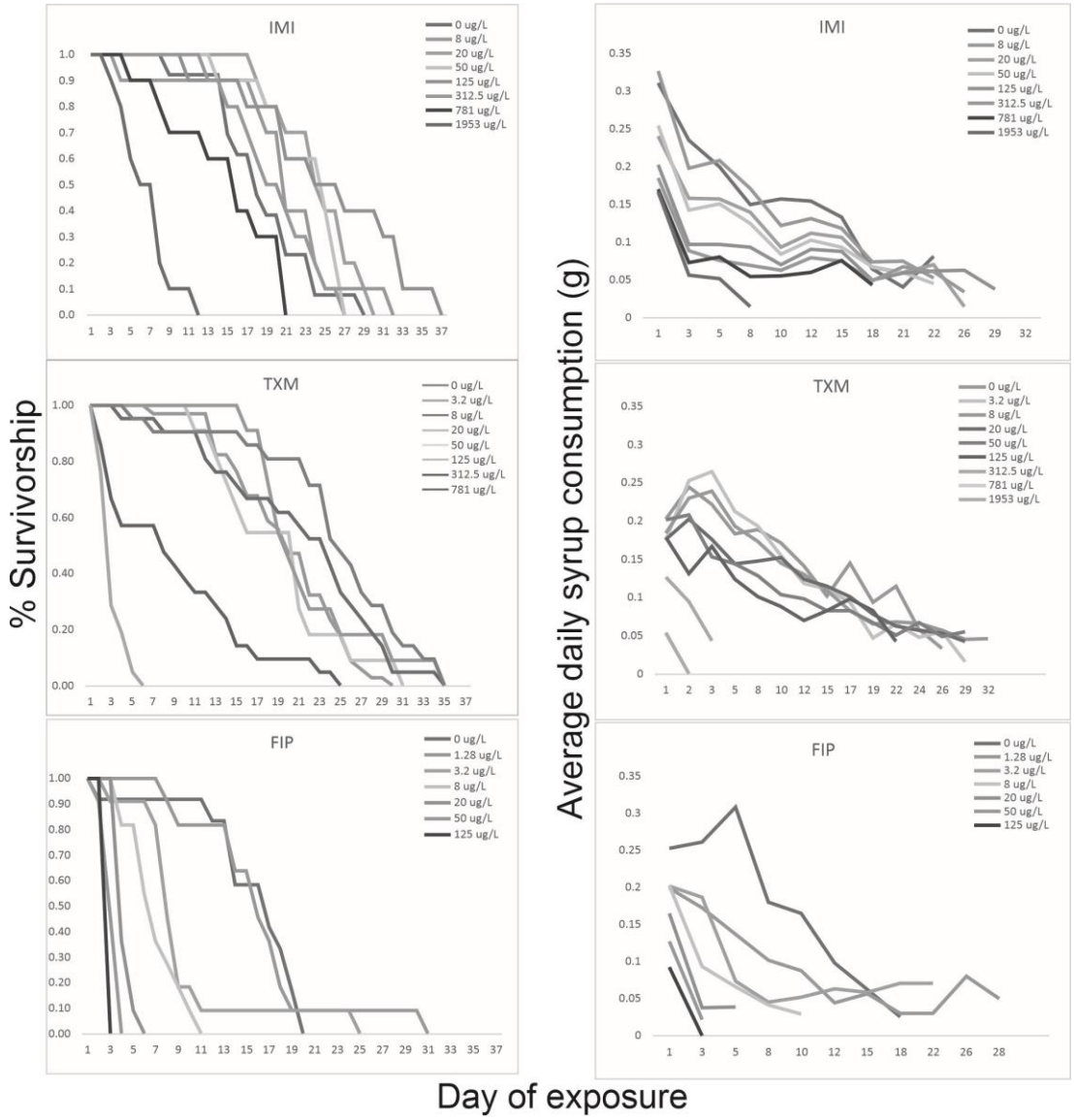
2.6 Supplementary Information



***SI Figure 2.1**: Averaged lifespan, in days, per dose (ppb) with standard error bars for (a) imidacloprid, (b) thiamethoxam, and (c) fipronil. Groupings reflect post-hoc Tukey tests, with all three pesticides having significant ANOVAS for tests of dose vs. longevity (α<0.05). Different groupings (A, B, C) on top of error bars reflect significant differences (α<0.05). Controls are shown slightly offset for ease of examination, as is the 1.01 ppb dose in fipronil (second point from the left).*



***SI Figure 2.2**: Averaged syrup consumption per bee per day per dose (ppb) with standard error bars in grams of syrup for (a) imidacloprid (b) thiamethoxam, and (c) fipronil. Groupings on top of error bars (A.B,C) reflect post-hoc Tukey test results, after significant ANOVAS (α<0.05) for all three pesticides. Doses in different groups represent consumption that was signficantly different (α<0.05). Controls are shown slightly offset for ease of examination, as is the 1.01 ppb dose in fipronil (second point from the left).*



SI Figure 2.3*: Survivorship curves (left) and averaged syrup consumption per dose per day, in grams of syrup (right) for imidacloprid (IMI), thiamethoxam (TXM), fipronil (FIP). Survivorship (in days) is based on proportion survival within each dose.*

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Chapter 3: Pulse Exposures Help Assess the Pace of Pesticide Toxicokinetics in Bumblebees

3.1 Abstract

Neonicotinoid pesticides help protect crops from damaging pests but have been linked to the decline of certain non-target bee species. The potential mismatch between laboratory and field condition exacerbate debates over the extent of harm posed to bees by pesticide use. ‘Recovery’ is a reversal of effect and can be used as an indicator of the speed of toxicokinetics. Pulse-exposure studies provide an opportunity to test recovery, while better mimicking the likely realistic oscillations of pesticide exposures in the field. Using fipronil as a positive control, this study compared the effects of 96-hour pulsed exposures against half-dose constant exposures to examine toxicokinetics and recovery for the neonicotinoid, thiamethoxam. Doses expected to reduce lifespan were selected (fipronil: 2.5 ppb (static) and 5.4 ppb (pulse); thiamethoxam: 98.4 ppb (static) and 196.8 ppb (pulse)). In fipronil exposures, similar reductions occurred in syrup consumption and lifespan for both pulse and consistent doses, indicating that recovery did not occur. In thiamethoxam exposures, bees under pulsed regimes lived twice as long as those on consistent dose, despite ingesting three times as much active ingredient. These results indicate thiamethoxam is unlikely to bioaccumulate. This study also points to the potentially wider applicability of pulse studies for elucidating pesticide toxicokinetics, which may help reduce uncertainty surrounding pesticide risks.

3.2 Introduction

Systemic neonicotinoids are used as plant protection products (PPPs) to shield crops against biting and sucking insect pests (Halm *et al.*, 2006; EFSA, 2012b). While PPPs may help maintain agricultural yields in an era of increasing demands (Zilberman *et al.*, 1991; Lobell, Cassman and Field, 2009), they have been implicated as a threat to wild pollinators, including bumblebees (Pimentel *et al.*, 1992; Allen-Wardell *et al.*, 1998; Goulson, 2013). Despite the widespread use of neonicotinoids as PPPs since the early 1990s (Liu *et al.*, 2005), the degree of harm rendered by their application remains contested (Alkassab and Kirchner, 2016; Sánchez-Bayo *et al.*, 2016; Holder *et al.*, 2018), with increasing evidence from laboratory experiments that neonicotinoids do not enhance mortality of bees at field realistic doses being apparently at odds with continued population declines among bees in the field (Blacquière and van der Steen, 2017). This may be reflective of sub-lethal or synergistic impacts of neonicotinoids on wild populations of bees, or the inability of laboratory experiments to capture the varied nuances of the natural environment. Moreover, while much focus has been given to the relationship between bees and imidacloprid (Cresswell, 2011; Arena and Sgolastra, 2014; Sánchez-Bayo *et al.*, 2016), newer neonicotinoids, including thiamethoxam still need further evaluation, particularly in relation to the threat they may pose to the health of bumblebee species (Pisa *et al.*, 2015).

One reason for the continued uncertainty over the threat to bees posed by neonicotinoids may arise from the mismatch between laboratory testing and field realistic conditions. In the wild, bees are exposed to a variety of threats

(e.g. habitat loss, Varroa mites, viruses, climate change) (Brown and Paxton, 2008; Vanbergen and the Insect Pollinator Initiative, 2013), synergistic effects from pesticides in mixture (Damalas and Eleftherohorinos, 2011; Raimets *et al.*, 2018), and the variation in exposure levels to PPPs due to the timing and intensity of flowering in pesticide treated crops. Foraging bumblebees live approximately 20 days (Doums *et al.*, 2002), and their colonies continue to forage typically for 2-3 months. Crops, which bees may forage on (Cutler *et al.*, 2014; Kessler *et al.*, 2015), flower in differing synchrony and abundance - with rapeseed, for instance, flowering for a month in April or May (Westcott and Nelson, 2001). Such scenarios, where bee lifespan and the flowering of treated crops do not entirely overlap, may create pulses of pesticide exposure in wild bee species (I. Laycock *et al.*, 2014). Moreover, foraging on treated crops has been shown to either possibly deter further consumption of pesticide-containing flowers (Detzel and Wink, 1993; Jager, Barsi and Ducrot, 2013), or encourage feeding on treated crops (REF), either of which could lead to short-term pulse-exposures in bees who mixed foraging on crops and alternative flower sources, such as wildflowers. Examining laboratory bees under pulse-exposure regimes may help give a more realistic picture of the threat posed by neonicotinoid applications in the field.

Pulse-exposures have already been used to examine recovery from pesticide consumption. The ability to ‘recover’ is non-specific (OECD, 2007), and can be measured at a variety of endpoints, including such measures as activity, reproduction rate, lifespan, or feeding rate (Azevedo-Pereira, Lemos and Soares, 2011; Laycock and Cresswell, 2013; Agatz, Ashauer and Brown,

2014). Recovery necessarily implies that, with cessation of exposure, bees are able to regain some function lost through pesticide ingestion due to depuration (i.e. clearance or elimination or detoxification). Ability to recover quickly from exposure would indicate that the pesticide does not have slow toxicokinetics and is not bioaccumulative, as accumulating toxins would persist in the subject's system and continue to impact function even after exposure ended (Franke *et al.*, 1994). This recovery could be made apparent by applying a pulse-exposure treatment to bees and comparing the effects to bees given a consistent exposure to pesticides. Thus, pulse studies may be able to serve the dual purpose of providing a more environmentally realistic exposure in the laboratory, and also be useful as a tool for investigating the speed of toxicokinetics by comparing the effects on bees under pulse and consistent-exposure treatments.

Bioaccumulation remains a contested aspect of neonicotinoid toxicokinetics (Rondeau *et al.*, 2015), and no conclusive evidence exists to suggest they exhibit slow toxicokinetics (Cresswell *et al.*, 2014; Holder *et al.*, 2018). However, the potential ramifications of bioaccumulative substances (Jaga and Dharmani, 2003; Chopra, Sharma and Chamoli, 2011) warrant a thorough investigation to ensure they do not pose the risk of harm posed by known bioaccumulative substances, such as fipronil (Carvalho *et al.*, 2014). Specifically, slow toxicokinetics enable xenobiotics present even at trace dietary amounts to accumulate to severely toxic levels in an exposed subject's body. Fipronil has been widely banned partly in acknowledgement of its bioaccumulative tendencies, and may have been responsible for some of the

impacts on bee health previously attributed to neonicotinoids (Holder *et al.*, 2018). Consequently, fipronil provides a useful positive control in bioaccumulation studies. Here, I tested the effect on bumblebees of pulse-exposures as a tool for examining bioaccumulation in scenarios better mimicking the natural oscillation of pesticide exposure in the field in order to: (i) characterize the toxicokinetics of thiamethoxam and fipronil; (ii) evaluate the correspondence of varied bioassays of toxicokinetics; (iii) assess threats in the field posed by focal pesticides; and (iv) propose regulatory development using laboratory exposures to evaluate threats from bioaccumulative toxicity.

3.3 Methods

3.3.1 Provenance and Husbandry of Bees

Experiments were conducted on commercial colonies of *B. terrestris* (Biobest, Westerlo, Belgium). Each experiment was conducted on a separate shipment of bees (N=1 per experiment). Bees were placed individually in softwood boxes (0.07 x 0.07 x 0.035 m) fitted with mesh sides and given syrup feeders (punctured micro-centrifuge tubes containing 1.27 Kg/L fructose/glucose/saccharose solution; Attracker, Koppert B.V.). The caged bees were kept in a semi-controlled environment (approximately 25 °C, 45% relative humidity). The bees were fed *ad libitum* undosed syrup for 72 hours prior to experimental dosing for acclimation.

3.3.2 Treatments

Treatments were divided into ‘static’ ‘pulse’ and ‘control’, which were delivered by dosing feeder syrup. Bees in the static treatment were given the lowest

dose with a clearly observable effect on lifespan, which was determined from previous experiments on these pesticides (thiamethoxam: 125 ug/L, fipronil: 3.2 ug/L). Bees in the pulse treatment were given double the static dose (thiamethoxam: 250 ug/L; fipronil: 6.4 ug/L) for 48-hours, followed by 48-hours of undosed syrup, and I repeated the pulse pattern for the lifespan of the specimens. Control bees were given undosed syrup for the duration of the experiment.

The thiamethoxam experiment used 60 bees, 20 each for pulse, static and control treatments. The fipronil treatments used 45 bees, 15 each in pulse, static and control.

3.3.3 Preparation of Doses

3.3.3.1 Thiamethoxam

Powdered thiamethoxam (Pestanal 37924; Sigma-Aldrich, Gillingham, UK) was dissolved in deionized water to produce a primary stock which was further diluted to produce a 100X concentrated ‘pulse’ stock of 25,000 ug/L (19,684 ppb). This was diluted by 50% to produce the ‘static’ stock of 12,500 ug/L (9,843 ppb). 1 mL aliquots of those stocks were added to 99 mL of clean syrup to produce the “pulse” (250 ug/L) and “static” (125 ug/L) doses. Control syrup was prepared with a 1:99 ratio of deionized water to syrup.

3.3.3.2 Fipronil

Powdered fipronil (Pestanal 46451.; Sigma-Aldrich, Gillingham, UK) was dissolved in acetone to create a primary stock, further diluted to produce a

100X concentrated ‘pulse’ stock of 640 ug/L (504 ppb). This was then diluted by 50% to produce the ‘static’ concentrated stock of 320 ug/L (2.52 ppb). 1 mL aliquots of stock were added to 99 mL of clean syrup to produce the “pulse” (6.4 ug/L) and “static” (3.2 ug/L) doses. Control syrup was prepared with a 1:99 ratio of acetone to syrup.

3.3.4 Experimental Exposures and Statistical Analysis

Bees were monitored daily for mortality. Syrup was changed precisely every 48-hours to ensure an accurate pulse regime, and new batches of treated syrup were made weekly. Pulse bees were given treated syrup for the first 48-hours after clean syrup acclimation, then 48-hours of clean syrup, repeating for the duration of the lifespan.

All analysis was conducted using SPSS (IBM SPSS, v. 25, Chicago, Illinois, USA). ANOVAS with post hoc Tukey tests were used to assess variation among treatments in consumption and lifespan, while t-tests were used to compare nanograms (ng) of Active Ingredient (AI) consumed between treatments.

3.4 Results

3.4.1 Differential Toxicokinetic Pace: Survivorship and Consumption

Comparative analysis of pulse and static treatments reveals a differential pace of toxicokinetics in bees exposed to thiamethoxam or fipronil (Fig, 3.1). As expected, continuous exposures to both pesticides reduced syrup consumption and lifespan in relation to controls, which verifies the presence of pesticide in treatment syrups. However, only bees exposed to thiamethoxam had a less severe response to the pulse treatment, which is consistent with fast-toxicokinetic recovery. In contrast, pulse and static exposures were equivalent in bees exposed to fipronil (Fig. 3.1).

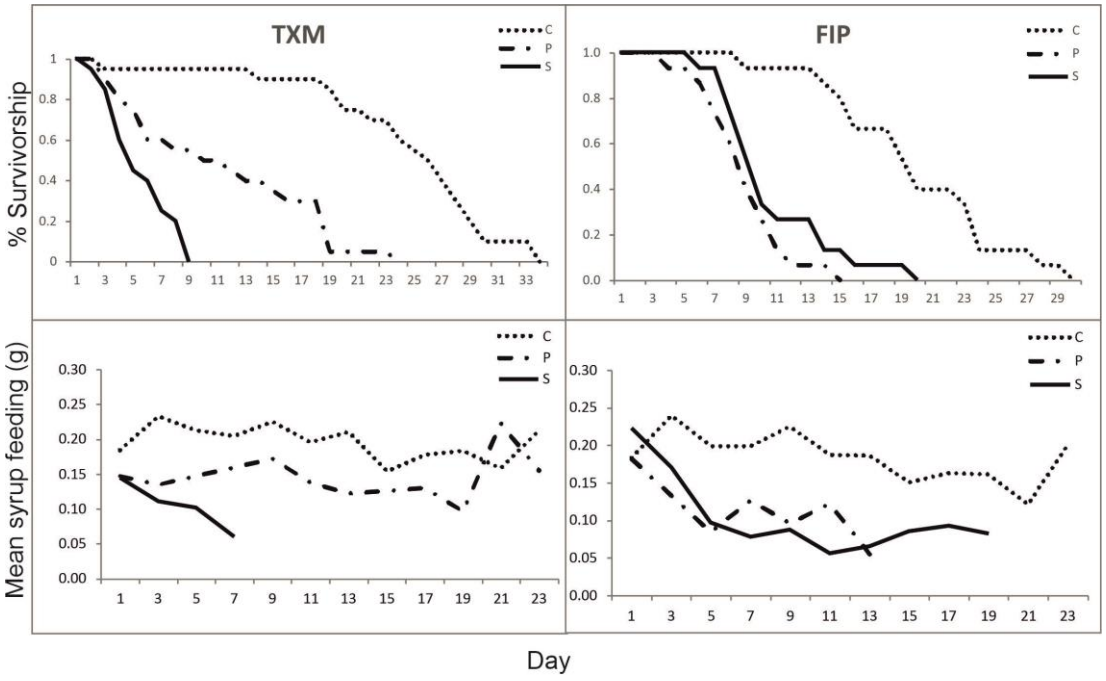

*Figure **3.1a** (top)**:** Survivorship, based on proportion alive per dose per day.. **3.1b** (bottom): Average daily syrup consumption (g) per treatment.*

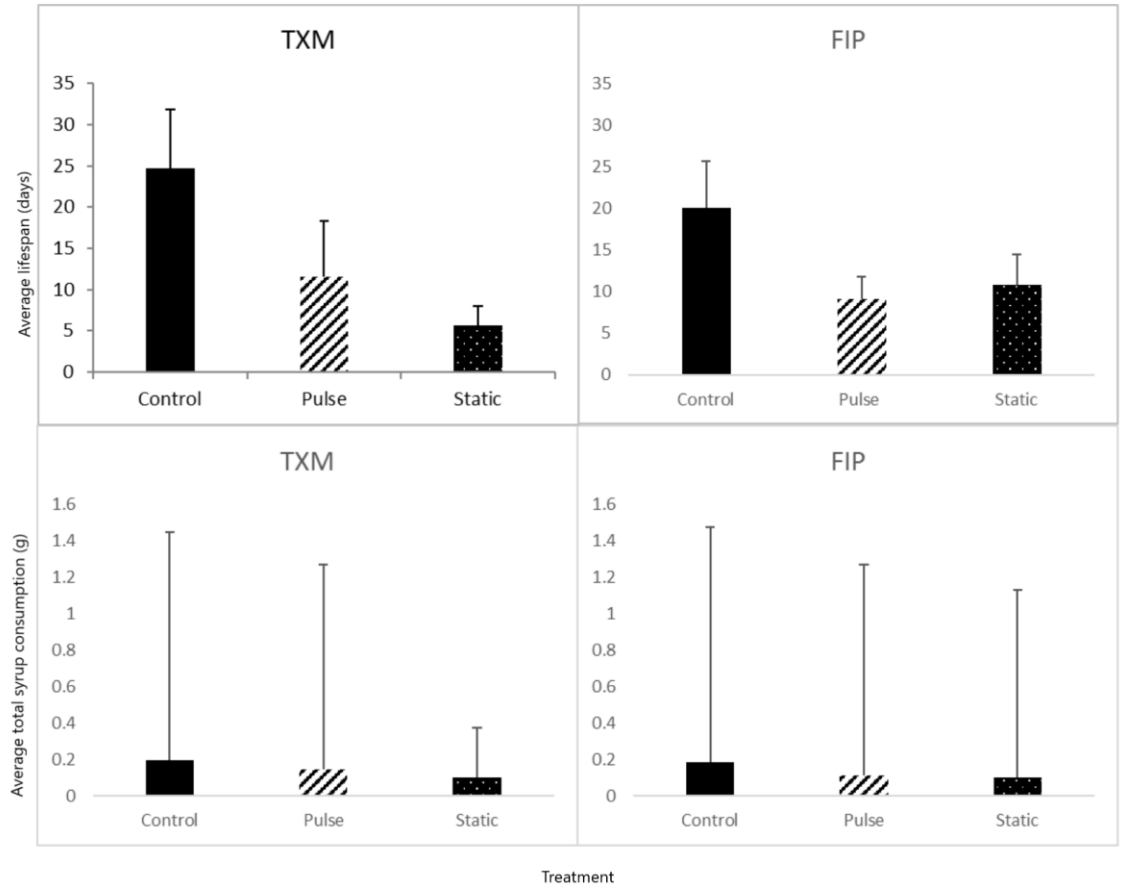

*Figure **3.2a** (top): Mean lifespan by treatment (days) with SE bars. **3.2b** (bottom): Average lifetime syrup consumption (g) by treatment with SE bars.*

In thiamethoxam, variation amongst treatment existed for syrup consumption (Fig. 3.2; ANOVA: $F(2,57)=70.36$, $p<0.001$), and longevity (Fig 3.2; ANOVA: $F(2,57)=55.24$, $p<0.001$). Tukey tests ($p<0.05$) revealed that while both treatments ate less and lived shorter lives than control bees, pulse-treatment bees not only ate more than static-treatment bees, but also lived significantly longer, with an average lifespan of 11.5 ± 6.8 days, versus only 5.7 ± 2.3 days in static-treatment bees. I discovered differential toxicity between pulse and static treatments, with pulse-exposure bees maintaining higher consumption and longevity despite higher levels of exposure during treated periods

indicates fast toxicokinetics, because it appears that the 48-hour respite on undosed syrup was sufficient to allow some clearance of the pesticide.

Fipronil treatments varied significant in consumption (Fig 3.2; ANOVA: $F(2,42)=28.13$, $p<0.001$) and lifespan (Fig 3.2; ANOVA: $F(2,42)=30.34$, $p<0.001$). However, post hoc Tukey tests ($p<0.05$) showed no difference in consumption (P=1.34 ± 0.35g, S=1.11 ± 0.14g) or lifespan (P=10.8 ± 3.7, S=9.1 ± 2.7 days) between pulse and static treatments. The consistency of toxic effects between pulse and static treatments indicates that bees were unable to recover during clean-pulse periods, indicative of slow toxicokinetics of fipronil when ingested by bumblebees.

3.4.2 Differential Toxicokinetic Pace: Lifetime AI Ingestion

A similar pattern emerges when examining the mass of active ingredient ingested between pulse and static treatments. In thiamethoxam, despite pulse doses being twice as concentrated, they were able to live longer and ingest significantly more active ingredient (Fig. 3.3; P: lifetime ingestion of active ingredient=177.8 ± 118.1 ng; S= 59.1 ± 26.8 ng) than static doses (t-test: $t_{39}=7.22$, $p<0.001$). Only a toxicant with fast toxicokinetics, whereby clearance is providing recovery of lifespan, could account for higher ingestion rates while maintaining longevity in pulse-treatment bees.

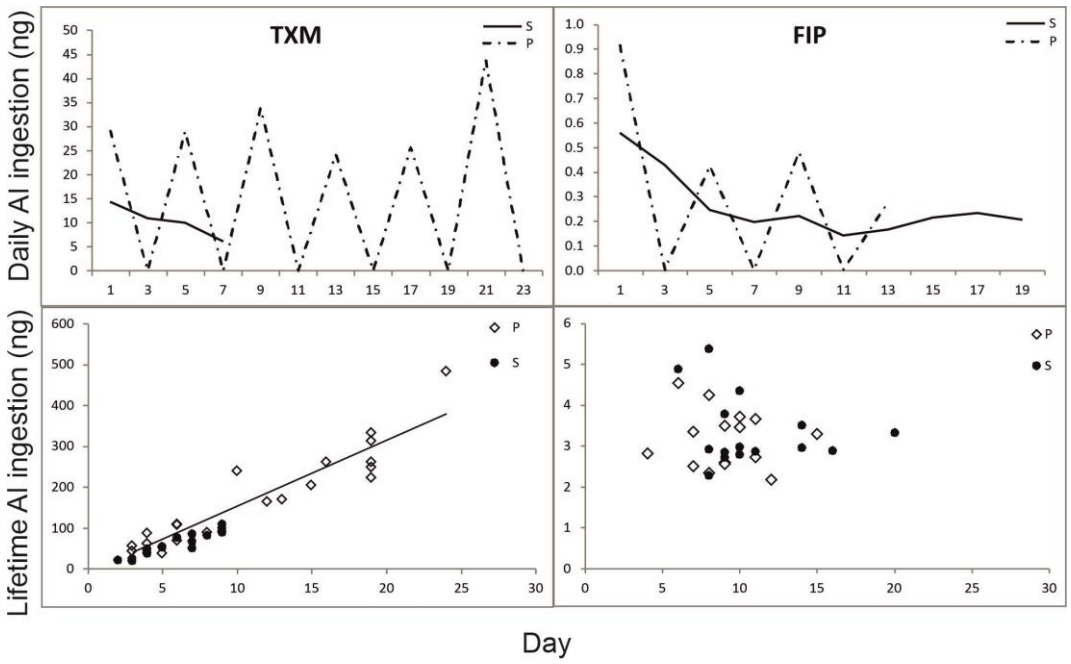

*Figure **3.3a** (top): Daily ingestion (ng) of active ingredient by pulse (p) and static (s) treatments, reflecting the 48-hour pulse of clean and dosed syrup in pulse. **3.3b** (bottom): Lifetime ingestion of active ingredient of pesticide (ng for lifespan in days) for both pulse (open dots) and static (closed dots) treatments for thiamethoxam (left) and fipronil (right).*

With fipronil, however, the pulse bees exhibited similar lifespans and active ingredient ingestions as static bees (t-test: t_{29}=22.81, p=0.14), where cumulative ingestion of AI of approximately 3ng (Fig 3.3; P: lifetime ingestion of active ingredient=3.2 ± 0.9 ng; S=3.4 ± 0.7 ng). The consistency amongst treatment indicates an inability to recover in respite periods of the pulse-treatment characteristic of slow toxicokinetics, whereby fipronil persists in the bee system until a fatal ‘toxic threshold’ of approximately 3 ng is reached.

3.5 Discussion

3.5.1 Toxicokinetic Inferences

This study demonstrates a differential response to pulse-exposures for bumblebees ingesting fipronil or thiamethoxam, which appears to be symptomatic of the different toxicokinetic paces of the two pesticides. The usefulness of pulse experiments as a measurement of toxicokinetic pace arises from the comparative response between pulse and static treatments, as substances with slow toxicokinetics would be characterized by a persistence of the toxicant in the system, manifested as an equivalent lifespan of pulse-treatment and static-treatment bees. By contrast, substances with fast toxicokinetics would be expected to experience minimal in-body residence of the pesticide, and clearance during the respite periods of the pulse treatment would allow for recovery, measured here as increased lifespan and syrup consumption, when compared to consistently exposed bees. Here, thiamethoxam exhibits the signs of fast toxicokinetics – evidenced by increased consumption and longevity in pulse-exposed bees despite higher overall active ingredient ingestion when compared to static-treatment bees. In contrast, fipronil exhibits the indicators of slow toxicokinetics as lifespan, syrup consumption and active ingredient ingestion were equal amongst experimental treatments.

Bees exposed to thiamethoxam showed increased consumption and lifespan in the pulse-exposures compared to consistently exposed bees, despite consuming significantly more active ingredient. Such results indicate that the 48-hour pulse periods are ample to allow some recovery from thiamethoxam

ingestion in bumblebees. These results are consistent with previous findings, as they are similar to reports of 48-hour clearance of imidacloprid in the same species (Laycock and Cresswell, 2013). That is not to say that the high exposures did not cause some injury (i.e. contribute to toxic load), as pulse-treated bees did consume less syrup (a known effect of high dose thiamethoxam (Elston, Thompson and Walters, 2013; Overmyer *et al.*, 2018)), and live shorter lives than control bees. However, bees in the pulse regime were able to recoup function and even exceed expected lifespans with oscillating exposure, even at doses much higher than ever to be found in the field (EFSA, 2016), indicating the pesticides are moving quickly through the bee's system. While the pace of toxicokinetics for ingested thiamethoxam has not been sufficiently researched, the closely related neonicotinoid imidacloprid has been widely scrutinized for its potential to accumulate (Sánchez-Bayo, Belzunces and Bonmatin, 2017), with the increasing consensus that recovery is possible from exposure (Cresswell *et al.*, 2014; Holder *et al.*, 2018), even in more susceptible bumblebees (Laycock and Cresswell, 2013). In summary, the literature review in Chapter 1 and the experiments of Chapters 2 and 3 indicate that neonicotinoids are likely to exhibit fast toxicokinetics in bees.

By contrast, fipronil exhibits signs of slow toxicokinetics, as lifespan, consumption and ng of active ingredient ingested were equal amongst the static and pulse exposures. The consistency of effect between pulse and static exposures despite the 48-hour clean-syrup periods indicates persistence of fipronil in the system, which reached a toxic threshold (here reported as 3 ng of active ingredient or 10 days of exposure at the experimental doses), which

induced mortality. These observations suggest that 48-hours is insufficient to clear fipronil, and that the accretion of the toxicant to a toxic threshold is expected by a slow-toxicokinetic bioaccumulative substance, one that is unlikely to be cleared in relevant time scales to bee species. These findings are consistent with the known bioaccumulative nature of fipronil, which has long been widely banned (Gunasekara *et al.*, 2007; Lu *et al.*, 2015).

3.5.2 Correspondence of Various Toxicokinetic Bioassays

Pulse exposures were used here to characterize the pace of toxicokinetics and to detect bioaccumulative tendencies. Thus, the pulse-exposure provides an additional assessment of TRT, along with the Haber's exponent and ingestion:longevity relationship evaluated in Chapter 2. Where the three metrics align, a toxicokinetic bioassay is established that is highly suggestive of the pace of toxicokinetics within the given study subject. Here, all three indicators identified the positive control (fipronil) as exhibiting slow toxicokinetics, and thereby generating TRT. This lends validity to the findings that thiamethoxam exhibits fast toxicokinetics when ingested by bumblebees, consistent amongst the Haber's exponent, the ingestion:longevity relationship and the pulse-exposure experiment. Therefore, it appears thiamethoxam is unlikely to be either bioaccumulative, or capable of generating TRT.

The proposition that a pulse-exposure can serve as a toxicokinetic bioassay gains when correspondence is also found in other measures derived from chronic paradigm laboratory testing. As chronic paradigm experiments are conducted for the lifespan of the subject, a more realistic examination of

pesticide effects can be determined than those of the acute paradigm LC/NOEC studies often used in regulatory testing. As the Haber's exponent also allows for the calculation of LC/NOEC regulatory metrics, the pulse experiments additionally provide an opportunity to determine the minimum recovery period for fast toxicokinetic substances, as the pulses could be fine-tuned to give a more precise measure of recovery timescales for pesticide exposures. Here, the power of the toxicokinetic bioassay lends further support to the widespread ban on fipronil, while helping to dispel some concerns regarding thiamethoxam application, as the fast toxicokinetic properties and 48-hour recovery make thiamethoxam unlikely to harm bees at trace levels in the field.

3.5.3 Threats Posed to Bee Health by Focal Insecticides

Our findings indicate that thiamethoxam is unlikely to accumulate due to fast toxicokinetics, which agrees with studies concluding that doses of thiamethoxam within the field-realistic range (Elston, Thompson and Walters, 2013) do not reduce the lifespan of bees (Thompson *et al.*, 2015). It is important to reiterate that while fipronil treatments were comparable to doses applied to crops (Zaluski, Justulin Jr. and Orsi, 2017), thiamethoxam exposures were 10 and 20 times the field-realistic range of 1-11 ppb (Cresswell, 2016; Overmyer *et al.*, 2018), and still clearance was apparently effective for thiamethoxam-dosed bumblebees.

At the colony level, thiamethoxam has been shown not to affect honeybee adult or larval mortality, overwintering success or consumption, even at doses

exceeding field realistic levels (Overmyer *et al.*, 2018), which are unexpected characteristics if a substance is highly bioaccumulative. Thiamethoxam may even be less toxic even than other neonicotinoids, with some reports of no effect at field-realistic doses on flight activity and homing (Thompson *et al.*, 2016), behavior and olfactory learning (El Hassani *et al.*, 2008), colony development and health (Pilling *et al.*, 2013), which have been reported for other neonicotinoids. However, as with many neonicotinoids, there are conflicting reports that do show sub-lethal harm to bees from thiamethoxam (Alkassab and Kirchner, 2017), making it difficult to assert whether or not thiamethoxam renders harm to bees. Ultimately more scrutiny will be required to fully assess the safety of its widespread use as an insecticide application.

3.5.4 Potential Regulatory Development to Include Bioassays for TRT

Measures of bioaccumulation including pulse-exposure studies, are needed to fully understand the potential harm to non-target species from pesticide applications. However, measures of bioaccumulation, especially in the field, are challenging, often lacking statistical power (EFSA, 2014) and sometimes require specialist equipment (Feng *et al.*, 2016; Coulon *et al.*, 2017). Pulse studies represent a simple, cost-effective, and widely applicable format for examining toxicokinetics and bioaccumulation which may help aid the recent push to include measures of bioaccumulation in regulatory testing (Gaylor, 2000; EFSA, 2012b; Holder *et al.*, 2018). By using single bees in laboratory conditions, the number of bees needed for testing is minimized, while also preventing potential contamination of field-study environment, and eliminating confounding factors that may give confusing impressions of toxicokinetics

(EFSA, 2014). While further testing, including thorough field testing, is needed to understand the full scope of pesticide-bee interactions, utilizing simple tests that comprise the toxicokinetic bioassay may prevent use of unexpectedly toxic pesticides in the field in the future (Cresswell, 2018). Here I have successfully used the pulse-exposure format to corroborate the bioaccumulative tendencies of fipronil, while ascertaining that thiamethoxam is unlikely to bioaccumulate within bumblebees, with 48-hour rest periods sufficient to recover some lost function in consumption and lifespan, even with continued, oscillating, high dose exposure. Implementation of the toxicokinetic bioassay in regulatory testing could provide a simple method for filling a gap in regulatory testing for bioaccumulative toxicity and to better understand the threats posed by toxicants to non-target species.

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Concluding Discussion

4.1 Fast Toxicokinetics of Neonicotinoids and the Chronic Paradigm

Continued declines in wild pollinators keeps alive research into the threats posed by neonicotinoid pesticides. Studies have previously tried to establish whether neonicotinoids have slow toxicokinetics, whereby toxic effects are amplified by TRT (Walker *et al.*, 2012). If present in pesticides currently in use, slow toxicokinetics and bioaccumulation could possibly account for unanticipated declines in non-target species seen in recent years (Holder *et al.*, 2018). While seemingly compelling evidence exists on both sides of the debate, a closer examination of the current literature regarding the toxicokinetic properties of neonicotinoid pesticides, coupled with the execution of two 'chronic paradigm' experiments intentionally designed to address bioaccumulative toxicity have helped shed insight into the propensity of these substances to accumulate, I conclude that it is unlikely neonicotinoids exhibit TRT. Moreover, the experiments done here provide a framework for chronic testing that may aid future pesticide regulation by providing quantifiable metrics of slow-toxicokinetic bioaccumulation.

From reviewing the literature, I have established that bees are equipped with robust metabolic detoxification mechanisms (CYPs) and that neonicotinoids are liable to successful elimination by these metabolic pathways (Olsen, Oostenbrink and Jørgensen, 2015). The metabolic detoxification may account for reports that imidacloprid, the most researched and most debated of the neonicotinoid pesticides, can be cleared from bumblebee systems in 48-hours
(Laycock and Cresswell, 2013), and by hardier honey-bees in 24-hours (Cresswell *et al.*, 2014). These results may also stem from the reversibility of

ligand-receptor binding due to weak electrostatic bonds that neonicotinoids use to bond to their target sites (Kezić, Lucić and Sulimanović, 1992). With a complex acetylcholine network in the insect nervous system, and the reversible ligand bonding of neonicotinoids, this leads to the conclusion that neonicotinoids are unlikely to persist at target sites in bees, aiding in rapid recovery from exposure. However, while neonicotinoids are unlikely to exhibit TRT, pesticides invariably contribute to exposed-bees toxic load, and injuries may nevertheless manifest as sub-lethal endpoints, or as a result of secondary metabolites created during the detoxification process (Alkassab and Kirchner, 2017).

To quantify bioaccumulative toxicity, I employed Haber's Law to analyse the results of dose-dependence experiments that used the highly toxic phenylpyrazole pesticide fipronil as a positive control against bees exposed to either imidacloprid, or the more recently developed neonicotinoid, thiamethoxam. The Haber's Law exponent (*b*), derived from the regression line of the log transformed dose against log transformed longevity of exposed bees, acts as a quantifier for TRT, whereby pesticides that are easily cleared behave with $b=1$ regression, meaning bees lifespan corresponded proportionally to dose (Rozman, 2000; Holder *et al.*, 2018). Both thiamethoxam and imidacloprid had Haber's exponents approaching 1, and both only statistically impacted lifespan at doses more than 100-times field-realistic exposures, indicating fast toxicokinetics in both neonicotinoids. Pesticides with steeper regression lines ($b>1$, approaching 2) are having toxic effect (reduction in lifespan) faster than predicted by dose, and are considered to have slow toxicokinetics. Fipronil, known to bioaccumulate (Connelly, 2001; Holder *et al.*, 2018), had a Haber's exponent

approaching 2, and began diminishing lifespan well within the field-realistic exposure range. The slow toxicokinetics of fipronil and fast toxicokinetics of the two neonicotinoids were further confirmed by the ingestion:longevity relationships. Comparing dose-dependence trends of the neonicotinoids with the highly toxic fipronil, I found the neonicotinoids to be relatively benign, and in low-doses (those reflective of the higher-end of field possible doses) even a stimulant for longevity – a hormetic response previously recorded for imidacloprid (Holder *et al.*, 2018). Neonicotinoids may affect sub-lethal endpoints, but neither thiamethoxam nor imidacloprid showed indications of reducing longevity at trace-levels, nor did they show indications of bioaccumulative toxicity, and are unlikely to pose a threat to wild bees in these respects.

While pulse-exposure experiments have been used to better reflect field-realistic fluctuations in pesticide uptake (Ian Laycock *et al.*, 2014), they also provide another platform for chronic paradigm toxicokinetic testing. In fact, of the neonicotinoids previously examined, only thiamethoxam could be tested using this paradigm, as doses as high as 1500 ppb (compared to the 1-11 ppb field realistic range) of imidacloprid were insufficient to induce desired longevity effects in the Haber's Law experiments of Chapter 2, and thiamethoxam doses had to be as strong as 200 ppb for the same purpose. Here, I compared the lifespan of bees given a steady amount of pesticide to those given a pulse of twice the amount for 48-hours, followed by 48-hours of clean syrup, a pattern that repeated for the lifespan of the experimental bees. For bees given pesticides with slow toxicokinetics, the accumulation of the toxicant and eventual arrival at a toxic threshold would induce death, so despite periods of respite, bees exposed to TRT

substances (and twice the AI), would have similar lifespans to bees given a steady amount as the toxins persist in the body during recovery periods. Substances that do not accumulate, however, and which could be successfully cleared within 48-hours, would allow bees to ingest higher amounts of AI without the corresponding loss of lifespan. I estimate that bees exposed to fipronil (at doses within the field realistic range) reached a toxic threshold of 3 ng of AI, which was accumulated within a lifespan of 10 days, whether on pulse or consistent dose exposures, a marker of slow toxicokinetics. In contrast, bees exposed to dietary thiamethoxam bees under pulse conditions were able to live twice as long despite ingesting three times the amount of AI in their lifetimes than consistently exposed bees, corroborating the Haber's finding that thiamethoxam is unlikely to exhibit TRT.

Beyond validating the toxicokinetic evidence that neonicotinoids likely do not exhibit bioaccumulative toxicity, both the Haber's and pulse experiments demonstrated here provide reproducible frameworks for testing substances for TRT. These experiments are simple, cheap, easy to replicate, and minimize equipment, potential contamination, and study subjects. They can be adapted for myriad substances and study subjects. They also represent a vital gap in the current regulation industry. While governmental reports recognize the significance and the potential to test for bioaccumulation (EFSA, 2012a), they often continue to rely on acute paradigm metrics of NOEC and LC_{50}, testing regimes that are inherently ill-prepared to account for TRT. Moreover, the merits of the Haber's analysis extend beyond TRT, as the regression also allows for the calculation of NOECs and LC_{50}s. Using chronic paradigm dose-dependence

experiments, these metrics can be calculated from results grounded in the study subject's biology, rather than as statistical extrapolations (Chapman, Caldwell and Chapman, 1996). The pulse experiments, beyond measuring TRT and potential toxic thresholds, also could be fine-tuned to determine times needed to clear substances that do not exhibit bioaccumulative toxicity, providing further insights into the toxicokinetics of substances. I therefore propose the use of Haber's Law and pulse-exposure experiments demonstrated here can aid regulatory testing and potentially safeguard from the use of slow toxicokinetic substances that may be missed by acute paradigm testing regimes.

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