

**Biological effects and effect mechanisms of
neonicotinoid pesticides in the bumble bee
*Bombus terrestris***

**Submitted by Ian Laycock to the University of Exeter
as a thesis for the degree of
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Abstract

Bumble bees provide valuable pollination services to many agricultural crops and wild flower species. Consequently, evidence that wild populations are in decline has caused widespread concern. Among multiple causal factors, some have singled out neonicotinoid pesticides as potentially a major contributor to these declines. Bumble bees are exposed to neonicotinoids, such as imidacloprid and thiamethoxam, whilst foraging for nectar and pollen from treated crops. For neonicotinoids to cause population decline, the typical residues that bumble bees encounter in the field (defined here as between 1–12 µg kg⁻¹) should be capable of reducing colony success by detrimentally impacting demographically relevant endpoints such as reproduction and worker performance. Whether field-realistic neonicotinoids are capable of causing such effects is yet to be fully established. The overall aim of this thesis was to investigate the effects of field-realistic neonicotinoids on endpoints of demographic importance and improve understanding of the effect mechanisms of neonicotinoids in bumble bees. Laboratory experiments were conducted with *Bombus terrestris* L. exposed to dietary neonicotinoids up to 98 µg kg⁻¹.

Results showed that food consumption and production of brood (eggs and larvae) in queenless *B. terrestris* microcolonies were significantly reduced by the two highest concentrations of imidacloprid and thiamethoxam tested (39, 98 µg kg⁻¹), but only imidacloprid produced a negative effect when concentrations were in the typical field-realistic range. Imidacloprid's affect on microcolonies was mirrored in queenright colonies where field-realistic concentrations substantively reduced both feeding and brood production. It was postulated that the detrimental effects of imidacloprid on brood production emerge principally

from nutrient limitation imposed by the failure of individuals to feed. Removing imidacloprid from the bees' diet resulted in the recovery of feeding and brood production in queenright colonies, even when previously exposed to high doses ($98 \mu\text{g kg}^{-1}$). Investigation into the effect mechanisms of imidacloprid in *B. terrestris* revealed that cytochrome P450 enzymes are not important for metabolism of the neonicotinoid in adult workers. A transcriptomic analysis indicated *B. terrestris* exhibit a general stress response to imidacloprid, characterised by the alteration in expression of genes involved in, for example, metabolism and storage of energy.

The thesis findings raise further concern about the threat of imidacloprid to wild bumble bees. However, they also suggest that some demographically important endpoints are resilient to imidacloprid as a realistic pulsed exposure, and that bumble bees may be less sensitive to field-realistic concentrations of thiamethoxam. Further research, which is required to fully establish the demographic consequences for bumble bees of exposure to neonicotinoids, can be developed based on the foundation of work presented here.

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List of Definitions

Brood production	Production of eggs and larvae in a colony or microcolony
Continuous exposure	An exposure period in which bees ingest neonicotinoid-contaminated food only, and do not have access to uncontaminated food
Microcolony	Nests comprising a small number of bumble bee workers that develop in the absence of their queen until a worker becomes dominant and lays eggs
More-than-additive effect	Describes the effect of two toxins that when combined produce a toxicity greater than expected from the sum of their individual toxicities
'On dose'	14-day period of pulsed exposure in which bees are fed on syrup dosed with neonicotinoid pesticide
'Off dose'	14-day period of pulsed exposure that follows 'on dose' period in which doses are removed and all bees feed exclusively on clean syrup
Pulsed exposure	A exposure in which bees feed on pesticide-contaminated food for a period of time, and subsequently switch feeding on uncontaminated food
Recuperation	The recovery of performance in bees when neonicotinoid exposure ends
Repression	The reduction of performance in bees following exposure to a neonicotinoid pesticide
Syrup	A solution of sugar(s) fed to bees as a nectar substitute
Terminal oocyte	Oocyte closest to ovipositor, i.e. the most mature oocyte that is yet to be laid
'Typical field-realistic' range	Concentration range of neonicotinoids typically detected in nectar or pollen of treated crops (~1–12 ppb)
'Worst-case field-realistic' range	Concentration range of neonicotinoids that are occasionally detected in nectar or pollen, but fall outside of the typical field-realistic range (~12–100 ppb)

List of Abbreviations and Uncommon Units

$\mu\text{g kg}^{-1}$	Micrograms per kilogram
$\mu\text{g L}^{-1}$	Micrograms per litre
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
BHM	Bayesian Hierarchical Model
CDD	Conserved domain database
cDNA	Complementary deoxyribonucleic acid
CO1	Cytochrome c oxidase subunit 1
d	Days
DEFRA	Department for Environment Food and Rural Affairs
EBI	Ergosterol biosynthesis inhibitor
EC ₅₀	Median effective concentration
EFSA	European Food Safety Authority
ELO	Elongation of very long chain fatty acid
ES	Enrichment score
ETC	Electron transport chain
FAR	Fatty acyl-CoA reductase
FERA	Food and Environmental Research Agency
FPKM	Fragments per kilobase of transcript per million mapped fragments
GLD	Glucose dehydrogenase
GLM	General linear model
GO	Gene ontology
h	Hours
HSP	Heat shock protein
IMI	Imidacloprid
IPI	Insect Pollinators Initiative
JHBP	Haemolymph juvenile hormone binding protein
LCMS	Liquid chromatography-mass spectrometry
LD ₅₀	Median lethal dose
min	minute
MTA	More-than-additive

m/z	Mass-to-charge ratio
nAChRs	Nicotinic acetylcholine receptors
NADK	NAD kinase
NCBI	The National Centre for Biotechnology Information
NERC	Natural Environment Research Council
OSR	Oilseed rape (also known as <i>canola</i>)
P450	cytochrome P450 monooxygenases (enzymes)
PBO	Piperonyl butoxide
ppb	Parts per billion
psi	Pounds per square inch
R ²	R-squared (coefficient of determination)
RM-ANOVA	Repeated measures analysis of variance
RNA	Ribonucleic acid
RNA-seq	Ribonucleic acid sequencing
s	Second
SD	Standard deviation
SE	Standard error
SPE	Solid phase extraction
TMX	Thiamethoxam
THC	Thiacloprid
USDA	United States Department of Agriculture
VLCFA	Very long chain fatty acid
ZIP	Zero-inflated Poisson

Prescribed introduction

In accordance with section 2.2 of the University of Exeter document ‘Presentation of Theses/Dissertations for Degrees in the Faculty of Graduate Research: Statement of Procedures’, I present here a prescribed introduction to a ‘thesis including published papers’. Specifically, the research presented in chapters two, three and four has previously been published in peer-reviewed journals. Additionally, the thesis contains research papers that at the time of writing were yet to be submitted to their intended journals (chapters five and six). Each research paper is co-authored, and each therefore includes a unique statement of contribution at its beginning. I briefly state the aims and results of the thesis research below (these are fully described, along with all research methodology, in the chapters that follow).

The overall aim of this thesis was to investigate the effects and effect mechanisms of dietary neonicotinoid pesticides in the buff-tailed bumble bee, *Bombus terrestris*. Characterised by its white-tipped abdomen, *B. terrestris* is abundant across Europe and is currently expanding its range in both Northern and Southern Hemispheres. The success of this species may be due in large part to its capability, as a generalist forager, to feed on the nectar and pollen from a variety of plants, including many of those grown as crops in agriculture. Whilst they feed from the flowers of certain bee-attractive crops such as oilseed rape, *B. terrestris* (along with several other species of bumble bee) are potentially exposed to neonicotinoid pesticides. This group of neurotoxic chemicals are applied to protect crops against damage from insect herbivores, but bees are unintentionally exposed to trace levels of neonicotinoid that appear in nectar and pollen following application. Evidence of the harm that neonicotinoids could cause to bees has led to widespread concern, with some singling out these pesticides as contributors to declines in wild and managed populations. Consequently, it was important to test the effects of field-realistic levels of neonicotinoid pesticides in *B. terrestris* (see chapter one for a full discussion of neonicotinoids, the concentrations to which bees are exposed in the field, and evidence of their effects on endpoints of importance to colony success). Specifically, in chapters two and three I demonstrated that food consumption and brood production in worker-only microcolonies (small groups

of worker bumble bees that reproduce asexually when maintained in the laboratory in the absence of the mother queen bee from their original colony) were more susceptible to field-realistic exposures of imidacloprid than thiamethoxam (specifically to concentrations in the range up to 12 µg kg⁻¹). In chapter four, I found that field-realistic imidacloprid also reduced feeding and brood production in queenright colonies (those colonies containing their original mother queen bee and her daughter workers); however, the performance of bumble bees recovered when the neonicotinoid was removed from their diet. In chapter five, I found that piperonyl butoxide, an insecticide synergist and cytochrome P450 enzyme inhibitor, did not enhance the toxicity of imidacloprid in individual workers, and I thereby demonstrated that bumble bee P450 enzymes are probably not an important mechanism for metabolism of imidacloprid. In chapter six I conducted a transcriptomic analysis of imidacloprid-treated workers, and in doing so identified the bees' molecular response to dietary imidacloprid. The already published research in this thesis adds to a growing body of evidence that suggests imidacloprid is a threat to wild bumble bee populations. However, the papers' findings also demonstrate that bumble bees may be somewhat resilient to imidacloprid as a realistic pulsed exposure, and that they may also be less susceptible to thiamethoxam than to imidacloprid when the two neonicotinoids are presented at realistic concentrations.

Chapter One

Introducing bees and the three Ps:
Pollination, population, pesticides

1.1 The value of bees

1.1.1 *Eight thousand years, Man and Bee*

The story of man and bee could justifiably be labelled ‘an epic’. The relationship spans millennia. First evidenced in prehistoric artwork (a honey-hunter known as the ‘Man of Bicorp’, who was painted on the walls of a cave around eight thousand years ago; Crane 1997), humans long ago learned to exploit the nutritional and medicinal value of honey (Bogdanov et al. 2008). Today, bees are a global industry. Over 1.5 million metric tonnes of commercial honey is produced annually (Fintrac 2012) and products such as beeswax and propolis (a resinous substance collected by bees from plants) are valued for their use in cosmetics and medicine (Marcucci 1995; Al-Waili 2005). Despite its great value, it is not the bees’ produce but their services to agriculture that are most important to mankind. For humans, bees equal food. They pollinate hundreds of crop plants that we consume or feed to the livestock we eat (Delaplane and Mayer 2000; Klein et al. 2007). Indeed, an estimated one-third of the human diet in developed countries can be traced back to pollination by bees (Delaplane and Mayer 2000) and consequently their services have multi-billion dollar value (Morse and Calderone 2000; Losey and Vaughan 2006; Allsopp et al. 2008). Furthermore, while many staple foods such as cereals and potatoes are not reliant on the pollination of bees (Ghazoul 2005), without animal pollinators our currently diverse diet would be severely threatened. More eloquently put by Delaplane and Mayer (2000), “bees may not be necessary for human life, but they are necessary for life as we know it”.

1.1.2 *Apis* pollination

Commercial pollination has become a large-scale industry and managed stocks of the European honey bee, *Apis mellifera* L. are responsible for more than 90 percent of current activity (Allsopp et al. 2008). Some apiarists manage thousands of hives, earning a living by transporting them to agricultural areas and renting them to farmers for crop pollination (Morse and Calderone 2000). In the USA, apiarists are estimated to manage more than two million honey bee hives (vanEngelsdorp et al. 2010), while in Europe 620,000 beekeepers are thought to be active (Chauzat et al. 2013). Aside from producing saleable products such as honey, as commercial pollinators honey bees have the distinct advantage of forming vast perennial colonies comprising thousands of foraging workers that pollinate a variety of flower types. They can pollinate substantial areas of land throughout the flowering season and in some studies have been shown to increase yields of certain fruit, seed and nut crops by more than 90 percent (Klein et al. 2007). However, despite their advantages, it has become clear in recent years that over-reliance on honey bees as the single pollinator species in agriculture is both risky and potentially inefficient (Westerkamp and Gottsberger 2000; Garibaldi et al. 2013).

Despite global stocks of commercial hives increasing in the past 50 years by ~45 percent (in-line with global *per capita* demand for honey), managed honey bee numbers have not kept pace with a more-than 300 percent increase in the area of insect pollinated crops (Aizen and Harder 2009) and this has led to a potential shortfall in hives. Additionally, abnormally high mortality rates in some regions of the USA (vanEngelsdorp et al. 2008, 2010; Ellis et al. 2010) and Europe (Aston 2010; Potts et al. 2010b; Soroker et al. 2010) have led to

regional declines in the number of managed honey bee colonies. The primary driver of these declines has yet to be identified, though several potentially interacting culprits are currently under investigation including, but not limited to, disease, pesticide poisoning, the parasitic *Varroa* mite (*V. destructor* Anderson & Trueman), loss of forage, climate change, and decreased profitability in beekeeping (vanEngelsdorp and Meixner 2010). Even when their colonies are abundant and healthy, honey bees will not necessarily pollinate important crops. For example, Westerkamp (1991) describes how experienced honey bee foragers can learn to take nectar from apple trees (*Malus spp.*) and alfalfa (*Medicago sativa* L.) without being struck by adhering pollen that normally necessitates substantial grooming (primarily by avoiding the anthers or pollen-releasing trigger mechanisms), and thus avoid pollinating the flowers. The honey bees' relatively short tongue and inability to release pollen by bodily vibration (buzz pollination) also prevent them from pollinating clover (*Trifolium spp.*) or Solanaceae plants (e.g. tomatoes), respectively (Goulson 2003a). Indeed, Garibaldi et al. (2013) recently reported that honey bees significantly increased the fruit set (the proportion of a plant's flowers that develop into mature fruits or seeds) in only 14 percent of forty-one crop systems studied worldwide. Where honey bees are efficient pollinators, their pollination service may be limited in regions prone to spells of poor Spring/Summer weather (such as the UK) because they are fair-weather foragers (Willmer et al. 1994) and tend not to be active at temperatures below 15 °C (Heinrich 2004). When taken in combination, concerns such as these begin to point to the value of alternative non-*Apis* pollinators that can add value to the commercial pollination industry with their own pollination services.

1.1.3 Non-*Apis* pollination

A small number of solitary bees are now commercially reared and are typically used to supplement or replace the pollination services of honey bees (Velthuis and van Doorn 2006): the alkali bee (*Nomia melanderi* Cockerell) and the leafcutter bee (*Megachile rotundata* Fabricius) are used for alfalfa pollination; the hornfaced bee (*Osmia cornifrons* Radoszkowski) and the mason bee (*Osmia lignaria* Say) are used to pollinate apples and almonds. However, it is *Bombus spp.* that is now the predominant alternative to honey bees, with around one million *Bombus* colonies produced and used annually in crop pollination (Velthuis and van Doorn 2006). Commercial *Bombus* colonies mainly comprise the buff-tailed bumble bee, *Bombus terrestris* L., and the common eastern bumble bee, *Bombus impatiens* Cresson, which is used in North America where the import of *B. terrestris* is banned (Goulson 2003a). Commercial bumble bees now pollinate an increasingly long list of agricultural crops, including thousands of hectares of greenhouse tomatoes (*Lycopersicon esculentum* L.) (Velthuis and van Doorn 2006). In contrast to honey bees, bumble bees make excellent pollinators of tomatoes, as well as many other greenhouse fruits and vegetables, because of their ability to buzz pollinate (Buchmann 1983): they are capable of vibrating their body at a frequency of 400 Hz (Harder and Barclay 1994) close to the flower's anther, causing the flower to release pollen. In addition to their effectiveness as greenhouse pollinators (Morandin et al. 2001), commercial bumble bees also improve fruit yields when deployed alongside native pollinators in the field (Lye et al. 2011). However, some have questioned the importation of commercial bumble bee colonies and their release into the environment (Ings et al. 2006) because they can become non-native invasive species (Matsumura et al. 2004) or transfer

pathogens (Colla et al. 2006; Graystock et al. 2013b) and parasites (Goka et al. 2006) to native populations.

1.1.4 Wild bees

In contrast to the small number of commercialised bee species, there are reportedly 17,000 species of native wild bees worldwide (Michener 2007). With the potential problems attached to commercial pollination, there is an increasing appreciation for the importance of wild bees as crop pollinators. Indeed, evidence suggests that the substantial contribution wild bees make to the pollination of several important crops (Kremen et al. 2002; Morandin and Winston 2005; Greenleaf and Kremen 2006a; Winfree et al. 2007) could in some cases outweigh the contribution of managed honey bees (Willmer et al. 1994; Westerkamp and Gottsberger 2000; Garibaldi et al. 2013). Furthermore, the presence of foraging wild bees can actually change the foraging behaviour of honey bees, essentially increasing the effectiveness of honey bee flower visitation and thereby enhancing their efficiency as crop pollinators (Greenleaf and Kremen 2006b; Brittain et al. 2013). Of the native wild bees, *Bombus* are arguably the most important wild pollinators in the Northern hemisphere (Corbet et al. 1991; Goulson 2003a). They are highly efficient foragers that remain active at low temperatures and during inclement weather (Willmer et al. 1994; Heinrich 2004) and are thought to pollinate at least 35 major crop species (Goulson 2003a), whilst also playing a key role in the pollination of many wild flower species (Motten 1986; Goulson 2003a). Growing evidence that wild pollinator populations, and in particular bumble bee populations, are declining in many regions has therefore led to widespread concern (Potts et al. 2010a; Vanbergen and the Insect Pollinators Initiative 2013).

1.2 Population decline in bumble bees

Evidence of wild pollinator declines has resulted in the implementation of initiatives to investigate and tackle the issue (e.g. the International Pollinator Initiative or IPI, <http://www.internationalpollinatorsinitiative.org>), and the decline in wild bees is of particular concern (Goulson 2003b; Biesmeijer et al. 2006; Winfree 2010; Burkle et al. 2013). With data lacking on populations of solitary bee species (Brown and Paxton 2009), evidence for wild bee decline comes primarily from studies of bumble bees that in recent decades have shown ongoing declines in local abundance and species diversity across their native northern hemispheric range (Goulson et al. 2008; Williams and Osborne 2009). In the UK, for example, Williams (1982) reported post-1960 reductions in the distribution of several bumble bee species such that the mainland contained a ‘Central Impoverished Region’ suffering from a significant loss of species diversity. Recently, Biesmeijer et al. (2006) highlighted declines in UK bee diversity post-1980, which suggests that the pre- to post-1960 trend has continued. For example, Biesmeijer et al. (2006) report that bee species richness has decreased significantly in over 50 percent of the regions studied in the UK, which the authors suggest reflects shifts in the distributions of many species. Furthermore, they show that there has been an increase in the domination of UK bee communities by a smaller number of species, with about 30 percent fewer species accounting for half of the post-1980 records, indicating that the density of certain species is in decline (Biesmeijer et al. 2006). In addition, the future sustainability of specific UK species such as *Bombus sylvarum* L. and *Bombus muscorum* L., which already exist in a series of small fragmented populations, is threatened by their low effective population sizes with low genetic diversity (Darvill et al. 2006; Ellis et al. 2006).

Across the rest of Europe, population declines have occurred in Ireland (decreased distribution; Fitzpatrick et al. 2007), the Netherlands (decreased distribution and density; Biesmeijer et al. 2006), and a number of other central and western European countries (loss of species richness and decreased distribution: Kosior et al. 2007; Williams and Osborne 2009), while several extinctions have also been reported since the beginning of the 20th century (Kosior et al. 2007). However, not all European *Bombus* species are in decline; a small number have expanded their range in recent decades (Goulson 2003a). Successful species tend to be short-tongued generalists such as *B. terrestris* and *Bombus lapidarius* L., which have expanded where long-tongued specialists – adapted to increasingly rare flowers with deep corollas – have declined (Goulson et al. 2005; Dupont et al. 2011; Bommarco et al. 2012; but see Williams 2005).

Decline is also an issue in North America where the relative abundance and geographic range of four particular species, namely *Bombus occidentalis* Greene, *Bombus pensylvanicus* De Geer, *Bombus affinis* Cresson, *Bombus terricola* Kirby, decreased by up to 96% in the last four decades (Colla and Packer 2008; Cameron et al. 2011). However, historical records show that range decline and even local extirpation of certain species was occurring in the USA as far back as 1940 and coincided with large-scale agricultural intensification (Grixti et al. 2009). Across the planet, the cause of declines and loss of diversity in bumble bees is widely debated (Goulson et al. 2008; Williams and Osborne 2009; Winfree 2010). Agricultural intensification is currently seen as the most likely cause (Goulson 2003a), but no single culprit

has yet been identified. Indeed, bumble bee declines are most likely driven by multiple and interacting detrimental agents (Williams and Osborne 2009).

In general, for any detrimental agent to precipitate population decline in a species it must be capable of reducing *per capita* birth rate, increasing *per capita* death rate, or altering lifespan (Gotelli 2001). In bumble bees, effective population size may be small despite an apparent abundance of individuals because female worker bees are largely infertile (Chapman and Bourke 2001). The bumble bee colony is essentially a single breeding pair because queens are usually mated only once (Schmid-Hempel and Schmid-Hempel 2000) and are principally responsible for a colony's reproductive output (Lopez-Vaamonde et al. 2004). The number of successful colonies determines the size of future populations (Darvill et al. 2006) and success can be defined by the production and survival of new queens and males that go on to produce new colonies (Heinrich 2004). Detrimental agents acting directly on the birth and death rate of new queens and males are therefore most likely to produce a population-level impact in bumble bees. However, effects on bumble bee workers as the colony develops – on their birth and death rate, or performance as foragers for example – may also be important because the number of new queens and males a colony produces depends on the size and efficiency of its workforce (Owen et al. 1980; Müller and Schmid-Hempel 1992a; Heinrich 2004). Indeed, the ingredients of a successful bumble bee colony are manifold: access to nesting sites, consistent availability of food resources, a strong and abundant workforce – these elements in particular must be in place for colonies to succeed.

Before discussing the detrimental agents that are thought to cause colony failure and population decline in bumble bees, it is necessary to first outline the elements and endpoints that should be in place to ensure colony success. These are the vulnerable endpoints on which detrimental agents act – the elements that when stressed can initiate colony failure. In the following section, these elements are described in the context of the natural history of the bumble bee colony.

1.3 The ingredients for colony success

1.3.1 *Beginning at the end*

The majority of bumble bee species worldwide have an annual colony cycle from which only new queens survive each year (Heinrich 2004). In the autumn, at the end of the cycle, the old queen and all of her workers die. Male offspring survive long enough to inseminate new queens. In most species, queens are mated only once during a single mating flight in which they receive and store enough sperm to produce all of their future offspring (Baer et al. 2003). During this period an abundance of available males is probably important; unfertilised queens are generally not found in over-wintering sites (Cumber 1954), so too few males could theoretically hinder colony success at an early stage. Once fertilized, new queens seek out sites in which they spend the winter in a state of diapause. These sites are usually underground in well-drained and shaded banks or slopes, or under trees or leaf litter (Alford 1969). Many queens will die before or during the winter, and only a small proportion will go on to produce their own colony (Heinrich 2004). Increasing their body weight raises a queen's chance of surviving diapause (Beekman et al. 1998), with increased weight

likely linked to the stores of fat and glycogen accumulated prior to over-wintering and utilized during the winter months (Alford 1969). At this early stage in the cycle, an abundance of new queens, suitable over-wintering sites and available forage are all critical to foundation of new colonies.

1.3.2 Colony foundation

Queens emerge from diapause in the spring, making their new nests in any suitable cavity and commonly in deserted mouse holes (Heinrich 2004). They forage alone and fill their nests with pots full of nectar and a pollen clump into which they lay a small batch of approximately ten eggs that progress into larvae, pupae and eventually female worker bees (Heinrich 2004). In the early stages of establishing a nest, queens incubate eggs continually whilst maintaining a high thoracic and abdominal temperature that is energetically costly to maintain (Heinrich 1974). To fuel these processes queens must have continuous access to a high-energy food source; they take all their energy from the carbohydrates in nectar and take protein for egg production from pollen (Vogt et al. 1998; Heinrich 2004). Indeed, from this stage onwards the colony runs on pollen and nectar: larvae derive all nutrients from a mixture of the two (Pereboom 2000), while workers eat a little pollen but mostly subsist on sugars from nectar throughout their life (Heinrich 2004).

1.3.3 An abundant and efficient workforce

Once hatched and as the colony progresses, new workers acquire the majority of nectar and pollen resources and feed the larvae. Unlike honey bees, bumble bees do not store large food surpluses and so they necessarily employ an abundant and efficient foraging workforce at all times. Colonies that suffer a

sustained loss of forage are likely to fail. For example, when their food supply is completely exhausted bees will enter a state of torpor – a drowsiness resembling suspended animation – during which all activity and meaningful biological events cease (Heinrich 2004). The size of the workforce dictates the quantity of resources available to the colony, which is all-important because the availability of resources determines the production rate of new workers, which in-turn determines the acquisition of future resources that are needed to produce new queens and males. For example, a queen requires the protein in the pollen brought by her workers to produce new eggs (Vogt et al. 1998) and when sufficiently provisioned she lays eggs at a constant rate (Duchateau and Velthuis 1988). However, she also ensures that the number of larvae to be fed remains roughly proportional to number of workers available to forage and feed her young (Heinrich 2004). Therefore, both the queen's egg laying rate and future colony growth are contingent on the size of the workforce.

An abundance of workers is critical to bumble bee colony success. There is evidence that the number of new queens and males a colony produces directly depends on the size of its workforce (Owen et al. 1980; Müller and Schmid-Hempel 1992a). Queens and males are fed more as larvae than workers (Ribeiro et al. 1999): to develop into queens rather than workers, larvae require more than three times more food (Duchateau and Velthuis 1988) and queens are approximately twice as costly to rear as males (Owen et al. 1980; Owen and Plowright 1982). Rearing of sexual offspring is therefore feasible only when the colony's workforce is sufficiently large enough to feed larvae at an increased rate (Duchateau and Velthuis 1988). Indeed, artificially doubling the number of workers in young colonies can bring forward production of sexuals (Bloch 1999)

and larger colonies may produce more queens (Owen et al. 1980; Owen and Plowright 1982; Müller et al. 1992). When resources are limited, the smaller colonies that emerge are likely to produce fewer queens. However, these colonies may still produce a large number of males (Beekman and van Stratum 1998) because they are smaller and cheaper to produce (Owen et al. 1980; Owen and Plowright 1982) and can represent colony fitness in the absence of queens (Bourke 1997). Furthermore, colonies of greater size respond faster and more effectively to perturbations in their environment (Weidenmüller et al. 2002) and smaller colonies may be more susceptible to stress-related failure caused by an Allee effect (Bryden et al. 2013).

Along with abundance, the quality of the workforce is also important to bumble bee colony success. In most colonies there exists very little division of labour among workers: they work either in-hive or as foragers and all bees can perform all tasks where necessary (Cartar 1992; O'Donnell et al. 2000; Jandt et al. 2009). Smaller bees tend to work in-hive (Heinrich 2004; Jandt and Dornhaus 2009) while larger bees are more likely to forage (Goulson et al. 2002b; Spaethe and Weidenmüller 2002). The size of workers is determined by nutrition; bees of any size can be produced by food deprivation (Plowright and Jay 1977) and underfed workers become the smallest (Sutcliffe and Plowright 1988). Producing smaller bees has some value because: a) they can navigate the nest effectively (Heinrich 2004); and b) a size range among workers allows specialisation appropriate to their morphology, either on different types of flower or as nectar vs. pollen gatherers, (Goulson 2003a). However, larger bees are thought to be better foragers. They bring back more forage with greater efficiency (Goulson et al. 2002b; Spaethe and Weidenmüller 2002), and forage

over greater distances (Free 1955) and during poorer weather (Heinrich 2004). Therefore, a workforce containing an abundance of large individuals is essential for gathering resources over a foraging range that in bumble bees can extend from 200 metres to ten kilometres (Osborne et al. 1999; Cresswell et al. 2000; Goulson and Stout 2001).

Larger individuals are also beneficial because they thermoregulate their own bodies more effectively, which is crucial to foraging because bumble bees cannot fly if their muscle temperature drops below 30 °C (Heinrich 2004). Additionally, large workers contribute more to thermoregulation of the nest (Jandt and Dornhaus 2009). Established colonies must maintain the nest at an optimum temperature around 30 °C by producing heat or by fanning when the temperature gets too high (O'Donnell and Foster 2001). Outside of this optimum temperature larvae can develop stunted growth or developmental defects (Heinrich 2004). In-hive workers therefore invest a significant proportion of their energy maintaining nest temperature. The efficiency of thermoregulation increases with colony size (Heinrich 2004) and heat from the collective metabolism of bees in larger colonies can be sufficient to incubate larvae without direct contact (Vogt 1986), allowing workers to perform additional brood maintenance whilst the temperature of the nest remains stable.

1.3.4 Low mortality

For a bumble bee colony to remain at a size and level of efficiency that permits production of sexual offspring, the mortality of workers throughout the cycle should be low. The natural mortality rate of workers in a colony is approximately 25–35 percent per week (Schmid-Hempel and Heeb 1991). However, foraging

is an inherently dangerous task wherein workers risk death from predators (Morse 1979) and parasites (Schmid-Hempel et al. 1990), and mortality increases with their rate of foraging (da Silva-Matos and Garófalo 2000). Colonies are apparently resilient to moderate rates of additional mortality (Schmid-Hempel and Heeb 1991), but high mortality rates restrict colony growth and affect sexual reproduction. For example, where mortality is artificially increased colonies produce fewer males and smaller queens (Müller and Schmid-Hempel 1992b). While high mortality obviously reduces colony size, it may also reduce the foraging efficiency of the colony because experienced foragers are lost. There is evidence that inexperienced foragers often visit low-reward flowers, handle high-reward flowers ineffectively (Heinrich 1979), or take longer to collect resources (Heinrich 1979; Raine and Chittka 2007). Bees learn to specialise on flowers with foraging experience, which increases their efficiency, but in the face of high mortality these bees are replaced with inexperienced in-hive workers that forage less efficiently and are also at higher risk of predation (Heinrich 2004).

1.3.5 Success is sexual reproduction

At the end of the season, the bumble bee colony naturally collapses. If by this point it has reached a critical size, with workers sufficiently able to provision and thermoregulate the nest, then new queens and males – the determinants of colony fitness – are produced and the colony can be deemed a success. In order to reach this state, the production of new workers in the colony must remain high, while mortality remains low. Furthermore, the efficiency of workers must be high – their ability to forage, thermoregulate, and care for brood are all important determinants of success. In actuality, the natural rate of colony failure

is high; predation, parasitism, disease, and starvation all disrupt the colony cycle so that only the strongest colonies will succeed in producing new queens and males (Goulson 2003a). Moreover, colonies are under additional pressure from anthropogenic stressors, primarily associated with the intensification of agriculture (Pywell et al. 2005; Winfree et al. 2009; Kennedy et al. 2013), which are believed to interact with each other and with natural stressors to become the agents of population decline (Goulson et al. 2008; Williams and Osborne 2009; Vanbergen and the Insect Pollinators Initiative 2013). Indeed, recent research suggests that bumble bee colonies can reach a critical stress level, beyond which additional stress can mean the difference between success and failure (Bryden et al. 2013). The following section outlines a selection of the most important natural and anthropogenic stressors, the detrimental agents that can impact on the success of bumble bee colonies and potentially affect population decline.

1.4 Bumble bees under stress: the agents of colony failure

1.4.1 Predators and parasitoids

A few species of insectivorous bird, including shrikes, spotted flycatchers and bee-eaters, will eat bumble bees (Goulson 2003a). Mammalian predators such as badgers, skunks, and voles (Selko 1937; Boyle and Whelan 1990; Vepsäläinen and Savolainen 2000) will also raid ground-nesting colonies and consume their brood, collected resources, and in-hive adults. Although the numbers of some mammalian predators appear to have risen in recent decades (e.g. the European badger, *Meles meles*, in the UK; Macdonald and Newman 2002), which could have increased predation rates, actual data on avian or

mammalian predation of bumble bees are lacking. The relationship between changes in predator abundance and bee population declines is therefore unknown. Other arthropods such as crab spiders and the bumblebeewolf (*Philanthus bicinctus* Mickel, a predatory wasp) also prey on *Bombus* spp. (Morse 1979; Gwynne 1981). Rates of predation appear to be relatively low, perhaps because bees can learn to avoid high-risk areas (Abbott 2006; Ings and Chittka 2009), but there is some evidence that the presence of arthropod predators can reduce the density of bumble bees (Dukas 2005). Larvae of the wax moth *Aphomia sociella* L. occur only in *Bombus* nests and voraciously consume the nests' contents, effectively devastating the colony while adult bees offer little or no defence (Goulson 2003a). Infestation appears more prevalent in urban environments and artificial nest boxes (Goulson et al. 2002a; Goulson 2003a), but is rare in underground nests (Goulson et al. 2002a).

Conopid flies are parasitoids that lay their eggs in the abdomen of adult bumble bees whilst the bees visit flowers (Goulson 2003a). Once hatched, conopid larvae consume haemolymph and tissues until their host dies (Schmid-Hempel and Schmid-Hempel 1988). Conopid attack can: a) severely reduce worker efficiency (Schmid-Hempel and Schmid-Hempel 1991; Müller and Schmid-Hempel 1993); b) increase worker mortality (Müller and Schmid-Hempel 1992a); and c) reduce the size of queens reared in parasitized colonies (Müller and Schmid-Hempel 1992b). Despite their potentially severe effects on colonies, incidence of parasitisation by conopids is highly variable (Goulson 2003a) and evidence that they have impacted populations is currently lacking.

1.4.2 Mites

Elevated populations of the parasitic *Varroa* mite in honey bee colonies is a key factor in colony collapse, probably because of the mite's impact on the health of individual bees (Dainat et al. 2012). Of the fifteen genera of mites associated with bumble bees (Goulson 2003a), the tracheal mite (*Locustacarus buchneri* Stammer) poses the most obvious threat to its host. These mites live and reproduce in the bees' tracheae, feed on haemolymph, and infect several hosts as the colony develops (Husband and Sinha 1970). Heavy infection can cause diarrhoea, lethargy and an inability to forage (Husband and Sinha 1970), alter the bees' behaviour (Otterstatter et al. 2005), and reduce lifespan (Otterstatter and Whidden 2004). Although the prevalence of tracheal mites in field-caught bumble bees is low (Otterstatter and Whidden 2004), there is evidence that the mite has now invaded native colonies from commercially imported colonies (Goka et al. 2006) and could negatively impact these previously unaffected populations.

1.4.3 Parasites

Several protozoans parasitize bumble bees, with the primary route of transmission through ingestion of spores that multiply in the host's gut (Goulson 2003a). For example, the trypanosome protozoa *Critidium bombi* is passed out in the host's faeces and quickly spreads within the colony (Schmid-Hempel 2001). Infection between colonies occurs through shared use of flowers (Durrer and Schmid-Hempel 1994) and the majority of colonies in the field are infected (Schmid-Hempel 2001). Despite high infectivity, *C. bombi* normally has low virulence (Schmid-Hempel 2001), but when bees become stressed the parasite can become a serious problem. In bumble bees, coupling *C. bombi* infection

with starvation can increase host mortality by 50 percent (Brown et al. 2000). Pesticide exposure can result in *C. bombi*-infected mother queens surviving fewer days (Fauser-Misslin et al. 2014), whilst infection coupled with a stressful over-wintering period can result in queens that found colonies that are 40 percent more likely to fail (Brown et al. 2003).

The microsporidian protozoa *Nosema bombi* also appears to be relatively common (Schmid-Hempel 2001), infecting bumble bee hosts across a wide geographic area (Tay et al. 2005). Results of previous investigations into *N. bombi*'s effects are inconsistent. For example, infected workers and males suffered reduced survival in laboratory trials (Otti and Schmid-Hempel 2007) and queens produced significantly smaller colonies and zero sexual offspring in controlled field trials (Otti and Schmid-Hempel 2008). However, infection was associated with increased production of sexuals in an uncontrolled field trial (Imhoof and Schmid-Hempel 1999). Schmid-Hempel (2001) states that there is no doubt *N. bombi* can devastate individuals and colonies, but that there is no simple pattern to these effects. Recently, a higher prevalence of *N. bombi* was reported in rapidly declining North American bumble bee species than in stable species (Cameron et al. 2011), but it remains unclear whether this observation supports the hypothesis that *N. bombi* is affecting population decline (Brown 2011).

Closely related to *N. bombi*, *Nosema ceranae* usually infects honey bees. It can suppress immune response (Antúnez et al. 2009), alter feeding behaviour (Naug and Gibbs 2009), and increase mortality (Paxton et al. 2007) in its host, and has been linked to honey bee colony collapse in Spain (Higes et al. 2009,

2010). Consequently, the detection of *N. ceranae* in several bumble bee species worldwide (Plischuk et al. 2009; Li et al. 2012; Graystock et al. 2013a) is an issue of great concern. A recent study of *N. ceranae* in seven UK *Bombus* spp. reported it to be more infective and more virulent to bumble bees than honey bees (Graystock et al. 2013a). Thus, this microsporidian poses a significant health risk to its new *Bombus* host, particularly in populations that are already small and lacking genetic diversity and are therefore more vulnerable to parasites (Whitehorn et al. 2011). Infection of *Bombus* spp. with *N. ceranae* may be an example of pathogen spillover (Meeus et al. 2011), and such a movement of pathogens to wild bumble bees from other native taxa (Singh et al. 2010; Graystock et al. 2013a; Fürst et al. 2014), non-native invasive species (Goulson 2003c), and commercially imported colonies (Colla et al. 2006; Goka et al. 2006; Otterstatter and Thomson 2008; Graystock et al. 2013b) can debilitate wild populations (Cameron et al. 2011, but see Brown 2011) and is becoming an issue of increasing concern for bumble bee conservationists (Williams and Osborne 2009; Vanbergen and the Insect Pollinators Initiative 2013).

1.4.4 Viruses

Many of the viruses identified as threats to honey bees, may also represent a threat to bumble bees. *Deformed wing virus* (DWV) either kills developing honey bee pupae or causes the emergence of deformed and unviable adults (de Miranda and Genersch 2010) and has been linked to colony collapse and overwintering colony losses (Martin 2001; Highfield et al. 2009; Nazzi et al. 2012; Schroeder and Martin 2012). Worryingly, DWV has now been detected in several bumble bee species (Genersch et al. 2006; Singh et al. 2010; Li et al.

2011; Levitt et al. 2013; Fürst et al. 2014), in which it also causes deformities and loss of viability in individuals, and therefore poses a significant threat to colonies (Genersch et al. 2006). The prevalence of DWV in wild bumble bees is linked to its prevalence in sympatric managed honey bees, suggesting that *Apis* is the likely source of the disease and that pathogen transmission from managed to wild bees is a possible contributing cause of population declines (Fürst et al. 2014).

Israeli acute paralysis virus (IAPV) has also been linked colony collapse disorder in honey bees (Cox-Foster et al. 2007; Johnson et al. 2009a): it disrupts normal behaviour, for example by depressing the homing ability of foraging bees (Li et al. 2013), and increases mortality (Xun et al. 2009). IAPV has recently been detected in *B. impatiens*, *Bombus vagans* Smith, and *Bombus ternarius* Say (Singh et al. 2010; Levitt et al. 2013) and replication of the virus has been demonstrated in *B. impatiens* (Levitt et al. 2013). Little is known about the virus' impact on *Bombus* spp. and so further research is required to determine if IAPV is linked to bumble bee declines.

1.4.5 Intensification of agriculture

In section 1.3 we saw that wild bumble bees are entirely dependent on nectar and pollen as a food resource: for colonies to thrive they need a constant supply of flowers on which to forage throughout their cycle. Indeed, in experimental trials, field-colonies that received food supplementation reach larger sizes and had higher reproductive success than colonies that fed naturally (Pelletier and McNeil 2003). Most researchers agree that land-use changes driven by the modernization and intensification of agriculture in the

latter part of the 20th century – for example, the growth of field sizes, the removal of unimproved grassland and hedgerows, the predomination of monoculture crops – are likely to have impoverished bumble bee habitats and limited food supply to the extent that some species can no longer thrive in agricultural environments (Goulson 2003a; Goulson et al. 2008; Williams and Osborne 2009). In general, agricultural landscapes that are: a) naturally florally diverse; b) in close proximity to natural habitat; or c) managed to improve their floral diversity will support pollinator communities better than those that are not (Kells et al. 2001; Kremen et al. 2002, 2004; Potts et al. 2003; Öckinger and Smith 2007; Winfree et al. 2011). For bumble bees, species richness is usually low in intensively farmed landscapes (Mänd et al. 2002; Pywell et al. 2005). Short-tongued species with generalist foraging strategies continue to survive on farmland, but long-tongued species that have undergone dramatic declines in recent years are rarely found (Pywell et al. 2005). The loss of flower-rich habitat supporting clovers and other flowers with long corollas from the family Fabaceae is thought to be a key driver in the decline of the long-tongued *Bombus* spp. (Goulson 2003a; Colla and Packer 2008; Dupont et al. 2011; Roulston and Goodell 2011; Bommarco et al. 2012) that tend to specialise on these increasingly rare plants (Goulson and Darvill 2004; Goulson et al. 2005). Farms that are in close proximity to flower-rich habitat (Kremen et al. 2002; Kennedy et al. 2013) or implement management schemes to increase floral diversity (Carvell et al. 2004, 2011; Pywell et al. 2005) show improved abundance and diversity of bumble bee species. Away from impoverished agricultural environments, florally diverse urban environments and suburban gardens are habitats that increasingly provide a stronghold for bumble bees (Goulson et al. 2002a, 2010; Ahrné et al. 2009).

Certain modern agricultural practices can be beneficial for bumble bees. The planting of mass-flowering monoculture crops such as the oilseed rape can be advantageous because it effectively concentrates a massive food resource into large blocks that are available while the crop blooms (Westphal et al. 2003). Indeed, the abundance, early colony growth, and foraging efficiency of bumble bees may be improved where mass-flowering crops are present (Westphal et al. 2003, 2006, 2009; Hanley et al. 2011; Stanley et al. 2013). However, only species with longer foraging ranges and generalist foraging strategies such as *B. terrestris* are likely to benefit significantly from monocultures (Walther-Hellwig and Frankl 2000; Knight et al. 2009; Diekötter et al. 2010) because they offer resources in short pulses lasting from one to eight weeks whilst the crop is in bloom (Hoyle et al. 2007; Westphal et al. 2009; Pilling et al. 2013) after which bees must find alternative forage. Furthermore, queens that emerge before or after flowering (Pyke et al. 2011) will not survive in monoculture landscapes unless additional floral resources are also available (Goulson 2003a).

1.4.6 Pesticides

In addition to reducing the availability of food, modern agriculture has had impacts on bees' health through unintentional exposure to pesticides and other toxicants used to protect the crops on which they forage. The actual threat posed to bees by pesticides is strongly debated (Stokstad 2012; Connolly 2013; Dicks 2013; Walters 2013), partly because there are difficulties disentangling their effects from those of other aspects of agriculture (Brittain and Potts 2011). However, there is an unquestionable need to understand the population level impacts of pesticides because the exposure of bees to a variety of agrochemicals is likely to be widespread (Chauzat et al. 2006; Mullin et al.

2010; Stoner and Eitzer 2013) and could be expected to increase as pesticide usage increases in the next 50 years (Tilman et al. 2001).

Bees are primarily exposed to pesticides either by contact with residues sprayed onto the crop, from ingestion of contaminated nectar and pollen, or (for honey bees) in apiaries from in-hive treatments (Johnson et al. 2010; Blacquière et al. 2012). Pesticide residues also appear in guttation fluid exuded by treated plants (Reetz et al. 2011), and while some laboratory trials have been used to suggest that bees risk fatal exposure from these fluids (Girolami et al. 2009) there is little evidence from the field to support this claim (Thompson 2010; Reetz et al. 2011). Historically, honey bees have been fatally exposed to pesticides in contaminated dust produced during seed-drilling into soil (Alix et al. 2009; Forster 2010), but improvements to drilling procedures and technology are thought to have minimized this risk in Europe (Thompson 2010; but see Tapparo et al. 2012). In North America, however, experts agree that insecticide contaminated dust produced during planting with pneumatic seeders could still be contributing to incidents of bee mortality, and that risks need to be minimised (Cutler et al. 2013).

Prior to their use in the field, pesticides are routinely risk assessed for their lethality to honey bees and, in general, appropriate use ensures that field-realistic exposure are unlikely to cause direct mortality (Goulson 2013). However, the results of honey bee risk assessments may not be directly applicable to bumble bees (Thompson and Hunt 1999; Mommaerts and Smagghe 2011; Decourtye et al. 2013; Arena and Sgolastra 2014). Indeed, in some cases *Apis* is more sensitive to specific pesticides than *Bombus*, and in

other cases *Bombus* appears most sensitive (Hardstone and Scott 2010; Cresswell et al. 2012b; Arena and Sgolastra 2014). Extrapolating the results of honey bee risk assessments to deduce the risk posed to wild bumble bees from pesticides is therefore problematic. Whereas incidence of substantial direct mortality in the field is now rare (Barnett et al. 2007), the sublethal effects of pesticides in bees are well documented (Desneux et al. 2007). The majority of evidence comes from research conducted on honey bees (Desneux et al. 2007; Cresswell 2011; Blacquière et al. 2012), but researchers are beginning to report the sublethal effects of pesticides on bumble bees at both individual and colony levels. For example, detrimental effects on brood production (Tasei et al. 2000; Mommaerts et al. 2006, 2010a, b; Besard et al. 2010; Gradish et al. 2010, 2012), food consumption and foraging efficiency (Gels et al. 2002; Gradish et al. 2010, 2012; Gill et al. 2012), and production of sexual offspring (Whitehorn et al. 2012; Larson et al. 2013; Fauser-Misslin et al. 2014) in bumble bees were investigated following exposure to: acaricides, abamectins, *Bacillus thuringiensis*, carbamates, chitin synthesis inhibitors, neonicotinoids, organophosphates, and pyrethroids. In several of these studies effects are demonstrated principally at dosages above the range bumble bees encounter in the field (EFSA 2012) and in other studies the same pesticides are reported to have little or no effect (Tasei et al. 2000; Morandin and Winston 2003; Franklin et al. 2004; Alarcón et al. 2005; Thompson et al. 2013). As evidence of their impact is inconsistent among studies it remains uncertain whether exposure to environmental pesticide residues is capable of affecting population decline in bumble bees.

1.4.7 A summary of the threats to bumble bee populations

The factors threatening bumble bee populations are likely multiple and interacting, and may differ among regions making the precise causes of decline difficult to establish (Williams and Osborne 2009; Bryden et al. 2013; Vanbergen and the Insect Pollinators Initiative 2013). Regarding the factors discussed here, there is little evidence that natural predators or parasitoids could have driven declines (Williams and Osborne 2009). In contrast, incidence of infection with mites and pathogens has increased and is linked by some as a contributing factor, if not a driver, of declines (Dicks 2013). In addition, agricultural intensification has reduced the availability of forage for several *Bombus* spp. and is likely to have contributed to the observed loss of species diversity in many regions. The precise threat of pesticides to wild populations remains uncertain. The residues to which bees are exposed to in the wild are unlikely to cause substantial levels of direct mortality, but they produce sublethal effects that can contribute to reductions in colony fitness. Therefore, more research is clearly required to establish the demographic impact of pesticides on bumble bees.

Recently, a selection of researchers, regulatory agencies and campaign groups have singled out one particular group of pesticides – the neonicotinoids – as culprits for bee declines (EFSA 2013e; Maxim and van der Sluijs 2013; Shardlow 2013; van der Sluijs et al. 2013). Arising from this criticism, and largely driven by the precautionary principle (Alemanno 2013), a Europe-wide 2-year moratorium on the use of certain neonicotinoids as seed-treatments on bee-attractive crops was implemented on 1st December 2013 (European Commission 2013). Although some evidence for the detrimental impact of

neonicotinoids on bee health has accumulated over the last decade (reviewed in: Decourtye and Devillers 2010; Blacqui  re et al. 2012), the association between these pesticides and bumble bee declines is far from certain. In the sections that follow, a critical analysis is given on our current knowledge of neonicotinoid pesticides in the environment and their effects on endpoints that contribute to colony success in bumble bees – particularly mortality, worker performance, and reproduction.

1.5 Neonicotinoid pesticides in the environment

1.5.1 *Introducing neonicotinoids*

With the launch of the imidacloprid in 1991, the neonicotinoids emerged onto the crop protection market and were quickly established as one of the best selling insecticide families worldwide (Nauen et al. 2008). In the ten years that followed, six additional neonicotinoids were launched (Elbert et al. 2008) – acetamiprid, nitenpyram, thiamethoxam, thiacloprid, clothianidin and dinotefuran – and by 2008 the family had gained a quarter share of the total insecticide market (Jeschke et al. 2011). The rapid success of these pesticides is attributable to their flexibility of use (Jeschke et al. 2011) – they are used in seed treatments, soil and foliar sprays, drip or drench irrigation systems, bait formulations, flea treatments in companion animals – and their effective control of insect pests including aphids, whitefly, leaf- and planthoppers, thrips and some Lepidoptera and coleopteran species, that could otherwise cause significant damage to crops (Elbert et al. 2008). In terms of crop use, imidacloprid remains most popular with registrations for over 140 uses in more than 120 countries worldwide (Jeschke et al. 2011) and is used on major crops

including cotton, cereals, rice, fruit, vegetables and oilseed rape (Elbert et al. 2008). Although its use is declining in favour of other neonicotinoids in some regions (Walters 2013), imidacloprid is now off patent and generic products have entered the market leading to a broad scale use of the compound (Elbert et al. 2008). Thiamethoxam is also registered for over a hundred crop uses in at least 65 countries (Jeschke et al. 2011). Clothianidin is derived from thiamethoxam as a toxic metabolite (Nauen et al. 2003), and is an increasingly popular crop protection product with, for example, applications making up over 70 percent of the total weight of neonicotinoids applied to UK crops in 2012 (FERA 2014). Of the remaining neonicotinoids, acetamiprid, dinotefuran and thiacloprid are primarily used as foliar sprays, with the latter compound declared largely ‘bee safe’ and available for use on flowering crops, whilst nitenpyram is primarily used for flea control in pets (Jeschke et al. 2011).

Neonicotinoids are synthetic derivatives of nicotine (Jeschke and Nauen 2008), the naturally occurring alkaloid found in the nightshade plants (Andersson et al. 2003). Like nicotine, neonicotinoids mimic the action of acetylcholine (Thany 2010), the major neurotransmitter in the insect central nervous system (CNS). They act selectively as agonists of the postsynaptic nicotinic acetylcholine receptors (nAChRs) (Matsuda et al. 2001), which are their molecular target site and the receptors responsible for rapid neurotransmission in the insect CNS (Casida and Quistad 2004). Unlike nicotine, the binding affinity of neonicotinoids at the nAChRs in mammals is much less than that of insect nAChRs (Tomizawa and Casida 2003), giving them relatively low mammalian toxicity (Casida and Quistad 2004). In insect pests, however, exposure quickly results in paralysis and death because the neonicotinoid binds strongly to insect

nAChRs, causing overstimulation and blockage of receptors (Tomizawa and Casida 2005). Indeed, neonicotinoids are effective against pest insects in very small quantities. LD₅₀ values (the dose that kills 50 percent of individuals) vary with body size from 0.82–88 ng neonicotinoid insect⁻¹ and concentrations <10 parts per billion (ppb) in plant tissue are usually sufficient to protect crops (reviewed by Goulson 2013). In comparison to longer-established pesticide groups such as pyrethroids, developed resistance to neonicotinoids in target species remains relatively low (Jeschke and Nauen 2008) and as a result of their efficient and unique mode of action there is minimal cross-resistance between neonicotinoids and older classes of pesticide (Jeschke et al. 2011). However, the systemic activity of neonicotinoids is perhaps the major factor in their success (Elbert et al. 2008): when applied directly to the soil or seeds as a dressing, neonicotinoids are taken up via roots and distributed systemically throughout the plant; when applied as a foliar spray they penetrate leaves and are distributed acropetally to new growing shoots. This systemic action enables direct targeting of sucking, piercing, chewing and boring pests as they attack the plant and feed on its tissues. Protection is extended throughout the entire plant and can be sustained for months (Jeschke et al. 2011). With these advantages, neonicotinoids were seen for many years as an unmitigated success (Nauen et al. 2008), being described by some as new milestone in modern agriculture (Jeschke and Nauen 2008). However, over the last decade their safety has been brought into question by the finding that where bees forage on neonicotinoid-treated crops, their health may be at risk.

1.5.2 Environmental exposure of bees to neonicotinoids

The potential health risk posed by neonicotinoids to bees was first highlighted in 1994 after imidacloprid (under the brand name Gaucho®) was used in France in sunflower farming (reviewed by Maxim and van der Sluijs 2013). The following year, beekeepers reported serious problems in their honey bee colonies – foragers appeared disorientated and some were found dead or did not return to the hive – and implicated imidacloprid. In 1999, after years of research that raised suspicions about its effects without formally proving its responsibility, the French government applied the precautionary principle (Alemanno 2013) and banned the use of imidacloprid in sunflower seed dressing. In 2013, the same ban is still in force in France and, following a risk analyses conducted by the European Food Safety Authority (EFSA 2013a, b, c), the EU have recently followed suit by restricting the use of imidacloprid, thiamethoxam and clothianidin on bee-attractive plants and cereals (European Commission 2013).

Although a two-year moratorium on the use of neonicotinoids is currently in place in Europe, there is no universal consensus about the impact of these pesticides on bees (Cresswell et al. 2012a; Cutler et al. 2013; Dicks 2013; Walters 2013) and they are used widely across North America and Asia where bees continue to be exposed (Jeschke et al. 2011; Statistics Canada 2012; USDA 2012). Because neonicotinoids are systemic, trace residues appear in the nectar and pollen of treated crops and bees are exposed when foraging from flowers or consuming in-hive food stores (Rortais et al. 2005). Currently, data on the actual concentrations of neonicotinoid found in environmental nectar and pollen is surprisingly limited (reviewed in: Blacqui  re et al. 2012; EFSA 2012; Goulson 2013). Back in 2002, Bayer (the manufacturers of

Gaucho[®]) declared bees were likely exposed to imidacloprid at concentrations ranging from zero to 5 ppb (Maxim and van der Sluijs 2013). Summarized in Table 1, current data from twenty published studies suggests Bayer's estimated range remains typical today, with mean residues of imidacloprid measured in nectar and pollen calculated at 2.5 and 8.1 ppb, respectively. The imidacloprid concentration range reportedly peaks at over 900 ppb (Mullin et al. 2010), but the highest mean residue value in any one study, 33 ppb in pollen (Dively and Kamel 2012), is probably more indicative of typical worst-case environmental exposures. Measured residues of thiamethoxam tend to be higher than those of imidacloprid: mean thiamethoxam residues in nectar and pollen are calculated at 4.2 and 12.3 ppb, respectively (Table 1). The range for thiamethoxam extends to 127 ppb in pollen, but again the highest mean residue value (for pollen) is below 40 ppb (both values from Dively and Kamel 2012). Residues of clothianidin are typically lower than both imidacloprid and thiamethoxam, with mean values of 1.5 ppb for nectar and 5.7 ppb for pollen (Table 1). Clothianidin residues as high as 88 ppb have been found in pollen, but the highest mean value for clothianidin in a single study is much lower at approximately 17 ppb in pollen (Krupke et al. 2012). Limited studies on the foliar-applied neonicotinoids suggest that acetamiprid and dinotefuran residues in nectar and pollen are variable but generally low (Table 1). The mean residue value across studies for thiacloprid in pollen is the highest of all neonicotinoids at 54.2 ppb (Table 1). However, even at high concentrations such as these, thiacloprid is thought to be largely safe for bees (Jeschke et al. 2011).

Clearly, the concentrations of neonicotinoid to which bees are exposed in the environment vary with compound and have the capacity on rare occasions to

rise to levels approaching 1000 ppb. However, the typical field-realistic range for imidacloprid, thiamethoxam and clothianidin – those compounds identified by the EU as a risk to bee health – appears to fall approximately between 1 and 12 ppb (Table 1). Mean residues do occasionally rise above this typical range, but principally remain below 40 ppb, and the maximum concentration of each of the restricted compounds rarely exceeds 100 ppb. These neonicotinoid concentration ranges are defined here subsequently as the ‘typical field-realistic’ range (1–12 ppb) and the ‘worst-case realistic’ range (>12–100 ppb).

1.6 The effects of neonicotinoids in bees

Using the term ‘neonicotinoid’ to search the database ‘Web of Knowledge’ results in a list of 1,218 scientific articles, with more than half of these (771 articles) published in the last five years (search conducted 3rd December 2013). Regarding those neonicotinoid-related papers published in the last five years, more than 15 percent discuss the effect of these pesticides on bees. The majority of published studies focus on imidacloprid (Walters 2013) and *A. mellifera* honey bees (reviewed in: Decourtye and Devillers 2010; Cresswell 2011; Blacqui  re et al. 2012). There is a dearth of literature on non-*Apis* bees, however, where they are considered the primary focus is *Bombus* spp. Both *Apis* and *Bombus* literature is reviewed below.

1.6.1 Effects of neonicotinoids on mortality in bees

A meta-analysis based on 14 studies of imidacloprid’s impact on adult honey bees found that dietary field-realistic concentrations in the range from 1 to 10

ppb had virtually no effect on rates of mortality, nor did concentrations up to 100 ppb produce significant lethal effects (Cresswell 2011). Recent experimental work adds support to this evidence (Cresswell et al. 2012b). However, these findings are not consistent across all studies because, for example, Decourtey et al. (2003) observed a significant increase in honey bee mortality following an 11-day dietary imidacloprid exposure at 48 ppb. In honey bees, the non-lethal effect of imidacloprid in the field-realistic range is one that generally translates to other dietary neonicotinoids. For example, thiamethoxam increased mortality at 100 ppb (Laurino et al. 2011) but not below 50 ppb (Aliouane et al. 2009; Laurino et al. 2011; Oliveira et al. 2013). Clothianidin produced a significant lethal effect at 75 ppb but not below 37.5 ppb, while neither acetamiprid nor thiacloprid increased mortality despite bees feeding on residues far exceeding the ‘worst-case realistic’ range (i.e. 100,000–144,000 ppb; Laurino et al. 2011). All of the above results come from laboratory or semi-field (a mix of laboratory and field settings) trials, but in full-field trials honey bee mortality was also unaffected by maximum residues of thiamethoxam (3 ppb) and clothianidin (2.6 ppb) in the nectar and pollen from treated maize or oilseed rape crops (Cutler and Scott-Dupree 2007; Pilling et al. 2013).

Unsurprisingly, very large dietary concentrations of imidacloprid (200–200,000 ppb) also substantively reduce the life span of laboratory maintained bumble bees (*B. terrestris* and *B. impatiens*) (Gradish et al. 2010; Mommaerts et al. 2010b). However, the effects of smaller concentrations on mortality are inconsistent among studies. For *B. terrestris* workers maintained individually, longevity was unaffected by dietary imidacloprid concentrations up to 100 ppb (Cresswell et al. 2012b), whereas in small groups of workers kept in queenless

microcolonies, survival was reduced by concentrations between 10 and 20 ppb in one study (Tasei et al. 2000) but not in another (Mommaerts et al. 2010b). However, in the latter study (Mommaerts et al. 2010b), when queenless microcolonies were presented with an additional foraging task, workers suffered 50 percent mortality following an 11-week exposure at 20 ppb. In queenright *B. terrestris* colonies, exposure to dietary imidacloprid at 10 ppb did not affect worker survival when the colony was also able to forage naturally for uncontaminated nectar and pollen (Gill et al. 2012), but it significantly increased mortality when colonies were confined to the laboratory (Bryden et al. 2013) or had no access to alternative forage (Mommaerts et al. 2010b).

In those studies that tested neonicotinoids other than imidacloprid in bumble bees, large dietary concentrations of thiamethoxam (100–1000 ppb; Mommaerts et al. 2010b), clothianidin (171 ppb; Larson et al. 2013), and thiacloprid (60,000–12,000 ppb; Mommaerts et al. 2010b) were found to be lethal in *B. terrestris* or *B. impatiens*, whereas exposure to concentrations of ~10 ppb lasting up to 11 weeks had no substantive affect on mortality (Sechser and Freuler 2003; Mommaerts et al. 2010b; Elston et al. 2013). A notable exception was a recent study where it was observed that worker longevity was reduced by approximately one week in *B. terrestris* colonies exposed simultaneously to thiamethoxam and clothianidin at 4 and 1.5 ppb, respectively, in both syrup and pollen for nine weeks (Fauser-Misslin et al. 2014). The authors of this study justified a mixed dietary dose of these two compounds because where thiamethoxam is converted by metabolism into clothianidin in treated plants (Nauen et al. 2003) both neonicotinoids could jointly be encountered in the field (Fauser-Misslin et al. 2014).

Aside from directly increasing mortality or reducing longevity, evidence exists that the sublethal effects of neonicotinoids could eventually lead to death in bees. For example, Henry et al. (2012a) showed that thiamethoxam fed to foraging honey bees in a single dose at $67 \mu\text{g L}^{-1}$ disrupted navigation and increased the risk of homing failure, thereby increasing loss of foragers. The authors claimed these losses could precipitate colony collapse and their findings were a key factor in EU's decision to restrict neonicotinoids. However, since its publication the paper has received significant criticism. First, the exposure regime used to demonstrate the effect was deemed 'unrealistic' because bees consumed their entire estimated daily exposure of thiamethoxam in a single dose rather than over several foraging trips (Cresswell and Thompson 2012; Campbell 2013; Guez 2013a; Walters 2013). Indeed, the authors themselves recently acknowledged this lack of environmental realism, citing the technical difficulty of repeatedly exposing bees whilst also monitoring their navigation over kilometres (Henry 2013). Secondly, the method by which Henry et al. (2012a) calculated mortality due to homing failure and the parameters with which they populated their model of colony population dynamics have each been questioned by other authors, who suggest estimates of honey bee mortality and colony collapse due to thiamethoxam are unduly inflated (Cresswell and Thompson 2012; Guez 2013a, b). The original authors dispute these specific criticisms (Henry et al. 2012b; Henry 2013; Henry and Decourtye 2013) and the discussion surrounding their paper continues. Recently, a similar study to Henry et al. (2012a) was published in which honey bees that were fed an acute dose of either imidacloprid at 75 ppb or thiacloprid at 12,500 ppb suffered significant difficulty navigating back to their hive (Fischer et al. 2014). In terms of its environmental relevance, this study suffered from the

same methodological flaw demonstrated in Henry et al. (2012a), namely the administration in a single meal of unrealistically high doses designed to represent a bee's daily intake of neonicotinoid. Interestingly, when Fischer et al. (2014) fed honey bees a somewhat more realistic acute dose of clothianidin (25 ppb), fewer detrimental effects on navigation were observed. In another study where honey bees were dosed with more realistic acute dietary exposures (11.5 ppb imidacloprid and 38 ppb clothianidin), no detrimental affect on homing ability was observed (Schneider et al. 2012). With regards to homing failure in bumble bees, *B. terrestris* workers exposed to imidacloprid residues ≤ 10 ppb failed to return to the colony more frequently than unexposed workers in one study (Gill et al. 2012), but not in another (Tasei et al. 2001).

1.6.2 Effects of neonicotinoids on worker performance

In general, where field-realistic neonicotinoid exposures are incapable of causing substantive direct mortality in bees, they are often capable of causing sublethal effects on worker performance. Indeed, in one comparative study, sublethal performance biomarkers in honey bees were more sensitive than mortality to dietary imidacloprid in a range up to $125 \mu\text{g L}^{-1}$ (Cresswell and Laycock 2012). Furthermore, in the Cresswell (2011) meta-analysis that reported dietary imidacloprid in the range up to $100 \mu\text{g L}^{-1}$ to be non-lethal in honey bees, concentrations between 1 and $10 \mu\text{g L}^{-1}$ reduced worker performance (encompassing various aspects of behaviour) by between 6 and 20 percent.

The receptor targets of neonicotinoids, the nAChRs, play an important role in learning and memory in bees (Gauthier 2010), and bees' ability to forage

successfully and efficiently depends on how well these processes function (Raine and Chittka 2008; Lihoreau et al. 2011). To date, researchers have tested for effects of neonicotinoids on learning and memory in *A. mellifera* only, and primarily by employing classical conditioning of the proboscis extension reflex (PER) (Blacquière et al. 2012). In acute exposure trials using PER, olfactory learning and memory in honey bees was detrimentally affected by neonicotinoids when doses were unrealistically high (i.e. imidacloprid and acetamiprid at 12 and 100 ng bee⁻¹, respectively: Decourtye et al. 2004a; El Hassani et al. 2008), but not when doses approached realistic levels (i.e. 0.12–1.28 ng imidacloprid or thiamethoxam bee⁻¹: Decourtye et al. 2004a; El Hassani et al. 2008; Williamson et al. 2012). Where PER and chronic, rather than acute, exposure are employed, imidacloprid affects honey bee learning at lower concentrations. For example, learning and/or memory were significantly impaired following chronic exposures lasting up to two weeks to imidacloprid in the range from 12 to 48 ppb (Decourtye et al. 2003, 2004b; Han et al. 2010; Williamson and Wright 2013). In contrast, thiamethoxam and acetamiprid had no observable effect on honey bee learning following 11-day exposures at 3 µg L⁻¹ and 3 mg L⁻¹, respectively (Aliouane et al. 2009). The environmental relevance of PER trials testing neonicotinoids is difficult to extrapolate because the PER process generally involves: a) anesthetization (by chilling or CO₂) and b) immobilization of the bee throughout the testing procedure (Bitterman et al. 1983). Theoretically, the PER assay could increase stress and impair metabolism in the bees, resulting in increased neurotoxic impacts (discussed in Cresswell et al. 2012b). It is therefore likely that results from trials in which learning is tested whilst bees are free to move or fly are more indicative of natural conditions. In two such studies, negative impacts on learning were

demonstrated following chronic exposure to imidacloprid at 24 ppb and 48 ppb in honey bees that were free to fly (Decourtye et al. 2004b) or were placed in a maze (Han et al. 2010), respectively.

Equally intrinsic to a worker bees' foraging or in-hive performance is the ability to move freely within the environment. Neonicotinoids cause paralysis and death in pest species (Tomizawa and Casida 2005), and so their affect on locomotory activity in bees has also been studied. In chronically exposed honey bees, imidacloprid concentrations \geq 50 ppb reduced locomotion and speed of movement in 24-hour trials (Medrzycki et al. 2003; Teeters et al. 2012), but in trials $>$ 24 hours concentrations ranging from 0.1 to 98 ppb had no effect on movement (Aliouane et al. 2009; Cresswell et al. 2012b). For thiamethoxam, an acute dose of 1 ng honey bee⁻¹ (equivalent to 100 ppb) had no significant impact on locomotor activity (El Hassani et al. 2008). In *B. terrestris* bumble bees, two studies have quantified locomotion during imidacloprid exposure and each observed decreased movement at 98 ppb (Cresswell et al. 2012b, 2013).

Several studies have measured actual foraging performance and feeding rates in worker bees exposed to neonicotinoids. In honey bees, food consumption and foraging activity are reduced when food contains residues of imidacloprid $>$ 20 ppb (Decourtye et al. 2004b; Yang et al. 2008; Decourtye and Devillers 2010; Schneider et al. 2012). Conversely, collection, consumption or storage of honey and pollen is largely unaffected by imidacloprid \leq 20 ppb (Schmuck et al. 2001; Cresswell et al. 2012b; Schneider et al. 2012). In field and semi-field trials, 'typical field-realistic' concentrations of thiamethoxam (<0.5–3 ppb) and clothianidin (<1–3.8 ppb) produced no significant affect on honey bee foraging

behaviour or food storage (Cutler and Scott-Dupree 2007; Schneider et al. 2012; Pilling et al. 2013), but concentrations of clothianidin at 38 ppb reduced forager efficiency (Schneider et al. 2012). In bumble bees, the effects of neonicotinoids on feeding and foraging behaviour are inconsistent among studies and compounds. In laboratory trials, exposure to dietary imidacloprid, thiamethoxam, or a mixture of thiamethoxam and clothianidin at concentrations between 1 and 100 ppb reduced the rate at which *B. terrestris* consumed syrup and/or pollen (Cresswell et al. 2012b, 2013; Elston et al. 2013; Fauser-Misslin et al. 2014) and impacted the foraging performance of *B. impatiens* in one study (Morandin and Winston 2003) but not in another (Franklin et al. 2004). In a semi-field trial also, pollen-foraging performance was significantly reduced in *B. terrestris* colonies fed syrup dosed with 10 ppb imidacloprid (Gill et al. 2012). However, forager efficiency suffered no ill affect when *B. terrestris* colonies visited imidacloprid-treated sunflowers containing residues <10 ppb (Tasei et al. 2001).

In two recent studies, researchers inferred that the neurophysiological affects produced by neonicotinoids on the brains of bees might be correlated with the effects that these pesticides have on cognition, locomotion and behaviour. Mushroom bodies are structures in the insect brain that mediate multisensory integration, learning and memory (Zars 2000). Using recordings from *A. mellifera* Kenyon cells (the major neuronal component of mushroom bodies), Palmer et al. (2013) showed that by modulating the activity of nAChRs, imidacloprid and clothianidin can inhibit neuronal firing and significantly impair brain function. Similarly, imidacloprid reduced the growth of neurites in mushroom body Kenyon cells isolated from the brains of 13-day old *B.*

impatiens nurse and forager bumble bees, indicating that the neonicotinoid could diminish the capacity for brain, and therefore behavioural, plasticity in dosed bees (Wilson et al. 2013). In both studies, effects were observed at typical field-realistic concentrations of ~2.5 ppb, but Wilson et al. (2013) acknowledge that interpreting the environmental relevance of these results is difficult. Directly exposing bees' brains or brain-cells to a full field-realistic dose is not a natural situation because it bypasses both the metabolic and biological barriers that usually moderate the concentration to which the brain is exposed (Suchail et al. 2004a, b; Cresswell et al. 2013), and these barriers potentially reduce the level of pesticide to <5 percent of the initial ingested dose (Suchail et al. 2004a). As suggested by Wilson et al. (2013), the findings of these studies could prove environmentally relevant if trace residues of neonicotinoid accumulate in the bees' head over time. However, an accumulation such as this is yet to be experimentally demonstrated.

1.6.3 Effects of neonicotinoids on reproduction in bees

We have seen that concentrations of neonicotinoid as low as one ppb can detrimentally affect feeding behaviour in bumble bees, but production of brood could be even more sensitive (Cresswell and Laycock 2012). Clearly, reproduction – here encompassing the production of healthy brood, workers and sexual offspring – is vital to colony success and several studies have already investigated to what extent this endpoint is affected by neonicotinoids. Fewer studies have tested for effects of neonicotinoids on reproduction in honey bees than bumble bee, perhaps because of the difficulty inherent in measuring the former Hymenopterans' very large perennial colonies, and once again results across studies are inconsistent.

In honey bee colonies, chronic exposure to dietary imidacloprid at ~20 ppb reduced brood production in one study (Decourtye et al. 2004b), but had no effect on the reproductive capacity of the hive in another (Schmuck et al. 2001). Imidacloprid may also affect honey bee larval development because Decourtye and Devillers (2010) note that larvae exposed at 5 ppb take longer to emerge. However, Yang et al. (2012) observed no effect on rates of pupation or eclosure following an acute 0.4 ng imidacloprid larvae⁻¹ dose (administered by means of four consecutive once-daily exposures at 100 µg L⁻¹). Although they observed no effect on larval development, Yang et al. (2012) reported that treatment with 0.04 ng imidacloprid larvae⁻¹ (10 µg L⁻¹ on four consecutive days) impaired olfactory associative learning ability when the larvae reached adulthood, thus revealing a delayed detrimental effect not instantly obvious in the larval stage. Once again, neonicotinoids in full-field trials produced no effect on honey bees; specifically, clothianidin at maximum residues of 2.6 ppb in oilseed rape did not affect brood production either directly following a 3-week exposure or in the subsequent spring following over-wintering (Cutler and Scott-Dupree 2007).

In bumble bees, brood production in queenless microcolonies containing small groups of workers exposed to neonicotinoids has been used to measure the pesticides' impact on reproduction. In the most comprehensive study of this kind, Mommaerts et al. (2010b) found that *B. terrestris* microcolonies required to complete a foraging task produced fewer male offspring following an 11-week dietary exposure in syrup to imidacloprid at 10 ppb, thiamethoxam at 100 ppb or thiacloprid at 12,000 ppb. Similarly, dietary exposure in syrup and pollen reduced brood production in *B. terrestris* microcolonies presented with

imidacloprid (10 ppb syrup, 6 ppb pollen; Tasei et al. 2000) or thiamethoxam (10 ppb in both syrup and pollen; Elston et al. 2013).

Whilst microcolony studies provide a valuable first approximation of how neonicotinoids affect bumble bee reproduction, their environmental relevance is somewhat limited by the removal of the queen, who in the wild is primarily responsible for reproductive output (Lopez-Vaamonde et al. 2004), and so studies using queenright colonies are necessary. In the laboratory, *B. terrestris* colonies monitored for six weeks showed reduced rates of worker eclosure (emergence from the pupa case) for the first three weeks and virtually zero eclosures in the final three weeks when fed 10 ppb imidacloprid in syrup (Bryden et al. 2013). In contrast, laboratory exposures to imidacloprid or clothianidin lasting ten weeks produced no effect on the number of workers, males, queens, and amount of brood produced in colonies of *B. occidentalis* or *B. impatiens* when concentrations in pollen were between 7 and 36 ppb (Morandin and Winston 2003; Franklin et al. 2004). However, when clothianidin was combined with thiamethoxam (at 1.5 and 4 ppb, respectively), *B. terrestris* colonies exposed in syrup and pollen for nine weeks showed reductions in worker production (between weeks four and seven) and overall investment in sexual offspring (Fauser-Misslin et al. 2014).

In semi-field trials, dietary imidacloprid in the typical field-realistic range had impacts on some, but not all, elements of reproduction in *B. terrestris* colonies. Specifically, dietary imidacloprid at 10 ppb reduced the number of larvae, pupae and workers produced in colonies exposed in-hive for four weeks but also allowed to forage naturally for environmental nectar and pollen (Gill et al. 2012).

Conversely, in *B. terrestris* colonies that foraged in the field for nine days on imidacloprid-treated sunflowers containing residues <10 ppb and were then transferred to the lab where colonies were closed and fed pesticide-free syrup and pollen for a further 17 days, the number of workers and new queens produced was not significantly different from control colonies (Tasei et al. 2001). Furthermore, the number of pupae, workers and males produced was unaffected in colonies that were laboratory-exposed to imidacloprid for two weeks at 6 ppb in pollen and 0.7 ppb in syrup and then allowed to forage naturally for a further six weeks (Whitehorn et al. 2012). However, in the latter study, the number of queens produced in imidacloprid-treated colonies was reduced by 85 percent (Whitehorn et al. 2012).

When trials are conducted with colonies entirely in the field, results conflict with laboratory and semi-field trials. For example, where granular or spray residues of imidacloprid were applied to weedy turf as recommended, i.e. at the maximum label rate followed by irrigation, they did not affect the number or weight of workers produced, nor the amount of brood, in *B. impatiens* colonies foraging on white clover for 30 days (Gels et al. 2002). However, colonies that consumed non-irrigated residues produced fewer brood and workers, highlighting the need to follow closely recommended application instructions (Gels et al. 2002). Unsurprisingly, when clothianidin was applied contrary to label precautionary statements, i.e. as a spray to white clover in bloom to the extent that residues in nectar reached 171 ppb, *B. impatiens* colonies that foraged on the clover did not produce new queens (Larson et al. 2013). Finally, in a study described by its own authors as an investigation of a real-life field situation and not a statistically robust experiment, Thompson et al. (2013)

tested for effects on *B. terrestris* colonies placed near imidacloprid- and clothianidin-treated oilseed rape crops. The study suffered from contamination of ‘control’ colonies (or more specifically, colonies placed near oilseed rape crops grown from untreated seeds) with thiamethoxam residues because bees had foraged in thiamethoxam-treated oilseed rape approximately one kilometre from the study site. There were also systematic differences in colony size between study sites, arising from differences in flowering phenology and study start times, that brought the validity of the results further into question. However, despite its significant failings, the study of Thompson et al. (2013) did show that colonies containing maximum thiamethoxam residues of 2.4 ppb in nectar and 0.7 ppb in pollen, or imidacloprid and clothianidin at levels below quantification, were capable of producing new queens at a minimum mean rate of 17 per colony. As Thompson et al. (2013) highlight, this rate of queen production is comparable to the mean number of queens produced in the untreated control colonies of a semi-field trial where queen production in treated colonies was reduced by dietary residues of imidacloprid (6 ppb in pollen, 0.7 ppb in syrup; Whitehorn et al. 2012).

1.6.4 Summarising the effects of neonicotinoids on demographically relevant endpoints in bees

Although the effect of neonicotinoids on bee mortality appears somewhat dependent on factors such as the length of exposure (Tasei et al. 2000; Moncharmont et al. 2003; Bryden et al. 2013), experimental procedure (Mommaerts et al. 2010b; Bryden et al. 2013), age and species of bee or colony (Decourtye and Devillers 2010), or even the season in which testing occurs (Decourtye et al. 2003), the majority of evidence suggests that ‘typical field-

'realistic' concentrations are not directly lethal to either honey bees or bumble bees. Direct mortality is more likely to occur in honey bees as neonicotinoid concentrations rise above ~50 ppb and in bumble bees above ~20 ppb. The sublethal effects of neonicotinoids may also lead to death, for example when foragers do not return to the colony because of homing failure. Such effects have been demonstrated in honey bees at concentrations above ~40 ppb only, but have been observed in bumble bees where the dietary exposure falls within the 'typical field-realistic' range (i.e. ~10 ppb).

Locomotory activity is the worker performance endpoint least sensitive to neonicotinoids because detrimental effects typically occur in *Apis* and *Bombus* spp. when dietary concentrations are >50 ppb. While data are lacking for bumble bees, learning and memory is impaired in honey bees primarily at realistically-high concentrations (i.e. 12–48 ppb). Detrimental effects on feeding and foraging behaviour tend to occur in honey bees when concentrations rise above ~20 ppb, but occur in bumble bees exposed to neonicotinoids in the 'typical field-realistic' range (i.e. between 1 and 10 ppb).

In honey bees, detrimental effects on brood production can occur where dietary neonicotinoids rise above ~20 ppb. For bumble bees, the results of laboratory, semi-field and field studies are inconsistent, but 'typical field-realistic' neonicotinoid concentrations are capable of having impacts on reproduction: in queenless microcolonies production of brood decreases following exposures at ~10 ppb; in queenright colonies the amount of brood and the number workers, males and queens produced is reduced by concentrations between ~6–10 ppb.

1.7 Effect mechanisms of neonicotinoids in bees

While the various effects of neonicotinoids begin to be elucidated, the effect mechanisms of these pesticides in bees remain largely unstudied. Further research into, for example, the metabolism pathways involved in detoxification of neonicotinoids, or the general molecular response of bees to exposure, could help to better establish the pesticides' toxic effect mechanisms. Research into effect mechanisms is in its infancy in bumble bees. A study of whole-body clearance has shown that bumble bees can eliminate bodily imidacloprid within 48 hours, but the mechanism of elimination was not revealed (Cresswell et al. 2013). The use of cell cultures from bumble bee brains has also revealed that imidacloprid can disrupt brain cell growth (Wilson et al. 2013). The study of effect mechanisms in honey bees is more advanced. Methodologically, these studies vary from the purely computational – comparing xenobiotic detoxifying enzymes between the genomes of honey bees and other insects (Claudianos et al. 2006) – to the purely experimental – using enzyme inhibitors to investigate mechanisms of pesticide metabolism (Iwasa et al. 2004). Somewhere in between, studies that combine these approaches have informed our current knowledge of synthetic and natural xenobiotic metabolism in honey bees (Mao et al. 2009, 2011, 2013; Johnson et al. 2012). The effect mechanisms of neonicotinoids have been covered in two studies in which: a) an enzyme inhibitor assay was used to demonstrate that honey bee cytochrome P450 enzymes are an important mechanism for metabolism of cyano-substituted neonicotinoids such as thiacloprid, but not for nitro-substituted neonicotinoids such as imidacloprid in adult honey bees (Iwasa et al. 2004); b) transcriptomics was used to reveal the genome-wide RNA transcriptional response of honey bee larvae exposed to field-realistic concentrations of dietary imidacloprid

(Derecka et al. 2013). Similar work is necessary to begin establishing the effect mechanisms of neonicotinoids in bumble bees.

1.8 Knowledge gaps and thesis research objectives

Existing literature suggests that neonicotinoids have detrimental impacts on demographically relevant endpoints in bumble bees at lower concentrations than in honey bees (see section 1.6). Specifically, while there is little evidence that exposures in the ‘typical field-realistic’ range from 1–12 ppb have a significant impact on honey bees, several studies demonstrate the detrimental effects of concentrations in this range on feeding behaviour and reproduction in bumble bees. As detailed in section 1.3, both feeding performance and production of brood and workers influence the amount of sexual offspring produced in bumble bee colonies, and so it is possible that detrimental effects on these particular endpoints could have impacts on colony success and potentially contribute to population declines. However, whilst negative effects on feeding and reproduction have thus far principally been demonstrated when bumble bees are exposed to dietary neonicotinoid concentrations at the upper end of the ‘typical field-realistic’ range between 6–12 ppb (section 1.6), exposure in the field will most often occur at levels <6 ppb. For example, the mean level of imidacloprid, thiamethoxam and clothianidin (the compounds now restricted for use in Europe) is below 6 ppb in 77 percent of studies where residues were measured (Table 1). Consequently, in order to establish a fuller understanding of the impact that neonicotinoids could have on wild bumble bees, it will be necessary to test the performance of bees exposed to dietary

concentrations at the lower end of the ‘typical field-realistic’ range from >0–6 ppb. Therefore, in the thesis work that follows (chapters two, three and four), a further investigation into the effects of dietary neonicotinoids on food consumption and brood production in *B. terrestris* bumble bees is presented, and this includes the testing of concentrations in the range ≤6 ppb as well as residues up to 100 ppb to account for the ‘worst-case field-realistic’ range. Establishing not only the effects of neonicotinoids, but also the effect mechanisms of these pesticides is also important if we are to gain a better understanding of their toxicity to bees. Currently, very little is known about the effect mechanisms in bumble bees, and so this thesis also includes experiments designed to investigate the effect mechanisms of neonicotinoids in *B. terrestris* workers (chapters five and six). In the next five sub-sections, the specific research objectives of each of the five experimental chapters contained within this thesis are outlined.

*1.8.1 Chapter two: effects of imidacloprid in *B. terrestris* microcolonies*

In chapter two, in order to determine whether environmentally realistic levels of imidacloprid are capable of making an impact on demographically relevant endpoints in bumble bees, queenless microcolonies (comprising small groups of *B. terrestris* workers isolated from their queen) were exposed *via* their diet to a range of concentrations between zero and 98 ppb. The microcolony assay is recommended for use in ‘higher tier’ risk assessment studies of bumble bees (EFSA 2013d) because it enables a convenient evaluation of both lethal and sublethal effects of dietary pesticides using multiple replicates (Blacquière et al. 2012). The assay was therefore used in chapter two to examine the effects of imidacloprid on ovary development, oviposition, brood production, consumption

of syrup and pollen, and survivorship in bumble bees following an exposure lasting approximately two weeks.

*1.8.2 Chapter three: effects of thiamethoxam in *B. terrestris* microcolonies*

Having established the effects of imidacloprid in microcolonies in chapter two, a comparable experiment was conducted in chapter three to establish the effects of thiamethoxam on demographically relevant endpoints in bumble bees. Specifically, a microcolony assay was used to examine the effects of dietary thiamethoxam in the range from zero to 98 ppb on oviposition, brood production, consumption of syrup and pollen, and survivorship in *B. terrestris* microcolonies. The same dosages were applied in chapters two and three, and similar endpoints and exposure periods were adopted, in order to enable a comparison of the relative sensitivity of bumble bees to imidacloprid and thiamethoxam.

*1.8.3 Chapter four: effects of a pulsed exposure to imidacloprid in queenright *B. terrestris* colonies*

Having established the relative effects of imidacloprid and thiamethoxam in microcolonies, the effects of a pulsed exposure to dietary imidacloprid on oviposition, brood production and food consumption in queenright *B. terrestris* colonies were investigated in chapter four. Colonies at an early stage of development (comprising a queen and four workers) were exposed to imidacloprid in feeder syrup in the range from zero to 98 ppb for two weeks. Imidacloprid was subsequently removed from their diet and bees were fed on clean syrup for a further two weeks – thus creating a 28-day pulsed exposure (14 days ‘on dose’, 14 days ‘off dose’). A pulsed imidacloprid exposure scenario

was adopted because it has environmental relevance. For example, a pulsed exposure could arise in the environment where wild bees forage on the flowers of a treated mass-flowering crop during its transient bloom and subsequently switch to foraging on pesticide-free wild flowers (see chapter four for a full discussion of pulsed exposures in the environment). For each endpoint, the performance of colonies was determined during both 'on dose' and 'off dose' periods, thereby enabling an evaluation of bumble bees' capacity for recovery from imidacloprid's effects once an exposure has ceased.

*1.8.4 Chapter five: effects of piperonyl butoxide on the toxicity of neonicotinoid pesticides in *B. terrestris* workers*

In chapter five, an investigation of the role that cytochrome P450 enzymes play in the metabolism of neonicotinoids in bumble bees was undertaken. Specifically, the effects of piperonyl butoxide (PBO), an insecticide synergist and cytochrome P450 enzyme inhibitor, in combination with imidacloprid or thiacloprid were studied. PBO increases the toxicity of thiacloprid but not imidacloprid when applied topically to honey bees (Iwasa et al. 2004), and so the hypothesis that bumble bees would exhibit similar effects following oral or topical exposure to these compounds was tested in chapter five. The usual neonicotinoid concentrations (zero to 98 ppb) were tested alone or in combination with PBO in individual *B. terrestris* workers, and the effects on syrup consumption, locomotory activity, and longevity were observed. If bumble bees metabolized neonicotinoids in the tested range using P450 enzymes, we expected to observe an interactive effect between PBO and the neonicotinoid that would raise the pesticide's toxicity and increase the severity of effects on the bee.

*1.8.5 Chapter six: a transcriptomic analysis of the effect mechanisms of imidacloprid in *B. terrestris* workers*

In chapter six, in order to investigate the effect mechanisms of imidacloprid in bumble bees, a transcriptomic analysis was applied to *B. terrestris* workers. RNA was extracted from the abdomens of bumble bees that for 12 hours were either exposed to syrup dosed with 98 µg imidacloprid kg⁻¹ or fed clean syrup. The relatively high concentration of imidacloprid was chosen for its capacity to produce behavioural and physiological effects on individual bumble bees and not for environmental relevance. RNA sequencing was applied and the resultant data analyzed for the alteration in expression of genes between treatments. Genes that were differentially expressed in the imidacloprid treatment were characterized in order to establish a preliminary profile of the neonicotinoid's toxic effect mechanisms in bumble bees.

1.8.6 A note on the denomination of neonicotinoid concentrations and their comparability with other studies

In the experiments described here, bees were dosed orally and chronically (but see chapter five, which also includes acute topical dosing). Specifically, neonicotinoids were presented to bees in dietary syrup on which they were able to feed *ad libitum*. In general, to produce the most concentrated dietary dose, neonicotinoids were first dissolved in a solvent and then mixed into syrup at a concentration of 125 µg L⁻¹. This most concentrated dose was then serially diluted down using clean syrup to produce a full neonicotinoid concentration range. In the chapters that follow, the concentration range is described in µg L⁻¹, but also converted and described in µg kg⁻¹. The latter is conventional in the current literature (see section 1.6), and is often also converted into parts per

billion or ppb. However, readers should note that because doses are administered in syrup, while $\mu\text{g kg}^{-1}$ and ppb are interchangeable, $\mu\text{g L}^{-1}$ and ppb are not (in other words, $\mu\text{g kg}^{-1} \neq \mu\text{g L}^{-1}$). Quite simply, this is because the mass of one litre of syrup is usually greater than one kilogram; here, the mass of one litre of syrup is equal to 1.27 kg. Therefore, in the current work a conversion from $\mu\text{g L}^{-1}$ to $\mu\text{g kg}^{-1}$ (or ppb) is calculated as follows (using 125 $\mu\text{g L}^{-1}$ as an example): $125 \mu\text{g L}^{-1} / 1.27 = 98.4 \mu\text{g kg}^{-1}$. Performing these conversions not only increases the thesis work's comparability with studies that use $\mu\text{g kg}^{-1}$ or ppb, but also facilitates a calculation of the mass of neonicotinoid consumed bee^{-1} (or colony^{-1}) day^{-1} . This additional data is somewhat useful for comparison with studies in which bees are dosed acutely (e.g. ng neonicotinoid bee^{-1}), but also for comparison with the estimated mass of neonicotinoid consumed by bees that forage on contaminated nectar and pollen in the field (Rortais et al. 2005).

Chapter One: Tables

Table 1.1 Overview of data on neonicotinoid residues in nectar and pollen

Neonicotinoid	Nectar and honey ^a		Pollen ^{a, b}		Reference
	Mean residue (ppb)	Residue range (ppb)	Mean residue (ppb)	Residue range (ppb)	
Imidacloprid	-	-	3.0	1.0 – 11.0	Bonmatin et al. (2003)
	-	-	2.1	0.3 – 18.0	Bonmatin et al. (2005)
	0.7	0.3 – 1.8	0.9	0.2 – 5.7	Chauzat et al. (2006, 2009, 2011)
	5.7	0 – 16.0	33.3	0.1 – 101.0	Dively and Kamel (2012) ^c
	0.4	0 – 0.8	2.4	0 – 7.6	EFSA (2012)
	-	-	3.1	0 – 912.0	Mullin et al. (2010)
	0	0	-	-	Pareja et al. (2011)
	-	-	2.8	0 – 36.5	Pettis et al. (2013)
	0.6	0 – 2.0	0	0	Pohorecka et al. (2012)
	-	-	30.8	3.5 – 216.0	Rennich et al. (2012)
	10	5.0 – 20.0	14	5.0 – 35.0	Stoner and Eitzer (2012)
	-	-	5.2	1.0 – 70.0	Stoner and Eitzer (2013)
	0.1	0 – 0.5	0	0	Thompson et al. (2013)

Table 1.1 Overview of data on neonicotinoid residues in nectar and pollen (*Continued*)

Neonicotinoid	Nectar and honey ^a		Pollen ^{a, b}		Reference
	Mean residue (ppb)	Residue range (ppb)	Mean residue (ppb)	Residue range (ppb)	
Thiamethoxam	5.7	0.1 – 15.1	38.1	0.1 – 127.0	Dively and Kamel (2012) ^c
	2.8	<1.0 – 5.2	14.0	<1.0 – 51.0	EFSA (2012)
	0	0	4.5	0 – 20.4	Krupke et al. (2012)
		<0.5 – 4.0	-	<1.0 – 6.0	Pilling et al. (2013)
	4.2	0 – 12.9	3.8	0 – 9.9	Pohorecka et al. (2012)
	11	5.0 – 20.0	12	5.0 – 35.0	Stoner and Eitzer (2012)
Clothianidin	-	<0.5 – 2.2	-	<0.5 – 2.6	Cutler and Scott-Dupree (2007)
	3.4	0 – 8.6	3.6	1 – 11.4	EFSA (2012)
	0	0	17.4	0 – 88.0	Krupke et al. (2012)
	-	<1.0 – 1.0	-	<1.0 – 4.0	Pilling et al. (2013)
	2.3	0 – 10.1	1.8	0 – 3.7	Pohorecka et al. (2012)
	0.1	0.1 – 0.3	0.1	0 – 0.7	Thompson et al. (2013)
Thiacloprid	-	<1.0	-	<1.0	EFSA (2012)
	-	-	1.3	0 – 115.0	Mullin et al. (2010)
	-	-	1.1	0 – 12.4	Pettis et al. (2013)
	6.5	0 – 208.8	89.1	0 – 1002.2	Pohorecka et al. (2012)

Table 1.1 Overview of data on neonicotinoid residues in nectar and pollen (*Continued*)

Neonicotinoid	Nectar and honey ^a		Pollen ^{a, b}		Reference
	Mean residue (ppb)	Residue range (ppb)	Mean residue (ppb)	Residue range (ppb)	
Thiacloprid	-	-	187.6	49.1 – 326.0	Rennich et al. (2012)
(Continued)	-	-	24.0	<10.0 – 90.0	Škerl et al. (2009)
	-	-	22.3	1.0 – 68.0	Stoner and Eitzer (2013)
Acetamiprid	-	-	1.9	0 – 134.0	Mullin et al. (2010)
	-	-	59.1	0 – 401.0	Pettis et al. (2013)
	2.4	0 – 13.3	4.1	0 – 26.1	Pohorecka et al. (2012)
Dinotefuran	6.1	0.1 – 16.0	41.4	8.0 – 147.0	Dively and Kamel (2012) ^c
	-	-	4.0	2.1 – 7.6	Stoner and Eitzer (2013)

^a Residues measured in: oilseed rape, *Phacelia* spp., alfalfa, sunflower, pumpkin, squash, honey, bumble bee colonies

^b Residues measured in: maize, various pollen collected by honey bees

^c Contains residues detected following foliar-applied treatments, which are typically higher than with seed and soil treatments

Chapter Two

Effects of imidacloprid, a neonicotinoid
pesticide, on reproduction in worker bumble
bees (*Bombus terrestris*)

Preliminary introduction

The following chapter is based on a paper submitted to *Ecotoxicology* in January 2012 for which I was first author. The paper was accepted and first published in the journal online in May 2012. The final publication is available at <http://link.springer.com/article/10.1007/s10646-012-0927-y> (paper) and <http://link.springer.com/article/10.1007/s10646-012-0974-4> (erratum to paper). The content below is presented in the style of *Ecotoxicology* and is identical to the published paper, with the following exceptions. The original publication was accompanied by an erratum, which corrected a number of concentration conversions (from $\mu\text{g L}^{-1}$ to parts per billion). Here, the corrected conversions are provided within the main text and are reflected in an amendment to *imidacloprid intake* in Figure 2. I also make a small number of qualitative amendments in order to improve the clarity of the work. Additionally, ambiguous references are lettered (a, b, etc) and the numbering of sections, figures and tables has been altered in accordance with their final position in the thesis.

Statement of contribution

As first author, I was primarily responsible for conception and design of the work, acquisition and analysis of the data, and writing the paper. My supervisor and co-author Dr James Cresswell provided advice and guidance on all of the above, including revision notes on early manuscript drafts. Co-author Andrew Barratt provided his expertise in Bayesian analysis and conceived the Bayesian model in the paper. He also provided the training that enabled me to understand and run the model in WinBugs and R (see Methods). Kate Lenthall was involved in the work as part of her undergraduate research project and dissertation, and is credited as a co-author because of her assistance with related pilot work (not included here) and data collection in the first of three trials that comprise the final experiment. Additional contributors are acknowledged at the end of the paper: Dr Hannah Florance provided LC-MS expertise, running samples through the mass-spectrometer and providing training in sample preparation and analysis of LC-MS data; Bobbi Hope assisted with maintenance of microcolonies; two anonymous reviewers provided comments on the manuscript during the *Ecotoxicology* review process.

Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (*Bombus terrestris*)

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Keywords

Bombus terrestris microcolonies; demographic toxicology, fecundity; neonicotinoid pesticides; ovary development; sublethal effects

Abstract

Bumble bees are important pollinators whose populations have declined over recent years, raising widespread concern. One conspicuous threat to bumble bees is their unintended exposure to trace residues of systemic neonicotinoid pesticides, such as imidacloprid, which are ingested when bees forage on the nectar and pollen of treated crops. However, the demographic consequences for bumble bees of exposure to dietary neonicotinoids have yet to be fully established. To determine whether environmentally realistic levels of imidacloprid are capable of making a demographic impact on bumble bees, we exposed queenless microcolonies of worker bumble bees, *Bombus terrestris*, to a range of dosages of dietary imidacloprid between zero and 125 µg L⁻¹ (= 0-98 ppb) and examined the effects on ovary development and fecundity. Microcolonies showed a dose-dependent decline in fecundity, with environmentally realistic dosages in the range of 1 µg L⁻¹ capable of reducing brood production by one third. In contrast, ovary development was unimpaired by dietary imidacloprid except at the highest dosage. Imidacloprid reduced feeding on both syrup and pollen but, after controlling statistically for dosage, microcolonies that consumed more syrup and pollen produced more brood. We therefore speculate that the detrimental effects of imidacloprid on fecundity emerge principally from nutrient limitation imposed by the failure of individuals to feed. Our findings raise concern about the impact of neonicotinoids on wild bumble bee populations. However, we recognize that to fully evaluate impacts on wild colonies it will be necessary to establish the effect of dietary neonicotinoids on the fecundity of bumble bee queens.

2.1 Introduction

Animal pollinators play an important role in global food production (Klein et al. 2007) and in maintaining wild plant communities (Kearns et al. 1998; Ashman et al. 2009). Wild and managed bees are important pollinators whose populations have declined over recent years (Goulson et al. 2008; vanEngelsdorp et al. 2008; De la Rúa et al. 2009) raising widespread concern (Allen-Wardell et al. 1998; Potts et al. 2010a). The detrimental factors affecting bee populations are likely to be multiple and interacting (Williams and Osborne 2009), but one conspicuous threat is their unintended exposure to agricultural pesticides that protect crops from pest herbivores (Desneux et al. 2007). Neonicotinoids, such as imidacloprid, are among the most effective and widely used pesticides employed to control common insect pests such as aphids and whiteflies (Elbert et al. 2008). They are synthetic neurotoxins that act as agonists of nicotinic acetylcholine receptors to disrupt the nervous system of pests to lethal effect (Matsuda et al. 2001). Applied as a seed dressing or foliar spray, neonicotinoids are taken up by plants and distributed systemically (Sur and Stork 2003) to target pest herbivores that consume sap and plant tissues. Bees are non-target organisms that ingest dietary residues of neonicotinoids in the nectar and pollen of treated mass-flowering crops (Rortais et al. 2005). Bees are exposed to dietary trace residues, defined here as the range up to 10 µg insecticide kg⁻¹ (= parts per billion or ppb), when foraging on several crops (reviewed in Blacquièvre et al. 2012). For example, imidacloprid residues ranging from 1.1 ppb to 5.7 ppb were detected in pollen collected from French honey bee colonies (Chauzat et al. 2006). In treated sunflowers, *Helianthus annuus* L., imidacloprid residues in pollen averaged 3 ppb in field crops and reached 1.9 ppb in nectar and 3.3 ppb

in pollen of greenhouse plants (Schmuck et al. 2001; Bonmatin et al. 2003). Pollen from imidacloprid-treated maize, *Zea mays* L., and oilseed rape, *Brassica napus* L., contained residues of 2.1 ppb (Bonmatin et al. 2005) and 1.0 ppb (Cresswell, pers. obs.) respectively, whilst the nectar of *Phacelia tanacetifolia* Benth. contained imidacloprid residues up to 10 ppb when sampled from the honey sac of foraging bees (Decourtye et al. 2003). Clothianidin, another major neonicotinoid, was detected in pollen from treated maize and in wild flowers growing near to treated fields at levels of 3.9 ppb and 9.4 ppb, respectively; however, residues reached 88 ppb (nine times our defined field-realistic range) in pollen collected by honeybees foraging on treated crops (Krupke et al. 2012).

Quantifying population-level responses to a xenobiotic provides an important basis for assessing its potential for ecological impact (Walthall and Stark 1997; Forbes and Calow 1999; Herbert et al. 2004). In protecting the sustainability of a non-target species, we are particularly interested in establishing whether a realistic level of exposure to a pesticide is capable of causing the population to decline. In the past, certain pesticides have proven capable of causing population declines in non-target species and have been implicated as culprits by their evident detrimental effects on demographically relevant variables. For example, the insecticide dichlorodiphenyltrichloroethane (DDT) caused population decline in predatory bird species through reduced fecundity (Grier 1982). By analogy with such cases, some have asserted that neonicotinoids are a cause of bee declines (Hansard 2011), but in actuality the demographic consequences for bees of exposure to trace dietary neonicotinoids have yet to

be fully established. In laboratory and semi-field trials on honey bees (*Apis mellifera* L.), trace dietary imidacloprid reduced performance on a variety of measures by between 6 and 20% (Cresswell 2011), but uncertainty remains over the population-level implications of these effects. In field trials on honey bees, exposure to dietary neonicotinoids impacted on forager survival and colony dynamics in one recent study (Henry et al. 2012a), whereas colony persistence was unaffected by neonicotinoids in other studies (Faucon et al. 2005; Cutler and Scott-Dupree 2007; but note, Cresswell (2011) showed these trials only had sufficient statistical power to detect severely detrimental impacts). We therefore further investigated the potential for neonicotinoids to make a demographic impact and, specifically, we examined the effects of trace dietary intake on reproduction in bees.

We focus on bumble bees, *Bombus spp.*, which are important pollinators of both agricultural crops (Goulson 2003b) and wild plants (Goulson et al. 2008). While declines among managed honey bee populations in some regions have received widespread recognition (vanEngelsdorp et al. 2008; De la Rúa et al. 2009), evidence of population decline among bumble bees has also accumulated (Cameron et al. 2011). In the UK, for example, more than half of extant bumble bee species are rare or in decline (Williams and Osborne 2009). However, it is unclear whether dietary neonicotinoids could be implicated in bumble bee declines because the results of previous investigations are inconsistent. Following laboratory exposure to dietary imidacloprid at 6 or 12 ppb, colonies of the buff-tailed bumble bee, *Bombus terrestris* L., suffered reduced colony growth and queen production whilst developing under field

conditions (Whitehorn et al. 2012). Where laboratory assays are similar, dietary imidacloprid in the range between 6 and 25 ppb affected the survivorship of *B. terrestris* in one study (Tasei et al. 2000), but not in another (Mommaerts et al. 2010b). In colonies of the western bumble bee, *Bombus occidentalis* G., reproduction was unaffected by dietary imidacloprid (Morandin and Winston 2003), but in contrast imidacloprid reduced reproductive output in *B. terrestris* (Tasei et al. 2000; Mommaerts et al. 2010b). Furthermore, these detrimental effects have been demonstrated principally at dosages above the range that bumble bees encounter in the nectar and pollen of imidacloprid treated crops, so it remains uncertain whether environmentally realistic exposures are capable of making a demographic impact on wild bumble bee populations. We therefore investigated the effect of dietary imidacloprid on brood production in bumble bees, and we tested a range of dosages that included the environmentally realistic range.

In order to investigate the influence of dietary neonicotinoids on brood production, we made use of the capacity of worker bumble bees to produce unfertilized eggs that mature into males (Amsalem et al. 2009). In this eusocial species, bumble bee queens normally dominate the reproductive output of the colony and the workers make only a small contribution (Alaux et al. 2004; Lopez-Vaamonde et al. 2004). However, bumble bee workers adaptively upregulate their reproduction in colonies rendered queenless (Alaux et al. 2007). ‘Microcolonies’ are nests comprising a small group of worker bees that are allowed to develop, in the absence of a queen, until a worker becomes dominant and begins laying eggs while the others forage and care for brood

(Blacquière et al. 2012). This method enables the convenient evaluation of both lethal and sub-lethal effects of dietary neonicotinoids using multiple replicates (Blacquière et al. 2012). In this study, we used queenless microcolonies to evaluate the effects of imidacloprid on ovary development and fecundity in *B. terrestris*.

2.2 Methods

2.2.1 Microcolonies

We obtained three domesticated queenright colonies of *B. terrestris*, each consisting of a single queen, approximately 150 workers, and brood at various stages of development (Natupol Beehive; Koppert B.V., Berkel en Rodenrijs, Netherlands). Groups of *B. terrestris* workers rendered queenless develop their ovaries and begin to oviposit after approximately seven days (Alaux et al. 2007; Amsalem et al. 2009) and we made use of this reproductive plasticity in the laboratory by grouping together workers into queenless microcolonies. We placed 328 individual workers into microcolony boxes in groups of four (second and third trials) or five (first trial). The allocation of workers to microcolonies was randomised, but each microcolony contained workers from the same original queenright colony. Microcolonies were housed in a softwood box (internal dimensions: 120 × 120 × 45 mm) with a plywood base and a transparent acrylic cover with ventilation holes. A central wooden partition separated each box into two equal sized compartments, but workers had access to either compartment through a centrally drilled hole. Additional holes in the side of the box accommodated 2 mL microcentrifuge tubes (Simport, Beloeil, Canada) that

were punctured so as to function as sugar syrup feeders. We maintained microcolonies for 14 days in a controlled environment (24-27 °C, 23-43 % relative humidity, 10:14 h light:dark period). Before exposure to imidacloprid, workers were given 24 h to forage *ad libitum* on control sugar syrup (Attracker: fructose/glucose/saccharose solution, 1.27 kg L⁻¹; Koppert B.V., Berkel en Rodenrijs, Netherlands). During this period we removed a small number of dead bees and replaced them with workers from the same original queenright colony.

Imidacloprid was obtained as a solution in acetonitrile (Dr. Ehrenstorfer GmbH, Augsburg, Germany, product code L 14283700AL). Acetonitrile was removed by evaporation in a vacuum concentrator (ScanSpeed MaxiVac Beta; LaboGene ApS, Lynge, Denmark) and the imidacloprid was resuspended in deionised water before being mixed into syrup. After feeding on control syrup for 24 h, each microcolony was provided with a pollen ball, which was not dosed with imidacloprid, and feeders containing either control syrup or a syrup with one of the following dosages of imidacloprid (units are µg imidacloprid L⁻¹): 125.00, 50.00, 20.00, 8.00, 3.20, 1.28, 0.51, 0.20, 0.08. The level of replication was such that we had a total of 6, 3, 5, 5, 7, 17, 7, 5, 6, and 15 microcolonies treated with dosages of 125.00, 50.00, 20.00, 8.00, 3.20, 1.28, 0.51, 0.20, 0.08, and 0 µg L⁻¹ imidacloprid, respectively. Once dosing began, we monitored microcolonies daily for mortality and brood production and dead bees were no longer replaced. Feeders were weighed each day to measure the consumption of syrup and fresh syrup at the appropriate dosage was provided as required. Pollen balls were prepared by grinding pollen pellets collected from honey bee hives (Werner Seip Bioprodukte, Butzbach, Germany) into a powder and mixing

the mass with water to form dough. The pollen balls (mean mass = 5.4 g, SE = 0.03 g) provided workers with a protein source and a substrate for nest building, and they were weighed before and after the experiment to assess pollen consumption. In our analysis, we corrected for evaporation of water from syrup and pollen based on the mass change of several feeders and pollen balls kept in empty microcolony boxes under identical experimental conditions. Three trials each comprising 14 days (one day of acclimatisation and 13 days of imidacloprid exposure) were conducted between November 2010 and March 2011. Across the entire study, the number of microcolonies originating from a single queenright parent colony was distributed approximately evenly within dosage treatments and across trials.

To verify the concentration of imidacloprid in our doses, we prepared the usual range of experimental dosages, but in water rather than syrup to facilitate analysis. Samples were analysed in an Agilent 1200 series liquid chromatograph interfaced *via* an electrospray ionisation source to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) and using methods adapted from Takino and Tanaka (2006). Specifically, samples and standards (10 µl) were injected onto an Eclipse Plus (Agilent Technologies, Santa Clara, CA, USA) C₁₈ reverse phase column (150mm x 2.1mm, 3.5µm). Mobile phase A was 2% acetonitrile + 0.1% formic acid. Mobile phase B was 95% acetonitrile + 0.1% formic acid. The elution conditions were: 0 min – 0% B, 1 min – 70% B, 10 min – 80% B, 10.2 min – 100% B, 12 min – 100% B; with a flow rate of 0.3 ml min⁻¹ increasing to 0.45 ml min⁻¹ at 10 min. The source N₂ gas temperature was held at 350°C with a flow

of 11 L min⁻¹ and a nebulizer pressure of 35 psi. The capillary voltage was 4kV. Fragmentor and collision energy voltages were 40V and 20V respectively. Imidacloprid was identified and quantified by selected reaction monitoring (SRM) using the product ion *m/z* 209 derived from the precursor ion of *m/z* 256. Samples of each dosage were spiked with a reference standard of 100 µg L⁻¹ [²H]imidacloprid (Sigma-Aldrich, Gillingham, UK). The deuterated imidacloprid was detected using a precursor ion *m/z* of 260 and a product ion *m/z* of 213. Imidacloprid concentrations in the dilution series were quantified by comparing peak areas from [²H]imidacloprid to peak areas of non-labelled imidacloprid in SRM chromatograms. The instrument response was linear over the range 0.061 to 125 µg L⁻¹ imidacloprid and we found that all dosages contained appropriate levels of imidacloprid (*measured imidacloprid* = 1.13 × *nominal dosage* + 0.84: $r^2 > 0.99$).

2.2.2 Ovary development and fecundity

After 14 days, we killed the worker bees and removed their laid eggs and larvae. The bees were dissected in phosphate-buffered saline to remove their ovaries. Each worker ovary contained four ovarioles and in each ovariole there were several oocytes. Using image analysis software (ImageJ; <http://rsbweb.nih.gov/ij/>), we measured the length, width and area of each intact terminal oocyte from each dissected ovary (mean number of oocytes measured per ovary = 3.8, SE = 0.04) and each laid egg. The mean size of all intact terminal oocytes per ovary and the size of the largest terminal oocyte per bee were taken as measures of ovary development. We removed a forewing from each worker and recorded the length of the radial cell as a proxy for body size

(Medler 1962). In the first trial, we also dissected and measured the oocytes of 10 workers taken directly from their original queenright colony in order to calculate the change in ovary development of workers during their time in the microcolony. These 10 workers were removed from the colony at the same time as all of the other workers subsequently placed into microcolonies, but were freeze-killed immediately. All microcolony dissections, ovary dissections, and oocyte measurements were performed by operators who were unaware of the imidacloprid dosage that corresponded to the specimens.

2.2.3 Statistical analyses

To test whether the fecundity of *B. terrestris* microcolonies responded to dietary imidacloprid, we fitted a relationship between brood production and dosage with a Poisson error structure and a ‘random effects’ term to account for overdispersion. Specifically, we fitted a Bayesian Hierarchical Model (BHM) as follows: $\text{brood} \sim \text{Poisson}(\mu)$; and $\log(\mu) \sim \alpha + \beta \times \log(\text{dosage} + 1) + \lambda$. Here, α and β are fitted coefficients, which are analogous to the conventional regression coefficients of slope and intercept; and the ‘random effects’ term, λ , has a normal distribution with a mean of zero. We fitted the BHM using WinBugs (Lunn et al. 2000) and obtained 95 % confidence intervals with 20,000 iterations of Bayesian inference using a Markov Chain Monte Carlo method with Gibbs sampling.

To estimate the number of workers in each microcolony with mature oocytes inside their ovaries, we compared the longest terminal oocyte inside each bee’s ovaries to the mean length of eggs laid in microcolonies fed control syrup

(mean length = 3.0 mm, SE = 0.02 mm, N = 146). A worker was deemed to have mature oocytes if the length of the longest terminal oocyte in its ovaries was at least 3 mm.

Among microcolonies, we analyzed variation in mean oocyte size and number of workers with mature oocytes due to imidacloprid dosage using one-way Analysis of Variance (ANOVA) with dosage treated as a categorical variable because the dose-response relationship was non-linear. Where ANOVA detected a significant response to dosage, we compared between selected dosage groups with orthogonal contrasts.

In order to test for dosage-independent effects on fecundity and ovary development of variation among microcolonies in feeding rate and body size, we used partial correlation analysis to control for imidacloprid dosage. All statistical analyses were conducted in R version 2.10 (R Core Team 2013).

2.3 Results

In our experiment, microcolonies began to lay eggs after approximately seven days. After 14 days, microcolonies had laid up to 39 eggs and some offspring had progressed to a larval stage. In our analyses, we take the fecundity of a microcolony during the experimental period to be the total number of the brood it produced comprising both laid eggs and larvae. Levels of fecundity and the effects of dosage were highly similar in the three experimental trials and the data were pooled for analysis. During the 13 days of imidacloprid exposure,

total mortality comprised one dead worker in a single microcolony exposed to imidacloprid at $125 \mu\text{g L}^{-1}$.

2.3.1 Dosage effects

Worker fecundity declined significantly with increasing dosage of dietary imidacloprid (Spearman's $\rho = -0.61$, $N = 76$, $P < 0.001$; Fig. 2.1). Using BHM, the best description of the dose-response relationship was $\text{brood} = \exp[2.49 - 1.84 \times \log(\text{dosage} + 1)]$ and the standard deviation of the overdispersion parameter was estimated as $\text{SD}(\lambda) = 1.39$. Based on this relationship, exposure to imidacloprid at an environmentally realistic level of $1 \mu\text{g L}^{-1}$ ($= 0.8 \text{ ppb}$) results in a 42 % reduction in worker fecundity (95 % CI: 33 %, 51 %; Fig. 2.1). There was no effect of dosage on the number of days elapsing before the first oviposition was observed in a microcolony (mean elapsed days = 7.5, SE = 0.5; Spearman's correlation: *elapsed days vs. dosage*, $\rho = -0.04$, $N = 57$, $P = 0.75$).

In the microcolonies, *per capita* daily rates of feeding declined with increasing dosage of imidacloprid for both syrup and pollen (Spearman's correlation: *syrup feeding rate vs. dosage*, $\rho = -0.63$, $N = 76$, $P < 0.001$; *pollen feeding rate vs. dosage*, $\rho = -0.63$, $N = 76$, $P < 0.001$; Fig. 2.2). Despite consuming less syrup, bees exposed to higher dosages nevertheless ingested larger amounts of imidacloprid (Fig. 2.2).

Compared to bees collected from the queenright colony at the beginning of the experiment, individuals examined after the 14-day experimental period had

ovaries with larger oocytes at all but the highest dosage of imidacloprid (Fig. 2.3). All measures of oocyte size in microcolonies (mean length, width and area) and the number of workers with mature oocytes per microcolony were affected significantly by imidacloprid dosage (One-way ANOVA: *oocyte size*, $F_{9, 42} \leq 7.7$, $P < 0.001$; *number of workers with mature oocytes*, $F_{9, 42} = 3.7$, $P < 0.01$). By any measure, oocytes were smaller in bees from microcolonies exposed to imidacloprid at $125 \mu\text{g L}^{-1}$ (98 ppb) when compared to all other dosages (Orthogonal contrast: $t \leq -6.6$, $P < 0.001$; Fig. 2.3). However, no dose-dependent variation in oocyte size was evident among bees exposed to dosages below $125 \mu\text{g L}^{-1}$ on any measure (One-way ANOVA: $F_{8, 37} \leq 1.5$, $P \geq 0.20$; Fig. 2.3). There were fewer workers with mature oocytes in microcolonies at $125 \mu\text{g L}^{-1}$ compared to all other dosages (Orthogonal contrast: $t = -4.2$, $P < 0.001$), but no significant difference was detectable among the numbers of workers with mature oocytes in microcolonies exposed to imidacloprid at dosages below $125 \mu\text{g L}^{-1}$ (mean number of workers with mature oocytes per microcolony = 1.39, SE = 0.11; One-way ANOVA: $F_{8, 37} = 1.12$, $P = 0.38$).

2.3.2 Dosage-independent effects

After controlling statistically for the effects of imidacloprid and mean body size in a microcolony, fecundity increased significantly in microcolonies with higher *per capita* daily rates of feeding for both syrup and pollen (Pearson's partial correlation: *fecundity vs. syrup feeding rate*, $r = 0.36$, $df = 50$, $P < 0.01$; *fecundity vs. pollen feeding rate*, $r = 0.40$, $df = 50$, $P < 0.01$). There was no effect of mean body size on fecundity, independent of imidacloprid dosage and daily feeding rates (Pearson's partial correlation: $r = -0.10$, $df = 50$, $P = 0.50$).

Daily rates of feeding did not significantly affect the mean size of terminal oocytes in a microcolony (Pearson's partial correlation: *mean oocyte area vs. syrup feeding rate*, $r = 0.24$, $df = 50$, $P = 0.08$; *mean oocyte area vs. pollen feeding rate*, $r = 0.26$, $df = 50$, $P = 0.06$), nor was there a correlation between mean terminal oocyte size and body size of individual bees (Spearman's correlation: $\rho = 0.01$, $N = 231$, $P = 0.89$).

2.4 Discussion

The key result emerging from our work is that ingestion of imidacloprid at environmentally realistic levels substantively reduced the fecundity of worker bumble bees. This finding is consistent with those of previous studies, which have shown that exposure of *B. terrestris* workers to dietary imidacloprid at 10 ppb in feeder syrup reduced larval production by 43% (Tasei et al. 2000) and drone production by between 41-62 % (Tasei et al. 2000; Mommaerts et al. 2010b). However, wild bees are probably exposed to imidacloprid residues lower than 10 ppb when they consume the nectar and pollen of treated crops (Bonmatin et al. 2003; Bonmatin et al. 2005; Chauzat et al. 2006). We have now demonstrated that dietary trace residues of imidacloprid in the range of one ppb can reduce worker fecundity by at least one third.

Our methodology is likely to have produced realistic exposures to dietary imidacloprid. The amount of imidacloprid ingested by nectar and pollen foraging honey bees is estimated to be between 49 pg and 610 pg per day (Rortais et al. 2005). In our experiments, *B. terrestris* workers ingested on average 376 pg of

imidacloprid per day when feeding on syrup dosed with imidacloprid at 1 ppb ($1.28 \mu\text{g L}^{-1}$), which is in the lower range of field-realistic concentrations. In actuality, individual bumble bees probably consume more nectar in a day than honey bees (Thompson and Hunt 1999); therefore, our observations may be reasonably used as a minimum estimate of the effects on the fecundity of worker bumble bees that feed exclusively on real nectars containing imidacloprid residues.

Our findings raise further concern about the impact of systemic neonicotinoids on wild bumble bee populations. A recent review summarising 15 years of research on the hazards of neonicotinoids to bees highlighted the sub-lethal effects of exposure in the laboratory to neonicotinoids ≥ 6 ppb on reproduction and behaviour in bumble bees (Blacqui  re et al. 2012). We have now shown that dietary neonicotinoids in the range <6 ppb can cause substantive sub-lethal effects on bumble bee reproduction. However, we recognize that to fully evaluate impacts on wild colonies it will also be necessary to establish whether the fecundity of bumble bee queens is as sensitive to a dietary neonicotinoid as that of workers. Whitehorn et al. (2012) demonstrated that exposure of young *B. terrestris* colonies to dietary imidacloprid at 6 ppb for 14 days reduced colony growth after 8 weeks by 8 % and queen production by 85 %. The underlying mechanism was not studied, but we speculate that reduced fecundity in queens during imidacloprid exposure could account for these observations. Additionally, it will be necessary to evaluate the capacity of bumble bees to recover from the short-term pulsed exposure to dietary neonicotinoids that is likely to occur during the synchronous bloom of a mass-flowering neonicotinoid-treated crop.

Consider, for example, the interaction between bumble bees and neonicotinoid-treated oilseed rape, which probably provides the most widespread exposure of bees to dietary neonicotinoids in Europe. In the UK, a field of winter-sown oilseed rape blooms for around 28 days with approximately 75 % of the flowering occurring over a peak period of about 18 days in April and May (Hoyle et al. 2007). A bumble bee colony is initiated in spring; it develops over several months and typically delays the production of new queens and males until its latter stages (Goulson 2003a), which are therefore likely to emerge after oilseed rape has flowered. Other insects, such as aphids, whitefly and midges, are able to recover once a neonicotinoid disappears from their diet (Nauen 1995; Azevedo-Pereira et al. 2011; He et al. 2011). If the fecundity of a bumble bee colony recovers as the levels of dietary neonicotinoid diminish, the impact on reproduction and colony growth may be less severe than otherwise, but this speculation awaits further research.

In our study, the strongly detrimental effects of imidacloprid on fecundity at dosages of 39 ppb ($50 \mu\text{g L}^{-1}$) or lower were not due to impaired ovary development. Similarly, ovary development in the Eastern bumble bee, *B. impatiens* C., was sensitive only to very high dietary concentrations of the alkaloid gelsemine, which occurs naturally in the nectar of *Gelsemium sempervirens* L. (Carolina jessamine) (Manson and Thomson 2009). We therefore speculate that ovary development in bumble bees may be somewhat resilient to dietary toxins in general.

Except at relatively high dosages (i.e. above 98 ppb), the detrimental effect of imidacloprid on worker fecundity also cannot be explained by delayed brood production. When brood was produced, we observed egg cells in microcolonies after approximately one week, regardless of imidacloprid dosage, and this timescale is entirely typical of *B. terrestris* workers in queenless colonies (Alaux et al. 2007; Amsalem et al. 2009).

Indeed, the precise toxicological mechanisms that caused the detrimental effects of imidacloprid on bumble bee fecundity at dosages below 98 ppb are not revealed by our study. An individual bee's physiological function is tightly integrated with its nervous system and therefore the effects of a dietary neurotoxin are probably manifold. However, we observed that dietary imidacloprid reduced feeding on both syrup and pollen and that microcolonies that consumed more syrup and pollen produced more brood. Carbohydrates (Murphy et al. 1983; Boggs 1997; O'Brien et al. 2000) and protein (Webster et al. 1979; Wheeler 1996) are essential components for brood production in insects and we therefore speculate that reduced feeding imposed nutrient limitation on reproduction.

Reduced feeding on dosed syrup could be an indication that dietary imidacloprid is an aversive stimulus to workers or that imidacloprid reduced the bees' ability or need to feed. However, the initial reduction in feeding rate due to imidacloprid intensifies over successive days (Cresswell et al. 2012b), which suggests that it has a basis in toxicity rather than aversion. In our experiment, feeding on dosed syrup was accompanied by reduced feeding on untreated

pollen. This phenomenon may be an adaptive response by workers that are attempting to maintain a constant protein to carbohydrate (P:C) ratio, because honey bee workers rendered queenless and fed a choice of diets are known to maintain strict P:C ratios (Altaye et al. 2010). In summary, we have shown dietary imidacloprid at levels up to approximately 39 ppb fails to prevent bumble bee workers from developing their reproductive organs and we hypothesize that its detrimental effects on fecundity emerge in whole or in part from nutrient limitation imposed by the failure of individuals to feed.

We found that bumble bee workers feeding on syrup at the highest dosage, 98 ppb, neither developed their ovaries fully nor laid eggs, and that microcolonies feeding on syrups at dosages of 16 ppb ($20 \mu\text{g L}^{-1}$) or less both developed ovaries to the same degree as those feeding on undosed syrup and were capable of laying eggs. However, the workers in microcolonies exposed to an intermediate dosage of imidacloprid, 39 ppb, developed their ovaries, but did not lay eggs. This situation is similar to that observed among isolated *B. terrestris* workers (Amsalem et al. 2009), who require a social stimulus to initiate brood production. We therefore speculate that imidacloprid at 39 ppb may have disrupted social interactions and thereby repressed oviposition in these workers; however, we acknowledge the possibility that imidacloprid may be involved in repression of egg laying at the individual level via a non-social mechanism. While dietary neonicotinoids are able to affect behavioural performance in honey bees (Lambin et al. 2001; Decourtye et al. 2003, 2004b), further investigation is necessary to establish whether dietary neonicotinoids are capable of disrupting behavioural aspects of sociality in bees.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical standards

The work reported here conforms to the regulatory requirements for animal experimentation in the UK and has been approved by the Biosciences Ethics Committee at the University of Exeter.

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Chapter Two: Figures

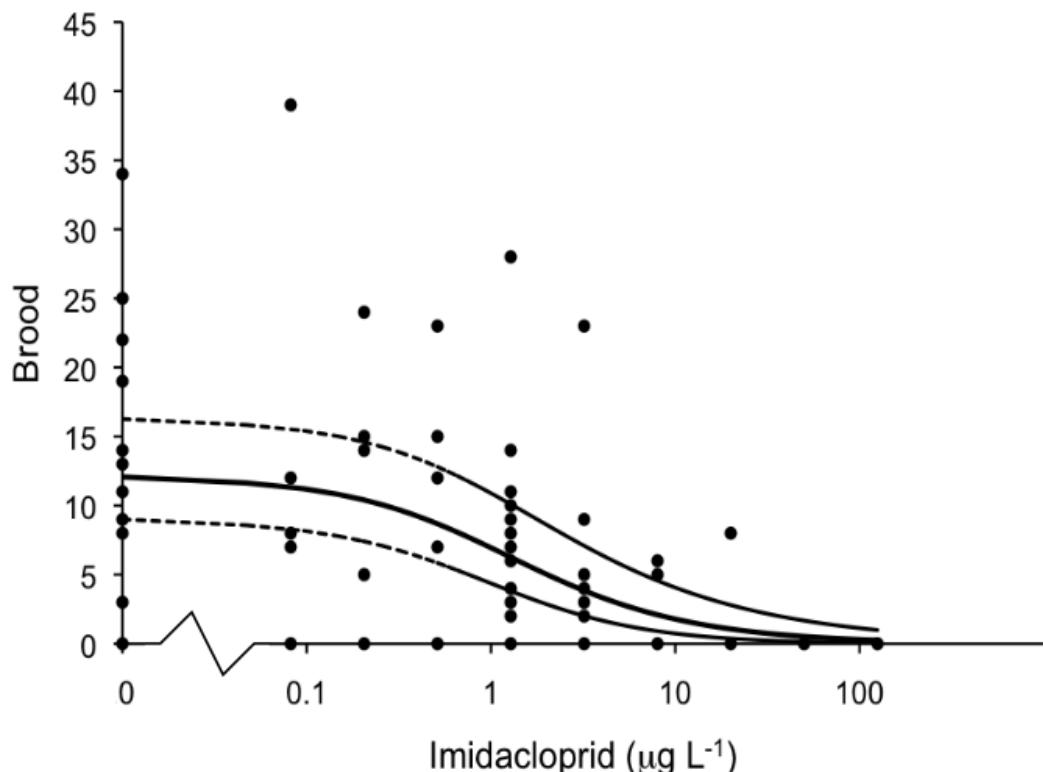


Fig. 2.1 Fecundity (y-axis: number of brood individuals per microcolony) of *B. terrestris* worker microcolonies ($N = 76$) after 14 days, including 13 days of exposure to dietary imidacloprid in dosed syrups (x-axis: concentration of imidacloprid in syrup in $\mu\text{g L}^{-1}$). The solid line indicates the best-fit dose-response relationship and the dashed lines indicate the relationship's 95 % confidence intervals

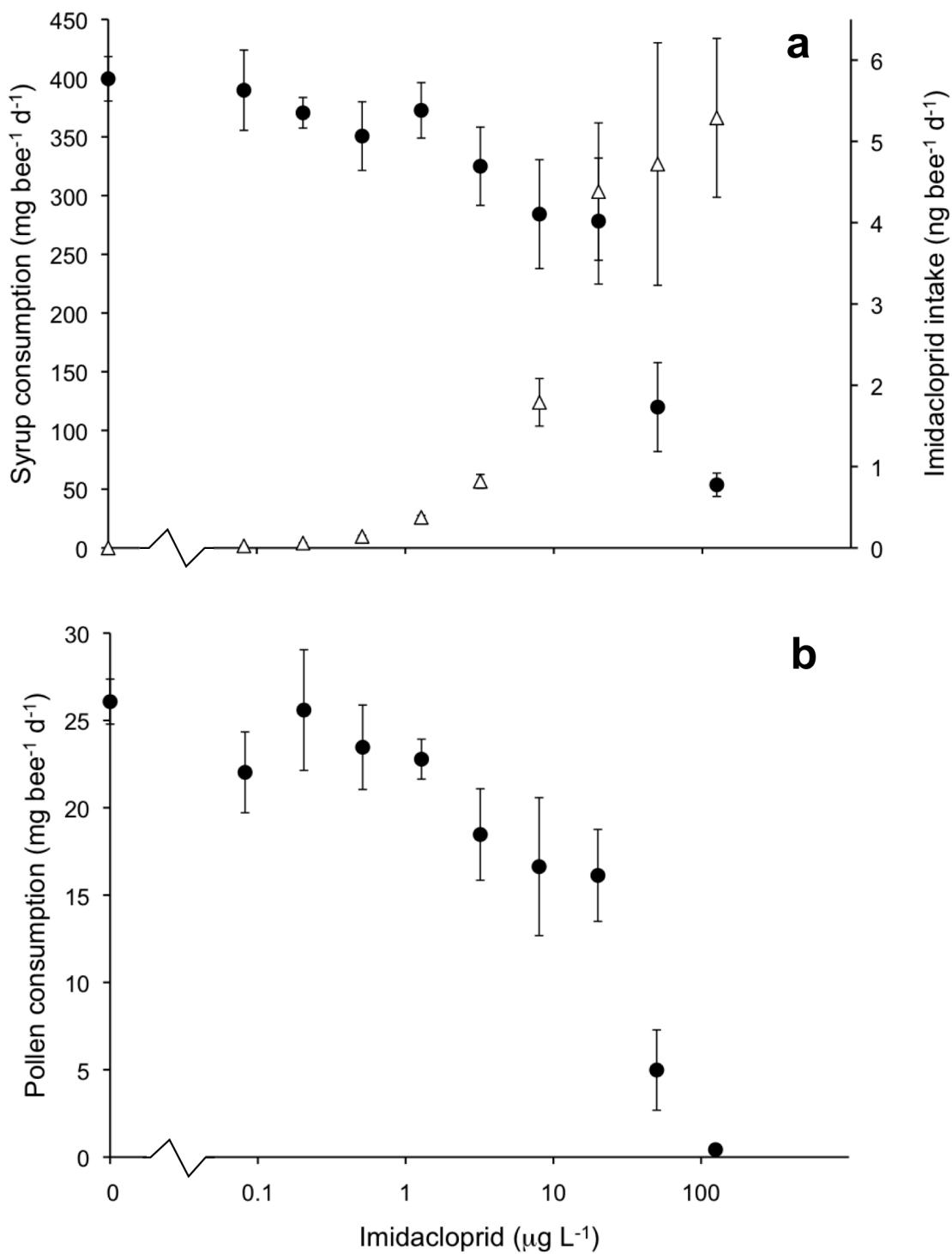


Fig. 2.2 Upper panel (a) Daily syrup feeding rate (left *y*-axis: mean *per capita* feeding rate of microcolony in mg, denoted by circular symbol) and daily imidacloprid intake (right *y*-axis: mean *per capita* imidacloprid intake in microcolony in ng, triangle) in *B. terrestris* worker microcolonies ($N = 76$) fed for 13 days on imidacloprid-treated syrup (x-axis: concentration of imidacloprid in syrup in $\mu\text{g L}^{-1}$) and untreated pollen. Lower panel (b) Daily pollen feeding rate (*y*-axis: mean *per capita* feeding rate in microcolony in mg) in *B. terrestris* worker microcolonies ($N = 76$) fed for 13 days on imidacloprid-treated syrup and untreated pollen. Error bars indicate 1 SE

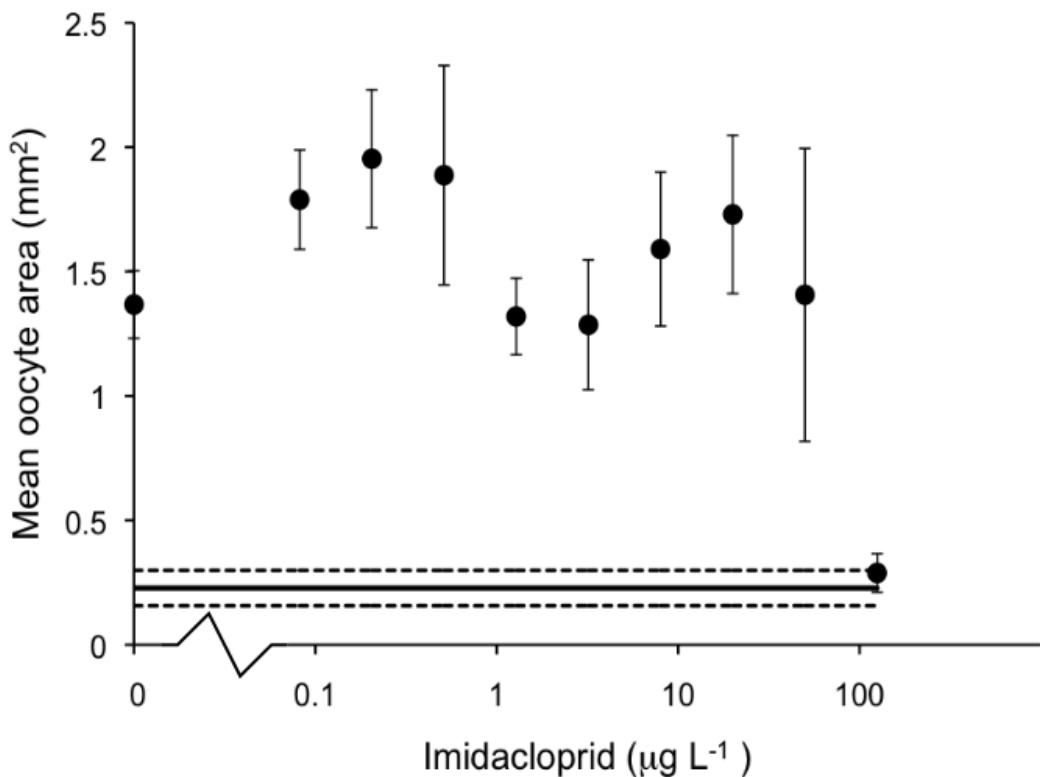


Fig. 2.3 Terminal oocyte area (y-axis: mean area of terminal oocytes in individuals from a microcolony) of *B. terrestris* workers ($N = 231$) in microcolonies ($N = 52$) after 14 days, including 13 days of exposure to dietary imidacloprid in dosed syrup (x-axis: concentration of imidacloprid in syrup in $\mu\text{g L}^{-1}$). Error bars indicate 1 SE. The solid horizontal line indicates the mean terminal oocyte area of workers ($N = 10$) in the queenright colony before the microcolony experiment was conducted and the associated dashed lines indicate 1 SE

Chapter Three

Effects of the neonicotinoid pesticide
thiamethoxam at field-realistic levels on
microcolonies of *Bombus terrestris* worker
bumble bees

Preliminary introduction

In the following chapter I present work based on a paper submitted to the journal *Ecotoxicology and Environmental Safety* in June 2013 for which I was first author. The paper was accepted for publication in October 2013 and first published online in November 2013 (available in its original form at <http://dx.doi.org/10.1016/j.ecoenv.2013.10.027>). The content below is presented in the format dictated by *Ecotoxicology and Environmental Safety* and is identical to that published in the journal, with the exception of a small number of qualitative amendments made to improve the clarity of the work. Additionally, ambiguous references are lettered (a, b, etc) and the sections, figures and tables in the paper are numbered according to their position within the thesis. Where Laycock et al. (2012) is cited, I am referring to chapter two of this thesis.

NOTICE: this is the author's version of a work that was accepted for publication in *Ecotoxicology and Environmental Safety*. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in *Ecotoxicology and Environmental Safety*, [Vol 100, 153-158, (February 2014)] <http://dx.doi.org/10.1016/j.ecoenv.2013.10.027>.

Statement of contribution

As first author, I was primarily responsible for conception and design of the work, acquisition and analysis of the data, and writing the paper. My supervisor and co-author Dr James Cresswell advised on data analysis and provided revision notes during the writing process. My other co-authors, Katie Cotterell and Thomas O'Shea-Wheller, were involved in collection of data from microcolonies that contributed towards this paper and their own undergraduate research projects. Additionally, Dr Hannah Florance ran thiamethoxam samples through the LC-MS. Prof. Charles Tyler and three anonymous reviewers also provided comments on the manuscript during the *Ecotoxicology and Environmental Safety* review process.

Effects of the neonicotinoid pesticide thiamethoxam at field-realistic levels on microcolonies of *Bombus terrestris* worker bumble bees

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Keywords

bee health; *Bombus*; field-realistic; imidacloprid; neonicotinoid; thiamethoxam

Abstract

Neonicotinoid pesticides are currently implicated in the decline of wild bee populations. Bumble bees, *Bombus* spp., are important wild pollinators that are detrimentally affected by ingestion of neonicotinoid residues. To date, imidacloprid has been the major focus of study into the effects of neonicotinoids on bumble bee health, but wild populations are increasingly exposed to alternative neonicotinoids such as thiamethoxam. To investigate whether environmentally realistic levels of thiamethoxam affect bumble bee performance over a realistic exposure period, we exposed queenless microcolonies of *Bombus terrestris* L. workers to a wide range of dosages up to 98 µg kg⁻¹ in dietary syrup for 17 days. Results showed that bumble bee workers survived fewer days when presented with syrup dosed at 98 µg thiamethoxam kg⁻¹, while production of brood (eggs and larvae) and consumption of syrup and pollen in microcolonies were significantly reduced by thiamethoxam only at the two highest concentrations (39, 98 µg kg⁻¹). In contrast, we found no detectable effect of thiamethoxam at levels typically found in the nectars of treated crops (between 1 and 11 µg kg⁻¹). By comparison with published data, we demonstrate that during an exposure to field-realistic concentrations lasting approximately two weeks, brood production in worker bumble bees is more sensitive to imidacloprid than thiamethoxam. We speculate that differential sensitivity arises because imidacloprid produces a stronger repression of feeding in bumble bees than thiamethoxam, which imposes a greater nutrient limitation on production of brood.

3.1 Introduction

The pollination services of wild bees help to maintain plant species in natural ecosystems and are worth billions of dollars annually to agriculture (Williams and Osborne, 2009; Winfree, 2010). Evidence of declining wild bee populations (Biesmeijer et al., 2006) and the extirpation of certain species (Burkle et al., 2013) are therefore issues of increasing concern (Vanbergen and IPI, 2013). It is widely acknowledged that several factors are driving declines in wild bees (Williams and Osborne, 2009; Potts et al., 2010a). However, a group of neurotoxic pesticides, the neonicotinoids, have specifically been singled out for blame (Shardlow, 2012), which has led to calls for restrictions on their use in agricultural (EFSA, 2013e; Maxim and van der Sluijs, 2013) that have recently been implemented across the European Union (European Commission, 2013). The neonicotinoids, which include imidacloprid, thiamethoxam and clothianidin, are systemic and so the pesticide is distributed throughout plant tissues to control sucking insect pests (Elbert et al., 2008). Consequently, trace residues can appear in nectar and pollen (Blacquière et al., 2012) and bees are exposed to dietary neonicotinoids by foraging from the flowers of treated agricultural crops (Elbert et al., 2008).

Bumble bees are important wild pollinators that are detrimentally affected by neonicotinoids in laboratory studies, where dietary residues reduce food consumption and brood production of *Bombus terrestris* L. workers (Tasei et al., 2000; Mommaerts et al., 2010b; Cresswell et al., 2012b; Laycock et al., 2012), and in semi-field studies, where *B. terrestris* colonies under exposure exhibit reduced production of brood, workers and queens (Gill et al., 2012; Whitehorn

et al., 2012). The majority of these studies focus solely on imidacloprid, which has historical relevance because it was the first neonicotinoid in widespread use (Elbert et al., 2008) and was identified publicly as a potential threat to bee health in 1999 (Maxim and van der Sluijs, 2013). However, newer neonicotinoid varieties, such as thiamethoxam and its toxic metabolite clothianidin, are increasingly preferred to imidacloprid in crop protection. For example, in 2011 imidacloprid made up just 10 % of the total 80,000 kg of neonicotinoid applied to UK crops (FERA, 2014). Consequently wild bumble bees are at increased risk of exposure to these alternative neonicotinoids. We therefore chose to further investigate the effects of dietary thiamethoxam on bumble bees.

Residues of thiamethoxam ranging from 1 to 11 $\mu\text{g kg}^{-1}$ (= parts per billion or ppb) have been detected in nectar from treated crops including alfalfa, oilseed rape, pumpkin, sunflower, squash and *Phacelia tanacetifolia* (Dively and Kamel, 2012; EFSA, 2012; Stoner and Eitzer, 2012). In pollen, residues are typically higher, ranging from 1 to 12 $\mu\text{g kg}^{-1}$ in sunflower, oilseed rape and squash, but reaching 39, 51 and 95 $\mu\text{g kg}^{-1}$ in *Phacelia*, alfalfa, and pumpkin, respectively (Dively and Kamel, 2012; EFSA, 2012; Stoner and Eitzer, 2012). For bees, exposure to residues such as these probably occurs in transient pulses; for example, during the mass-flowering of treated oilseed rape that lasts for approximately one month and peaks over a period of around two weeks (Hoyle et al., 2007; Westphal et al., 2009). Detrimental effects on honey bees of dietary thiamethoxam at 67 $\mu\text{g L}^{-1}$ have already been demonstrated (Henry et al., 2012), but the effects on bumble bees in a similar dosage range are unclear. For example, in one *B. terrestris* microcolony study 100 $\mu\text{g kg}^{-1}$ thiamethoxam

presented to workers in sugar solution increased mortality and reduced drone production while residues at 10 µg kg⁻¹ had no detectable effect (Mommaerts et al., 2010b). However, in another study 10 µg kg⁻¹ thiamethoxam reduced workers' production of drone brood (the workers' eggs and larvae), while microcolony feeding rates were reduced at both 1 and 10 µg kg⁻¹ (Elston et al., 2013). With evidence of thiamethoxam's effects currently inconsistent, it remains uncertain whether environmentally realistic residues are capable of having a detrimental impact on bumble bee populations. We therefore present an experiment designed to test the performance of bumble bees presented with dietary thiamethoxam at a wide range of concentrations, including dosages within the field-realistic range for nectar.

In this study, we made use of the reproductive capacity of *B. terrestris* workers in queenless microcolonies to investigate the effects of thiamethoxam on bumble bee performance. In microcolonies, small groups of bumble bee workers are maintained in the absence of a queen and, over a period of days, a dominant worker lays eggs that will develop into drones while the others forage and care for brood (Tasei et al., 2000). In a recent guidance document for risk assessment of plant protection products on bees (EFSA, 2013d), the use of microcolonies was recommended as part of 'higher tier' risk assessment studies in bumble bees. Using *B. terrestris* microcolonies, we characterised dose-response relationships that described thiamethoxam's effects on brood (eggs and larvae) production, food consumption and days survived by workers (Laycock et al., 2012) over an exposure lasting 17 days. Following laboratory exposure periods of similar length, imidacloprid produced substantive sublethal

effects on feeding and brood production in *B. terrestris* microcolonies (Laycock et al., 2012) and reduced colony growth and production of new queens in queenright colonies allowed to develop for a further six weeks in pesticide-free conditions (Whitehorn et al., 2012). Here we applied dosages and some endpoints that were adopted in the imidacloprid microcolony study (i.e. Laycock et al., 2012) to enable us to compare the relative sensitivity of bumble bees to the two neonicotinoids.

3.2 Materials and methods

3.2.1 Microcolonies

We obtained four colonies of *B. terrestris* (subspecies *audax*) (Biobest, Westerlo, Belgium) each consisting of a queen and approximately 150 workers. One hundred queenless microcolonies were established by placing 400 individual workers (100 from each queenright colony) into softwood boxes (120 × 120 × 45 mm) in groups of four. The allocation of workers to boxes was randomized, but each microcolony contained workers from the same queenright colony. Each box was fitted with two 2 mL microcentrifuge tubes (Simport, Beloeil, Canada) that were punctured so as to function as syrup (artificial nectar) feeders. We maintained microcolonies for 18 days under semi-controlled conditions (23–29 °C, 20–40 % relative humidity) and in darkness except during data collection. Specifically, all microcolonies were acclimatised to experimental conditions by feeding *ad libitum* on undosed control syrup (Attracker: 1.27 kg L⁻¹ fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) for 24 h prior to 17 days of exposure to

thiamethoxam. The 18-day experimental period employed here differs from the 14-day experimental period used in Laycock et al. (2012) because bumble bees in the current study were slower to begin oviposition (see Results) and required additional time to produce brood. A single bee that died during acclimatisation was replaced with a worker from its queenright source colony.

3.2.2 *Thiamethoxam dosages*

To produce a primary thiamethoxam stock solution ($10^5 \mu\text{g thiamethoxam L}^{-1}$), we dissolved 5 mg thiamethoxam powder (Pestanal[®]; Sigma-Aldrich, Gillingham, UK) in 50 mL purified water. Primary stock solution was further diluted (to $10^4 \mu\text{g L}^{-1}$) in purified water and an aliquot of diluted stock was mixed into feeder syrup to produce our most concentrated dietary solution of $125 \mu\text{g thiamethoxam L}^{-1}$ (or $98.43 \mu\text{g kg}^{-1} = \text{ppb}$). By serial dilution from the highest concentration we produced nine experimental dosages at the following concentrations: 98.43, 39.37, 15.75, 6.30, 2.52, 1.01, 0.40, 0.16, 0.06 $\mu\text{g thiamethoxam kg}^{-1}$. Following acclimatisation, microcolonies were fed *ad libitum* for 17 days with undosed pollen balls (ground pollen pellets, obtained from Biobest, mixed with water; mean mass = 5.3 g, SE = 0.1 g) and either undosed control syrup (19 control microcolonies) or syrup dosed with thiamethoxam (9 dosed microcolonies per thiamethoxam concentration, listed above). This level of replication (i.e. a minimum of nine replicates per concentration) is consistent with similar microcolony studies (Mommaerts et al., 2010b; Laycock et al., 2012; Elston et al., 2013). Pollen balls were weighed before and after placement into microcolonies to quantify pollen consumption and syrup feeders were weighed each day to measure syrup consumption. We corrected for evaporation of water

from syrup and pollen based on the mass change of syrup feeders and pollen balls maintained under experimental conditions, but not placed into microcolonies. Additionally, where syrup or pollen was collected by bees but not consumed, for example where syrup was stored in wax honey pots, its mass was determined and subtracted from consumption accordingly. We monitored microcolonies daily for individual worker mortality and the appearance of wax covered egg cells that indicate the occurrence of oviposition. To assess brood production, at the end of the experiment we freeze-killed workers in their microcolony boxes and collected all laid eggs and larvae from the nests. In our previous microcolony study (Laycock et al., 2012), we also investigated the effect of imidacloprid on ovary development because imidacloprid produced a dose-dependent decline in workers' brood production. Except at the highest dosages, thiamethoxam had no effect on brood production (i.e. microcolonies laid eggs at a statistically equivalent rate, see section 3.3) and we therefore chose not to measure ovary development here. The experiment was conducted in two replicate trials between October and December 2012. Each trial comprised 50 microcolonies and dosage groups were approximately equally represented in both. We tested whether the effects of dosage on brood production, food consumption and worker survival were materially affected by the inclusion of 'trial' as a factor in the statistical analyses (see section 3.2.3) and found that they were not. Therefore, data from the two trials were pooled for further analysis.

We verified the concentration of thiamethoxam in our doses using solid phase extraction (SPE) and liquid chromatography-mass spectrometry (LCMS) as

follows. First, we dissolved our dosed syrups in LCMS-grade water (Fisher Scientific, Loughborough, UK). To extract thiamethoxam from syrup, the diluted samples were processed through 1 mL Discovery[®] DSC-18 SPE tubes (Sigma-Aldrich, Gillingham, UK) under positive pressure. Specifically, we conditioned the SPE tube with 1 mL LCMS-grade methanol (Fisher Scientific, Loughborough, UK) followed by 1 mL LCMS-grade water, prior to passing through a 1 mL diluted sample. The tube was washed with 1 mL LCMS-grade water and the thiamethoxam was eluted from the column with three separate, but equivalent, aliquots of LCMS-grade methanol, totalling 450 µL. Methanol was removed by evaporation in a ScanSpeed MaxiVac Beta vacuum concentrator (LaboGene ApS, Lyngé, Denmark) and the remaining thiamethoxam was dissolved in 500 µL of LCMS-grade water. Extracted thiamethoxam samples were analysed in an Agilent 1200 series liquid chromatograph interfaced via an electrospray ionisation source to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), along with a calibration curve consisting of nine known thiamethoxam concentrations that ranged from 0.1 to 125 µg L⁻¹, using methods described in Laycock et al. (2012). The instrument response was linear over the range 0.1–125 µg L⁻¹, with the relationship of the calibration curve given by *instrument response = 228.42 × thiamethoxam concentration + 265.87, R² > 0.99*). We used the calibration equation to determine the concentration values of our extracted samples and found that all dosages contained appropriate levels of thiamethoxam (*measured thiamethoxam = 1.16 × nominal dosage + 1.57, R² > 0.99*).

3.2.3 Statistical analyses

In our experiments, endpoints responded only to the two highest dosages of thiamethoxam (see section 3.3). We therefore analysed the variation in food consumption and days survived by workers in microcolonies that was due to thiamethoxam using one-way ANOVA, with *dosage* (dosage of thiamethoxam in $\mu\text{g kg}^{-1}$) treated as a categorical variable, and compared the highest dosage groups to those below using orthogonal contrasts.

We tested whether the two highest thiamethoxam dosages were associated with an increased frequency of oviposition failure (zero brood produced) using a 2×2 contingency table and Pearson's Chi-squared test with Yates' continuity correction.

To determine whether brood production was dose-dependent below the two highest dosages, we used zero-inflated Poisson regression (ZIP) because of an excess of zero counts in our data (Lambert, 1992). We tested the appropriateness of the ZIP model by comparing it to a standard Poisson model using a Vuong non-nested test and confirmed that the ZIP model was the superior choice (*Vuong test statistic* = -5.17, $P < 0.001$).

In our analysis, the total number of eggs and larvae produced in microcolonies during the 17-day exposure period represents *brood* (brood were not produced during pre-dose acclimatisation). Where necessary, we log-transformed *dosage* to $\log(\text{dosage} + 1)$ to meet test assumptions. All statistical analyses were conducted in R v3 (R Core Team 2013).

3.3 Results

Per capita consumption of syrup and pollen in microcolonies was significantly affected by thiamethoxam (ANOVA: *syrup consumption*, $F_{9, 90} = 9.29$, $P < 0.001$; *pollen consumption*, $F_{9, 90} = 15.14$, $P < 0.001$; Fig. 3.1). Specifically, a significant reduction in food consumption was evident only in microcolonies exposed at the two highest dosages, $39 \mu\text{g kg}^{-1}$ and $98 \mu\text{g kg}^{-1}$ (orthogonal contrast: *syrup consumption*, $t = -8.87$, $P < 0.001$; *pollen consumption*, $t = -11.22$, $P < 0.001$). No dose-dependent variation was detectable among microcolonies exposed to dosages $\leq 16 \mu\text{g kg}^{-1}$ (ANOVA: *syrup consumption*, $F_{7, 74} = 0.39$, $P = 0.91$; *pollen consumption*, $F_{7, 74} = 0.90$, $P = 0.51$). Despite consuming less syrup, microcolonies exposed to higher dosages nevertheless ingested larger amounts of thiamethoxam (Table 3.1).

In microcolonies, the frequency of oviposition failure at the two highest thiamethoxam dosages (94 % failure) was greater than at lower dosages (48 %) and these frequencies differed significantly (Chi-squared contingency table analysis: $X^2 = 11.33$, $df = 1$, $P < 0.001$; Fig. 3.2). Excluding the two highest dosages, thiamethoxam did not significantly affect the number of brood produced (ZIP regression: *brood count*, $z = -1.26$, $P = 0.21$; *zero brood production*, $z = 0.45$, $P = 0.65$; Fig. 3.1).

Among microcolonies that produced brood, there was no effect of dosage on the number of brood produced or on the timing of first oviposition (Spearman's correlation: *brood vs. dosage*, $\rho = -0.03$, $N = 44$, $P = 0.85$; *days until oviposition vs. dosage*, $\rho = 0.08$, $N = 44$, $P = 0.63$; Table 3.1).

The number of days survived by workers in microcolonies varied significantly with thiamethoxam dosage (ANOVA: $F_{9, 90} = 27.43$, $P < 0.001$; Fig. 3.1), but it was reduced only at $98 \mu\text{g kg}^{-1}$ (orthogonal contrast: $t = -15.44$, $P < 0.001$) and did not differ at lower dosages (ANOVA: $F_{8, 82} = 1.25$, $P = 0.28$).

3.4 Discussion

3.4.1 Thiamethoxam effects

We found that thiamethoxam reduced feeding and brood production in *B. terrestris* microcolonies that fed on syrup with a dietary concentration of $39 \mu\text{g kg}^{-1}$ or above for 17 days. At lower dosages, microcolonies consumed syrup and pollen at normal control rates and brood production was not detectably dose-dependent. These results are consistent with those of a previous *B. terrestris* microcolony study in which dietary thiamethoxam produced an EC₅₀ (half maximal effective concentration) for drone production of $35 \mu\text{g kg}^{-1}$ and had no observable effect on workers at $10 \mu\text{g kg}^{-1}$ (Mommaerts et al., 2010b). However, another recent study reported that $10 \mu\text{g kg}^{-1}$ thiamethoxam was capable of reducing syrup feeding and brood production in microcolonies (Elston et al., 2013). These contrasting results may have arisen because bumble bees consumed different amounts of thiamethoxam in nominally equivalent treatment groups, with Elston et al. (2013) having dosed both syrup and pollen at $10 \mu\text{g kg}^{-1}$, whereas Mommaerts et al. (2010b), like us, dosed only syrup. Additionally, our results correspond with studies of clothianidin, which is thiamethoxam's primary toxic metabolite and becomes active during thiamethoxam exposure (Nauen et al., 2003). Specifically, dietary clothianidin at

$38 \mu\text{g kg}^{-1}$ negatively influenced honey bee foraging behaviour (Schneider et al., 2012), but lower dosages had no adverse effects on colonies of *Bombus impatiens* Cresson bumble bees (Franklin et al., 2004).

Where thiamethoxam was presented to microcolonies at $39 \mu\text{g kg}^{-1}$ or above, we observed an association between impaired feeding on syrup and pollen and failure to produce brood. A similar association was observed in *B. terrestris* microcolonies fed imidacloprid across a range of dosages (Laycock et al., 2012). The hypothesis proposed by Laycock et al. (2012), that nutrient limitation imposed by an imidacloprid-induced reduction of feeding may be responsible for repression of brood production in bumble bees, can also be applied in our current study to explain thiamethoxam's detrimental effect on brood production at higher dosages. We therefore postulate that the capacity to impair bumble bee feeding behaviour is common amongst neonicotinoids, particularly at high dosages, and this may provide a general mechanism for reduced brood production (Gill et al., 2012; Laycock et al., 2012; Elston et al., 2013).

Consistent with previous findings (Mommaerts et al., 2010b), the number of days survived by workers was significantly reduced in microcolonies fed approximately $100 \mu\text{g kg}^{-1}$ thiamethoxam. For honey bees, relatively large dosages of thiamethoxam ($67 \mu\text{g L}^{-1}$) also impact on worker survival (Henry et al., 2012). Apparently, these relatively high concentrations of dietary thiamethoxam are highly toxic to bees in general.

3.4.2 Differential sensitivity of bumble bees to thiamethoxam and imidacloprid

In other toxicology studies the biological efficacy of thiamethoxam is said to be comparable to other neonicotinoids (Nauen et al., 2003), but relative toxicity is somewhat inconsistent among studies and species. For example, the LC₅₀ for bumble bees (Mommaerts et al., 2010b) and LD₅₀ for honey bees (Iwasa et al., 2004) was lower for imidacloprid than thiamethoxam (i.e. imidacloprid was more potent) in oral and topical toxicity studies, respectively, but higher when other beneficial arthropods and pest species were tested (Magalhaes et al., 2008; Prabhaker et al., 2011). Our study indicates that bumble bees may be less sensitive to thiamethoxam than imidacloprid at dosages in the realistic range typically found in nectars of treated crops (approximately 1–11 µg kg⁻¹; Dively and Kamel, 2012; EFSA, 2012; Stoner and Eitzer, 2012). Whereas we found no detectable effect on *B. terrestris* microcolonies of thiamethoxam in this range, a previous study conducted under approximately identical conditions found that dietary imidacloprid was capable of substantively reducing brood production and food consumption in microcolonies at concentrations as low as 1.0 and 2.5 µg kg⁻¹, respectively (Laycock et al., 2012). Similar differences in sensitivity have been demonstrated in aphids, *Myzus spp.*, with imidacloprid repressing feeding at concentrations as low as 6 µg L⁻¹ (Nauen, 1995; Devine et al., 1996) and thiamethoxam failing to repress feeding even at higher dosages (Cho et al., 2011). However, we note that these two *B. terrestris* microcolony studies offer only an approximate comparison. For example, in the present study brood production was lower overall than that observed by Laycock et al. (2012), perhaps because of the intrinsic variation in reproductive success that exists between bumble bee colonies (Müller and Schmid-Hempel, 1992a). In future

work it will be important to compare the sensitivity of bumble bees from the same colony.

Differential sensitivity may be due to imidacloprid producing a stronger repression of feeding in bumble bees than thiamethoxam at field-realistic dosages (Cresswell et al., 2012b; Laycock et al., 2012). Such differences perhaps arise because of thiamethoxam binding to target sites that are distinct from those of imidacloprid (Kayser et al., 2004; Wellmann et al., 2004; Thany, 2011) or because imidacloprid has a greater affinity for insect nicotinic acetylcholine receptors (nAChRs) (Wiesner and Kayser, 2000). However, while imidacloprid is only a partial agonist of native nAChRs in several insects including honey bees (Déglise et al., 2002; Brown et al., 2006; Ihara et al., 2006), clothianidin is a ‘super’ agonist of *Drosophila* nAChRs (Brown et al., 2006) and has a higher agonist efficacy than imidacloprid in cockroach nAChRs (Ihara et al., 2006). We assume that thiamethoxam is metabolised to clothianidin in bumble bees as it is in other organisms (Nauen et al., 2003), but whether the metabolite is a superior agonist of bumble bee nAChRs is currently unknown. If clothianidin has the higher agonist efficacy in bumble bees, the differential sensitivity we observe may be attributable to the superior hydrophobicity of imidacloprid (Ihara et al., 2006), which could determine the neonicotinoids’ accessibility to the receptor and therefore its insecticidal potency (Ihara et al., 2006). While our results show that differential sensitivity of bumble bees to neonicotinoids is possible, further research is required to understand the mechanistic basis of this phenomenon.

3.4.3 Environmental relevance

In our study, realistic dietary residues of thiamethoxam between one and 11 µg kg⁻¹ had no detectable effect on the performance of bumble bee workers in microcolonies. We extrapolate our results to wild bumble bee populations with caution because additional work is clearly necessary to determine the impact of thiamethoxam on bumble bee queens and their colonies. We also note that our study considers only the effects of dietary thiamethoxam in nectar and not pollen. Furthermore, we test an exposure period of 17 days, whereas environmental exposure could extend across a month or more as bumble bees forage on mass-flowering crops throughout their bloom (Westphal et al. 2009). Consequently, we may underestimate the effects of field-realistic exposures. However, our failure to detect an effect in this range is consistent with a recent field study in which *B. terrestris* colonies produced new queens successfully despite being found to contain stored forage comprising thiamethoxam at an average of 2.4 µg kg⁻¹ in nectar and 0.7 µg kg⁻¹ in pollen (Thompson et al., 2013).

Our findings suggest that environmentally realistic residues of imidacloprid have the potential to make a greater impact on bumble bees than residues of thiamethoxam, which could have important implications for future neonicotinoid usage in agriculture. However, further research is required to establish thiamethoxam's impact on queenright colonies in wild populations.

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Disclosure statement

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Chapter Three: Figures and Tables

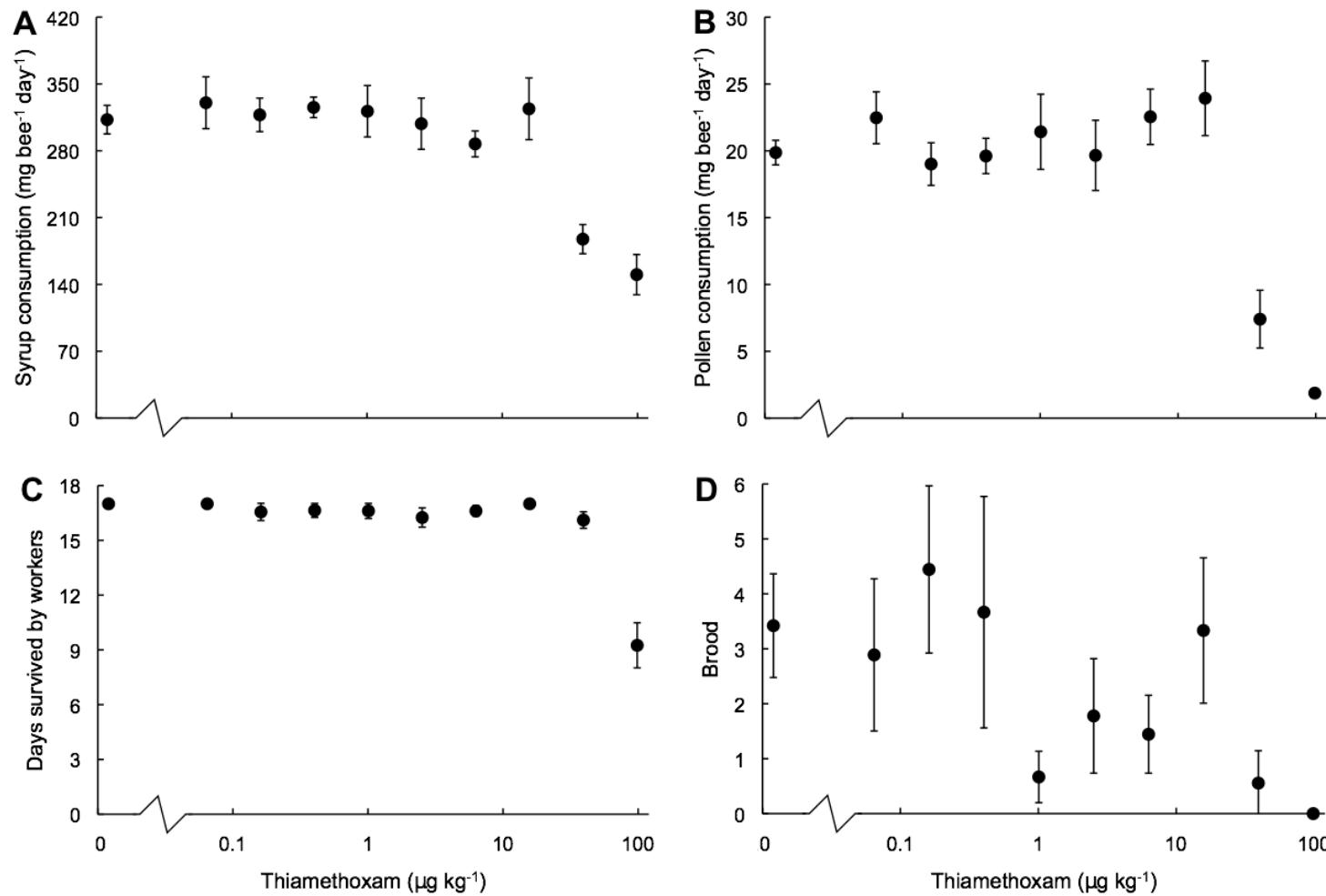


Fig. 3.1. Daily *per capita* feeding rates, days survived by workers, and brood production in *Bombus terrestris* bumble bee microcolonies following 17 days of exposure to thiamethoxam in dosed syrup ($\mu\text{g kg}^{-1}$ = parts per billion). (A) Daily *per capita* consumption of dosed syrup; (B) daily *per capita* consumption of undosed pollen; (C) number of days workers survived while under exposure (maximum = 17 days); and (D) brood production (eggs and larvae produced; data includes microcolonies that failed to oviposit). Data represent the means and error bars indicate $\pm \text{SE}$ (replicates per dosage group: control, $N = 19$ microcolonies; dosage treatments, $N = 9$ microcolonies per concentration). Control data (zero $\mu\text{g kg}^{-1}$) are displayed slightly displaced on the x-axis for ease of inspection.

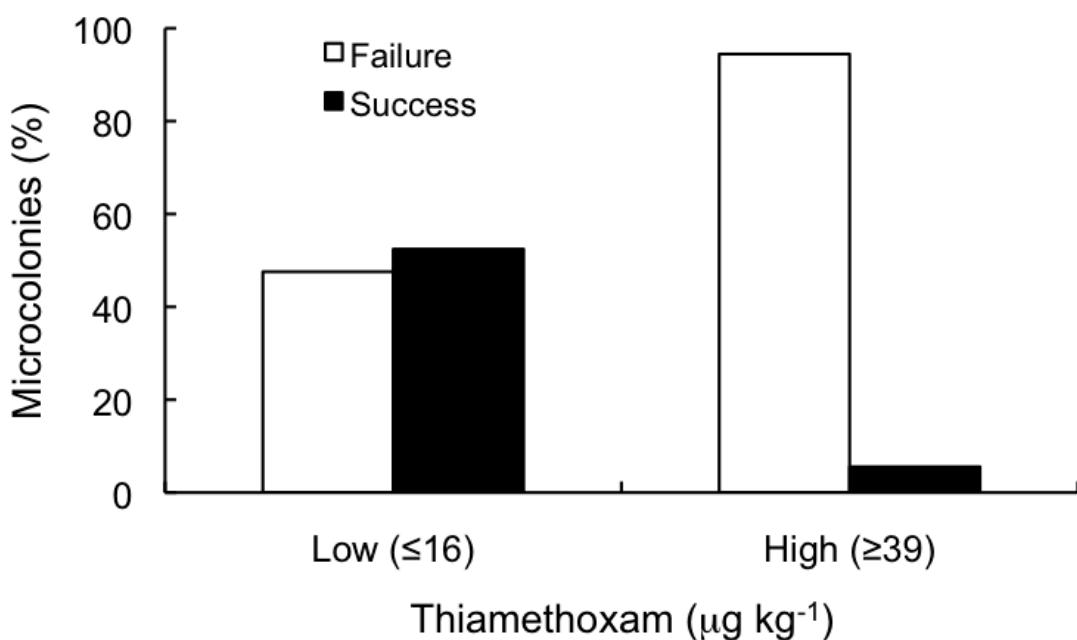


Fig. 3.2. Frequency of oviposition failure and success in *Bombus terrestris* bumble bee microcolonies presented for 17 days with thiamethoxam in dosed syrup ($\mu\text{g kg}^{-1}$ = parts per billion). Low dosage group ($N = 82$) and high dosage group ($N = 18$) consist of microcolonies exposed to dietary thiamethoxam at concentrations of ≤ 16 and $\geq 39 \mu\text{g kg}^{-1}$, respectively. Open bars represent failure to produce brood (zero brood produced) and filled bars represent success (\geq one brood individual, i.e. egg or larvae, produced). Frequency of oviposition failure in the high dosage group (94 %) differed significantly from that in low dosage group (48 %; Chi-squared contingency table analysis, $P < 0.001$).

Table 3.1 Frequency of successful oviposition in *Bombus terrestris* bumble bee microcolonies, with the number of brood (eggs and larvae) produced by successful ovipositors and the time at which first oviposition occurred. Microcolonies ($N = 100$) were presented with thiamethoxam (TMX) in feeder syrup at given dosages for 17 days (replicates per dosage group: control, $N = 19$; dosage treatments, $N = 9$ per concentration). *Per capita* consumption of TMX in microcolonies is provided for each dosage treatment. Only data from the 44 % (44 from 100) of microcolonies that produced brood is provided in successful oviposition, brood given oviposition and day of first oviposition columns. Except for successful oviposition, data represent the mean \pm SE. We found no detectable effect of dosage on brood production or timing of oviposition in successfully ovipositing microcolonies (Spearman's correlation, $P > 0.05$).

TMX dosage ($\mu\text{g kg}^{-1}$ /ppb)	TMX consumed (ng bee^{-1} day $^{-1}$)	Successful oviposition (%)	Brood, given oviposition	Day of first oviposition
Control	0.000 ± 0.000	63	5.4 ± 1.1	10.7 ± 0.7
0.1	0.021 ± 0.002	67	4.3 ± 1.8	11.0 ± 0.5
0.2	0.051 ± 0.003	78	5.7 ± 1.6	13.1 ± 1.5
0.4	0.131 ± 0.004	22	11.0 ± 3.2	9.8 ± 3.5
1.0	0.324 ± 0.027	22	3.0 ± 0.0	9.5 ± 1.5
2.5	0.777 ± 0.068	33	5.3 ± 1.8	11.3 ± 3.6
6.3	1.809 ± 0.085	44	3.3 ± 1.0	12.8 ± 2.6
15.7	5.101 ± 0.509	67	5.0 ± 1.6	11.3 ± 0.6
39.4	7.379 ± 0.602	11	5.0 ± 0.0	12.0 ± 0.0
98.4	14.785 ± 2.076	0	—	—
All ovipositing microcolonies			5.3 ± 0.6	11.4 ± 0.5

Chapter Four

Repression and recuperation of brood
production in *Bombus terrestris* bumble bees
exposed to a pulse of the neonicotinoid
pesticide imidacloprid

Preliminary introduction

The following chapter contains work based on a paper submitted to *PLoS One* in March 2013 for which I was first author. The paper was accepted for publication in September 2013, published online in November 2013, and is available in its original form at <http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.007987>. The content below is identical to that published in *PLoS One*, with the exception of a small number of qualitative amendments that have been made to improve clarity. Additionally, ambiguous references are lettered (a, b, etc), and the sections, figures and tables are numbered according to their position within the thesis. Where Laycock et al. (2012) is cited, I am referring to chapter two of this thesis.

Statement of contribution

As first author, I was primarily responsible for conception and design of the work, acquisition and analysis of the data, and writing the paper. My supervisor and co-author Dr James Cresswell contributed advice and assistance during the design of the experiment and analysis of data, and provided constructive criticism and revision notes during the writing process. Additional contributors are acknowledged at the end of the paper: Dr Hannah Florance ran imidacloprid samples through the LC-MS; Dr Chris Pook provided advice on solid phase extraction methods; James Smith provided his expertise in Bayesian analysis to help obtain confidence intervals on best-fit dose-response relationships produced by Bayesian modelling. Prof. Charles Tyler and three anonymous reviewers also provided comments on the manuscript during the review process with *PLoS One*, and a further five anonymous reviewers commented on earlier drafts following submission to journals other than *PLoS One*.

Repression and Recuperation of Brood Production in *Bombus terrestris* Bumble Bees Exposed to a Pulse of the Neonicotinoid Pesticide Imidacloprid

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Abstract

Currently, there is concern about declining bee populations and some blame the residues of neonicotinoid pesticides in the nectar and pollen of treated crops. Bumble bees are important wild pollinators that are widely exposed to dietary neonicotinoids by foraging in agricultural environments. In the laboratory, we tested the effect of a pulsed exposure (14 days 'on dose' followed by 14 days 'off dose') to a common neonicotinoid, imidacloprid, on the amount of brood (number of eggs and larvae) produced by *Bombus terrestris* L. bumble bees in small, standardised experimental colonies (a queen and four adult workers). During the initial 'on dose' period we observed a dose-dependent repression of brood production in colonies, with productivity decreasing as dosage increased up to 98 µg kg⁻¹ dietary imidacloprid. During the following 'off dose' period, colonies showed a dose-dependent recuperation such that total brood production during the 28-day pulsed exposure was not correlated with imidacloprid up to 98 µg kg⁻¹. Our findings raise further concern about the threat to wild bumble bees from neonicotinoids, but they also indicate some resilience to a pulsed exposure, such as that arising from the transient bloom of a treated mass-flowering crop.

4.1 Introduction

Currently, there is concern about declines in bee populations [1,2] and some implicate neonicotinoid pesticides as culprits [3,4]. Neonicotinoids disrupt the insect nervous system [5] and their dietary intake can reduce the expected performance of bees [6,7]. For example, neonicotinoids may increase worker losses while reducing reproductive output and foraging performance in bumble bees, *Bombus* spp. [8,9], and induce homing failure and suppress colony growth in honey bees, *Apis mellifera* L. [10] (but see [11,12] for further discussion). Whether neonicotinoids are a principal cause of bee declines is unclear [13,14], but in regions where they are not banned [4] bees are certainly exposed to them on a massive spatial scale by foraging from treated agricultural crops. For example, oilseed rape (or canola), *Brassica napus* L., is the principal mass-flowering crop in many areas of North America (> 8 million hectares [15,16]) and Northern Europe (e.g. ~0.7 million hectares in the UK [17]) and many of its fields are protected from pests by neonicotinoids [18,19]. Neonicotinoids are systemic pesticides, so they are distributed throughout the plant following application [18] and bees are exposed to dietary residues by consuming nectar and pollen [20]. For oilseed rape in the USA, residues of a widely used neonicotinoid, imidacloprid, have been detected in nectar at 0.8 parts per billion (ppb) and in pollen at 7.6 ppb [21]. Other bee-attractive crops such as sunflower and alfalfa are often protected with neonicotinoids [18,21], and so the exposure of bees to these pesticides is widespread. To understand whether a widespread exposure to neonicotinoids is capable of causing bee populations to decline, we must understand their demographic toxicity, which

occurs when a toxic agent detrimentally affects the birth and death rates of the exposed species [22].

The lethality of imidacloprid to bees appears to be dependent on the time of exposure [23,24]. However, in some laboratory trials the trace levels of imidacloprid typically found in nectar and pollen (≤ 10 ppb [21], but see [25]) have negligible effects on mortality in honey bees [7] and bumble bees [26,27], but they can substantively affect birth rates in bumble bees [28]. Specifically, dietary imidacloprid at levels as low as one ppb may reduce the number of eggs and larvae produced by adult bumble bee workers by one third [28], but the demographic implications of this are unclear because queens are principally responsible for a colony's reproductive output [29]. Because the number of new queens and males that a bumble bee colony produces depends on its size [30,31], the number of workers produced by a queen during a colony's development can determine colony fitness. We therefore examined the effects of dietary imidacloprid on brood production (specifically, the numbers of eggs and larvae destined to become workers) by queen bumble bees at dosages that spanned the environmentally realistic range.

We investigated the effects of a 14-day exposure to dietary imidacloprid on the performance of small, standardised experimental colonies of the buff-tailed bumble bee, *Bombus terrestris* L., in the laboratory. We found a dose-dependent decrease in brood production up to 98 ppb imidacloprid (see Results) and so we extended our experiment to create a pulsed exposure, feeding bees for an additional 14 days on an imidacloprid-free diet, because a

scenario such as this may be relevant to wild bumble bee colonies. For example, a pulsed exposure may be caused by the synchronized bloom of imidacloprid-treated oilseed rape fields that normally flower for approximately four weeks in April or May [32] (where the crop is winter-sown) and the exposure subsides when the bees subsequently switch to foraging on pesticide-free wildflowers [33]. Recuperation from some imidacloprid-induced effects has been reported following an exposure in honey bees [34], coccinellids [35], aphids [36], whitefly [37], and the aquatic larvae of midges [38], but our study is the first to explore the potential for such a recovery in bumble bees.

4.2 Materials and methods

4.2.1 Ethics statement

The protocol reported here conforms to the regulatory requirements for animal experimentation in the UK and was approved by the Biosciences Ethics Committee at the University of Exeter.

4.2.2 Bees, experimental colonies and imidacloprid diets

We obtained colonies of *B. terrestris* (subspecies *audax*) at an early stage of development (Biobest, Westerlo, Belgium). In order to create small, standardised experimental colonies for testing, we removed each queen and randomly chose four of her adult workers from their pre-experimental source colony and placed them together in a softwood box (120 × 120 × 45 mm) fitted with two 2 mL microcentrifuge tubes (Simport, Beloeil, Canada) that were punctured so as to function as syrup (artificial nectar) feeders [28]. Experimental

colony size (a queen and four adult workers) was chosen to simulate early-stage bumble bee colonies, consistent with those used in similar studies [8]. We maintained these experimental colonies for 28 days in a semi-controlled environment (23–27 °C, 21–47 % relative humidity).

We obtained imidacloprid as a solution in acetonitrile (Dr. Ehrenstorfer GmbH, Ausberg, Germany). Acetonitrile was removed by evaporation and the imidacloprid was dissolved in purified water before being mixed into feeder syrup (Attracker: 1.27 kg L⁻¹ fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) to produce our most concentrated dosage of 125 µg imidacloprid L⁻¹ (or 98.43 µg kg⁻¹ = ppb). By serial dilution from 125 µg L⁻¹ (dilution factor = 0.4) we produced the following nine experimental dosages: 125.00, 50.00, 20.00, 8.00, 3.20, 1.28, 0.51, 0.20, and 0.08 µg imidacloprid L⁻¹ (= 98.43, 39.37, 15.75, 6.30, 2.52, 1.01, 0.40, 0.16, and 0.06 µg imidacloprid kg⁻¹). A fresh dilution series containing all nine concentrations was produced at the beginning of each pulsed exposure trial (see below) and kept inside a dark fridge at 5 °C. Dosed syrup from the second pulsed exposure trial was used in the continuous exposure experiment (below).

4.2.3 Exposure to dietary imidacloprid

To create a pulsed exposure, the 28-day experimental period was split into two successive periods of 14 days. During the ‘on dose’ period (days 1–14), 60 experimental colonies were provided *ad libitum* with either undosed control syrup (6 control colonies) or dosed syrup (6 colonies per dosage treatment, listed above). Fresh syrup at the appropriate dosage was provided to colonies

daily. For the 'off dose' period (days 15–28), the bees were transferred to new softwood boxes and fed *ad libitum* with only undosed control syrup. At the beginning of each 14-day period, each experimental colony was provided with a fresh ball of undosed pollen (Biobest, Westerlo, Belgium) to which bees had *ad libitum* access. Pollen balls (mean mass = 6.1 g, SE = 0.02) were prepared from ground pollen pellets mixed with water to form dough and were weighed before and after placement in colonies to quantify pollen consumption. We corrected for evaporation of water from syrup and pollen based on the mass change of several feeders and pollen balls kept in empty colony boxes under experimental conditions. Experimental colonies were kept in darkness except when monitored daily for the appearance of wax covered egg cells (indicating that oviposition had occurred), syrup consumption and individual mortality. To minimise disturbance to bees, we assayed brood production by collecting all laid eggs and larvae from experimental colony boxes only at the end of each 14-day period, (i.e. on days 14 and 28). The experiment was conducted in two replicate trials, one between October–November 2011 and the other between January–February 2012. Each trial comprised 30 experimental colonies and treatment groups were equally represented in both (3 colonies per treatment).

To establish that the observed recuperation from imidacloprid-induced effects under pulsed exposure (see Results) was caused by the removal of dietary imidacloprid rather than from acclimation to exposure over elapsed time, we conducted a separate continuous exposure experiment. Using the same husbandry techniques described above, we randomly assigned 12 experimental colonies to either 28 days feeding on control syrup (7 colonies) or 28 days

feeding on syrup dosed at 98.43 µg imidacloprid kg⁻¹ (5 colonies) and we used the same interruption to collect brood on days 14 and 28. This continuous exposure trial was conducted between March–April 2012. This protocol is an adequate test because the highest level of recuperation was observed at 98.43 µg kg⁻¹ in the previous experiments (see Results).

To verify the concentration of imidacloprid in our doses, we first dissolved the dosed syrup in liquid chromatography-mass spectrometry (LCMS)-grade water (Fisher Scientific UK Ltd, Loughborough, UK) spiked with a reference standard of imidacloprid-d₄ (Dr. Ehrenstorfer GmbH, Augsburg, Germany) at 100 µg L⁻¹ (ratio of syrup to water = 5:7). We used solid phase extraction (SPE) to extract imidacloprid and imidacloprid-d₄ from the syrup as follows. Diluted dosed syrup samples were processed through 1 mL Discovery® DSC-18 SPE tubes (Sigma-Aldrich, Gillingham, UK) under positive pressure. We first conditioned the SPE tube with 1 mL pure LCMS-grade methanol (Fisher Scientific UK Ltd, Loughborough, UK) followed by 1 mL pure LCMS-grade water. A 1 mL sample was passed through the tube, before the tube was washed with 1 mL pure LCMS-grade water and the imidacloprid was eluted from the column with three separate, but equivalent, aliquots of pure LCMS-grade methanol totalling 450 µL. We removed the methanol by evaporation and the remaining imidacloprid was dissolved in 500 µL of pure LCMS-grade water. Imidacloprid samples were analysed in an Agilent 1200 series liquid chromatograph interfaced via an electrospray ionisation source to an Agilent 6410 triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) using methods described in Laycock et al. [28]. The instrument response was linear over the

range 0.06–125 µg L⁻¹ for imidacloprid and imidacloprid-d₄ and we found that dosages in all trials contained appropriate levels of imidacloprid (pulsed exposure trial 1, *measured imidacloprid* = 0.989 × *nominal dosage* + 0.204, $R^2 > 0.99$; pulsed exposure trial 2 and continuous exposure trial, *measured imidacloprid* = 1.035 × *nominal dosage* – 0.205, $R^2 > 0.99$).

4.2.4 Statistical analyses

In our analyses, ‘brood’ represents the total number of eggs and larvae produced in an experimental colony in a given period. We tested whether the ‘brood’ dose-response relationships differed between our two pulsed exposure trials by analysis of covariance (ANCOVA), with ‘dosage’ (dosage of imidacloprid in µg kg⁻¹) log-transformed to log(‘dosage’ + 1) as the covariate and ‘trial’ as the fixed factor, and detected no significant difference between the two trials and so the data were pooled for further analysis (ANCOVA: ‘on dose’ brood, dosage × trial, $F_{1, 56} = 0.99, P = 0.32$; ‘off dose’ brood, dosage × trial, $F_{1, 56} = 0.03, P = 0.86$; total brood, dosage × trial, $F_{1, 56} = 0.34, P = 0.56$). The size of the pre-experimental source colony (mean number of workers = 16.4, SE = 1.1; mean number of brood = 101.8, SE = 7.5) from which the members of an experimental colony (queen and four workers) originated did not explain variation in brood production among the experimental colonies and it was disregarded in the analyses below (Spearman’s correlation: ‘on dose’ brood vs. source colony size, $\rho = -0.10, N = 60, P = 0.44$; ‘off dose’ brood vs. source colony size, $\rho = 0.07, N = 60, P = 0.59$; total brood vs. source colony size, $\rho = -0.01, N = 60, P = 0.91$).

We tested for dose-dependent brood production, timing of oviposition and food consumption during each period of the pulsed exposure using Spearman's correlation analyses. We tested for dose-dependent recuperation by analysing the differences in performance in experimental colonies between the 'on dose' and 'off dose' periods as follows. For a given variable X , denote the 'on dose' performance of a colony by X_{on} and its 'off dose' performance by X_{off} . For each colony we calculated $(X_{off} - X_{on})$, so that a positive value indicates that a colony produced more brood during the 'off dose' period, i.e. it showed recuperation. We investigated recuperation by testing whether $(X_{off} - X_{on})$ increased with imidacloprid dosage using Spearman's correlation analysis.

For brood production, once the statistical significance of the dose-response relationship was established by correlation we used Bayesian Hierarchical Models (BHM) to fit a relationship between 'brood' and 'dosage'. In each BHM, we fitted: $brood \sim \text{Poisson}(\mu)$; $\log(\mu) \sim \alpha + \beta \times \log(dosage + 1) + \lambda$. Here, α and β are fitted coefficients analogous to the conventional regression coefficients of slope and intercept, and λ is a 'random effects' term to accommodate overdispersion (λ has a normal distribution with a mean of zero). Each model was fitted with 40,000 iterations of Bayesian inference using a Markov Chain-Monte Carlo method with Gibbs sampling after a burn-in period that discarded the first of 7000 iterations on each chain. We obtained confidence intervals on this relationship as follows. The pairs of α and β values from the final 40,000 iterations of the Bayesian inference estimate the posterior joint probability distribution of the two coefficients; we therefore plotted the 40,000 relationships corresponding to these pairs and extracted the upper and lower percentiles (2.5

(%, 97.5 %) of the fitted brood values that corresponded to each imidacloprid intake across the range of interest. For brood production, we estimated the EC₅₀ (half maximal effective concentration) and EC₁₀ using the BHM best-fit relationships. We estimated EC values for the imidacloprid-induced reduction in food consumption by using GraphPad Prism v6.0c and evaluated the goodness of fit based on R². BHM procedures were implemented in WinBUGS v1.4.3 [39], while all other statistical analyses were conducted in R v3 [40].

4.3 Results

In both pulsed and continuous exposure experiments, *B. terrestris* queens in experimental colonies began producing eggs after approximately two days and some brood progressed to a larval stage within the 14-day periods. No queens died during the experiments and there was negligible worker mortality (one dead worker at 98 ppb, two dead at 39 ppb in the same colony, two dead at 16 ppb in separate colonies).

During the 14-day ‘on dose’ period of pulsed exposure, colonies exhibited dose-dependent repression of brood production such that fewer brood were produced as dosage increased up to 98 ppb imidacloprid (Spearman’s correlation: ‘on dose’ brood vs. dosage, $\rho = -0.45$, $N = 60$, $P < 0.001$; Figure 4.1). The dose-response relationship for brood and imidacloprid dosage during the ‘on dose’ period was given by $\text{brood} = \exp[2.002 - 1.788 \times \log(\text{dosage} + 1)]$ and the standard deviation of the overdispersion parameter was $\text{SD}(\lambda) = 1.89$ (Figure

4.2). Based on this relationship, the EC₅₀ and EC₁₀ values for imidacloprid's affect on brood production were 1.44 ppb and 0.15 ppb, respectively.

During the 14-day 'off dose' period, brood production showed dose-dependent recuperation (Spearman's correlation: ($Brood_{off} - Brood_{on}$) vs. dosage, $\rho = 0.32$, $N = 60$, $P = 0.01$; Figure 4.3). Dosage did not significantly affect brood production during the 'off dose' period (Spearman's correlation: 'off dose' brood vs. dosage, $\rho = 0.10$, $N = 60$, $P = 0.47$; Figure 4.1) and, taken over the entire 28-day pulsed exposure, total brood production was not significantly correlated with imidacloprid dosage (Spearman's correlation: total brood vs. dosage, $\rho = -0.13$, $N = 60$, $P = 0.32$; Figure 4.1). However, we note that based on the 28-day dose-response relationship for brood and imidacloprid, given by $brood = \exp[2.770 - 0.198 \times \log(dosage + 1)]$ with $SD(\lambda) = 1.25$ (Figure 4.2), recuperation of brood production was incomplete at higher dosages. For example, a 32% reduction remained apparent in colonies dosed with imidacloprid at 98 ppb (Figure 4.2). The EC₅₀ value for reduced brood production over the entire 28-day pulsed exposure was beyond our tested dosage range (> 98 ppb), while the EC₁₀ was estimated at 2.5 ppb.

Based on the fitted dose-response relationships (Figure 4.2), we estimate that 14-day exposures to dietary imidacloprid at environmentally realistic levels of between 0.3 ppb and 10 ppb may reduce brood production in *B. terrestris* colonies by between 18–84 % (Table 4.1). However, the effects of recuperation in this residue range are such that given a further 14 days without exposure the drop in brood is ameliorated to between 2–19 % (Table 4.1).

Recuperation is unlikely to be attributable to acclimation over time because brood production remained repressed under continuous exposure at 98.4 ppb over 28 days (Figure 4.1). Specifically, colonies dosed at 98.4 ppb imidacloprid exhibited significantly reduced brood production over 28-days compared to control colonies (ANOVA: dosage, $F_{1, 21} = 6.33, P < 0.05$), but brood production did not differ between successive 14-day periods (days 1–14 and 15–28) of continuous exposure (ANOVA: period, $F_{1, 21} = 2.22, P = 0.15$) and the effect of dose on brood production did not depend on period (ANCOVA: dosage × period, $F_{1, 20} = 0.47, P = 0.50$).

Where brood were produced, imidacloprid did not affect the timing of first oviposition during the ‘on dose’ period (Spearman’s correlation: days until oviposition vs. dosage, $\rho = 0.11, N = 35, P = 0.5$; Table 4.2), but it delayed oviposition in the subsequent ‘off dose’ period (Spearman’s correlation: days until oviposition vs. dosage, $\rho = 0.53, N = 45, P < 0.001$; Table 4.2).

During pulsed exposure, we observed dose-dependent reductions in the daily consumption of syrup and pollen by experimental colonies whilst they were ‘on dose’ (Spearman’s correlation: ‘on dose’ syrup consumption vs. dosage, $\rho = -0.59, N = 60, P < 0.001$; ‘on dose’ pollen consumption vs. dosage, $\rho = -0.77, N = 60, P < 0.001$; Figure 4.4). Based on these results, the EC₅₀ and EC₁₀ values for reduced pollen consumption were 4.4 ppb ($R^2 = 0.95$) and 0.2 ppb ($R^2 = 0.96$), respectively, while the equivalent values for reduced syrup consumption were > 98 ppb ($R^2 = 0.90$) and 23.6 ppb ($R^2 = 0.97$).

During the ‘off dose’ period, colonies demonstrated dose-dependent recuperation of both syrup consumption (Spearman’s correlation: ($Syrup_{off} - Syrup_{on}$) vs. dosage, $\rho = 0.60$, $N = 60$, $P < 0.001$) and pollen consumption (Spearman’s correlation: ($Pollen_{off} - Pollen_{on}$) vs. dosage, $\rho = 0.81$, $N = 60$, $P < 0.001$). Dosage did not significantly affect syrup consumption during the ‘off dose’ period (Spearman’s correlation: ‘off dose’ syrup consumption vs. dosage, $\rho = 0.21$, $N = 60$, $P = 0.11$; Figure 4.4), but pollen consumption significantly increased among colonies previously exposed to higher dosages (Spearman’s correlation: ‘off dose’ pollen consumption vs. dosage, $\rho = 0.40$, $N = 60$, $P = 0.001$; Fig. 4.4).

Taken over the entire 28-day pulsed exposure period, the amount of syrup and pollen consumed in experimental colonies declined as imidacloprid dosage increased (Spearman’s correlation: syrup consumption vs. dosage, $\rho = -0.47$, $N = 60$, $P < 0.001$; pollen consumption vs. dosage, $\rho = -0.25$, $N = 60$, $P = 0.05$; Figure 4.4), demonstrating that recuperation of food consumption was incomplete. From these results, EC₅₀ values were calculated to be 43.7 ppb ($R^2 = 0.50$) for reduced pollen consumption and > 98 ppb ($R^2 = 0.68$) for reduced consumption of syrup, while EC₁₀ values were 16.2 ppb ($R^2 = 0.60$) and 32.4 ppb ($R^2 = 0.78$) for pollen for syrup, respectively.

After using partial correlation analysis to control for the effects of dosage, brood production in experimental colonies increased with higher daily consumption of both syrup and pollen (Pearson’s partial correlation: brood vs. syrup

consumption, $r = 0.32$, $df = 58$, $P = 0.01$; brood vs. pollen consumption, $r = 0.59$, $df = 58$, $P < 0.001$).

4.4 Discussion

Under pulsed exposure to dietary imidacloprid, standardized colonies of *B. terrestris* bumble bees ‘on dose’ for 14 days exhibited dose-dependent repression of brood production, such that their productivity decreased as dosage increased up to 98 ppb. The removal of imidacloprid from colonies during the subsequent 14-day ‘off dose’ period produced dose-dependent recuperation of brood production to the extent that total productivity under pulsed exposure was not correlated with dosage up to 98 ppb. Pulsed exposure of colonies to dietary imidacloprid at 98 ppb produced the largest observed recuperation, but continuous exposure to the same concentration repressed brood production without recuperation during a separate experiment of equal duration. We therefore argue that recuperation is primarily achieved by the reversibility of imidacloprid-induced effects rather than acclimation to imidacloprid over time.

The dose-dependent decrease in brood production we observed in queenright colonies mirrors the effect on brood production in queenless microcolonies of *B. terrestris* workers over the same period of time [28]. Similarly, our EC₅₀ value for a 14-day exposure (1.44 ppb) is comparable to the EC₅₀ for imidacloprid’s effect on drone production in *B. terrestris* microcolonies exposed over eleven weeks (3.7 ppb) [26]. However, the recuperation of brood production in bumble

bee colonies we observed under pulsed exposure is a new finding. Other insects show recuperation from some imidacloprid-induced effects during pulsed exposure [35-38], but we are the first to demonstrate the resilience of an important demographic endpoint in bees. In our study, when imidacloprid exposure ceased, the ameliorating effect of recuperation on bumble bee brood production was such that the EC₅₀ for a 28-day pulsed exposure was raised beyond 98 ppb. However, we note that recuperation remained incomplete at higher doses, with overall brood productivity still reduced by between 19–32 % at dosages between 10–98 ppb. According to a recent guidance document for the risk assessment of plant protection products on bees [41], a reduction in this range would constitute a ‘medium’ colony-level-impact and could translate into a similar effect on colony size. Additionally, we found that oviposition was delayed during the ‘off dose’ period of pulsed exposure in colonies that were first presented with imidacloprid at higher dosages. Our results suggest that where bumble bees experience a pulsed exposure to residues of imidacloprid above 10 ppb [25], incomplete recuperation of brood production and delayed oviposition could detrimentally impact colony size and thereby influence colony fitness [30,31].

Consumption of syrup and pollen in our experimental colonies also underwent dose-dependent repression and recuperation during the ‘on dose’ and ‘off dose’ periods of pulsed exposure, respectively. Repression was most severe in pollen consumption, with an EC₅₀ of just 4.4 ppb, and both feeding endpoints showed incomplete recuperation at the two highest dosages (39 and 98 ppb). This result is somewhat consistent with a previous study of recovery in honey bees, in

which recuperation of foraging activity was incomplete in colonies exposed to imidacloprid at 48 ppb [34]. Since the pollen in our experiment was not dosed, the imidacloprid in the syrup reduced the bees' overall ability or desire to feed during the 'on dose' period. In a previous study, *B. terrestris* workers exposed to dietary imidacloprid in microcolonies exhibited dose-dependent feeding reductions that were also linked to reductions in brood production [28]. Consequently, it was hypothesized that imidacloprid-induced nutrient limitation might play some part in repressing bumble bee egg production during exposure [28]. Our data lend support to this hypothesis because they demonstrate that: a) queenright colonies that consumed more syrup and pollen produced more brood; b) bees showed dose-dependent reductions in feeding whilst 'on dose'; c) repression of brood production coincided with repressed feeding. Additionally, recuperation of food consumption and brood production in colonies occurred simultaneously when exposure ceased and we therefore suggest that removal of imidacloprid from the bees' diet caused feeding rates to recover, which re-established sufficient nutrient intake to facilitate reproduction in bumble bee queens. Although the mechanism for recuperation of food consumption was not studied here, we speculate that it has its basis in the metabolic elimination of the toxicant [42], which in a previous study appeared to take place within 48 hours in bumble bees fed imidacloprid at 98 ppb [43].

4.4.1 Comparison with results of semi-field trials

In our study, a two-week exposure to dietary imidacloprid at 10 ppb in syrup substantively reduced brood production in *B. terrestris* colonies. In a semi-field trial, Gill et al. [8] found that *B. terrestris* colonies also dosed with 10 ppb

imidacloprid solely in artificial nectar produced significantly fewer workers at the end of a four-week exposure, without suffering elevated levels of in-colony worker mortality. Although they did not measure egg production, Gill et al. found that imidacloprid-dosed colonies accumulated fewer larvae and pupae over 4 weeks and speculated that this was due to imidacloprid's effect on brood survival. Based on our findings, we hypothesize that repressed brood production may have been an important cause of Gill et al.'s observations.

In a second semi-field study, Whitehorn et al. [9] exposed *B. terrestris* colonies to field-realistic dosages of dietary imidacloprid for two weeks in the laboratory and monitored colony development for a further six weeks in the field. We exercise caution when comparing our observations to Whitehorn et al.'s because pollen was their principle delivery vehicle for imidacloprid. However, following a similar exposure duration and an extended imidacloprid-free period, Whitehorn et al. found no significant effect of imidacloprid on the number of pupae and workers in colonies, but a strong negative effect on the number of queens. Potentially, recuperation of brood and worker production occurred in Whitehorn et al.'s colonies when exposure ceased, but for some unknown reason any recovery was insufficient to sustain normal levels of queen production. Their observations may originate in either increased intoxication of the existing queen caused by consumption of contaminated pollen during lab exposure or the impact of a longer exposure to imidacloprid in the stored nectar and pollen within in the nest, which is important for successful development of new queens [44]. Additionally, if imidacloprid reduces the foraging efficiency of workers [8] then exposed colonies may lack sufficient resources to produce the

normal quota of queens, each of which comprises almost twice the biomass of a male bumble bee [30]. Furthermore, brood and worker production in bumble bee colonies may recover better following imidacloprid exposure than other important endpoints. We therefore suggest that the potential for recuperation of performance in demographically important endpoints other than brood production is an area requiring further research in bumble bees.

4.4.2 Environmental relevance

Whilst our study raises further concerns about the threat to wild bumble bees from imidacloprid it also indicates some resilience to a pulsed exposure that could arise during the synchronized bloom of a treated mass-flowering crop. However, when interpreting the environmental relevance of our findings we recognize the limitations of our study, which are as follows. First, the pollen consumed in our colonies was not dosed. There is no reason to suspect different levels of toxicity arising due to ingestion of imidacloprid in nectar vs. pollen, but a bumble bee queen is likely to eat a substantial pollen load whilst producing eggs [45] and consequently her exposure in the wild may be more severe than tested here.

Second, the duration of exposure in the environment may differ from our experiment. Exposure for 14 days is a reasonable first approximation because, for example, roughly 75 % of the flowering of winter-sown oilseed rape in the UK occurs over a peak period of about two weeks [32]. However, total flowering duration can extend across five weeks or more and bumble bee colonies may continue to forage on mass-flowering crops throughout their blooming period

[46]. Conversely, colonies will vary in the extent to which their development intersects with the blooming period of mass-flowering crops because bumble bee queens emerge from their overwinter sites and initiate colonies at various times in spring [47]. Consequently, colonies of later-emerging queens may develop after the crop's bloom has largely or completely declined and could broadly escape neonicotinoid effects.

Third, our study may underestimate the severity of imidacloprid's effects. For example, we focus primarily on brood production, but there are other demographically important endpoints such as mortality. A diet dosed with imidacloprid at realistically high levels (10 ppb) appears to raise mortality in colonies by increasing the risk that workers become lost whilst foraging and in addition exposed foragers tend to return to the nest with less pollen less often [8]. If these impacts also occur at lower dosages (< 10 ppb), which are more typically found in environmental nectar and pollen [21], they could certainly add to the stress on wild bumble bee colonies and diminish their reproductive output. Additionally, while the amount of brood and workers produced in a bumble bee colony can influence the quantity of new queens and males that are produced [30,31], the quality of sexual offspring produced may also be critical for colony fitness. For example, body mass predicts whether a young queen will survive diapause [48] and body size may impact on a male's mating success [49]. Furthermore, wild colonies are likely to be under additional stresses from pathogens [50], parasites [51] and other agrochemicals [8], which could augment the severity of a neonicotinoid's impact and the potential for recovery. Additive [8] and synergistic [52] effects of certain neonicotinoids and other

agrochemicals have been reported for bees, but further study into combinatorial effects of neonicotinoids and other potential stressors is necessary. Finally, under laboratory conditions winter honey bees appear to be less sensitive to imidacloprid than summer honey bees [53]. Although winter active bumble bees have been observed at latitudes as far north as southern England [54], unlike winter honey bees they are unlikely to be social foragers because bumble bee colonies typically perish in the autumn before newly mated queens enter hibernation [55]. Therefore, if seasonal differences in sensitivity exist in wild bumble bees, foragers from spring and late summer colonies would have to be compared. Commercially bred bumble bees, which were used in autumn and winter in our current study, are produced throughout the year. As these bees are reared under standardised conditions, it is unlikely that they would show seasonal variation in sensitivity to imidacloprid. However, the effects reported here could be more severe in wild colonies and in future work it would be important to compare the sensitivity of commercially reared and wild bumble bees.

4.5 Conclusions

Our study provides further evidence that dietary neonicotinoid pesticides in the environmentally realistic range can have detrimental effects on bumble bee health, specifically by repressing brood production and nutritional intake in queenright colonies. We also show, however, that bumble bees may be somewhat resilient to a pulsed exposure because they exhibit dose-dependent recuperation of brood production when exposure ends. We acknowledge that to

interpret the environmental relevance of our findings for wild bumble bee colonies additional studies are necessary. These should seek to establish whether recuperation from pulsed exposure to neonicotinoids occurs during extended exposures and for other demographically important endpoints besides brood production. Finally, the severity of imidacloprid's impact on bumble bees appears to be highly sensitive to its dietary level even within the currently recognized environmentally realistic range [21]. Unfortunately, this range is based on scant published data [56] and more widespread surveys of residues in crops and colonies, such as those recently begun in the USA [25], are therefore urgently required.

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Chapter Four: Figures and Tables

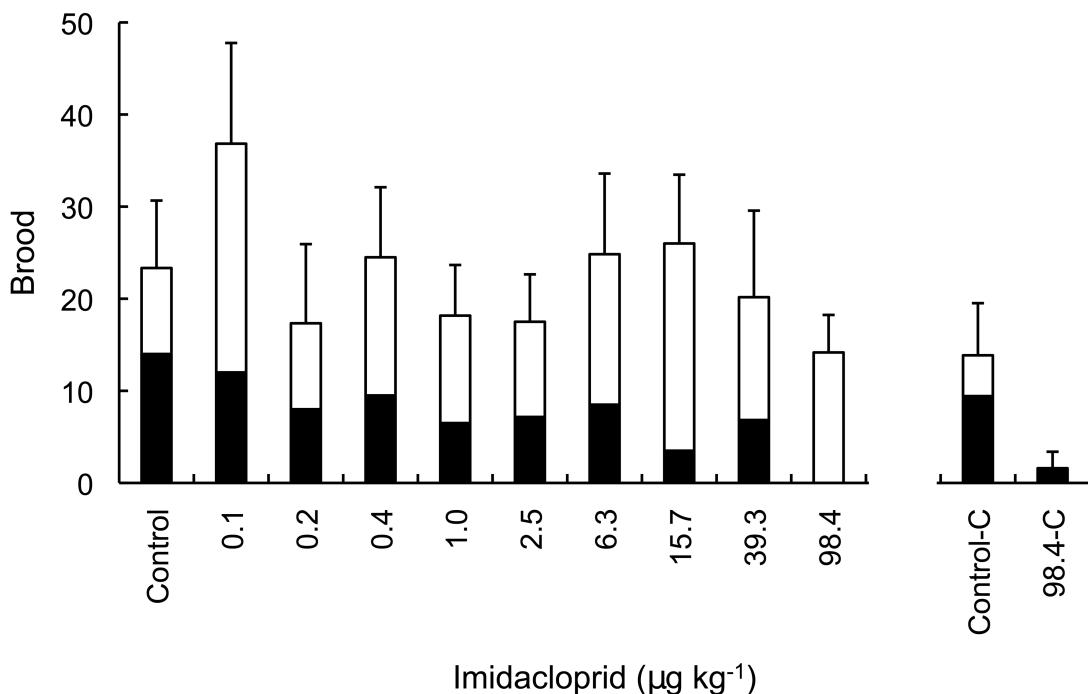


Figure 4.1. Brood production in *Bombus terrestris* colonies during a pulsed or continuous exposure to imidacloprid.

Mean number of brood produced in standardised *Bombus terrestris* colonies ($N = 60$) during 28-day pulsed or continuous exposure to dietary imidacloprid. For pulsed exposure (from left to right, 'Control' to '98.4'): brood produced during the 14-day 'on dose' period (black bars), during which colonies were exposed to imidacloprid in syrup at the specified dosage (in $\mu\text{g kg}^{-1}$ = parts per billion); and brood produced during the subsequent 14-day 'off dose' period (white bars), during which all colonies fed exclusively on control syrup. For continuous exposure ('Control-C' and '98.4-C'): brood produced during first 14 days of exposure (black bars) and brood produced during second 14 days of exposure (white bars). Where a column does not contain a black bar or a white bar, zero brood were produced during days 1-14 or days 15-28, respectively. Error bars indicate $\pm \text{SE}$ of mean brood production over 28 days.

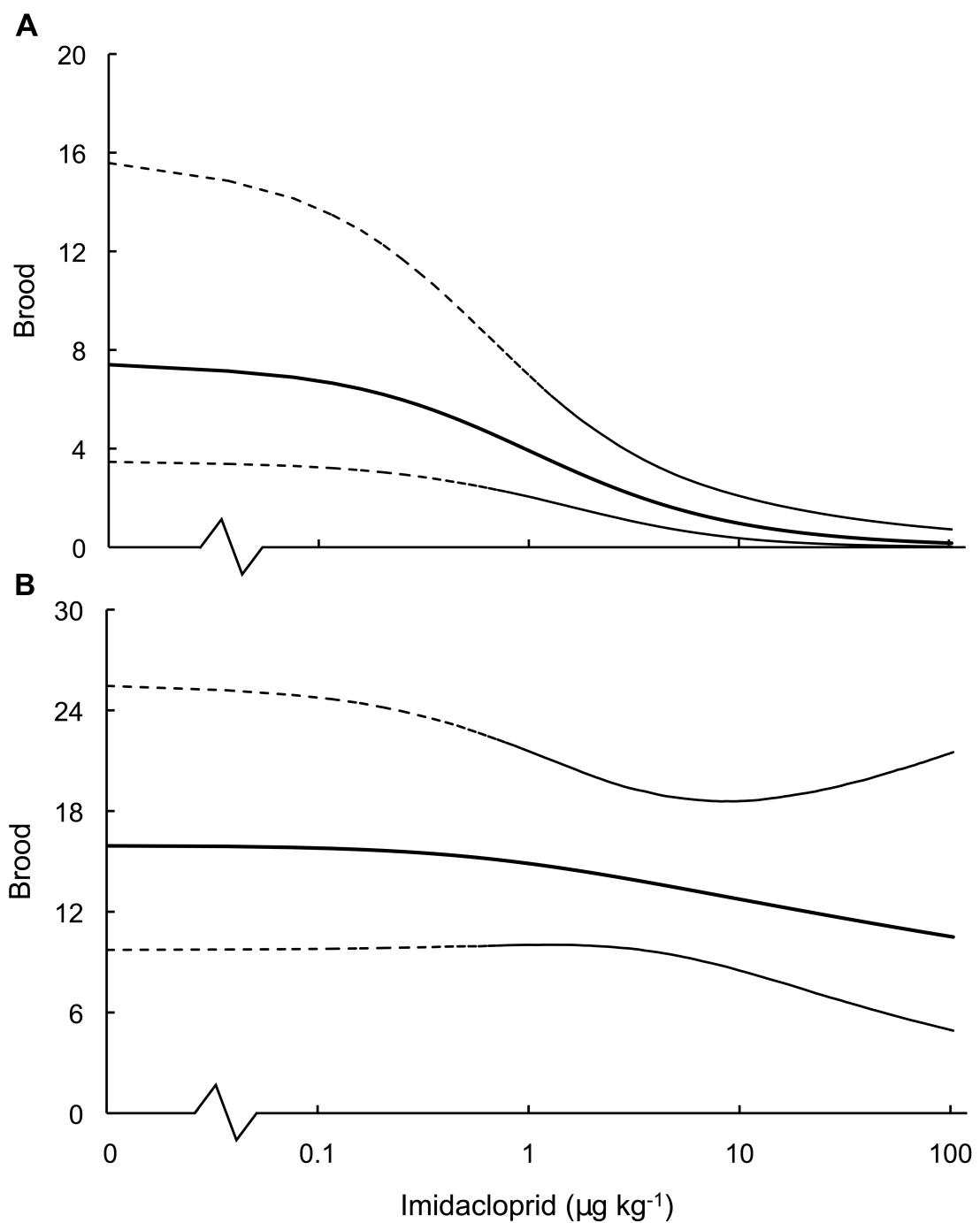


Figure 4.2. Best-fit dose-response relationships of brood production in *Bombus terrestris* colonies under pulsed exposure to imidacloprid.

Dose-response relationships of brood production in standardised *Bombus terrestris* colonies ($N = 60$) following a 28-day pulsed exposure to dietary imidacloprid in syrup. Specifically, (A) brood production during the 14-day ‘on dose’ period of pulsed exposure in which bees fed on syrup dosed with imidacloprid and (B) total brood production taken over the entire 28-day pulsed exposure (including brood produced during the 14-day ‘on dose’ period and during the subsequent 14-day ‘off dose’ period in which imidacloprid was removed from the bees’ diet). Solid lines indicate the best-fit dose response relationship (obtained using Bayesian Hierarchical Modelling of the data summarized in Figure 1, see Methods) and dashed lines indicate the relationship’s 95 % confidence intervals.

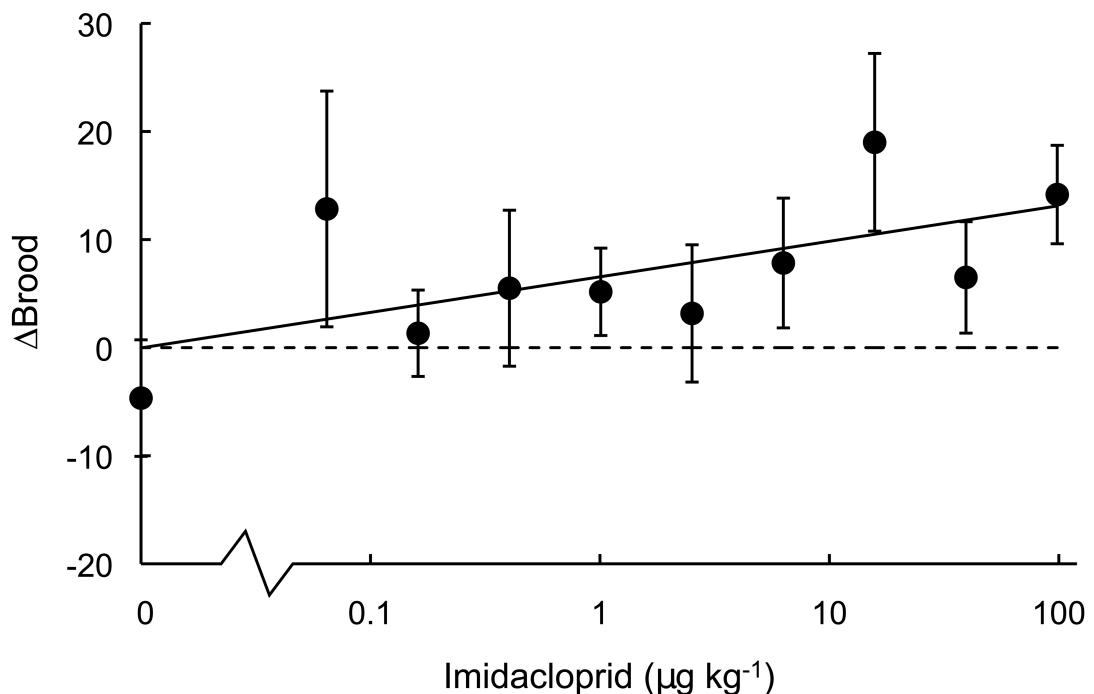


Figure 4.3. Recuperation of brood production in *Bombus terrestris* colonies during a pulsed exposure to imidacloprid.

Recuperation of brood production in standardised *Bombus terrestris* colonies ($N = 60$) during the 14-day ‘off dose’ period of pulsed exposure, wherein bees fed exclusively on undosed control syrup. The ‘off dose’ period followed a 14-day ‘on dose’ period during which bees fed on syrup dosed with imidacloprid at the given concentrations (in $\mu\text{g kg}^{-1}$ = parts per billion). Recuperation (ΔBrood) is determined by analyzing the difference in brood production between the ‘on dose’ (days 1–14) and ‘off dose’ (15–28) periods, specifically: $\Delta\text{Brood} = \text{Brood}_{\text{off}} - \text{Brood}_{\text{on}}$, with a positive value indicating increased production of brood when ‘off dose’. Data represent the means and error bars indicate $\pm \text{SE}$. The solid line indicates the following logarithmic trend: $\Delta\text{Brood} = 1.428 \times \ln(\text{dosage}) + 6.533$, $R^2 = 0.38$. Dashed line indicates $\Delta\text{Brood} = 0$.

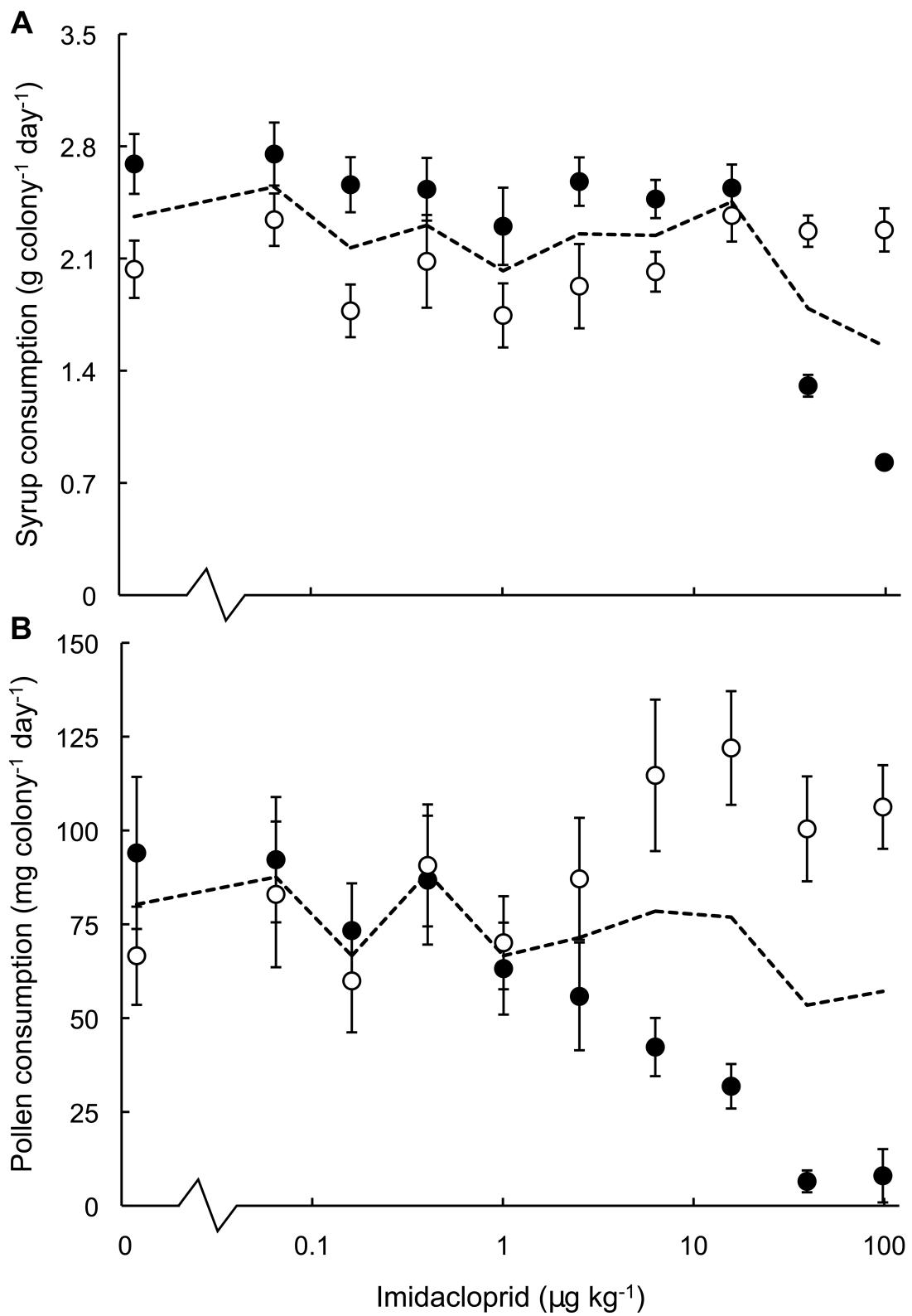


Figure 4.4. Food consumption in *Bombus terrestris* colonies during a pulsed exposure to imidacloprid.

Feeding responses of standardised *Bombus terrestris* colonies ($N = 60$) during a 28-day pulsed exposure to dietary imidacloprid. Specifically, (A) mean daily syrup and (B) mean daily pollen consumption during the initial 14-day ‘on dose’ period feeding on imidacloprid dosed syrup (filled circles) and during the subsequent 14-day ‘off dose’ period feeding on undosed control syrup (unfilled circles). Dashed lines connect the mean consumption rates of colonies over the entire 28-day pulsed exposure. Error bars indicate \pm SE. Control data (zero $\mu\text{g kg}^{-1}$) are displayed slightly displaced on the x-axis for ease of inspection.

Table 4.1. Estimated decrease in brood production exhibited by *Bombus terrestris* colonies during pulsed exposure to realistic imidacloprid residues, equivalent to those previously detected in nectar of treated crops.

Realistic exposure scenario	Imidacloprid residue (ppb)	14-day 'on dose' brood reduction (%) ^a	28-day pulsed exposure brood reduction (%) ^b
OSR–Europe ^c	0.3	18 (14–24)	2 (0–6)
OSR–USA ^c	0.8	37 (30–45)	5 (0–12)
Mean max. level ^d	1.9	56 (51–64)	9 (0–19)
Gill et al. ^e	10.0	84 (84–86)	18 (9–27)

Reductions are relative to the number of brood produced in undosed control colonies and were obtained using the appropriate BHM best-fit dose-response relationship from Figure 2. The reduction's 95% confidence intervals, given in parentheses, were also obtained from BHMs in Figure 2

^a Refers to the estimated decrease in brood production expected after a 14-day exposure to imidacloprid at the given dosage

^b Refers to the estimated total decrease in brood after a 28-day pulsed exposure at the given dosage (14 days 'on dose', 14 days 'off dose')

^c Maximum imidacloprid residues detected in the nectar of oilseed rape [21]. Data originates from studies conducted only in Member States of the European Union (OSR–Europe) and from studies including North America (OSR–USA)

^d Mean maximum level of neonicotinoid residues in nectar calculated from 20 studies [56]

^e Residues in dosed syrup used in a semi-field trial conducted by Gill et al. [8]

Table 4.2. Mean number of days taken by *Bombus terrestris* queens to undertake oviposition during pulsed exposure to dietary imidacloprid.

Imidacloprid dosage ($\mu\text{g kg}^{-1}$ = ppb)	On dose: day of first oviposition ($\pm \text{SE}$)	Off dose: day of first oviposition ($\pm \text{SE}$)
Control	4.2 (1.1)	1.3 (0.3)
0.1	2.6 (1.1)	2.8 (0.9)
0.2	5.0 (1.5)	6.0 (1.9)
0.4	2.8 (1.2)	1.5 (0.4)
1.0	3.0 (1.3)	4.2 (1.4)
2.5	10.3 (0.3)	6.0 (2.1)
6.3	3.8 (1.9)	6.0 (2.1)
15.7	11.0 (0.0)	5.7 (1.6)
39.4	2.3 (1.0)	7.8 (1.7)
98.4	— ^a	7.2 (1.2)

Oviposition occurred in standardised experimental colonies (queen and four workers) during either the 14-day 'on dose' period of pulsed exposure (during which bees fed on syrup dosed with dietary imidacloprid at the given concentration) or the subsequent 14-day 'off dose' period (when all imidacloprid dosages were removed from the bees' diet)

^a Oviposition did not occur during the 'on dose' period in colonies exposed at 98.4 ppb

Chapter Five

The effects of piperonyl butoxide, a cytochrome P450 enzyme inhibitor, on the toxicity of neonicotinoid pesticides at field-realistic concentrations in bumble bees (*Bombus terrestris*) and honey bees (*Apis mellifera*)

Preliminary introduction

The following chapter contains a paper intended for submission to the journal *Apidologie*. The content below is therefore presented in the *Apidologie* format, except that ambiguous references are lettered (a, b, etc) and the sections, figures and table of the paper are numbered according to their position within this thesis. Where I cite Laycock et al. (2012) or Laycock and Cresswell (2013), I indicate that I am also referring to chapters two and four of the thesis, respectively.

Statement of contribution

As first author, I was primarily responsible for conception and design of the work, acquisition and analysis of the data, and writing the paper. My supervisor and co-author Dr James Cresswell provided advice on experimental design and data analysis. Will Morgan and Kacie Thomson received co-author credits because they were involved in the work as part of their undergraduate research projects. Specifically, they assisted with data collection during the dietary imidacloprid trial in which bumble bees were tested (see Materials and methods). Additional contributors are acknowledged at the end of the paper: Prof. Colin Walker was consulted prior to the experiment for his ecotoxicology expertise, providing advice on the correct and safe use of piperonyl butoxide as an insecticide synergist; MSc students Yueru Li and Jonathan Wheeler were involved in data collection for the pilot studies that preceded (and contributed towards the design of) the main experiment presented here; Prof. Charles Tyler provided constructive comments on the final draft of the manuscript.

The effects of piperonyl butoxide, a cytochrome P450 enzyme inhibitor, on the toxicity of neonicotinoid pesticides at field-realistic concentrations in bumble bees (*Bombus terrestris*) and honey bees (*Apis mellifera*)

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Keywords

Apis / Bombus / cytochrome P450 / neonicotinoid / piperonyl butoxide

Abstract

While the resilience of honey bees to certain pesticides involves cytochrome P450 enzymes (P450s) that metabolise absorbed or ingested toxicants, detoxification mechanisms in other bee species, including bumble bees, remain largely unstudied. Although neonicotinoid pesticides can detrimentally affect their health, both honey bees and bumble bees are somewhat resilient to ingested residues in the field-realistic range and above ($\sim 0.1\text{-}100 \mu\text{g kg}^{-1}$). In order to investigate the role of P450s in the metabolism of neonicotinoids, we assessed the effects of a P450 inhibitor in combination with imidacloprid or thiacloprid in *Bombus terrestris* bumble bees, and we studied *Apis mellifera* honey bees in parallel for comparison. We found that: (a) bumble bees were more sensitive than honey bees to ingestion of imidacloprid or thiacloprid; (b) bumble bees were more sensitive to imidacloprid when exposed *via* their diet compared to topical exposure; and (c) piperonyl butoxide (PBO), the P450-inhibiting insecticide synergist, did not substantively enhance the toxicity of either imidacloprid or thiacloprid in bees that ingested concentrations up to $98 \mu\text{g kg}^{-1}$. Our results suggest that P450s are not an important mechanism in bees for metabolism of dietary imidacloprid or thiacloprid in the field-realistic range.

5.1 Introduction

In an evolutionary arms race spanning four hundred million years, the deleterious effects of toxins defensively deployed by plants are continuously countered by insect herbivores *via* the development of resistance to these toxins (Herrera and Pellmyr 2009). Taking inspiration from natural plant toxins (so called allelochemicals), the agricultural industry develops synthetic toxins that are deployed as pesticides on crops vulnerable to insect pests. The widespread, often prophylactic, use of these agrochemicals has exerted additional selection pressure on grazing insect populations to the extent that hundreds of species have developed pesticide resistance (Berenbaum 1995; Feyereisen 1995; Heckel 2012). In insects, mechanisms of resistance manifest as changes in: a) behaviour (e.g. avoidance of the toxin); b) physiology (e.g. degradation of the toxin); or c) a combination of both (Després et al. 2007). Commonly, pesticide resistance involves the insect increasing its metabolic capability, for example by overproduction of detoxification enzymes that catabolise the xenobiotic to a less toxic form (Li et al. 2007). These enzymes include the cytochrome P450 monooxygenases (P450s, or CYPs for encoding genes), which are particularly well characterised in insects (Schuler 2011; Feyereisen 2012).

When taken together, the genomes of several insect species reveal almost two thousand CYP gene sequences (Schuler 2011). The P450 enzymes that they encode are responsible for the oxidative metabolism of a diverse variety of substrates, including endogenous substrates such as juvenile hormone and pheromones, and exogenous substrates such as allelochemicals and

xenobiotics (Scott and Wen 2001). In several insects, resistance to specific pesticides results from the activity of these genes and enzymes. For example, in *Drosophila melanogaster* Meigen a single CYP gene, *CYP6G1*, confers resistance to the organochlorine insecticide DDT (Daborn et al. 2002). Likewise in the house fly *Musca domestica* L., up-regulation of *CYP6D1* provides resistance to pyrethroid insecticides (Scott et al. 1998). Genes from the CYP6 and CYP9 superfamilies are also involved in resistance to pyrethroids in agricultural pest species such as the corn earworm *Helicoverpa zea* Boddie (Li et al. 2004) and disease vectors such as the mosquitoes *Anopheles gambiae* Giles and *Aedes aegypti* L. (Poupardin et al. 2010; David et al. 2013). While many of these insects have a large repertoire of P450s providing them excellent protection from pesticides (e.g. over one hundred in *A. aegypti*; Strode et al. 2008), others insects apparently lack this diversity. The genome of the honey bee *Apis mellifera* L. contains just 46 P450 encoding genes (Claudianos et al. 2006). It has been speculated that this apparent paucity of detoxification genes contributes to the honey bee's sensitivity to agricultural chemicals (Claudianos et al. 2006). However, the P450s that honey bees do possess clearly function to metabolise some pesticides, including pyrethroids, in-hive miticides and certain neonicotinoids (Pilling et al. 1995; Iwasa et al. 2004; Johnson et al. 2006, 2009b). The number of P450s available to other bee species is largely unstudied. The genome of the buff-tailed bumble bee, *Bombus terrestris* L., has recently been sequenced and published (Stolle et al. 2011) and this particular bumble bee is beginning to emerge as a model species. Like *A. mellifera*, *B. terrestris* is an important pollinator that appears to be susceptible to agricultural pesticides (Mommaerts and Smagghe 2011). Whether *B. terrestris* deploy

P450s in response to pesticide exposure is unknown, but in our previous work we found that colonies were somewhat resilient to dietary concentrations of the neonicotinoid pesticide imidacloprid up to $98 \mu\text{g kg}^{-1}$ (= parts per billion or ppb) (Laycock and Cresswell 2013 [see chapter four]). Furthermore, individual *B. terrestris* workers can eliminate these large concentrations of imidacloprid from their bodies within 48 hours (Cresswell et al. 2013), suggesting that a system of metabolic degradation exists within the bumble bee. We therefore investigated the possibility that P450 enzymes are involved in metabolic detoxification of neonicotinoid pesticides in *B. terrestris* bumble bees.

Neonicotinoid pesticides provide plant protection across many agricultural landscapes because, as agonists on the insect nicotinic acetylcholine receptor (nAChR), they cause paralysis and death to insect pests when absorbed or ingested (Jeschke et al. 2011). Until recently neonicotinoids were used in 120 countries (Jeschke et al. 2011), but mounting evidence of their detrimental affect on the health and performance of bees (Cresswell 2011; Blacquière et al. 2012; Gill et al. 2012; Henry et al. 2012a; Whitehorn et al. 2012) has led to restrictions on their use in agriculture (European Commission 2013; Maxim and van der Sluijs 2013). Despite these restrictions, neonicotinoids are considered by some to be highly successful pesticides because they provide effective pest control with a low incidence of resistance compared to older classes of pesticide (Jeschke and Nauen 2008). While resistance to neonicotinoids remains relatively low, there is the potential to develop resistance in species that represent the core target for the pesticides (Jeschke et al. 2011). For example, some resistance to imidacloprid is reported in the Colorado potato beetle

Leptinotarsa decemlineata Say (Zhao et al. 2000), the greenhouse whitefly *Trialeurodes vaporariorum* Westwood (Gorman et al. 2007), and the brown planthopper *Nilaparvata lugens* Stål (Gorman et al. 2008). Where resistance develops, it is more commonly associated with enhanced metabolic detoxification by P450s – largely responsible for Phase I metabolism of neonicotinoids – than with nAChR mutants or variants (Casida 2010). Resistance to neonicotinoids is rarely studied in bees. However, metabolic studies in honey bees report that imidacloprid and its toxic metabolites, olefin and 5-hydroxy-imidacloprid, are metabolized using only Phase I enzymes (Suchail et al. 2004a). These enzymes may include P450s because pre-treatment with piperonyl butoxide (PBO), a well-known insecticide synergist and P450 enzyme inhibitor (Hodgson and Levi 1998), can increase the toxicity of neonicotinoids in individual honey bees (Iwasa et al. 2004). The extent to which the toxicity of a neonicotinoid is enhanced by PBO in honey bees appears to vary according to the chemical structure of the compound because the toxicity of *N*-cyanoamidine neonicotinoid varieties (e.g. thiacloprid and acetamiprid) is greatly enhanced compared to *N*-nitroguanidines (e.g. imidacloprid) (Iwasa et al. 2004). Here, we present a study of the role that P450 enzymes play in the metabolism of neonicotinoids in *B. terrestris*. Specifically, we investigate the effects of inhibiting P450s with PBO whilst exposing bumble bees to imidacloprid and thiacloprid; two neonicotinoids with different chemical structures.

In previous studies in which P450s were inhibited, bees were tested in LD₅₀ assays where mortality was assessed 24 hours after topical treatment with PBO

followed by a pesticide at a relatively high dose (Iwasa et al. 2004; Johnson et al. 2006, 2009b). However, in the current study we are particularly interested in understanding whether P450s contribute towards the resilience we have previously observed in bees to environmentally relevant dietary concentrations of neonicotinoid in the range from 0.1 to 98 $\mu\text{g kg}^{-1}$ (Cresswell et al. 2012b, 2013; Laycock and Cresswell 2013 [see chapter four]). Using a typical LD₅₀ mortality/ topical exposure assay was therefore not appropriate here because: a) neonicotinoids in the field-realistic concentration range (EFSA 2012) produce sublethal rather than lethal effects, even following an exposure that lasts several weeks (Mommaerts et al. 2010b; Cresswell 2011; Laycock et al. 2012 [see chapter two]; Whitehorn et al. 2012; Laycock and Cresswell 2013 [see chapter four]); b) we will primarily dose *via* diet; a route of neonicotinoid exposure that is typical in the wild *via* the nectar and pollen consumed by bees while they forage from treated crops (Blacquière et al. 2012). The alternative assay tested here therefore involved chronic oral exposure to syrup dosed with PBO and with neonicotinoids in the concentration range up to 98 $\mu\text{g kg}^{-1}$. If bumble bees metabolize dietary neonicotinoids in the realistic range using P450 enzymes, we would expect to find an interactive effect, and possibly synergism, between PBO and the neonicotinoid that raises the pesticide's toxicity and increases the severity of sublethal effects on the bee. Since an equivalent study incorporating realistic dietary concentrations is lacking in honey bees, we also conduct a similar but separately run assay with *A. mellifera*. In addition, because information on topically applied neonicotinoids and PBO in bumble bees is limited, we also included a small topical dosage experiment in order to

compare P450 activity in bumble bees exposed to neonicotinoids *via* different routes (i.e. oral *vs.* topical).

5.2 Materials and methods

5.2.1 Chemicals

Imidacloprid was obtained as a solution in acetonitrile (Dr. Ehrenstorfer GmbH, Ausberg, Germany). Acetonitrile was removed by evaporation and the residuum imidacloprid was dissolved in purified water to produce a stock concentration of $10^4 \mu\text{g L}^{-1}$. Analytical standard thiacloprid and PBO (both Pestanal, Sigma-Aldrich, UK) were obtained as powder and liquid, respectively. Thiacloprid was dissolved in purified water to stock concentrations of 10^4 and $10^5 \mu\text{g L}^{-1}$, while PBO was dissolved in acetone (Fisher Scientific UK Ltd, Loughborough, UK) to $10^8 \mu\text{g L}^{-1}$.

5.2.2 Bees

Bumble bees, *B. terrestris* (subspecies *audax*), were obtained from a commercial supplier in colonies consisting of approximately 150 workers (Natopol Beehive; Koppert B.V., Berkel en Rodenrijs, Netherlands). In total, five colonies were obtained and used as described in separate experiments, below. To collect bumble bees, colonies were released into a small flight cage (dimensions: 100 cm × 75 cm × 75 cm) from which adult workers were caught in glass vials and individually caged in wooden boxes (cage dimensions: 65 mm × 50 mm × 35 mm, with the two largest faces made of plastic mesh; Cresswell et al. 2012b).

Groups of adult honey bee workers, *A. mellifera*, were collected in plastic vials on their day of use from hives maintained at the University of Exeter, UK. In the laboratory, these vials were chilled on ice and when the bees were immobile they were removed from the vials and caged in wooden boxes (dimensions: 100 mm × 80 mm × 18 mm, with the two largest faces made of fine netting; Cresswell et al. 2012b). Honey bees were caged in groups of approximately eight (mean = 8.2, SE = 0.1) because they are somewhat dependent on a social context for survival in the laboratory (Cresswell et al. 2012b). Once caged, honey bees were given approximately 1 hour to recover from the chilling process and any bees that were not walking within that time were removed from their box. Each honey bee group contained individuals from a single hive.

All bumble bee and honey bee boxes were fitted with a single 2 mL microcentrifuge tube that was punctured to function as a syrup (artificial nectar) feeder. In all bumble bee assays described below, we first quantified the intrinsic variation in individual feeding rate due to variation in body size (Cresswell et al. 2012b) by maintaining bees on a diet of undosed control syrup (Attracker: 1.27 kg L⁻¹ fructose/glucose/saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) for three days before neonicotinoid dosing took place. The intrinsic variation in feeding rate among individual honey bees is negligible (Cresswell et al. 2012b), so they were dosed on the day they were caged. In all experimental assays, bees were maintained in similar semi-controlled environments (24-27 °C, 23-43 % relative humidity, 10:14 h of light:darkness).

5.2.3 Combined dietary imidacloprid and PBO assay

In the dietary imidacloprid assay, we used 120 individually boxed bumble bees taken from two separate source colonies and 120 boxes of honey bees collected from hives at University of Exeter. Bees were assigned at random to one of four treatment groups – *imidacloprid plus PBO*, *imidacloprid only*, *PBO only*, and *solvent control*. In each of the four treatments, bees were fed *ad libitum* on syrup dosed at one of ten concentrations (nine experimental doses and an undosed control group) as described below. Each treatment consisted of 30 boxes, comprising three boxes per concentration. The experiment was conducted for honey bees in June 2011 and bumble bees in October 2011.

In the *imidacloprid plus PBO* treatment, we produced our most concentrated dosage by mixing imidacloprid stock and PBO stock together into syrup at a concentration ratio of 1:10,000, imidacloprid:PBO, with imidacloprid at $125 \mu\text{g L}^{-1}$ (or $98.43 \mu\text{g kg}^{-1}$) and PBO at $1,250,000 \mu\text{g L}^{-1}$. The imidacloprid:PBO ratio was approximately equivalent to the ratio of pyrethroid:PBO that produced LD₅₀ values in a previous study of honey bees (Johnson et al. 2006). By serial dilution (dilution factor = 0.4) beginning with the most concentrated dosage we produced the following nine experimental dosages with imidacloprid in the mixture at the following concentrations (and PBO concentrations 10,000 times higher than imidacloprid): 125.00, 50.00, 20.00, 8.00, 3.20, 1.28, 0.51, 0.20, and $0.08 \mu\text{g imidacloprid L}^{-1}$ ($= 98.43, 39.37, 15.75, 6.30, 2.52, 1.01, 0.40, 0.16$, and $0.06 \mu\text{g imidacloprid kg}^{-1}$ or ppb). We kept the imidacloprid:PBO ratio constant across the concentration range to account for competitive interactions between imidacloprid and PBO as substrates for the P450 enzymes (Ankley et

al. 1991), i.e. each P450 had an equal probability of binding a neonicotinoid or PBO molecule at each concentration in the range. In the *imidacloprid only* treatment we mixed imidacloprid stock and acetone into syrup and by serial dilution produced nine dosages at the same concentrations described above, but without PBO. The acetone was added because the *imidacloprid plus PBO* treatment contained a small amount as a solvent from the PBO stock. In the most concentrated *imidacloprid only* dosage (125 µg imidacloprid L⁻¹) acetone was added at 11.25 mL acetone L⁻¹ syrup, which was equivalent to the concentration of acetone in the highest dosage of *imidacloprid plus PBO*, before the acetone and imidacloprid mixture was serially diluted (dilution factor = 0.4; this produced the following acetone concentrations: 11.25, 4.5, 1.8, 0.72, 0.29, 0.12, 0.05, 0.02, and 0.01 mL L⁻¹). In the *PBO only* treatment we mixed PBO stock into syrup to produce the most concentrated dosage of 1,250,000 µg L⁻¹ and serially diluted from this dosage in the usual way to produce the nine experimental dosages equivalent to PBO concentrations in *imidacloprid plus PBO* treatment. In the *solvent control* treatment we established the effect of acetone in syrup at the nine concentrations of acetone described above (i.e. 11.25–0.01 mL L⁻¹).

In all treatments, feeders were weighed daily to measure syrup consumption. We stopped measuring syrup consumption because of high mortality rates in some assays after 10 days for bumble bees and 6 days for honey bees, but continued to provide fresh syrup at the appropriate concentration for as long as bees survived. Syrup consumption was corrected for evaporation of water from syrup based on the mass change of ten feeders kept in empty boxes under

experimental conditions. We monitored individual mortality daily and removed dead honey bees from their groups within 24 h of death. To quantify locomotory activity, bumble bees were observed on seven successive occasions at 30 minute intervals and each bee was scored as moving or stationary on each occasion. Bumble bee locomotion was observed on the 10th day of dosing, and following locomotory scoring we calculated the proportion of total observations in which the bumble bee was in motion. For honey bee locomotion, we filmed each box for approximately 1 min and, during playback, counted the number of honey bees in motion at 10 s intervals. We calculated the proportion of honey bees in motion in each cage at each 10 s interval and then calculated the mean proportion of honey bees in motion over 1 min. We filmed honey bee locomotory activity on the 2nd dosage day (sooner than in bumble bees because of the honey bees' shorter life expectancy in the laboratory, see Results).

5.2.4 Combined dietary thiacloprid and PBO assay

In the dietary thiacloprid assay, we used 72 individually caged bumble bees from two source colonies and 72 boxes of honey bees. The experiment was conducted using identical methods and husbandry techniques as described in the dietary imidacloprid assay, except that in each of the four treatment groups (*thiacloprid plus PBO*, *thiacloprid only*, *PBO only*, and *solvent control*) we used six (rather than ten) concentrations (five experimental dosages and an undosed control) replicated three times each. The concentrations of thiacloprid in the mixture were: 125.00, 50.00, 8.00, 1.28, and 0.20, µg thiacloprid L⁻¹ (= 98.43, 39.37, 6.30, 1.01, and 0.16 µg kg⁻¹). Where they were tested, concentrations of PBO were again 10,000 times higher than those of the neonicotinoid and

concentrations of acetone were 11.25, 4.5, 0.72, 0.12, and 0.02 mL L⁻¹. The dietary thiacloprid assay was conducted in November 2012 for bumble bees and June 2013 for honey bees.

Thiacloprid has a low toxicity when topically applied to honey bees (Iwasa et al. 2004), but information on dietary thiacloprid's toxicity to both bumble bees and honey bees is lacking. Therefore, in addition to the four treatment groups described above, we included a fifth treatment in the thiacloprid assay – *thiacloprid without acetone*. This treatment group was included to test the effects of dietary thiacloprid without the addition of acetone on bumble bees and honey bees at concentrations up to 32,298 µg L⁻¹ (= 25,431 µg thiacloprid kg⁻¹). In bumble bees, the *thiacloprid without acetone* treatment consisted of 30 bees feeding on syrup dosed at the eight concentrations (those six already described plus 9,688 and 25,431 µg kg⁻¹). In honey bees, 23 boxes of bees fed at seven thiacloprid concentrations (the usual six doses plus 25,431 µg kg⁻¹). In the *thiacloprid without acetone* treatment each thiacloprid concentration was replicated at least three times for each species.

5.2.5 Combined topical neonicotinoids and PBO assay

We included a small topical neonicotinoid dosage experiment in order to facilitate a comparison of P450 activity in topically and orally exposed bumble bees. We used a total of 87 *B. terrestris* workers taken from a single source colony. Before we began dosing, the intrinsic variation in feeding rate was measured over three days (as described above) and then bees were randomly assigned to one of the following ten treatment groups: 7 ng imidacloprid; 7 ng

imidacloprid + 100 µg PBO; 18 ng *imidacloprid*; 18 ng *imidacloprid* + 100 µg PBO; 15 ng *thiacloprid*; 15 ng *thiacloprid* + 100 µg PBO; 14.6 µg *thiacloprid*; 14.6 µg *thiacloprid* + 100 µg PBO; 100 µg PBO control; acetone control.

Dosage treatments were produced by dissolving stock solutions in acetone to obtain the appropriate dosage in 1 µL of acetone. For each treatment that included a neonicotinoid, we determined the amount of active ingredient to be applied to a bee as follows. The lowest doses (i.e. 7 ng *imidacloprid*, 15 ng *thiacloprid*) were equivalent to a bumble bee's mean daily intake of imidacloprid or thiacloprid whilst feeding on syrup dosed at 98 µg kg⁻¹ during the dietary exposure assays presented here. The highest doses (18 ng *imidacloprid*, 14.6 µg *thiacloprid*) were equivalent to the LD₅₀ reported for a topical application of the appropriate neonicotinoid in honey bees (Iwasa et al. 2004). In order to topically dose bumble bees, they were removed from their box and immobilised inside a marking cage (Dadant & Sons Inc, Hamilton, USA). Topical applications at the appropriate dosage were delivered in 1 µL of acetone to the dorsal thorax using a Gilson MICROMAN M10 positive-displacement pipette (Gilson Inc., Middleton, USA). Bees were kept immobilised in the marking cage for approximately 2 min until the applied solvent was no longer visible on the thorax and was assumed to have been absorbed. The acetone control received 1 µL of acetone only. PBO was topically applied at 100 µg per bee, which is a dosage equivalent to the maximum sublethal topical dosage previously determined for honey bees (Johnson et al. 2006) and similar to the daily intake of PBO in bumble bees feeding on syrup dosed at 984,300 µg PBO kg⁻¹ (the highest concentration in the PBO only treatment) in the dietary assays presented here. Once dosed, bees were re-caged and fed *ad libitum* for 24 h on

control syrup. Syrup consumption and individual mortality were assessed after 24 h. To quantify locomotory activity, bumble bees were observed on ten successive occasions at 30 minute intervals and each bee was scored as moving or stationary on each occasion. Scoring locomotion took place in the final 5 h of the 24 h observation period. Each treatment group began with a minimum of 8 bees and bees were approximately equally represented across treatments. The topical exposure assay was conducted in March 2013.

5.2.6 Statistical analyses

For the dietary exposure assays, we tested whether syrup consumption and longevity responded to the concentration of neonicotinoid/PBO/acetone (*dosage*) and, where appropriate, whether dose-response relationships differed between treatment groups (*treatment*: e.g. *neonicotinoid plus PBO*, *neonicotinoid only*) using analysis of covariance (ANCOVA). Where dose-response relationships did not meet ANCOVA test assumptions, we instead used analysis of variance (ANOVA) with *dosage* treated as a categorical variable. For locomotory activity, we tested whether the proportion of locomotion varied with *dosage* and *treatment* by generalised linear model (GLM) using methods described by Crawley (2007), including a quasibinomial error structure to account for overdispersion in data. Where the locomotory activity dose-relationship was manifestly non-linear, *dosage* was treated as a categorical variable in the GLM. In the models, the main effects were *dosage*, which was transformed to $\log_{10}(\text{dosage in } \mu\text{g kg}^{-1} + 1)$ and used as the covariate, and *treatment*, which was the fixed factor. We included the interaction between the two (*dosage* \times *treatment*) in the model to determine whether the effects of

dosage on syrup consumption/longevity/locomotion depended on *treatment* (i.e. depended on the presence or absence of PBO). Where we found a significant interaction, we concluded that adding PBO to a neonicotinoid changed the neonicotinoid's dose-response relationship and, therefore, altered its toxicity. Where appropriate, we conducted model simplification as described in Crawley (2007).

For the topical exposure assay, we tested whether syrup consumption and locomotion varied with treatment in bumble bees using Mann-Whitney U tests to account for the low sample size and use of proportion data.

For bumble bees, syrup consumption was corrected for intrinsic variation among individuals as described by Cresswell et al. (2012b). Specifically, for each experiment we regressed the post-dose mean daily feeding rate ($\text{mg bee}^{-1} \text{ day}^{-1}$) on the pre-dose feeding rate, which explained approximately 25% of variation in the dietary imidacloprid assay ($R^2 = 0.26$, $F_{1,124} = 43.7$, $P < 0.001$), 60% in the dietary thiacloprid assay ($R^2 = 0.61$, $F_{1,82} = 130.4$, $P < 0.001$), and 45% in the topical assay ($R^2 = 0.45$, $F_{1,82} = 66.3$, $P < 0.001$). The adjusted post-dose feeding rate for each individual was expressed relative to the performance of an average bee by adding the individual's residual from this least-squares regression to the mean rate of post-dose feeding among all bees (Cresswell et al. 2012b).

In order to determine whether P450s were likely to be involved in metabolism of imidacloprid and thiacloprid, we first tested whether PBO altered the toxicity of a

neonicotinoid to bees by modelling the interaction *dosage* × *treatment* (see above). Where a significant interaction was detected, we then tested whether the change in toxicity was produced additively (by addition of the individual toxicities of the neonicotinoid and PBO) or synergistically (by interaction between the neonicotinoid and PBO to produce a toxicity substantially greater than sum of the individual toxicities). However, whilst interaction effects were detected in our analyses (see Results), we concluded that the magnitude of these effects was not sufficient to justify the term ‘synergy’. Therefore, here we defined an interaction that produced toxicity greater than expected from the sum of individual toxicities as ‘more-than-additive’, and we detected and determined the magnitude of ‘more-than-additive’ effects in two scenarios as follows. In the first scenario, for any given endpoint, where the dose-response relationships for *neonicotinoid only* and *neonicotinoid plus PBO* treatments were significantly different (i.e. there was a significant interaction for *dosage* × *treatment*, and therefore PBO significantly altered the toxicity of the neonicotinoid), but where variation in the dose-response relationship for the *PBO only* treatment was not statistically significant (i.e. PBO alone produced no individual toxic effect in bees), we considered the interaction effect to be ‘more-than-additive’. We then determined the magnitude of the ‘more-than-additive’ effect at each concentration in the dose-response relationship by calculating the ‘more-than-additive ratio’ (MTA ratio = performance in *neonicotinoid only* treatment/performance in *neonicotinoid plus PBO* treatment; analogous to the synergism ratio described by Iwasa et al. 2004). In the second scenario, where we detected a significant interaction of *dosage* × *treatment* as above, but the dose-response relationship for the *PBO only* treatment varied significantly with

dose (i.e. PBO alone exerted an individual toxic effect in bees), we proceeded as follows. First, we took the mean performance of bees at each PBO concentration in the *PBO only* dose-response relationship and subtracted this from the mean performance of bees in the undosed control group (zero PBO) to give the individual toxic effect of PBO on performance at each tested concentration. Second, for each concentration we combined the derived individual effect of PBO with the effect of the neonicotinoid alone in the *neonicotinoid only* dose-response relationship to give a new dose-response relationship describing the estimated mean additive effect of the two chemicals (i.e. a new *treatment*, which we defined *estimated additive effect*). Third, we determined whether the dose-response relationships of the *neonicotinoid plus PBO* and *estimated additive effect* treatments differed by testing for a significant interaction (in the model *endpoint ~ dosage × treatment*) using ANCOVA, ANOVA or GLM as appropriate. Where the analysis confirmed that these dose-response relationships were significantly different (i.e. an interaction was detected), we concluded that PBO had enhanced the toxicity of the neonicotinoid by more-than-additive means. We then calculated the MTA ratio as described above. All statistical analyses were conducted in R v3.0 (R Core Team 2013).

5.3 Results

5.3.1 Effects of dietary imidacloprid and PBO

In the dietary imidacloprid assays, the concentration of acetone in the *solvent control* had no significant effect on syrup consumption, locomotory activity or longevity in either bee species (Table S5.1).

The rate at which bumble bees consumed syrup responded to the concentration of imidacloprid, but this relationship was not altered by mixing the neonicotinoid with PBO (two-way ANOVA: *dosage*, $F_{8,43} = 11.7$, $P < 0.001$; *dosage* × *treatment*, $F_{1,43} = 0.8$, $P = 0.36$; Fig. 5.1). In honey bees, dietary imidacloprid had no significant effect on syrup consumption (two-way ANOVA: *dosage*, $F_{8,44} = 1.4$, $P = 0.23$; Fig. 5.2), but honey bees dosed with imidacloprid plus PBO ate significantly less than those dosed with imidacloprid alone (two-way ANOVA: *dosage* × *treatment*, $F_{1,44} = 17.7$, $P < 0.001$; Fig. 5.2). Where honey bees ingested imidacloprid and PBO, the observed reduction in their consumption of syrup, relative to bees that ingested imidacloprid alone, was not attributable to the individual toxicity of PBO because honey bees dosed with PBO only displayed no significant change in feeding rate (one-way ANOVA: *dosage*, $F_{1,24} < 0.1$, $P = 0.95$; Fig. S5.1 and Table S5.2). The toxicity of imidacloprid was therefore more-than-additively enhanced by PBO in honey bees, which resulted in the bees' decreased feeding. However, this effect was low across all nine concentrations (mean MTA ratio = 1.1, SE = 0.02; Table 5.1).

Dietary imidacloprid significantly affected locomotory activity in bumble bees (GLM: *dosage*, $F_{8,45} = 2.4$, $P < 0.05$; Fig. 5.1), but it had no effect on the activity

of honey bees (GLM: *dosage*, $F_{8,45} = 0.8$, $P = 0.61$; Fig. 5.2). Adding PBO to imidacloprid produced no additional effect on locomotion in either species (GLM: bumble bee, *dosage* \times *treatment*, $F_{1,44} = 3.5$, $P = 0.07$; honey bee, *dosage* \times *treatment*, $F_{1,44} = 1.6$, $P = 0.21$; Fig. 5.1 and Fig. 5.2, respectively).

The longevity of bumble bees in the laboratory varied significantly with imidacloprid concentration, but adding PBO did not alter imidacloprid's effect (two-way ANOVA: *dosage*, $F_{8,44} = 3.4$, $P < 0.01$; *dosage* \times *treatment*, $F_{1,44} < 0.1$, $P = 0.86$; Fig. 5.1). Imidacloprid did not affect honey bee longevity (two-way ANOVA: *dosage*, $F_{8,44} = 1.9$, $P = 0.09$; Fig. 5.2), but across the concentration range bees that fed on imidacloprid plus PBO survived fewer days than those feeding on imidacloprid alone (two-way ANOVA: *dosage* \times *treatment*, $F_{1,44} = 9.1$, $P < 0.01$; Fig. 5.2). It was necessary to test whether this observed reduction in longevity was produced additively or more-than-additively because ingesting PBO alone was itself sufficient to significantly affect honey bee longevity (one-way ANOVA: *dosage*, $F_{8,18} = 3.1$, $P < 0.05$; Fig. S5.1 and Table S5.2). We found that the dose-response relationship describing the *estimated additive effect* of imidacloprid and PBO was significantly different from the dose-response relationship describing the actual effect of *imidacloprid plus PBO* in a mixture (two-way ANOVA: *dosage* \times *treatment*, $F_{1,44} = 5.1$, $P < 0.05$). We therefore concluded that PBO increased the toxicity of imidacloprid more-than-additively so that honey bee longevity was reduced across all concentrations with a maximum MTA ratio of 2.4 at 98 µg imidacloprid kg⁻¹ (mean MTA ratio = 1.4, SE = 0.1; Table 5.1).

5.3.2 Effects of dietary thiacloprid and PBO

Similar to the dietary imidacloprid assay, the acetone solvent control of the dietary thiacloprid assay did not significantly affect syrup consumption, locomotory activity or longevity in either bee species (Table S5.1).

In bumble bees, syrup consumption was significantly affected by thiacloprid only when concentrations $\geq 9,688 \mu\text{g kg}^{-1}$ were included in the dose-response relationship (one-way ANOVA: dosage, $F_{6,20} = 4.4$, $P < 0.01$; Fig. S5.2). At concentrations $\leq 98 \mu\text{g kg}^{-1}$, thiacloprid had no effect on bumble bee feeding, nor did PBO produce any interaction with thiacloprid (two-way ANOVA: dosage, $F_{4,24} = 0.3$, $P = 0.87$; dosage \times treatment, $F_{1,24} = 2.9$, $P = 0.10$; Fig. 5.3). In honey bees, concentrations of thiacloprid as high as $25,431 \mu\text{g kg}^{-1}$ had no effect on syrup consumption (one-way ANOVA: dosage, $F_{5,14} = 1.5$, $P = 0.24$; Fig. S5.2) and PBO did not significantly interact with thiacloprid at concentrations up to $98 \mu\text{g kg}^{-1}$ (two-way ANOVA: dosage \times treatment, $F_{1,24} = 3.2$, $P = 0.09$; Fig. 5.4).

Dietary thiacloprid at concentrations up $25,431 \mu\text{g kg}^{-1}$ had no significant effect on the locomotory activity of either bee species (GLM: bumble bees, dosage, $F_{6,20} = 2.3$, $P = 0.07$; honey bees, dosage, $F_{5,14} = 1.8$, $P = 0.21$; Fig. S5.2). When combined with thiacloprid concentrations $\leq 98 \mu\text{g kg}^{-1}$, PBO did not produce any additional effect on bumble bee locomotion (GLM: dosage \times treatment, $F_{1,22} = 1.1$, $P = 0.31$; Fig. 5.3), but honey bees that ingested PBO mixed with thiacloprid exhibited significantly reduced locomotory activity relative to honey bees that ingested thiacloprid only (GLM: dosage \times treatment, $F_{1,24} =$

20.9, $P < 0.001$; Fig. 5.4). Locomotory activity in honey bees was not significantly affected by exposure to PBO alone (GLM: *dosage*, $F_{4,10} = 3.0$, $P = 0.07$; Fig. S5.1 and Table S5.2), and so the interaction between thiacloprid and PBO that reduced locomotion in honey bees was more-than-additive and the effect occurs at all concentrations, with MTA peaking at 2.5 for 39 µg thiacloprid kg⁻¹ (mean MTA ratio = 1.8, SE = 0.2; Table 5.1).

Neither the longevity of bumble bees nor honey bees was affected by thiacloprid in the range up to 25,431 µg kg⁻¹ (one-way ANOVA: bumble bees, *dosage*, $F_{6,20} = 1.6$, $P = 0.37$; honey bees, *dosage*, $F_{5,14} = 1.0$, $P = 0.45$; Fig. S5.2). PBO had no additional effects on longevity when added to concentrations of thiacloprid ≤ 98 µg kg⁻¹ (two-way ANOVA: bumble bees, *dosage × treatment*, $F_{1,24} < 0.1$, $P = 0.83$; honey bees, *dosage × treatment*, $F_{1,24} = 0.7$, $P = 0.42$; Fig. 5.3 and 5.4, respectively).

5.3.3 Effects on bumble bees of topically applied neonicotinoids and PBO

In comparison with undosed bees, thiacloprid significantly reduced syrup consumption in bumble bees when topically applied at 14.6 µg (Mann-Whitney U test: $U = 53$, $P = 0.05$; Table 5.2). Syrup feeding was not significantly different in undosed control and dosed bees for all other dosages of imidacloprid and thiacloprid (Mann-Whitney U test: 7 ng imidacloprid, $U = 43$, $P = 0.09$; 18 ng imidacloprid, $U = 43$, $P = 0.28$; 15 ng thiacloprid, $U = 30$, $P = 0.89$; Table 5.2). When applied alone, PBO significantly reduced bumble bee feeding (Mann-Whitney U test: *PBO control*, $U = 84$, $P < 0.05$; Table 5.2). However, when topically applied in combination with each of the tested neonicotinoid doses,

PBO did not have a more-than-additive effect on syrup consumption (Mann-Whitney U test: *neonicotinoid only* vs. *neonicotinoid plus PBO*, $U \leq 50$, $P \geq 0.06$; Table 5.2).

In comparison with undosed bees, the locomotory activity of dosed bumble bees was not significantly affected by topical applications of imidacloprid or thiacloprid at any dosage (Mann-Whitney U test: *imidacloprid*, $U \leq 29$, $P \geq 0.42$; *thiacloprid*, $U \leq 31$, $P \geq 0.24$; Table 5.2). PBO applied alone did not significantly affect locomotion (Mann-Whitney U test: *PBO control*, $U = 67$, $P = 0.28$; Table 5.2), and had no more-than-additive effects when combined with either neonicotinoid (Mann-Whitney U test: *neonicotinoid alone* vs. *neonicotinoid plus PBO*, $U \leq 42$, $P \geq 0.32$; Table 5.2).

Bumble bee mortality under topical exposure was negligible. Only two bees died following dosing; one bee in the 7 ng imidacloprid plus PBO treatment and one in the 14.6 µg thiacloprid treatment.

5.4 Discussion

5.4.1 Effects on bees of neonicotinoid pesticides with different chemical structures

We found that dietary imidacloprid at concentrations of up to 98 µg kg⁻¹ affected syrup consumption, locomotory activity and longevity in individual bumble bees, but had no effect on the performance of honey bees. These results are consistent with previous findings (Mommaerts et al. 2010b; Cresswell et al.

2012b, 2013) and the differential sensitivity may have arisen in our study because bumble bees ingested at least twice as much imidacloprid than honey bees in equivalent dosage groups (Cresswell et al. 2013). Similar to another study of differential sensitivity to imidacloprid in bees (Cresswell et al. 2012b), we housed bumble bee alone, but kept honey bees in small groups to avoid the elevated mortality that rapidly occurs when they are isolated (Laycock, pers. obs.). It is possible that the absence of sociality imposed on our bumble bees was an additional stressor not imposed on honey bees, and that the effects of isolation may have interacted with the effects of imidacloprid to increase the potency of the neonicotinoid. However, bumble bee workers kept in small groups exhibit similarly reduced performance when exposed to dietary imidacloprid in concentration range tested here (Mommaerts et al. 2010b; Laycock et al. 2012 [see chapter two]). The effects of imidacloprid on bumble bees therefore appear somewhat generalisable between social conditions, but to test this hypothesis properly new research should be conducted on single- and group-housed bumble bees taken from the same source colony.

In contrast to our dietary results, we found that bumble bees were unaffected by topical applications of imidacloprid at 18 ng bee^{-1} ; a dose more than twice as large as the mean daily intake of imidacloprid in bees feeding on syrup dosed at $98 \mu\text{g kg}^{-1}$. Topical doses of this magnitude are toxic to honey bees, with a previous study reporting a value of $18 \text{ ng imidacloprid bee}^{-1}$ as the LD₅₀ for *A. mellifera* (Iwasa et al. 2004). Therefore, considered alongside the findings of Iwasa et al. (2004), our results suggest that bumble bees and honey bees may be differentially sensitive to imidacloprid depending on the route of exposure.

Similar to our observation, differential sensitivity between bees dosed orally and topically with the same pesticide has previously been observed in bumble bees, where certain insecticides (spinetoram and deltamethrin) are more toxic by ingestion than by direct contact (Gradish et al. 2012), and honey bees, where insecticides including imidacloprid can be more toxic by contact than oral exposure (Bailey et al. 2005). Indeed, there is some evidence that bumble bees may be generally less sensitive than honey bees to topical applications of various pesticides (Hardstone and Scott 2010), and other authors have speculated that this could originate in differences in absorption due to cuticle properties (Cresswell et al. 2012b).

In contrast to imidacloprid, dietary thiacloprid in the range up to 98 $\mu\text{g kg}^{-1}$ had no observable effect on bumble bee performance. Diminished syrup consumption was observed only in bumble bees exposed orally to concentrations of thiacloprid approaching 10,000 $\mu\text{g kg}^{-1}$ or a topical dose at 14.6 $\mu\text{g bee}^{-1}$ (an amount approximately 1,000 times greater than the daily dietary intake of thiacloprid in bumble bees fed syrup dosed at 98 $\mu\text{g kg}^{-1}$).

Similar to previous studies (Hawthorne and Dively 2011; Laurino et al. 2011), we found that honey bees were unaffected by dietary thiacloprid at concentrations greater than 25,000 $\mu\text{g kg}^{-1}$. However, our results contrast with a recent study in which the ability to navigate was impaired in free-flying honey bees fed an acute dose of thiacloprid equivalent to 12,500 ppb (Fischer et al. 2014). It is well known that cyano-substituted neonicotinoids, such as thiacloprid, are substantively less toxic to honey bees than nitro-substituted varieties, such as imidacloprid (Iwasa et al. 2004; Laurino et al. 2011), and we

have now shown experimentally that this is also true for bumble bees. Our results have positive implications for the use of thiacloprid in agriculture because dietary concentrations equivalent to the residues detected in field pollen (typically <200 µg thiacloprid kg⁻¹; Škerl et al. 2009; Mullin et al. 2010; EFSA 2012; Pohorecka et al. 2012; Pettis et al. 2013; Rennich et al. 2013) were apparently harmless to both bee species. We note, however, that while field-realistic exposure to thiacloprid alone appears to have no impact on bees, studies have shown that dietary thiacloprid at ~5000 µg L⁻¹ or µg kg⁻¹ (Vidau et al. 2011; Doublet et al. 2014) elevates mortality in adult honey bees infected with the microsporidian parasite *Nosema ceranae*. If these impacts also occur at lower concentrations more typically found in environmental nectar and pollen (<200 µg kg⁻¹, see above) they could increase the stress on colonies of managed honey bees and wild bumble bees that are often infected with *N. ceranae* (Graystock et al. 2013a) and thereby reduce their performance. Furthermore, larval honey bees infected with black queen cell virus (BQCV) exhibit increased BQCV-induced mortality when fed thiacloprid at 100 µg kg⁻¹ (Doublet et al. 2014), and therefore more research is required into the effects of thiacloprid in larval bee stages when administered alone or in combination with other stressors.

5.4.2 Enhancement of neonicotinoid toxicity by PBO

For bumble bees, the toxicity of imidacloprid was not enhanced by PBO when the neonicotinoid was administered orally at concentrations ≤ 98 µg kg⁻¹ or topically at doses ≤ 18 ng bee⁻¹. To our knowledge, we are the first to report an investigation into the interactive effects of PBO with neonicotinoids in bumble

bees and our results demonstrate that the P450-inhibitor has no effect on the toxicity of imidacloprid in *B. terrestris*. In honey bees, we observed a small ‘more-than-additive’ effect between dietary imidacloprid and PBO that reduced feeding and longevity by a maximum of approximately 2-fold. This finding is consistent with a previous study (Iwasa et al. 2004), in which PBO enhanced the toxicity of imidacloprid by a ratio of just 1.7 in honey bees treated topically with relatively large doses of the neonicotinoid ($18 \text{ ng bee}^{-1} = \text{LD}_{50}$ dose).

In bumble bees, we found that a ‘more-than-additive’ effect did not occur between PBO and either dietary thiacloprid in the range up to $98 \mu\text{g kg}^{-1}$ or topically applied thiacloprid at doses as high as $14.6 \mu\text{g bee}^{-1}$. The lack of interaction between thiacloprid and PBO is a new finding for bumble bees, and contrasts with the findings of a previous honey bee study (Iwasa et al. 2004). Specifically, Iwasa et al. (2004) reported a synergy between topically applied thiacloprid and PBO that increased the toxicity of the neonicotinoid approximately 150-fold (reducing the LD_{50} from $14.6 \mu\text{g bee}^{-1}$ to 90 ng bee^{-1}). In our study, PBO produced a small ‘more-than-additive’ enhancement of dietary thiacloprid toxicity in honey bees that resulted in the bees’ locomotory activity decreasing approximately 2-fold at concentrations $\leq 98 \mu\text{g kg}^{-1}$. There is, therefore, some disparity between our results and those of Iwasa et al. (2004) and this may have arisen for a number of reasons, which are as follows. First, the lack of synergy between thiacloprid and PBO in our dietary exposure assays could be explained by the field-realistic concentrations of thiacloprid that were used. For example, Iwasa et al. (2004) observed a synergy with a dose of thiacloprid approaching $\sim 90 \text{ ng bee}^{-1}$, while in our study bumble bees that were

fed thiacloprid at 98 µg kg⁻¹ ingested the neonicotinoid at ~15 ng bee⁻¹ day⁻¹. Thiacloprid has a relatively low toxicity to bees (Elbert et al. 2000; Schmuck et al. 2003; Iwasa et al. 2004; Laurino et al. 2011), so it is plausible that field-realistic dietary residues in the range up to 98 µg kg⁻¹ show little or no toxicity at the endpoints we measured even when they are combined with a P450-inhibitor. Second, comparing our results to those of Iwasa et al. (2004) suggested bumble bees may be less sensitive than honey bees to imidacloprid administered topically – this may also be true for thiacloprid. If so, honey bees would exhibit the effects of a synergism between topically applied thiacloprid and PBO (e.g. increased mortality; Iwasa et al. 2004) more readily than bumble bees. Third, P450-inhibitors may enhance the toxicity of thiacloprid to honey bees in LD₅₀ bioassays where the neonicotinoid is topically applied (Iwasa et al. 2004), but fail to produce a synergy in other assays such as the chronic dietary exposure tested in our study. For example, a finding comparable to our own has previously been reported for thiacloprid in honey bees by Schmuck et al. (2003). Specifically, when thiacloprid was used in combination with an ergosterol biosynthesis inhibitor (EBI) fungicide known to disrupt P450 activity, the EBI fungicide strongly enhanced the neonicotinoid's toxicity to honey bees only in topical LD₅₀ laboratory tests and not in field-relevant tunnel tests incorporating realistic dietary exposures in treated plants.

5.4.3 The role of P450 enzymes in metabolism of imidacloprid and thiacloprid in bees

Our results suggest that cytochrome P450 enzymes are not responsible for metabolism of imidacloprid in *B. terrestris* bumble bees. Furthermore, the

negligible enhancement by PBO of dietary imidacloprid's toxicity in honey bees, suggests that P450s are not an important mechanism for metabolism of imidacloprid at concentrations $\leq 100 \mu\text{g kg}^{-1}$ in honey bees. This is new finding for bumble bees, and in honey bees has previously only been demonstrated in topical dosing assays (Iwasa et al. 2004). In other insects such as the house fly *M. domestica* (Liu et al. 1993) and cat flea *Ctenocephalides felis* Bouche (Richman et al. 1999) the toxicity of imidacloprid is increased more than 10-fold by PBO, suggesting that metabolism pathways for imidacloprid may vary between insect species. In bees, detoxification of imidacloprid may instead involve metabolic proteins such as the glutathione-S-transferases or carboxyl/cholinesterases – both encoded by the honey bee genome (Claudianos et al. 2006) and known to take part in insecticide metabolism (Yu et al. 1984) – and further research is required to investigate their role in imidacloprid's metabolism.

We note that a scenario exists in which P450s could play a role in metabolism of imidacloprid in bees, but where PBO may not produce an enhancement of the neonicotinoid's toxicity. For example, P450 enzymes could perhaps be involved in the biotransformation of imidacloprid into several metabolites, two of which, 5-hydroxyimidacloprid and olefin, exhibit a toxicity of similar magnitude to that of the parent compound (Suchail et al. 2004a, b). In this hypothetical scenario, P450s could be said to 'activate' imidacloprid by rapidly reducing exposure to the parent compound, but introducing toxic metabolites that are linked with the appearance of mortality in bees (Suchail et al. 2000). In such a scenario, introduction of a P450-inhibitor would be unlikely to enhance the

effects of a neonicotinoid but instead reduce its toxicity to bees by minimising the production of toxic metabolites. Although in the current study we found no evidence that PBO reduced the toxicity of imidacloprid in bees, further investigation is necessary to determine whether P450 enzymes are indeed entirely unimportant in detoxification of imidacloprid or whether they could in fact ‘activate’ the compound by releasing its toxic metabolites. In future work, a re-designed protocol – that perhaps involved chemical analysis of bees exposed to imidacloprid with and without PBO, and included quantification of imidacloprid and its metabolites in bee tissues – would be needed to discriminate between these hypotheses.

Our results suggest that neither bumble bees nor honey bees rely upon P450 enzymes as a system to reduce the toxicity of dietary thiacloprid in the range up to 98 µg kg⁻¹. This is consistent with a previous study (Schmuck et al. 2003), in which no adverse effects occurred against honey bees exposed to a P450-inhibitor and realistic levels of thiacloprid. Although the mechanism of bees' resistance to dietary thiacloprid is not revealed by our study, it may result in part from the metabolites of *N*-cyanoamidine neonicotinoids, such as thiacloprid and acetamiprid, having a low toxicity to bees (Iwasa et al. 2004). Additionally, other proteins working in concert with metabolic enzymes may mediate resistance. For example, members of the ABC transporter protein family move toxins such as pesticides towards excretion (Buss and Callaghan 2008), and inhibiting ABC transporters in honey bees increases the toxicity of neonicotinoids including thiacloprid (Hawthorne and Dively 2011).

The results of our topical assay suggest that bumble bees may not rely on P450s to metabolise large doses of thiacloprid absorbed through their cuticle, which is in contrast to previous findings for honey bees (Iwasa et al. 2004). Not unlike the metabolism pathway for imidacloprid in bees vs. other insect species (Liu et al. 1993; Richman et al. 1999; Iwasa et al. 2004), bumble bees and honey bees may metabolise topical doses of thiacloprid differently. However, we acknowledge that our small topical study in bumble bees is insufficient to confirm this hypothesis. A larger study, incorporating simultaneous testing of bumble bees and honey bees topically dosed with thiacloprid at a range of concentrations, would be necessary to fully investigate this apparent difference.

5.4.4 Conclusions

In this study, we have highlighted the differential sensitivity to neonicotinoid pesticides that exists between bumble bees and honey bees (Cresswell et al. 2012b, 2013) and now shown that susceptibility to these agrochemicals may differ between the species based on route of exposure. Although our study did not reveal the mechanism of this differential sensitivity, we have further exposed the dangers, previously expressed by other authors (Thompson 2001; Decourtye et al. 2013), of risk assessing pesticides based solely on their toxicity to honey bees or on their affects during LD₅₀ assays. We therefore join with Decourtye et al. (2013) in advocating the use of more rigorous pesticide testing, incorporating a broad range of exposure scenarios that are appropriate to the organism.

Our results suggest that P450 enzymes are not important for metabolism of dietary imidacloprid or thiacloprid in the range up to ~100 µg kg⁻¹ in either bumble bees or honey bees. However, we also acknowledge that our work has limitations that make the findings preliminary. For example, PBO may exert unforeseen impacts on an insect that affect its tolerance to a pesticide. In *Drosophila*, for example, PBO not only inhibits P450s (Hodgson and Levi 1998), but also results in the induction of several putative detoxification genes that could potentially increase pesticide metabolism (Willoughby et al. 2007). In addition, prolonged exposure to PBO increases P450 activity to levels above those measured prior to treatment in some organisms such as mice (Hodgson and Levi 1998), although this finding is yet to be reported in insects. Further research to validate the results of our current study is therefore necessary and where possible the need for an insecticide synergist such as PBO should be eliminated. For example, future studies could incorporate functional toxicogenomics (Waters and Fostel 2004) of bees (Johnson 2013), where results would not be reliant on a synergist, but rather on techniques such as RNA-sequencing and transcriptome profiling that could be employed to identify the underlying molecular mechanisms of neonicotinoid toxicity and resistance in bees.

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Chapter Five: Figures and Tables

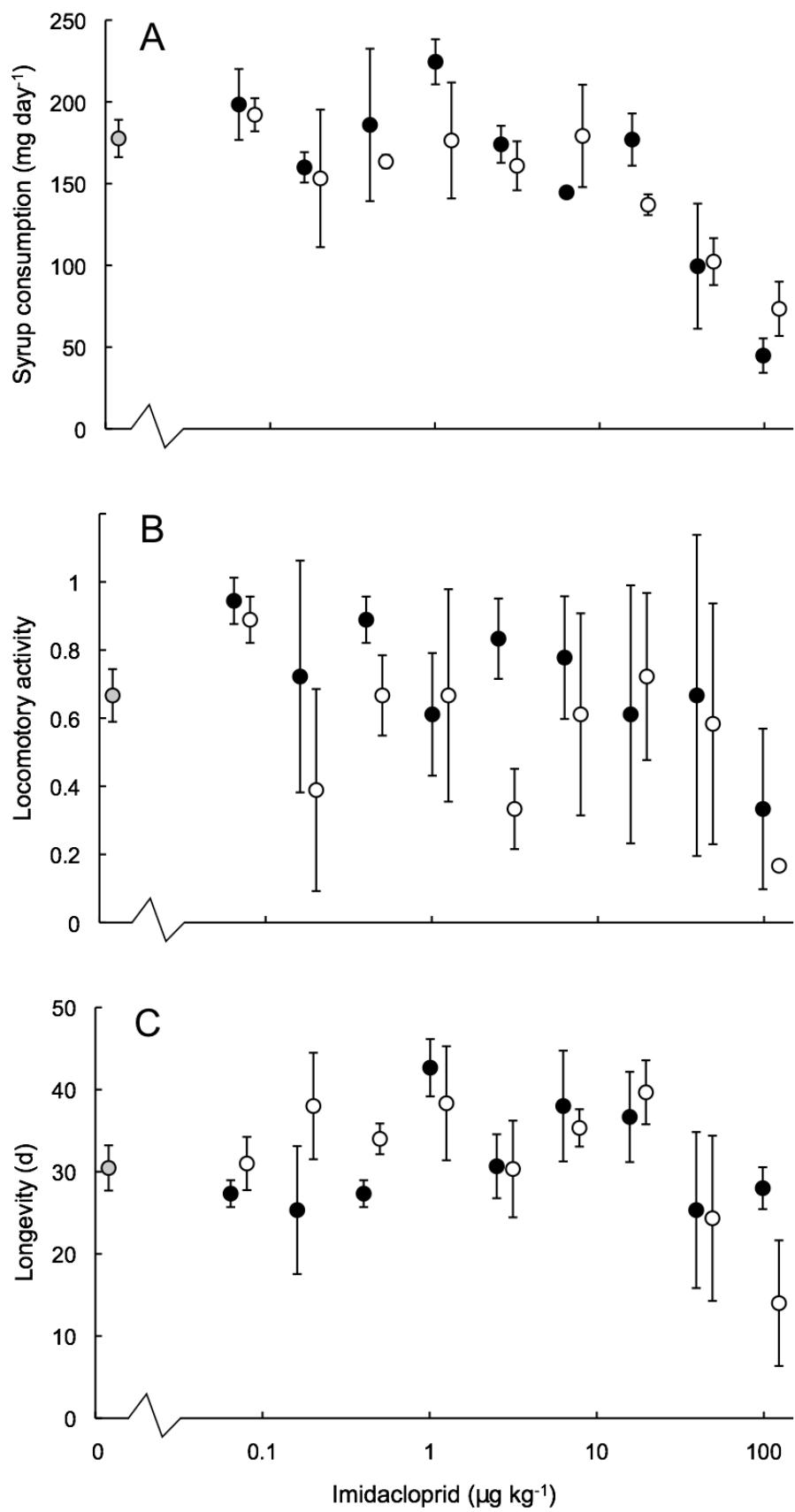


Fig. 5.1 Effects of imidacloprid alone and in combination with PBO in *Bombus terrestris* bumble bees exposed to dietary residues in syrup. Specifically, the effect of imidacloprid in the range from zero to up to 98 µg kg⁻¹ (black filled circles) and the effect of the same concentration of imidacloprid combined with PBO (unfilled circles) on (A) syrup consumption, (B) locomotion and (C) longevity in bumble bees. Ratio of imidacloprid to PBO was kept constant at 1:10,000 (imidacloprid:PBO, see Methods). All data represent the mean and error bars indicate ± SE. Control data (grey filled circles, representing zero µg imidacloprid kg⁻¹) and imidacloprid plus PBO data are displayed slightly displaced on the x-axis for ease of inspection. Bumble bee performance varied significantly with imidacloprid concentration for all three endpoints in A, B and C ($P < 0.05$), but PBO did not enhance imidacloprid toxicity

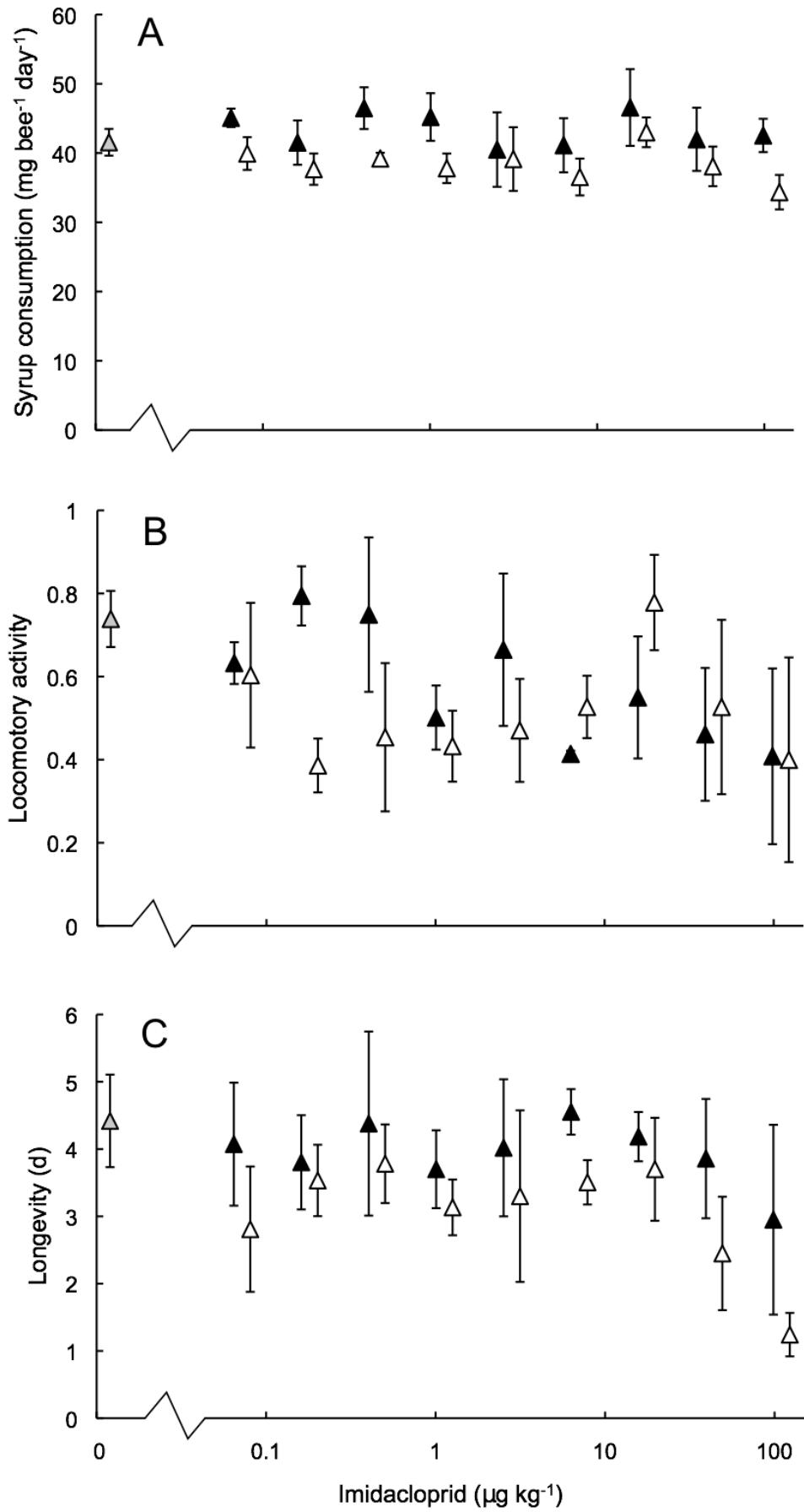


Fig. 5.2 Effects of imidacloprid alone and in combination with PBO in *Apis mellifera* honey bees exposed to dietary residues in syrup. Specifically, the effect of imidacloprid in the range from zero to up to 98 µg kg⁻¹ (black filled triangles) and the effect of the same concentration of imidacloprid combined with PBO (unfilled triangles) on (A) syrup consumption, (B) locomotion and (C) longevity in honey bees. Ratio of imidacloprid to PBO was kept constant at 1:10,000 (imidacloprid:PBO, see Methods). All data represent the mean and error bars indicate ± SE. For ease of inspection, control data (grey filled triangles, representing zero µg imidacloprid kg⁻¹) and imidacloprid plus PBO data are displayed slightly displaced on the x-axis. Honey bee performance was not significantly affected by imidacloprid concentration, but PBO produced a more-than-additive effect in combination with imidacloprid that reduced (A) consumption of syrup ($P < 0.001$) and (C) longevity ($P < 0.01$) in bees

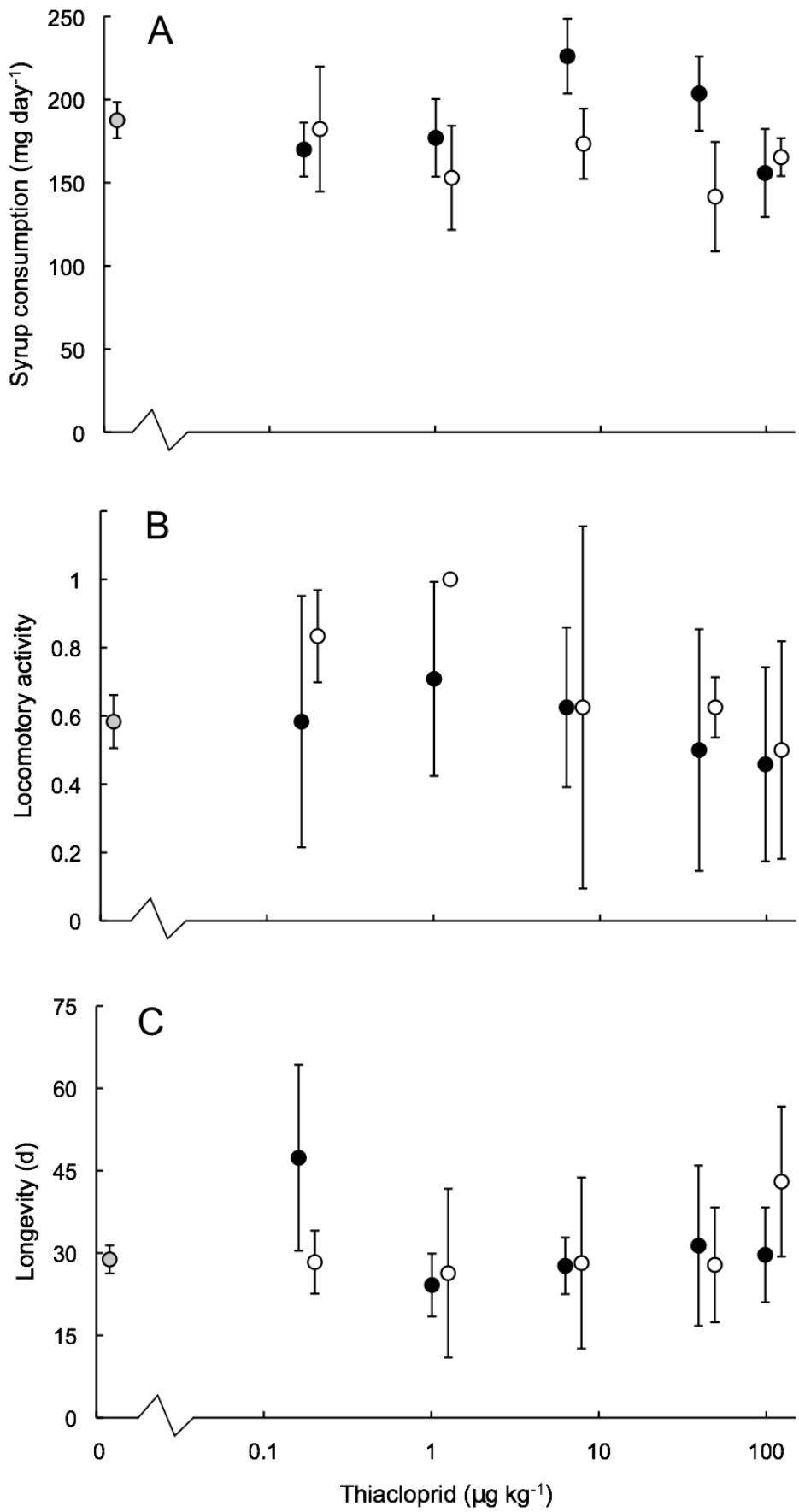


Fig. 5.3 Effects of thiacloprid alone and in combination with PBO in *Bombus terrestris* bumble bees exposed to dietary residues in syrup. Specifically, the effects of thiacloprid in the range from zero to up to 98 $\mu\text{g kg}^{-1}$ (black filled circles) and the effect of the same concentration of thiacloprid combined with PBO (unfilled circles) on (A) syrup consumption, (B) locomotion and (C) longevity in bumble bees. Ratio of thiacloprid to PBO kept constant at 1:10,000 (thiacloprid:PBO, see Methods). All data represent the means and error bars indicate $\pm \text{SE}$. Control data (grey filled circles, representing zero $\mu\text{g thiacloprid kg}^{-1}$) and thiacloprid plus PBO data are displayed slightly displaced on the x-axis for ease of inspection. Dietary thiacloprid had no significant effect on bumble bee performance and PBO did not enhance thiacloprid toxicity

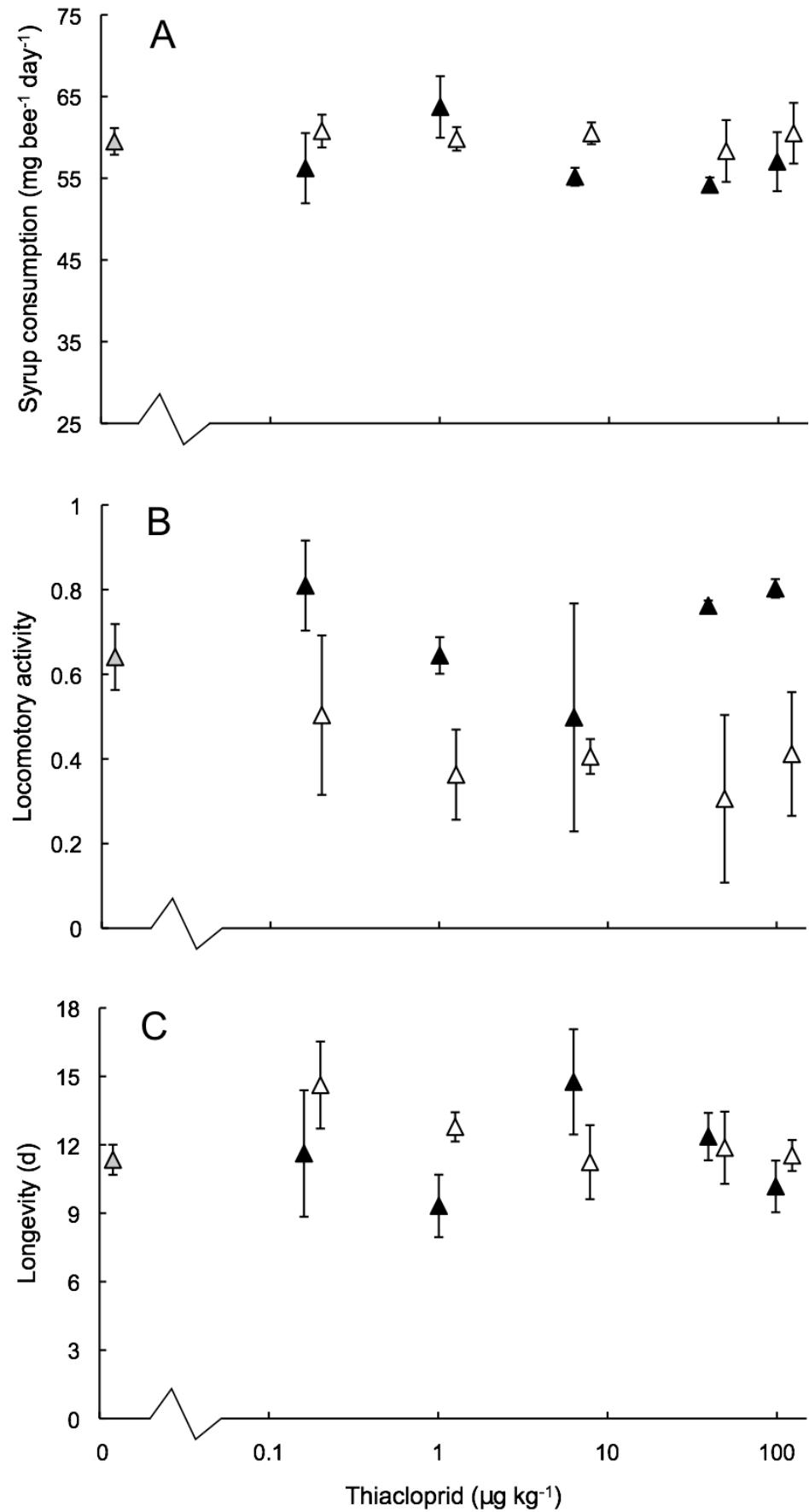


Fig. 5.4 Effects of thiacloprid alone and in combination with PBO in *Apis mellifera* honey bees exposed to dietary residues in syrup. Specifically, the effects of thiacloprid in the range from zero to up to 98 $\mu\text{g kg}^{-1}$ (black filled triangles) and the effect of the same concentration of thiacloprid combined with PBO (unfilled triangles) on (A) syrup consumption, (B) locomotion and (C) longevity in honey bees. Ratio of thiacloprid to PBO kept constant at 1:10,000 (thiacloprid:PBO, see Methods). All data represent the means and error bars indicate $\pm \text{SE}$. For ease of inspection, control data (grey filled triangles, representing zero $\mu\text{g thiacloprid kg}^{-1}$) and thiacloprid plus PBO data are displayed slightly displaced on the x-axis, while y-axis representing (A) syrup consumption begins at 25 $\text{mg bee}^{-1} \text{ day}^{-1}$ rather than zero. Performance of honey bees was not significantly affected by thiacloprid, but PBO produced a more-than-additive effect in combination with thiacloprid that (B) reduced locomotory activity in bees ($P < 0.001$)

Table 5.1 More-than-additive effect ratios (MTA) describing the more-than-additive enhancement by PBO of dietary imidacloprid (IMI) and thiacloprid (THC) effects on syrup consumption, worker longevity and locomotion in *Apis mellifera* honey bees.

Dietary neonicotinoid and affected endpoint			
Concentration ($\mu\text{g kg}^{-1}$)	IMI on syrup consumption (MTA) ^a	IMI on longevity (MTA) ^a	THC on locomotory activity (MTA) ^a
0.1	1.13	1.45	–
0.2	1.10	1.08	1.61
0.4	1.19	1.16	–
1.0	1.20	1.18	1.78
2.5	1.03	1.22	–
6.3	1.13	1.30	1.23
15.7	1.08	1.13	–
39.4	1.10	1.58	2.49
98.4	1.24	2.37	1.95

^a MTA ratio = (mean performance of bees in neonicotinoid treatment group)/(mean performance of bees in neonicotinoid plus PBO treatment group). For thiacloprid, where a dash (–) appears rather than MTA, the dosage was not tested.

Table 5.2 Effects of imidacloprid and thiacloprid topically applied alone or in combination with PBO on syrup consumption, locomotory activity and mortality in *Bombus terrestris* bumble bees. Neonicotinoids were topically applied to the dorsal thorax at the specified dose in 1 µL of acetone, either alone or in combination with 100 µg of the P450-inhibitor PBO. Bees in the acetone control received a topical dose of 1 µL of acetone only. Apart from mortality, data represent the mean ± SE. Where a chemical administered alone produced a significant effect, this is indicated (see footnotes). PBO did not enhance the effect of topically applied neonicotinoids for any endpoint.

Treatment	<i>n</i>	Syrup	Locomotory	Mortality
		consumption (mg)	activity	(%)
Acetone control	8	213 ± 13	0.88 ± 0.06	0
PBO control	13 ^a	167 ± 13 ^c	0.85 ± 0.10	0
Imidacloprid: 7 ng				
Alone	8	191 ± 17	0.86 ± 0.12	0
With PBO	8	169 ± 14	0.83 ± 0.13	13
Imidacloprid: 18 ng				
Alone	8	189 ± 18	0.91 ± 0.04	0
With PBO	8	143 ± 15	0.91 ± 0.04	0
Thiacloprid: 15 ng				
Alone	8	205 ± 27	0.89 ± 0.11	0
With PBO	7 ^b	157 ± 15	0.93 ± 0.04	0
Thiacloprid: 14.6 µg				
Alone	8	187 ± 11 ^c	0.80 ± 0.11	13
With PBO	8	143 ± 19	0.68 ± 0.11	0

^a Two bees died during pre-dose feeding period

^b One bee died during pre-dose feeding period

^c Significantly different ($P < 0.05$) from performance in acetone solvent control

Chapter Five: Supplementary material

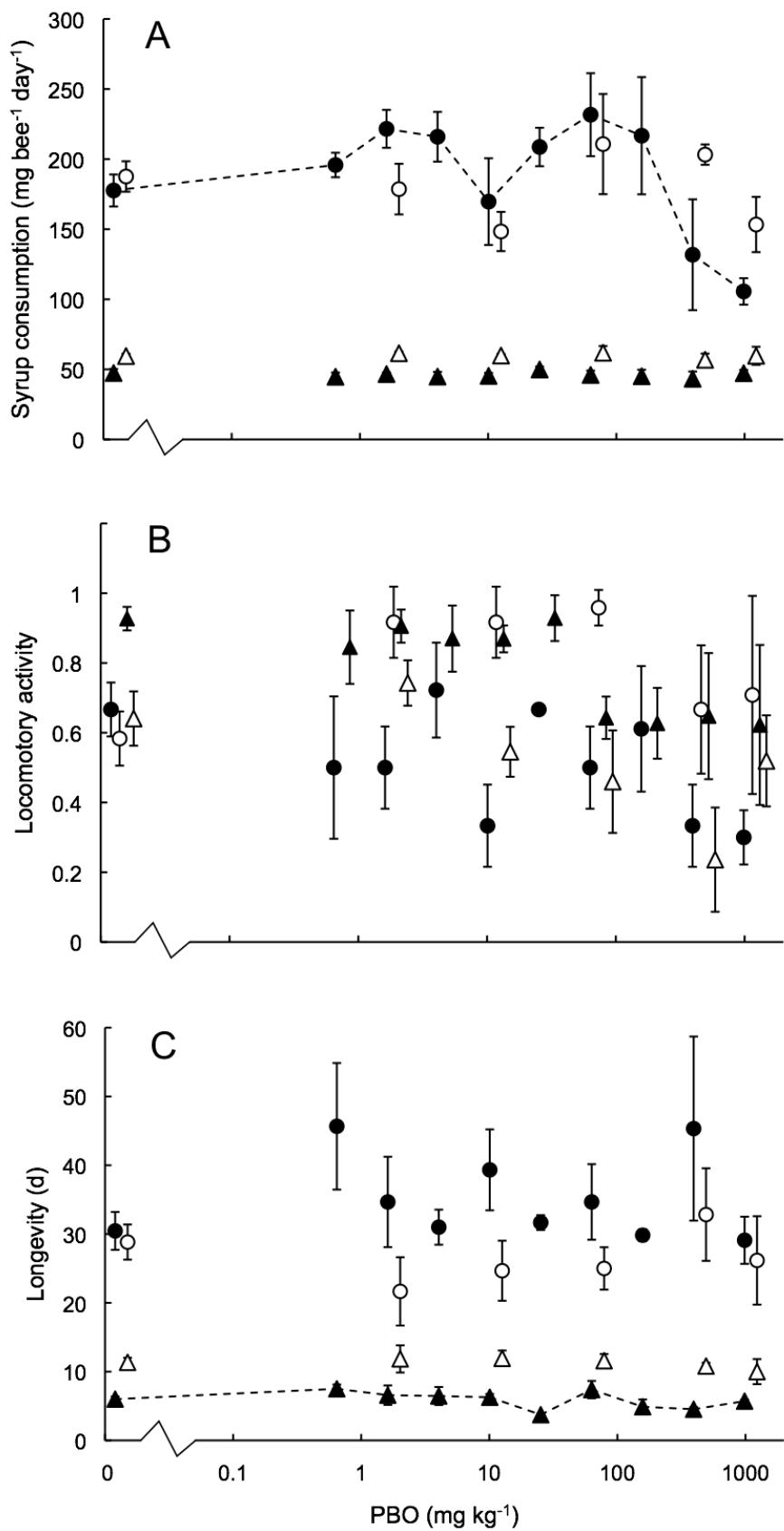


Fig. S5.1 Effects of piperonyl butoxide (PBO) on the performance of *Bombus terrestris* bumble bees (circles) and *Apis mellifera* honey bees (triangles). Specifically, the effect of PBO on: (A) syrup consumption, (B) locomotory activity and (C) longevity in trials involving exposure to imidacloprid (filled symbols) and thiacloprid (unfilled symbols). All data represent mean performance under PBO exposure at a given concentration and error bars indicate \pm SE. Control data (zero mg PBO kg $^{-1}$), all data from thiacloprid trials, and the locomotion data from honey bees in imidacloprid trials (filled triangles in B) are displayed slightly displaced on the x-axis for ease of inspection. A dashed line, included for inspection purposes only, indicates a significant dosage effect of PBO (bumble bee syrup consumption (A), imidacloprid trial, $P < 0.001$; honey bee longevity (C), imidacloprid trial, $P < 0.05$)

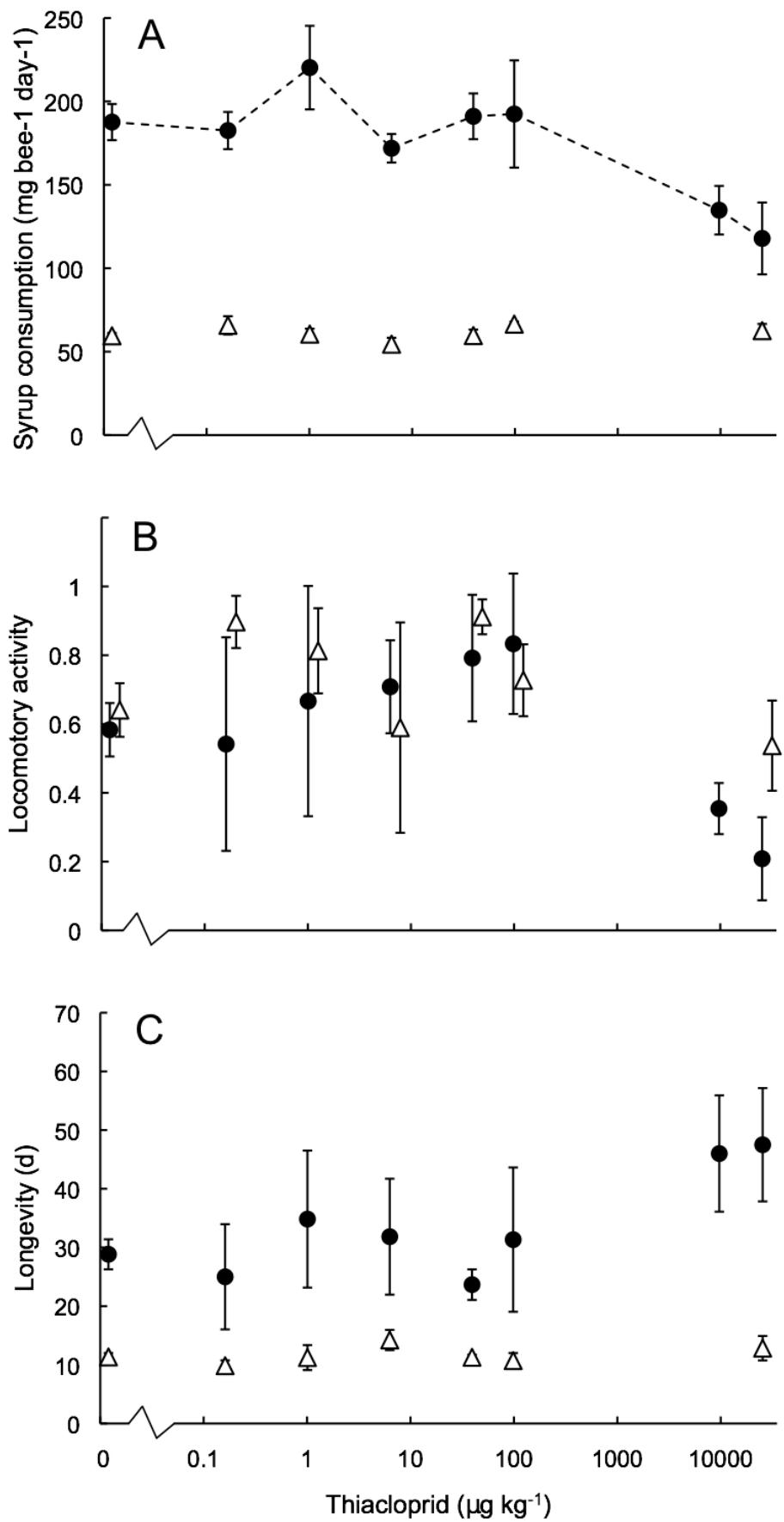


Fig. S5.2 Effects of thiacloprid without acetone in *Bombus terrestris* bumble bees (filled circles) and *Apis mellifera* honey bees (unfilled triangles) exposed to dietary residues in syrup. Specifically, the effects of thiacloprid in the range from zero to up to 25,431 µg kg⁻¹ on: (A) syrup consumption, (B) locomotory activity, and (C) longevity in bees. All data represent the means and error bars indicate ± SE. Control data (zero µg thiacloprid kg⁻¹ in A, B and C) and honey bee locomotion data (B) are displayed slightly displaced on the x-axis for ease of inspection. The dashed line is for inspection purposes only and connects the mean syrup consumption of bumble bees (A), which was significantly affected by thiacloprid concentration ($P < 0.01$)

Table S5.1 Statistical analysis of *solvent control* treatment in bumble bee and honey bee dietary neonicotinoid assays. Values are from analysis of acetone dose-response relationships using one-way analysis of variance (ANOVA) or generalised linear model (GLM) in assays including imidacloprid (IMI) and thiacloprid (THC), with acetone in syrup at concentrations ranging from zero to 11.25 ml L⁻¹ (see Methods). Degrees of freedom (dosage treatment, residuals) are given in parentheses.

Species (assay) / endpoint	Statistical test	F value	P value
Bumble bee (IMI)			
Syrup consumption	ANOVA	1.53 (9, 19)	0.21
Locomotory activity	GLM	1.02 (9, 17)	0.46
Longevity	ANOVA	0.91 (9, 20)	0.54
Bumble bee (THC)			
Syrup consumption	ANOVA	2.76 (5, 12)	0.07
Locomotory activity	GLM	0.33 (5, 12)	0.89
Longevity	ANOVA	1.74 (5, 12)	0.20
Honey bee (IMI)			
Syrup consumption	ANOVA	0.39 (9, 20)	0.93
Locomotory activity	GLM	1.25 (9, 20)	0.32
Longevity	ANOVA	0.61 (9, 20)	0.78
Honey bee (THC)			
Syrup consumption	ANOVA	2.39 (5, 12)	0.10
Locomotory activity	GLM	0.20 (5, 12)	0.95
Longevity	ANOVA	0.71 (5, 12)	0.63

Table S5.2 Statistical analysis of *PBO control* treatment in bumble bee and honey bee dietary neonicotinoid assays. Values are from analysis of PBO dose-response relationships using one-way analysis of variance (ANOVA) or generalised linear model (GLM) in assays including imidacloprid (IMI) and thiacloprid (THC), with PBO in syrup at concentrations ranging from zero to 1,250,000 µg L⁻¹ (see Methods). Degrees of freedom (dosage treatment, residuals) are given in parentheses.

Species (assay) / endpoint	Statistical test	F value	P value
Bumble bee (IMI)			
Syrup consumption	ANOVA	8.57 (8, 25)	<0.001 ^a
Locomotory activity	GLM	2.04 (8, 25)	0.08
Longevity	ANOVA	1.53 (8, 25)	0.20
Bumble bee (THC)			
Syrup consumption	ANOVA	0.03 (1, 13)	0.86
Locomotory activity	GLM	3.75 (1, 13)	0.08
Longevity	ANOVA	1.91 (1, 13)	0.19
Honey bee (IMI)			
Syrup consumption	ANOVA	0.01 (1, 24)	0.95
Locomotory activity	GLM	2.00 (8, 18)	0.11
Longevity	ANOVA	3.12 (8, 18)	<0.05 ^a
Honey bee (THC)			
Syrup consumption	ANOVA	0.34 (1, 10)	0.85
Locomotory activity	GLM	2.97 (4, 10)	0.07
Longevity	ANOVA	2.44 (1, 13)	0.14

^a Significant effect of PBO dosage (*P* < 0.05)

Chapter Six

Application of transcriptomics to investigate
effect mechanisms of the neonicotinoid
pesticide imidacloprid in the bumble bee

Bombus terrestris

Preliminary introduction

The following chapter comprises a paper intended for submission to the journal *Pesticide Biochemistry and Physiology* and is therefore presented in the journal's prescribed format, except that ambiguous references are lettered (a, b, etc) and the sections, figures and tables of the paper are numbered according to their position within the thesis. Where I cite Laycock et al. (2012) or Laycock and Cresswell (2013) I indicate that I am also referring to chapters two and four of this thesis, respectively.

Statement of contribution

As first author, I was primarily responsible for conception and design of the work, acquisition and analysis of the data, and writing the paper. Other researchers worked on the experiment and their contribution was as follows. Dr James Cresswell, Dr Karen Moore, Dr Konrad Paszkiewicz and Dr Ronnie Van Aerle were consulted on the design of the experiment and all offered their expert advice. Dr Moore also provided training in RNA extraction and quality control techniques, and helped to refine the protocol for extraction of RNA from bumble bees described in section 6.2.4. The University of Exeter Sequencing Service team prepared libraries from the extracted RNA and sequenced all samples as described in section 6.2.5. Dr Paszkiewicz performed the initial bioinformatics analysis described in section 6.2.6 and was also consulted, along with Dr Van Aerle, for advice regarding functional analysis of differentially expressed genes. F.X. Robert extracted imidacloprid from bee tissue as described in section 6.2.5, and ran extracted samples through the LC-MS. Prof. Charles Tyler provided constructive comments on final draft of the manuscript.

Application of transcriptomics to investigate effect mechanisms of the neonicotinoid pesticide imidacloprid in the bumble bee *Bombus terrestris*

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Keywords

bee health; *Bombus*; insecticide; neonicotinoid; toxicogenomics; transcriptomics

Abstract

With evidence of the risk posed to bees from agrochemicals continuing to mount, mechanism-based risk assessment has been proposed as a method to better establish the effects of pesticide exposure. Studies investigating the molecular response of honey bees to toxicants are underway, but similar studies in other bee species are lacking. Neonicotinoid pesticides, such as imidacloprid, have been implicated in declines of wild bumble bee populations. Imidacloprid produces toxic effects in bumble bees, but the species *Bombus terrestris* L. shows some resilience to dietary exposures in the range up to 100 µg kg⁻¹. Here, we applied transcriptomic analyses to investigate the effect mechanisms of imidacloprid in *B. terrestris* workers. We extracted RNA from the abdomens of bees fed for 12 h on clean syrup (control) or syrup dosed with 98 µg imidacloprid kg⁻¹. Following high-throughput RNA sequencing, we found that twenty-six genes were differentially expressed in imidacloprid-treated bees and these included genes involved in carbohydrate metabolism (e.g. *glucose dehydrogenase*, *L-lactate dehydrogenase*), fatty acid and fatty alcohol synthesis (e.g. *elongation of very long chain fatty acids protein 4 and 6*, *putative fatty acyl-CoA reductase*), and xenobiotic metabolism (e.g. *cytochrome P450 6a13*). One gene, *niscarin*, was only expressed in dosed bees and, although its function in bumble bees is unclear, it may therefore represent a biomarker for imidacloprid exposure in bumble bees. The results presented here provide a preliminary insight into the molecular mechanisms of imidacloprid toxicity and resilience in *B. terrestris* bumble bees. Our work represents a solid foundation on which future toxicogenomic research in bumble bees can be built and, theoretically, a

basis for distinguishing differences in susceptibility to neonicotinoid pesticides between bee species.

6.1 Introduction

In recent years, our understanding of how poisonous chemicals affect biological systems has rapidly increased in parallel with the evolution of sequencing technology and the adoption of gene expression analysis [1] as a tool for toxicology. From the incorporation of toxicology with genomic profiling a field of science has emerged, *toxicogenomics*, which aims to understand an organism's molecular response to a toxicant [2,3]. Notable advocates of the field include the pharmaceutical industry, which uses toxicogenomics to identify chemicals with a greater likelihood of success in clinical trials [4], and regulators such as the Environmental Protection Agency that support the use of appropriate toxicogenomic data in risk assessment [5]. Indeed, despite some scepticism [6], most believe that mechanistic data collected using computational and molecular tools can support traditional chemical assessments and ultimately inform regulatory decision-making and future environmental health policy [5,7,8].

Currently, the risk posed to bees from agricultural chemicals is an environmental issue causing widespread concern [9,10]. Controversy surrounds the present risk assessment procedure for bees and pesticides, which some suggest should be overhauled to incorporate modern techniques and developments [11]. With the genome sequences of five bee species — namely *Apis mellifera* L. [12], *Apis florea* Fabricius [13], *Bombus impatiens* Cresson [14], *Bombus terrestris* L. [15], and *Megachile rotundata* Fabricius [16] — now publicly available, a move towards mechanism-based risk assessment incorporating functional toxicogenomics has been proposed [17]. If such a move should occur it would likely be born from toxicogenomic studies of the honey

bee *A. mellifera*, several of which have been conducted in the last decade. For example, in an early study Cladinos et al. [18] reported a deficit of genes encoding detoxification enzymes in the honey bee genome relative to other insect genomes, which they speculated could account for the species' sensitivity to pesticides. Subsequent studies identified cytochrome P450 monooxygenases (P450s) as one family of detoxification enzymes that metabolise specific pesticides in honey bees, including acaricides used to control *Varroa destructor* [19]. Recent findings suggest that regulation of these P450s may be tuned to naturally occurring constituents of the honey bees' pollen and honey diet, such as flavonoids [19-21] and *p*-coumaric acid [22], and this may have implications for apiculture. For example, these naturally occurring P450-inducing compounds are absent in honey substitutes such as high-fructose corn syrup; therefore the practice of feeding these substitutes to commercially farmed honey bees could compromise their ability to cope with pesticides [22]. Collectively, these studies begin to create a picture of honey bee toxicogenomics, but the molecular response of other bee species to pesticides is less well understood. We therefore examined the toxicogenomics of the bumble bee *B. terrestris*, specifically in response to a dietary neonicotinoid pesticide, imidacloprid.

Bumble bees are economically important wild pollinators [23] facing population decline and loss of species diversity in some Northern territories [24-26]. Among several factors likely threatening bumble bee species [27], pesticides have been highlighted as potential culprits and neonicotinoid pesticides in particular have been singled out for blame [28,29]. Current evidence suggests that some

neonicotinoids are toxic to bumble bees: concentrations of dietary imidacloprid \geq 100 parts per billion (ppb) significantly increase worker mortality [30] and concentrations below 100 ppb produce sub-lethal effects on feeding and reproduction in queenless and queenright colonies [30-35] [see chapter two and four]. However, bumble bees are somewhat resilient to imidacloprid at dosages as high as 98 ppb, as demonstrated by recovery of feeding rate and brood production when the toxin is removed from their diet [35] [see chapter four]. Furthermore, bumble bee workers appear to eliminate imidacloprid entirely from their bodies within 48 hours [36]. Together, this evidence suggests that an underlying metabolic system capable of detoxifying imidacloprid at relatively high concentrations is at work in bumble bees. If such a system exists, its molecular mechanisms are currently unknown.

In a previous study, the cytochrome P450 enzyme inhibitor piperonyl butoxide (PBO) did not synergize dietary imidacloprid (\leq 98 ppb) in bumble bee workers [see chapter five], suggesting that P450s may not be responsible for detoxification of this neonicotinoid. Similarly, P450s appear to play little part in detoxifying topically [37] or orally [see chapter five] applied imidacloprid in adult honey bee workers. Conversely, honey bee larvae upregulate nine P450 genes when fed imidacloprid at low concentrations ($2 \mu\text{g L}^{-1}$), although currently it is not known whether these genes form part of a specific detoxification response targeting the neonicotinoid [38]. In the latter study, RNA sequencing (RNA-seq) techniques were employed to examine the transcriptome of imidacloprid-exposed honey bee larvae [38]. Here, we employ a similar technique but use larger doses: feeding imidacloprid at 98 ppb to adult *B. terrestris* bumble bee

workers before obtaining their genome-wide RNA transcriptional response using high-throughput sequencing methods. From analysis of the resultant transcriptome, specifically the genes differentially expressed in response to the pesticide, we provide insight into the underlying biological and molecular processes initiated in bumble bees exposed to large doses of imidacloprid.

6.2 Materials and methods

6.2.1 *Imidacloprid diets*

Imidacloprid was obtained as a solution in acetonitrile (Dr. Ehrenstorfer GmbH, Augsburg, Germany). Acetonitrile was removed by evaporation in a ScanSpeed MaxiVac Beta vacuum concentrator (LaboGene ApS, Lynge, Denmark) and the imidacloprid was dissolved in purified water to produce a primary stock solution ($10^4 \mu\text{g imidacloprid L}^{-1}$). An aliquot of primary stock solution (1 mL) was mixed into Attracker sugar syrup (1.27 kg L^{-1} fructose + glucose + saccharose solution; Koppert B.V., Berkel en Rodenrijs, Netherlands) to produce a solution containing imidacloprid at $125 \mu\text{g L}^{-1}$ ($= 98 \mu\text{g kg}^{-1}$ or ppb). This dosage was chosen for its capacity to produce behavioural and physiological effects on individual bumble bees [31,36] and not for environmental relevance. A single preparation of 98 ppb dietary imidacloprid solution (80 mL) was made at the beginning of the experiment and kept refrigerated in the dark at 5°C when not in use.

6.2.2 Preliminary study to determine the appropriate experimental exposure period

In previous chronic exposure studies [31,33,35] [see chapters two and four], the rate at which bumble bees feed on syrup dosed with 98 ppb imidacloprid is a consistently sensitive endpoint that reflects the behavioural and physiological performance of individuals and colonies in the laboratory and may also be indicative of metabolic activity [36]. For individual bumble bees exposed at 98 ppb the detrimental effect of imidacloprid on feeding rate intensifies over days [31], but the effect over hours is currently unknown. Hourly feeding rate may be relevant here because bumble bees can exhibit a rapid genetic response to stress that may be largely complete within 24 h [39]. We therefore conducted a preliminary experiment to establish the temporal pattern of feeding in imidacloprid-exposed bumble bees measured at 3-hourly intervals over 36 h, with the aim of establishing an appropriate time point at which to sample RNA.

Twenty adult *B. terrestris* workers (subspecies *audax*, age unknown) were chosen in pseudo-random fashion from a queenright colony containing approximately 150 workers (Biobest, Westerlo, Belgium). These bees were removed and individually caged in wooden boxes (cage dimensions: 65 mm × 50 mm × 35 mm, with the two largest faces made of plastic mesh; [31]) fitted with a single 2 mL microcentrifuge tube that was punctured to function as a syrup (artificial nectar) feeder. For acclimatisation, all bees fed *ad libitum* on undosed syrup for 12 h before being randomly assigned to one of two treatments: 36 h feeding on undosed control syrup ($N = 10$); 36 h feeding on syrup dosed with imidacloprid at 98 ppb ($N = 10$). Once imidacloprid exposure

had begun, syrup consumption was measured at 3 hourly intervals between 0–12 h and 24–36 h by weighing syrup feeders. Bees were maintained in a semi-controlled environment (26 °C; 40 % relative humidity) throughout the experiment, which took place in July 2012. Statistical analysis of the resulting data was conducted in R v3.0 [40]. Specifically, we used repeated measures analysis of variance (RM-ANOVA) and determined that imidacloprid significantly reduced syrup consumption (RM-ANOVA: $F_{1,18} = 6.93$, $P < 0.05$; Fig. S6.1), while feeding also declined over time (RM-ANOVA: $F_{4,72} = 5.55$, $P < 0.001$; Fig. S6.1). From visual inspection of the data (Fig. S1), we concluded that bees fed imidacloprid-dosed syrup were already consuming less than bees in the control group following 3 h of exposure and the feeding rate of dosed bees reached a plateau by 12 h.

6.2.3 *Exposure of bees to dietary imidacloprid prior to RNA-sequencing*

Based on the results of the preliminary experiment, we chose to expose bees to dietary imidacloprid for 12 h prior to RNA-seq. The experiment was conducted in the same semi-controlled environment described in section 6.2.2 and as follows. We obtained a young *B. terrestris* (subspecies *audax*) colony consisting of a single queen, 10 adult workers, and brood at various stages of development (Biobest, Westerlo, Belgium). On the day the colony arrived, each of the workers was placed inside a marking cage (Dadant & Sons Inc, Hamilton, USA) and marked on the dorsal side of the thorax with queen marking paint (Solway Bee Supplies, Twynholm, UK) before being placed back inside the colony. To control for the possible effects of age on imidacloprid-induced gene expression, the colony was monitored daily for the emergence of new

(unmarked) workers and newly emerged bees (within 24 h of eclosion) were collected and individually caged as described in section 2.2. For acclimatisation, all caged workers were provided with undosed syrup prior to dosing and we determined ‘pre-dose’ syrup consumption by weighing feeders after 12 h. Following 12 h acclimatisation, bees were assigned at random to one of two treatment groups: 12 h feeding on undosed control syrup; 12 h feeding on syrup dosed with imidacloprid at 98 ppb. Although, ultimately, the RNA of six bees was sequenced (see section 6.2.5), at this stage of the experiment 26 bees were removed from the colony and assigned to either the control ($N = 13$) or imidacloprid ($N = 13$) treatments to account for possible issues arising with, for example, RNA-extraction or sequencing that would require repeated work and therefore a surplus of identically treated replicates. Once exposure began, we measured syrup consumption after 12 h by weighing feeders. For each bee, we calculated the difference in feeding rate between acclimatisation and exposure periods as: *reduction in feeding* = *pre-dose feeding* – *post-dose feeding*. We also measured locomotory activity by scoring each bee as stationary or in-motion at 10-min intervals in the period between 11–12 h ‘post-dose’. Using R v3.0 [40], we tested whether *reduction in feeding* or *locomotory activity* varied with imidacloprid by one-way analysis of variance (one-way ANOVA). The exposure experiment was conducted over 17 days during July 2012, with replicates (individual bees) staggered as newly eclosed workers emerged. Individual bees were frozen and stored at -80 °C at the end of each experimental replicate in preparation for RNA extraction.

6.2.4 RNA extraction

Total RNA was extracted from the abdomens of ten bumble bees (five bees selected at random from the control treatment and five from the imidacloprid treatment). Again, not all 26 bees were used for RNA extraction in order to have spare replicates should problems with extraction arise. Similar to previous RNA-seq studies in bees [21,39,41,42], we assayed the abdomen because it contains the gut: the principal site of pesticide detoxification and an integral component of immune defence in bees [41]. We used components of the RNeasy Lipid Tissue Mini Kit and MaXtract High Density (both from Qiagen, Hilden, Germany) to extract RNA from each bee as follows. Abdomens were placed individually into 2 mL microcentrifuge tubes containing Lysing Matrix A (MP Biomedicals, California, USA). In order to release RNA, abdominal tissue was disrupted and the lysate was homogenized in 1 mL of QIAzol Lysis Reagent by using a FastPrep®-24 homogenizer (MP Biomedicals, California, USA) for a total of 100 s (four 25 s repeats, with specimens placed on ice for 2 min between repeats). Following 5 min incubation at room temperature, sample tubes were centrifuged (5 min at 12,000 g and 20 °C) to pellet the matrix and insoluble material. The resultant homogenate was transferred into a MaXtract tube and mixed by inversion with 100 µL 1-bromo-3-chloropropane (BCP; Sigma-Aldrich, Gillingham, UK). Following 3 min incubation at room temperature and a further centrifugation (15 min at 12,000 g and 4 °C), the upper aqueous phase containing RNA was transferred to a new 2 mL tube. This solution was mixed with one volume (approximately 500 µL) of isopropanol (Sigma-Aldrich, Gillingham, UK) by vortexing before transfer to an RNeasy Mini spin column in a 2 mL collection tube. Following further centrifugation (1 min at

12,000g and 20 °C), a DNase digestion treatment was performed ‘on-column’ using an RNase-free DNase set (Qiagen, Hilden, Germany) including a DNase I working solution prepared in advance (DNase I = 8:1 mixture of Buffer RDD:DNase I stock) and as follows. First, the RNeasy spin column was washed with 350 µL Buffer RW1 (centrifuged for 1 min at 8000 g and 20 °C). Second, 80 µL of DNase I working solution was added directly to the membrane and the spin column was incubated as room temperature for 15 min. Finally, the column was washed with 700 µL Buffer RW1 (centrifuged for 15 s at 8000 g and 20 °C). Following DNase digestion, the RNeasy spin column membrane was washed (two aliquots of 500 µL prepared Buffer RPE separately applied and centrifuged at 8000 g; the first for 15 s, the second for 2 min) before RNA was eluted from the column (two aliquots of 50 µL RNase-free water separately added to the column and separately centrifuged at 8000 g for 1 min). Extracted RNA was stored at -80 °C until sequencing.

6.2.5 Quality control and sequencing of RNA

The concentration of nucleic acid in a 1 µL aliquot of undiluted RNA was initially quantified using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, USA) and all ten samples were found to contain RNA at levels \geq 850 ng µL⁻¹ (mean = 995 ng µL⁻¹, SE = 35). The quality and integrity of 1 µL total RNA diluted in RNase-free water (dilution factor = 4) was also assessed on an Agilent 2100 Bioanalyzer using the Agilent RNA 6000 Nano Chip kit according to the manufacturers guidelines (Agilent Technologies, Santa Clara, USA). The resulting Bioanalyzer ribosomal RNA (rRNA) profile was consistent with the endogenous composition of insect rRNA described by

Winnebeck et al. [43] and each sample contained RNA at a concentration \geq 420 ng μL^{-1} (mean = 902 ng μL^{-1} , SE = 92).

We also verified that bees in each treatment group had consumed or not consumed imidacloprid as appropriate by quantifying pesticide residues in their head and thorax. These body parts were used to extract and quantify imidacloprid because: a) they had not been used for RNA extraction (i.e. the tissue was readily available) and; b) residues detected in the head and thorax would indicate distribution of the pesticide throughout the body, whereas residues in the abdomen could contain newly ingested and unprocessed imidacloprid in syrup stored in the bumble bees' honey stomach (Cresswell et al. 2013). Bee tissue was homogenized and centrifuged, before imidacloprid was extracted and quantified using solid phase extraction and LC-MS analysis, respectively, following methods described in Cresswell et al. [36]. Imidacloprid was detected in the head and thorax of four out of five bees in the imidacloprid treatment, but at levels below the level of quantification (mean detected imidacloprid residue = 0.01 ng bee $^{-1}$; level of quantification = 0.16 ng, [36]). The single imidacloprid-dosed bee that was found not to contain imidacloprid was not chosen for sequencing (below). Imidacloprid was not detected in control bees.

Based on the results of quality control, we selected the RNA of six individual bumble bees for sequencing (three control bees, three imidacloprid-treated); the maximum sample size given the financial scope of the experiment. In the final samples, RNA concentration was $>$ 650 ng μL^{-1} (mean = 1014 ng μL^{-1} , SE =

100) according to Bioanalyzer analysis. For sequencing, we started with 4 µg of RNA from each sample. We synthesised cDNA to create a library using the low sample protocol described in the ‘TruSeq RNA Sample Prep. v2 Guide’ (http://support.illumina.com/sequencing/sequencing_kits/truseq_rna_sample_prep_kit_v2/documentation.ilmn) and performed sequencing on an Illumina HiSeq 2000 (Illumina, San Diego, USA). All six bumble bee samples were sequenced on a single lane using paired-end sequencing and by the University of Exeter’s sequencing service team.

6.2.6 RNA-seq analysis

We used FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) to determine the quality of sequence data and we filtered out and removed low quality reads (≤ 20 bases) or adaptors using the default settings of RobiNA [44]. RNA-seq analysis was conducted in TopHat [45] and Cufflinks [46] using the protocol described in Trapnell et al. [47] to perform read alignment, transcript assembly and quantification of differential gene expression. Specifically, TopHat was used to map the filtered reads to the *B. terrestris* reference genome (Bter_1.0; which is available for download at the NCBI website: http://www.ncbi.nlm.nih.gov/genome/2739?project_id=68545). The resulting alignment files were passed to Cufflinks to generate transcriptome assemblies for each individual bumble bee. Prior to differential expression analysis, the transcriptome assemblies were merged in Cufflinks using the Cuffmerge utility to provide a uniform basis for calculating gene expression in both treatments. Within the Cufflinks environment, Cuffdiff was used to calculate the expression levels in dosed and undosed bees and differences in expression were tested for

statistical significance. First, expression levels were normalised by Cufflinks to account for transcript length and yield. Normalised expression levels for each identified transcript were given as ‘fragments per kilobase of transcript per million mapped fragments’ or FPKM [47]. We used $P < 0.05$ to represent a statistically significant difference in FPKM between treatments, with the P -value adjusted by Cufflinks for the false discovery rate using the Benjamini-Hochberg correction.

6.2.7 Identification of differentially expressed genes

Where a transcript was differentially expressed between treatments, we retrieved its FASTA sequence using the gene expression output from Cuffdiff. Specifically, we took a sequence’s start and end position from within a specific linkage group and extracted the full sequence from the Bter_1.0 *B. terrestris* genome assembly (see Table S1). Using these sequences, we identified the differentially expressed transcripts at the gene level with BLASTn [48] by searching for sequence matches in the nucleotide (nr/nt) database. Within BLASTn we set an E-value cutoff set of 1e-20 and chose only the best *B. terrestris* gene match for each sequence. Where a gene match in *B. terrestris* did not exist, we chose the best *Bombus* spp. match instead.

6.2.8 Functional analysis of differentially expressed genes

In order to infer the function of differentially expressed genes, we first identified the conserved domains in their putative protein products that could give an initial indication of molecular and cellular function. For each gene, we used its entry in NCBI (<http://www.ncbi.nlm.nih.gov/gene>) to identify its related protein

product. We took the FASTA sequence of each protein from its NCBI entry (<http://www.ncbi.nlm.nih.gov/protein>) and submitted these sequences to the NCBI Conserved Domain Database (CCD) [49]. We searched for domains in CDD using an E-value cut-off of 1e-2 with low complexity filtering switched off.

Little or no functional annotation was attached to the differentially expressed *B. terrestris* genes and so we further investigated their function by identifying orthologs in organisms with better-annotated genomes; namely the honey bee *A. mellifera*, another Hymenoptera, and *Drosophila melanogaster* Meigen, another arthropod. Orthologs of differentially expressed *B. terrestris* proteins were identified by submitting their sequences to BLASTp [48] and searching against all known proteins for *A. mellifera* and *D. melanogaster*. We used an E-value cut-off 1e-10 and selected only the best-matched ortholog in each organism for further analysis (see Table S2). Several Gene Ontology (GO) terms [50] were attached to the *Apis* orthologs (see Table S3), however the level of annotation was insufficient for further analysis. In contrast, *D. melanogaster* orthologs were extensively annotated and so we investigated GO term enrichment in these genes/proteins. Specifically, we analysed over-represented GO terms and clusters of functional annotation in *Drosophila* orthologs using the web tool DAVID v6.7 [51]. When testing for enrichment of GO terms, we set the count threshold (minimum number of genes per GO term) in DAVID at 2 and the EASE (DAVID's modified Fisher Exact *P*-value) threshold at $P \leq 0.05$. When testing for clusters of functional annotation (a group of annotation consisting of closely related GO terms), we used the following settings in DAVID: similarity term overlap = 4; similarity threshold = 0.35; group

membership = 2; multiple linkage threshold = 0.5; Ease = 0.2. We ranked functional clusters by DAVID's group Enrichment Score (ES, the geometric mean, in –log scale, of the cluster member's P-values; [51]).

6.3 Results and Discussion

6.3.1 Effects of imidacloprid on feeding and locomotion in bumble bees

Prior to RNA-seq, we measured two behavioural endpoints, syrup consumption and locomotory activity, to determine if there were any associations between specific gene responses and adverse phenotypes. For bumble bees that fed for 12 h on syrup dosed with imidacloprid at 98 ppb there was an apparent reduction in syrup consumption between the pre- and post-dose periods, but this was not statistically significant (ANOVA: $F_{1,24} = 2.95$, $P = 0.10$; Fig. 6.1). Dosed bees did, however, show significantly reduced locomotory activity in comparison to undosed bees (ANOVA: $F_{1,24} = 16.11$, $P < 0.001$; Fig. 6.1). These results are consistent with previous studies in which exposure to imidacloprid at 98 ppb over several days reduced feeding and locomotory activity in individual *B. terrestris* workers [31,36] [also see chapter five]. Even a small reduction in sugar-intake could force bumble bees into a low energy state in which they stop moving and enter torpor [52]. The decreased locomotory activity of dosed bees could therefore be a product of imidacloprid's detrimental effect on carbohydrate intake.

6.3.2 Differentially expressed transcripts in imidacloprid-exposed bees

By mapping the sequenced reads obtained from our six bumble bees to the *B. terrestris* Bter_1.0 genome, we identified a total of 21,400 transcripts. Our *B. terrestris* transcriptome is similar in size to the transcriptome produced by Woodward et al. [53] (19,485 contigs), but smaller than that produced by Colgan et al. [54] (36,000 contigs, with ~96 % mapping to the *B. terrestris* genome). The contrast in transcriptome size may have arisen because, similar to Woodward et al. [53], we generated transcripts from abdomens of workers only whereas Colgan et al. [54] generated transcripts from whole bodies of individuals from several *B. terrestris* life stages.

In our *B. terrestris* transcriptome, we identified 26 transcripts that were expressed at a significantly different level in imidacloprid-exposed bumble bees (i.e. $P < 0.05$; see Fig. 6.2 and Table S6.1 for level of expression between treatments; see Fig. 6.3 for expression levels between individual bees). To our knowledge, we are the first to present RNA-seq data from bumble bees exposed to imidacloprid. However, 300 differentially expressed transcripts were identified in honey bee larvae fed for 15 days on syrup dosed with imidacloprid at 2 $\mu\text{g L}^{-1}$ [38]. The disparity in abundance of differentially expressed transcripts is likely to have arisen primarily because of differences in experimental protocol (e.g. exposure period, imidacloprid concentration, organism life-history stage). However, the disproportionate response to imidacloprid observed in the transcriptome of bees in these two studies could begin to explain the differential sensitivity to the pesticide reported in previous

comparative studies of bumble bees and honey bees [31,36]. Of course, this speculation requires further research.

In differentially expressed transcripts, 35 % (9/26) showed elevated levels of RNA (up-regulated genes) in imidacloprid-treated bees relative to control bees, while 65 % (17/26) showed reduced levels of RNA (down-regulated genes). In all differentially expressed transcripts, the fold-change in level of expression (or $\log_2[\text{FPKM}_{\text{dosed}} / \text{FPKM}_{\text{undosed}}]$) was ≥ 0.9 (Table S6.1). Notably, the ratio of up-to down-regulated transcripts we observed (~1:2) was entirely consistent with the expression profile of imidacloprid-exposed honey bee larvae [38].

6.3.3 Functional analysis of differentially expressed genes

Using BLAST, we identified each differentially expressed transcript to gene level, with a significant match made in all cases (BLASTn: E-values $\leq 1e-38$, identities ≥ 73 %; Table 6.1). We identified 96 % of transcripts (25/26) as putative or hypothetical protein encoding genes in the *B. terrestris* genome, while a single transcript without a gene match in *B. terrestris* was instead matched in another bumble bee species, *Bombus impatiens* Cresson (Table 6.1).

Genes are categorized by their molecular functions and involvement in biological processes, and are annotated in relevant databases with GO terms that reflect their known or predicted roles. GO analysis can therefore reveal the likely function of a gene or groups of genes. GO analysis of our differentially expressed *Bombus* genes revealed that annotation was almost entirely lacking,

thus functional analysis of these specific genes was not possible. However, it was possible to analyse GO annotation in orthologs of these genes (or more specifically, their protein products) in closely related and well-annotated organisms. In *A. mellifera*, we identified orthologs for 23 of our differentially expressed proteins (Blastp: E-values $\leq 4e-20$; Table S6.2) and 48 % of these (11/23) were associated with at least one GO term (Table S6.3). The GO terms in these *Apis* orthologs provide some insight into the general function of proteins differentially expressed during imidacloprid exposure (Table S6.3). For example, a number of up-regulated proteins function in aerobic metabolism (with processes involving oxidation-reduction and the electron transport chain featuring most heavily), perhaps reflecting the bees' increased need for energy. Down-regulation of proteins involved in glycolysis and carbohydrate metabolism may be attributable to the reduced consumption of sugars that we observe in dosed bees. Overall, however, GO annotation in *Apis* orthologs was too low to identify significantly enriched GO terms. We therefore investigated orthologs in another insect with arguably the best characterised genome of any organism, namely *Drosophila*.

In *Drosophila*, we identified orthologs for 22 of our differentially expressed *Bombus* proteins (Blastp: E-values $\leq 3e-15$; Table S6.2) and 73 % (16/22) of these were associated with ten significantly enriched GO terms (DAVID: $P \leq 0.05$; Table 6.2). Of these GO terms, 90 % were simultaneously associated with both up- and down-regulated gene products, highlighting the complexity and overlap of processes involved in the bumble bee molecular response to imidacloprid. Nevertheless, GO analysis revealed that differentially expressed

proteins in imidacloprid-exposed bees were orthologs of *Drosophila* proteins operating in metabolism and behaviour (Table 6.2). Specifically, oxidation-reduction activity (Table 6.2) suggests metabolic networks affecting energy release are altered by imidacloprid, while the cellular aromatic compounds (Table 6.2) that are metabolised may include the aromatic ring structure contained within imidacloprid and some of its metabolites [55]. The neurotoxic effect of imidacloprid [56] could explain the altered expression of genes involved in behaviour, cognition and detection of stimuli (Table 6.2). In addition to the enrichment of ten individual GO terms in *Drosophila* orthologs, we identified four significantly enriched GO clusters consisting of functionally related terms (Table 6.3). The first three clusters reconfirmed the results of the initial GO analysis: the highest ranking cluster ($ES = 2.2$) collected orthologs of differentially expressed proteins involved generally in catalysis and metabolic processes; cluster two collected proteins operating in oxidation-reduction ($ES = 1.5$); cluster three contained proteins involved in behavioural ($ES = 1.3$) processes. In the final cluster, nucleotide/nucleoside binding ($ES = 0.8$), the majority of proteins are down-regulated (Table 6.3). This finding suggests that imidacloprid can affect the signalling transduction pathways that are regulated by nucleotide binding proteins. The proper regulation of these pathways can be the difference between health and disease in humans and inhibition of nucleotide binding proteins can have therapeutic benefit [57]. Presumably deviation from normal regulation of these pathways could also have negative consequences for the health of bumble bees, and the apparent down-regulation of proteins involved in nucleotide binding could serve to limit the impact.

In addition to GO analysis of *Apis* and *Drosophila* orthologs, we investigated the conserved domains contained within the proteins encoded by our differentially expressed *Bombus* genes. We found that 85 % (22/26) of these genes encoded proteins that contained known functional domains (CDD: E-values \leq 9e-3; Table 6.1). As we show in Table 6.1, these domains begin to provide a general indication of the molecular and cellular function of individual genes differentially expressed in response to imidacloprid. Among the identified domains were those suggesting the involvement of proteins in several stress-related cellular processes and, below, we discuss the putative role in these processes of all 26 *Bombus* genes differentially expressed in response to dietary imidacloprid.

6.3.3.1 *Nischarin: imidazoline receptor and potential biomarker for imidacloprid exposure*

We found that the protein *nischarin* (XM_003402935) was expressed only in those bees exposed to imidacloprid (see Table 6.1, Fig. 6.2, Fig. 6.3). Functionally, *nischarin* is an intracellular protein known to regulate cell migration [58]. The orchestrated migration of cells to specific locations is integral to the homeostasis of multicellular organisms, but it can also contribute to the progression of harmful pathological processes [59]. In humans, overexpression of *nischarin* leads to profound inhibition of cell migration [58]. Although the role of *nischarin* in bumble bees is currently unknown, it is conceivable that its overexpression in response to imidacloprid also inhibits cell migration, although to what end is unclear. *Nischarin* can also act as a functional imidazoline receptor, mediating cell signalling [60] and binding numerous imidazoline ligands. Imidazoline derivatives are known products of imidacloprid metabolism,

and they can be highly toxic to insect pests and have a high affinity to their nicotinic acetylcholine receptors [61,62]. It is possible that up-regulated *nischarin* proteins in bumble bees bind imidacloprid's harmful metabolites and may therefore have an important role in detoxification of the neonicotinoid.

Additionally, *nischarin* may represent a potential biomarker for imidacloprid exposure in *B. terrestris* bumble bees. An underlying goal of toxicogenomics is to identify a unique profile or 'genetic fingerprint' corresponding with exposure to a given chemical [2]. Once established, the response of organisms exposed to unknown chemicals can be compared to known chemical profiles in order to predict the properties or identity of the unknown. In our experiment, *nischarin* was expressed only in imidacloprid-exposed bumble bees. However its suitability as a biomarker for imidacloprid exposure would depend on further research into its expression over differing exposure periods and concentrations. *Nischarin* is not differentially expressed in imidacloprid-exposed honey bee larvae [38] and the up-regulation we observe may therefore be species or life-stage specific.

6.3.3.2 *Expression of cytochrome P450 enzymes*

In insects, cytochrome P450 enzymes catalyse the oxidation of several compounds including lipids, hormones and xenobiotics such as synthetic insecticides. For example, in *Drosophila* up-regulation of particular P450 genes can confer resistance to certain pesticides [63]. In imidacloprid-exposed bumble bees, we did not observe an up-regulation of P450 enzymes; rather the down-regulation of a single P450 gene (*probable cytochrome P450 6a13*,

XM_003393814; Table 6.1, Fig. 6.2, Fig. 6.3). In response to imidacloprid, honey bee larvae up-regulate a group of nine P450s, although the role of these enzymes in detoxification or resistance to the pesticide is unconfirmed [38]. In contrast, adult honey bee workers do not appear to utilize P450s for detoxification of imidacloprid [37] [and see chapter five]. The apparent disparity between honey bee life stages in regulation of detoxification genes is mirrored in the mosquito *Anopheles gambiae*, which developmentally regulates approximately a quarter of its detoxification genes [64], and probably arises because of differences in exposure, diet and biosynthetic requirements between larvae and adults. Our observation that bumble bee workers do not up-regulate P450s in response to imidacloprid is consistent with our previous work [see chapter five], in which a P450-inhibitor (PBO) had no effect on imidacloprid's toxicity at concentrations up to 98 ppb. We therefore suggest that P450s are not involved in detoxification of imidacloprid in adult bumble bees, although we acknowledge that more studies are required to establish the expression of P450s under differing exposure scenarios. However, it is unclear whether, like in honey bee larvae [38], P450s are expressed in imidacloprid-exposed bumble bee larvae and this is an area that requires further research.

6.3.3.3 Carbohydrate-metabolising genes

Three genes associated with the metabolism of carbohydrates were down-regulated in bumble bees exposed to imidacloprid (XM_003396723, XM_003403329, XM_003398955; Table 6.1, Fig. 6.2, Fig. 6.3). We also observed apparent reduction of syrup consumption in imidacloprid-exposed bees relative to undosed bees. In *Drosophila*, diminished expression of

carbohydrate metabolising genes is caused by nutrient deprivation imposed by starvation [65]. We suggest that the observed down-regulation of carbohydrate-metabolising genes in imidacloprid-exposed bumble bees may be a response to carbohydrate limitation imposed by a toxic repression of syrup feeding [33,35] [see chapters two and four].

The observed down-regulation of carbohydrate-metabolising genes in bumble bees is consistent with previous RNA-seq studies of xenobiotic stress in *Drosophila* [66] and *Apis* [38], in which phenobarbital (a GABA agonist and an effective inducer of detoxification genes) and imidacloprid, respectively, were associated with reduced expression of genes involved in carbohydrate metabolism. Carbohydrates are involved in a wide variety of metabolic processes, but importantly oxidation of sugars provides quick and easy access to energy in species capable of aerobic respiration. Where energy is required but oxygen is limited, anaerobic glycolysis can be used to convert glucose into pyruvate and release the energy necessary to form the high-energy compounds ATP and NADH. *L-lactate dehydrogenase* (XM_003396723) is an enzyme that catalyses the final step of glycolysis. The down-regulation of *L-lactate dehydrogenase* in imidacloprid-exposed bumble bees suggests that the neonicotinoid could compromise the bees' capability to produce energy *via* glycolysis.

In addition to energy production, carbohydrate metabolism drives several other processes. The down-regulated gene *glucose dehydrogenase* (GLD; XM_003403329) is a glucose-metabolizing enzyme from the GMC-FAD

oxidoreductase family [67]. *GLD* plays a major role in insect development and immunity [68] and, for example, is an essential gene for exoskeleton metabolism in early-stage *Drosophila* [69]. The down-regulated enzyme *UDP-glucuronosyltransferase* (XM_003398955) is a major component of Phase II metabolism that catalyses the transfer of the glycosyl group from a nucleotide sugar to specific hydrophobic molecules, resulting in more hydrophilic compounds that are efficiently excreted [70]. In insects, UDP-glucuronosyltransferases have roles in metabolism of endogenous and exogenous substrates, cuticle formation, and olfaction [71]. Whether glucose dehydrogenase or UDP-glucuronosyltransferases have similar functions in the bumble bee remains to be shown, but analogous to other insects diminished regulation of carbohydrate-metabolising genes could compromise important functions other than energy production.

6.3.3.4 Genes involved in oxidative phosphorylation

Two genes with a putative role in energy production *via* oxidative phosphorylation were up-regulated in imidacloprid-exposed bees, namely *NADH-ubiquinone oxidoreductase chain 5* (*NADH*, XM_003493500) and *cytochrome c oxidase subunit 1* (*CO1*, XM_003394378) (Table 6.1, Fig. 6.2, Fig. 6.3). Specifically, both genes are involved in generation of chemical energy in the mitochondrial electron transport chain (ETC): the *NADH* protein is the first enzyme (Complex I) of the ETC; *CO1* is the main subunit of the final enzyme complex (Complex IV). This finding is consistent with previous studies in honey bees, which report increased activity of cytochrome oxidase in the brains of bees dosed with imidacloprid [72,73]. The up-regulation of genes involved in

oxidative phosphorylation is likely to reflect the bees' increased demand for energy when challenged with the metabolic stress imposed by imidacloprid.

6.3.3.5 *Reduced expression of fatty acid and fatty alcohol-synthesising genes*

Three genes with a putative involvement in fatty acid biosynthesis showed reduced transcriptional activity in response to imidacloprid (XM_003399574, XM_003402652, XM_003400541; Table 6.1, Fig. 6.2, Fig. 6.3). Among these were two genes from the fatty acid elongase family: elongation of very long chain fatty acids (VLCFAs) proteins 4 (*ELO4*, XM_003399574) and 6 (*ELO6*, XM_003402652). The fatty acid elongases catalyse the extension by two carbons or more of fatty acids that begin with a chain length \geq 16-carbons [74] and the resultant VLCFAs are incorporated into the cuticles, waxes and sphingolipids of various organisms [75,76]. Down-regulation of fatty acid elongases may be part of a general response to imidacloprid in bees because transcription of *ELO6* is also reduced in imidacloprid-exposed honey bee larvae [38]. Also down-regulated in imidacloprid-exposed bumble bees, the enzyme NAD kinase (*NADK*, XM_003400541) converts nicotinamide adenine dinucleotide (NAD) into phosphorylated NAD (NADP). NADP is used in lipid synthesis and fatty acid chain elongation [77], but is also vital for protection against oxidative stress [78]. In most organisms fatty acids play a major role as energy storage molecules and decreased expression of fatty acid synthesising genes could therefore disrupt energy storage in imidacloprid-exposed bumble bees. Energy storage is probably not a priority during pesticide exposure, rather stored energy is likely used to metabolise the chemical or, in the case of bumble

bees dosed with imidacloprid, in response to reduced nutrient intake imposed by toxic repression of feeding [33,35] [see chapters two and four]. Similar to bumble bees, starvation in *Drosophila* can alter the transcriptional activity of lipid synthesising genes including the *ELO6* ortholog, *baldspot* [79].

The down-regulated gene *fatty acyl-CoA reductase* (*FAR*; XM_003399896; Table 6.1, Fig. 6.2, Fig. 6.3) produces an enzyme that catalyzes the reduction of fatty acyl-CoAs to the fatty alcohols. *FAR* genes have a role in lipid biosynthesis [80] and, in mammals and plants, the production of wax [81,82]. In contrast, insect *FAR* genes function primarily in pheromone, and not wax, biosynthesis [83,84]. For example, the structure and expression profile of the gene *AMFAR1* in honey bees suggests its involvement in pheromone or lipid biosynthesis rather than beeswax production [85]. It is not known whether *FAR* genes are also involved in pheromone production in bumble bees, but if so their reduced transcription in response to imidacloprid could detrimentally affect colonies. Bumble bee workers use pheromones to signal the availability of food [86] and recruit nest mates as foragers [87], so disruption to pheromone biosynthesis could detrimentally affect a colony's foraging capability.

6.3.3.6 *Putative response to stress: up-regulated genes*

In addition to *nischarin*, *NADH* and *CO1*, we identified a further six *Bombus* genes that were up-regulated in response to imidacloprid. Two of these were uncharacterized (XM_003394522, XM_003400362; Table 6.1) and could not be identified because of a lack of functional annotation. As research into the genomes of Hymenopterans progresses [88], the function of these

uncharacterized genes may be revealed. The remaining four genes were identified as having putative roles in response to stress as follows.

Similar to honey bee larvae [38], the uncharacterised gene XM_003397772 is up-regulated over 4-fold in imidacloprid-dosed bumble bees (Table 6.1, Fig. 6.2, Fig. 6.3). The protein product of XM_003397772 contains a glycine rich domain and is therefore part of the glycine rich family of proteins induced in response to various stressors [89]. In stressful environments, adult insects express glycine rich cuticular proteins that induce a change in the nature of their cuticle allowing them to adapt to harsh conditions [90]. For example, the Colorado potato beetle *Leptinotarsa decemlineata* Say expresses glycine rich insect cuticular proteins in response to organophosphorous insecticides and dry environments [90].

The protein product of *aromatic-L-amino-acid decarboxylase* (XM_003399613; Table 6.1, Fig. 6.2, Fig. 6.3) catalyses several different decarboxylation reactions including those involved in synthesis of the neurotransmitter dopamine [91]. Being agonists of nicotinic acetylcholine receptors, neonicotinoids can induce dopamine release as demonstrated in rats exposed to thiamethoxam and clothianidin [92], and *aromatic-L-amino-acid decarboxylase* may have a role in synthesising this neurotransmitter. In *Drosophila*, the ortholog of *aromatic-L-amino-acid decarboxylase* (the gene *Ddc*) plays an important role in learning ability [93], probably because dopamine synthesis is necessary for the formation of the flies' memories of certain aversive stimuli [94]. In honey bees too, dopamine mediates learning aversive stimuli including learning to avoid toxic food [95]. It is conceivable that the

aromatic-L-amino-acid decarboxylase gene is up-regulated in bumble bees as part of dopamine synthesis and in response to imidacloprid being an aversive stimulus that the bees must learn to avoid.

The uncharacterised gene XM_003401440 encodes a protein in the Rab GTPase family with a conserved domain indicative of the Rab38/Rab32 subfamily (Table 6.1, Fig. 6.2, Fig. 6.3). The Rab GTPases are proteins localised to the cytosolic face of intracellular membranes where they function as regulators of membrane traffic, transporting lipids and proteins between membrane-bound organelles [96]. In humans, Rab32 and Rab38 are known to participate in regulation of mitochondrial dynamics [97] and maturation of phagosomes used to engulf and kill pathogenic organisms [98]. However, in *Drosophila* half of all Rabs, including Rab32, are active at neurons [99]. Here they regulate synapse-specific membrane trafficking including synaptic recycling [99], presumably an important function during exposure to a neurotoxicant such as imidacloprid that targets the insect central nervous system.

Finally, the uncharacterised gene XM_003397958 encodes a protein with a domain indicative of the *haemolymph juvenile hormone binding protein* (*JHBP*). In insects, this protein transports juvenile hormone from its site of synthesis to target tissues. A quantitative change in *JHBP* has been linked to insecticide resistance in the house fly *Musca domestica* L. [100]. Whether altered transcription of *JHBP* is related to the bumble bees' resilience to imidacloprid remains to be seen.

6.3.3.7 Putative response to stress: down-regulated genes

In addition to those already discussed, we found a further nine genes down-regulated in bumble bees exposed to imidacloprid. Two of these genes were uncharacterised (XM_003399821, XM_003402117; Table 6.1) and were unidentifiable. The remaining seven genes have putative roles in the response to stress and are discussed in more detail below.

Consistent with the response of honey bee larvae [38], a heat shock protein (*heat shock protein 83 (HSP83)*, XM_003396849; Table 6.1, Fig. 6.2, Fig. 6.3) is down regulated in bumble bees exposed to imidacloprid. Functionally, HSPs are chaperone proteins that assist other proteins to fold and establish conformation and stability. Their expression is often triggered in response to stresses that have the potential to disrupt proteins structure and affect cellular metabolism [101]. For example, expression of *HSP83* protects *Drosophila* against the detrimental effect of sleep deprivation [102] and heat shock [103]. In midges [104] and rats [105] pesticides induce overexpression of HSPs and it is therefore unclear why expression of *HSP83* was lowered in imidacloprid-exposed bumble bees. In dosed honey bee larvae, reduced transcription of HSPs may be a developmental buffering strategy in response to a new stressor [38]. Specifically, it could allow the expression of genetic and epigenetic variants, some of which may improve larval development while others increase sensitivity to imidacloprid [38]. However, whether down-regulation of *HSP83* in bumble bees reflects the effect of imidacloprid on the bees developmental buffering system is not known.

Another gene putatively involved in protein folding, *peptidyl-prolyl cis-trans isomerase FKBP4*, is also down-regulated in both bumble bees (XM_003402688; Table 6.1, Fig. 6.2, Fig. 6.3) and honey bee larvae dosed with imidacloprid [38]. The encoded protein contains a conserved domain indicative of proteins in the FKBP immunophilin family that play an important role in immunity, including defence against neurodegenerative disorders [106]. The protein also contains a tetratricopeptide repeat motif associated with protein-protein interactions [107] that is likely to preferably interact with WD-40 repeat containing proteins such as the down-regulated *WD repeat-containing protein 75* (XM_003394530; Table 6.1, Fig. 6.2, Fig. 6.3). Proteins containing WD-40 domains are abundant in eukaryotes and commonly coordinate assembly of multi-protein complexes involved in a variety of processes, including transcriptional regulation and cell signalling [108].

The four remaining down-regulated genes all have putative roles in transcriptional regulation. The *zinc finger protein 235* (XM_003396949; Table 6.1, Fig. 6.2, Fig. 6.3) contains a C2H2-type zinc finger domain, which indicates its likely involvement as a transcriptional activator or repressor that binds DNA [109]. Its encoding gene has been implicated in phagocytosis and immunity [110]. The enzyme encoded by *S-adenosylmethionine synthase* (SAM; XM_003403220; Table 6.1, Fig. 6.2, Fig. 6.3) catalyzes the formation of S-adenosylmethionine, a methyl donor that enables DNA methylation [111]. The methylation of DNA locks genes in the ‘off’ position and therefore has an important role in genes expression [111,112]. Additionally, SAM genes are putatively involved in translational regulation in *Drosophila* [113]. The *n-terminal*

kinase-like protein (XM_003395781; Table 6.1, Fig. 6.2, Fig. 6.3) is a member of the protein kinase enzyme family that phosphorylate proteins. Phosphorylation functions as an off/on switch for many cellular processes, including transcription, metabolism and immunity [114]. In *Drosophila*, the knockdown of *n-terminal kinase-like* protein's ortholog (the gene *yata*) induces deterioration of the nervous system and decreases lifespan [115]. Also linked to transcription, the down-regulated *u4/U6 small nuclear ribonucleoprotein Prp3-like* (XM_003402816; Table 6.1, Fig. 6.2, Fig. 6.3) is involved in pre-mRNA slicing and spliceosome formation. The protein therefore forms part of the machinery by which primary RNA transcripts are processed to their functional form [116,117]. Together, the reduced expression of these genes suggests that exposure to imidacloprid affects transcriptional and translational regulation in bumble bee workers, which could have negative implications for several important processes.

6.4 Conclusions

Using RNA-seq to analyse the genome-wide transcriptional response of adult *B. terrestris* workers, we have identified several genes that are differentially expressed in bumble bees exposed to dietary imidacloprid. The interpretations of our results are provisional because the actual levels of protein encoded by these genes are yet to measured, but under the assumption that RNA expression levels largely predict protein levels (mRNA sequence and concentration explains two-thirds of protein abundance in human cells, [118]) we have begun to elucidate the toxicogenomics of imidacloprid-exposed

bumble bees. We observed altered transcription of several genes involved in energy metabolism and storage. Similar to honey bee larvae [38], we saw carbohydrate metabolizing and lipid synthesizing genes down-regulated, while genes involved in chemical energy production were up-regulated in response to imidacloprid. Our results suggest that imidacloprid increases the bees' demand for energy, but also diminishes their supply of fuel in the form of metabolized sugars and deposited fat stores. Coupled with nutrient limitation imposed by imidacloprid's toxic repression of feeding [33,35] [see chapter two and chapter four], disruption to energy metabolism could impact the delicate energy economy of individual bumble bees or, more importantly, whole colonies. For example, energy from carbohydrates fuels much of the colony's most important activity, including thermoregulation, flight and foraging [52]. If the energy available is insufficient to maintain these tasks then the colony's fitness will suffer, potentially leading to colony failure. Previous studies have demonstrated that dietary imidacloprid reduces bumble bee colony growth, forager efficiency and queen production, while increasing forager losses [32,34]. Additionally, imidacloprid-exposed bumble bees forced to forage by flying or walking some distance for food suffered increased mortality compared to bees that were supplied with food in-house [30]. The mechanistic basis of these effects is yet to be fully explained, but it seems likely that imidacloprid's capacity to limit both intake and production of energy plays some part. However, we note that environmental exposure of bumble bees to imidacloprid occurs at concentrations much lower than those investigated here (typically < 12 ppb; [119], but see [120]). It is possible that the gene expression profiles identified here are specifically indicative of exposure to large concentrations of

imidacloprid. For example, exposure to imidacloprid at 98 ppb can substantively reduce food consumption in bumble bees (Cresswell et al. 2012; Laycock et al. 2012; Laycock and Cresswell 2013 [see chapters two, four and five]) and therefore the changes in gene expression we have identified could reflect starvation rather than (or as well as) the direct effects of imidacloprid. Whether dietary imidacloprid at concentrations lower than 98 ppb can alter expression of, for example, energy metabolizing genes in bumble bees is currently unknown and is clearly an area for future research.

Our study also raises the question: how do adult bees metabolically detoxify imidacloprid? Honey bee larvae up-regulate detoxification genes in the cytochrome P450 monooxygenase superfamily in response to the neonicotinoid [38], but neither adult honey bees [37] nor bumble bees [also see chapter five] appear to make use of these enzymes. Rather, we observed a molecular response to stress involving up- and down-regulation of several genes with no specific group of detoxification proteins over-represented. Currently, the question of detoxification mechanisms remains unanswered, but further research could uncover the mechanism of imidacloprid toxicity in bees and our work represents a solid foundation of toxicogenomics on which this research can be built.

We also note that because gene and protein expression patterns can be highly dependent on toxicant concentration and time of exposure [2] our study provides only a snapshot of the full toxicogenomic profile. In future studies, the toxicogenomics of imidacloprid-exposed bumble bees should be assessed over

time and dose space. Here we investigated a short-term chronic exposure (12 h) at a relatively high concentration (98 ppb) known to produce physiological effects on bumble bees [31,36]. However, to establish a full understanding of imidacloprid toxicity in bumble bees it will be necessary to investigate toxicogenomic responses over a period of hours, days and possibly weeks and at several other dosages. Furthermore, because some variation in response to imidacloprid is exhibited among individual bees we suggest that, wherever possible, the sample size in future investigations is increased (i.e. more than $N = 3$ per treatment) and this will help to improve the robustness of future findings. Not only will such experiments minimize the misinterpretation of potentially transient responses [2], they will provide insight into changes in the molecular environment that may be indicative of the *B. terrestris* bumble bee's apparent resilience to the neonicotinoid [35] [see chapter four]. Additionally, such studies would better reflect environmental exposures, which occur at low concentrations and potentially extend over several weeks [35] [see chapter four]. Incorporating environmentally relevant exposures into future toxicogenomic testing of bumble bees would signify a step forwards for mechanism-based risk assessment, potentially provide a basis for distinguishing differences in susceptibility to pesticides between bee species, and could contribute a vital insight into the capabilities of important wild pollinators to cope effectively with the ever-changing demands of the agricultural environment.

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Chapter Six: Figures and Tables

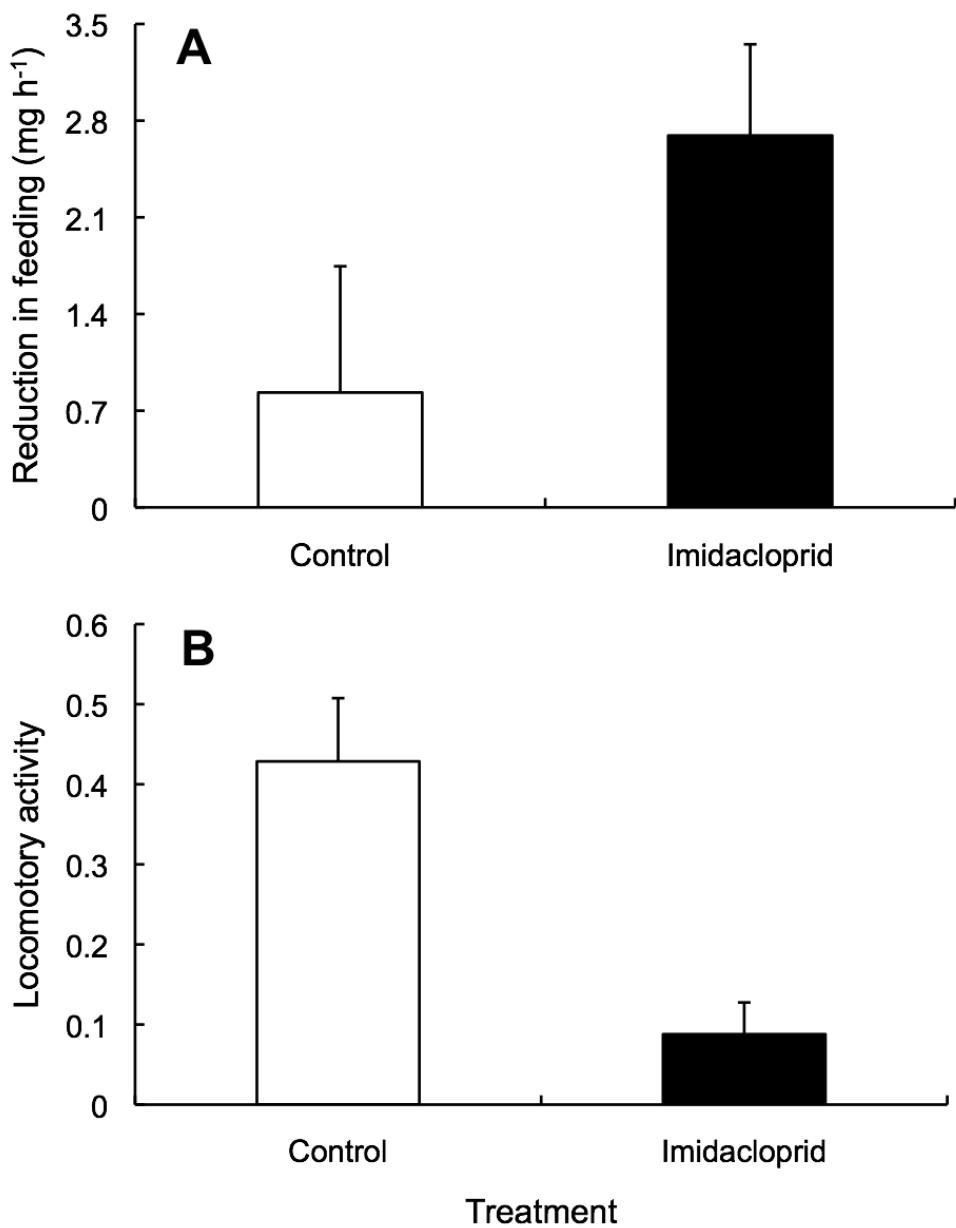


Fig. 6.1 Locomotory activity and reduction in syrup consumption in *B. terrestris* bumble bees exposed to imidacloprid.

All bumble bees (adult *B. terrestris* workers, $N = 26$) were provided with undosed syrup for 12 hours prior to dosing. Once dosing began, bees in the imidacloprid treatment ($N = 13$) were fed syrup dosed at $98 \mu\text{g kg}^{-1}$ for 12 hours, while control bees ($N = 13$) were fed undosed syrup over the same period of time. Reduction in feeding was calculated by the drop in syrup consumption between the pre- and post-dose periods (i.e. *reduction* = *pre-dose feeding* – *post-dose feeding*). Locomotory activity represents the proportion of time bees spent in motion, where bees were scored as either stationary or moving at 10-minute intervals over one hour (between 11–12 h post-dose). Data represent means \pm SE. Imidacloprid significantly affected locomotory activity ($P < 0.001$), but not reduction in feeding rate ($P = 0.1$).

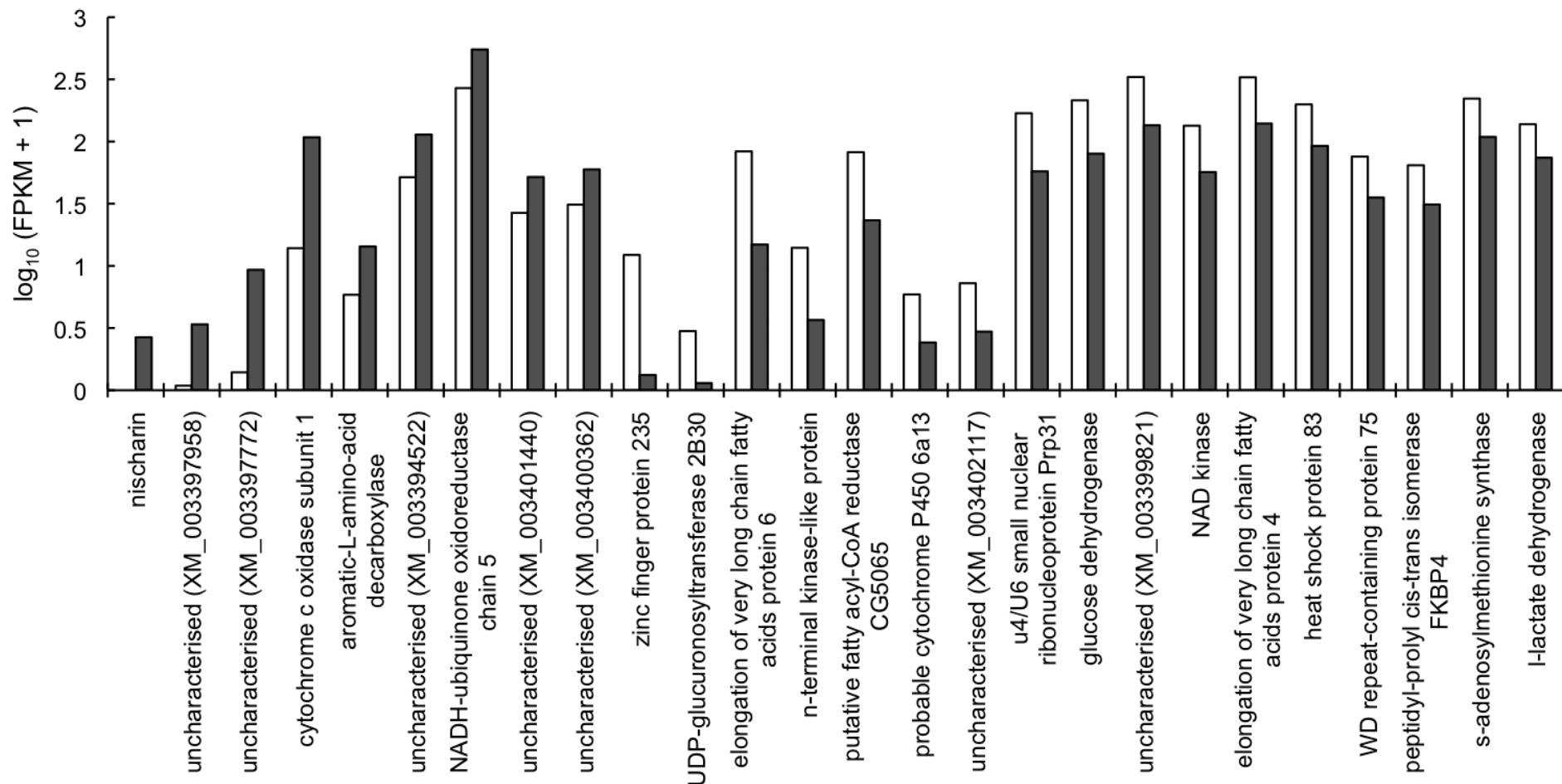


Fig. 6.2 Relative expression levels of 26 significantly differentially expressed transcripts in undosed vs. imidacloprid-exposed bumble bees.

Bumble bees (adult *B. terrestris* workers, $N = 6$) were either fed for 12 hours on syrup dosed with imidacloprid at 98 ppb ($N = 3$: dosed bees, filled bars) or fed undosed syrup over the same period of time ($N = 3$: control bees, unfilled bars). Expression values (y-axis) are given as normalised ‘fragments per kilobase of transcript per million mapped fragments’ (or \log_{10} FPKM + 1). Transcripts (x-axis) are labelled with their gene names as identified in BLAST ([48]; see Table 6.1 for more information).

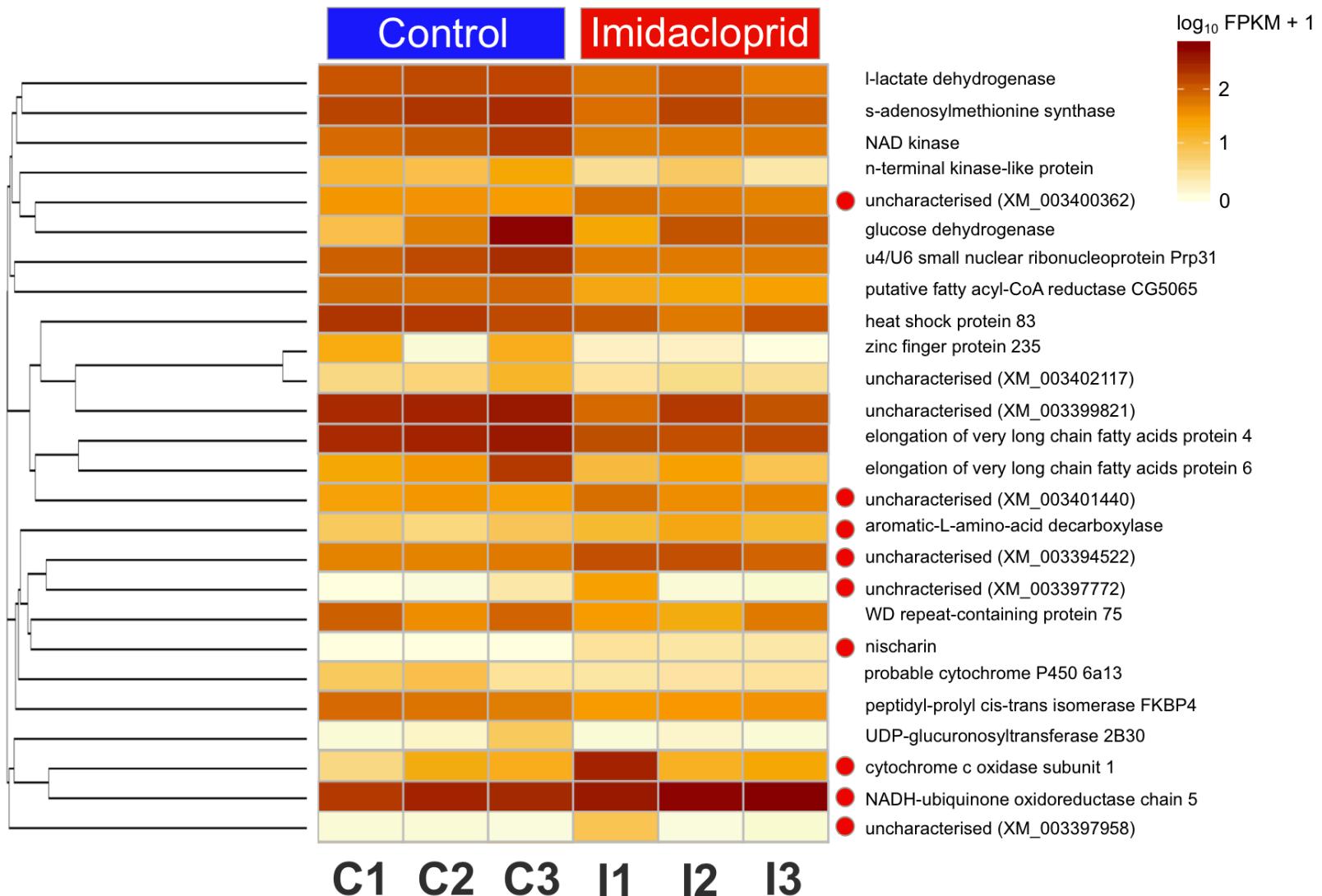


Fig. 6.3 Heatmap of expression values for 26 significantly differentially expressed transcripts in individual imidacloprid-exposed bumble bees.

Bumble bees (adult *B. terrestris* workers, $N = 6$) were either fed for 12 hours on syrup dosed with imidacloprid at 98 ppb ($N = 3$: dosed bees I1, I2, I3) or fed undosed syrup over the same period of time ($N = 3$: control bees C1, C2, C3). Expression values are given as normalised FPKM (fragments per kilobase of transcript per million mapped fragments), with the lightest colour shade indicative of zero expression (e.g. see control bees for the gene *nisharin*) and a continuum of darker shades indicating increasing expression levels. Transcripts up-regulated in imidacloprid-exposed bees are marked with a red circle for ease of inspection and all transcripts are labelled with gene names identified in BLAST ([48]; see Table 6.1 for more information). Transcripts are clustered according to their position in a phylogenetic tree that is structured according to the similarity between protein sequences and therefore begins to describe the evolutionary distance between these sequences. To generate the phylogenetic tree, we used the default parameters in Clustal Omega [121] to generate a multiple sequence alignment of the transcripts putative protein products (with FASTA sequences obtained from NCBI protein database; <http://www.ncbi.nlm.nih.gov/protein>) and passed the output to ClustalW2 [122].

Table 6.1 Genes differentially expressed in *B. terrestris* bumble bees exposed to dietary imidacloprid.

A list of transcripts significantly differentially expressed in imidacloprid-exposed *B. terrestris* bumble bee workers relative to undosed control bees, following a 12 h exposure to imidacloprid at 98 µg kg⁻¹ in dietary syrup. List includes gene identification from BLAST, functional protein domain identification from CDD and predicted protein function.

Best BLAST match in <i>Bombus</i>		Conserved protein domains and function		
Gene name	Accession number (RefSeq)	Identity ^a (%)	Functional domains ^b	Putative protein function
↑ nischarin	XM_003402935	73	Phosphoinositide binding Phox Homology domain of the Imidazoline Receptor Antisera-Selected: cd06875, 1e-63	Imidazoline receptor; cell signalling; cell migration
↑ hypothetical protein	XM_003397958	100	Haemolymph juvenile hormone binding protein: cl12117, 3e-04	Hormone transport; xenobiotic resistance
↑ hypothetical protein	XM_003397772	100	Glycine rich protein family: cl06274, 3e-03	Response to stress
↑ cytochrome c oxidase subunit 1	XM_003394378	94	Heme-copper oxidase subunit I: cl00275, 5e-130	Oxidative phosphorylation; ATP synthesis

Table 6.1 Genes differentially expressed in *B. terrestris* bumble bees exposed to dietary imidacloprid. (Continued)

Best BLAST match in <i>Bombus</i>		Conserved protein domains and function		
Gene name	Accession number (RefSeq)	Identity ^a (%)	Functional domains ^b	Putative protein function
↑ aromatic-L-amino-acid decarboxylase	XM_003399613	84	DOPA decarboxylase family: cd06450, 3e-121	Dopamine synthesis; learning and memory
↑ hypothetical protein	XM_003394522	100		Unknown
↑ NADH-ubiquinone oxidoreductase chain 5 ^c	XM_003493500	88	NADH dehydrogenase subunit 5: MTH00165, 8e-43	Oxidative phosphorylation; ATP synthesis
↑ hypothetical protein	XM_003401440	100	Rab GTPase family, Rab38/Rab32 subfamily: cd00154, 1e-119	Protein trafficking; synaptic recycling
↑ hypothetical protein	XM_003400362	100		Unknown
↓ zinc finger protein 235	XM_003396949	100	Zinc finger, C2H2 type: cl15478, 9e-03	Transcriptional regulation; immune response
↓ UDP-glucuronosyltransferase 2B30	XM_003398955	76	GT1_Gtf_like: cd03784, 2e-13; Ecdysteroid UDP-glucosyltransferase: PHA03392, 1e-49	Carbohydrate metabolism; Phase II xenobiotic metabolism
↓ elongation of very long chain fatty acids protein 6	XM_003402652	100	ELO GNS1/SUR4 family: pfam01151, 3e-58	Fatty acid biosynthesis

Table 6.1 Genes differentially expressed in *B. terrestris* bumble bees exposed to dietary imidacloprid. (Continued)

Best BLAST match in <i>Bombus</i>		Conserved protein domains and function		
Gene name	Accession number (RefSeq)	Identity ^a (%)	Functional domains ^b	Putative protein function
↓ n-terminal kinase	XM_003395781	78	Catalytic domain of Protein Kinases: cd00180, 1e-13	Protein phosphorylation; regulation of cellular response
↓ putative fatty acyl-CoA reductase CG5065	XM_003399896	100	fatty acyl CoA reductases (FARs), extended SDRs: cd05236, 2e-112; C- terminal domain of fatty acyl CoA reductases: cd09071, 7e-34	Fatty alcohol biosynthesis; pheromone production
↓ probable cytochrome P450 6a13	XM_003393814	100	Cytochrome P450: cl12078, 5e-53	Oxidative metabolism; xenobiotic resistance
↓ hypothetical protein	XM_003402117	87		Unknown
↓ u4/U6 small nuclear ribonucleoprotein Prp31	XM_003402816	81	Putative snoRNA binding domain: pfam01798, 5e-40; Prp31 C terminal domain: pfam09785, 4e-28; SIK1: COG1498, 3e-50	mRNA slicing; RNA processing
↓ glucose dehydrogenase [acceptor]	XM_003403329	100	GMC_oxred_N: pfam00732, 4e-51	Carbohydrate metabolism; exoskeleton metabolism

Table 6.1 Genes differentially expressed in *B. terrestris* bumble bees exposed to dietary imidacloprid. (Continued)

Best BLAST match in <i>Bombus</i>		Conserved protein domains and function		
Gene name	Accession number (RefSeq)	Identity ^a (%)	Functional domains ^b	Putative protein function
↓ hypothetical protein	XM_003399821	100		Unknown
↓ NAD kinase	XM_003400541	100	NAD kinase: COG0061, 2e-60	NAD metabolism; fatty acid biosynthesis
↓ elongation of very long chain fatty acids protein 4	XM_003399574	100	ELO: GNS1/SUR4 family: pfam01151, 1e-67	Fatty acid biosynthesis
↓ heat shock protein 83	XM_003396849	100	HATPase_c: cd00075, 1e-06, Hsp90 protein: pfam00183, 0	Response to stress; protein folding and stability
↓ WD repeat-containing protein 75	XM_003394530	100	WD40: cd00200, 4e-05	Transcriptional regulation
↓ peptidyl-prolyl cis-trans isomerase FKBP4	XM_003402688	99	Tetratricopeptide repeat domain: cd00189, 10e-15; FKBP-type peptidyl-prolyl cis-trans isomerase: pfam00254	Protein folding, trafficking, transcription; immune response
↓ s-adenosylmethionine synthase	XM_003403220	100	S-AdoMet_synt_C: pfam02773, 9e-82; S-AdoMet_synt_M: pfam02772, 9e-69; S-AdoMet_synt_N: pfam00438, 6e-61	Transcriptional and translational regulation

Table 6.1 Genes differentially expressed in *B. terrestris* bumble bees exposed to dietary imidacloprid. (Continued)

Best BLAST match in <i>Bombus</i>		Conserved protein domains and function		
Gene name	Accession number (RefSeq)	Identity ^a (%)	Functional domains ^b	Putative protein function
↓ L-lactate dehydrogenase	XM_003396723	100	L-lactate dehydrogenase_1: cd05293; L-LDH-NAD: TIGR01771, 3e-137	Carbohydrate metabolism; glycolysis

^a BLAST maximum identity: percent similarity between the query and subject sequences over the length of the coverage area [48]

^b Functional domains identified from putative gene product in Conserved Domain Database [49]. Data represent domain name, domain accession number and E-value for the domain appearing in the putative protein

^c An appropriate gene match in *B. terrestris* genome was not identified, match is therefore taken from *B. impatiens* genome

Table 6.2 Gene Ontology of differentially expressed *Bombus* genes based on annotation of orthologs in *D. melanogaster*.

Gene Ontology (GO)	GO IDs	P-value ^a	Up-regulated ^b	Down-regulated ^b
Biological Process (unknown)			4 (2)	12 (4)
Metabolism	GO:0008152	0.003	4	12
Behaviour	GO:0007610	0.009	3	2
Cellular aromatic compound metabolism	GO:0006725	0.011	2	1
Cognition	GO:0050890	0.030	2	2
Oxidation-reduction	GO:0055114	0.030	2	3
Alcohol metabolism	GO:0006066	0.045	1	2
Cellular amino acid derivative biosynthesis	GO:0042398	0.049	1	1
Detection of light stimulus	GO:0050908; GO:0050962; GO:0009584	≤ 0.05	0	2
Molecular function (unknown)			4 (5)	11 (6)
Catalytic activity	GO:0003824	0.001	4	11
Oxidoreductase activity	GO:0016491	0.027	2	3

^a Significantly enriched ($P < 0.05$) GO terms [50] taken from 22 *Drosophila* genes identified as orthologs of differentially expressed *Bombus* genes

^b Expression patterns within the gene set are represented as the number of genes significantly up- or down-regulated. Genes with unknown biological process or molecular function are in parentheses

Table 6.3 Functional clusters of differentially expressed *Bombus* genes based on GO annotation in *D. melanogaster* orthologs.

Gene Ontology ^a	GO IDs	P-value	Up-regulated ^b	Down-regulated ^b
Cluster 1 (Enrichment score: 2.20)				
Catalytic activity	GO:0003824	0.001	4	11
Metabolism	GO:0008152	0.003	4	12
Cellular metabolism	GO:0044237	0.059	4	7
Cluster 2 (Enrichment score: 1.54)				
Oxidoreductase activity	GO:0016491	0.027	2	3
Oxidation-reduction	GO:0055114	0.030	2	3
Cluster 3 (Enrichment score: 1.34)				
Behaviour	GO:0007610	0.009	3	2
Cognition	GO:0050890	0.030	2	2
Cellular metabolism	GO:0044237	0.059	4	7
Response to stimulus	GO:0050896	0.071	3	3
Neurological system process	GO:0050877	0.090	2	2
System process	GO:0003008	0.095	2	2
Cluster 4 (Enrichment score: 0.82)				
Purine nucleotide binding	GO:0017076	0.093	1	4

Table 6.3 Functional clusters of differentially expressed *Bombus* genes based on GO annotation in *D. melanogaster* orthologs.
 (Continued)

Gene Ontology ^a	GO IDs	P-value	Up-regulated ^b	Down-regulated ^b
Nucleotide binding	GO:0000166	0.161	1	4
Adenyl nucleotide binding	GO:0030554	0.173	1	3
Purine nucleoside binding	GO:0001883	0.175	1	3
Nucleoside binding	GO:0001882	0.178	1	3

^a Functional clusters (groups of annotation that consist of closely related GO terms; [50] were produced using the DAVID web tool v6.7 [51] and 22 *Drosophila* genes identified as orthologs of differentially expressed *B. terrestris* genes. The DAVID group Enrichment Score (the geometric mean, in -log scale, of the cluster member's P-values; [51] is used to rank cluster (see section 6.2.8 for full details)

^b Expression patterns within the gene set are represented as the number of genes significantly up- or down-regulated

Chapter Six: Supplementary material

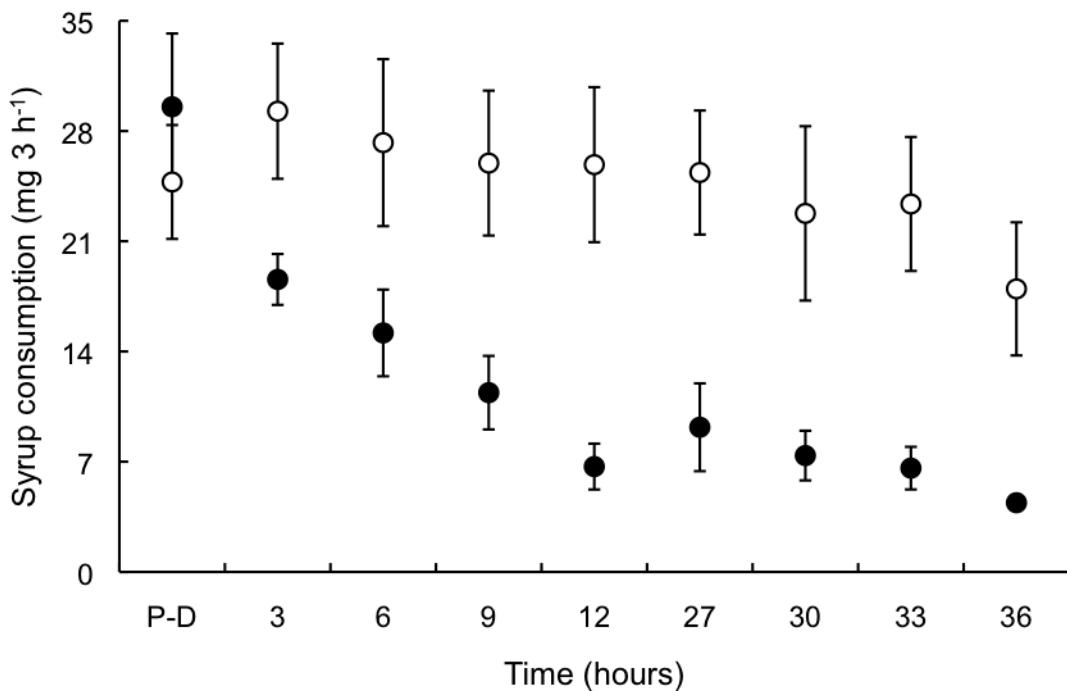


Fig. S6.1 Syrup consumption in *B. terrestris* bumble bee workers exposed to dietary imidacloprid for 36 hours.

Feeding response of individual bees ($N = 20$), fed undosed control syrup (unfilled circles, $N = 10$) or syrup dosed with imidacloprid at $98 \mu\text{g kg}^{-1}$ (filled circles, $N = 10$), which was measured at three-hourly intervals over an exposure period of 36 hours. Before dosing began, all bees fed exclusively on undosed syrup for 12 hours and the estimated pre-dose (P-D) three-hourly feeding rate over this period is displayed. Once dosing began, syrup consumption was measured between 0-12 hours and 24-36 hours. Data represent mean \pm SE.

Table S6.1 Significantly differentially expressed transcripts in imidacloprid-exposed *B. terrestris* bumble bee workers.

Differential expression of bees exposed for 12 hours to dietary imidacloprid at 98 µg kg⁻¹ relative to undosed control bees. List includes the position of the transcript in the *B. terrestris* genome assembly, the fold-change in expression level and the gene match from BLAST.

Linkage group (accession no.) ^a	Start position ^b	Length ^c	Fold change ^d	P-value ^e	<i>Bombus</i> gene accession ^f
LG B09 (NC_015770)	1,725,205	6552	1.79e+308	.018	XM_003402935
LG B09, Group9.6 (NW_003565363)	618,874	5883	4.77	<.001	XM_003397958
LG B09, Group9.4 (NW_00356536)	912,414	2084	4.40	<.001	XM_003397772
GroupUn3310 (NW_003568713)	728	236	3.06	<.001	XM_003394378
LG B07, Group7.3 (NW_003565351)	10,258,262	1407	1.46	.031	XM_003399613
LG B03, Group3.5 (NW_003565335)	2,125,206	4048	1.15	.001	XM_003394522
GroupUn4769 (NW_003570172)	0	551	1.03	.001	XM_003493500
LG B15, Group15.6 (NW_003565395)	5,864,513	16453	0.99	.004	XM_003401440
LG B14 (NC_015775)	892,386	4221	0.96	.003	XM_003400362
LG B07, Group7.3 (NW_003565351)	13,202,848	558	-5.11	.005	XM_003396949
GroupUn609 (NW_003566012)	93,451	2749	-3.80	.001	XM_003398955
GroupUn609 (NW_003566012)	77,732	15254	-2.57	<.001	XM_003402652
LG B02, Group2.2 (NW_003565328)	819,741	3085	-2.28	<.001	XM_003395781
LG B12, Group12.2 (NW_003565374)	6,898,772	4436	-1.86	<.001	XM_003399896

**Table S6.1 Significantly differentially expressed transcripts in imidacloprid-exposed *B. terrestris* bumble bee workers.
(Continued)**

Linkage group (accession no.) ^a	Start position ^b	Length ^c	Fold change ^d	P-value ^e	<i>Bombus</i> gene accession ^f
LG B02, Group2.3 (NW_003565329)	3,176,169	1549	-1.78	.042	XM_003393814
LG B05 (NC_015766)	4,145,837	10767	-1.67	.012	XM_003402117
LG B14, Group14.5 (NW_003565386)	2,347,825	16942	-1.57	.009	XM_003402816
GroupUn1737 (NW_003567140)	0	1769	-1.44	.010	XM_003403329
LG B12, Group12.2 (NW_003565374)	5,372,033	4166	-1.30	<.001	XM_003399821
LG B14, Group14.5 (NW_003565386)	2,365,934	3813	-1.25	.002	XM_003400541
LG B12 (NC_015773)	38,647	3916	-1.24	.001	XM_003399574
LG B07, Group7.3 (NW_003565351)	10,374,884	3304	-1.12	.001	XM_003396849
LG B03, Group3.5 (NW_003565335)	2,919,604	12862	-1.11	.026	XM_003394530
GroupUn633 (NW_003566036)	153,424	6754	-1.07	.012	XM_003402688
GroupUn1054 (NW_003566457)	116,662	11014	-1.03	.020	XM_003403220
LG B07, Group7.3 (NW_003565351)	5,414,398	4170	-0.90	.026	XM_003396723

^a Whole genome and linkage groups in the *B. terrestris* genome assembly (Bter_1.0)

^b Transcript start position in linkage group and ^c transcript length

^d Fold change in gene expression between treatments defined as $\log_2(\text{FPKM}_{\text{dosed}} / \text{FPKM}_{\text{undosed}})$. FPKM represents fragments per kilobase of transcript per million mapped fragments

^e Significance level for differential expression of transcripts between treatments ($P < 0.05$ following a false discovery rate adjustment using the Benjamini-Hochberg correction)

^f Gene accession number of best BLAST match for the transcript in appropriate *Bombus* genome

Table S6.2 Orthologs of differentially expressed *B. terrestris* genes identified in *A. mellifera* and *D. melanogaster*.

Orthologs were identified by searching against all proteins in appropriate organism using BLASTp with the putative protein products of differentially expressed *Bombus* genes.

Bombus gene acc. no.	Bombus protein acc. no.	<i>A. mellifera</i> ortholog (gene, Entrez GenID)	BLAST match ID	<i>D. melanogaster</i> ortholog (gene name; Entrez GenID)	BLAST match (E-value; % ID)
XM_003402935	XP_003402983	nischarin-like; 411145	0; 96	CG11807; 36683	3e-101; 38
XM_003397958	XP_003398006	uncharacterized; 100576902	4e-151; 83		
XM_003397772	XP_003397820	uncharacterized; 100578625	5e-28; 64	CG16885; 34811	3e-15; 44
XM_003394378	XP_003394426	cytochrome oxidase 1; 807695	6e-153; 84	mitochondrial Cytochrome c oxidase subunit I; 192469	2e-111; 64
XM_003399613	XP_003399661	dopa decarboxylase; 410638	0; 89	Ddc Dopa decarboxylase; 35190	0; 73
XM_003394522	XP_003394570	uncharacterized; 100577178	4e-20; 52		
XM_003493500	XP_003493548	NADH dehydrogenase subunit 5; 807698	4e-114; 44	mitochondrial NADH-ubiquinone oxidoreductase chain 5; 192478	2e-71; 36
XM_003401440	XP_003401488	uncharacterized; 726887	0; 79	Itd lightoid; 35940	4e-115; 72
XM_003400362	XP_003400410				

Table S6.2 Orthologs of differentially expressed *B. terrestris* genes identified in *A. mellifera* and *D. melanogaster*. (Continued)

Bombus gene acc. no.	Bombus protein acc. no.	A. mellifera ortholog (gene, Entrez GenID)	BLAST match ID	D. melanogaster ortholog (gene name; Entrez GenID)	BLAST match (E-value; % ID)
XM_003396949	XP_003396997	zinc finger protein 235-like; 552585	0; 99	CG7368; 39301	1e-98; 64
XM_003398955	XP_003399003	glucuronosyltransferase; 409304	0; 66	CG15661; 37421	1e-72; 29
XM_003402652	XP_003402700	elongation of very long chain fatty acids protein 6-like; 725031	1e-121; 60	Baldspot; 39860	9e-106; 55
XM_003395781	XP_003395829	catalase; 411758	0; 93	Yata; 43508	0; 63
XM_003399896	XP_003399944	putative fatty acyl-CoA reductase CG5065-like; 100578329	0; 67	CG1443; 43420	2e-127; 38
XM_003393814	XP_003393862	cytochrome P450 336A1; 724211	5e-169; 52	Cyp6a14; 35835	3e-52; 27
XM_003402117	XP_003402165				

Table S6.2 Orthologs of differentially expressed *B. terrestris* genes identified in *A. mellifera* and *D. melanogaster*. (Continued)

Bombus gene acc. no.	Bombus protein acc. no.	A. mellifera ortholog (gene, Entrez GenID)	BLAST match ID	D. melanogaster ortholog (gene name; Entrez GenID)	BLAST match (E-value; % ID)
XM_003402816	XP_003402864	u4/U6 small nuclear ribonucleoprotein Prp31-like; 410907	0; 98	Prp31; 39655	0; 69
XM_003403329	XP_003403377	GMC oxidoreductase; 410748	0; 70	CG9503; 32424	0; 59
XM_003399821	XP_003399869			norpA no receptor potential A; 31376	2e-00; 25
XM_003400541	XP_003400589	NAD kinase-like; 408470	0; 87	CG33156; 36507	0; 68
XM_003399574	XP_003399622	elongation of very long chain fatty acids protein 4-like; 552205	3e-123	James bond; 42657	2e-95; 53
XM_003396849	XP_003396897	heat shock protein 90; 408928	0; 98	heat shock protein 83; 38389	0; 82
XM_003394530	XP_003394578	WD repeat-containing protein 75-like; 550808	0; 84	CG12050; 35374	2e-89; 34

Table S6.2 Orthologs of differentially expressed *B. terrestris* genes identified in *A. mellifera* and *D. melanogaster*. (Continued)

Bombus gene acc. no.	Bombus protein acc. no.	A. mellifera ortholog (gene, Entrez GenID)	BLAST match ID	D. melanogaster ortholog (gene name; Entrez GenID)	BLAST match (E-value; % ID)
XM_003402688	XP_003402736	FK506-binding protein FKBP59; 412287	0; 90	FK506-binding protein FKBP59; 47762	5e-143; 50
XM_003403220	XP_003403268	s-adenosylmethionine synthase-like; 551102	0; 96	S-adenosylmethionine synthetase; 48552	0; 73
XM_003396723	XP_003396771	L-lactate dehydrogenase; 411188	0; 77	mpL3 Ecdysone-inducible gene L3; 45880	3e-176; 72

Table S6.3 Gene Ontology of differentially expressed *Bombus* genes based on annotation of orthologs in *A. mellifera*.

Gene Ontology ^a	GO IDs	Up-regulated ^b	Down-regulated ^b	Gene (Refseq) accession number ^c
Biological Process (unknown)		4 (4)	7 (8)	
Oxidation-reduction	GO:0055114	2	2	XM_003396723; XM_003393814; XM_003394378; XM_003493500
Aerobic respiration	GO:0009060	1	0	XM_003394378
Electron transport chain	GO:0022900; GO:0042773	2	0	XM_003394378; XM_003493500
Oxidative phosphorylation	GO:0006119	1	0	XM_003394378
Carboxylic acid metabolism	GO:0006520; GO:0019752	1	0	XM_003399613
Cell communication	GO:0007154	1	0	XM_003402935
Metabolism	GO:0008152	0	1	XM_003398955
Carbohydrate metabolism	GO:0005975; GO:0044262	0	1	XM_003396723
Glycolysis	GO:0006096	0	1	XM_003396723
Protein phosphorylation	GO:0006468	0	1	XM_003395781
Protein folding	GO:0006457	0	2	XM_003396849; XM_003402688
Response to stress	GO:0006950	0	1	XM_003396849
mRNA splicing	GO:0000244; GO:0000398	0	1	XM_003402816

**Table S6.3 Gene Ontology of differentially expressed *Bombus* genes based on annotation of orthologs in *A. mellifera*.
(Continued)**

Gene Ontology ^a	GO IDs	Up-	Down-	Gene (Refseq) accession number ^c
		regulated ^b	regulated ^b	
Molecular function (unknown)		4 (5)	5 (12)	
Oxidoreductase activity	GO:0016491; GO:0016616; GO:0016705; GO:0004497	2	2	XM_003396723; XM_003393814; XM_003394378; XM_003493500
Electron carrier activity	GO:0009055	1	1	XM_003393814; XM_003394378
Ion binding	GO:0005506; GO:0020037; GO:0046872; GO:0008270	1	2	XM_003393814; XM_003394378; XM_003396949;
Catalytic activity	GO:0006520; GO:0016829; GO:0016831	1	1	XM_003399613; XM_003396723
Phosphate binding	GO:0030170; GO:0035091	2	0	XM_003399613; XM_003402935
Cytochrome-c oxidase activity	GO:0035091	1	0	XM_003394378
NADH dehydrogenase activity	GO:0004129	1	0	XM_003493500
Transferase activity	GO:0016740; GO:0016757; GO:0016758; GO:0016772	0	2	XM_003398955; XM_003395781
Nucleic acid binding	GO:0003676	0	1	XM_003396949
Protein binding	GO:0051082	0	1	XM_003396849

**Table S6.3 Gene Ontology of differentially expressed *Bombus* genes based on annotation of orthologs in *A. mellifera*.
(Continued)**

Gene Ontology ^a	GO IDs	Up-regulated ^b	Down-regulated ^b	Gene (Refseq) accession number ^c
Molecular function (Cont.)				
ATP binding	GO:0005524	0	2	XM_003396849; XM_003395781
Protein kinase activity	GO:0004672	0	1	XM_003395781
L-lactate dehydrogenase activity	GO:0004459	0	1	XM_003396723

^a Listed GO terms, not significantly enriched ($P < 0.05$) in 23 *A. mellifera* genes identified as orthologs of differentially expressed *Bombus* genes

^b Expression patterns within the gene set are represented as the number of genes significantly up- or down-regulated. Genes with unknown biological process or molecular function are in parentheses.

Chapter Seven

Discussion: Findings, implications, caveats and
future research

7.1 Overall thesis aims and objectives

The overall aim of the thesis research was two-fold: a) to further investigate the effects of neonicotinoids on endpoints of demographic importance in bumble bees, particularly at field-realistic exposures; and b) to improve understanding of the effect mechanisms of neonicotinoids in bumble bees. In order to achieve this aim, laboratory experiments were conducted on *B. terrestris* at the individual, microcolony, and colony levels. Chapters two and three comprised investigations into the effects of dietary imidacloprid and thiamethoxam on feeding behaviour and brood production in queenless microcolonies of *B. terrestris* workers. From these studies conclusions were drawn about the differential sensitivity of bumble bees to the two neonicotinoid compounds. Chapter four built upon the work of chapter two with an investigation into effects of dietary imidacloprid in queenright *B. terrestris* colonies and, for the first time, the capacity for recovery from imidacloprid-induced effects in bumble bees. Chapters five and six focussed on the effect mechanisms of neonicotinoids in bumble bees, specifically with an investigation into the role of cytochrome P450 enzymes in metabolism of neonicotinoids in *B. terrestris* and a transcriptomics study of imidacloprid's effect mechanisms. Here in chapter seven, the final discussion chapter, key findings of the thesis work are presented, together with a description of the environmental implications and the limitations of the research. Furthermore, suggestions are made for future research.

7.2 Key findings

7.2.1 Field-realistic concentrations of dietary imidacloprid repress feeding and brood production in bumble bees

In chapter two, queenless microcolonies of *B. terrestris* workers exposed to dietary imidacloprid for approximately two weeks exhibited a dose-dependent decrease in brood production as concentrations increased from zero to 98 ppb.

In chapter four, the effect was mirrored in queenright colonies exposed over a similar period of time and to the same imidacloprid concentrations. In both studies, dietary concentrations of approximately one ppb imidacloprid – an exposure at the lower end of the ‘typical field-realistic’ range – were capable of reducing brood production, and doing so by around 50 percent. These studies were the first to test measured concentrations in range of one ppb, and the results are comparable to the EC₅₀ value for imidacloprid’s effect on brood production in *B. terrestris* microcolonies (3.7 ppb) in the study by Mommaerts et al. (2010b). With the exception of the highest exposure concentration (98 ppb), the detrimental impact of imidacloprid on brood production was not caused by impaired ovary development or delayed oviposition.

In microcolonies and queenright colonies, bumble bees exhibited a dose-dependent reduction in daily consumption of syrup and pollen, with feeding substantively reduced by concentrations of imidacloprid at the lower end of the ‘typical field-realistic’ range. For example, the EC₅₀ value for imidacloprid’s effect on pollen feeding in queenright colonies was 4.4 ppb. The observed reduction in brood production was likely linked with this reduction in feeding because: a) colonies that consumed more syrup and pollen produced more

brood individuals (independent of dose); b) bees showed reduced feeding when exposed to imidacloprid; and c) reduced production of brood coincided with reduced feeding. The carbohydrate and protein that insects obtain from nectar (or syrup) and pollen are essential components for reproduction generally (Webster et al. 1979; Murphy et al. 1983; Wheeler 1996; Boggs 1997; O'Brien et al. 2000). In bumble bees specifically these nutrients are used in egg production and contribute towards the energetically costly maintenance of high abdominal temperatures necessary for successful incubation of ovaries and brood (Vogt et al. 1998; Heinrich 2004). From the results in chapters two and four it was therefore suggested that imidacloprid, by reducing bees' consumption of food, imposed a nutrient limitation on colonies that repressed production of brood.

Reduced feeding on dosed syrup could indicate that imidacloprid is an aversive stimulus in bumble bees. Imidacloprid has anti-feeding effects at concentrations above $40 \mu\text{g L}^{-1}$ in honey bees (DEFRA 2007) and below $6 \mu\text{g L}^{-1}$ in *Myzus* spp. (Devine et al. 1996), and produces a repellent effect at around $1 \mu\text{g L}^{-1}$ in various Dipterans and Coleopterans (Easton and Goulson 2013). In bumble bees fed field-realistic doses, a previous laboratory study has shown that a mixture of thiamethoxam and clothianidin produced a possible anti-feeding effect in *B. terrestris* colonies (Fauser-Misslin et al. 2014). In another laboratory study, however, anti-feeding effects of imidacloprid were ruled out as a cause of reduced feeding in individual bumble bees because the effect intensified over time (Cresswell et al. 2012b). Furthermore, anti-feedant effects were not observed in laboratory-maintained *B. terrestris* colonies fed imidacloprid in

sucrose solution at 10 ppb, even though bees were also able to forage for uncontaminated nectar in the environment (Gill et al. 2012). In chapter four, queenright colonies of *B. terrestris* that ingested imidacloprid ≥ 39 ppb exhibited reduced syrup consumption from the outset (Laycock pers. obs., see Fig. S7.1), and so anti-feeding effects cannot be excluded for exposure to the highest concentrations. However, in the experiments conducted in this thesis the pollen was not dosed yet bees showed a dose-dependent reduction in consumption of pollen. Therefore, field-realistic concentrations of imidacloprid consumed in syrup produced a toxic effect that reduced the bumble bees' overall ability or 'desire' to feed themselves and their brood. Although the mechanism underlying this toxic effect was not investigated, in chapter six an exposure to 98 ppb imidacloprid induced a molecular response in bumble bees indicative of increased energy demand with reduced energy supply as metabolism of carbohydrate and storage of fat were apparently curtailed. If a similar molecular response is exhibited by bumble bees exposed to 'typical field-realistic' concentrations it would in part explain the impact on feeding in this work, because disruption to the bees' energy balance could, for example, induce a lethargy in which they fail to feed (Heinrich 2004). However, further research would be required to confirm whether this is indeed the case.

7.2.2 Feeding and brood production in queenright bumble bee colonies are resilient to imidacloprid as a pulsed exposure

Wild bees are likely to experience a pulsed exposure to neonicotinoids in the wild. It occurs wherever bees forage on the nectar and pollen of a treated mass-flowering crop during its transitory bloom (Westphal et al. 2003; Stanley et al.

2013) and subsequently revert to foraging on pesticide-free wildflowers (Goulson and Darvill 2004). In chapter four, following a 14-day imidacloprid exposure that reduced feeding and brood production in queenright colonies, the neonicotinoid was removed from the bees' diet for a further 14 days – thereby creating a 28-day pulsed exposure. Following removal of imidacloprid from their diet, bumble bees showed dose-dependent recuperation of feeding and brood production that occurred even after exposure to concentrations as high as 98 ppb. Although the bees' performance did not always recover fully, the effects of the recuperation were such that colonies previously exposed to field-realistic residues (0.3-10 ppb) saw, for example, an 18-84 percent drop in brood production ameliorated to a 2-19 percent drop. Honey bees and other insects show full or partial recovery from certain imidacloprid-induced effects in pulsed exposure trials (Nauen 1995; Ramirez-Romero et al. 2005; Azevedo-Pereira et al. 2011; He et al. 2011, 2012), but the work presented in chapter four is the first to demonstrate the resilience to pulsed exposure of demographically important endpoints in bumble bees. Recovery of food consumption and brood production occur simultaneously when exposure ends, and it is therefore likely that removal of imidacloprid allowed the bees' feeding rate to recover, thereby increasing their nutrient intake to levels sufficient for normal brood production.

*7.2.3 *B. terrestris* microcolonies are more sensitive to field-realistic imidacloprid than thiamethoxam*

Chapter three showed that thiamethoxam could reduce food consumption and brood production in *B. terrestris* microcolonies exposed to dietary concentrations of 39 or 98 ppb. However, at lower concentrations, including

those in the ‘typical field-realistic’ range, effects on feeding and brood production were not seen. These results are consistent with a previous microcolony study, in which the EC₅₀ value for brood production was 35 ppb where bees were exposed to thiamethoxam in syrup (Mommaerts et al. 2010b), but not another, where 10 ppb thiamethoxam in both syrup and pollen reduced brood size (Elston et al. 2013). A further study demonstrated negative effects in a ‘worst-case’ realistic scenario (nine weeks continuous exposure to field-realistic doses of both thiamethoxam and clothianidin in nectar and pollen; Fauser-Misslin et al. 2014), but typically the detrimental effects of thiamethoxam and its primary toxic metabolite clothianidin (Nauen et al. 2003) in bees are observed only where exposures rise above ~40 ppb (Franklin et al. 2004; Cutler and Scott-Dupree 2007; El Hassani et al. 2008; Aliouane et al. 2009; Laurino et al. 2011; Henry et al. 2012a; Schneider et al. 2012; Larson et al. 2013; Oliveira et al. 2013; Pilling et al. 2013; Thompson et al. 2013).

The experiments in chapters two and three were similar in design, and taking the results collectively demonstrates that during an exposure in *B. terrestris* microcolonies lasting approximately two weeks (13 vs. 17 days, chapter two vs. chapter three), brood production and food consumption are more sensitive to field-realistic concentrations of imidacloprid than thiamethoxam. For example, imidacloprid substantively reduced brood production and pollen consumption at approximately one and three ppb, respectively, whilst thiamethoxam in this range had no measurable effect on microcolonies. A similar difference in sensitivity to these neonicotinoids has also been shown in other insects, primarily *Myzus* spp. (Nauen 1995; Devine et al. 1996; Cho et al. 2011). This

differential sensitivity could arise for a number of reasons that may include the following: a) the two neonicotinoids bind to distinct target sites (Kayser et al. 2004; Wellmann et al. 2004; Thany 2011); b) imidacloprid has a greater affinity for insect nAChRs than thiamethoxam (Wiesner and Kayser 2000); c) the higher hydrophobicity of imidacloprid, compared with thiamethoxam's toxic metabolite clothianidin, enables imidacloprid better access to receptors and gives it greater insecticidal potency (Ihara et al. 2006); d) imidacloprid weakens or blocks the currents induced by acetylcholine at the nAChRs at lower concentrations than clothianidin (Ihara et al. 2006).

*7.2.4 Cytochrome P450 enzymes are not an important mechanism for metabolism of imidacloprid in adult *B. terrestris* bumble bees*

In chapter five, it was demonstrated that bumble bee workers might be more sensitive to imidacloprid *via* the diet compared with a topical exposure. Differential sensitivity to pesticides as a function of their route of exposure has previously been shown in species of both *Bombus* and *Apis* (Bailey et al. 2005; Gradish et al. 2012). However, regardless of exposure route, the toxicity of imidacloprid was not enhanced by the cytochrome P450 enzyme (P450) inhibitor piperonyl butoxide (PBO). This result suggests that P450s – a superfamily of enzymes known to be heavily involved in metabolism of various non-neonicotinoid pesticides in honey bees (Pilling et al. 1995; Johnson et al. 2006; Johnson et al. 2009b) and other insects (Scott et al. 1998; Daborn et al. 2002; Li et al. 2004; Poupardin et al. 2010; David et al. 2013) – are not responsible for metabolism of imidacloprid in bumble bees. This is a new finding for bumble bees, although a similar finding has previously been reported in

honey bees dosed topically with imidacloprid (Iwasa et al. 2004). In non-Hymenoptera insects the toxicity of imidacloprid can be enhanced 10-fold or more by PBO (Liu et al. 1993; Richman et al. 1999), which suggests that metabolic pathways for this neonicotinoid may vary between insect genera.

In chapter six, a transcriptomic analysis of *B. terrestris* workers was used to investigate their molecular response to relatively large concentrations of imidacloprid (i.e. 98 ppb). Following a 12-hour exposure, 26 genes were found to be differentially expressed in imidacloprid-treated bumble bees, one of which was related to P450s: *probable cytochrome P450 6a13*. However, this gene was down-regulated, providing further evidence that P450s are probably not important for metabolism of imidacloprid in adult bumble bees. Whether this finding generalises to other bumble bees life-stages and castes is unclear, but adult honey bees also appear not to use P450s to metabolise imidacloprid (see chapter five; Iwasa et al. 2004) whilst honey bee larvae up-regulate nine P450 genes following dietary exposure (Derecka et al. 2013). The specific mechanism by which adult bees metabolically detoxify concentrations of imidacloprid known to produce detrimental effects on their performance is thus still unknown. The transcriptomic analysis conducted here indicated a more general response to stress, which involved up- or down-regulation of several genes likely to be involved in a variety of biological processes including (but not limited to) energy production (e.g. carbohydrate metabolism, oxidative phosphorylation) and energy storage (e.g. fatty acid synthesis). Further work is therefore necessary to establish the metabolism pathways of imidacloprid, and the effect mechanisms of the neonicotinoid, in bumble bees.

7.3 Environmental relevance of the research

7.3.1 Implications for wild bumble bees exposed to dietary imidacloprid

The finding that ‘typical field-realistic’ concentrations of dietary imidacloprid can substantively reduce food consumption and brood production in *B. terrestris* colonies raises further concern about this particular neonicotinoid. In chapter one we saw that the carbohydrate and protein in nectar and pollen fuels the colony, and ample collection and consumption of these food resources is critical to colony success. For example (Heinrich 2004): queens require substantial food resources to produce, incubate and feed their young; workers use large amounts of energy in order to forage and thermoregulate the nest; and larvae derive all their nutrients from a nectar-pollen mixture. If field-realistic residues of imidacloprid reduce food consumption in wild bumble bees to the same extent observed in the laboratory studies of this thesis, it is likely that the success of their colonies would be at risk. Furthermore, the rate of brood production in a bumble bee colony is likely to influence the overall number of workers it contains, and therefore its size. The size of a colony’s workforce dictates the quantity and quality of its future food resources (Macevicz and Oster 1976), which in-turn dictates the number of new workers and sexual offspring that can be produced (Duchateau and Velthuis 1988; Heinrich 2004). Typically only the largest colonies succeed in producing new queens (Owen et al. 1980; Müller and Schmid-Hempel 1992a), and so, by reducing feeding and brood production, exposure to field-realistic imidacloprid could theoretically become the catalyst for smaller colonies that produce fewer queens. In short, the findings of this thesis suggest that imidacloprid has the potential to reduce colony fitness in bumble bees and negatively impact populations.

In general, the results of the thesis work are consistent with those of other laboratory and semi-field studies that suggest imidacloprid in the ‘typical field-realistic’ range presents a potential threat to bumble bee colonies. In the laboratory, the feeding rate of individual *B. terrestris* declined rapidly as imidacloprid in their diet increased above one ppb (Cresswell et al. 2012b), and at 10 ppb fewer brood were produced in queenless microcolonies (Tasei et al. 2000; Mommaerts et al. 2010b) and fewer workers eclosed in queenright colonies (Bryden et al. 2013). In one semi-field trial, the pollen-foraging efficiency of *B. terrestris* workers was significantly reduced when their colony could access syrup dosed with imidacloprid at 10 ppb, and the same colonies produced fewer larvae, pupae and workers during the 4-week experiment (Gill et al. 2012). In another semi-field trial, *B. terrestris* colonies that fed for two weeks in the laboratory on syrup and pollen dosed with imidacloprid at 0.7 and 6 ppb, respectively, then foraged freely in the wild for a further six weeks were smaller (by mass) at the end of the experiment than control colonies, and produced significantly fewer queens (Whitehorn et al. 2012). Where it was measured, collection or consumption of food was negatively affected by imidacloprid in these studies, where it was not, an imidacloprid-induced reduction in food consumption is a plausible explanation for the observed negative effects on colony growth and production of brood, workers, and queens. However, the results presented in this thesis conflict with a laboratory study in which 7 ppb imidacloprid had no effect on pollen consumption or reproduction in colonies of *B. occidentalis* or *B. impatiens* (Morandin and Winston 2003). Notably, in contrast to studies in which detrimental effects were observed, Morandin and Winston (2003) used species other than *B. terrestris*.

Similarly, Franklin et al. (2004) found that field-realistic concentrations of clothianidin had no effect on *B. impatiens* colonies in the laboratory. The difference in these findings raises the important question of whether *B. terrestris*, the bumble bee species most commonly used in toxicology research, is more susceptible to dietary neonicotinoids than other bumble bee species.

Whilst some results in this thesis add to the body of evidence that suggest imidacloprid is a threat to wild bumble bees, other findings demonstrate bumble bees have resilience to the effects of this neonicotinoid that emerge for a pulsed exposure. Specifically, reductions in feeding and brood production suffered during a two-week exposure largely recovered once imidacloprid was removed from the bees' diet. As previously highlighted, pulsed exposure – comprising a few weeks exposure to dietary neonicotinoids in treated mass-flowering crops followed by a return to foraging on primarily pesticide-free wild flowers – is a likely scenario for wild bees foraging in agricultural landscapes. If the ability of bumble bee colonies to recover from imidacloprid-induced effects during pulsed exposure translates from the laboratory to the field, the detrimental impact of the neonicotinoid may be somewhat ameliorated. The potential for recovery in bumble bees may go some way to explaining the inconsistent results observed in continuous laboratory exposure trials (where effects may be severe) and field trials in which bees have the opportunity to forage on both neonicotinoid-treated and untreated forage (where residues <10 ppb have little or no effect on colonies, e.g. Tasei et al. 2001; Thompson et al. 2013). However, in a semi-field trial, recovery was not immediately obvious where a two-week imidacloprid exposure was followed by a six-week recovery period because strong negative

effects on colony mass and number of new queens were still observed (Whitehorn et al. 2012). Perhaps the endpoints that showed recovery in chapter four, feeding and brood production, recover better following imidacloprid exposure than other endpoints such as production of queens. Given that the production of sexual offspring is the most important endpoint for bumble bee colony fitness (see chapter one), if queen production cannot recover following pulsed-exposure to imidacloprid then wild colonies would remain under threat despite the apparent recovery in performance in other areas. Therefore, more research is clearly required to understand the extent of bumble bee resilience to imidacloprid as a pulsed exposure.

7.3.2 Implications for wild bumble bees exposed to dietary thiamethoxam

In contrast to imidacloprid, concentrations of thiamethoxam in the ‘typical field-realistic’ range had no detectable effect on the performance of bumble bee workers in microcolonies (chapter three). Although these results are similar to another microcolony study that tested thiamethoxam on bumble bees (Mommaerts et al. 2010b), the findings of research based on microcolony assays should be extrapolated to wild bumble bee populations with caution because the absence of a reproductive queen somewhat limits their environmental relevance. Nevertheless, the failure to detect a substantive negative effect of field-realistic thiamethoxam in this thesis is consistent with the results of full-field trials using queenright colonies of *B. terrestris* (Thompson et al. 2013) and colonies of *A. mellifera* honey bees (Pilling et al. 2013).

Comparing the results derived from chapters two and three, it was possible to infer that residues of imidacloprid likely to be encountered in the field will have a greater impact on wild bumble bees than thiamethoxam. This could have important implications for the future of neonicotinoid use in agriculture. Along with clothianidin, the application of both compounds to bee-attractive crops is currently restricted in Europe (European Commission 2013). However, the focus of the existing ‘bees vs. neonicotinoids’ literature on which the EU’s decision was largely made is extremely narrow with imidacloprid featuring in 75 and 90 percent of laboratory and semi/full-field studies, respectively (Walters 2013). Whereas the accumulation of evidence for imidacloprid can arguably be used to justify its restricted use, the evidence against thiamethoxam and clothianidin is less persuasive. There is currently little evidence that field-realistic thiamethoxam and clothianidin exposures cause significant harm to bees, and the findings of chapter three further highlight that effects of imidacloprid cannot necessarily be extrapolated to other neonicotinoids. Therefore, whilst the two-year moratorium is in effect, more robust and environmentally relevant research into effects of neonicotinoids other than imidacloprid should be a priority.

7.4 Caveats to the environmental relevance of the research

Questions have often been raised about the environmental relevance of neonicotinoid exposures in the laboratory (e.g. Pilling et al. 2013; Walters 2013). Considering the implications of the findings from this thesis for wild bumble bees requires the acknowledgement of certain limitations that were

inherent in the laboratory studies conducted. In addition to the limitations of microcolony assays discussed in section 7.3.2, these caveats include the following.

First, the pollen consumed by bees in the thesis experiments was not dosed with neonicotinoid. Primarily, pollen was not dosed here because of the technical difficulty inherent in the process of distributing neonicotinoid evenly throughout a pollen ball in order to ensure that all bees in a treatment group ingested equal concentrations of neonicotinoid. Instead, feeder syrup (artificial nectar) alone was dosed; a medium in which dispersion of a dissolved neonicotinoid can be achieved with ease (evidenced by the measurement of imidacloprid and thiamethoxam residues in syrup; see chapters two, three, and four). Additionally, by dosing one food source but not the other, it was possible to infer the antifeedant effects of neonicotinoids. Although there is no reason to suspect different levels of toxicity arising due to ingestion of neonicotinoids in syrup *vs.* pollen, the relative importance of these nutrient sources to individual bumble bees may differ. For example, whereas queens and their brood are likely to eat a substantial pollen load as the colony develops and reproductive workers will consume more pollen than their unreproductive sisters as they upregulate their ovaries (Vogt et al. 1998; Pereboom 2000; Heinrich 2004), foraging workers will mainly subsist on nectar throughout their lives (Heinrich 2004). Consequently, neonicotinoid exposure *via* pollen may be relatively more important for investigation of effects on reproduction, while exposure *via* nectar may have more importance to worker performance and longevity. Therefore, by

dosing only syrup here, the effects of neonicotinoids on reproductive wild bumble bees may have been underestimated.

Secondly, the duration of exposure may differ in the environment from that tested here. An exposure period of around two weeks was chosen as a reasonable approximation of realistic environmental exposure because: a) the total expected flowering duration of several neonicotinoid-treated crops is approximately 2–4 weeks (Morandin and Winston 2005; Cutler and Scott-Dupree 2007; Hoyle et al. 2007; Westphal et al. 2009; Pilling et al. 2013; Thompson et al. 2013); and b) roughly 75 percent of the flowering of one such crop, winter-sown oilseed rape in the UK, occurs over a peak period of about two weeks (Hoyle et al. 2007). However, mass-flowering can extend beyond the typical four weeks (Westphal et al. 2003; Thompson et al. 2013) and crops will not always bloom in synchrony (Fauser-Misslin et al. 2014), thereby increasing the potential for an extended neonicotinoid exposure where bumble bees forage on crops throughout the blooming period (Westphal et al. 2009; Diekötter et al. 2010). Conversely, mass-flowering sometimes lasts only a few days (Pilling et al. 2013) and some colonies could therefore be exposed for less than two weeks. Additionally, certain colonies could broadly escape exposure to neonicotinoids, for example where their founding queens emerge from overwintering later in spring (Pyke et al. 2011) and they develop after the bloom of mass-flowering crops has declined or where their foraging range does not contain treated fields, such as in urban or suburban habitats (Goulson et al. 2002a; Goulson et al. 2010). Consequently, the results of a two-week laboratory

exposure could be considered conservative in certain environmental situations and unrealistic in others.

Thirdly, continuous exposure to neonicotinoids without access to alternative pesticide-free forage is an unlikely environmental scenario. For example, it is rare for bumble bees to forage solely on a mass-flowering crop such as oilseed rape even when the crop is in full bloom – foragers visit not only the crop's flowers but also those of the wild plant species growing in the surrounding field margins and hedgerows (Stanley 2013). Foraging from wild flowers will therefore dilute the exposure to neonicotinoids that bees receive in the nectar and pollen from treated crops. Consequently, a continuous two-week laboratory exposure could overestimate the severity of the neonicotinoids' effects on wild bees. However, where soil or non-target vegetation growing near to treated crops also becomes contaminated with neonicotinoid (Krupke et al. 2012; Goulson 2013) a continuous exposure such as this would be possible. Furthermore, one semi-field study has demonstrated that free access to alternative forage during exposure to dietary imidacloprid (10 ppb) does not necessarily prevent detrimental effects on *B. terrestris* colonies (Gill et al. 2012).

Fourth, where alternative forage is available, bumble bees in the field may choose to avoid flowers contaminated with imidacloprid. Other insects, including species of both Diptera and Coleoptera, exhibit avoidance of pan traps contaminated with imidacloprid at concentrations as low as $0.01 \mu\text{g L}^{-1}$ (Easton and Goulson 2013). Whether insects are repelled in a similar way by neonicotinoid residues in nectar and pollen is not clear (Easton and Goulson

2013), nor have any studies been conducted on the feeding behaviour of *Bombus* spp. faced with a choice between contaminated and uncontaminated food resources. Bumble bees are known to recognise and avoid flowers contaminated with the *C. bombi* parasite (Fouks and Lattorff 2011), but whether this holds true for contamination with neonicotinoids is not known and therefore more investigation in this area is necessary.

7.5 Suggestions for future research

The research presented in this thesis adds to a growing body of evidence that will be used by stakeholders to evaluate the risks posed to bees by exposure to neonicotinoid pesticides in the environment. Although certain questions are answered in this work, research gaps that remain, and certain limitations for interpreting environmental relevance, should engender further research. In Europe, the two-year moratorium on the use of specific neonicotinoids in crops attractive to bees provides the perfect opportunity to complete at least some of the research required to fill these gaps. Here are some suggestions for the direction of this research.

7.5.1 Conducting field-relevant research

In chapter four, imidacloprid in the range of one ppb substantively reduced the feeding and brood production performance of bumble bees. Wild bees are likely exposed to concentrations in this range because mean residues of neonicotinoid detected in environmental nectar and pollen are below six ppb in 77 percent of reported studies (see chapter one). Indeed, Goulson (2013)

recently reported that, when applied as a seed dressing, mean maximum levels of neonicotinoid in nectar and pollen are two and six ppb, respectively. Other authors have acknowledged that in order to establish robust conclusions on the effects of neonicotinoids in the field, experimental design should wherever possible reflect realistic exposure scenarios (Walters 2013). It is surprising then, that this thesis work is some of the first to test the effects of dietary neonicotinoids at measured concentrations of ≤ 6 ppb in queenright bumble bee colonies (see also Franklin et al. 2004; Whitehorn et al. 2012; Fauser-Misslin et al. 2014). Clearly, more research is required at the colony level to understand the effects of neonicotinoid exposures in the range ≤ 6 ppb, particularly on endpoints critical for colony success such as: brood development and survivorship; production, survivorship and size of workers and sexuals; and feeding behaviour and foraging efficiency. Additional research on effects at the individual level would also be beneficial. For example, the impact of realistic neonicotinoid exposure on learning and foraging behaviour in workers and young queens is yet to be sufficiently studied in bumble bees. To reflect behaviour in the field, the study of these endpoints in free-flying bees rather than those that are harnessed for PER assays or confined to cages would be most useful. For example, it is possible to simulate ecologically relevant foraging tasks using artificial flowers in laboratory-based flight arenas (Raine and Chittka 2008; Evans and Raine 2014) or in greenhouses (Lihoreau et al. 2011, 2012). With the introduction of neonicotinoid treatments these assays could easily be adapted to assess effects on foraging efficiency and learning ability in free-flying bumble bee queens and workers. Additional environmental

realism could be introduced in greenhouse experiments by examining the behaviour of bees foraging from neonicotinoid-treated plants (Tasei et al. 2001).

Future research into bee health should strive not only to test realistic concentrations of neonicotinoid, but also to test them over realistic exposure periods. What constitutes a realistic exposure period is debatable, and more work is required to obtain baseline data on, for example, the extent to which bumble bees visit and forage from neonicotinoid-treated crops. However, in the absence of baseline exposure data, the typical blooming periods of mass-flowering crops offer a good first approximation. Previously discussed in section 7.4, the mass flowering of crops such as oilseed rape or maize typically lasts 2–4 weeks and bumble bees will supplement their foraging visits to crops with visits to pesticide-free wild flowers. To better reflect realistic environmental scenarios, rather than exposing colonies continuously in ‘worst-case’ scenarios (e.g. Fauser-Misslin et al. 2014), it will be necessary to produce more research in which colonies are: a) exposed to transitory pulses of neonicotinoid (with, for example, dietary dosing lasting no more than four weeks and preceding a period of pesticide-free recovery; e.g. chapter four, Whitehorn et al. 2012); and/or b) given the option to forage on uncontaminated nectar and pollen in addition to neonicotinoid-treated food (e.g. Gill et al. 2012). In chapter four, both food consumption and brood production in *B. terrestris* colonies showed strong levels of recovery when imidacloprid was removed from the bees diet and further research is required to investigate the extent to which other demographically relevant endpoints are also resilient to pulsed exposure.

Laboratory and semi-field simulations of realistic neonicotinoid exposures that take into account field-relevant residue levels and temporal exposure profiles are clearly the first step towards a robust understanding of the real risks posed by these pesticides to bumble bees and other pollinator species; however, full-field trials in natural conditions should be the ultimate goal for researchers. Conducting statistically robust field studies has thus far proved challenging because, for example, it was previously difficult to avoid neonicotinoid-contamination in ‘control’ colonies (from which bumble bee workers travelled great distances away from untreated fields to forage in fields that were apparently treated with neonicotinoid; Thompson et al. 2013). Since a 2-year moratorium on the use of neonicotinoids in bee attractive crops is now in place in the EU (European Commission 2013), if agencies can obtain permission to plant neonicotinoid-treated crops in Europe, now may be the time to conduct controlled field-trials because contamination with neonicotinoids outside of controlled fields will be unlikely. While full-field studies in North America and Asia may still be feasible, these studies would likely be dogged with ‘control contamination’ problems because neonicotinoid usage is apparently ubiquitous. Regardless of geography, eliminating ‘control contamination’ from full-field trials in agricultural settings will always remain difficult because of the risk of exposure to other pesticides used alongside, or as alternatives to, neonicotinoids. Similar to previous honey bee studies (e.g. Pilling et al. 2013), one possible solution would be containment of replicate bumble bee colonies and plants within large mesh covered tunnels during exposure. An experimental design such as this would: a) enable several replicates (both treated and untreated) on a single study site in multiple tunnels, and thus minimise variation

in, for example, climatic conditions; b) prevent cross-contamination between treatments; and c) allow a comparison of performance in neonicotinoid-treated and untreated colonies that could provide valuable insight into field-level effects. Furthermore, the assay could be adapted to introduce a choice of treated and untreated forage to bumble bees within a single tunnel and thereby permit field-relevant research in bees into the repellent effects of neonicotinoids, which have previously been observed in other insects (Easton and Goulson 2013).

7.5.2 More research into effects of neonicotinoids and other agrochemicals in bees

Analysis of the collective work presented in chapters two and three suggested *B. terrestris* bumble bees are more susceptible to field-realistic concentrations of imidacloprid than thiamethoxam. Despite the methodology in the experiments being very similar, the comparison made between these two studies provides only a strong indication of differential sensitivity because bumble bees came from different colonies and were tested at different times. In order to verify this result it will be important to compare the sensitivity of individuals from the same colony. Furthermore, comparative toxicology studies in which the effects of imidacloprid, thiamethoxam and clothianidin are examined in queenright colonies are necessary before it is possible to draw robust conclusions on how, or if, each compound differentially impacts bumble bees in the natural environment. Completion of studies in which thiamethoxam and clothianidin are tested in realistic exposure scenarios, such as those discussed above, is currently of particular importance because research of this kind is generally

lacking, even though use of both compounds is restricted in the EU, together with imidacloprid (European Commission 2013).

While the 2-year moratorium restricts the use of the aforementioned neonicotinoids in Europe, whether it benefits bumble bees and other pollinators will largely depend on the alternative pesticides that can and will be used on bee attractive crops instead. Similar to neonicotinoids, we are somewhat lacking in knowledge about the impact of alternative pesticides on bees. However, there is some evidence that *Apis* and *Bombus* could be harmed by exposure to common alternatives to neonicotinoids such as pyrethroids, acetylcholinesterase inhibitors, and organochlorines. Previous studies suggest that while most pyrethroids (axonic excitotoxins that prevent closure of sodium channels in axonal membranes) are highly toxic to honey bees, some can be tolerated but are not harmless (Johnson et al. 2010). For example, while high concentrations of tau-fluvalinate do not kill honey bees they affect the health of reproductive castes; reducing the size of queens and the reproductive capacity of drones (Johnson et al. 2010). In colonies of *B. terrestris*, the pyrethroid λ-cyhalothrin is capable of reducing the size (body mass) of new workers (Baron et al. 2014) and increasing worker mortality (Gill et al. 2012) during the early stages of colony development. In honey bees, chronic exposure to acetylcholinesterase inhibitors (including organophosphates, carbamates, and the acaricide coumaphos) can increase symptoms of malaise and have negative effects on memory and motor function (Williamson and Wright 2013; Williamson et al. 2013). A small number of bee poisoning incidents have also been reported in the UK following application of organochlorines (neurotoxic

pesticides that act on sodium ion channels) to oilseed rape or as wood treatments (Barnett et al. 2007). Alongside the harm alternative pesticides may cause to bees, insect pests have previously been more likely to develop resistance to compounds such as pyrethroids than to neonicotinoids (Jeschke and Nauen 2008). Increased usage of alternative compounds could therefore increase pesticide resistance, and without neonicotinoids the agricultural industry will be left with fewer options to manage its spread (Walters 2013). Thus, decisions on the future of neonicotinoids should not only be based on the evidence of their impact on bees, but also balanced with data on the impact of alternative pesticides and their efficacy in crop protection. Currently, this data is badly lacking and field-relevant research into the impact of these alternative compounds in bees is therefore urgently required.

In addition to testing the comparative toxicology of neonicotinoids and other agrochemicals in the model bumble bee species *B. terrestris*, it is also important that we compare the sensitivity among other bumble bee species to these compounds. By comparing published data (including those presented here), it is possible to conclude that while field-realistic neonicotinoids can detrimentally affect *B. terrestris*, they have no apparent effect on other species such as *B. impatiens* and *B. occidentalis* (Morandin and Winston 2003; Franklin et al. 2004). If species within *Bombus* are differentially sensitive to neonicotinoids, extrapolation from data collected primarily on *B. terrestris* could over- or underestimate the actual species-level impact of these pesticides in wild bumble bees. However, in order to draw robust conclusions, it will be necessary to conduct further studies in which several bumble bee species are tested under

identical conditions for their sensitivity to neonicotinoids. Moreover, research should extend beyond *Bombus* (and *Apis*) to include the full range of pollinator groups including solitary bees, hoverflies and butterflies (Goulson 2013; Sandrock et al. 2013).

7.5.3 Combinatorial effects of agrochemicals and other stressors

Bees that forage in agriculturally intensive landscapes are likely exposed to multiple pesticides, but we are only just beginning to understand their combinatorial effects. For example, Gill et al. (2012) found that *B. terrestris* colonies exposed to both imidacloprid and λ -cyhalothrin suffered significantly greater worker loss (a combination of mortality and loss whilst foraging) and were more likely to fail than colonies exposed to either compound alone. In honey bees, imidacloprid and coumaphos produce additive effects on learning and memory (Williamson and Wright 2013), while the toxicity of thiacloprid and acetamiprid was significantly increased by certain fungicides (e.g. triflumizole and propiconazole; Iwasa et al. 2004). During the moratorium there is vital need to build upon this research, so that if neonicotinoids are reintroduced we more clearly understand the effects of imidacloprid, thiamethoxam and clothianidin in combination with other commonly used agrochemicals.

Other stressors, besides agrochemicals, could also combine with neonicotinoids to produce an additional impact on bees. Researchers have thus far focussed on the combinatorial effects of infection with common parasites and exposure to agrochemicals. In bumble bees, Fauser-Misslin et al. (2014) found that mother queens infected with *C. bombi* survived fewer days than uninfected queens

when exposed to thiamethoxam and clothianidin for nine weeks. In honey bees, *Nosema spp.* infection was higher in workers previously exposed in their colony to imidacloprid up to 20 ppb (Vidau et al. 2011), and exposure to high, but sublethal, concentrations of thiacloprid (and the phenylpyrazole insecticide fipronil) increased mortality in *N. ceranae*-infected workers compared to the uninfected (Pettis et al. 2012). Although these studies provide initial insight into the interactive impact of pesticides and parasites, effects are demonstrated using exposure regimes that are ‘worst case’ rather than ‘field-realistic’. Moving forward, ‘field-realistic’ experimental design should be applied to ‘multiple stressor’ research to support the establishment of environmentally relevant conclusions. Importantly, this research must not ignore what is arguably the greatest threat to wild bees – namely the intensification of agriculture that has impoverished their natural habitat: limiting food supply, and the availability of suitable nesting and over-wintering sites (Goulson 2003a; Goulson et al. 2008; Williams and Osborne 2008). For example, while the pesticide treatments applied to mass-flowering crops represent one form of stress, the loss of floral diversity in monoculture landscapes and the nutrient stress it imposes on wild bees could represent a far greater threat to colony success. Indeed, the loss of flower-rich habitat is thought to be key driver in the decline of several species of *Bombus* (Goulson 2003a; Colla and Packer 2008; Dupont et al. 2011; Roulston and Goodell 2011; Bommarco et al. 2012). Thus, research into the interplay of multiple stressors in bees must also consider how loss of habitat and nutrient limitation interact with exposure to agrochemicals, parasites and disease.

7.5.4 Future research into the effect mechanisms of neonicotinoids

In chapters five and six, the effect mechanisms of imidacloprid in bumble bees were studied. In a similar manner to adult honey bees (Iwasa et al. 2004) but unlike some other insects (Liu et al. 1993; Richman et al. 1999), cytochrome P450 enzymes were found not to be an important route of metabolism for imidacloprid in *B. terrestris* workers. Despite an apparent lack of P450 activity during exposure, both adult honey bees and bumble bees appear capable of metabolising and entirely eliminating high concentrations of imidacloprid and its toxic metabolites from their bodies in a relatively short period of time (within 48 hours: Suchail et al. 2004a, b; Cresswell et al. 2013). In contrast to adult bees, honey bee larvae appear to upregulate P450 genes when fed imidacloprid in the field-realistic range ($2 \mu\text{g L}^{-1}$; Derecka et al. 2013). Although it is not known whether these genes form part of a targeted detoxification response in honey bee larvae, it would be interesting to determine whether P450s are also upregulated in imidacloprid-treated bumble bee larvae. In general, further research into the metabolism pathways for imidacloprid in bees would help increase our understanding of its toxic effect mechanisms. Moreover, a better understanding of the action and metabolism of all neonicotinoid compounds is necessary to more accurately evaluate their potential impact on bee health. Indeed, some studies have already received criticism for limited consideration of metabolism within their experimental design (e.g. Henry et al. 2012a). Therefore, because metabolism could potentially ameliorate toxic effects of neonicotinoids in bees, this important aspect of exposure should not be ignored in future laboratory and field studies.

Future work into the action and effect mechanisms of neonicotinoids in bees can now be developed based on the foundation of transcriptomics research presented in chapter six of this thesis and by other authors (Derecka et al. 2013). A transcriptomics approach used to assess the toxicogenomics of neonicotinoid-treated bees over time and dose space (i.e. testing a range of environmentally relevant exposures over time periods ranging from hours to weeks) would help to establish a more complete understanding of the compounds' toxicity. Theoretically, such research could also provide a basis for distinguishing or understanding differences in susceptibility to neonicotinoids between genera, species or caste of bee (Johnson 2013). On a broader scale, the comparative toxicogenomic approach could ultimately be integrated into development, assessment and regulation of pesticides. For example, for a given pesticide, identifying the mechanistic connection between a molecular initiating event and an adverse outcome at a biological level (Ankley et al. 2010) could facilitate a more focused and efficient assessment of the risks it poses to beneficial arthropods.

7.5.5 Economic implications of restricting neonicotinoid usage

The moratorium provides an opportunity for researchers not only to expand their knowledge of the negative impact of neonicotinoids, but also to build a fuller picture of their benefits in European agriculture. Currently, opinion is polarised regarding the economic benefits of neonicotinoids. For example, Goulson (2013) argues that their prophylactic use has done little to improve crop yields and has instead curtailed the use of more environmentally sensitive practices such as integrated pest management, but in contrast Campbell (2013) argues

that neonicotinoid usage prevents losses to crops that would result in a 40 percent yield reduction. If the losses suggested by Campbell (2013) became a reality, European farmers would suffer a significant impact leading to greater import of food from outside the EU and, ultimately, higher food prices across the continent. However, as Goulson (2013) points out, evidence for the economic benefits of neonicotinoids is somewhat lacking and if such benefits exist we must balance them with the risks they pose to beneficial wildlife. It is clear that more research is required into the economic implications of restricting neonicotinoid usage, and the moratorium provides a perfect opportunity to closely monitor effects on farmers, their crops, the European agricultural industry, and the wider economy.

7.6 Concluding remarks

In the research presented in this thesis, field-realistic exposure to imidacloprid clearly produced detrimental effects in laboratory maintained individuals and colonies. This and other research (Gill et al. 2012; Whitehorn et al. 2012) therefore raises concerns about the threat of imidacloprid to wild bumble bees. It has been inferred that bumble bee colonies have a critical stress tolerance threshold, and even a small increase above this threshold level can make the difference between colony success and failure (Bryden et al. 2013). Wild colonies are subject to multiple stress factors including parasites, disease, loss of habitat and forage resources, and exposure to agrochemicals other than imidacloprid (Williams and Osborne 2009; Gill et al. 2012; Vanbergen and the Insect Pollinators Initiative 2013). Although the stress of imidacloprid alone can

detrimentally impact bumble bees, it is known that stressors in combination can have additive, interactive or even synergistic effects. The combinatorial impact of neonicotinoids and other stressors has been reported in bees (Vidau et al. 2011; Gill et al. 2012; Pettis et al. 2012; Fauser-Misslin et al. 2014; Williamson and Wright 2013), but further research in this area could be the key to understanding the pesticides' role, if any, in population declines.

The laboratory work in this thesis also indicates that some demographically important endpoints in bumble bees are resilient to imidacloprid in a realistic pulsed exposure scenario. Moreover, it shows that bumble bees may be less susceptible to other neonicotinoids. Conceivably, these findings go some way to explaining why in full-field trials bees show no adverse effects from foraging on imidacloprid-, thiamethoxam- or clothianidin-treated crops for the duration of their flowering period before switching to uncontaminated forage (Cutler and Scott-Dupree 2007; Pilling et al. 2013; Thompson et al. 2013). That the results of most other laboratory studies are currently in conflict with these field trials is perhaps indicative of the need for future experimental work to better reflect realistic and not 'worst-case' exposure scenarios.

Whether or not neonicotinoids are a cause of, or a contributing factor to, bumble bee declines remains unclear. However, if bees are in fact somewhat resilient to neonicotinoid pesticides as suggested here and in field trials, it seems unlikely that simply restricting or entirely banning their use will be sufficient to prevent further losses. Indeed, there is real a danger that focussing too narrowly on one specific pesticide group could detract attention from the many other threats

currently facing bees in the environment. An integrative approach to the problem, incorporating research on the interaction between multiple threats (Vanbergen and the Insect Pollinators Initiative 2013), should therefore be swiftly adopted. The future of wild bumble bees, the important pollination services they provide, and perhaps even “life as we know it” may be contingent on it.

Chapter Seven: Supplementary material

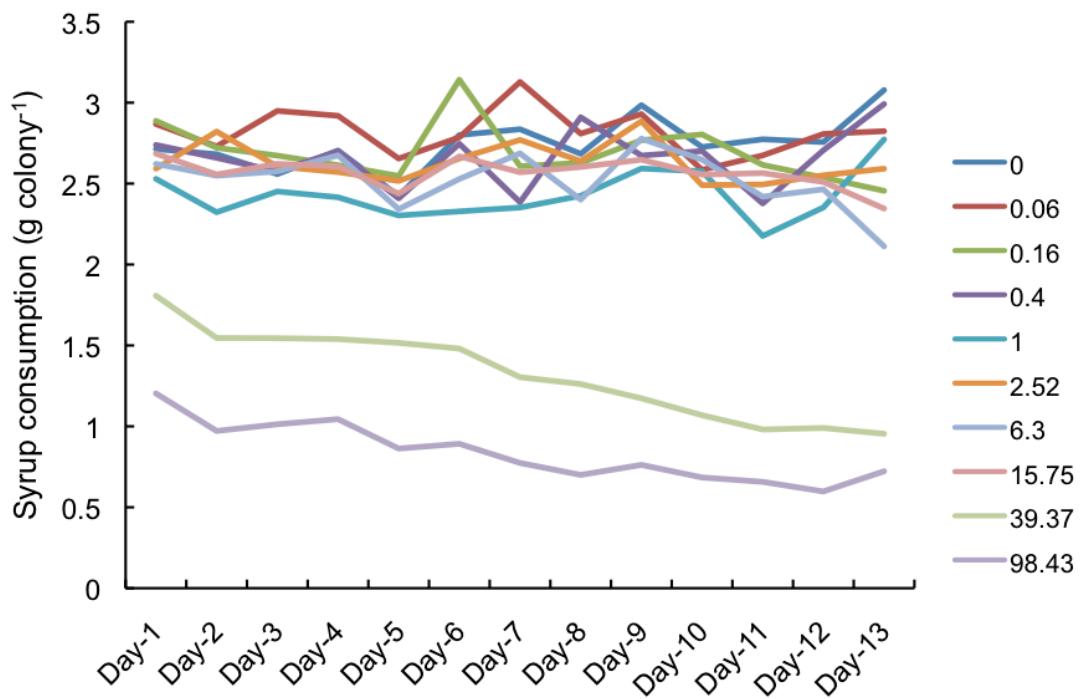


Fig. S7.1 Daily consumption of syrup in *Bombus terrestris* colonies ($N = 60$) during the 'on dose' period of pulsed exposure to imidacloprid. Data is taken from the first 13 days of the 14-day 'on dose' period of imidacloprid exposure described in chapter four. For each day (x-axis), data represent the mean syrup consumption (y-axis) of six colonies fed a particular concentration of imidacloprid ranging from zero to $98 \mu\text{g kg}^{-1}$ (= parts per billion). Data for each concentration is individually colour-coded (right-hand legend).

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