Combined impacts of pesticides and parasites on social bees: from the individual to colony level

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

Royal Holloway, University of London

June 2015

Gemma Louise Baron

Declaration of Authorship

I Gemma Baron hereby declare that this thesis and the work presented in it is entirely my own. Where I have consulted the work of others, this is always clearly stated.

Signed: _____

Date: _____

Acknowledgements

I would like to thank my supervisors Mark Brown and Nigel Raine for providing fantastic advice, support and teaching throughout my PhD. I have learned a huge amount and appreciate all the time and thought they have contributed.

My thanks also goes to all my friends and colleagues in the School of Biological Sciences at Royal Holloway, who have been a massive source of support, encouragement, and friendship over the last three and a half years. Particular thanks go to the Bees and Trees team: Catherine Jones, Matthias Fürst, Rich Gill, Dara Stanley, Fabio Manfredini, Lisa Evans, Karen Smith, Oscar Ramos-Rodriguez, Harriet Milligan, Eva Muiruri, Elli Leadbeater, James Hourston, Tina Steinbrecher, Callum Martin, Dylan Hodgkiss, and Liz Samuelson for help in the field and lab, proof reading chapters, statistics advice, great discussions in lab meetings and in the pub and some amazing cake! I would also like to thank the technicians, students and volunteers from Royal Holloway and elsewhere who assisted with field and laboratory work, in particular Kel Liu, Barry McCrea, Susan Baldwin, Tammy Mak, David Wells, Ruth Carter, Amber Dodd and Lucie Guirkinger.

For the Lactic Acid Bacteria experiment (Chapter 5), I collaborated with Alejandra Vasquez and Tobias Olofsson at Lund University, Sweden. I would like to thank them, and their research group in Lund, for welcoming me into their lab, teaching me about the bacteria, and for supplying equipment and consumables for this project.

I am extremely grateful to the Natural Environment Research Council for funding my PhD and enabling me to attend several excellent training courses. Also, I would like to thank the British Ecological Society, and the International Union for the Study of Social Insects (North West European Section) for providing funding for conference attendance. I am also grateful to The Crown Estate for access to Windsor Great Park, and for allowing collection of wild bumblebees.

Finally, I am immensely grateful to my amazing family and friends who have supported me, not only during my PhD, but throughout my education and life. In particular to my Mum and Dad and to Nic, for their love, encouragement and support.

Abstract

There is strong evidence for a decline in wild and managed bee populations. This is of concern both for conservation and agriculture, as bees play an important role in the pollination of wild plants and commercially grown crops. Agricultural intensification is likely to be one of several drivers of bee declines, in particular the habitat loss and increased use of agrochemicals with which it is associated. Bees in agricultural environments are therefore faced with a range of anthropogenic stressors such as pesticides in addition to the natural stressors to which they are normally exposed.

This thesis explores the impacts of pesticides on life-history traits of social bees, and particularly in combination with additional stress from naturally occurring parasites. The four research chapters explore impacts across a range of levels, from whole bumblebee colonies, to individual bumblebees, to the gut microbiota of honeybees.

In Chapter 2, chronic exposure to a pyrethroid insecticide resulted in the production of smaller workers by *Bombus terrestris* colonies, but had limited effects on other aspects of colony development. In Chapter 3, colony founding *B. terrestris* queens, an essential yet vulnerable stage of the colony cycle, showed a reduction in colony initiation after exposure to a neonicotinoid. However, no interactive effects with the parasite *Crithidia bombi* were found. In Chapter 4, a reduction in feeding and oocyte development in multiple species of bumblebee queens was observed after exposure to a neonicotinoid. Finally, in Chapter 5 symbiotic lactic acid bacteria found in honeybee guts were exposed to a range of pesticides in-vitro. Both inhibition and promotion of bacterial growth occurred, although no consistent patterns were detected.

Overall, I found variable impacts of environmentally realistic doses of pesticides on life-history traits of bees. Some of these have important implications for conservation and agricultural policy and management.

Table of Contents

List of Figures

List of Tables

List of Appendices

1 Introduction

1.1 Biodiversity declines

The diversity of life on earth is huge, and the number of described species currently exceeds 1.4 million (Mora *et al.* 2011). This represents only a fraction of the total number of species, which is notoriously difficult to estimate (May 1988; Mora *et al.* 2011), but is likely to be over 8 million (Mora *et al.* 2011). Declines in global biodiversity have been recognised (Heywood 1995; Pimm & Raven 2000; Butchart *et al.* 2010), and were first brought to international attention during the Earth Summit in Rio de Janeiro in 1992. The Convention on Biodiversity was an outcome of this, the aim of which is the conservation, sustainable use and equitable sharing of biodiversity and the benefits arising from it (Secretariat of the Convention on Biological Diversity 2015). Human activity is the major threat to ecosystems (Vitousek *et al.* 1997; Foley *et al.* 2005). Understanding the impacts of human activity on biodiversity and ecosystem functioning, and the consequences for human society is vital (Cardinale *et al.* 2012).

Many natural biological processes and organisms can be beneficial to human activities (Costanza *et al.* 1997). These benefits, widely termed 'ecosystem services', include processes such as nutrient cycling, biological control of pests, water regulation and crop pollination, and are considered a priority for global conservation efforts. Pollination is an essential part of reproduction in angiosperms, 87.5% of which are estimated to be pollinated by animals globally (Ollerton, Winfree & Tarrant 2011). Animal mediated pollination is extremely valuable for agriculture (Klein *et al.* 2007; Gallai *et al.* 2009), and in order to protect this, the International Initiative for the Conservation and Sustainable Use of Pollinators was established in 2000 as part of the Convention on Biological Diversity.

1.2 Bees

Amongst the insect pollinators, bees (Hymenoptera: Aculeata) are an important group, with global distribution from the tropics to temperate regions. Many bees have special adaptations to enable the collection of pollen and nectar from plants, for example a hairy body, a corbicula in females (long hairs present on the abdomen or hind legs in which pollen is collected), and a proboscis (elongated tongue which is used to suck up nectar from flowers) (Michener 2000). Pollen is generally fed to offspring during development as a source of protein and other

nutrients, whilst nectar is used as a direct fuel source for adults. Bees foraging on flowers often inadvertently pollinate the plant through the transfer of pollen from anther to stigma, which enables fertilisation. Consequently bees have a symbiotic relationship with angiosperms (Bronstein, Alarcón & Geber 2006).

The anthropogenic threats to biodiversity are also of concern for bees, with evidence for a decline in diversity and species ranges for several groups within the last century. The majority of data available for wild populations is for the bumblebees, which are found in temperate and mountainous regions around the world (Williams & Jepsen 2014). Many species are deemed to be of conservation concern (Williams & Osborne 2009), and nine species are currently classified as near threatened, vulnerable, endangered or critically endangered by the IUCN (IUCN). Bumblebee declines have been reported in the UK and Ireland (Williams 1982; Williams 1986; Fitzpatrick *et al.* 2007), Western and Central Europe (Kosior *et al.* 2007), North America (Colla & Packer 2008; Grixti *et al.* 2009; Cameron *et al.* 2011; Colla *et al.* 2012) and South America (Schmid-Hempel *et al.* 2014). Vulnerability in bumblebee species has been correlated with several specific traits, including a narrow climatic specialisation, proximity to the edge of the climatic range (Williams, Colla & Xie 2009) and later emergence of the queen (Fitzpatrick *et al.* 2007; Williams, Colla & Xie 2009).

There are far fewer data available for other wild bees, making the detection of trends over time more difficult. The status of European bees was recently assessed by the IUCN, and 9.2% of species are currently considered threatened (Nieto *et al.* 2015), although 1,535 species (79%) remain unclassified due to a lack of data. Extinctions of numerous solitary bee species have been recorded in the UK since the 19th Century (Ollerton *et al.* 2014). Historical records from the UK and Netherlands indicate that solitary bee diversity has declined in these regions since 1980 (Biesmeijer *et al.* 2006). A narrow habitat range, highly specialised diet, long tongue length and single generation per year all correlated with species declines, although these patterns were not consistent between the UK and the Netherlands (Biesmeijer *et al.* 2006).

The honeybee *Apis mellifera* L.*,* although domesticated and widely managed for agricultural and commercial purposes, has also experienced extensive colony losses in certain parts of the world (Aizen & Harder 2009; Potts *et al.* 2010b; vanEngelsdorp *et al.* 2011).

Whilst historical declines are apparent and some species remain at risk of extinction, recent evidence suggests that rates of decline in wild bees and other insect pollinators may have been slowing in Western Europe since the 1990's (Carvalheiro *et al.* 2013). This could be due to

conservation efforts, or possibly because particularly vulnerable species had already been lost. Whilst these results are encouraging, it is essential that we understand the threats to bees and the causes of declines, in order to minimise further risk to our existing bee fauna.

A decline in wild bee populations is of great concern from a conservation and biodiversity perspective. In addition, declines could have ramifications for the valuable ecological and economic benefits bees provide to humans, in their role as pollinators of wildflowers and commercially important crops (Corbet, Williams & Osborne 1991; Klein *et al.* 2007; Ollerton, Winfree & Tarrant 2011). Animal mediated pollination can increase productivity of many commercially important crop species by 10 % or more (Klein *et al.* 2007), and visitation by bees increases pollen deposition and fruit set in a range of crops worldwide (Garibaldi *et al.* 2013). In Europe, 43 commercially important crops have been identified as benefiting from bee pollination, along with many wildflowers (although numbers of these are harder to quantify) (Corbet, Williams & Osborne 1991). The value of these pollination services is difficult to define, although one estimate of the annual global economic value is $153 billion (Gallai *et al.* 2009). The nutritional value of animal-pollinated crops is thought to be particularly high, as they contribute large amounts of lipids, Vitamins A, C and E, along with Carotenoids and several minerals, to the global production of these nutrients (Eilers *et al.* 2011). There is increasing reliance on animal pollinated crops in the agricultural industry (Aizen *et al.* 2008), and this, coupled with declines in pollinators such as bees, has led to concerns over a 'pollinator crisis' (Allen-Wardell *et al.* 1998; Kremen, Williams & Thorp 2002; Potts *et al.* 2010a; Vanbergen *et al.* 2013; Regan *et al.* 2015). Whilst some debate remains around the true extent of this problem (Ghazoul 2005a; Ghazoul 2005b; Steffan-Dewenter, Potts & Packer 2005), it is widely agreed that more research is needed, in order to establish the anthropogenic threats to pollinator species such as bees, and the implications of these for both conservation and agriculture.

1.3 Drivers of bee declines

Understanding the factors driving bee declines is extremely important for conservation (Brown & Paxton 2009). Several key factors have been implicated in declines, and these are discussed below.

1.3.1 Habitat loss and a lack of floral resources

Bees need suitable flowering plants throughout the spring and summer, as well as suitable habitat for nesting (Osborne, Williams & Corbet 1991). Floral abundance is positively

correlated with bumblebee nest density (Knight *et al.* 2009), and is thought to be an important factor in regulating bee populations (Roulston & Goodell 2011). Loss of these resources through changes in land use, and in particular agricultural intensification, is widely considered to be one of the biggest drivers of bee declines (Osborne, Williams & Corbet 1991; Brown & Paxton 2009). For example, in the UK, loss of natural habitats (Howard *et al.* 2003), and declines in wild forage plants used by bumblebees (Carvell *et al.* 2006) have occurred during the 20th century, likely due to changes in farming practices during this time. Fragmentation of suitable habitat may also be important. Habitat patch size is correlated with wild bee species richness, and smaller habitat patches have fewer species (Bommarco *et al.* 2010).

1.3.2 Pesticides

During the last century, there has been a rapid expansion in agriculture, and an increase in the pollutants associated with this (Tilman *et al.* 2001). Pesticides are widely used in agricultural environments around the world (Figure 1.1), and the large range of substances found in pollen and nectar (Chauzat *et al.* 2006; Mullin *et al.* 2010) indicate that bees are very likely to be exposed. Several recent studies have implicated low level pesticide exposure with negative changes in bee behaviour, reproduction, and social bee colony success (Gill, Ramos-Rodriguez & Raine 2012; Henry *et al.* 2012; Whitehorn *et al.* 2012; Bryden *et al.* 2013), which could have important consequences for wild bee populations.

1.3.3 Invasive species

Invasive species, including plants and insects, can have negative consequences for native bees (Stout & Morales 2009). Commercial rearing of bees has led to widespread transportation of some species around the world, and accidental release into the wild (Macfarlane & Gurr 1995; Inoue, Yokoyama & Washitani 2008; Schmid-Hempel *et al.* 2014). In Japan, *B. terrestris*, introduced as a commercial pollinator, has become established in the wild, and is considered a threat to local bumblebee species (Goka 2010). Similarly in South America, introduced *B. terrestris* and *B. ruderatus* have become established, and the range expansion of *B. terrestris* in particular (up to 200 km per year) is associated with the disappearance of the native *B. dahlbomii* (Schmid-Hempel *et al.* 2014).

1.3.4 Parasites and disease

Another consequence of the global commercial trade in bees is the spread of emergent and invasive parasites (Meeus *et al.* 2011). Many microbes and parasites are found in commercially reared honeybee (Cox-Foster *et al.* 2007; vanEngelsdorp & Meixner 2010) and bumblebee (Whittington & Winston 2003; Colla *et al.* 2006; Otterstatter & Thomson 2008; Graystock *et al.*

2013; Murray *et al.* 2013) colonies. Whilst these can have damaging consequences for managed populations (vanEngelsdorp & Meixner 2010), of more pressing concern is the potential spillover of these parasites to wild bee populations. A range of viruses and other parasites which are known to infect honeybees have been detected in wild bee populations (Fürst *et al.* 2014; McMahon *et al.* 2015), and at least one, Deformed Wing Virus (DWV), is known to infect wild bumblebee species, and to have negative impacts on fitness (Fürst *et al.* 2014). Spillover from commercially reared *B. terrestris* colonies to wild populations is also thought to occur (Colla *et al.* 2006; Murray *et al.* 2013; Graystock, Goulson & Hughes 2014).

1.3.5 Climate change

Climate change is often cited as a threat to biodiversity by increasing extinction risk (Thomas *et al.* 2004). Changes in climate could alter the phenology of bees and their food-plant species, and as such impact on the plant-pollinator interactions between them (Hegland *et al.* 2009). Simulating pollinator and plant phenological responses to a doubling of atmospheric CO_2 indicated that disruption of these interactions could occur, and would be most likely to affect specialised species (Memmott *et al.* 2007).

1.4 Study System

Understanding the importance and impacts of these threats to bees is vital if we are to protect managed and wild populations. In this thesis, I investigate the impacts of pesticides on bees, particularly in combination with stress from natural parasites. Given the extent of pesticide use, high probability of exposure to bees, and negative impacts which have previously been found, pesticides can be considered an important threat to bees. Understanding this threat has been identified as a priority by conservation practitioners and insect pollinator scientists (Dicks *et al.* 2013). Most studies have investigated the impacts of pesticides in isolation, and yet in a field setting, bees are likely to be exposed to a range of stressors simultaneously. Parasites can exert varying degrees of pressure on their bee hosts, and this may be modulated depending on the condition of the host. Therefore, we might expect that pesticides could have an impact on a bee's ability to cope with parasitism, or vice versa. It is important to study the impacts of pesticides on bees in different natural contexts, in order to understand the pressures they face in the field.

The focal species used in my research are social bees, specifically bumblebees (*Bombus* spp.) and honeybees (*Apis mellifera)*. As social species, they form large highly related colonies, with

a queen, sterile workers, and males. Honeybee colonies are extremely large (up to several thousand individuals) and perennial, whilst wild bumblebees form smaller (up to several hundred individuals) annual colonies. Wild bumblebees forage on a wide range of flowering crop species (Corbet, Williams & Osborne 1991), and both bumblebees and honeybees are managed commercially for pollination services. The natural range of bumblebees in temperate and mountainous regions (Figure 1.2) coincides with areas where high levels of pesticides are used, such as the Americas and Europe (Figure 1.1). Wild bumblebees and commercially reared bees are therefore particularly likely to be exposed to pesticides. Whilst other social bees and solitary bees could also be at risk from the impacts of pesticides and parasites, there are far fewer data currently available for these groups. The literature review below therefore primarily covers the impacts of pesticides and parasites on bumblebees and honeybees (collectively referred to as bees for the remainder of this chapter).

1.5 Pesticides

1.5.1 Historical and current use

Since the mid-20th Century, global agriculture has rapidly increased in order to support a growing population (Meyer & Turner 1992). Increases in crop production are coupled with an escalation in the use of nitrogen and phosphorous fertilizers, irrigation and pesticides (Tilman *et al.* 2001), each of which has benefits to agriculture, as well as serious environmental consequences (Tilman *et al.* 2002). Pesticides are now used worldwide (Figure 1.1), and the global use of plant protection products exceeds 2.2 million kg annually, at a cost of more than $35 billion (Fishel 2007). A pesticide can be defined as a substance used to kill or control organisms that are harmful to cultivated plants or animals. Pesticides used in agriculture include herbicides, fungicides, insecticides, molluscicides and rodenticides, all of which are designed to kill the target pest. Other substances such as plant and insect growth regulators, repellents and pheromones can alter the growth or behaviour of the target pest, reducing the damage to the crop. As such, pesticides perform an important role in crop protection and yield enhancement (Webster, Bowles & Williams 1999; Cooper & Dobson 2007). The financial benefits of applying pesticides, in terms of reduction of crop losses, are thought to be four times as high as the costs (Pimentel & Lehman 1993). However, cost-benefit analyses of pesticide use often fail to take into account indirect costs, for example, to human health and the environment (Bowles & Webster 1995). Furthermore, continued use of pesticides can lead to resistance in the target species (Denholm & Rowland 1992), which poses further challenges for control.

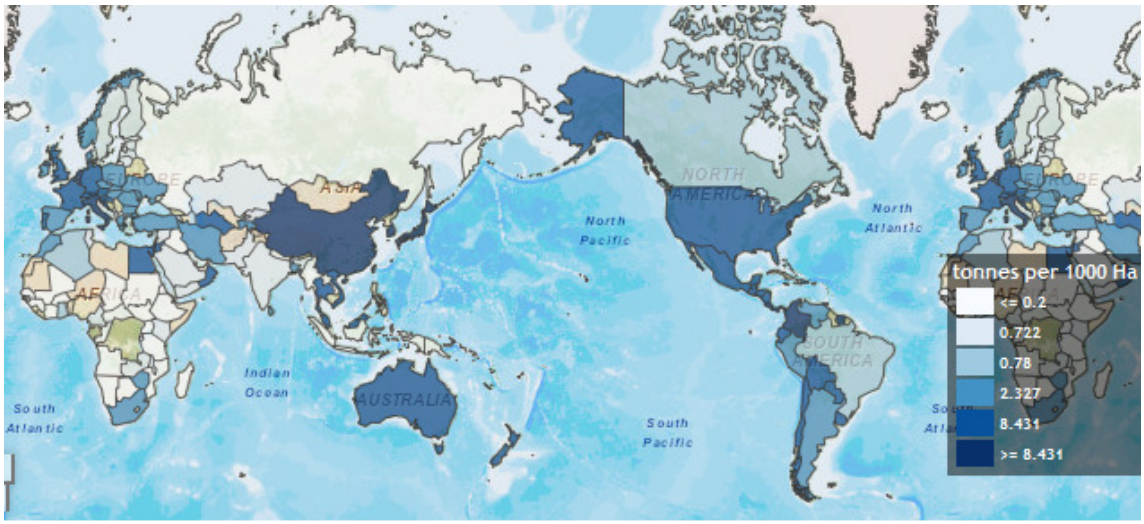


Figure 1.1: Global pesticide usage on arable and permanent crops between 1990 and 2010
(Tonnes of active ingredient per 1000 Ha). Image reproduced from FAO (2015)

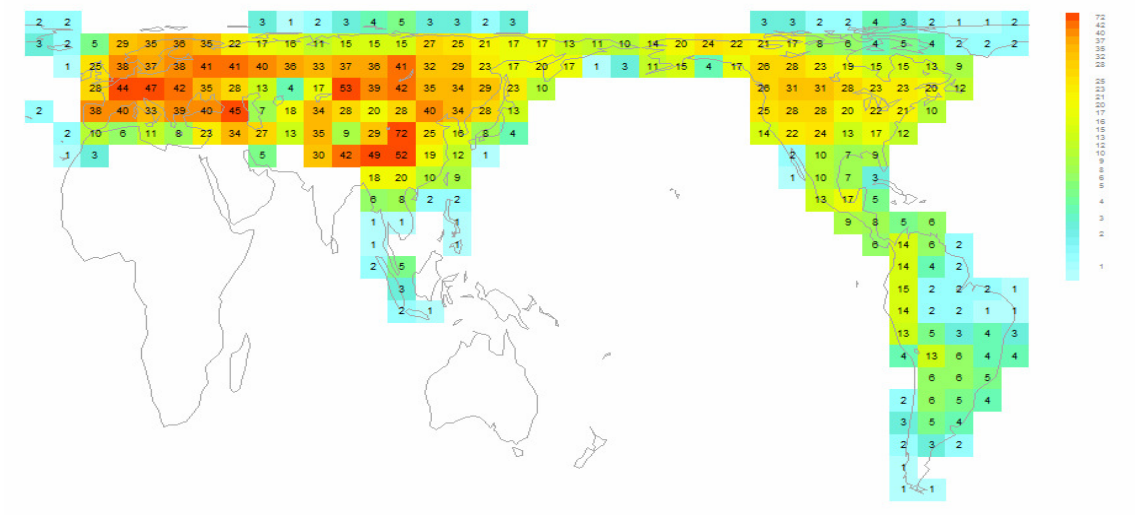


Figure 1.2: Species richness of bumblebees around the world
Red indicates high numbers of species, blue low numbers. Image reproduced from Williams & Jepsen (2014)

1.5.2 Environmental issues

Rachel Carson's landmark publication 'Silent Spring' (Carson 1962) highlighted the damaging effects pesticides could have on the environment. Some of the major themes remain a concern today, such as contamination of aquatic and terrestrial habitats and bioaccumulation of pesticides. Possibly the best known example of the latter was observed in the 1960's when raptor populations in Europe and the USA suffered declines in reproductive success as a result of egg-shell thinning (Ratcliffe 1967; Porter & Wiemeyer 1969). This was linked to the use of organochloride insecticides such as dichlorodiphenyltrichloroethane (DDT), which accumulated

in prey items. Top predators are not the only group at risk of exposure. Pesticides can accumulate in water bodies (Navarro *et al.* 2010; Beketov *et al.* 2013; Hladik, Kolpin & Kuivila 2014; Ippolito *et al.* 2015), and soil (Gevao, Semple & Jones 2000; Goulson 2013; Jones, Harrington & Turnbull 2014), which can lead to exposure of a wide range of non-target organisms through their natural environment or diet. High levels of pesticide contamination in water systems has been correlated with declines in stream invertebrate diversity (Beketov *et al.* 2013), whilst a range of soil invertebrates (Jänsch *et al.* 2006) and microbiota (Johnsen *et al.* 2001) can also be affected. Many invertebrates and plants that are controlled with pesticides provide a food source for birds and animals, and a reduction in these caused by pesticide use could have indirect effects on their predators. For example, reductions in farmland bird populations have been correlated with high levels of imidacloprid in water bodies in the Netherlands (Hallmann *et al.* 2014). This also correlates with declines in the invertebrates these birds feed to their young. Similarly, grey partridge (*Perdix perdix* L.) population declines in the mid-1900s were found to be attributed to a pesticide induced reduction in invertebrate prey for the chicks (Rands 1985).

Pesticide related declines in biodiversity have clear implications for conservation, particularly given the scale of pesticide use worldwide. Biodiversity loss could also have repercussions for agriculture, as many natural biological processes and organisms can be beneficial to agriculture (Costanza *et al.* 1997). Negative impacts of pesticides on these beneficial organisms or processes can have implications for the crops they are designed to protect (Chagnon *et al.* 2014). For example, systemic insecticides (which are water soluble and therefore taken up into plant tissue during growth) can be transmitted up the food-chain from treated soybean plants to non-target herbivores (slugs), and then to natural slug predators (ground beetles) (Douglas, Rohr & Tooker 2014). Whilst the slugs did not show any adverse reaction to pesticide exposure, the beetles displayed a range of responses from disruption of the motor system through to death. By disrupting the predator-prey interactions in this system, the pesticide had the additional effect of increasing slug density, and as a result, herbivory of the crop. This lead to a decrease in establishment and yield of the soybean (Douglas, Rohr & Tooker 2014).

Bees and other pollinating insects, although not a target for pesticides, can also come into contact with them if foraging in agricultural environments. The risk of exposure of bees to pesticides, coupled with recent bee declines and the potential for negative impacts on pollination services, has led to a growing interest in the impacts of pesticides on bees.

1.5.3 Exposure of bees to pesticides

Bees can be exposed to pesticides in a myriad of ways. Whilst foraging on treated crops, pesticide residues can be encountered orally in contaminated pollen and nectar (Chauzat *et al.* 2006; Mullin *et al.* 2010; Pettis *et al.* 2013; Thompson *et al.* 2013), as well as via contact exposure on the plant surface. Direct exposure during application can also occur during the spraying of crops (Greig-Smith *et al.* 1994), or through contact with dust generated from seed treatments during sowing (Pistorius *et al.* 2009; Krupke *et al.* 2012). Species such as honeybees which require additional sources of water can be at risk of exposure through contaminated liquids, for example, guttation fluid (Girolami *et al.* 2009; Thompson 2010), or possibly even puddles (Samson-Robert *et al.* 2014). Residues in soil (Krupke *et al.* 2012; Jones, Harrington & Turnbull 2014) or drift during application, could result in wildflowers that grow in agricultural areas also becoming contaminated (Thompson 2001; Krupke *et al.* 2012; Goulson 2013; Stewart *et al.* 2014), and additionally could be a route of exposure to wild ground nesting bee species. In addition to pesticide exposure through agricultural practices, managed bee colonies are often intentionally treated with pesticides, such as acaricides (substances toxic to ticks and mites), in order to control pests within the hives.

Whilst some of these exposure routes can be managed by improving the pesticide delivery processes, for example, by spraying pesticides at times when bees are not active (Thompson 2001), or controlling the spread of dust during and after planting treated seeds (Thompson 2010), bees are still likely to encounter low residues throughout their lifecycle. Figure 1.3 shows the pesticide treatment of two crops in the UK on which bees are known to forage: oilseed rape *Brassica napus* and field bean *Vicia faba*. This snapshot of seasonal pesticide use highlights the number and wide variety of compounds used during the flowering period of crops, many of which coincide with times when bees are most active. In addition to the compounds shown in Figure 1.3, a range of systemic insecticides are applied as seed treatments (Garthwaite *et al.* 2012a), residues of which will be found in plant tissue throughout growth. It is not surprising therefore that a huge array of pesticides of many classes have been identified in bee collected pollen and nectar (Chauzat *et al.* 2006; Mullin *et al.* 2010; Pettis *et al.* 2013), showing the extent to which bees are exposed, not only to individual pesticides, but to a combination of multiple pesticides throughout the year.

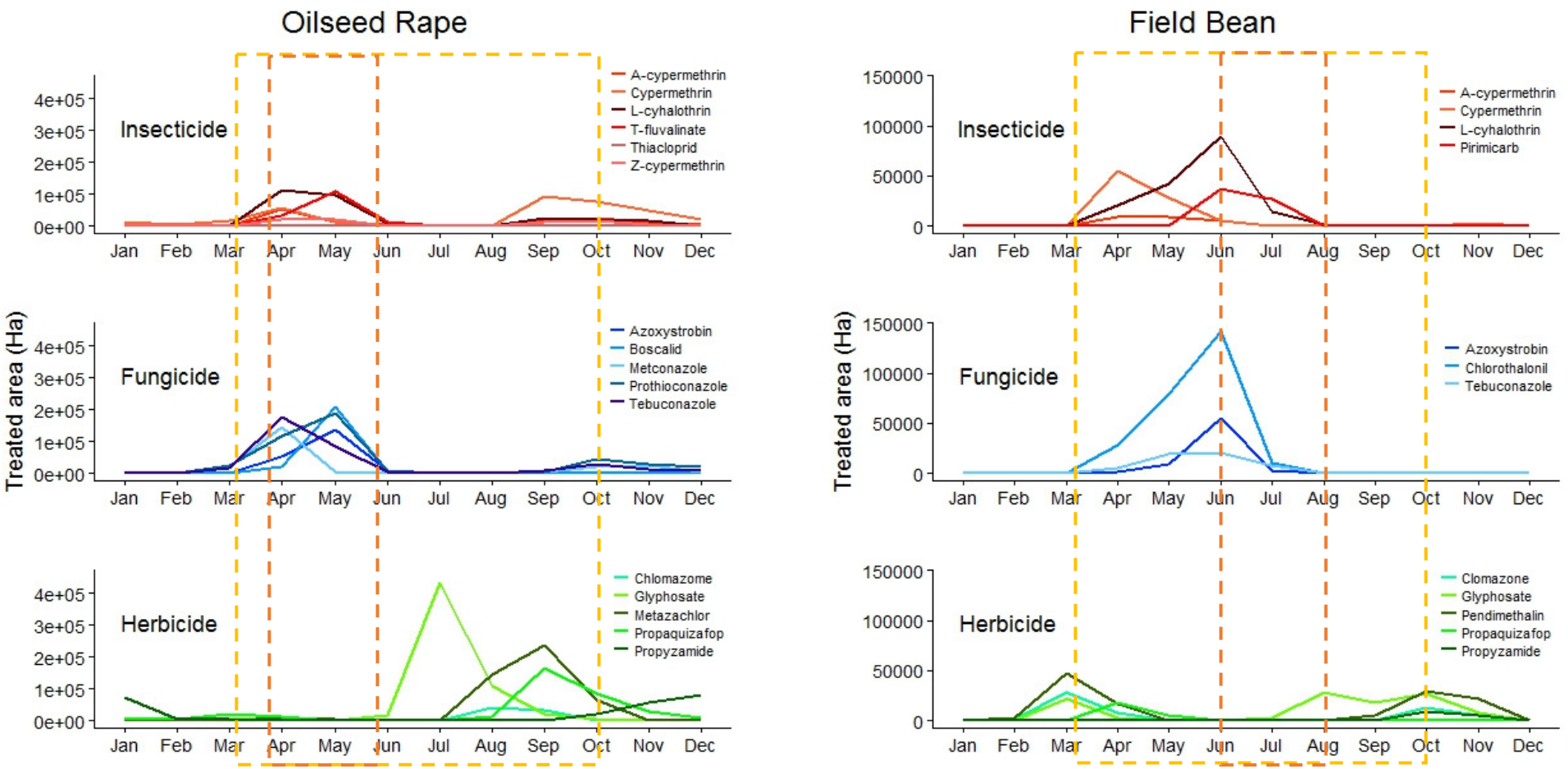


Figure 1.3: Monthly pesticide application of two crops attractive to bees in the UK in 2010

Dashed boxes indicate the period when most wild bee species are active (yellow box) and crop flowering period (orange box). Data from Garthwaite, D.G. *et al.* (2010), and personal communication from Garthwaite, D. G.

Understanding both the exposure profile and specific impacts of pesticides is important for quantifying the risk to bees. Whilst bees are exposed to a range of pesticide classes, insecticides are of particular concern, as they are specifically designed to target insects. Therefore the discussion below primarily focuses on insecticides, although it is noted that other pesticide classes are also a potential risk to bees.

1.5.4 Toxicity to bees

Many of the insecticides used in agriculture today are neurotoxins that target molecular processes in the insect nervous system (Belzunces, Tchamitchian & Brunet 2012). For example, acetylcholinesterase (AChE) inhibitors (organophosphates, carbamates) prevent the hydrolysis of acetylcholine (Ach), a neurotransmitter, leading to continuous stimulation of the nerve fibre (Fukuto 1990). Pyrethroids, which are derived from naturally occurring pyrethrins (found in *Crysanthemum cinerariifolium* flowers), prevent the sodium channels of neurons from closing, causing hyperexcitation (Soderlund & Bloomquist 1989). Neonicotinoids, the most widely used class of insecticide worldwide (Goulson 2013), are agonists of nicotinic acetylcholine receptors (nAChR). Initial activation of nAChR by neonicotinoids causes nervous stimulation, and overstimulation can occur at high doses, blocking the receptors and leading to paralysis and death (Tomizawa & Casida 2003).

Bees and other insects are naturally able to detoxify xenobiotics (including pesticides) using proteins such as glutathione-S-transferases (GSTs) (Enayati, Ranson & Hemingway 2005). Interestingly, honeybees are known to have fewer of these than other insects (Claudianos *et al.* 2006), but they are nonetheless able to metabolise ingested pesticides (Suchail, Guez & Belzunces 2000; Cresswell *et al.* 2014), and can clear a relatively high dose (98 µg/Kg) of the insecticide imidacloprid from body tissue within 24 hours (Cresswell *et al.* 2014). However, exposure of bees to high enough doses of pesticides can lead to severe neuronal disruption, and death (Stevenson & Racey 1966; Stevenson 1978).

Testing the toxicity of most modern insecticides to bees is currently part of the regulatory process for pesticide registration in the EU (EU 2013), USA (EPA 2014), and other parts of the world. The first level of testing in the EU (Tier I), involves finding the acute toxicity of the compound, measured using the LD_{50} (lethal dose required to kill 50% of test subjects under controlled laboratory conditions). A Hazard Quotient (HQ) is calculated based on the LD50 and application rate of the pesticide (for spray products). If the HQ exceeds a value considered to be safe, higher tier testing (semi-field studies (Tier II) and field trials (Tier III)) are undertaken to assess the risk to bees (European Commission, 2002). The risk is calculated by assessing how

hazardous the substance is and the likelihood of exposure. Honeybees are often used as a model bee species in such regulatory processes (Godfray *et al.* 2014). Such regulations ensure that the lethal dose of a pesticide is considerably higher than the residues bees are likely to encounter in the field.

Whilst a useful standardised test for toxicity, the LD50 provides only a basic understanding of the impacts of pesticides on bees. Most LD50 tests focus on the acute exposure (brief exposure to a high dose of pesticide) of individual adult worker bees. Chronic exposure (repeated long term exposure to low doses of pesticide) is a likely scenario in the field, and should also be tested. Furthermore, for the social bees, colony level impacts, as well as specific impacts on brood and different castes (queens and males) should also be taken into account when assessing risk. Honeybees, and to some extent bumblebees (*B. terrestris* and *B. impatiens)* are often used as model species in toxicity testing, but differences between species in sensitivity (Cresswell *et al.* 2012; Arena & Sgolastra 2014) and exposure to pesticides are likely to occur, and this should also be taken into consideration. The European Food Safety Authority (EFSA) recently provided guidance relating to this, advocating higher tier testing, particularly of chronic pesticide exposure, long term effects on bees, multiple pesticide exposure and effects on a range of bee species (honeybees, bumblebees and solitary bees) (EFSA 2013).

1.5.5 Sublethal effects

Sublethal exposure (exposure to doses which are below the lethal dose) can also impact on bees, both at the neural level and beyond. Impairment of the function of Kenyon cells (neurons which form a major component of bee mushroom bodies – an area of the brain responsible for learning and memory) in honeybees (Palmer *et al.* 2013) and bumblebees (Moffat *et al.* 2015) have been found in response to neonicotinoid and organophosphate exposure at field-relevant doses. The impairment of neural pathways by pesticides and their metabolites, can lead to a range of behavioural and physiological consequences (Belzunces, Tchamitchian & Brunet 2012). Olfactory learning (Decourtye *et al.* 2001; Decourtye, Lacassie & Pham-Delegue 2003; Decourtye *et al.* 2004; Decourtye *et al.* 2005; Williamson & Wright 2013; Stanley, Smith & Raine In Prep) and memory (Williamson & Wright 2013; Stanley, Smith & Raine In Prep) can be impaired by pesticide exposure, as well as changes in motor responses, such as hyperactivity (e.g. increased grooming and abdominal spasms (Williamson *et al.* 2013)), and gustatory and olfactory responses (e.g. a reduction in sucrose responsiveness (Eiri & Nieh 2012)).

Cognitive and motor functions are important for a range of behaviours vital to individual and colony level success. Navigation and homing ability require complex memory function (Menzel & Müller 1996). Neonicotinoids can disrupt homing ability in honeybees (Henry *et al.* 2012; Fischer *et al.* 2014), leading to an increase in worker mortality (Henry *et al.* 2012). Foraging is an essential activity in order to meet the nutritional needs of the individual bee and colony. Both nectar and pollen foraging are impaired after insecticide exposure (Mommaerts *et al.* 2010; Gill, Ramos-Rodriguez & Raine 2012; Feltham, Park & Goulson 2014; Gill & Raine 2014), and both brood production and colony growth can be reduced as a result (Gill, Ramos-Rodriguez & Raine 2012; Whitehorn *et al.* 2012; Fauser-Misslin *et al.* 2014). This can lead to smaller colonies and a reduction in their reproductive output (Whitehorn *et al.* 2012; Goulson 2015).

As well as colony level impacts on reproduction, pesticide exposure can also impair fecundity and nest building in individual worker bumblebees (Laycock *et al.* 2012; Elston, Thompson & Walters 2013; Laycock *et al.* 2013), although only imidacloprid has been found to have impacts at field relevant doses (Laycock *et al.* 2012). Whilst workers provide a useful model for investigating the impacts of pesticides on brood production by bees, they differ considerably in biology and life-history from queens, which are the primary egg laying caste within the colony. Egg laying by colony founding queens is a crucial stage of the colony cycle, yet little is known of the impacts of pesticides on queens at this stage. Furthermore, males, which are likely to have a very different pesticide exposure profile compared to workers (due to emergence later in the year), have also been relatively understudied with respect to pesticides.

1.5.6 Current issues

The studies discussed above show that sublethal doses of a range of insecticides can have a cascade of impacts on bees, from the neural to behavioural level, which have important consequences for survival and reproductive success. One class of insecticides, the neonicotinoids, are currently under particular scrutiny due to potential threats to bees and other organisms (Goulson 2013; Chagnon *et al.* 2014; Gibbons, Morrissey & Mineau 2014; Gross 2014; Pisa *et al.* 2014). There is currently a two year moratorium within the EU on the use of 3 neonicotinoids as seed treatments for crops on which bees forage. The moratorium is currently in place until December 2015. Evidence for the relative environmental safety, cost and efficacy of these compounds, and pest control methods which may be used to replace them, is urgently needed in order to assess their suitability for future use.

1.5.7 Field relevance

It is necessary to consider the effects of pesticides on bees in a field relevant context, particularly in relation to dose and length of exposure to the pesticide (Carreck & Ratnieks 2014; Godfray *et al.* 2014). Whilst some field and semi-field studies have been conducted (Gill, Ramos-Rodriguez & Raine 2012; Whitehorn *et al.* 2012; Pilling *et al.* 2013; Thompson *et al.* 2013), these are generally very difficult to control given the extent of pesticide usage in most landscapes, as well as potentially confounding environmental factors. More field studies are required, not only to test the effects of exposure to bees in natural settings, but also to confirm pesticide levels present in the pollen and nectar of crops and wildflowers throughout the year. For the purpose of this thesis, I have used parts per billion (ppb: the number of parts of active ingredient per billion parts of solvent) as a measure of pesticide dose, which is equivalent to µg/Kg (or µg/L mass: volume). This allows comparison of doses used in this thesis and in other studies, to residues found in the field.

Another issue related to field relevance is the presence of multiple stressors to which bees are likely to be exposed in agricultural environments. Exposure to multiple pesticides simultaneously can have a greater impact on bees than exposure to individual pesticides (Pilling & Jepson 1993; Gill, Ramos-Rodriguez & Raine 2012). When pyrethroid insecticides are combined with certain fungicides, the inhibition of detoxification processes by the fungicide can drastically increase the amount of time required for bees to metabolise the insecticide, resulting in increased toxicity (Pilling *et al.* 1995). The impacts of pesticides on bees could also be modulated by other environmental stressors. In a study of homing behaviour, Henry *et al.* (2014) found that homing failure due to pesticide exposure is context dependent, with ambient temperature and landscape complexity altering the level of impact. Stress from low temperature and toxin exposure (Archer *et al.* 2014) as well as from parasites (Brown, Loosli & Schmid-Hempel 2000), is known to have a greater impact on bees which are nutritionally challenged. It is likely therefore that the ability of bees to cope with pesticide exposure is dependent on a range of other environmental factors that influence their health. This thesis focuses on parasites as a potentially interacting factor, the impacts of which are summarised below, followed by the current extent of knowledge on interactions between pesticides and parasites in bees.

1.6 Parasites

Bumblebees and honeybees host a wide range of parasites, including viruses, protozoa, and arthropods (Schmid-Hempel 1998). They range from highly virulent organisms that can have significant effects on survival and fitness, to relatively low impact parasites (Schmid-Hempel 1998). Here I will discuss some examples of parasites that are widespread and prevalent in wild and managed bee populations, and thus could be encountered by bees in combination with other stressors such as pesticides.

1.6.1 Parasites of wild bees

Amongst the most prevalent of bumblebee parasites is *Crithidia bombi* Lipa and Triggiani, a trypanosomatid gut parasite with multiple *Bombus* host species. This parasite is found in wild bumblebee populations around the world (Shykoff & Schmid-Hempel 1991b; Colla *et al.* 2006; Rutrecht & Brown 2008a; Gillespie 2010; Kissinger *et al.* 2011; Jones & Brown 2014), and local prevalence can be extremely high (for example, up to 82% of individuals collected from North American populations were infected in 2007 (Gillespie 2010)). Sublethal impacts of this parasite on fitness (Brown, Loosli & Schmid-Hempel 2000; Brown, Schmid-Hempel & Schmid-Hempel 2003; Yourth, Brown & Schmid-Hempel 2008), worker reproduction (Shykoff & Schmid-Hempel 1991c) and foraging behaviour (Otterstatter *et al.* 2005) have been identified. Furthermore, *C. bombi* is known to have a context dependent effect, whereby virulence is increased when the host is under nutritional stress (Brown, Loosli & Schmid-Hempel 2000), or during particularly stressful stages of the life cycle (Brown, Schmid-Hempel & Schmid-Hempel 2003).

Nosema bombi Fantham and Porter is a microsporidian parasite of bumblebees which generally has a lower prevalence in the field than *C. bombi* (Shykoff & Schmid-Hempel 1991b; Kissinger *et al.* 2011), but can have severe fitness impacts (Schmid-Hempel & Loosli 1998; Otti & Schmid-Hempel 2007; Otti & Schmid-Hempel 2008). Increases in mortality were observed in laboratory reared *B. terrestris* colonies infected with *N. bombi* (Schmid-Hempel & Loosli 1998; Otti & Schmid-Hempel 2007). In field studies, significantly fewer infected colonies produced reproductive offspring compared to uninfected colonies (Otti & Schmid-Hempel 2008). However, colony level (Rutrecht & Brown 2008b) and species level (Rutrecht & Brown 2009) differences in infection dynamics and virulence have been observed, and horizontal transmission is thought to be low (Rutrecht, Klee & Brown 2007).

The bumblebee parasites with the highest impacts on their host include the nematode *Sphaerularia bombi* Dufour, and the neogregarine *Apicystis bombi*. Both of these parasites prevent reproduction in queens, *S. bombi* by preventing ovary development in the host, (Alford 1969; Poinar & Van Der Laan 1972), and *A. bombi* by dramatically reducing survival and thus preventing colony initiation by queens (Rutrecht & Brown 2008a; Jones & Brown 2014). These parasites are generally less prevalent than those discussed above (Colla *et al.* 2006; Rutrecht & Brown 2008a; Gillespie 2010; Jones 2014).

1.6.2 Parasites of managed bees

Intensive management of several bee species by humans for pollination (particularly *A. mellifera* and *B. terrestris*), has led to the movement of commercially reared bees around the globe. The consequences of this include infection of commercial bees with new parasites, and the transmission of non-native parasites from commercial colonies to native bees (Meeus *et al.* 2011).

Perhaps the best known and most commercially important example for the honeybee is the mite *Varroa destructor*. This is a natural parasite of the Eastern honeybee *Apis cerana*, which transferred to a new host, *A. mellifera*, most likely after transport of *A. mellifera* to Eastern Russia early in the 1900's (Oldroyd 1999). Co-evolution of parasite and host has resulted in *A. cerana* being well adapted to control levels of the mite through various mechanisms (Oldroyd 1999, Rath 1999), whilst the impacts on the new host *A. mellifera* are much more severe (Oldroyd 1999). The female mites feed on both adult bees and pupae, and ingest haemolymph which can lead to weight loss and nutritional problems in the bees (De Jong, De Jong & Goncalves 1982). In addition, *V. destructor* acts as a vector for several honeybee viruses (Genersch & Aubert 2010), such as DWV, the prevalence of which is vastly increased when the mite is present (Martin *et al.* 2012). Viruses such as these cause a range of symptoms, and can have further impacts on honeybee mortality (Genersch & Aubert 2010).

Several species of *Nosema* infect honeybees. *Nosema apis* is a natural parasite of *Apis mellifera,* whilst *N. ceranae*, native to Asian honeybees (*A. cerana),* has recently been discovered in *A. mellifera* colonies globally (Klee *et al.* 2007). This novel parasite is thought to be highly virulent to *A. mellifera.* Some evidence suggests it has a greater impact on individual survival than *N. apis* (Higes *et al.* 2007; Paxton *et al.* 2007). Immune suppression of *A. mellifera* was observed after infection by *N. ceranae*, but not *N. apis* (Antunez *et al.* 2009), a possible explanation for the differences in virulence observed. In addition to the impacts on survival

and health, *Nosema* is known to alter honeybee behaviour, for example by reducing homing ability (Kralj & Fuchs 2010; Wolf *et al.* 2014).

Whilst *V. destructor* and the associated viruses can have severe financial consequences for the apiculture industry, of greater concern for conservation is the transmission of parasites and diseases to wild bee populations (Meeus *et al.* 2011). Several viruses, primarily associated with honeybees, have been detected in commercially reared bumblebees (Genersch *et al.* 2006), and wild bee populations (Singh *et al.* 2010; Fürst *et al.* 2014; Ravoet *et al.* 2014; McMahon *et al.* 2015). Furthermore, several of these viruses are now known to be infective to bumblebees, produce overt symptoms and reduce survival and reproduction (Genersch *et al.* 2006; Fürst *et al.* 2014; Meeus *et al.* 2014). Commercially reared bumblebees (*B. terrestris*) may also act as pathogen reservoirs for wild bees. Commercial colonies and pollen supplied with them can contain high levels of bumblebee parasites, and prevalence of these parasites in wild bee populations is often higher when commercial bee colonies are located nearby (Colla *et al.* 2006; Otterstatter & Thomson 2008; Murray *et al.* 2013; Graystock, Goulson & Hughes 2014).

As well as the extensive range of bee parasites which have detrimental impacts, bees are also known to host an array of mutualistic microorganisms (Olofsson & Vásquez 2008; Koch & Schmid-Hempel 2011a; Martinson *et al.* 2011; Moran *et al.* 2012), which can be beneficial to the bee. The gut microbiota of honeybees and bumblebees can have positive health impacts and help to inhibit invading parasites (Forsgren *et al.* 2010; Koch & Schmid-Hempel 2011b). It is important to consider these interactions when studying the impacts of parasites and other stressors on bees.

1.7 Interactions between pesticides and parasites

Both pesticides and parasites can have substantial negative impacts on wild and managed bee species individually. Less is known about their combined impacts, despite the fact that bees foraging or nesting in agricultural areas are extremely likely to encounter multiple pesticides and parasites throughout their life. Interactions between pesticides and parasites are known in a range of other taxa (Kiesecker 2002; Coors *et al.* 2008; Kelly *et al.* 2010). In fact, interactions between pathogens and pesticides can be advantageous in the control of insect pests, for example, pesticide exposure can increase host susceptibility to entomopathogenic fungi (Ramakrishnan *et al.* 1999; Purwar & Sachan 2006). It is therefore likely that pesticides and

parasites could also have interactive impacts in bees, and understanding this is essential for assessing the risks of pesticide use, and making appropriate policy and regulatory decisions.

Recent findings suggest that the mortality rates of honeybees are higher when exposed to the microsporidian *N. ceranae* and imidacloprid in combination, compared to controls or each treatment alone (Alaux *et al.* 2010; Vidau *et al.* 2011; Aufauvre *et al.* 2012). The neonicotinoid thiacloprid can elevate the mortality rates of honeybee workers and larvae infected with black queen cell virus and *N. ceranae* (Doublet *et al.* 2014). Much less is known for bumblebees, although founding *B. terrestris* queens have reduced longevity when colonies are chronically exposed to *C. bombi* and thiamethoxam in combination (Fauser-Misslin *et al.* 2014).

There are several possible mechanisms for the interactive effects observed. Impairment of the immune system by pesticides could make bees more susceptible to parasites. Boncristiani *et al.* (2012) found that honeybee genes related to immunity were affected by exposure to a range of acaricides, altering metabolic responses. Di Prisco *et al.* (2013) found that pesticide exposure inhibited the immune function of honeybees, and led to increased replication of the virus DWV. Studies measuring other aspects of immunity were less conclusive. For example, Alaux *et al*. (2010), found that individual immunity (measured by phenol oxidase and haemocyte activity) was not altered by imidacloprid exposure. However, processes involved in social immunity such as glucose oxidase activity (which is involved in the production of antiseptic products secreted into larval food) were significantly reduced in the combined treatment. *Nosema ceranae* loads in honeybees were increased after larval exposure to imidacloprid (Pettis *et al.* 2012). Honeybees were also more susceptible to *N. ceranae* after exposure to pollen containing a range of fungicides (Pettis *et al.* 2013). Although no clear mechanisms were identified in these studies, it is possible that impairment of immune function could be involved. An additional hypothesis is that immune challenge by parasites could prevent other processes, such as detoxification, from functioning effectively. However, Vidau *et al.* (2011) found no change in the detoxification enzyme 7-ethoxycoumarin-O-deethylase, and an increase in GSTs in *N. ceranae* infected honeybees compared to controls, suggesting that these aspects of detoxification are not negatively impacted by this parasite.

Other mechanisms for interactive effects, although not thoroughly tested, could be plausible. For example, pesticides could have direct impacts on the parasites themselves. Conflicting results have been found on the proliferation of *N. ceranae* in bees treated with pesticides. Several of the studies above found an increase in spores after pesticide exposure (Vidau *et al.* 2011; Pettis *et al.* 2012; Pettis *et al.* 2013), whilst others found a decrease (Alaux *et al.* 2010;

Vidau *et al.* 2011). A decrease in parasite load could be attributed to a direct inhibition of parasite growth by the pesticide, although as all of these studies examined spore counts in vivo, it is impossible to separate direct impacts and host mediated impacts. In vitro studies would be informative to clarify this. Behavioural modification by the pesticide or parasite could also change the way bees are exposed to other stressors. For example, both parasites (Mayack & Naug 2009; Vidau *et al.* 2011) and pesticides (Thompson *et al.* 2014) can change the feeding behaviour of bees, by increasing or decreasing appetite, or through repellency. Vidau *et al.* (2011) suggest that an increase in feeding observed in *N. ceranae* infected bees could have resulted in greater exposure to the pesticide, resulting in higher impacts. An area which has not yet been explored is the impact of pesticides on the microbiota of bees. The gut flora of honeybees and bumblebees is known to inhibit invading parasites (Forsgren *et al.* 2010; Koch & Schmid-Hempel 2011b). These beneficial microbes could be directly exposed to a huge range of pesticides, including anti-microbial agents, after ingestion of contaminated pollen or nectar. If bee microbiota are affected by pesticides, this could in turn have an impact on the bee's ability to cope with parasites.

The examples above show that bees are faced with multiple sources of stress in their environment, and that these can interact to have an even greater impact on survival and health. The limited data available for interactive effects of pesticides and parasites on bees is almost exclusively focused on honeybee workers, and bumblebees remain largely unstudied in this context. Investigations into these issues in bumblebees and in different castes would be valuable.

1.8 Aims of this Thesis

From the literature review discussed above, I have identified several gaps in our knowledge and understanding of the impacts of pesticides on bees, and interactions with stress from natural parasites:

- Combined impacts of pesticides and parasites on bumblebees.
- Impacts of combined stressors on different castes, and at different stages of the bumblebee life cycle.
- Comparative impacts of pesticides on different species of wild bees.
- Interactions of pesticides with the mutualistic microbiota of bees.

The aim of the following four research chapters is to address these gaps. A brief summary of the main issues addressed in each chapter is given below:

Chapter Two explores the impact of a widely used pyrethroid insecticide on bumblebee colony development in the laboratory. The impacts of the pesticide in combination with parasite infection were also investigated. The survival and infection status of individual workers exposed to the pesticide as larvae within the colony, and subsequently infected as adults with the gut parasite *C. bombi,* were monitored.

Chapter Three looks at the impacts of combined stressors on a potentially vulnerable stage of the bumblebee colony cycle: founding queens. *B. terrestris* queens were hibernated in the laboratory, and the impacts of parasitism with *C. bombi*, hibernation length, and the neonicotinoid thiamethoxam on survival and colony founding were monitored.

Chapter Four focuses on the response of multiple species of spring caught bumblebee queens to pesticide exposure in the lab. The impacts of thiamethoxam on survival, ovary development and colony founding were explored.

Chapter Five looks at the potential for indirect impacts of pesticide exposure on honeybees, via changes to the gut microbiota. Thirteen phylotypes of honeybee lactic acid bacteria (LAB) were cultured in vitro, mixed with multiple doses of four different types of pesticide, and a combination of all four, and the growth of each phylotype was measured.

Finally, in **Chapter Six** I summarise the results of my research, and discuss the implications of these for bee conservation, management, and pesticide regulation and policy.

2 Impact of chronic exposure to a pyrethroid pesticide on bumblebees and interactions with a parasite

2.1 Abstract

Bees are exposed to pesticides when foraging in agricultural areas and growing evidence suggests that such compounds can be harmful to managed and wild populations. Given the economic and ecological importance of bees, and the evidence of widespread population declines, the full impacts of pesticides and their interactions with other stressors in the environment need to be investigated. Here I focus on the impacts of chronic exposure to the commonly used pyrethroid pesticide Lambda (λ)-cyhalothrin on the bumblebee *Bombus terrestris* at both the individual and colony level. Furthermore, I investigated the interactions of pesticide exposure with a highly prevalent trypanosome parasite *Crithidia bombi*. Colonies were exposed to λ-cyhalothrin in the laboratory and colony growth and reproductive output were monitored for up to 14 weeks. The potential interactions between the pesticide and *C. bombi* were investigated by quantifying the impact of pesticide treatment on susceptibility to, and success of experimental infections, as well as the survival of workers. Male survival after larval pesticide exposure was also monitored. Pesticide-treated colonies produced workers with a significantly lower body mass. However, out of the twelve variables of colony development measured this was the only metric that was significantly affected by pesticide treatment and there was no subsequent significant impact on the reproductive output of colonies. Lambda-cyhalothrin had no significant impact on the susceptibility of workers to *C. bombi,* or intensity of parasitic infection. Pesticide exposure did not cause differential survival in workers or males, even when workers were additionally challenged with *C. bombi*. Chronic exposure to λ-cyhalothrin has a significant impact on worker size, a key aspect of bumblebee colony function. This could indicate that under times of resource limitation, colonies exposed to this pesticide in the field may fail. However, the lack of other impacts found in this study, indicate that further field trials are needed to elucidate this.

The results of this chapter were published in January 2014 in the Journal of Applied Ecology: Baron, G.L., Raine, N.E., Brown, M.J.F. (2014) Impact of chronic exposure to a pyrethroid pesticide on bumblebees and interactions with a trypanosome parasite. Journal of Applied Ecology, 51: 460–469.

2.2 Introduction

Wild bee populations are declining at a global scale (Williams 1982; Biesmeijer *et al.* 2006; Brown & Paxton 2009; Williams & Osborne 2009; Cameron *et al.* 2011). Given the economic and ecological importance of pollinating insects such as bees (Klein *et al.* 2007; Ollerton, Winfree & Tarrant 2011), an understanding of the underlying causes of these declines is vital (Potts *et al.* 2010a; Dicks *et al.* 2013; Vanbergen *et al.* 2013). Several factors have been implicated in declines, including habitat loss (Williams 1986; Osborne, Williams & Corbet 1991; Carvell *et al.* 2006), parasites and disease (Colla *et al.* 2006; Cameron *et al.* 2011; Meeus *et al.* 2011), and the introduction of non-native species (Thomson 2004; Stout & Morales 2009). There is also mounting evidence that bees are regularly exposed to pesticides (Chauzat *et al.* 2009; Mullin *et al.* 2010) and that some of these compounds are detrimental to bees, even at sub-lethal levels (Johnson *et al.* 2010; Cresswell 2011; Gill, Ramos-Rodriguez & Raine 2012; Henry *et al.* 2012; Whitehorn *et al.* 2012; Bryden *et al.* 2013).

Most research into the impacts of pesticides on bees has focused on honeybees *Apis mellifera*, due to their extensive use in commercial pollination globally, and concerns over widespread honeybee losses in the USA and Europe (vanEngelsdorp *et al.* 2008; Potts *et al.* 2010b; Aizen & Harder 2009). However, protecting the diverse wild bee community is equally important for commercial pollination and maintaining wild ecosystems (Westerkamp & Gottsberger 2000; Klein *et al.* 2007; Breeze *et al.* 2011, Garibaldi *et al.* 2013). Bumblebees are key pollinators of agricultural crops and wild plants (Corbet, Williams & Osborne 1991), but their annual lifecycle, relatively small colony size, and different foraging strategies to honeybees, are traits which are likely to make them more vulnerable to pesticide exposure (Thompson 2001). Furthermore, recent evidence suggests that honeybees and bumblebees vary in their sensitivity to a neonicotinoid pesticide (Cresswell *et al.* 2012). Recent studies have demonstrated sub-lethal effects of pesticides on bumblebee fecundity (Laycock *et al.* 2012), queen production (Whitehorn *et al.* 2012), and foraging ability (Gill, Ramos-Rodriguez & Raine 2012).

The vast majority of recent available data on the sublethal impacts of pesticides on bumblebees focuses on neonicotinoids, whilst other pesticide classes remain relatively understudied. This stands in contrast to the fact that the usage of pesticides such as pyrethroids is widespread and increasing, for example, pyrethroid usage in the UK has nearly doubled since the early 1990s (FERA 2012), and given the recent EU moratorium on neonicotinoid usage for crops attractive to bees, use of alternative pesticides is likely to

increase further. Here, I investigate the impacts on *Bombus terrestris* colonies of exposure to a widely used pyrethroid insecticide, lambda-cyhalothrin (λ-cyhalothrin). This pesticide is sprayed during the flowering period on a range of crops, such as oilseed rape *Brassica napus*, which provide an important bumblebee foraging resource (Westphal, Steffan-Dewenter & Tscharntke 2003; Knight *et al.* 2009). Lambda-cyhalothrin is applied to large areas of agricultural crops in the UK throughout the spring and summer (for example, 43 % of oilseed rape was treated with this pesticide in 2012; Garthwaite *et al.* 2012a). Bumblebee colonies in agricultural landscapes are therefore likely to be exposed to low levels of this compound over extended periods of time (chronic exposure) whilst foraging on flowering crops. Gill *et al.* (2012) found that *B. terrestris* colonies exposed to λ-cyhalothrin had higher levels of worker mortality during the early stages of colony development. Our study expands on this by exploring the long-term impact of chronic exposure to λ-cyhalothrin on *B. terrestris* colony growth and the production of queens and males.

In order to understand the full impacts of pesticides on bumblebees in the wild we also need to consider other stressors, such as parasites, which are likely to influence colony success. Interactions between pesticides and parasites could result in a greater impact than the sum of each stressor acting individually (a synergistic interaction), which has been demonstrated in both vertebrates (Kiesecker 2002) and invertebrates (Coors *et al.* 2008). Such interactions have received some attention in honeybees (Alaux *et al.* 2010; Vidau *et al.* 2011; Aufauvre *et al.* 2012; Pettis *et al.* 2012, Di Prisco *et al.* 2013), and more recently, bumblebees (Fauser-Misslin *et al.* 2013). Whilst the above studies explore the impacts of chronic pesticide exposure in adult bees, little is known about how larval exposure to a pesticide impacts on adult survival, or how this interacts with parasite infection. Here I address these important questions in the bumblebee *B. terrestris.* Bumblebees are hosts to a wide range of parasites (Schmid-Hempel 1998), the most prevalent of which in Europe is *Crithidia bombi* (Shykoff & Schmid-Hempel 1991b). This gut parasite infects a range of bumblebee species (Ruiz-González *et al.* 2012), and is transmitted via contaminated faeces within the natal colony and on flower surfaces when foraging (Durrer & Schmid-Hempel 1994). *Crithidia bombi* occurrence in wild bumblebee populations varies spatio-temporally, and across species and caste, but prevalence levels of up to 47.5% have been reported in spring *B. terrestris* queens and up to 80% in workers (Shykoff & Schmid-Hempel 1991b). This parasite has been shown to increase mortality in nutritionally stressed *B. terrestris* workers (Brown, Loosli & Schmid-Hempel 2000) and reduce queen fitness after a stressful hibernation period (Brown, Schmid-Hempel & Schmid-Hempel 2003; Yourth, Brown & Schmid-Hempel 2008). The likelihood of bumblebees encountering stress from a

combination of parasite and pesticide exposure in the field is therefore high and the interactions between these stressors need to be determined.

In this study I addressed the following questions:

1. How does chronic exposure to λ-cyhalothrin affect *B. terrestris* colony growth and reproductive output?
2. Are workers exposed to λ-cyhalothrin as larvae more susceptible to infection by *C. bombi*?
3. Do larval exposure to λ-cyhalothrin, *C. bombi* or a combination of both have an impact on the survival of workers?
4. Is male survival affected by larval exposure to λ-cyhalothrin?

2.3 Materials and methods

2.3.1 Bumblebee colonies

Thirty early stage *B. terrestris* colonies (containing a queen, brood, and a mean of 8 (± 0.55 SE) workers were obtained from Syngenta Bioline (Weert, Netherlands). Colonies were kept in a dark room (red light was used for colony manipulation) at 25 °C. To ensure that colonies were healthy and developing normally, they were monitored for 18 days prior to allocation to a treatment group. All colonies were screened for the common parasites, *C. bombi*, *Nosema bombi,* and *Apicystis bombi*, by microscopic examination of faecal samples from queens (19 out of 24 colonies), and by dissection of 10 % of workers present at the time of sampling (mean = 2 ± 0.2 SE, range = 0-3). No infections were found in any colonies at this stage. A laboratory setup was used to ensure that colonies remained parasite-free throughout the experiment.

The number of workers per colony was counted, and each colony matched to another of equivalent size. One colony in each pair was then randomly allocated to the 'pesticide' treatment group and the other to the 'control' group. Six of the 30 queens (control = 4, pesticide = 2) died within the first four weeks of treatment, due to damage caused to these colonies during transit. These colonies were excluded from the rest of the experiment.

2.3.2 Pesticide treatment

Colonies were exposed to λ-cyhalothrin (Technical grade λ-cyhalothrin PESTENAL, Sigma-Aldrich) via the pollen feed provided, which was sprayed at a concentration of 37.5 ppm (the recommended application rate for oilseed rape: Syngenta Crop Protection UK, 2011), following the methods of Gill *et al.* (2012). A stock solution of λ-cyhalothrin in acetone was prepared and a sample of this was diluted each week with distilled water to obtain the required concentration. The same concentration of acetone was used for the control treatment. Pollen treatment took place at the same time every seven days (the minimum interval between applications to a single crop: Syngenta Crop Protection UK, 2011). Defrosted frozen pollen pellets (Koppert Ltd UK) were weighed into 10 g portions to create a single layer in a Petri dish (diameter 8.6cm). Pollen was sprayed with the λ-cyhalothrin or control solution from a distance of 20 cm using a fine mist sprayer to ensure even coverage. Each Petri dish was then closed and kept in dry dark conditions for 15 hours (overnight) at 22 °C to ensure that the solution was absorbed into the pollen. All pesticide-treated pollen was combined and mixed, before being weighed into clean Petri dishes. The same process was repeated with the control-treated pollen. Two samples of pollen treated in this way were analysed for λ-cyhalothrin residues using gas chromatography-mass spectrometry (GC-MS) (Food and Environment Research Agency, Sand Hutton, York). Two 1 g portions of each of these were extracted with methanol (20 mL). A portion of the extract was evaporated to dryness, re-dissolved in ethyl acetate and the λ-cyhalothrin residue was determined by GC-MS, with a limit of detection of 0.05mg/kg. Method performance was assessed by fortifying a control sample with 1 mg/kg of λ-cyhalothrin, recovery was 93 %. The average residue detected in the pollen samples was 0.247 mg/kg (± 0.021 SE), which is approximately a 100-fold reduction from the application rate. This is a similar reduction to that found by Choudhary & Sharma (2008) in mustard pollen treated with λ-cyhalothrin within a similar time frame.

A standardized amount of treated pollen was provided to each colony once per week, based on an estimate of colony size (allowing 0.5 g per bee each week). The weekly treatment represents the minimum time interval between treatments of individual crops (Syngenta Crop Protection UK, 2011). Treated pollen was provided to the colony in a Petri dish for 3 days, and then replaced with *ad libitum* untreated pollen for the remaining 4 days, this simulated the field scenario where bees will forage for pollen on pesticide treated crops and untreated plants. This temporal protocol was chosen to account for daily fluctuations in pollen intake (observed in a pilot experiment, GB unpublished data). Colonies were also provided with *ad*

libitum Ambrosia (E H Thorne Ltd), an inverted sugar syrup solution, which was diluted with equal parts water to make a 50% solution. The mass of treated and untreated pollen removed from the feeding dishes by each colony was weighed to the nearest 0.1 g, on a weekly basis. In order to check that workers would forage on treated pollen and feed this to larvae, I undertook a trial using micro-colonies, observing the behaviour of individual workers when provided with treated and untreated pollen (see Appendix 1).

2.3.3 Colony development

Workers and males that died in the colony were counted and discarded, whilst live males were kept for a survival experiment (see below), or were frozen. All gynes (unmated queens) were removed from the colonies and frozen. The dates of the first male and gyne eclosion, foundress queen death, and the onset of worker egg laying (competition point) were all recorded, as they represent the main phases of colony development (Duchateau & Velthuis 1988; Lopez-Vaamonde *et al.* 2009).

Pesticide treatment continued for 14 weeks. The peak time of λ-cyhalothrin application to crops in the UK is from April to July (in 2010, more than 100,000 ha of crops were treated with λ-cyhalothrin in each of these months; Garthwaite *et al.* 2010). As such, a 14 week period represents a worst case scenario, and mimics a situation where bumblebee colonies are collecting pollen over an extended period, from a range of treated crops which are treated at different times, with each crop potentially being treated multiple times.

Each colony was removed from the experiment and frozen four weeks after the queen's death, ensuring that all queen-laid offspring had eclosed. At this point a final count of workers, males and gynes within the colony was made. All living bees removed from the colonies were frozen at -20 °C. Frozen workers and males from each colony (when available), were randomly sub-sampled, and twenty of each caste were dried at 60 °C for 5 days, from which the average dry mass of workers and males was calculated for each colony (see Appendix 2 for an explanation of this procedure). All gynes produced were dried in the same way and weighed. The total dry mass of workers and sexual offspring (males and gynes) produced by each colony could then be estimated, by multiplying the total number of bees produced by their average dry mass.

2.3.4 Worker infection and survival

This stage of the experiment began four weeks after the start of pollen treatment to ensure that any workers removed from the colonies were exposed to the treated pollen throughout

their larval development (average worker development time is 22 days (Duchateau & Velthuis 1988)). Callow workers were only removed from colonies on days when untreated pollen was provided. Workers removed from each colony were allocated sequentially to a parasite or control treatment group, resulting in a fully crossed design (Table 2.1). Throughout the rest of the experiment, these workers were kept in plastic boxes (13 x 11 x 6.8 cm) containing a small amount of recycled paper cat litter (Waitrose) to remove excess moisture, and *ad libitum* untreated food (pollen and 50 % Ambrosia solution) in a dark room at 22 °C. After three days each worker was removed from its box, starved for three hours and transferred into a vial containing a 20 µl droplet (inoculum) of 50 % Ambrosia solution containing either 10,000 *C. bombi* cells or a control solution (acquisition and purification of *C. bombi* and the control solution are described below). Only bees which consumed all of the inoculum were included in the experiment. A dose of 10,000 cells lies within the range of *C. bombi* cells shed by infected workers which has been reported in previous studies (5000 cells/µl (Ruiz-González & Brown 2006) to 25000 cells/µl (Logan, Ruiz-González & Brown 2005)). Therefore, workers in an infected colony will be exposed to this level of the parasite if they ingest food contaminated with faeces.

Seven days after inoculation, faeces were collected from each bee, diluted with 0.9 % insect Ringer solution (Thermo Fisher, Basingstoke, UK) to a concentration of 10 %, thoroughly mixed, and the number of *C. bombi* cells per microlitre of faeces were counted using a Neubauer chamber. Workers were monitored every day until death. Dead workers were placed into a -20 °C freezer within 24 hours. The hindgut of each worker was dissected out and checked microscopically for the presence of *C. bombi*.

2.3.5 Male survival

Males which had been exposed to λ-cyhalothrin throughout their development were removed from colonies in the same way as described above for workers. Males were kept in groups of up to ten in communal wooden boxes (24 x 14 x 10.5 cm), provided with *ad libitum* pollen and sugar water, and monitored every day until death.

2.3.6 Crithidia bombi purification protocol

Wild *B. terrestris* queens, naturally infected with only *C. bombi* (queens were also screened for *N. bombi,* and *A. bombi)* were collected from Windsor Great Park, Surrey, UK (Latitude: 51.417432, Longitude: -0.60481256). Local adaptations of a parasite to its host can cause variability in infectiveness to different host populations (Imhoof & Schmid-Hempel 1998;

Yourth & Schmid-Hempel 2006). To select strains that would infect the commercial colonies used in our experiment, we infected workers from a commercial colony with a multitude of wild *C. bombi* strains, and used only strains infective to these stock bees for subsequent experimental infections. Faeces from uninfected queens from the same wild population were fed to stock bees from the same colony to provide a control. Stock bees were kept in groups of up to 20 individuals in wooden boxes (24 x 14 x 10.5 cm) and fed *ad libitum* pollen and 50 % Ambrosia solution. On the day of inoculation of experimental workers, faeces was collected from at least ten stock bees, then combined and diluted with 0.9 % insect Ringer solution to make a 1 ml solution (dilution 1). Using a modified protocol for purification developed by Cole (1970), the faeces solution was centrifuged at 0.4 G for two minutes, the supernatant separated, and the remaining pellet re-suspended with Ringer solution to a volume of 1 ml. This process was repeated 8 times, each time the supernatant from each tube being used to dilute the pellet from the proceeding tube so that 8 dilutions were produced. The three centre dilutions (dilutions 4-6) were then centrifuged at 8 G for 1 minute and the pellets combined with 100 µl Ringer solution and mixed thoroughly. The *C. bombi* cells in the resulting solution were counted using a Neubauer chamber and the volume of solution that contained 10,000 cells/bee was diluted with 50 % Ambrosia solution. The same protocol was followed for the control solution, using faeces from uninfected stock bees.

Table 2.1: Numbers of workers and males from either λ-cyhalothrin treated colonies, or control treated colonies that were removed from their colonies and included in survival experiments. Workers were either infected with the parasite *Crithidia bombi*, or uninfected.

Treatment	N workers (colonies)	Infected workers per colony	Uninfected workers per colony	N males (colonies)	Males per colony
Control	52 (6)	3, 3, 6, 3, 7, 2 (total **n = 24**)	4, 3, 9, 4, 6, 2 (total **n = 28**)	27 (5)	8, 6, 6, 3, 4
Pesticide	41 (5)	7, 3, 4, 4, 2 (total **n = 20**)	3, 4, 6, 5, 3 (total **n = 21**)	50 (5)	9, 5, 12, 17, 7

2.3.7 Analysis

Colony development and productivity

Data on the dry mass of workers were not available for four colonies due to low numbers of living workers in these colonies at the end of the experiment. In order to perform an analysis

including data from all 24 colonies, and also to analyse the worker mass data from the 20 colonies for which data were available, two separate multivariate ANOVAs (MANOVAs) were performed. Data that did not meet the assumptions of normally distributed residuals, or equality of variance, were transformed. The first MANOVA, using data from 20 colonies, included the following dependent variables: total number of workers produced, mean worker dry mass (g), total worker dry mass (g), total number of males produced (log_{10} transformed), mean male dry mass (g), and total dry mass of sexual offspring (log_{10} transformed). The dry mass of sexual offspring was calculated by combining the total dry mass of gynes with the total dry mass of males. As such, colonies that produced no gynes (control = 7, pesticide = 11) had the same total male dry mass as total sexual dry mass, and so total male dry mass was not included in the analysis. Worker mortality (number of workers found dead in the colony: log_{10} transformed) was also included as a dependent variable as an indicator of colony health. Pesticide treatment was used as a fixed factor and the number of workers at the start of the experiment as a covariate. The second MANOVA, using data from all 24 colonies, was performed in the same way and included the following dependent variables: total number of workers produced (log_{10} transformed), total number of males produced, mean male dry mass (g), total dry mass of sexual offspring (g), worker mortality (log_{10} transformed) and foundress queen survival (days from the start of the experiment: reciprocal transformation). When a MANOVA was significant, but none of the variables or direction of the trends explained this, a discriminant analysis was used to follow up and explore the underlying factors driving this effect.

Due to the large size of some colonies, accurate monitoring of certain events, such as the competition point and date of first male eclosion, was not always possible. Therefore the data for these variables were incomplete for some colonies, and were not included in either MANOVA. Instead, individual ANOVAs were used to analyse the data available, including treatment as a fixed factor and the number of workers at the start of the experiment as a covariate.

Individual ANOVAs were performed on the number of gynes produced, mean dry mass of gynes, and total dry mass of gynes. Treatment was included as a fixed factor and the number of workers at the start of the experiment as a covariate. As these data did not conform to the assumptions of normality or equality of variance, they were bootstrapped 1000 times to provide a robust estimate of significance and 95 % confidence intervals.

The effect size and 95 % confidence intervals for this were calculated for each variable in the analysis, in order to assess the reliability of the data, and its power to detect differences between the treatment groups.

Pollen consumption

In order to examine the difference in pollen consumption between pesticide and control treatment groups, and differences within colonies in consumption of treated and untreated pollen, a mixed design ANOVA was used. The mean weekly pollen consumption (total pollen consumption per colony each week / number of days on which each type of pollen was provided (treated pollen was provided for 3 days, and untreated for 4 days)) was used as the dependent variable, the pollen treatment (treated or untreated) as the within subject factor, and treatment type (pesticide or control) as the between subject factor. Pollen consumption was recorded each week throughout the 14 weeks of pesticide treatment. However due to variation in the length of experiment for each colony the sample size after week 9 was reduced and data beyond this point did not conform to the assumptions of normality or equality of variance. As such, only weekly pollen consumption data up to and including week 9 were included in this part of the analysis. In a separate analysis, the mean daily consumption of treated or untreated pollen (total pollen consumption for the whole experiment / number of days of experiment) for each colony was analysed using a mixed design ANOVA including the pollen treatment (treated or untreated) as the within subject factor, treatment type (pesticide or control) as the between subject factor, and the total number of bees produced as a covariate. When sphericity was violated, the degrees of freedom were corrected using the Greenhouse-Geisser estimates of sphericity (Field 2009).

Worker infection experiment

A G-test was used to test for differences among treatment groups in the prevalence of *C. bombi* both seven days post exposure and at death. A nested ANOVA was used to analyse the infection intensity of *C. bombi* (based on cell counts in faeces samples 7 days after parasite exposure) with the natal colony of each bee nested within the pesticide treatment.

A Generalised Linear Mixed Model (GLMM) was used to test for differences among treatment groups in worker survival. The model used a gamma (log-link) distribution, and included survival time (days) as the response variable, pesticide and parasite treatment as fixed factors, and colony as a random factor. Male survival was analysed in the same way, with only pesticide treatment as a fixed factor

All data analyses were performed using IBM SPSS versions 19 and 20.

2.4 Results

2.4.1 Colony development and productivity

Pesticide treatment had a significant overall effect in both MANOVAs (MANOVA 1, $F_{(7, 11)} = 3.406$, $P = 0.034$; MANOVA 2, $F_{(6, 16)} = 3.331$, $P = 0.025$). In the first MANOVA (Table 2.2) this was driven by a significantly lower mean worker dry mass in pesticide treated colonies compared to control colonies (ANOVA, $F_{(1, 17)} = 9.846$, $P = 0.006$: Figure 2.1). In the second MANOVA no uniform trend in the effects of pesticide treatment on the dependent variables was apparent (Table 2.3), so a discriminant analysis was used to explore the underlying drivers of the difference between treatment groups. One significant discriminant function (Wilk's Lambda = 0.435, $X^2_{(6)} = 15.798$, $P = 0.015$) was identified: the number of males produced, the total dry mass of sexual offspring produced and the difference between these were the major factors driving this discriminant function. This is likely to be due to differences in male and gyne production between pesticide and control colonies; on average pesticide treated colonies produced a greater number of males with a higher mean dry mass (Table 2.3) but fewer gynes with a lower mean dry mass (Table 2.4) compared to controls. However these differences were not individually significant within the MANOVA. Similarly, neither the overall dry mass of sexual offspring produced (Tables 2.2 and 2.3), nor the timing of key colony developmental events, such as the competition point (ANOVA, $F_{(1, 16)} = 0.616$, $P = 0.444$) and the number of days until the first male emerged (ANOVA, $F_{(1, 20)} = 2.563$, $P = 0.125$), were affected by pesticide treatment (Table 2.5). In both MANOVAs, the number of workers at the start of the experiment had a significant overall effect (MANOVA 1, $F_{(7, 11)} = 3.601$, $P = 0.029$; MANOVA 2, $F_{(6, 16)} = 3.178$, $P = 0.030$), with individually significant effects on the number of workers produced, number of males produced, the total dry mass of sexual offspring, and the number of worker mortalities (Tables 2.2 and 2.3).

The power of our data to detect differences between treatment groups may differ across variables (Appendix 3). Whilst effect sizes for the mean dry mass of workers, mean dry mass of males, and number of days until male production have tight confidence intervals, suggesting that these results are reliable, effect sizes for other variables measured (see Appendix 3 for details), have much larger confidence intervals which cross zero, suggesting that larger samples may be needed to definitively ascertain the impact of pesticide treatment.

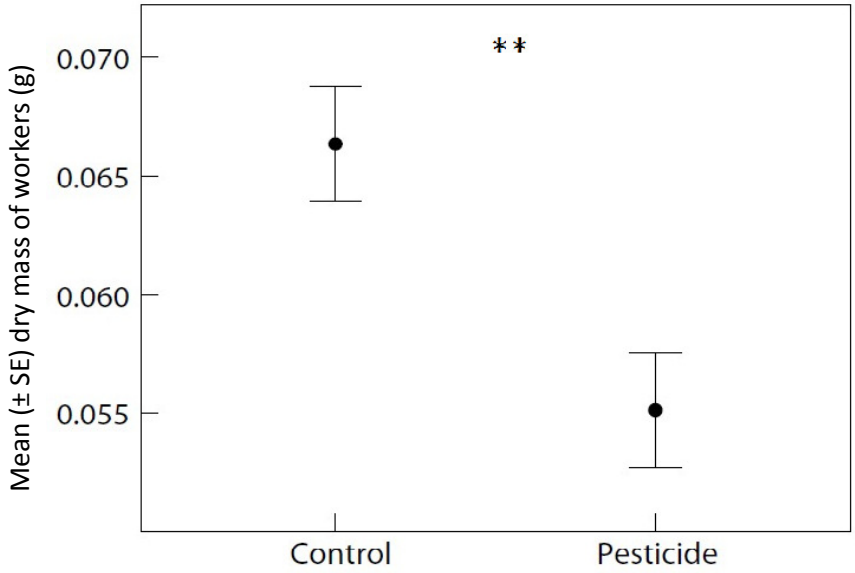


Figure 2.1: Mean dry mass of *Bombus terrestris* workers sub-sampled from colonies treated with a control or pesticide (λ-cyhalothrin). ** indicates significant difference (P = 0.006)

2.4.2 Pollen consumption

Results from the foraging and feeding trial indicate that workers from micro-colonies will collect pesticide treated pollen and feed this to larvae (Appendix 1). No rejection of treated pollen was observed.

In the main experiment, pollen consumption increased in both treatment groups over the first 8–9 weeks as colonies grew and then decreased as they began to senesce (mixed design ANOVA, $F_{(2.268, 45.361)} = 51.970$, $P < 0.005$). Pesticide treatment did not significantly affect pollen consumption in the first 9 weeks (mixed design ANOVA, $F_{(1, 20)} = 0.053$, $P = 0.821$) or the full 14 weeks of the experiment (mixed design ANOVA, $F_{(1, 21)} = 0.331$, $P = 0.571$). There was no significant effect of whether the pollen was treated (with acetone or λ-cyhalothrin) or untreated on average daily consumption (mean (g) ± SE pesticide treated = 5.77 ± 0.94; pesticide untreated = 5.97 ± 0.94; control treated = 6.72 ± 1.24; control untreated = 6.21 ± 1.28: repeated measures ANOVA, $F_{(1, 21)} = 0.001$, $P = 0.972$) when the total number of bees produced by each colony was controlled for.

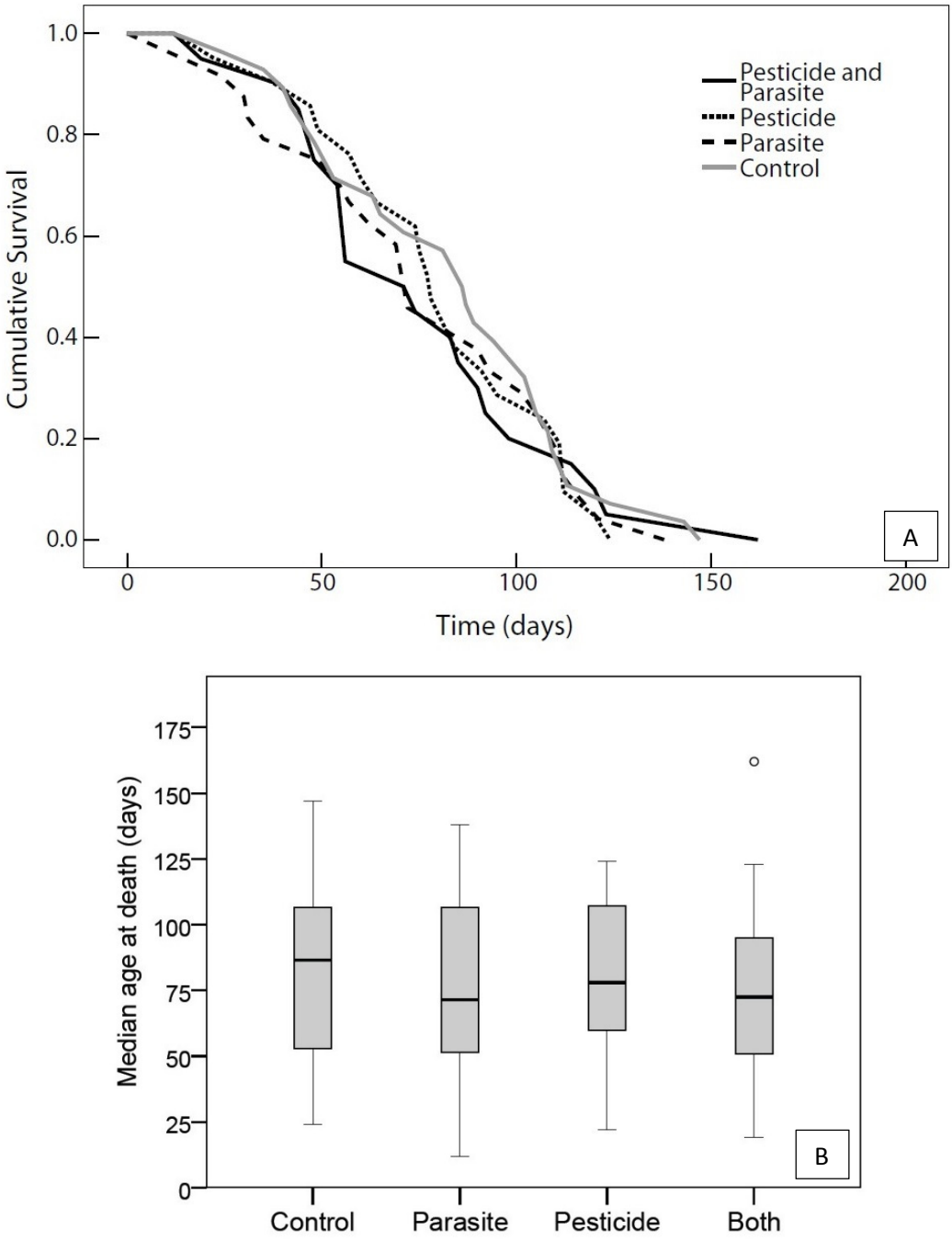


Figure 2.2: The cumulative survival (A) and median age at death (B) of *Bombus terrestris* workers exposed to a pesticide (λ-cyhalothrin), a parasite (*Crithidia bombi*), both pesticide and parasite, or neither (control). In the box and whisker plots, the thick horizontal bar is the colony median, the top and bottom of the box indicate the first and third quartile, and the whiskers show the minimum and maximum values.

2.4.3 Worker infection experiment

Seven days after exposure to *C. bombi*, faeces samples were available for 93 % (41 of 44) of workers. Of these, 91 % (20 of 22) of workers from control treated colonies were infected, and 95 % (18 of 19) from pesticide treated colonies. There was no significant effect of pesticide treatment (G = 10.007, d.f. = 10, p = 0.440) or colony (G = 17.852, d.f. = 20, p = 0.957) on the prevalence of *C. bombi* after 7 days. Results from the analysis of workers post mortality were qualitatively the same (data not shown).

There was no effect of pesticide treatment on infection intensity in workers 7 days after exposure to *C. bombi* (mean ± SE (*C. bombi* cells/µl faeces) pesticide = 33,809 ± 8,065; control = 27,942 ± 5,957: Nested ANOVA, F (1, 33) = 0.204, p = 0.75). There was also no significant effect of natal colony on infection intensity (Nested ANOVA, F (12, 33) = 1.763, p = 0.25).

Worker survival was not significantly affected by pesticide treatment (GLMM, $F_{(1, 89)} = 0.006$, $P = 0.936$), parasite treatment (GLMM, $F_{(1, 89)} = 1.371$, $P = 0.245$), or the interaction between these factors (GLMM, $F_{(1, 89)} = 0.391$, $P = 0.532$) (Figure 2.2). Similarly, male survival was not significantly affected by pesticide treatment (mean ± SE (days) pesticide = 32 ± 1 days; control = 31 ± 2: GLMM, $F_{(1, 7)} = 0.352$, $P = 0.555$).

2.5 Discussion

In this experiment, chronic exposure to λ-cyhalothrin resulted in the production of smaller workers by *B. terrestris* colonies. However, there were no significant impacts on the production of gynes or males, the susceptibility of individual workers to *C. bombi*, or any interactive effects of the pesticide and parasite on worker survival.

2.5.1 Worker size

Whilst the smaller size of workers in pesticide treated colonies did not result in any effects on sexual offspring production in this study, this is unsurprising, as previous laboratory studies also using *ad libitum* food showed that bumblebee colonies are able to compensate under such conditions (E.g. Müller & Schmid-Hempel 1992). However, a reduction in worker size is likely to have impacts on colony productivity in the field. Larger workers have greater visual acuity (Spaethe & Chittka 2003), higher antennal sensitivity (Spaethe *et al.* 2007), are better able to fly under lower light conditions (Kapustjanskij *et al.* 2007), and are more efficient foragers (Goulson *et al.* 2002; Spaethe & Weidenmuller 2002). Consequently, a colony

producing smaller workers may be less able to collect sufficient food resources, which will impact on the production of sexual offspring, and make the colony more vulnerable to the costs associated with an energy shortfall (Cartar & Dill 1991).

The mechanism underlying the reduced mass of workers produced by λ-cyhalothrin treated colonies is unknown, but could be due to differences in larval feeding. In bumblebees the size of an adult worker is determined by how much it is fed during development (Sutcliffe & Plowright 1988), and so a difference in larval feeding between treatment groups might account for the difference in adult worker mass. The results of the foraging trial (Appendix 1) indicate that *B. terrestris* workers readily forage on λ-cyhalothrin treated pollen and feed it to larvae. Furthermore, there was no significant effect of pesticide treatment on pollen consumption by colonies, indicating that if reduced feeding of larvae occurred it was not due to any repellent or anti-feedant effect of the pesticide. Previous research has identified behavioural changes in worker honeybees and bumblebees after exposure to a range of doses of pesticides (Henry *et al.* 2012; Schneider *et al.* 2012; Gill, Ramos-Rodriguez & Raine 2012) suggesting we could also see behavioural changes relating to within nest tasks, like brood care, potentially resulting in reduced larval feeding by workers. Interestingly, the mass of males and gynes produced during the current experiment was not significantly affected by the pesticide treatment, possibly suggesting that the pesticide had a stronger effect earlier in colony development, when most larvae developed into workers. The ratio of workers to brood is lower earlier in the colony cycle (Duchateau & Velthuis 1988), and so male and gyne larvae could have been buffered from any pesticide induced reduction in larval feeding, as there would have been more workers available for brood care.

2.5.2 Field relevance

Gill *et al.* (2012) found that some impacts of pesticide exposure on bumblebee colonies only became apparent several weeks after exposure began, highlighting a need for longer-term studies into chronic exposure to pesticides (EFSA, 2013). However, the profile of pesticide exposure bees experience in the field remains unknown. Lambda-cyhalothrin is applied to a wide range of crops in the spring and summer (Garthwaite *et al.* 2012a; 2012b), on several of which bumblebees are known to forage (Thompson & Hunt 1999). Bumblebees are likely to be exposed to this pesticide on a range of crops which flower at different times. There is a paucity of data on how compounds such as λ-cyhalothrin persist in floral tissue such as pollen, which makes it difficult to predict how long bee colonies may be exposed to residues. Furthermore, it is unknown whether bumblebees will actually take contaminated pollen back to the colony –

acute effects of the pesticide may cause death of workers in the field. However, this compound has been detected in stored pollen in honeybee hives (Mullin *et al.* 2010) and pollen collected from foraging honeybees (Choudhary & Sharma 2008), showing that honeybees collect pyrethroid-contaminated pollen, and may subsequently be exposed to residues in the hive for some time. In addition, our data show that bumblebee workers will collect pollen treated with pesticide at the dose provided in our experiment with no significant impact on mortality. Individual crops can be treated up to four times during flowering (Syngenta Crop Protection UK, 2011), and it is likely that different crops will be sprayed at different times dependent on the pest being targeted. Consequently, the 14-week exposure period used in this study explores a potential worst case scenario. Interestingly, the significant effect of pesticide exposure (a 16% reduction in worker mass) occurred during the first 5–6 weeks of the experiment. Not only does this correspond to an ecologically realistic timeline, it coincided with one of the most vulnerable stages of colony development. This suggests that assessments of colony level impacts should match field relevant pesticide exposure with appropriate developmental stages of the focal species' lifecycle.

2.5.3 Colony development

Despite the extensive period of exposure in our experiment, the impacts on colony development and reproductive output under laboratory conditions were minimal. However, interpretation of the effect size and confidence intervals for the variables measured in this study (Appendix 3), suggest that larger sample sizes may be required to fully understand any impacts of λ-cyhalothrin exposure on some aspects of colony development (e.g. worker mortality) and reproductive output of colonies. In addition, our study only takes into account pesticide exposure of bees and brood within the colony via contaminated food resources. There is also a chance that foraging bees may encounter pyrethroids at higher doses outside the colony, for example if they are sprayed during pesticide application, and these impacts should be taken into account when considering the potential risks of pyrethroid use to wild bees.

2.5.4 Worker infection

In order to fully understand the pesticide impacts on beneficial arthropods in the wild, it is crucial to understand how pesticides interact with other stressors such as parasites. This is the first study to address this question in bumblebees using a pyrethroid pesticide. We found no effect of pesticide treatment during larval development on the susceptibility of adult workers to *C. bombi* infection, or on the intensity of infection. Larval exposure of workers to λ-

cyhalothrin did not have an impact on adult survival even under subsequent challenge with *C. bombi.* Individuals in this study were provided with *ad libitum* food, and different results may be found if individuals are placed under nutritional stress (Brown, Loosli & Schmid-Hempel 2000). Additionally, there was no impact of larval λ-cyhalothrin exposure on male survival. Previous studies on honeybees have found that several pesticides interact synergistically with *N. ceranae* resulting in an increased worker mortality (Alaux *et al.* 2010; Vidau *et al.* 2011; Aufauvre *et al.* 2012), although these studies exposed adult workers directly to an acute dose of pesticide. Given the differential susceptibility of bumblebees and honeybees to pesticides and differences in parasite virulence our results suggest that the simple extrapolation of studies across taxa, across stressors, or between exposure scenarios is unwarranted.

2.5.5 Conclusions

The growing evidence that neonicotinoid pesticides have a detrimental impact on bumblebees (Cresswell *et al.* 2012; Gill, Ramos-Rodriguez & Raine 2012; Laycock *et al.* 2012; Whitehorn *et al.* 2012, Bryden *et al.* 2013) and other non-target organisms (Goulson 2013), and the recent moratorium on the use of three major neonicotinoid pesticides in Europe is likely to result in an increase in demand for alternative crop protection products such as pyrethroids. If this shift in pesticide usage is to take place, it is important that we understand potential impacts on essential wild pollinators. Our study shows that field research into the exposure profile and impacts on vulnerable life stages of these pollinators is urgently needed. Such studies should inform risk assessments and policy guidelines for the future application and usage of pesticides.

Table 2.2: Colony development data from 20 *B. terrestris* colonies treated with either the pesticide λ-cyhalothrin or a control solution, used in statistical analysis including worker mass as a variable. Data shown are colony means (± SE), n indicates the number of colonies per treatment group. Test statistics are from individual ANOVAs for the variable in each row. The overall MANOVA was significant (see Results for details). † Data were log_{10} transformed prior to analysis. 'Trend' indicates whether the pesticide treatment had a negative or positive (but not necessarily significant) effect on each variable. Significant p values are shown in bold: * = p < 0.05, ** = p < 0.01

Dependent Variable	**Control colonies Mean** (± SE) n=11	**Pesticide colonies Mean** (± SE) n=9	**Trend**	**ANOVA test statistics (including colonies with data available)**							
				Pesticide treatment				**Number of workers at start**			
				F	**d.f.**	**Error d.f.**	***P***	**F**	**d.f.**	**Error d.f.**	***P***
Number of workers produced	196 (± 35)	184 (± 47)	-	0.136	1	17	0.717	5.879	1	17	**0.027***
Average dry mass of workers (g)	0.066 (± 0.002)	0.055 (± 0.002)	-	9.846	1	17	**0.006****	0.075	1	17	0.787
Total dry mass of workers (g)	13.221 (± 2.520)	10.624 (± 3.004)	-	0.684	1	17	0.42	3.904	1	17	0.065
Number of males produced †	207 (± 47)	192 (± 54)	-	0.022	1	17	0.884	7.138	1	17	**0.016***
Average dry mass of males (g)	0.109 (± 0.008)	0.128 (± 0.007)	+	2.915	1	17	0.106	1.124	1	17	0.304
Total dry mass of sexual offspring (g) †	28.057 (± 7.296)	27.059 (± 8.911)	-	0.017	1	17	0.898	5.357	1	17	**0.033***
Worker mortalities †	57 (± 13)	57 (± 20)	0	0.306	1	17	0.587	3.569	1	17	0.076

Table 2.3: Colony development data from 24 *B. terrestris* colonies treated with either the pesticide λ-cyhalothrin or a control solution, used in statistical analysis which did not include worker mass as a variable. Data shown are colony means (± SE), n indicates the number of colonies per treatment group. Test statistics are from individual ANOVAs for the variable in each row. The overall MANOVA was significant (see Results for details). † Data were Log $_{10}$ transformed. ‡ Data were transformed with a reciprocal transformation prior to analysis. 'Trend' indicates whether the pesticide treatment had a negative or positive (but not necessarily significant) effect on each variable. Significant p values are shown in bold: * = p < 0.05, ** = p < 0.01

Dependent Variable	**Control colonies Mean** (± SE) n=11	**Pesticide colonies Mean** (± SE) n=13	**Trend**	**ANOVA test statistics (including all colonies)**							
				Pesticide treatment				**Number of workers at start**			
				F	**d.f.**	**Error d.f.**	***P***	**F**	**d.f.**	**Error d.f.**	***P***
Queen longevity (days from treatment start) ‡	59 (± 5)	50 (± 6)	-	2.465	1	21	0.131	1.656	1	21	0.212
Number of workers produced †	196 (± 35)	165 (± 33)	-	1.517	1	21	0.232	3.798	1	21	0.065
Number of males produced	207 (± 47)	239 (± 49)	+	0.035	1	21	0.854	9.413	1	21	**0.006****
Average dry mass of males (g)	0.109 (± 0.008)	0.124 (± 0.005)	+	2.085	1	21	0.163	0.294	1	21	0.593
Total dry mass of sexual offspring (g)	28.057 (± 7.296)	31.457 (± 7.162)	+	0.035	1	21	0.853	5.289	1	21	**0.032***
Worker mortalities †	57 (± 13)	70 (± 16)	-	0.084	1	21	0.775	8.024	1	21	**0.010***

Table 2.4: Gyne production data from *B. terrestris* colonies treated with either the pesticide λ-cyhalothrin or a control solution. The Bootstrapping column shows the significance and confidence intervals after bootstrapping the data 1000 times. 'Trend' indicates whether the pesticide treatment had a negative or positive (but not necessarily significant) effect on each variable

Dependent Variable	**Control colonies Mean** (± SE)	**Pesticide colonies Mean** (± SE)	**Trend**	**Bootstrapping**		
				P	**95% Confidence Intervals**	
					Lower	**Upper**
Number of gynes produced	9 (± 7) n=11	1 (± 1) n=13	-	0.380	-25.143	1.408
Average dry mass of gynes (g)	0.302 (± 0.030)	0.240 (± 0.041)	-	0.181	-0.271	0.014
Total dry mass of gynes (g)	8.951 (± 6.480)	1.285 (± 0.689)	-	0.422	-33.882	1.739

Table 2.5: The timing of key events in colony development measured in *B. terrestris* colonies treated with either the pesticide λ-cyhalothrin or a control solution. Data shown are colony means (± SE), n indicates the number of colonies included in each analysis. Test statistics are from individual ANOVAs. † Data were square root transformed prior to analysis. 'Trend' indicates whether the pesticide treatment had a negative or positive (but not necessarily significant) effect on each variable. Significant p values are shown in bold: * = p < 0.05, ** = p < 0.01

Dependent Variable	**Control colonies Mean** (± SE)	**Pesticide colonies Mean** (± SE)	**Trend**	**ANOVA test statistics**							
				Pesticide treatment				**Number of workers at start**			
				F	**d.f**	**Error d.f**	**p**	**F**	**d.f.**	**Error d.f.**	**p**
Competition point (days from treatment start)	43 (± 4) n=9	38 (± 4) n=10	+	1.518	1	17	0.235	0.005	1	17	0.946
Male production (days from treatment start) †	45 (± 6) n=10	33 (± 3) n=13	+	2.563	1	20	0.125	14.521	1	20	**0.001****

3 Thiamethoxam reduces egg laying in overwintered *Bombus terrestris* queens.

3.1 Abstract

Bumblebees, an important group of pollinators, are declining on a global scale. Pesticides have been identified as a threat to bumblebees, and research into their impacts is vital. One stage of the bumblebee lifecycle which has been largely neglected in the assessment of pesticide risk to bees is colony founding queens. Having undergone an energetically demanding hibernation, and without a colony to buffer them from environmental stress, queens at this stage represent the most vulnerable phase of the cycle. Queens are likely to be exposed to pesticides and other stressors whilst foraging and nest-searching in the spring, and yet little is known about the impacts on their success in founding a colony. I investigated the combined impacts of a neonicotinoid insecticide and a prevalent bumblebee parasite on colony founding bumblebee queens.

Bombus terrestris queens were mated in the laboratory, exposed to the trypanosome parasite *Crithidia bombi*, and hibernated for a period of 6 weeks or 12 weeks. They were then exposed to a field relevant dose of the neonicotinoid thiamethoxam for two weeks, whilst maintained in ideal conditions to initiate a colony. A fully crossed design was used in order to explore the individual and combined effects of these treatments, and queens were monitored for 10 weeks for mortality and signs of colony initiation. There was no effect of the pesticide, parasite or hibernation treatment on survival of queens. Exposure to thiamethoxam caused a 26 % reduction in the overall proportion of queens that laid eggs, and resulted in a shift in the timing of colony initiation, but had no effect on subsequent colony development. A short hibernation also resulted in fewer egg laying queens. No effects of the parasite, or interactive impacts between stressors on survival or colony initiation were detected.

In this experiment, field relevant exposure of bumblebee queens to a widely used neonicotinoid pesticide resulted in a considerable reduction in colony initiation. This is the first indication that neonicotinoid pesticides impact upon this key stage in the bumblebee lifecycle, and thus may have a major impact on population dynamics in this group.

3.2 Introduction

Queen hibernation and colony founding represent a critical but vulnerable period in the lifecycle of bumblebees. At this stage of the colony cycle, success depends entirely upon the queen's survival and ability to initiate a colony. Queens can lose up to 80 % of their fat reserves during hibernation (Alford 1969), which may make them vulnerable to additional stress. In social bees, loss of individuals from the colony is buffered to some extent by the presence of many colony members. However, founding queens are functionally solitary at this stage, and do not have a colony to buffer them from environmental stress. This study investigates the impacts of multiple stressors on bumblebee queens at this vulnerable stage of colony development.

Little is known about overwintering survival of bumblebee queens in the wild, but studies in the laboratory have shown that a range of factors can influence the survival and fitness of queens during and immediately after hibernation, and these may also be important in wild hibernating queens. Wild queens build up fat and glycogen reserves prior to overwintering and these are utilised during hibernation (Alford 1969). Pre-hibernation weight is therefore, unsurprisingly, an important predictor of hibernation survival in the laboratory (Holm 1972; Beekman, van Stratum & Lingeman 1998). The duration of hibernation can also be important in survival (Beekman, van Stratum & Lingeman 1998), as longer hibernation periods (6 months or more) substantially decreased the survival of queens, whilst shorter durations (1-4 months) had much better rates of survival. Genotype of the queen and of her mate can both impact on queen survival (Korner & Schmid-Hempel 2003; Yourth, Brown & Schmid-Hempel 2008) and queen genotype also has implications for reproductive success (Yourth, Brown & Schmid-Hempel 2008).

Additional stress from environmental factors such as parasites and pesticides may further reduce the survival or fitness of queens during this time, and thus have an impact on bumblebee populations. Several bumblebee parasites are known to completely inhibit the reproductive success of bumblebee queens, either through castration, e.g. the nematode *Sphaerularia bombi*, or through mortality of queens, e.g. the neogregarine *Apicystis bombi* (Schmid-Hempel 1998; Rutrecht & Brown 2008a; Jones & Brown 2014). Less virulent parasites can also have impacts at a sub-lethal level, for example, *Crithidia bombi,* a prevalent trypanosome parasite of bumblebees, has a context-dependent impact on its queen host (Brown, Schmid-Hempel & Schmid-Hempel 2003). In laboratory conditions, parasitized queens lost up to 11 % more mass during hibernation (Brown, Schmid-Hempel & Schmid-Hempel

2003), and had up to 40 % reduction in fitness compared to uninfected queens (Brown, Schmid-Hempel & Schmid-Hempel 2003; Yourth, Brown & Schmid-Hempel 2008).

Exposure to agrochemicals has been implicated in bee declines, and there is growing evidence that pesticides have an impact on bumblebee colonies and individuals (Gill, Ramos-Rodriguez & Raine 2012; Laycock *et al.* 2012; Whitehorn *et al.* 2012; Bryden *et al.* 2013). Bumblebee queens are likely to be exposed to a range of pesticides throughout their lifecycle, particularly when foraging in the early spring on flowering crops such as oilseed rape (G. Baron – personal observation, see Chapter 4). To date, there has been no research into the impacts of pesticides on spring queens and their ability to initiate a colony. Results from later stages of the colony cycle suggest that queens could be susceptible to pesticide exposure. For example, exposure of bumblebee colonies to pesticides can reduce founding queen longevity (Fauser-Misslin 2013; Scholer & Krischik 2014) and the overall reproductive output of the colony (Whitehorn *et al.* 2012; Fauser-Misslin 2013). There is also evidence from bumblebee workers that fecundity is reduced after pesticide exposure (Tasei, Lerin & Ripault 2000; Laycock *et al.* 2012; Elston, Thompson & Walters 2013). None of these studies explicitly examine pre-colony founding queens, but do suggest that queens are also vulnerable to pesticides, and that reproductive function may be at risk. It is therefore vital that we understand the potential impacts of pesticides on bumblebee queens, and how this is likely to further impact on populations in the field.

Thiamethoxam is a neonicotinoid insecticide which is currently under a two year moratorium for use on flowering, bee attractive crops in the EU, particularly in relation to its potential risk to bees (EFSA 2013). This compound was widely in use in the UK prior to the moratorium (e.g. 300,547 hectares oilseed crops were treated in 2012 (FERA 2012)), and research into its impacts on bees is currently of particular importance for conservation and policy. This study investigates the effects of thiamethoxam exposure on *B. terrestris* queen survival and colony initiation, in combination with two other environmental stressors: infection with the parasite *C. bombi*, and variation in hibernation duration.

3.3 Methods

3.3.1 Colonies

Fifteen *Bombus terrestris audax* colonies were obtained from Koppert Ltd (Haverhill, UK). Colonies were kept in the laboratory in darkness (red light was used for colony manipulation), at 22 °C. Colonies were fed *ad libitum* with 50 % Ambrosia (E H Thorne Ltd), an inverted sugar

syrup solution (from now on referred to as syrup), and frozen pollen pellets (Koppert Ltd, Haverhill, UK)). On arrival, 10 % of the workers from each colony were dissected and screened microscopically for the parasites *C. bombi* (Trypanosomatidae)*, Nosema bombi* (Microsporidia), and *Apicystis bombi* (Neogregarinida)*,* using a Nikon eclipse (50i) compound microscope at 400x magnification. No parasite infections were detected at this stage.

3.3.2 Overall experimental design

Mated queens were exposed to the parasite *C. bombi*, or a control (see below). Equal numbers of queens from both parasite and control groups were hibernated for either a 6 week period (short hibernation) or 12 week period (long hibernation). After hibernation, queens were sequentially allocated to a pesticide or control group (ensuring a fully crossed design). This resulted in a total of eight treatment groups (summarised in Table 3.1), in which multiple queens per natal colony were represented.

3.3.3 Mating

Males and gynes (reproductive females) were removed from colonies as callows (newly emerged bees), and kept communally in single sex wooden boxes (24 x 14 x 10.5 cm), with nest mates of the same age, and fed *ad libitum* pollen and syrup.

Four days after eclosion, gynes were mated with unrelated males of at least four days of age. Mating took place in a 60 x 50 x 50 cm wooden framed arena, with plastic mesh sides, under natural light, at a temperature of 22 ° C. Up to 25 males from a single colony were placed into the arena, and left to acclimatise for 10 minutes. Unrelated gynes from another single colony and age group, were then added to the arena. Mating pairs were removed from the arena immediately, and the time, date, male and female colony, and age were recorded. Once mating was complete, the male was removed, and frozen at -20 °C. The mated queen was kept in an individual plastic box (13 x 11 x 6.8 cm) containing a small amount of tissue paper to remove excess moisture, and immediately provided with 100 µl of inoculum (see below for inoculum preparation). When this full amount had been consumed, the queens were provided with *ad libitum* food (pollen and 50% syrup), for between 2 and 4 days after mating (depending on how quickly the inoculum was consumed), at which point they were weighed, and placed into hibernation (see below). Queens that did not consume the full amount of inoculum within 4 days were excluded from the experiment.

Gynes that did not mate on the first attempt were kept in their communal boxes as described above, and further mating attempts (up to 5 attempts per gyne) were made (with different groups of males), until mating took place. Males were also kept until mating had occurred, and mating attempts continued until males were 2 weeks of age, at which point they were frozen at -20 °C.

3.3.4 Preparation and delivery of C. bombi inoculum

Crithidia bombi was obtained from naturally infected wild *B. terrestris* queens, collected from Windsor Great Park, Surrey, UK (Latitude: 51.417432, Longitude: -0.60481256) during the spring of 2013. Queens were also screened for *N. bombi*, *S. bombi,* and *A. bombi;* any queens co-infected by these parasites were removed. *Crithidia* infected queens were kept in the laboratory in Perspex queen rearing boxes (13.3 x 8 x 5.6 cm) with *ad libitum* syrup and pollen, and kept in a dark room at a constant temperature of 28 °C and 50 % humidity (conditions suitable for colony initiation). Eleven naturally infected queens (and their colonies in 6 cases) were available at the start of the experiment, and 10 µl of faeces was collected from each of these, combined, and used to infect 20 stock worker bees collected from each of the experimental colonies. This ensured that a wide range of naturally occurring strains of *C. bombi* was available for the infection of experimental queens. All faeces collected were combined, and diluted with 0.9 % Ringer's solution to make 1 ml of solution. *Crithidia bombi* cells were filtered using a modified protocol for purification developed by Cole (1970) (see Chapter 2 for details). This process was repeated, using wild caught queens from the same population that were not infected with *C. bombi, A. bombi*, *N. bombi*, or *S. bombi* in order to provide a control.

The stock bees were taken from the experimental colonies in order to account for any filtering of the parasite strains by workers prior to infection of the experimental queens (Ulrich, Sadd & Schmid-Hempel 2011). Workers were removed from each colony and starved for a period of four hours. Each stock bee was then individually fed a 10 µl drop of inoculum (containing 10,000 *C. bombi* cells), and observed until all of the liquid had been consumed. These stock bees were then kept communally in wooden boxes with their nest-mates, and fed *ad libitum* pollen and 50 % syrup. The same process was repeated using faeces from the uninfected wild queens, to create a control stock.

To make the inoculum for the experimental queens, an equal amount of faeces (10 µl) was collected from each box of stock bees each day that inoculation took place. This was combined

and purified as described above. The resulting solution was diluted with syrup, and 100 µl of this inoculum (containing at least 20,000 *C. bombi* cells) was provided in a feeding tube for each queen. The same process was repeated using the *C. bombi* free faeces from the control stock bees.

3.3.5 Hibernation

Mated queens (only those which had consumed the full amount of inoculum), were weighed and placed into 50 ml tubes (Falcon) with damp sterilised sand, and kept in a dark incubator at a constant temperature of 4 °C for either a six week period, or a 12 week period. After this hibernation period, the queens were removed from the tubes and re-weighed. Surviving queens were then placed into Perspex queen rearing boxes (13.3 x 8 x 5.6 cm) with *ad libitum* syrup and pollen, and kept in a dark room at a constant temperature of 28 °C and 50 % humidity.

3.3.6 Pesticide exposure

Three days after emergence from hibernation, queens in the pesticide treatment group were provided with syrup containing 2.4 ppb thiamethoxam, which is the equivalent to that found in stored nectar in bumblebee colonies located 1 km from treated oilseed rape crops (Thompson *et al.* 2013). Bumblebee queens emerging from hibernation in April are likely to be foraging when oilseed rape crops are in flower, and are known to forage on flowering crops such as these (Chapter 4). Therefore nectar or pollen from treated crops may be collected by queens as they establish a colony in the spring. The concentration of thiamethoxam used in this experiment represents a field relevant dose if queens are foraging on treated crops, as well as other untreated forage plants.

Analytical standard thiamethoxam (Pestenal, Sigma Aldrich) was mixed with acetone (Fluka, Sigma Aldrich) to give a stock solution of 100 mg/ml. Aliquots of this stock were diluted with syrup, to give a final concentration of 2.4 ppb thiamethoxam. Acetone alone was diluted in the same way, to provide a control. Solution was freshly made each day of the experiment. Samples of treated syrup from two dates in the experiment were collected and analysed for thiamethoxam residues using liquid chromatography–mass spectrometry (LC-MS) (FERA, Sand Hutton, York). Average residues were found to be 2.5 µg/Kg (ppb) ± 0.085 SE.

Queens were provided with the pesticide treated syrup for 14 days, and the amount consumed by the queen during this time measured twice (once after 7 days at which point the feeder was

replenished with fresh treated syrup, and once after 14 days) using a 25 ml measuring cylinder to an accuracy of 0.25ml. Average evaporation rate was measured by keeping feeders (n=10) in empty rearing boxes for a week, and calculating volume lost during this time – syrup consumption data was then corrected for evaporation. *Ad libitum* untreated syrup was provided for the remainder of the experiment.

3.3.7 Post-hibernation monitoring

All queens were provided with a pollen ball (ground pollen pellets mixed with syrup to form a soft dough, shaped into a cylinder of approximately 1 cm in height and diameter), in which to lay their eggs and as a source of food. Unused pollen balls (which did not contain eggs or brood) were changed twice a week, in order to provide a source of fresh pollen for the queens. Pollen balls containing brood were left in the box, and an additional pollen ball or dish of loose pollen provided twice a week.

Queens were monitored daily for mortality and egg laying. All bees which died during the experiment were frozen at -20 °C on the day of death. The first date of egg laying (colony initiation) was recorded, as was the date that the first adult worker eclosed. Queens which had not initiated a colony 10 weeks after emergence from hibernation were frozen at -20 °C. Queens which had brood were kept for an additional 4 weeks, in order to monitor development of the brood into adult workers.

Each queen was checked for the presence of *C. bombi* (by microscopic examination of a fresh faecal sample) three times during the experiment. The first check occurred 4 days after the end of hibernation, the second 11 days after hibernation, and the third check 30 days after hibernation.

3.3.8 Dissection

All dissections were performed using a Nikon microscope (SM2800) at a magnification of x10 to x30. At the end of the experiment, all queens were dissected, and checked microscopically for the presence of *C. bombi* (as described for the parasite screening above). Queens were also screened for *N. bombi* and *A. bombi* in order to verify the earlier colony screening results. Neither of these parasites were found at this stage. Queens that had not laid eggs were also assessed for ovary development. The presence or absence of developing oocytes was noted, and the length of each terminal oocyte was measured using an ocular graticule (at x20 magnification), and the number of terminal oocytes recorded.

3.3.9 Analysis

Models were constructed for each analysis using some or all of the following factors: **Hibernation** (short or long), **Pesticide** (pesticide or control), **Parasite** (exposed to the parasite or not exposed), **Infection** (Infected or uninfected – this was assessed through the four parasite checks – if *C. bombi* was detected during any of these, the individual was considered to be infected). The following covariates were also considered: **Preweight** (pre-hibernation weight), **Postweight** (post-hibernation weight), **Weightloss** (proportion of weight lost during hibernation), and **Thorax** (thorax width). The natal colony of the queen, and of her mate (**QColony** and **MColony**) were considered as random factors in mixed models, and compared to equivalent models without random factors. In analysis of ovary development, egg laying and colony development, all queens that died during the experiment were excluded, as they had not been present during the entire 10 (or 14) week observation period. In the analysis of oocyte presence or absence, queens which had laid eggs were included as having developed oocytes. Details of each analysis are summarised in Table 3.2.

All analyses were performed in R (Version 3.1.1, R Core Team (2014)) and RStudio (Version 0.98.501 (2012)). The packages lme4 (Bates *et al.* 2014), ggplot2 (Wickham 2009), MuMin (Bartoń 2014), survival (Therneau 2014), doBy (Højsgaard & Halekoh 2014) and gridExtra (Auguie 2012) were used for data summarisation, analysis, and construction of graphs.

Model selection

In order to select the optimal model for each analysis, Akaike Information Criterion (AIC) values, which measure of how well the model fits the data, were used (corrected for small sample sizes using the AICc). These were compared for a set of candidate models. Firstly, mixed models with one or both of the random factors Qcolony and Mcolony were compared to equivalent models with no random factors (Zuur *et al.* 2009). This was used to decide on the random structure used in further model selection (one random factor, both random factors, or no random factors). Candidate models were then constructed (see Appendix 4) including biologically meaningful combinations of the fixed factors listed above. When AICc values for different models were within two units of the lowest, model averaging was undertaken (Johnson & Omland 2004) (except in cases where the null model was amongst these, in which case the null was assumed to be optimal). Final models were verified graphically for fit and to ensure all assumptions had been met (Zuur *et al.* 2009; Zuur, Hilbe & Ieno 2013). Interpretation of the importance of factors within the final models was based on the size of

the estimate (the larger the estimate, the greater the effect size of that factor), and 95 % confidence intervals (those which did not cross zero were considered reliable and important to the model).

3.4 Results

3.4.1 Summary of data

A total of 319 mated queens were placed into hibernation. Of these, 20 died during hibernation, and a further 68 were excluded from the final analysis. Exclusion was due to a lack of replication for their natal colony (as a result of nest-mates being lost (n=60)), accidental infection with *C. bombi* (n=6), and accidental death (through drowning (n=2)). The distribution of the remaining 231 queens (from eight colonies) across the eight treatment groups is shown in Table 3.1.

Table 3.1: Summary of sample sizes for the eight treatment groups used in the experiment. Hibernation, Pesticide and Parasite are the three treatments, Infection status indicates the number and percentage for each Parasite group that was successfully infected by the end of the experiment.

Hibernation	Pesticide	Parasite	n	Infection	n	% Infected
Long	Pesticide	Parasite	31	Infected	20	64
		Control	27			
	Control	Parasite	29	Infected	18	62
		Control	27			
Short	Pesticide	Parasite	30	Infected	23	76
		Control	30			
	Control	Parasite	28	Infected	22	78
		Control	29			

3.4.2 Hibernation survival

Pre-hibernation weight of queens was the most important factor in predicting hibernation survival (estimate = -7.195, 95 % CI [-12.159, -2.231]). Surviving queens were heavier than those that died (mean (g) ± SE = 0.77 ± 0.007 (survived), 0.654 ± 0.028 (died)).

3.4.3 Weight loss during hibernation

Both hibernation treatment (estimate = -5.379, 95 % CI [-6.700, -4.059]) and parasite treatment (estimate = 1.323, 95 % CI [0.006, 2.641]) had an effect on weight loss during

hibernation. Queens lost more of their body weight after a long hibernation compared to a short hibernation (mean % weight loss ± SE = 17.2 % ± 0.50 (long), 11.8 % ± 0.45)). Parasite exposure also caused an increase in weight loss, although this was a much smaller effect (mean % weight loss ± SE = 15.19 % ± 0.55 (parasite), 13.86 % ± 0.53 (control)).

3.4.4 Post-hibernation survival

Of the 231 queens included in the post-hibernation analysis, 85 % survived (n=197) until the end of the experiment (10 weeks post-hibernation). Survival was not explained by any of the factors included in the model selection process, and the optimal model for survival and survival time, was the null model (no fixed or random factors). Thus the pesticide, parasite and hibernation treatments used in the experiment did not have an impact on queen survival to 10 weeks.

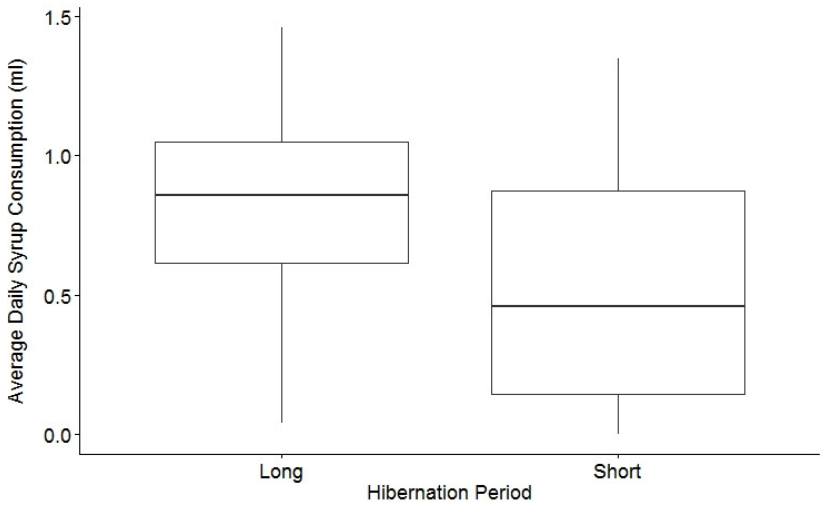


Figure 3.1: Average daily consumption of syrup containing thiamethoxam by *B. terrestris* queens during a two week pesticide exposure period. Boxplots show the median (central line), inter-quartile range (box), and range which lies within 1.5 times of the interquartile range from the box (whiskers).

3.4.5 Syrup consumption

Hibernation was an important factor in syrup consumption (estimate = -0.278, 95% CI [-0.373, -0.183]). Queens that had been hibernated for longer, consumed more syrup post hibernation (mean daily syrup consumption (ml) ± SE = 0.805 ± 0.031 (long), 0.527 ± 0.036 (short)) (Figure 3.1). The average daily amount of active ingredient consumed by pesticide treated queens was 1.685 ng. In the long hibernation group this was 1.977 ng per day, compared to 1.405 ng in the short hibernation group.

Due to correlations between weight loss during hibernation, and the hibernation and parasite treatments, this covariate was not included in the model above. Instead, a separate model was used, which showed that weight loss was positively correlated with syrup consumption (estimate = 0.018, 95 % CI [0.009, 0.027)]. As weight loss was higher during a long hibernation, this could be linked to the result found above.

3.4.6 Egg laying

Pesticide treatment (estimate = -0.628, 95 % CI [-1.240, -0.017]) and a short hibernation (estimate = -1.514, 95 % CI [-2.131, -0.898]) had a negative impact on the proportion of egg laying queens. At the end of the 10 week observation period, 38 % of queens in the pesticide treatment laid eggs compared to 52 % in the control treatment, whilst 28 % of queens had laid eggs after 10 weeks in the short hibernation group, compared to 61 % in the long group (Figure 3.3).

Pesticide and hibernation treatments were also important in the timing of egg laying. Pesticide treatment was found to violate the assumption of proportional hazards (i.e. that survival curves for the two categories must have hazard functions that are proportional over time) in initial models. In order to deal with this, episode splitting was used to estimate separate hazard ratios for different time intervals; during pesticide treatment (P1), and after treatment (P2) (Mills 2011). Both of these interaction terms were important in the final model (P1 estimate = 1.400, 95 % CI [0.275, 2.525], P2 estimate = -0.573, 95 % CI [-1.034, -0.112]), along with hibernation (estimate = -1.044, 95 % CI [-1.499, -0.590]). The estimates above show that the two interaction terms have opposite effects, which can be seen in Figure 3.2, whereby pesticide treated queens show increased egg laying early in the experiment, and decreased egg laying by the end of the experiment.

Syrup consumption was not included in models for egg laying, in order to avoid confounding effects due to its association with several of the treatment factors and covariates (see results above). However, egg laying queens consumed considerably more syrup that non-egg layers (average daily consumption (ml) ± SE = 0.934 ± 0.022 by egg layers, 0.432 ± 0.032 by non-egg layers).

3.4.7 Oocyte development

A short hibernation period resulted in a reduction in the proportion of queens with developing oocytes after 10 weeks (65 % of queens in the short hibernation group had developing

oocytes, compared to 87 % in the long hibernation group) (estimate= -1.354, 95 % CI [-2.084, -0.624]) (Figure 3.3). Thorax width was also important in the final model (estimate= -1.463, 95 % CI [-2.825, -0.102]), although the difference in thorax width between queens with oocyte development and those without was extremely small (7.894 mm ± 0.039 SE (no developing oocytes), 7.817 mm ± 0.022 SE (with developing oocytes)).

3.4.8 Development of colonies

Of the 197 queens which survived, 22 % (n = 44) successfully reared adult offspring, and the mean number of workers per queen by the end of the experiment was 11 ± 1.9 SE The average time between first laying eggs, and emergence of the first adult offspring was 32 days. When all queens in the experiment were considered, the null model was optimal for predicting the presence or absence of adult offspring. However, when only egg laying queens were considered, pesticide was an important factor (estimate = 1.214, 95 % CI [0.320, 2.107]), a higher proportion of egg laying queens in the pesticide treatment group had adult offspring by the end of the experiment (Figure 3.3).

The parameters and estimates for all of the final models used above are shown in Table 3.2. Candidate models and AICc values for each analysis can be found in Appendix 4.

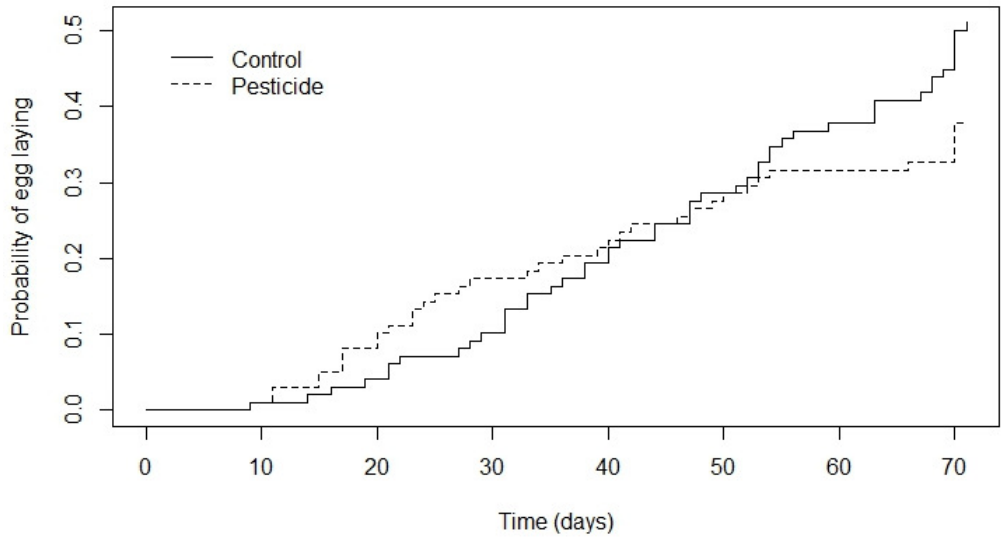


Figure 3.2: Event history curve for the time from the end of hibernation (time = 0 days) until the first egg was laid, by *B. terrestris* queens exposed to either the pesticide thiamethoxam, or a control.

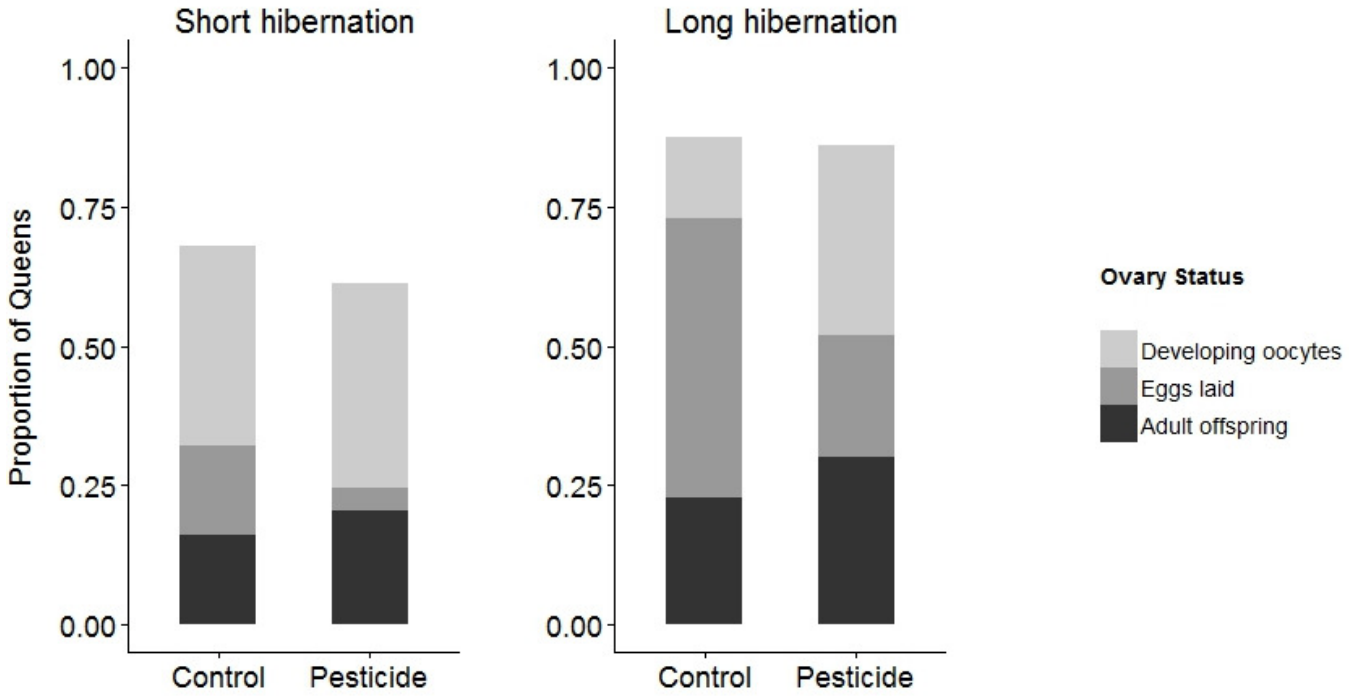


Figure 3.3: Ovary development, egg laying, and colony development of *B. terrestris* queens exposed to the pesticide thiamethoxam or a control, and to one of two hibernation periods (Short = 6 weeks, Long = 12 weeks). Pale grey bars are the proportion of queens which had developing oocytes present at week ten. Medium grey bars are the proportion of queens that had laid eggs by week 10, and dark grey bars are the proportion of queens with adult offspring by week 14.

3.5 Discussion

Exposure of *B. terrestris* queens to field relevant levels of thiamethoxam for two weeks resulted in a 26 % reduction in colony initiation by the end of the experiment. Pesticide exposure also caused egg laying to begin earlier. Whilst hibernation was also important in ovary development and egg laying, infection with *C. bombi* had a minimal impact in this study. No interactive effects of combinations of stressors were observed.

3.5.1 Colony initiation

Exposure to thiamethoxam resulted in a shift in the timing of colony initiation in queens, and ultimately a reduction of 26 % in the proportion of queens that had survived and laid eggs by the end of the experiment. Whilst pesticide impacts on reproduction have been found in bumblebee workers (Laycock *et al.* 2012; Elston, Thompson & Walters 2013; Laycock *et al.* 2013), and solitary bees (Sandrock *et al.* 2014) this is the first time this has been observed in bumblebee queens.

Interestingly, pesticide exposure caused an increase in the number of queens laying eggs early in the experiment (Figure 3.2). However, by day 40, colony initiation by pesticide and control treated queens had levelled off, and by the end of the experiment a higher proportion of control queens had laid eggs. There is evidence that individuals from various taxa respond to natural enemies by shifting reproduction effort earlier, for example the snail, *Biomphalaria glabrata,* increases oviposition soon after exposure to a trematode worm (Thornhill, Jones & Kusel 1986). *Daphnia* species lay larger clutches earlier when exposed to a microsporidian parasite (Chadwick & Little 2005), and mature at an earlier instar after exposure to predatory fish cues (Sakwińska 2002). This plasticity in life-history traits is thought to be an adaptive response to threats to survival or reproduction. Moret & Schmid-Hempel (2004) found that bumblebee colonies will shift reproduction earlier in response to immune challenge of workers, and also to harsh conditions (lower temperature). This resulted in an increase in the production of sexual offspring early on in these treatment groups, followed by a drop compared to controls later in the experiment. Although this effect was seen at the colony level rather than the individual level, it shows that social insects such as bumblebees also exhibit life-history changes in response to physiological stress. Whilst there is no evidence to date that similar processes may occur in response to pesticide exposure, it is known that pesticides can cause metabolic changes in honeybees, including the regulation of genes associated with immune function and detoxification (Boncristiani *et al.* 2012; Aufauvre *et al.* 2014). It is

possible therefore that the shift in timing of colony initiation observed in the current experiment is a response to physiological stress from pesticide exposure.

The mechanisms behind the overall reduction in egg laying by the pesticide treated queens are not clear. There was no effect of pesticide on ovary development 10 weeks after hibernation. It is possible that that any impairment to ovary development occurred earlier in the experiment, and that this was not detectable by week 10. Alternatively perhaps the binomial measurement used for the analysis of ovary development (presence or absence of developing oocytes) was not powerful enough to detect any pesticide induced differences. In bumblebee workers, impairment of ovary development occurred after two weeks of exposure to imidacloprid, but only at the relatively high dose of 125 ppb (Laycock *et al.* 2012). At doses lower than this, no impairment was observed. Further investigation into the impacts of pesticides on queen ovary development is needed, at a range of doses, and a range of time-points after hibernation.

Queens which underwent a 12 week hibernation period were more likely to lay eggs compared to queens in the 6 week hibernation group, something which has been observed in laboratory studies before (Beekman, van Stratum & Lingeman 1998; Brown, Schmid-Hempel & Schmid-Hempel 2003). In the current study, this is possibly related to an impairment in ovary development, as the shorter hibernation period resulted in a 25 % reduction in queens with developing oocytes present. The longer hibernation period therefore appears to be beneficial (at least in laboratory conditions) in the development of ovaries in *B. terrestris* queens. Wild *B. terrestris* queens in the UK are likely to need to hibernate for up to 6 – 9 months through the winter (Alford 1969), and so perhaps this reflects an adaptation allowing them to perform better under longer hibernation conditions.

3.5.2 Colony development

Queens from all treatment groups were able to rear their brood to adulthood. When all queens in the experiment were considered, there was no effect of any treatment on the emergence of adult offspring. However, when only egg laying queens were considered, a higher proportion of thiamethoxam treated queens had adult offspring by the end of the experiment. This is likely to be due to a higher proportion of treated queens laying eggs early in the experiment, giving them more time to rear adult offspring. It might therefore be expected that if the queens had been observed for longer, this trend would have been reversed, as for egg laying. These results suggest that whilst a pulse of pesticide exposure soon

after hibernation can prevent colony initiation in some queens, it does not prevent those that are able to lay eggs from developing their colony further. A two week exposure period was used in this experiment, to represent the period of foraging immediately after hibernation and before colony initiation is likely to begin, and therefore specifically targeting the queens rather than developing brood. However, wild foraging queens could be exposed to thiamethoxam and other pesticides for longer periods, and potentially well into the development of the colony. It would be useful to explore this further, as the impacts on colony initiation observed in this study may be greater still if queens were exposed for longer periods.

3.5.3 Survival

None of the treatments or covariates measured in this study had an impact on the survival of queens after hibernation, although queens which were heavier before hibernation were more likely to survive to the end of hibernation, as has been found in a number of previous studies (Holm 1972; Beekman, van Stratum & Lingeman 1998).

Neonicotinoids have been found to have an impact on queen survival in several previous studies (Fauser-Misslin *et al.* 2014, Scholer & Krischik 2014). However, in these cases, this effect was seen at much higher doses (20 ppb or higher in Scholer & Krischik 2014), or much later in the life cycle (Fauser-Misslin *et al.* (2014) found an interactive effect of thiamethoxam and *C. bombi* 15 weeks into their experiment, when colonies were well developed). The difference in survival between previous studies and the current results could therefore be age related. Queens in the current study were monitored for 10 weeks, as this is the period when queens would be expected to initiate a colony in the wild. However, it is much shorter than the natural lifespan of a bumblebee queen, and potential impacts of the treatments on longevity may not have been detected. Queen survival after pesticide exposure also appears to be dose dependent. Scholer & Krischik (2014) tested a range of doses of both thiamethoxam and clothianidin on *B. impatiens* colonies for 11 weeks. Effects on queen survival in this case were seen at doses of 20 ppb and higher after 6 weeks, whilst doses of less than 20 ppb had no impact on queen survival. The dose of thiamethoxam used in the current study represents a field relevant level for bumblebees foraging in a mosaic of treated crops and untreated plants in the UK (Thompson *et al.* 2013). My results therefore show that exposure to thiamethoxam at a dose queens are likely to encounter in the field, has no impact on queen mortality during the colony founding period. It is important to note however, that this result was found under optimal laboratory conditions, and in field conditions, other stressors (such as low food availability), may change the outcome.

3.5.4 Syrup consumption

There was no evidence for a reduction in feeding associated with thiamethoxam treatment in this study. Similarly, Laycock *et al.* (2013) found that bumblebee workers exposed to doses below 39 ppb did not show any change in feeding behaviour. However, Elston, Waters & Thompson (2013) found that worker consumption of syrup dosed with 1 and 10 ppb thiamethoxam was reduced compared to controls. This difference could be due to thiamethoxam exposure through both syrup and pollen (Elston, Thompson & Walters 2013) compared to syrup only (as in the current study and in Laycock *et al.* (2013)). Furthermore, queens are likely to have greater nutritional needs than workers, particularly after hibernation when their energy stores are depleted, which may override any inhibitory or repellent effect of pesticides in the food they encounter. In order to standardise the exposure of queens, there was no option of untreated nectar in the pesticide treated group in this experiment, so whether queens have a preference for pesticide free nectar, given a choice, is unknown. However, given the evidence that neonicotinoids are likely to be ubiquitous in agricultural environments, with residues found in soil, waterways, and non-target plants (Krupke *et al.* 2012; Goulson 2013; Bonmatin *et al.* 2014; Stewart *et al.* 2014), it is unlikely that wild queens foraging in these conditions will be able to avoid neonicotinoids in their diet.

Syrup consumption was influenced by both weight loss, and hibernation treatment. It is likely that the increased weight loss observed during the longer hibernation resulted in a greater need to replenish energy stores after hibernation. By consuming more syrup during this stage, queens in the long hibernation group were also consuming more pesticide. It might therefore be expected that this would result in a greater impact of the pesticide in queens hibernated for longer. There was a greater difference in the proportion of pesticide and control queens that initiated a colony in the long hibernation group, compared to the short group (Figure 3.3), which may be indicative of an interaction between these two treatments. However, this was not supported statistically, perhaps due to a lack of power in the analysis.

Pollen is an important source of nutrients, such as protein, which are vital for larval development, growth, and ovary development in bees (Haydak 1970; Duchateau & Velthuis 1989). As such, it is an extremely important resource for bumblebee queens as they prepare to lay eggs, and then feed their developing brood. Pollen consumption was not measured in this study, in order to avoid disruption of eggs and brood in pollen balls. However, this would be an interesting area to research further, particularly in the context of pesticide exposure. Worker bumblebees exposed to imidacloprid have a reduced ability to collect pollen in the field (Gill,

Ramos-Rodriguez & Raine 2012; Feltham, Park & Goulson 2014). If pesticide exposure effects bumblebee queens similarly, this could have serious consequences for colony initiation and development.

3.5.5 Impacts of C. bombi

Exposure to *C. bombi* resulted in greater weight loss during hibernation, as was the case in Brown, Schmid-Hempel & Schmid-Hempel (2003). However, the impacts on the other traits measured was minimal, and the final models in our analysis suggest that infection was much less important than the other treatments in predicting colony initiation. Brown, Schmid-Hempel & Schmid-Hempel (2003) found an impact of *C. bombi* on colony founding and development, with fewer infected queens initiating a colony, and those which did producing fewer workers, males, and gynes. Yourth, Brown & Schmid-Hempel (2008) also found that *C. bombi* had an impact on fitness of *B terrestris* queens in the laboratory, and Shykoff & Schmid-Hempel (1991c) found a reduction in ovary development in infected workers. Brown, Schmid-Hempel & Schmid-Hempel (2003), and Yourth, Brown & Schmid-Hempel (2008), both used considerably larger sample sizes than was possible in the current experiment (given the number of treatment groups). As such, perhaps these studies simply had more power to detect differences between infected and uninfected queens. The studies above also used queens reared from wild populations, and infected with parasites from these same populations, whilst the colonies used to source queens and males in the current study were obtained from a commercial bumblebee breeding centre. It is known that interactions between the genotype of *C. bombi* and its bumblebee host can result in variation in parasite virulence and transmission (Shykoff & Schmid-Hempel 1991a; Imhoof & Schmid-Hempel 1998). As such, perhaps the reduced impact of *C. bombi* in this study compared to previous studies is down to genetic differences between wild and commercially reared queens.

3.5.6 Conclusions

These results provide evidence that chronic exposure to thiamethoxam reduces colony initiation by bumblebee queens. This is the first time that the effects of pesticides on this vulnerable stage of the bumblebee lifecycle have been tested. Further research is needed to explore the long-term impacts of the observed reduction in egg laying on colony success, and population dynamics in the field. These results indicate the importance of considering all aspects of the bumblebee life cycle when assessing the risk of pesticides to bees.

Table 3.2: (Table shown on next two pages) Summary of models used in analysis of data on the impact of various factors and covariates on *B. terrestris* queens in the laboratory. Table includes details of models used in a model selection process (Appendix 4), and the specific R packages used, as well as the parameters and estimates from the final or composite models.

⁺ In Cox Regression models, random factors were included as frailty terms (Mills 2011), and model selection was undertaken as described for mixed models.

* Timing of egg laying was analysed using a cox regression with proportional hazards. Examination of the residuals showed that the pesticide factor did not meet the assumption of proportional hazards. To deal with this, the interaction between pesticide and time was considered, and separate hazard functions were calculated for the period during pesticide exposure (P1: the first 17 days), and for the period after exposure (P2: 17 days - end) (Mills 2011). These two interaction terms were included instead of pesticide in the model selection process.

† For analyses during hibernation, the fixed factor parasite indicates the exposure of queens to the parasite or a control (as infection status was unknown at this stage). All other analyses including parasite used data on infection status (whether the queen was successfully infected or not).

◊ Analysis included egg layers only.

Table 3.2 (Part 1)

	Model types	Data structure	Link function	Fixed Factors	Random Factors	R packages used	Final / Composite model			95% CI	
							Parameter	Estimate	SE	Lower	Upper
Weight lost during hibernation	lm, lme	Gaussian		Hibernation, Parasite†, Thorax	Qcolony, Mcolony	nlme	Intercept	17.895	3.284	11.491	24.298
							Hibernation	**-5.379**	**0.677**	**-6.700**	**-4.059**
							Parasite	**1.323**	**0.676**	**0.006**	**2.641**
							Thorax	-0.658	1.204	-3.006	1.691
Hibernation survival	GLM, GLMM	Binomial	Logit	Hibernation, Parasite†, Thorax, PreWeight	Qcolony, Mcolony	lme4	Intercept	2.355	1.597	-0.759	5.470
							Preweight	**-7.195**	**2.546**	**-12.159**	**-2.231**
							Parasite	0.447	0.497	-0.522	1.415
							Hibernation	0.778	2.786	-4.655	6.211
							Preweight*Hib.	-6.945	5.606	-17.877	3.987
Post-hibernation survival ⁺	Cox Regression	Event history		Hibernation, Pesticide, Parasite, Thorax	Qcolony, Mcolony	lme4	Null model				
Syrup consumption	lm, lme	Gaussian		Hibernation, Pesticide, Parasite	Qcolony, Mcolony	nlme	Intercept	0.802	0.037	0.729	0.874
							Hibernation	**-0.278**	**0.049**	**-0.373**	**-0.183**
							Pesticide	0.024	0.488	-0.927	0.974
				Thorax, Weightloss	Qcolony, Mcolony	nlme	Intercept	0.494	0.292	-0.075	1.063
							Weightloss	**0.018**	**0.004**	**0.009**	**0.027**
							Thorax	-0.042	0.089	-0.216	0.132

Table 3.2 (Part 2)

	Model types	Data structure	Link function	Fixed Factors	Random Factors	R packages used	Final / Composite model			95% CI	
							Parameter	Estimate	SE	Lower	Upper
Presence of egg laying	GLM, GLMM	Binomial	Logit	Hibernation, Pesticide, Parasite, Thorax	Qcolony, Mcolony	lme4	Intercept	5.969	5.200	-4.171	16.110
							Hibernation	**-1.514**	**0.316**	**-2.131**	**-0.898**
							Pesticide	**-0.628**	**0.313**	**-1.240**	**-0.017**
							Thorax	-0.990	0.582	-2.124	0.144
Timing of egg laying + *	Cox Regression	Event history		Hibernation, P1, P2, Parasite, Thorax	Qcolony, Mcolony	survival	**Hibernation**	**-1.044**	**0.233**	**-1.499**	**-0.590**
							P1	**1.400**	**0.577**	**0.275**	**2.525**
							P2	**-0.573**	**0.236**	**-1.034**	**-0.112**
							Parasite	0.096	0.228	-0.349	0.540
							Thorax	-0.553	0.378	-1.291	0.185
Oocyte development	GLM, GLMM	Binomial	Logit	Hibernation, Pesticide, Parasite, Thorax	Qcolony, Mcolony	lme4	Intercept	11.674	6.377	-0.762	24.109
							Hibernation	**-1.354**	**0.374**	**-2.084**	**-0.624**
							Thorax	**-1.463**	**0.698**	**-2.825**	**-0.102**
							Parasite	0.366	0.356	-0.329	1.061
Presence of adult offspring ◊	GLM, GLMM	Binomial	Logit	Hibernation, Pesticide, Parasite, Thorax	Qcolony, Mcolony	lme4	Intercept	-0.750	0.360	-1.453	-0.048
							Pesticide	**1.214**	**0.458**	**0.320**	**2.107**
							Hibernation	0.963	0.495	-0.003	1.928

4 Impacts of thiamethoxam on ovary development and feeding in queens of four wild bumblebee species.

4.1 Abstract

Pesticides are a potential threat to wild bees, and whilst there is a growing body of research into the impacts of pesticide exposure on a few model bumblebee species, little is known about the wider impacts on the majority of other wild species. Additionally, most pesticide research on bumblebees focuses on the worker caste or colony as a whole, whilst founding queens, which are vital to colony success, are often neglected. Given the likelihood of exposure of a range of bumblebee species to pesticides, it is important that we understand the potential risks, and potential differences among species and castes, and account for these in management practices and policy. This chapter investigates the impacts of thiamethoxam, a neonicotinoid insecticide, on four wild bumblebee species which differ in their life-history, biology and foraging behaviour. Spring caught queens of *Bombus terrestris*, *B. lucorum*, *B. pratorum* and *B. pascuorum* were exposed to field relevant doses (1 ppb or 4 ppb) of thiamethoxam or a control for two weeks in the laboratory, and survival, colony initiation and ovary development were assessed. Exposure to the higher dose of pesticide caused a reduction in feeding in some species, suggesting a species-specific repellency or anti-feedant effect. The higher dose of thiamethoxam also resulted in a reduction in the average length of terminal oocytes across all species. These results provide further evidence for impacts of thiamethoxam on reproduction in bumblebee queens. Furthermore, species-level differences in pesticide sensitivity were detected, which has important implications for assessing the risk of pesticides to pollinators.

4.2 Introduction

Pollination by wild insects is important for a vast array of crop systems and wild plants (Corbet, Williams & Osborne 1991; Klein *et al.* 2007; Garibaldi *et al.* 2013). Pollinator diversity is often critical for seed and fruit set (Kremen, Williams & Thorp 2002; Klein, Steffan-Dewenter & Tscharntke 2003; Hoehn *et al.* 2008) and maintaining diversity is therefore extremely important for agriculture and conservation. Bumblebees are pollinators of many crops (Corbet, Williams & Osborne 1991), and have traits which make them particularly efficient as pollinators, for example tolerance to cold temperatures (Corbet *et al.* 1993), and the ability to

forage for longer periods of the day than other pollinator species (Willmer, Bataw & Hughes 1994). Whilst bumblebees clearly benefit our agricultural systems, they in turn may benefit from the presence of mass flowering crops such as oilseed rape as a foraging resource (Westphal, Steffan-Dewenter & Tscharntke 2003; Knight *et al.* 2009). However, this also brings them into contact with anthropogenic threats, such as pesticides. The growing body of research into pesticide impacts on bumblebees generally focuses on *B. terrestris* as a model species within Europe. The ease of rearing this species in lab conditions, and wide availability through commercial rearing facilities make it a useful test organism. However, it is one of 24 extant species of bumblebee in the UK, and 68 in Europe (Williams & Jepsen 2014). These species have much variation in life-history traits, foraging behaviour and phenology, which may cause differences in their exposure and sensitivity to pesticides. Given these differences, extrapolating the effects of pesticides from one species to another is not always appropriate (Thompson & Hunt 1999; Cresswell *et al.* 2012; Arena & Sgolastra 2014), and testing effects of pesticides on a range of wild bumblebee species would be advisable.

Several species of bumblebee are known to forage on oilseed rape flowers (Cresswell & Osborne 2004; Stanley, Gunning & Stout 2013), as well as other flowering crops such as field beans (Corbet, Williams & Osborne 1991), which are often treated with an assortment of pesticides throughout the growth of the plant (Garthwaite *et al.* 2012a). Few studies have tested the toxicity of pesticides to bumblebee species other than *B. terrestris* (or *B. impatiens* in North America), but those which have, found differential sensitivity (in terms of the LD_{50}) between species, caste within a species, and between pesticides (Stevenson & Racey 1966; Drescher & Geusen-Pfister 1991; Wu *et al.* 2010). This variation in mortality in response to pesticide exposure may suggest that sub-lethal impacts could also vary between species, although this has not been tested. This study aimed to address this issue by testing sub-lethal impacts of the neonicotinoid thiamethoxam on four common bumblebee species, all of which are known to forage in agricultural areas. The four species selected; *B. terrestris*, *B. lucorum*, *B. pratorum* and *B. pascuorum* are all commonly found in agricultural areas, but differ in life-history and biological traits such as morphology, phenology, and behaviour (Appendix 5).

Queen reproduction is vital to bumblebee colony success. Having undergone an energetically demanding hibernation through the winter (Alford 1969), colony founding queens are then faced with additional pressure from environmental factors such as parasites (Schmid-Hempel 1998; Brown, Schmid-Hempel & Schmid-Hempel 2003; Rutrecht & Brown 2008a; Yourth, Brown & Schmid-Hempel 2008; Jones & Brown 2014) and pesticides (Chapter 3), which may

affect their ability to establish a colony. Results from the previous chapter of this thesis show that *B. terrestris* queens exposed to the neonicotinoid thiamethoxam are less likely to initiate a colony in the laboratory. This chapter builds on these results, and explores the potential impacts of thiamethoxam on multiple species of wild caught bumblebee queens. Queens of the four focal species (Table 4.2) were caught in the early spring, and exposed to a control or one of two field relevant doses of thiamethoxam. Impacts on feeding, survival, egg laying (colony initiation) and ovary development were monitored.

4.3 Methods

4.3.1 Field survey

In order to establish whether the target species of this experiment were likely to be foraging on treated crops in the spring, a field survey was undertaken. Two visits were made to two winter oilseed rape (*Brassica napus*) fields (variety PR46W21), at Shiplake Farm, Oxfordshire, UK (Latitude: 51.504696, Longitude: -0.90030080) during early April 2014 when the crop was in flower. Crop seeds had been treated with Modesto seed treatment (clothianidin and β-cyfluthrin, Bayer CropScience, Cambridge, UK), and planted the previous year. Transects around the edge of the fields (distance around each field = 2 km and 0.94 km) and through the centre of the crop (0.3 km and 0.4 km) were walked between 11am and 3pm on days when weather conditions were suitable (sunny and dry with minimal wind). Transects were walked once per visit, at a steady pace (total walking time per visit = 3 hours), and all bumblebee species within 2 metres of the transect were recorded, along with the caste and activity of each bee. Queens of the *B. lucorum* complex (*B. lucorum*, *B. cryptarum* and *B. magnus*) cannot be reliably separated using morphological features alone (Carolan *et al.* 2012), and so these were recorded as *B. lucorum agg.*

4.3.2 Lab trial

For the experimental component of this study, queens of four bumblebee species, *B. terrestris*, *B. lucorum*, *B. pratorum* and *B. pascuorum,* were collected between March and April 2014, from Windsor Great Park, Surrey, UK (Latitude: 51.417432, Longitude: -0.60481256). In total, 506 queens were collected (see Table 4.2 for breakdown of each species). As discussed above, species of the *B. lucorum* complex were treated as a group for the purpose of this study. These species were selected for the study as they are known to forage in agricultural habitats (Cresswell & Osborne 2004; Hanley *et al.* 2011; Stanley, Gunning & Stout 2013). Additionally, they are all abundant at the collection site, allowing sufficient samples to be collected with

minimal impacts on the local population. Individuals of each species were collected as early in the season as possible, and within a short time frame (Table 4.2). This minimised the time between emergence and capture, and to some extent standardised the previous experience of individuals. Furthermore, queens which were storing pollen in the corbiculae were not collected as they were likely to have already established a nesting site. Several pesticides are used at the collection site: triticonazole (a fungicide) and acetamiprid (a neonicotinoid) are used as a treatment for roses (Roseclear Ultra formulation). These are applied between June and September, which means that whilst queens collected would not have been exposed in the spring, they may have had exposure the previous summer when emerging from their natal colonies. Windsor Park is surrounded by agricultural and urban areas, where queens may also have come into contact with pesticides used in gardens or crops. As such it was not possible to control for the prior pesticide exposure of queens collected, but as queens were randomly allocated to treatment groups (see below), it was assumed that any individuals with previous exposure would be randomly distributed.

Queen faecal samples were screened microscopically for the parasites *Crithidia bombi* (Trypanosomatidae)*, Nosema bombi* (Microsporidia), and *Apicystis bombi* (Neogregarinida)*,* and for larvae of the nematode *Sphaerularia bombi,* using a Nikon eclipse (50i) compound microscope at a magnification of x400 (see Table 4.2 for prevalence of parasites in the queens collected). Only *C. bombi* was detected at this stage (n = 81), and infected queens were excluded from the experiment. Queens were then established in Perspex queen rearing boxes (13.3 x 8 x 5.6 cm), kept in a dark room at a constant temperature of 28 °C and 50 % humidity, and provided with *ad libitum* 50 % Ambrosia, an inverted sugar syrup solution (E H Thorne Ltd, from now on referred to as syrup), and pollen pellets (Koppert Ltd, Haverhill, UK).

4.3.3 Pesticide exposure

Queens were randomly allocated to one of three treatment groups, control, 1ppb thiamethoxam (low dose), and 4 ppb thiamethoxam (high dose). These doses represent the range of residues found in nectar stores in bumblebee colonies which were foraging in agricultural areas between April-June (Thompson *et al.* 2013). Analytical standard thiamethoxam (Pestenal, Sigma Aldrich), was mixed with acetone (Fluka, Sigma Aldrich) to give a stock solution of 100 mg/ml. Aliquots of this stock were diluted with syrup to give the final concentrations. The volume of acetone used in the high dose was diluted in the same way, to provide a control. Samples of treated syrup from two dates in the experiment were collected and analysed for thiamethoxam residues using liquid chromatography-mass spectrometry (LC-

MS) (Food and Environment Research Agency, Sand Hutton, York). The average residues were 1.87 ppb ± 0.065 SE (Low dose), and 5.32 ppb ± 0.579 SE (High dose). Control samples were also tested, and found to contain trace amounts of thiamethoxam (0.063 ppb ± 0.018 SE). Queens were provided with the pesticide treated syrup for 14 days. Oilseed rape can flower from early April in the UK, and flowering can last for 3-6 weeks (Delaplane, Mayer & Mayer 2000). Queens establishing a nest in the spring would need to forage for at least up to four weeks (until first adult workers emerge). If oilseed rape is considered a potential food source for queens (and could be one of several likely to contain pesticide residues), a two week exposure period represents a moderate exposure time. The weight of treated syrup consumed was measured to an accuracy of 0.1 g (once after 7 days, at which point freshly treated syrup was provided, and again after 14 days). Average daily consumption during this period was then calculated. The average evaporation rate was measured by keeping ten feeders in empty rearing boxes for a week, and calculating the weight of syrup lost during this time, syrup consumption data was then corrected for evaporation. *Ad libitum* untreated syrup was provided for the remainder of the experiment.

4.3.4 Monitoring

Following the pesticide exposure period, queens were observed for a further two weeks (four weeks in total), and checked daily for mortality, signs of waxing behaviour (wax is produced by queens as part of their natural nesting behaviour (Alford 1975)), and egg laying. A four week observation period was used in this experiment in order to assess both any immediate impacts of pesticide exposure on queens, and ovary development soon after exposure. Queens which died during the experiment were frozen at -20 °C. After four weeks, all remaining queens and brood were frozen.

4.3.5 Dissection

At the end of the four week experiment, all queens were dissected using a Nikon (SM2800) dissecting microscope at a magnification of x10 to x30. The abdomen contents were checked for internal mites (*Locustacaris buchneri)*, and adult and larval nematodes (*S. bombi*). A Nikon eclipse (50i) compound microscope at x400 magnification was used to screen samples from the hindgut, malpighian tubules, and fat body for the parasites *C. bombi*, *N. bombi*, and *A. bombi.* Queens which were found to have at least one of these parasites at this stage (n=235, Table 4.2 shows distribution between species and treatments), were excluded from the main analysis. The presence or absence of developing oocytes was also noted, and the length of each terminal oocyte was measured using an ocular graticule (at x20 magnification). The

thorax width was measured using digital calipers. The pesticide treatment group of each individual was concealed during the dissection process, in order to avoid operator bias.

4.3.6 Analysis

Models were constructed to test the impact of pesticide treatment on syrup consumption during pesticide treatment, survival to the end of the four week experiment, initiation of waxing, initiation of egg laying, and average oocyte length. For each analysis a model selection process was undertaken using the AICc value (the Akaike Information Criteria corrected for small sample sizes) to evaluate the best fitting model (Johnson & Omland 2004). Fixed factors included **Treatment** (control, low or high), **Species**, and size (which was adjusted for species differences by calculating the Z score for each individual (**SizeZ**= (individual size – mean size for that species)/ standard deviation for each species)). Models including individual fixed factors and combinations of these were compared against the null model (Appendix 6 contains all candidate models for each analysis). Where more than one model was considered a good fit (within two AICc units of the optimal model), model averaging was undertaken (Johnson & Omland 2004). Final models were verified graphically for fit and to ensure all assumptions had been met (Zuur *et al.* 2009; Zuur, Hilbe & Ieno 2013). Interpretation of the importance of factors within the final models was based on the 95 % confidence intervals (those which did not cross zero were considered reliable and important to the model) and on the size of the estimate (the larger the estimate, the greater the effect size of that factor). Where treatment effects were found, a post-hoc Tukey's test was used to compare treatment groups.

Linear models were used to analyse data on the average daily syrup consumption. In order to detect any species level differences which were not purely size related, the average daily syrup consumption was corrected to control for the size of the bee (syrup consumption / (thorax width)^3), giving a measure of consumption per unit volume of bee (g/mm^3). Model selection was undertaken as described above.

Survival was analysed both in terms of survival to the end of the experiment (28 days), using a binomial generalised linear model (GLM) with a log link, and also in terms of the timing of death using a Cox Regression.

The presence or absence of waxing behaviour and egg laying within the four week experiment were also analysed using binomial GLMs. A Cox Regression was used for the timing of egg laying. Only data for queens which had survived the whole experiment were used.

The average terminal oocyte length (corrected for species by using the Z score as described above) was analysed using a linear model. Again, only data for queens which had survived the whole experiment were used.

All analyses were performed in R (Version 3.1.1, R Core Team (2014)) and RStudio (Version 0.98.501 (2012)). The packages, ggplot2 (Wickham 2009), MuMin (Bartoń 2014), survival (Therneau 2014), doBy (Højsgaard & Halekoh 2014), multcomp (Hothorn, Bretz & Westfall 2008), and gridExtra (Auguie 2012) were used for data summarisation, analysis, and construction of graphs.

4.4 Results

4.4.1 Field survey

Seven species of queen were found in the vicinity of the oilseed rape crop during the two surveys, and of these six were observed foraging on oilseed rape flowers (Table 4.1). *B. terrestris* and *B. lapidarius* were the most commonly observed species foraging directly on the crop. Seventeen observations were made of queens foraging on other plants (predominantly *Lamium album*) within one metre of the crop, and 41 queens were observed nest searching within 1 metre of the crop.

Table 4.1: Species of bumblebee queen observed foraging in and around oilseed rape (OSR) fields during two visits in April 2014. Sample sizes are summed across the two fields. *Other flowers were *Lamium album, Glechoma hederacea, Veronica chamaedrys*.

Species	N observed foraging on OSR		N observed foraging on other flowers*		N observed nest searching	
	4.4.14	10.4.14	4.4.14	10.4.14	4.4.14	10.4.14
Bombus terrestris	12	8		2	8	3
Bombus lapidarius	28	22	1	7	9	14
Bombus lucorum agg.	1	1			2	1
Bombus hortorum	5	3	1	2		2
Bombus pratorum			1			
Bombus hypnorum	1			1		2
Bombus pascuorum	2		1	1		

Table 4.2: Summary of collection, treatment allocation, size, and infection status for the four focal species of queens used in this study. *Totals Include queens which had multiple parasites.

Species	Collection dates	Treatment	N	Average thorax width (mm) ± SE	Number of infected queens*					% Infected	N Uninfected
					C. bombi	*N. bombi*	*A. bombi*	*S. bombi*	*L. buchneri*		
B. lucorum	24 March - 11 April 2014	Control	41	7.351 ± 0.028	12		11	8		71	12
		Low	39	7.36 ± 0.034	14		7	17			5
		High	41	7.386 ± 0.035	11		6	15			10
B. pascuorum	9 April - 17th April 2014	Control	41	6.27 ± 0.043	6	3	12	2		53	17
		Low	41	6.33 ± 0.056	8		10	3			15
		High	41	6.297 ± 0.038	7	1	12	3	1		16
B. pratorum	4 March - 31 March 2014	Control	38	6.25 ± 0.035	4		1	1	7	39	22
		Low	39	6.254 ± 0.036	6		6	1	10		15
		High	39	6.29 ± 0.028	2	2	2	4	6		19
B. terrestris	11 March - 12 March 2014	Control	50	8.101 ± 0.034	8		1	5		27	35
		Low	48	8.042 ± 0.041	7		2	5			32
		High	48	8.094 ± 0.033	10		1	4			32
TOTAL			**506**		**95**	**6**	**71**	**68**	**24**		**230**

4.4.2 Lab trial

A total of 506 queens were collected, 12 of which escaped during the course of the experiment and were excluded. A further 235 were found during dissection to be infected with at least one of the following parasites; *C. bombi, A. bombi, N. bombi, S. bombi* or *L. buchneri* and were therefore not included in further analyses. Twenty nine queens had possible signs of infection, but molecular confirmation would be needed to verify this, and so these were also excluded from the analyses. The distribution across treatment groups of these infected queens, and the remaining 230 which were included in the analyses, is shown in Table 4.2.

4.4.3 Syrup consumption

The high dose of pesticide treatment had a negative impact on syrup consumption by *B. pascuorum* (estimate = -0.00114, 95% CI [-0.00219, -0.0000973]) and *B. pratorum* (estimate = -0.001300, 95% CI [-0.00229, -0.00030]) queens (Figure 4.1). The interaction between high dose and these species was important in the final model, but the treatment alone, and interactions with *B. terrestris* or *B. lucorum* were less important (Table 4.4). Despite the reduction in feeding by the queens in the high dose group, the consumption of the active ingredient was still higher on average compared to the low and control groups (Appendix 7).

There were species level differences in sucrose feeding, with *B. pratorum* consuming more syrup per mm^3 of body volume, compared to other species (estimate = 0.00159, 95% CI [0.00083, 0.00235]) (Figure 4.1).

4.4.4 Survival

Across all species, 88 % of queens (n = 203) survived for the four week observation period. Pesticide treatment was not important in the overall survival rate of queens, or in the time of death.

Size (corrected for species using the z-score) was an important factor in the binomial survival model (estimate = -0.655, 95% CI [-1.131, -0.178) (Table 4.5); queens which died during the experiment were slightly larger than average (Figure 4.2). The actual difference in size was fairly low (0.19mm for *B. lucorum,* 0.135mm for *B. pratorum,* 0.181mm for *B. terrestris)*, and *B. pascuorum* queens showed the opposite trend, (surviving queens were on average 0.079mm larger than those which died).

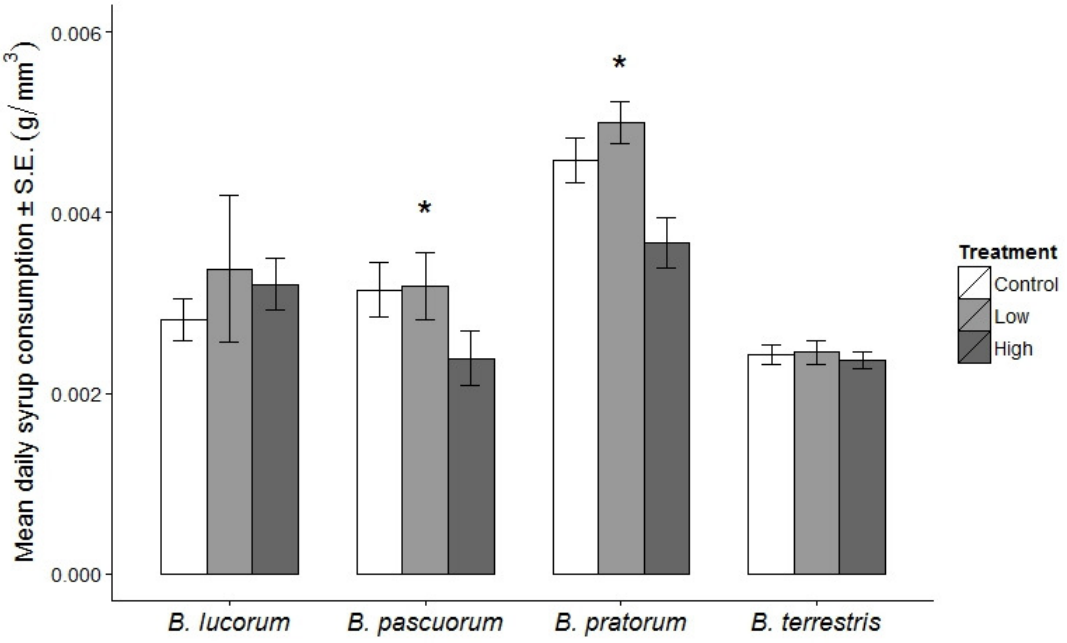


Figure 4.1: The average daily amount of syrup consumed by four species of bumblebee queen, treated with one of three doses of thiamethoxam (Control = no pesticide, Low = 1ppb, High = 4ppb). Bars show mean consumption (g) per unit volume of bee (mm^3). Error bars show the standard error. * indicates an important interaction between species and the high dose treatment (Table 4.4).

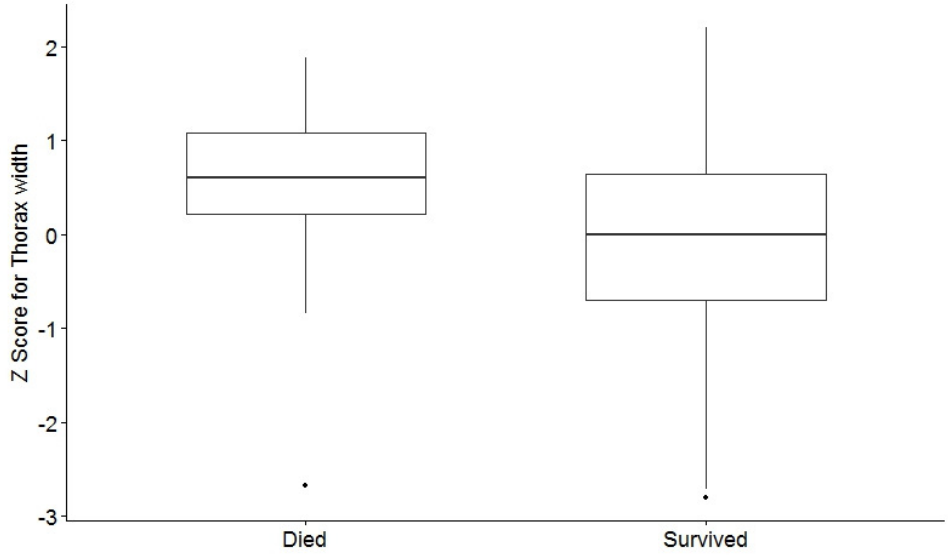


Figure 4.2: The relative size (Z score for thorax width) of bumblebee queens which died or survived during the four week experimental period. Boxplots show the median (central line), interquartile range (box), range which lies within 1.5 times of the interquartile range from the box (whiskers), and outliers (dots).

4.4.5 Waxing

Fifty three percent of queens exhibited waxing behaviour during the experiment. There were species level differences in the presence or absence of waxing (Table 4.5), but no treatment effects.

4.4.6 Egg Laying

There were differences in egg laying among species. More *B. terrestris* queens initiated a colony within 4 weeks than other species, and *B. pratorum* had the lowest colony initiation rate (Table 4.5). Treatment was not included in the optimal models for egg laying, or the timing of egg laying.

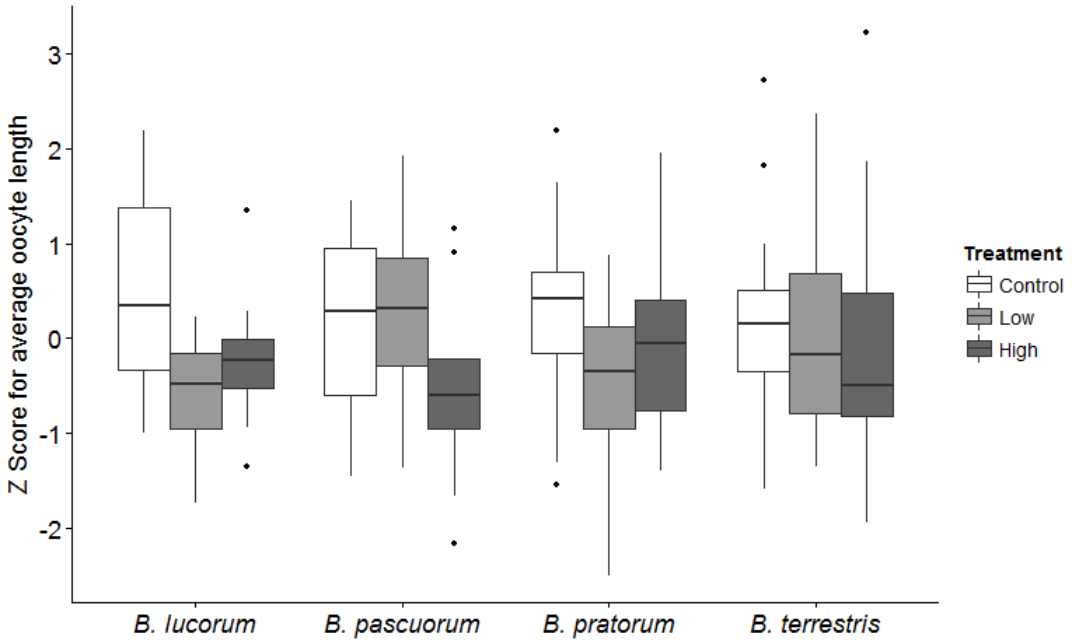


Figure 4.3: The relative oocyte length (Z score for mean oocyte length) of four species of bumblebee queen after exposure to three doses of thiamethoxam. Boxplots show the median (central line), interquartile range (box), range which lies within 1.5 times of the interquartile range from the box (whiskers), and outliers (dots).

4.4.7 Ovary development

The average length of terminal oocytes was smaller in the high treatment group compared to the control group, and this was the case across all four species (Figure 4.3). As the high pesticide treatment also caused a reduction in feeding, further analysis was undertaken to explore the influence of any effect this may have had on oocyte length. This involved further

model selection including models with a treatment by syrup interaction term. In this case, treatment was still an important factor (estimate = -1.2518, 95%CI [-2.2882, -0.2155]), and queens in the high dose had significantly smaller oocytes compared to both control and low groups ($p < 0.05$). The interaction term was also included in the final model, as was size, although these factors were less important (Table 4.6).

4.5 Discussion

Wild bumblebee queens are likely to be exposed to pesticides whilst foraging or nesting in agricultural areas. This study provides evidence, for the first time, that field relevant doses of thiamethoxam can have sub-lethal impacts on feeding and ovary development of queen bumblebees of multiple wild species. Furthermore, species level differences in response to pesticide exposure were observed; *B. pratorum* and *B. pascuorum* consumed less pesticide treated syrup compared to controls, whilst no pesticide-induced reduction in feeding was observed for *B. lucorum* and *B. terrestris*.

4.5.1 Reduction in feeding

The treatment of 4 ppb thiamethoxam in syrup resulted in a reduction in feeding by *B. pratorum* and *B. pascuorum* queens. No difference in feeding was found for *B. terrestris* and *B. lucorum*, suggesting that species may differ in their sensitivity to this compound. Previous species comparisons between honeybee and bumblebee workers (Cresswell *et al.* 2012), and between a bumblebee species and solitary bees (Scott-Dupree, Conroy & Harris 2009) have found differences in sensitivity to imidacloprid. The current results provide evidence that there are also differences among bumblebee species in response to sub-lethal doses of neonicotinoids. The mechanism behind the reduced feeding observed here could be related to a number of factors. Several pesticides are known to have a repellent effect on bees (Belzunces, Tchamitchian & Brunet 2012), which can result in a reduction in feeding. Cresswell *et al.* (2012) found reduced feeding on imidacloprid treated syrup by *B. terrestris* workers, and suggest that this was more likely to be due to toxicity rather than repellency, as the effect increased over time and with increasing dose. Toxicity may cause bees to learn to avoid a substance which has an adverse effect (Wright *et al.* 2010), or may disrupt the physiological, behavioural, or muscular processes involved in feeding (Belzunces, Tchamitchian & Brunet 2012). Further testing is needed to elucidate the mechanisms controlling the change in feeding observed in the current study.

4.5.2 Impacts on ovary development

The high dose of thiamethoxam treatment also caused a reduction in the length of terminal oocytes of queens. This was true across all species, and average oocyte length was reduced in queens from the high treatment group by 8.1 % (*B. lucorum),* 13.8 % (*B. pascuorum*), 5.9 % (*B. pratorum*), and 4.6 % (*B. terrestris*), when compared with controls (Table 4.3). In the previous chapter of this thesis, I found a reduction in colony initiation in thiamethoxam treated queens, but no detectable impact on the presence or absence of developing oocytes. The difference between the latter results and the current study could be due to the time frame of ovary observation. Here, ovaries were examined two weeks after pesticide treatment ended, compared to eight weeks in the previous chapter. If ovaries impaired by pesticide exposure can recover given enough time, changes in ovary development would only be observed soon after pesticide exposure. Several studies have found that bumblebees exposed to a pulse of dietary pesticide can recover after the pesticide is removed, both in terms of individual behaviour, and colony level brood production (Laycock & Cresswell 2013; Cresswell *et al.* 2014). However, results from the previous chapter show that colony initiation was affected at least up to 8 weeks after pesticide exposure ended, indicating that the pesticide was having long term impacts on queen reproduction. It is possible that the binomial assessment (presence or absence of oocytes) of ovary development used in the previous chapter was not sensitive enough to detect differences between treatment groups.

The inclusion of syrup consumption in the optimal model for oocyte length, may indicate an interaction between syrup consumption and dose (although the confidence intervals suggest that this interaction term was less important than other factors in the model (Table 4.6)). Given that the high dose of pesticide caused a decrease in syrup consumption in some species, the resulting reduction in nutrient intake could be responsible to some extent for the impact on ovary development. However, despite the interaction term being controlled for, the high dose of pesticide was still an important factor in the model. Furthermore, species which did not show reduced syrup feeding in response to the pesticide (*B. terrestris* and *B. lucorum*), still had a detectable reduction in oocyte length in the high treatment group compared to controls (Figure 4.5). These results suggest that whilst a reduction in syrup feeding caused by repellency or toxicity of the pesticide may partly explain the reduction in oocyte length, there are clearly also other mechanisms at play. Perhaps pollen consumption would help to explain this, as pollen contains essential nutrients for ovary development and brood production (Duchateau & Velthuis 1989). A reduction in untreated pollen consumption was observed in Laycock *et al.* (2012) by workers exposed to imidacloprid contaminated syrup. Pollen consumption was not

measured in the current experiment, due to the waxing behaviour of queens which made accurate measurement impractical. However, this would be an informative direction for further study.

4.5.3 Waxing and Egg laying

No impacts of pesticide treatment on either waxing behaviour or egg laying were found during this four week experiment. Interestingly in Chapter 3, there was a detectable difference between pesticide and control treated queens at four weeks, with a higher colony initiation rate in pesticide treated queens compared to controls (Figure 3.2). The difference could be related to sample sizes, as the current study had up to 35 queens of each species per treatment, compared to up to 118 in the previous chapter. Thus, perhaps the current study lacked the power to detect differences in this particular life history trait. In general the egg laying rate was higher in the current study compared to Chapter 3, with 23% of *B. terrestris* queens laying eggs by four weeks, compared to only 13% (at this time point) in the previous chapter. This is perhaps due to the use of wild caught queens in the current chapter, which would have already been feeding and developing their ovaries between emergence and capture. Also, weaker queens which may have been able to survive the relatively benign laboratory conditions during and post-hibernation in the previous chapter, could have been filtered out by more severe conditions in the field.

In Chapter 3, I found an overall reduction in egg laying by the end of the experiment in thiamethoxam treated queens. Whether the reduced size of oocytes observed in this experiment would have led to a long term reduction in egg laying, and ultimately in fitness, is unknown. Due to the invasive nature of measuring oocyte development, it is not possible to do this whilst keeping the queens alive, thus allowing monitoring of future success. However, a previous study on worker bumblebees sampled some individuals from the colony, whilst leaving other workers in the nest for future observation (Shykoff & Schmid-Hempel 1991c). Here, individuals from *C. bombi* infected colonies had reduced oocyte length compared to those from uninfected colonies, and this was correlated with a delay in worker egg laying. Given the long-term impacts of thiamethoxam (at a lower dose of 2.4ppb) on reproduction observed in the previous chapter, it is possible that further impacts would have been observed in this study, given a longer observation period.

4.5.4 Survival

No impact of thiamethoxam on survival was detected in the current study. As found in the previous chapter, survival rates of queens were high. As previously discussed (Chapter 3, Discussion), other studies on the impacts of thiamethoxam on bumblebee queens have found reduced survival, but at a much later stage in the colony cycle, or at a higher pesticide dose (Fauser-Misslin *et al.* 2014; Scholer & Krischik 2014). The current results support my previous findings that in the short term, exposure to a field relevant dose of this neonicotinoid does not reduce survival in queens.

Interestingly, there was a correlation between mortality and relative size of the queen. Queens which died during the experiment were on average larger than those which survived (Figure 4.2), although the absolute size differences were low (a maximum of 2.6% reduction). It might be expected that larger queens would be more likely to survive, and this is the case during hibernation (Chapter 3) (Holm 1972; Beekman, van Stratum & Lingeman 1998). The current result is therefore unexpected, and the cause unknown. Perhaps wild caught queens which are larger, experience more stress during the capture and rearing process, resulting in earlier death. Given the low sample sizes for queens which died (Table 4.3), further data is probably needed in order to elucidate this trend.

4.5.5 Design Limitations

This experiment used wild caught queens which had experienced natural hibernation conditions. Whilst this provided a more realistic model for assessing pesticide impacts on wild queens, it was not possible to standardise the past experiences of each individual, and so previous pesticide exposure and length of hibernation were unknown. However, queens were randomly allocated to each treatment group, and exposure to the pesticide in the lab was controlled to ensure these factors were randomly spread across treatments.

Whilst queens with a detectable parasite infection were excluded from the main analysis, it is possible that other infections were present and not detected. The incidence of parasitism in queens caught for this experiment was nearly 50% across all species, and was particularly high for *B. lucorum* (71%), which had fairly low sample sizes for parts of the analysis as a result. These levels of parasitism are generally comparable to previous data on bumblebee queens from England, Ireland, and Switzerland (Rutrecht & Brown 2008a; Jones 2014), although the prevalence of *A. bombi* in *B. pascuorum* and *B. lucorum*, and *S. bombi* in *B. lucorum* was unusually high. Queens with a detectable parasite infection were excluded from analysis in this

study due to low levels of replication for each parasite within each species. It would be interesting to investigate the pesticide impacts on naturally parasitized queens, as negative interactions between parasites and pesticides have been observed in laboratory studies (Fauser-Misslin *et al.* 2014). Furthermore, it would be useful to molecularly screen queens for the presence of other infections which are not visually detectable, such as viruses.

No effects on any of the traits measured were detected after exposure to the low dose of thiamethoxam used in this experiment (1.87 ppb ± 0.065 SE). This indicates that the impacts on feeding and oocyte development observed were dose dependent, with the lower dose being less toxic. This should be treated with caution though, as trace residues of thiamethoxam found in the control group (0.063 ppb ± 0.018 SE) may have masked impacts of the lower dose compared to the controls. The trace residues found in the control solution could be due to human error during the preparation of the solution, or could be due to trace amounts being present in other elements of the experimental setup. Testing of the Ambrosia syrup and water which are routinely used in the laboratory is needed in order to clarify this. Acetone was used as a solvent in stock solutions of thiamethoxam in this study. Solvent effects were controlled for by including acetone in the control solution at the same level as that found in the high dose solution. Due to sample size limitation, it was not possible to include a low dose acetone control as well. Acetone has been widely used as a solvent in similar experiments (Gill, Ramos-Rodriguez & Raine 2012; Elston, Thompson & Walters 2013; Doublet *et al.* 2014), and has been found to have no effect on bumblebee micro-colony feeding or colony initiation at a dose similar to that used in the current experiment (Elston, Thompson & Walters 2013). Whilst it is therefore unlikely that acetone was having any effect on queens in the current study, it is not possible to rule this out.

4.5.6 Field relevance

The results of the experimental section of this study show that sub-lethal doses of thiamethoxam have impacts on several species of bumblebee queen. These experiments, although carried out on wild caught queens, were conducted under controlled laboratory conditions. In order to assess the likelihood of exposure to such pesticides in the field, it is necessary to look at the field survey results. Three of the species used in this experiment were observed foraging directly on oil seed rape crops, and as such could be directly exposed to pesticides. *B. pratorum* was not observed foraging directly on the oilseed rape flowers, but was seen on *L. album* flowers within 1 metre of the crop. Several studies have found neonicotinoid residues, within the dose range used in this experiment, in wildflowers growing near to treated

crops (Krupke *et al.* 2012; Stewart *et al.* 2014). These compounds can accumulate in soil (Jones, Harrington & Turnbull 2014) and could potentially be taken up by non-target plants, as well as being present in areas where queens establish nests. It is therefore possible that all four of the species used in this study are exposed to neonicotinoids whilst foraging in the wild. The most abundant species found in the field survey was *B. lapidarius*. This species was not selected for the laboratory trial due to low abundance at the collection site (personal observation), but it would be interesting and pertinent to consider the impacts on this species as well.

It is difficult, given the current deficiency of data, to estimate the range of doses and length of exposure to pesticides that bumblebee queens will encounter in the wild. Residues of up to 3 ppb thiamethoxam have been found in nectar collected from winter oilseed rape in France (Pilling *et al.* 2013). Nectar stored in bumblebee colonies placed more than 1km from treated crops was found to contain up to 3.8 ppb (Thompson *et al.* 2013), and was likely to have originated from a mixture of treated and untreated plants. Furthermore, residues of up to 10.3 ppb have been found in nectar of spring oilseed rape (Pohorecka *et al.* 2012). These studies focus on periods when bumblebee colonies are already established, and workers are doing the majority of foraging. Neonicotinoid residues can vary in plant tissue over time as the plant develops (Huseth *et al.* 2014), which could result in different exposure levels for queens and workers which forage at different times of the year.

The doses used in the current experiment are generally comparable to the field residues reported, although the high dose of 5.32 ppb (± 0.579 SE) is slightly higher than reported values. However, considering that exposure in the field is likely to occur via both nectar and pollen (EFSA 2013), the doses used in the current study are likely to be comparable given that only the nectar was treated in this case. Furthermore, a two week exposure is relatively short compared to the flowering time of oilseed rape crops, which can flower for more than a month (Delaplane, Mayer & Mayer 2000). Each of the species used in the current experiment is likely to have a different exposure profile in the wild as a result of differences in foraging preferences, phenology and life history traits (Appendix 5). For example, species with early emerging queens such as *B. pratorum* and *B. terrestris* may only be exposed to pesticides in flowering crops at the end of their foraging career, when nests have already been established. On the other hand, later queens such as *B. pascuorum* emerge when crops such as oilseed rape are in full flower, and so may have a higher likelihood of exposure if foraging in agricultural environments. Some species (particularly of the subgenus Pyrobombus; e.g. *B.*

pratorum, B. jonellus) may be bivoltine, producing two generations per year (Alford 1975). In this case, queens may establish new colonies much later in the season, and experience a completely different pesticide exposure profile to queens establishing nests in the early spring. These issues should be taken into account during pesticide risk assessments, and if necessary, alternative forms of crop protection should be used at times when bumblebees and other wild species are most vulnerable.

4.5.7 Conclusions

This study provides evidence, for the first time, that exposure to field realistic doses of thiamethoxam has an impact on feeding and ovary development in multiple species of wild caught bumblebee queen. Bumblebee queens are rarely considered in pesticide safety testing, and yet these results indicate that queens are not only sensitive to pesticides, but also likely to encounter them in the wild. Furthermore, differential sensitivity between species highlights the importance of considering the impacts of pesticides on a range of wild bee species. More information is needed on residues and persistence of pesticides in crops, wild plants, and in wild bee nests in order to accurately assess the exposure risks for the full range of species and castes of bees which are likely to encounter them. This is essential for understanding and managing the threat to wild bees from agrochemicals, and preventing further declines.

Table 4.3: Summary of main results of impacts of three different doses of thiamethoxam (Treatment) on life-history traits of four species of bumblebee queen. As infected queens were excluded from analyses, the total number of uninfected queens represents the individuals used for analyses. The average daily syrup consumption was calculated for uninfected queens only. The average terminal oocyte length was calculated for uninfected queens which survived for the full four week experiment.

Species	Treatment	Total Collected	Average thorax width (mm) ± SE	Total Uninfected	Average daily syrup consumption (g)	N Died	N Waxing	N Egg laying	N with Oocytes	Average Oocyte length (mm) ± SE
B. lucorum	Control	41	7.351 ± 0.028	**12**	1.135 ± 0.080	3	3	3	9	3.103 ± 0.128
	Low	39	7.360 ± 0.034	**5**	1.328 ± 0.333	1	3	2	4	2.715 ± 0.140
	High	41	7.386 ± 0.035	**10**	1.187 ± 0.089	1	7	1	10	2.849 ± 0.077
B. pascuorum	Control	41	6.270 ± 0.043	**17**	0.767 ± 0.075	3	7	3	13	1.850 ± 0.103
	Low	41	6.330 ± 0.056	**15**	0.825 ± 0.111	1	10	2	14	1.884 ± 0.094
	High	41	6.297 ± 0.038	**16**	0.594 ± 0.076	1	6	1	12	1.594 ± 0.093
B. pratorum	Control	38	6.250 ± 0.035	**22**	1.107 ± 0.056	3	9	1	19	2.055 ± 0.074
	Low	39	6.254 ± 0.036	**15**	1.188 ± 0.053	1	10	0	14	1.785 ± 0.082
	High	39	6.290 ± 0.028	**19**	0.909 ± 0.064	3	6	2	16	1.933 ± 0.089
B. terrestris	Control	50	8.101 ± 0.034	**35**	1.291 ± 0.060	2	11	7	32	2.915 ± 0.063
	Low	48	8.042 ± 0.041	**32**	1.280 ± 0.058	5	8	7	27	2.848 ± 0.083
	High	48	8.094 ± 0.033	**32**	1.251 ± 0.047	3	8	6	27	2.780 ± 0.092

Table 4.4: Summary of the linear model for average daily syrup consumption by four species of bumblebee queen. Syrup consumption was measured during a two week period when queens were exposed to one of two doses (Low or High) of thiamethoxam or a control. Consumption was adjusted to control for size of the individual by dividing the amount consumed (g) by volume of the thorax (mm^3). Fixed factors are the factors which were included in the model selection process. Final model details are from the composite model after model selection using the AICc (see Appendix 6 for candidate models). Factors highlighted in bold are those which were most important to the model, based on the size of the estimate and confidence intervals.

	Model	Fixed factors	Final model	Estimate	Standard error	95% CI	
						lower	upper
Syrup consumption (g/mm^3)	lm	Treatment, Species	(Intercept)	2.94E-03	3.10E-04	2.34E-03	3.55E-03
			Treatment (Low)	4.28E-04	4.55E-04	-4.59E-04	1.31E-03
			Treatment (High)	1.31E-04	4.92E-04	-8.27E-04	1.09E-03
			Species (pasc)	1.34E-04	4.21E-04	-6.88E-04	9.56E-04
			Species (prat)	**1.59E-03**	**3.91E-04**	**8.29E-04**	**2.35E-03**
			Species (terr)	-5.03E-04	3.30E-04	-1.15E-03	1.41E-04
			Tment (Low) * Species (pasc)	-5.12E-04	6.31E-04	-1.74E-03	7.17E-04
			Tment (High) * Species (pasc)	**-1.14E-03**	**5.37E-04**	**-2.19E-03**	**-9.73E-05**
			Tment (Low) * Species (prat)	-1.36E-04	6.17E-04	-1.34E-03	1.07E-03
			Tment (High) * Species (prat)	**-1.30E-03**	**5.12E-04**	**-2.30E-03**	**-3.01E-04**
			Tment (Low) * Species (terr)	-5.32E-04	5.75E-04	-1.65E-03	5.90E-04
			Tment (High) * Species (terr)	-4.51E-04	4.76E-04	-1.38E-03	4.77E-04

Table 4.5: Summary of models used for survival, waxing behaviour and egg laying in queens of four species of bumblebee. All analyses were performed across all species, on uninfected queens. Model indicates the type of model used (GLM is a generalised linear model). Fixed factors are the factors included in a model selection process (SizeZ is the z score for thorax width as described in the Methods). Final model details are from the optimal or composite model after model selection using the AICc (see Appendix 6 for candidate models). Factors highlighted in bold are those which were most important to the model, based on the size of the estimate and confidence intervals.

	Model	Fixed factors	Final model	Estimate	Standard error	95% CI	
						lower	upper
Survival	GLM	Treatment, Species, SizeZ	*Intercept*	*2.17E+00*	*2.33E-01*	*1.71E+00*	*2.62E+00*
			SizeZ	**-6.55E-01**	**2.45E-01**	**-1.13E+00**	**-1.78E-01**
	Cox regression	Treatment, Species, SizeZ	Null model				
Waxing	GLM	Treatment, Species, SizeZ	*Intercept*	*1.28E+00*	*5.05E-01*	*2.96E-01*	*2.27E+00*
			Species (pasc)	-1.14E+00	5.91E-01	-2.29E+00	1.11E-02
			Species (prat)	-9.93E-01	5.82E-01	-2.13E+00	1.42E-01
			Species (terr)	**-1.51E+00**	**5.49E-01**	**-2.58E+00**	**-4.38E-01**
Egg laying	GLM	Treatment, Species, SizeZ	*(Intercept)*	*-1.03E+00*	*4.76E-01*	*-1.96E+00*	*-1.06E-01*
			Species (pasc)	-7.90E-01	6.49E-01	-2.05E+00	4.75E-01
			Species (prat)	**-1.70E+00**	**7.63E-01**	**-3.18E+00**	**-2.09E-01**
			Species (terr)	-1.88E-01	5.39E-01	-1.24E+00	8.64E-01
			Size	1.72E-01	2.00E-01	-2.19E-01	5.62E-01
	Cox regression	Treatment, Species, SizeZ	Results as for GLM				

Table 4.6: Summary of linear models used for the average terminal oocyte length of queens of four species of bumblebee (data was standardised for each species by using the Z score of Oocyte length as descibed in the Methods). Analyses include data from all four species, and include only queens which were uninfected and survived the full four week experiment. Fixed factors are factors which were included in a model selection process using the AICc value (Syrup is the raw average daily syrup consumption data, SizeZ is the z score for thorax width as described in the Methods). Final model details are from the optimal or composite model after model selection using the AICc (see Appendix 6 for candidate models). Factors highlighted in bold are those which were most important to the model, based on the size of the estimate and confidence intervals.

	Model	Fixed factors	Final model	Estimate	Standard error	95% CI	
						lower	upper
Average Oocyte length	lm	Treatment, Species, SizeZ	(Intercept)	2.56E-01	1.14E-01	3.40E-02	4.78E-01
			Treatment (Low)	-3.20E-01	1.71E-01	-6.54E-01	1.32E-02
			Treatment (High)	**-4.56E-01**	**1.64E-01**	**-7.76E-01**	**-1.36E-01**
			SizeZ	1.32E-01	7.15E-02	-7.18E-03	2.72E-01
	lm	Treatment, Syrup, Species, SizeZ	(Intercept)	8.19E-02	3.94E-01	-6.87E-01	8.51E-01
			Treatment (Low)	4.01E-02	5.71E-01	-1.07E+00	1.15E+00
			Treatment (High)	**-1.25E+00**	**5.31E-01**	**-2.29E+00**	**-2.16E-01**
			Syrup	1.50E-01	3.27E-01	-4.87E-01	7.88E-01
			SizeZ	1.35E-01	6.97E-02	-8.73E-04	2.71E-01
			Tment (Low)*Syrup	-8.27E-02	4.76E-01	-1.01E+00	8.46E-01
			Tment (High)*Syrup	7.57E-01	4.54E-01	-1.29E-01	1.64E+00

5 Responses of honeybee gut microbiota to a range of widely used pesticides.

5.1 Abstract

Honeybees are commercially reared for pollination and honey production on a global scale. Apiculture in Europe and the USA has suffered from severe colony losses, which are often attributed to disease. Other stressors such as pesticides have also been implicated, as colonies are frequently exposed to pesticides both as in-hive treatments for disease agents, and in pollen and nectar during foraging. Whilst the direct impacts of pesticides on honeybees have been widely studied, little is known about the effects on the symbiotic bacteria that colonise the honeybee guts. One such group are the lactic acid bacteria (LAB), which are known to benefit bees by inhibiting pathogens. It is therefore important that we understand how LAB and other bee microbiota interact with chemicals in the environment, and this may provide valuable insights into the mechanisms and processes involved when bees are exposed to pesticides. In this study, I investigated the direct impacts of four widely used pesticides (including a neonicotinoid, a pyrethroid, an in-hive acaricide, and a fungicide), on the growth of honeybee LAB in vitro. All 13 LAB phylotypes tested were able to grow in the presence of each pesticide, and a combination of all four pesticides. In some cases, pesticides inhibited the growth of LAB, and in other cases growth was promoted, overall, effects varied among LAB phylotypes, and no consistent patterns were detected. This study provides a first insight into interactions between honeybee LAB and pesticides, something which could have critical implications for honeybee health. Methodological considerations are discussed in order to aid future studies into this important system.

5.2 Introduction

Humans and animals host a diverse microbiota, which is known to influence many aspects of life, including digestion, immune function, and development (Fraune & Bosch 2010). Social bees are known to host a range of symbiotic bacteria in the gut, which develop in newly eclosed callow bees when they are exposed to these bacteria within their natal colony (Gilliam 1997; Olofsson & Vásquez 2008; Koch & Schmid-Hempel 2011a; Martinson *et al.* 2011; Moran *et al.* 2012). These microbial communities can provide their hosts with a range of benefits,

such as protection from parasites and diseases (Koch & Schmid-Hempel 2011b; Vásquez *et al.* 2012). Recent work on the honey stomach (an enlargement of the oesophagus) of honeybees has resulted in the discovery of a novel lactic acid bacteria (LAB) community (Olofsson & Vásquez 2008), which is found in *Apis mellifera* across a large geographical range (Vásquez, Olofsson & Sammataro 2009). Whilst the composition of the honeybee LAB community varies over time depending on the flowers visited by the bees (Olofsson & Vásquez 2008), it appears to be relatively robust to the changes in nutrient levels experienced in the honey stomach (caused by nectar and water being consumed and deposited by the bees), and this could be related to the formation of biofilms (Vásquez *et al.* 2012). LAB have been detected in all extant *Apis* species, as well as several related *Meliponini* species (Vásquez *et al.* 2012), and most recently in *Bombus terrestris* (Killer *et al.* 2014). As well as lactic acid, LAB produce a range of antibacterial compounds such as hydrogen peroxide, and bacteriocins (Klaenhammer 1988), which are beneficial to human health (Ouwehand, Salminen & Isolauri 2002). Recent evidence suggests that LAB also provide health benefits to bees, by inhibiting the growth of pathogenic bacteria such as *Paenibacillis larvae* (Forsgren *et al.* 2010), and *Melissococcus plutonius* (Vásquez *et al.* 2012).

Honeybees have historically been managed both for pollination services and for the production of honey, although declines in commercial apiaries have occurred in recent years in Europe and the USA (Aizen & Harder 2009; Potts *et al.* 2010b; vanEngelsdorp & Meixner 2010). Parasites and disease are often cited as playing a key role in declines and the collapse of colonies (Higes *et al.* 2008; Genersch 2010; vanEngelsdorp & Meixner 2010). Many in-hive products are used in apiculture in order to control disease agents, for example acaricides to control *Varroa destructor* mites (Karazafiris *et al.* 2008), and antibiotics to control bacterial infections (Mutinelli 1996). However, these compounds could also compromise symbiotic microbiota such as LAB, if ingested by honeybees. Recent evidence has found that honeybee LAB are sensitive to two antibiotics (oxytetracycline and tylosin) widely used in the apiculture industry (Vásquez *et al.* 2012). As well as exposure to pesticides in the hive, honeybees are exposed to a range of agrochemicals whilst foraging in agricultural areas (Chauzat *et al.* 2006; Mullin *et al.* 2010). Direct impacts of pesticide exposure on honeybee behaviour (Decourtye, Lacassie & Pham-Delegue 2003; Aliouane *et al.* 2009), navigation (Henry *et al.* 2012) and health (Di Prisco *et al.* 2013; Pettis *et al.* 2013) have been observed, but little is known about impacts on their gut flora. Pesticides are known to have an impact on other communities of microbiota, for example both inhibiting and in some cases promoting populations of bacteria in soil (Omar & Abdel-Sater 2001; Chu *et al.* 2008). It might therefore be expected that similar

effects could be seen with bee microbiota. Given the potential benefits of LAB to honeybee health, it is important that we understand the impacts of chemicals used for apiculture and agriculture, which honeybees, and therefore their gut microbiota, may be exposed to.

This study investigates how four commonly used pesticides (including one in-hive acaricide) impact honeybee LAB growth. Thirteen phylotypes (taxonomically similar groups) of LAB were directly exposed to the pesticides in vitro, and the impacts of each pesticide alone, and a combination of all pesticides were assessed.

5.3 Methods

Thirteen LAB phylotypes described in Olofsson & Vásquez (2008), and Vásquez *et al*. (2012) were used in this study: Fhon2, Fhon13 (*Lactobacillus kunkeei*), Hma3, Bin7 and Bin2 (*Bifidobacterium asteroides*), Bma6 (*Bifidobacterium coryneforme*), Hon2, Hma2, Biut2, Bma5, Hma8, Hma11 and Bin4 (other *Lactobacillus* phylotypes). Phylotypes were cultured individually in pollen broth (honeybee collected pollen mixed with water, pH 6.2) at a temperature of 35 °C for three days. A mixture of all phylotypes in equal volumes was also cultured in this way. Each phylotype was mixed with fresh pollen broth on the day of testing at a ratio of 1 part bacteria culture to 3 parts broth, providing fresh nutrients for growth.

Four pesticides were tested, including two insecticides (thiamethoxam and λ-cyhalothrin), one fungicide (boscalid), and one acaricide (coumaphos), as well as a mixture of all four. These compounds were selected as they have all been detected in nectar or pollen of flowering crops, or in bee pollen and nectar stores (Chauzat *et al.* 2006; Mullin *et al.* 2010). As such, they represent a range of pesticides which honeybees may ingest under natural conditions. Three doses of each pesticide were chosen based on the literature, and these represent a low, field realistic dose, slightly higher 'worst case scenario' dose, and a high dose (to detect any responses not found at field realistic doses). The mixed pesticide treatment used each of the four pesticides at the medium dose. Full details of the pesticides and the range of doses used can be found in Table 5.1.

Pesticides were obtained as analytical standards (PESTENAL) (Sigma-Aldrich, Schnelldorf, Germany). Stock solutions of pesticide in solvent were made using acetone (25 mg active ingredient (a.i.) / ml for coumaphos, 10 mg a.i. / ml for boscalid and λ-cyhalothrin) or water (1 mg a.i. /ml for thiamethoxam). Each pesticide was tested in a separate trial, and in each case

stock solutions were diluted with water, and added to the broth and bacteria culture to obtain the final dose range (Table 1). To detect any effects of the solvent, an equivalent volume of acetone as used in the highest treatment dose for each pesticide (acetone control), and water alone (blank control) were also included as treatments.

A novel method was used for assessing the honeybee LAB growth. Aliquots of the bacteria culture and pesticide mixtures (200 µl) were plated into 96 well microplates, with different phylotypes of bacteria on separate rows, and 6 replicates of each treatment (control, acetone-control, low, medium and high) distributed across several plates. Absorbance of the bacteria cultures was read using a Plate CHAMELEON V multilabel microplate reader (Hidex, Turku, Finland), using the software MicroWin 2000 (MICROTEL, Louisianna, USA). The plate was shaken for ten seconds at the start of processing, and then for two seconds between each reading. Two readings were taken per well, and these were averaged to give the final absorbance value. Readings were taken immediately after the bacteria cultures were mixed with the pesticide (baseline reading), and then at 12, 36 and 58 hours after the start.

The change in absorbance (compared to the baseline reading) was calculated and used as a measure of growth. The maximum growth throughout the trial was determined (highest difference between the baseline and subsequent readings), and these data were analysed separately for each pesticide phylotype combination, using two-way ANOVAs. Edge effects were observed in the data, whereby cultures in wells along the edges of each plate had lower growth compared to wells in the centre of the plate, regardless of treatment. In order to control for this, the first and last reading from each row was excluded from analysis. Treatment was included as a fixed factor, and in order to control for differences in growth between plates, plate number was also included as a fixed factor. Not all data conformed to the assumptions of normality or equal variance. However, as the F statistic in ANOVA is fairly robust (Field 2009) as long as sample sizes are equal (which was the case here), and also allows inclusion of multiple fixed factors (which is not the case for the equivalent non-parametric tests), it was selected as the most appropriate analysis for these data.

All analyses were done in R and RStudio, using the packages ggplot2 (Wickham 2009), nlme (Pinheiro *et al.* 2015) and doBy (Højsgaard & Halekoh 2014).

Table 5.1: Summary of pesticides used in the LAB assay, the recommended application rates are from commonly used formulations. The reported residues are from [1] Thompson et al. (2013), [2] Choudhary & Sharma (2008), [3] Wallner (2009), [4] Bogdanov (2006), [5] Mullin et al. (2010) and [6] Karazafiris et al. (2008). The application rate is shown in amount of active ingredient per hectare of crops, or amount applied per kg of seed (for seed treatments), or amount applied per colony (for in-hive treatments). The range of doses indicate the low, medium and high doses for each pesticide used in this study in parts per billion (ppb), and are based on residue levels from the literature.

Active Ingredient	Class	Application rate (product name)	Residues found in nectar (or pollen/honey stores)	Source of data	Range of doses (ppb)		
					Low	Medium	High
Thiamethoxam	Neonicotinoid	(Cruiser seed treatment) 4.2g/kg seeds	max = **3.87 ppb** mean = **2.397 ± 0.16 ppb**	Nectar collected from bumblebee colonies placed near treated OSR [1]	0.2	2	20
λ-Cyhalothrin	Pyrethroid	(Hallmark) 7.5g/Ha	**728 - 858 ppb** (0 hours after spraying) **4 - 13 ppb** (72 hours after spraying)	Honeybees collected on treated mustard crop, contents of honey stomach removed for analysis [2]	0.8	8	80
Boscalid	Fungicide	(Signum) 270g/Ha	**1430 ppb** (0 days after treatment) **25 ppb** (7 days after treatment)	Honeybees foraging on treated oilseed rape - honey stomachs removed. [3]	10	100	1000
Coumaphos	Acaricide	(Perizin) up to 50ml applied to large colony.	**15ppb** (honey)	Honey samples analysed in spring after winter treatment. [4]	1.5	15	150
		(Checkmite) 1.36g/strip. 2 Strips per colony	mean = **180.4 ± 30 ppb** (pollen) max = **129 ppb** (honey in brood comb) mean = **58 ppb** (honey in brood comb)	Pollen residues detected in pollen in hives in USA [5] Honey sampled from 4 hives from day 0 - day 145 [6]			

5.4 Results

All phylotypes were able to grow in the presence of all pesticide treatments. There were consistent differences in growth between phylotypes, and Bin2, Fhon2, Fhon13 and the mixed strains all had the highest growth across all trials (Figures 5.1-5.4). Treatment effects were found for each pesticide alone and the mixed treatment, however, these varied greatly among phylotypes and treatment dose (Table 5.2). For example, in the coumaphos trial the phylotypes Bma5 and Fhon2 both had higher change in absorbance at the medium and high doses compared to controls (Figure 5.2). Change in absorbance of the phylotypes Biut2 and Hma8 was lower in the thiamethoxam group compared to controls (Figure 5.4). The mixture of all pesticides had negative effects on three phylotypes (Bin2, Bin4, Hon2), and no significant positive effects. In several cases, inconsistencies between the effects of different doses were found, for example, the phylotype Bma5 had a higher change in absorbance after exposure to medium and low doses of boscalid compared to controls, whilst the highest dose had no significant effect (Figure 5.1).

The ANOVA results for each analysis are summarised in Appendix 8, whilst post-hoc results for significant differences between treatment groups are summarised in Table 5.2. In several cases, the acetone and blank controls differed significantly (Table 5.2). Plate number also had a significant effect in many pesticide phylotype combinations (Appendix 8).

5.5 Discussion

5.5.1 Pesticide impacts on LAB

Pesticide exposure did not consistently inhibit or promote growth in any of the LAB phylotypes included in this study, at any dose. Significant effects of pesticide treatment were found for some of the LAB, although the direction and size of the effects varied among treatments and phylotypes. Whilst many of the phylotypes did not show any variation in growth between treatments, growth of some appeared to be promoted, and in other cases inhibited. There are several mechanisms which could cause an increase or decrease in bacterial growth after exposure to pesticides, and these are discussed below.

Biodegradation of pesticides by microbes is thought to be one of the main routes of pesticide degradation in soil (Gavrilescu 2005). Bacteria are amongst those microbes which can

metabolise pesticides as a source of carbon and energy, resulting in increased bacterial growth (Rani *et al.* 2008). Although this hasn't been tested for bee LAB, this could be a possible explanation for the increased growth observed in the results. On the other hand, certain pesticides, for example the formulated herbicide Roundup (active ingredient glyphosate), are known to inhibit growth of *Lactobacillus* species used in the dairy industry (Clair *et al.* 2012), by blocking enzymes involved in amino acid synthesis. This demonstrates that pesticides can have toxic effects on organisms which were not the original target, but use similar biological processes. As such, it is possible that the decrease in growth observed in some of the LAB phylotypes in the current study could be due to toxic effects of the pesticides.

The differences in change in absorbance observed suggest that pesticide exposure can modulate LAB growth in vitro. However, the impacts observed were generally small and inconsistent, and whether such changes would have any major impacts on bee health is unclear. LAB naturally fluctuate in live bees in response to changes in diet (Olofsson & Vásquez 2008), and it is possible that the fluctuations in LAB growth of the magnitude observed in this study are not of cause for concern. However, it is also possible that by changing the balance of gut microbiota in bees, negative impacts of pesticides on bee health could be found. For example, pesticide induced changes in LAB could influence susceptibility to parasites and diseases. Di Prisco *et al.* (2013) found that neonicotinoid pesticides inhibit the immune function of honeybees, and promote replication of deformed wing virus. Pesticide exposure has also been shown to be associated with increased infection and growth of the gut parasite *Nosema ceranae* in honeybees (Pettis *et al.* 2012; Pettis *et al.* 2013). As the gut microbiota of bees also interact with invading parasites (Forsgren *et al.* 2010; Koch & Schmid-Hempel 2011b; Koch, Cisarovsky & Schmid-Hempel 2012; Koch & Schmid-Hempel 2012; Vásquez *et al.* 2012) it is possible that any pesticide induced changes in gut microbiota will result in changes to bees' natural response to pathogens.

Further testing of pesticide interactions with LAB, both in vitro and in vivo, would be valuable. In particular, it would be interesting to test the response of LAB at the community level in live bees exposed to pesticides. Changes in the levels of some phylotypes are likely to have knock-on effects on other phylotypes, and so pesticides may have an impact on the community dynamics of the LAB microbiota. It would also be interesting to investigate whether LAB are able to metabolise pesticides, by testing residues before and after bacterial growth in vitro, as this may further modulate the impacts on the bee host.

5.5.2 Methodological considerations

Whilst the results of this study provide an interesting first insight into the effects of pesticides on honeybee LAB, they should be treated with caution. Several methodological issues could have influenced the results, and should be taken into account when interpreting the results, and controlled for in future work. Firstly, edge effects and significant differences between plates were detected. As such, cultures were likely to have been growing differently, purely based on their plate, or position on the plate. In the future, this could be avoided by excluding the edge wells of each plate from the outset, and ensuring that every treatment was represented on each plate so that individual plates would form replicates. This may require reducing the number of treatments used in the experiment. Secondly, during the plate readings, condensation was observed forming on the cover of the microplates, which may have interfered with the absorbance reading. This is likely to be due to differences in temperature in the plate reader compared to the incubation temperature. One way to resolve this in future studies is to use a plate reader with temperature controls, in order to minimise any temperature differences during the course of the experiment. Thirdly, in this study, the maximum change in absorbance detected over the course of the experiment was used as a proxy for maximum growth. This allowed a large number of samples to be processed, as each plate was only read four times. However, it may be more informative to monitor each plate continuously over a set period, in order to obtain a growth curve over time. This would allow a range of information to be detected such as rate of growth, maximum growth, and time to reach a pre-defined threshold, allowing for a more detailed assessment of any pesticide impacts on growth (Brewster 2003). Finally, significant differences between the acetone controls and blank controls were observed in several of the trials in this study. The solvent could therefore also have been influencing growth of the LAB, and alternative solvents should be investigated in order to avoid this in future studies.

5.5.3 Conclusions

The LAB microbiota of honeybees plays a role in pathogen defence, and thus is important to the health of honeybees. My results indicate that four pesticides, widely used in agriculture and apiculture, do not consistently inhibit the growth of LAB in vitro, but may change the growth of some LAB phylotypes. Further work is needed to establish the extent of these effects both in vitro and in live bees, in order to assess the impacts of agrochemicals on an important element of honeybee biology.

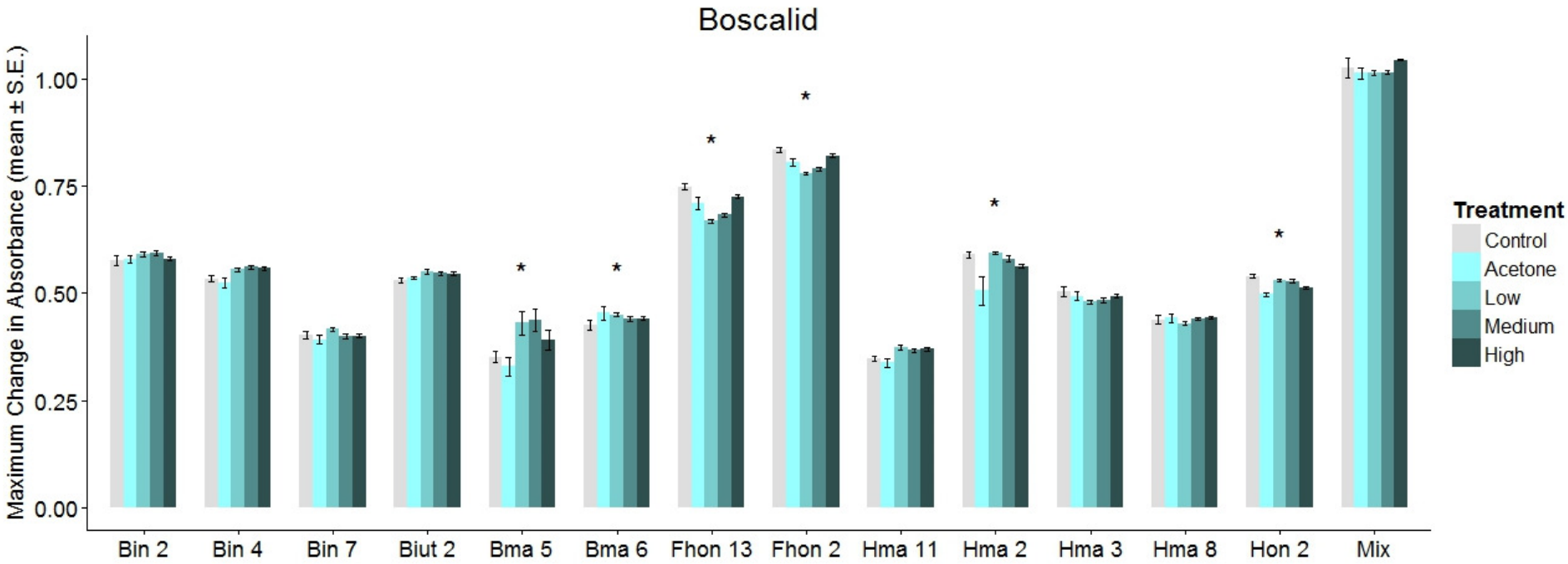


Figure 5.1: Maximum change in absorbance of 13 phylotypes of lactic acid bacteria over 3 days, after exposure to a blank control (water), an acetone control, or one of three doses of boscalid (10ppb, 100ppb, 1000ppb). An asterisk indicates significant treatment effect ($p<0.05$) for this phylotype. See Appendix 8 for full ANOVA results, and Table 5.2 for results of post hoc testing among treatment groups.

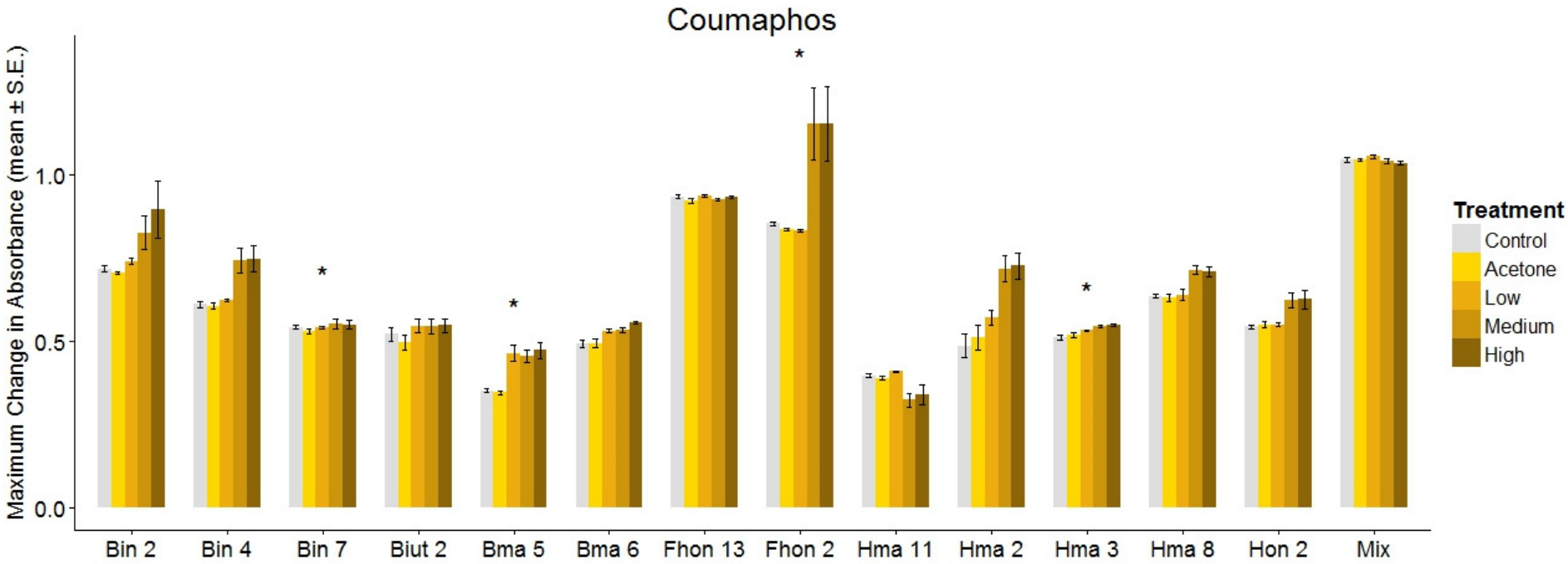


Figure 5.2: Maximum change in absorbance of 13 phylotypes of lactic acid bacteria over 3 days, after exposure to a blank control (water), an acetone control, or one of three doses of coumaphos (1.5ppb, 15ppb, 150ppb). An asterisk indicates a significant treatment effect ($p<0.05$) for this phylotype. See Appendix 8 for full ANOVA results, and Table 5.2 for results of post hoc testing among treatment groups.

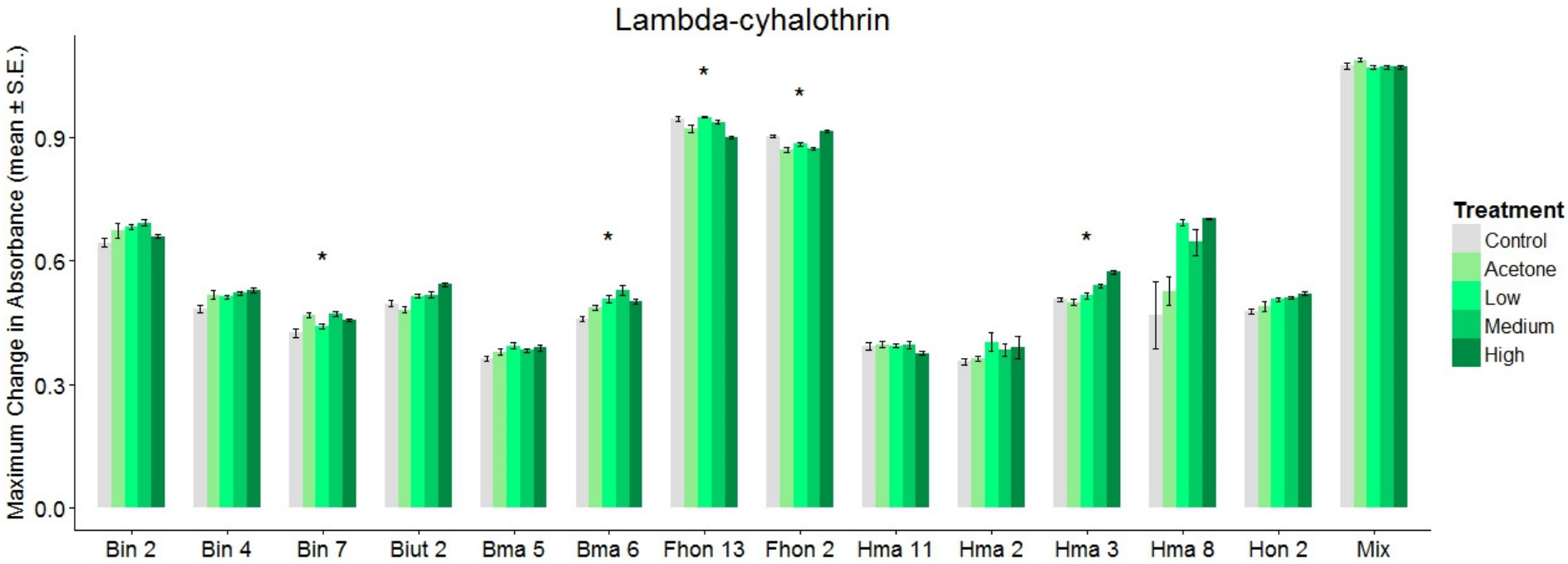


Figure 5.3: Maximum change in absorbance of 13 phylotypes of lactic acid bacteria over 3 days, after exposure to a blank control (water), an acetone control, or one of three doses of lambda-cyhalothrin (0.8ppb, 8ppb, 80ppb). An asterisk indicates a significant treatment effect ($p<0.05$) for this phylotype. See Appendix 8 for full ANOVA results, and Table 5.2 for results of post hoc testing among treatment groups.

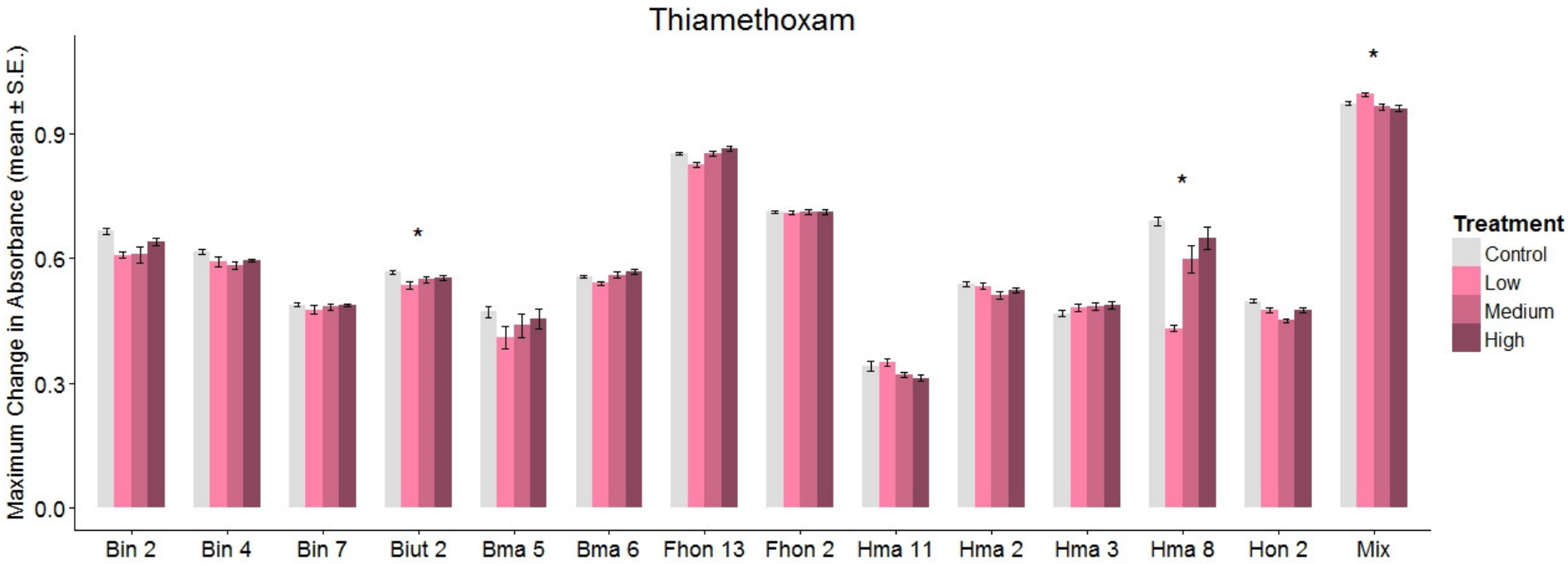


Figure 5.4: Maximum change in absorbance of 13 phylotypes of lactic acid bacteria over 3 days, after exposure to a blank control (water), or one of three doses of thiamethoxam (0.2ppb, 2ppb, 20ppb). An asterisk indicates a significant treatment effect ($p<0.05$) for this phylotype. See Appendix 8 for full ANOVA results, and Table 5.2 for results of post hoc testing among treatment groups.

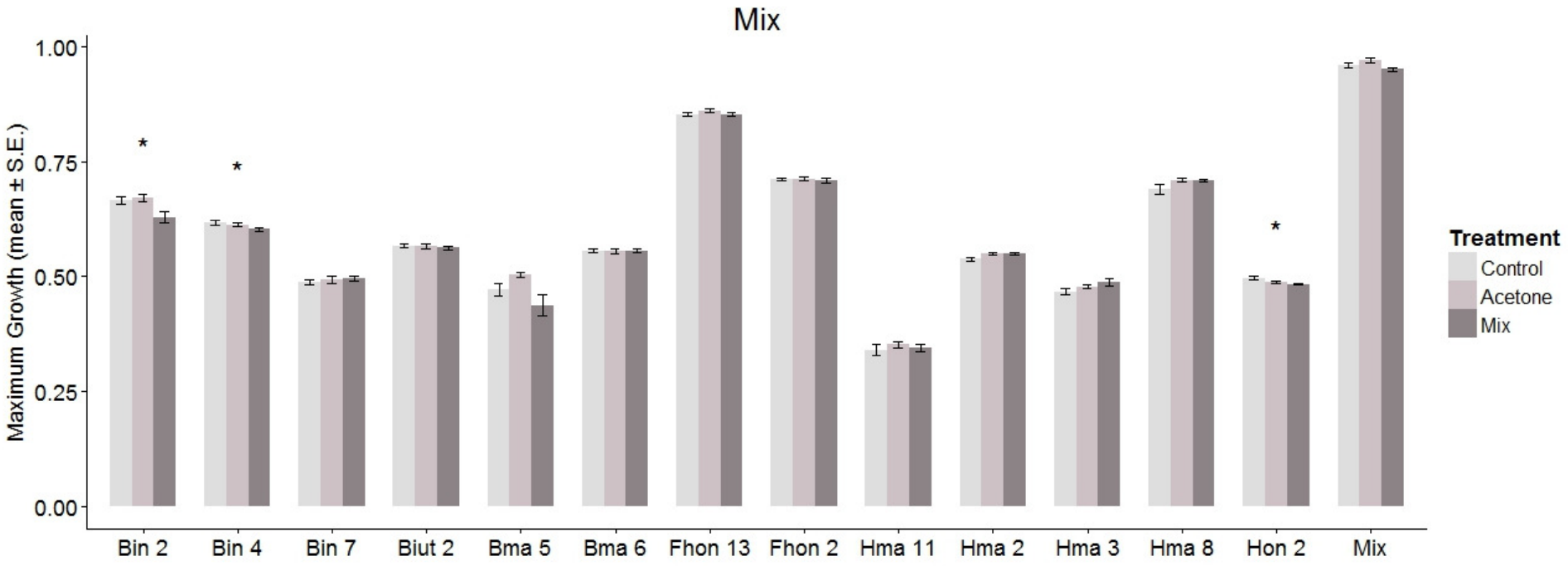


Figure 5.5: Maximum change in absorbance of 13 phylotypes of lactic acid bacteria over 3 days, after exposure to a blank control (water), an acetone control, or a mixture of the pesticides boscalid, coumaphos, lambda-cyhalothrin and thiamethoxam. An asterisk indicates a significant treatment effect ($p<0.05$) for this phylotype. See Appendix 8 for full ANOVA results, and Table 5.2 for results of post hoc testing among treatment groups.

Table 5.2: Results of Tukey's post hoc testing for differences between pesticide treatment group for each pesticide phylotype interaction. Only includes phylotypes where significant treatment effects were found, and pairs of treatments which differed significantly (see Appendix 8 for full ANOVA results). The treatment from each pair highlighted in bold had a higher change in absorbance (see Figures 5.1-5.4 for means and SE). Continued on following page.

Pesticide	Phylotype	Significant contrasts	
Boscalid	Bma5	Acetone	**Low**
		Acetone	**Medium**
	Bma6	Control	**Low**
	Fhon13	**Control**	Low
		Control	Medium
		Low	**High**
	Fhon2	**Control**	Acetone
		Control	Low
		Control	Medium
		Low	**High**
		Medium	**High**
	Hma2	**Control**	Acetone
		Acetone	**Low**
	Hon2	**Control**	Acetone
		Acetone	**Low**
		Acetone	**Medium**
		Acetone	**High**
Coumaphos	Bin7	Control	**Medium**
		Control	**High**
		Low	**Medium**
		Low	**High**
	Bma5	Control	**Low**
		Control	**High**
		Acetone	**Low**
		Acetone	**High**
	Fhon2	**Control**	Acetone
		Control	**Medium**
		Control	**High**
	Hma3	Control	**Medium**
		Control	**High**
		Low	**Medium**
		Low	**High**

Table 5.2 Continued – see above for full description.

Pesticide	Phylotype	Significant contrasts	
λ-cyhalothrin	Bin7	Control	**Acetone**
	Bma6	**Medium**	High
	Fhon13	**Control**	Acetone
		Control	High
		Acetone	**Low**
		Low	High
		Medium	High
	Fhon2	**Control**	Acetone
		Control	Low
		Control	Medium
		Acetone	**High**
		Low	**High**
		Medium	**High**
		Medium	**Low**
	Hma3	**Control**	Acetone
		Acetone	**High**
		Low	**High**
		Medium	**High**
Thiamethoxam	Biut2	**Control**	Low
		Control	High
	Hma8	**Control**	Low
	Mix	**Low**	Med
		Low	High
Mix	Bin2	**Control**	Medium
		Acetone	Medium
	Bin4	**Control**	Acetone
		Control	Medium
	Hon2	**Control**	Acetone
		Control	Medium

6 Discussion

The aims of this research were to investigate the sublethal impacts of pesticides on bumblebee and honeybee biology, from the individual to colony level, and in combination with parasites. The main questions addressed were:

- What are the impacts of pesticides on bees at an individual and colony level, and at different stages of the colony cycle?
- What are the combined impacts of pesticides and natural parasites on bees?
- Do different species of bumblebee have different tolerances to sublethal pesticide exposure?
- Does pesticide exposure have an impact on bee gut microbiota?

Bumblebees were the focal system for the first three questions. The impacts of pesticides and parasites at different stages of the bumblebee lifecycle were investigated. Chapter 2 focused on the development of colonies chronically exposed to a pyrethroid insecticide, whilst Chapters 3 and 4 investigated, for the first time, the impacts of neonicotinoid exposure on colony founding queens. Combined impacts of the pesticides and a prevalent gut parasite, *C. bombi,* on individual workers (Chapter 2) and queens (Chapter 3) were also explored. In an attempt to address the paucity of data on pesticide impacts on wild bee species, Chapter 4 compares the susceptibility of four UK bumblebee species to an insecticide during the colony founding period. Honeybee LAB were used in Chapter 5 to test for impacts of pesticide exposure on the bee gut microbiota.

In this final chapter, I will summarise and discuss the key results of my research, and make recommendations for future research and policy.

6.1 Key findings

6.1.1 Sublethal impacts of pesticides on bumblebee queens

The success of a bumblebee queen in surviving hibernation and laying eggs in the spring is crucial to the future of her colony. This period of the lifecycle has high energetic demands (Alford 1969), and additional stress at this time, for example from parasitism, can drastically reduce fitness (Brown, Schmid-Hempel & Schmid-Hempel 2003). Queens of several bumblebee species are likely to be exposed to pesticides during the spring whilst foraging and searching

for nest sites (as demonstrated in Chapter 4). However, no previous research has investigated the direct impacts of pesticide exposure on queens at this stage. My results indicate that field relevant doses of thiamethoxam can have sublethal impacts on colony initiation (Chapter 3), and ovary development (Chapter 4) in bumblebee queens. Furthermore, the timing of colony initiation was also affected by thiamethoxam treatment (Chapter 3). An early peak in egg laying by the pesticide treated queens was observed, but ultimately there was a 26% reduction in colony initiation compared to control queens by the end of the experiment. Previous studies into the effects of neonicotinoid exposure on bumblebee reproduction and egg laying have used worker micro-colonies as a proxy for the effects on queens (Laycock *et al.* 2012; Elston, Thompson & Walters 2013; Laycock *et al.* 2013). The results of these studies were mixed, but often showed minimal impacts on ovary development and brood production at low doses of thiamethoxam (Elston, Thompson & Walters 2013; Laycock *et al.* 2013). My experiments show that at doses of 2.4 ppb – 5.32 ppb thiamethoxam, queen reproduction is substantially impaired, perhaps due to the additional energy requirements of queens at this stage of the life cycle. These results indicate that worker micro-colony experiments are not sufficient for predicting the impacts of thiamethoxam on queens.

The mechanisms for the reduced ovary development and colony initiation observed remain unclear. Pollen intake is important as a source of protein, which is essential for the development of ovaries and production of eggs (Duchateau & Velthuis 1989). Whilst it was not possible to measure this in my experiments, future research could focus on pollen feeding and foraging in queens, which is known to be reduced in workers exposed to neonicotinoids (Laycock *et al.* 2012; Feltham, Park & Goulson 2014; Gill & Raine 2014). The detoxification of toxins such as pesticides involves metabolisation into less toxic substances, and may have high energy requirements. Cresswell, Merritt & Martin (1992), found that the detoxification of nicotine by the southern armyworm (*Spodoptera eridania*) imposed a significant metabolic cost, leading to a reduction in growth. Honeybees and bumblebees can clear ingested pesticide rapidly (Cresswell *et al.* 2014), however, the metabolic costs of the detoxification are unknown for bees. Chronic exposure could lead to the reallocation of nutrients such as proteins for detoxification, reducing nutrient availability for other processes such as ovary development. No impacts of exposure on the survival of queens, or on development of successfully initiated colonies were found. This is perhaps due to the period of pesticide exposure, which was relatively short (two weeks). Exposure of colonies to thiamethoxam for longer periods affects colony growth and the production of reproductive offspring (Fauser-Misslin *et al.* 2014). The two week pesticide exposure period used in my experiments could be considered a relatively

low exposure, as residues of thiamethoxam and other pesticides could be encountered by queens throughout the entire duration of their foraging, and also afterwards in pollen and nectar collected by their workers. On the other hand, queens were not given a choice in pesticide exposure, and under natural conditions may be exposed to much less pesticide in a two week timeframe. Until further data are available on the pesticide residues in crops and wildflowers on which bumblebees forage, it is not possible to ascertain a realistic exposure profile. This information is vital to understand and interpret the results of laboratory based studies.

Impairment of the reproductive output of queens could have important implications for wild bee populations. Bumblebee queens are faced with a range of stressors in the spring, including multiple parasite species, challenging weather conditions and variable food availability. The queens in my experiments were kept in constant conditions with unlimited food supplies. Given that effects of the pesticide could be detected even under these relatively undemanding conditions, it might be expected that queens exposed in the wild could be even more severely affected. My results clearly indicate the importance of considering bumblebee queens, in particular during colony founding, in the risk assessment for pesticides.

6.1.2 Interactions between pesticides and a prevalent gut parasite

The impacts of pesticides on bees could be modulated by exposure to additional sources of stress in the environment, and these potential interactions should be considered when assessing the risk of pesticides to bees. I tested the combined impacts of pesticide exposure with *C. bombi*, a prevalent trypanosome gut parasite, which is known to have a greater impact when its host is under additional stress (Brown, Loosli & Schmid-Hempel 2000; Brown, Schmid-Hempel & Schmid-Hempel 2003). In Chapter 2, I found that chronic exposure during larval development to a pyrethroid, λ-cyhalothrin, had no combined impacts with *C. bombi* infection on adult worker survival, or on infection success of the parasite. Infection of *B. terrestris* queens with *C. bombi* prior to hibernation, and subsequent post-hibernation exposure to thiamethoxam, did not have a greater impact on survival or colony initiation compared to each treatment alone (Chapter 3). Fauser-Misslin *et al.* (2014) found that whilst *C. bombi* infection and thiamethoxam exposure had an interactive impact on founding queen survival in *B. terrestris* colonies, no other colony level interactive effects were found.

It is possible that the experiments above lacked sufficient power to detect interactive effects. Studies that have found a fitness cost of *C. bombi* on queens have used sample sizes up to 106

per treatment group (Brown, Schmid-Hempel & Schmid-Hempel 2003), compared to a sample size of up to 31 per treatment group in Chapter 3, and ten colonies per group in Fauser-Misslin *et al.* (2014). Alternatively, perhaps *C. bombi* (or specifically the strains collected for these tests) were not sufficiently stressful to the hosts to have a detectable impact. Host-parasite genotype interactions are known to occur in this system (Imhoof & Schmid-Hempel 1998), and as the bumblebees (which were commercially reared), and parasites (harvested from wild bees) were from different populations, this could have reduced the impacts on the host. Compared to other bumblebee parasites, *C. bombi* is relatively low impact (see Chapter 1). It would be interesting to explore interactions with other, more virulent parasites, such as *A. bombi* (which severely decreases survival), *S. bombi* (which prevents queens from developing ovaries), or *N. bombi* (which can cause increases in mortality and reduced reproductive output in infected colonies). Impacts of pesticides on the ability of bumblebees to defend against these parasites, or direct impacts on the parasites themselves (either within the host, or at transmission sites), could alter the transmission dynamics, with potential consequences for bumblebee populations.

Results on pesticide interactions with parasite infection from the honeybee literature (Alaux *et al.* 2010; Vidau *et al.* 2011; Aufauvre *et al.* 2012; Pettis *et al.* 2012; Di Prisco *et al.* 2013; Pettis *et al.* 2013; Doublet *et al.* 2014), suggest that interactions can occur at the individual level, and that impairment of the immune function could be responsible (Di Prisco *et al.* 2013). Pesticide impacts on immune function have also been demonstrated for other insects (James & Xu 2012), and other taxa such as amphibians (Kiesecker 2002). It is likely therefore, that such processes also occur in wild bees, and further research in this area should be undertaken. Emergent infectious diseases (EIDs) are considered an important threat to wild bees (Meeus *et al.* 2011; Fürst *et al.* 2014; McMahon *et al.* 2015). The full impacts of these novel pathogens and parasites is largely unknown, although recent work shows that some, for example DWV, can be detrimental to bumblebee survival (Fürst *et al.* 2014). Further testing of EIDs in wild bees should consider the implications of additional stress, for example from pesticides, and how this may affect disease transmission and the impact of infection.

In the wild, bees could be exposed to a huge range of different sources of stress, including multiple pesticides, parasites, and nutritional stress (Goulson *et al.* 2015). Examining two-way interactions between pesticides and parasites in the lab is extremely useful for discovering mechanisms and specific sources of stress. However, using a modelling approach, Bryden *et al.* (2013) found that chronic sublethal stress of individual bees can potentially lead to colony

failure. As such, stress from different sources may have a cumulative effect, and larger scale experiments investigating this throughout the life cycle of bees would be valuable.

6.1.3 Gut microbiota

One avenue of research into interactions between pesticides and parasites that has not yet been explored, is the potential impact of pesticides on the gut microbiota of bees. The gut flora of honeybees and bumblebees is known to interact with invading parasites, by inhibiting development of infection (Forsgren *et al.* 2010; Koch & Schmid-Hempel 2011b). In vitro, growth of the LAB of honeybee guts varied with exposure to a range of pesticides. In some cases, growth was promoted, in other cases, inhibited, and this was often dose dependent (Chapter 5). If changes in the diversity and abundance of microbiota occur in live bees exposed to pesticides, this could have knock on effects for several aspects of bee biological functioning, such as defence against parasites and pathogens. Whilst the results from this chapter are not conclusive due to methodological considerations (see discussion of Chapter 5), further investigation both in vitro and in vivo would be valuable.

6.1.4 Effects of chronic pyrethroid exposure on bumblebee colony development

The systemic use of neonicotinoid insecticides could have important risks for bees and other wildlife (Goulson 2013; Chagnon *et al.* 2014; Gibbons, Morrissey & Mineau 2014; Gross 2014; Pisa *et al.* 2014). This is of particular interest at the current time due to the potential for policy changes. However, the range of pesticides used in agriculture today is huge, and understanding the impacts of other pesticide classes on bees is important in order to make informed decisions as to the most suitable pest control methods. In Chapter 2, I investigated the impact of a widely used pyrethroid insecticide, λ-cyhalothrin, on *B. terrestris* colony development. Chronic exposure to this compound resulted in the production of smaller workers, perhaps due to changes in brood care by the colony. Colonies producing smaller workers in the wild could have reduced foraging efficiency, with potential impacts for colony growth. However, in the current experiment, the impact of λ-cyhalothrin on other aspects of colony development was minimal. Further testing is needed, both in the field and on larger sample sizes, in order to confirm this. In contrast, *B. terrestris* colonies chronically exposed to neonicotinoids show reduced colony growth (Gill, Ramos-Rodriguez & Raine 2012; Bryden *et al.* 2013; Goulson 2015), and reduced production of sexual offspring (Whitehorn *et al.* 2012; Fauser-Misslin *et al.* 2014; Goulson 2015). The high impact of neonicotinoid exposure could be partially explained by the field or semi-field design of several of these studies (Gill, Ramos-Rodriguez & Raine 2012; Whitehorn *et al.* 2012; Goulson 2015), in which colonies were able to

forage outside of the laboratory. This would have incurred a greater cost to these colonies, as food availability may have been lower, and energetic demands higher. Furthermore, these semi-field studies used a relatively high dose of the pesticide compared to residues found in the field. However, even at lower doses, and under optimal laboratory conditions (Fauser-Misslin *et al.* 2014), the impacts of neonicotinoid exposure appear to be more severe than those found in my pyrethroid experiment. Comparative studies of different pest control regimes would be useful in order to fully understand the environmental costs of these, compared to their agricultural benefits.

6.1.5 Species level differences in susceptibility to pesticides

Apis mellifera, and more recently *B. terrestris,* are used as focal organisms for the majority of pesticide regulation testing and research. These species are easy to access and rear in the laboratory, and thus provide ideal model systems on which to test impacts of pesticides. However, a vast array of wild bee species are found in agricultural areas. It is likely that many species will come into contact with pesticides whilst foraging, and in Chapter 4, I established that a range of species of bumblebee queen will forage in and around pesticide treated oilseed rape crops. Little is known about how different species of bumblebee respond to stress from pesticide exposure, and by only testing one focal species, it is impossible to ascertain the full effects pesticides could be having on our wild bee fauna. I tested queens of four bumblebee species (*B. terrestris, B. lucorum, B. pascuorum and B. pratorum*), chronically exposing them to thiamethoxam in the laboratory. All species showed a decrease in ovary development in response to pesticide exposure. *B. pratorum* and *B. pascuorum* appeared to be more sensitive to the pesticide, indicated by a reduction in feeding on treated syrup compared to control queens. This effect was not detectable in the other two species, suggesting a species-specific response to this pesticide.

The results from Chapter 4 indicate the importance of considering a range of wild bee species in pesticide risk assessments. Whilst the focus of this thesis was on bumblebees and honeybees, many species of solitary and social bee are known to forage on and pollinate flowering crops, and could come into contact with pesticides as a result. These are often very different in their biology and life-history compared to *Apis* and *Bombus* species, and could differ in their susceptibility to pesticides as a result (Brittain & Potts 2010). The response of some of the more commercially important solitary bees (Tasei, Capou & Michaud 1977; Scott-Dupree, Conroy & Harris 2009; Gradish, Scott-Dupree & Cutler 2012), and sub-tropical stingless bees (Meliponini) (Valdovinos-Núñez *et al.* 2009) to pesticides has been tested. The toxicity of

several insecticides was found to be considerably higher for two solitary species (*Osmia lignaria* and *Megachile rotundata*) compared to a bumblebee species (*B. impatiens)* (Scott-Dupree, Conroy & Harris 2009). Furthermore, large differences in sensitivity to a range of pesticides were apparent when *A. mellifera* was compared to other bee species (Arena & Sgolastra 2014). This variation in sensitivity between species could have important implications for the levels of pesticide residues which are considered safe for bees. Further research into the sub-lethal impacts, and impacts throughout the life cycle of non *Apis* or *Bombus* bees is needed in order to address this issue.

Ultimately, the most important question regarding pesticide use and the impacts on bees, is whether current and future pest control strategies have long term negative impacts in the field, at a population and community level. Whilst controlled laboratory studies are extremely important for finding specific outcomes and mechanisms, more field studies are essential in order to put these results into context and find long term trends. Some field studies have been conducted, looking at species level performances (Pilling *et al.* 2013; Thompson *et al.* 2013), and population and community level changes in pollinators (Brittain *et al.* 2010; Tuell & Isaacs 2010) in response to pesticide use in individual crop systems. Whilst sufficient controls and replication are notoriously difficult to achieve in such field studies, developing experimental and observational studies in the field should be a priority.

6.2 Other directions for future research

In addition to the suggestions made above, there are several other areas of research which would be useful, but for which little is currently known. These are briefly discussed below.

6.2.1 Impacts on pollination

The increasing concern for pollinator species is often driven by the potential loss of the ecological services they provide (Allen-Wardell *et al.* 1998; Potts *et al.* 2010a). Whilst research into the impacts of pesticides has shown that pollinators such as bees may be negatively impacted by pesticide exposure, our understanding of how pesticides may directly have an impact on pollinators' ability to pollinate is limited. Given the behavioural changes induced by pesticide exposure which have been observed, in particular those relating to pollen foraging (Feltham, Park & Goulson 2014; Gill & Raine 2014), it seems highly possible that the pollination of crops and other flowering plants could be disrupted by pesticides.

6.2.2 Impacts on males

As for queens, the impacts of pesticides on male bees have not been widely studied. Males are required for the fertilisation of eggs, and subsequent production of female offspring. Any effect of pesticides on male survival or mating performance could therefore have serious implications for population dynamics. In social bees, males are generally in flight later in the summer, and so the exposure profile to pesticides will likely differ from that of workers and queens. Further research in this area is needed in order to assess the risk to another vital stage of the bee life cycle.

6.3 Conclusions and Recommendations

Agriculture is essential to feed the growing human population, and pesticides and agrochemicals have an important role to play in increasing crop productivity. However, the detrimental impacts on wildlife and ecosystems of our increasing dependence on chemicals for crop protection must be addressed. Bees are of immense importance in both wild and agricultural ecosystems, and the threats from agriculture; habitat loss, exposure to pesticides and spread of parasites and diseases, must be minimised. An essential part of this is understanding the nature of these threats, and ensuring appropriate policies are in place to protect bees. My research has highlighted several areas where policy could be improved, or where further research is needed in order to ensure that crop production and bee conservation are compatible. My main recommendations are summarised below:

- Policy decisions and risk assessments for pesticide use should include consideration of all life stages of bees which may be at risk of exposure. For social species, this should include the potential exposure of colony founding queens in the spring, as well as queens and males later in the year. Decisions such as timing and extent of application on certain crops should take this into account.

- In order to understand the full extent of exposure of non-target species to pesticides, testing for pesticide residues in the pollen and nectar of both crops and wildflowers is needed, in a range of environments, and at multiple time points throughout the year.

- Further research is needed into the combined impacts of multiple stressors. This should include research into specific interactions between stressors, such as pesticides and emergent infectious diseases, in order to determine mechanisms for interactions

and identify specific high risk combinations of stressors. However, studies investigating the long term impacts of cumulative exposure to a range of stressors in field conditions are also needed.

- Additional comparative studies of the impacts of different pest control strategies (including different classes of pesticide) on individuals, populations and communities are needed. These should include comparisons of the costs and benefits from an agricultural perspective (e.g.: financially), as well as from an environmental perspective. Policy decisions should focus on encouraging implementation of pest control strategies which provide a balance between agricultural productivity and environmental safety.

Ultimately, bees are fascinating and valuable creatures from both a conservation and economic perspective. It is essential that we balance the needs of a growing human population with the organisms and ecosystems that support it.

References

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

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

Alaux, C., Brunet, J.-L., Dussaubat, C., Mondet, F., Tchamitchan, S., Cousin, M., Brillard, J., Baldy, A., Belzunces, L.P. & Le Conte, Y. (2010) Interactions between *Nosema* microspores and a neonicotinoid weaken honeybees (*Apis mellifera*). *Environmental Microbiology,* **12,** 774-782.

Alford, D.V. (1969) A study of the hibernation of bumblebees (Hymenoptera:Bombidae) in Southern England. *Journal of Animal Ecology,* **38,** 149-170.

Alford, D.V. (1975) Bumblebees. Davis-Poynter Ltd, London.

Aliouane, Y., El Hassani, A.K., Gary, V., Armengaud, C., Lambin, M. & Gauthier, M. (2009) Subchronic exposure of honeybees to sublethal doses of pesticides: Effects on behavior. *Environmental Toxicology and Chemistry,* **28,** 113-122.

Allen-Wardell, G., Bernhardt, P., Bitner, R., Burquez, A., Buchmann, S., Cane, J., Cox, P.A., Dalton, V., Feinsinger, P., Ingram, M., Inouye, D., Jones, C.E., Kennedy, K., Kevan, P., Koopowitz, H., Medellin, R., Medellin-Morales, S. & Nabhan, G.P. (1998) The potential consequences of pollinator declines on the conservation of biodiversity and stability of food crop yields. *Conservation Biology,* **12,** 8-17.

Antunez, K., Martin-Hernandez, R., Prieto, L., Meana, A., Zunino, P. & Higes, M. (2009) Immune suppression in the honey bee (*Apis mellifera*) following infection by *Nosema ceranae* (*Microsporidia*). *Environmental Microbiology,* **11,** 2284-2290.

Archer, C.R., Pirk, C.W., Wright, G.A. & Nicolson, S.W. (2014) Nutrition affects survival in African honeybees exposed to interacting stressors. *Functional Ecology,* **28,** 913-923.

Arena, M. & Sgolastra, F. (2014) A meta-analysis comparing the sensitivity of bees to pesticides. *Ecotoxicology,* **23,** 324-334.

Aufauvre, J., Biron, D.G., Vidau, C., Fontbonne, R., Roudel, M., Diogon, M., Vigues, B., Belzunces, L.P., Delbac, F. & Blot, N. (2012) Parasite-insecticide interactions: a case study of *Nosema ceranae* and fipronil synergy on honeybee. *Scientific Reports,* **2,** 326.

Aufauvre, J., Misme-Aucouturier, B., Viguès, B., Texier, C., Delbac, F. & Blot, N. (2014) Transcriptome analyses of the honeybee response to *Nosema ceranae* and insecticides. *PLoS ONE,* **9**.

Auguie, B. (2012) gridExtra: Functions in grid graphics.

Bartoń, K. (2014) MuMin: Multi-model inference

Bates, D., Maechler, M., Bolker, B. & Walker, S. (2014) lme4: Linear mixed-effects models using Eigen and S4. R package version 1.1-7.

Beekman, M., van Stratum, P. & Lingeman, R. (1998) Diapause survival and post-diapause performance in bumblebee queens (*Bombus terrestris*). *Entomologia Experimentalis Et Applicata,* **89,** 207-214.

Beketov, M.A., Kefford, B.J., Schäfer, R.B. & Liess, M. (2013) Pesticides reduce regional biodiversity of stream invertebrates. *Proceedings of the National Academy of Sciences,* **110,** 11039-11043.

Belzunces, L.P., Tchamitchian, S. & Brunet, J.-L. (2012) Neural effects of insecticides in the honey bee. *Apidologie,* **43,** 348-370.

Biesmeijer, J.C., Roberts, S.P.M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., Schaffers, A.P., Potts, S.G., Kleukers, R., Thomas, C.D., Settele, J. & Kunin, W.E. (2006) Parallel declines in pollinators and insect-pollinated plants in Britain and the Netherlands. *Science,* **313,** 351-354.

Bogdanov, S. (2006) Contaminants of bee products. *Apidologie,* **37,** 1-18.

Bommarco, R., Biesmeijer, J.C., Meyer, B., Potts, S.G., Pöyry, J., Roberts, S.P., Steffan-Dewenter, I. & Öckinger, E. (2010) Dispersal capacity and diet breadth modify the response of wild bees to habitat loss. *Proceedings of the Royal Society B: Biological Sciences,* **277,** 2075-2082.

Boncristiani, H., Underwood, R., Schwarz, R., Evans, J.D., Pettis, J. & vanEngelsdorp, D. (2012) Direct effect of acaricides on pathogen loads and gene expression levels in honey bees *Apis mellifera*. *Journal of Insect Physiology,* **58,** 613-620.

Bonmatin, J.-M., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., Long, E., Marzaro, M., Mitchell, E.A.D., Noome, D.A., Simon-Delso, N. & Tapparo, A. (2014) Environmental fate and exposure; neonicotinoids and fipronil. *Environmental Science and Pollution Research,* **22,** 35-67.

Bowles, R. & Webster, J. (1995) Some problems associated with the analysis of the costs and benefits of pesticides. *Crop Protection,* **14,** 593-600.

Breeze, T.D., Bailey, A.P., Balcombe, K.G. & Potts, S.G. (2011) Pollination services in the UK: how important are honeybees? *Agriculture Ecosystems & Environment,* **142,** 137-143.

Brewster, J.D. (2003) A simple micro-growth assay for enumerating bacteria. *Journal of Microbiological Methods,* **53,** 77-86.

Brittain, C. & Potts, S.G. (2010) The potential impacts of insecticides on the life-history traits of bees and the consequences for pollination. *Basic and Applied Ecology,* **12,** 321-331.

Brittain, C.A., Vighi, M., Bommarco, R., Settele, J. & Potts, S.G. (2010) Impacts of a pesticide on pollinator species richness at different spatial scales. *Basic and Applied Ecology,* **11,** 106-115.

Bronstein, J.L., Alarcón, R. & Geber, M. (2006) The evolution of plant–insect mutualisms. *New Phytologist,* **172,** 412-428.

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

Brown, M.J.F. & Paxton, R.J. (2009) The conservation of bees: A global perspective. *Apidologie,* **40,** 410-416.

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

Bryden, J., Gill, R.J., Mitton, R.A.A., Raine, N.E. & Jansen, V.A.A. (2013) Chronic sublethal stress causes bee colony failure. *Ecology letters,* **16,** 1463-1469.

Butchart, S.H.M., Walpole, M., Collen, B., van Strien, A., Scharlemann, J.P.W., Almond, R.E.A., Baillie, J.E.M., Bomhard, B., Brown, C., Bruno, J., Carpenter, K.E., Carr, G.M., Chanson, J., Chenery, A.M., Csirke, J., Davidson, N.C., Dentener, F., Foster, M., Galli, A., Galloway, J.N., Genovesi, P., Gregory, R.D., Hockings, M., Kapos, V., Lamarque, J.F., Leverington, F., Loh, J., McGeoch, M.A., McRae, L., Minasyan, A., Morcillo, M.H., Oldfield, T.E.E., Pauly, D., Quader, S., Revenga, C., Sauer, J.R., Skolnik, B., Spear, D., Stanwell-Smith, D., Stuart, S.N., Symes, A., Tierney, M., Tyrrell, T.D., Vie, J.C. & Watson, R. (2010) Global biodiversity: Indicators of recent declines. *Science,* **328,** 1164-1168.

Cameron, S.A., Lozier, J.D., Strange, J.P., Koch, J.B., Cordes, N., Solter, L.F. & Griswold, T.L. (2011) Patterns of widespread decline in north american bumble bees. *Proceedings of the National Academy of Sciences of the United States of America,* **108,** 662-667.

Cardinale, B.J., Duffy, J.E., Gonzalez, A., Hooper, D.U., Perrings, C., Venail, P., Narwani, A., Mace, G.M., Tilman, D., Wardle, D.A., Kinzig, A.P., Daily, G.C., Loreau, M., Grace, J.B., Larigauderie, A., Srivastava, D.S. & Naeem, S. (2012) Biodiversity loss and its impact on humanity. *Nature,* **486,** 59-67.

Carolan, J.C., Murray, T.E., Fitzpatrick, Ú., Crossley, J., Schmidt, H., Cederberg, B., McNally, L., Paxton, R.J., Williams, P.H. & Brown, M.J.F. (2012) Colour patterns do not diagnose

species: Quantitative evaluation of a DNA barcoded cryptic bumblebee complex. *PLoS ONE,* **7,** 662-667.

Carreck, N.L. & Ratnieks, F.L. (2014) The dose makes the poison: have "field realistic" rates of exposure of bees to neonicotinoid insecticides been overestimated in laboratory studies? *Journal of Apicultural Research,* **53,** 607-614.

Carson, R. (1962) Silent spring. Fawcett Publications, Greenwich, Connecticut.

Cartar, R.V. & Dill, L.M. (1991) Costs of energy shortfall for bumble bee colonies: predation, social parasitism, and brood development. *Canadian Entomologist,* **123,** 283-293.

Carvalheiro, L.G., Kunin, W.E., Keil, P., Aguirre-Gutiérrez, J., Ellis, W.N., Fox, R., Groom, Q., Hennekens, S., Van Landuyt, W., Maes, D., Van de Meutter, F., Michez, D., Rasmont, P., Ode, B., Potts, S.G., Reemer, M., Roberts, S.P.M., Schaminée, J., WallisDeVries, M.F. & Biesmeijer, J.C. (2013) Species richness declines and biotic homogenisation have slowed down for NW-European pollinators and plants. *Ecology letters,* **16,** 870-878.

Carvell, C., Roy, D.B., Smart, S.M., Pywell, R.F., Preston, C.D. & Goulson, D. (2006) Declines in forage availability for bumblebees at a national scale. *Biological Conservation,* **132,** 481-489.

Chadwick, W. & Little, T.J. (2005) A parasite-mediated life-history shift in Daphnia magna. *Proceedings of the Royal Society B-Biological Sciences,* **272,** 505-509.

Chagnon, M., Kreutzweiser, D., Mitchell, E.A., Morrissey, C.A., Noome, D.A. & Van der Sluijs, J.P. (2014) Risks of large-scale use of systemic insecticides to ecosystem functioning and services. *Environmental Science and Pollution Research,* **22,** 119-134.

Chauzat, M.P., Faucon, J.P., Martel, A.C., Lachaize, J., Cougoule, N. & Aubert, M. (2006) A survey of pesticide residues in pollen loads collected by honey bees in France. *Journal of Economic Entomology,* **99,** 253-262.

Chauzat, M.P., Carpentier, P., Martel, A.C., Bougeard, S., Cougoule, N., Porta, P., Lachaize, J., Madec, F., Aubert, M. & Faucon, J.P. (2009) Influence of pesticide residues on honey bee (Hymenoptera: Apidae) colony health in France. *Environmental Entomology,* **38,** 514-523.

Choudhary, A. & Sharma, D.C. (2008) Dynamics of pesticide residues in nectar and pollen of mustard (*Brassica juncea* (L.) Czern.) grown in Himachal Pradesh (India). *Environmental Monitoring and Assessment,* **144,** 143-150.

Chu, X., Fang, H., Pan, X., Wang, X., Shan, M., Feng, B., Yu, Y. (2008) Degradation of chlorpyrifos alone and in combination with chlorothalonil and their effects on soil microbial populations. *Journal of Environmental Sciences,* **20,** 464-469.

Clair, E., Linn, L., Travert, C., Amiel, C., Séralini, G.-E. & Panoff, J.-M. (2012) Effects of roundup® and glyphosate on three food microorganisms: *Geotrichum candidum*, *Lactococcus lactis* subsp. *cremoris* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. *Current Microbiology,* **64,** 486-491.

Claudianos, C., Ranson, H., Johnson, R. M., Biswas, S., Schuler, M. A., Berenbaum, M. R., Feyereisen, R. and Oakeshott, J. G. (2006) A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. Insect Molecular Biology, 15: 615–636.

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

Colla, S.R., Gadallah, F., Richardson, L., Wagner, D. & Gall, L. (2012) Assessing declines of north american bumble bees (*Bombus* spp.) using museum specimens. *Biodiversity and Conservation,* **21,** 3585-3595.

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

Colla, S.R. & Packer, L. (2008) Evidence for decline in Eastern North American bumblebees (Hymenoptera: Apidae), with special focus on *Bombus affinis* Cresson. *Biodiversity and Conservation,* **17,** 1379-1391.

Cooper, J. & Dobson, H. (2007) The benefits of pesticides to mankind and the environment. *Crop Protection,* **26,** 1337-1348.

Coors, A., Decaestecker, E., Jansen, M. & De Meester, L. (2008) Pesticide exposure strongly enhances parasite virulence in an invertebrate host model. *Oikos,* **117,** 1840-1846.

Corbet, S.A., Fussell, M., Ake, R., Fraser, A., Gunson, C., Savage, A. & Smith, K. (1993) Temperature and the pollinating activity of social bees. *Ecological Entomology,* **18,** 17-30.

Corbet, S.A., Williams, I.H. & Osborne, J.L. (1991) Bees and the pollination of crops and wild flowers in the European community. *Bee World,* **72,** 47-59.

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R.V., Paruelo, J., Raskin, R.G., Sutton, P. & van den Belt, M. (1997) The value of the world's ecosystem services and natural capital. *Nature,* **387,** 253-260.

Cox-Foster, D.L., Conlan, S., Holmes, E.C., Palacios, G., Evans, J.D., Moran, N.A., Quan, P.-L., Briese, T., Hornig, M., Geiser, D.M., Martinson, V., vanEngelsdorp, D., Kalkstein, A.L., Drysdale, A., Hui, J., Zhai, J., Cui, L., Hutchison, S.K., Simons, J.F., Egholm, M., Pettis, S.J.

& Lipkin, W.I. (2007) A metagenomic survey of microbes in honey bee colony collapse disorder. *Science,* **318,** 283-287.

Cresswell, J.E. (2011) A meta-analysis of experiments testing the effects of a neonicotinoid insecticide (imidacloprid) on honey bees. *Ecotoxicology,* **20,** 149-157.

Cresswell, J.E., Merritt, S.Z. & Martin, M.M. (1992) The effect of dietary nicotine on the allocation of assimilated food to energy metabolism and growth in fourth-instar larvae of the southern armyworm, spodoptera eridania (lepidoptera: Noctuidae). *Oecologia,* **89,** 449-453.

Cresswell, J.E. & Osborne, J.L. (2004) The effect of patch size and separation on bumblebee foraging in oilseed rape: Implications for gene flow. *Journal of Applied Ecology,* **41,** 539-546.

Cresswell, J.E., Page, C.J., Uygun, M.B., Holmbergh, M., Li, Y., Wheeler, J.G., Laycock, I., Pook, C.J., Hempel de Ibarra, N., Smirnoff, N. & Tyler, C.R. (2012) Differential sensitivity of honey bees and bumble bees to a dietary insecticide (imidacloprid). *Zoology,* **115,** 365-371.

Cresswell, J.E., Robert, F.-X.L., Florance, H. & Smirnoff, N. (2014) Clearance of ingested neonicotinoid pesticide (imidacloprid) in honey bees (*Apis mellifera*) and bumblebees (*Bombus terrestris*). *Pest Management Science,* **70,** 332-337.

De Jong, D., De Jong, P. & Goncalves, L. (1982) Weight loss and other damage to developing worker honeybees from infestation with *Varroa jacobsoni* [parasitic mite]. *Journal of Apicultural Research,* **21,** 165-167.

Decourtye, A., Devillers, J., Cluzeau, S., Charreton, M. & Pham-Delegue, M.H. (2004) Effects of imidacloprid and deltamethrin on associative learning in honeybees under semi-field and laboratory conditions. *Ecotoxicology and Environmental Safety,* **57,** 410-419.

Decourtye, A., Devillers, J., Genecque, E., Le Menach, K., Budzinski, H., Cluzeau, S. & Pham-Delegue, M.H. (2005) Comparative sublethal toxicity of nine pesticides on olfactory learning performances of the honeybee *Apis mellifera*. *Archives of Environmental Contamination and Toxicology,* **48,** 242-250.

Decourtye, A., Lacassie, E. & Pham-Delegue, M.H. (2003) Learning performances of honeybees (*Apis mellifera* L) are differentially affected by imidacloprid according to the season. *Pest Management Science,* **59,** 269-278.

Decourtye, A., Le Metayer, M., Pottiau, H., Tisseur, M., Odoux, J.F. & Pham-Delegue, M.H. (2001) Impairment of olfactory learning performances in the honey bee after long term ingestion of imidacloprid. *Hazards of pesticides to bees* (eds L.P. Belzunces, C. Pelissier & G.B. Lewis), pp. 113-117.

Delaplane, K.S., Mayer, D.R. & Mayer, D.F. (2000) *Crop pollination by bees*. Cabi Publishing, Wallingford.

Denholm, I. & Rowland, M. (1992) Tactics for managing pesticide resistance in arthropods: Theory and practice. *Annual Review of Entomology,* **37,** 91-112.

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G. & Pennacchio, F. (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences,* **110,** 18466-18471.

Dicks, L.V., Abrahams, A., Atkinson, J., Biesmeijer, J., Bourn, N., Brown, C., Brown, M.J.F., Carvell, C., Connolly, C., Cresswell, J.E., Croft, P., Darvill, B., De Zylva, P., Effingham, P., Fountain, M., Goggin, A., Harding, D., Harding, T., Hartfield, C., Heard, M.S., Heathcote, R., Heaver, D., Holland, J., Howe, M., Hughes, B., Huxley, T., Kunin, W.E., Little, J., Mason, C., Memmott, J., Osborne, J., Pankhurst, T., Paxton, R.J., Pocock, M.J.O., Potts, S.G., Power, E.F., Raine, N.E., Ranelagh, E., Roberts, S., Saunders, R., Smith, K., Smith, R.M., Sutton, P., Tilley, L.A.N., Tinsley, A., Tonhasca, A., Vanbergen, A.J., Webster, S., Wilson, A. & Sutherland, W.J. (2013) Identifying key knowledge needs for evidence-based conservation of wild insect pollinators: A collaborative cross-sectoral exercise. *Insect Conservation and Diversity,* **6,** 435-446.

Doublet, V., Labarussias, M., de Miranda, J.R., Moritz, R.F. & Paxton, R.J. (2014) Bees under stress: Sublethal doses of a neonicotinoid pesticide and pathogens interact to elevate honey bee mortality across the life cycle. *Environmental Microbiology*. doi:10.1111/1462-2920.12426

Douglas, M.R., Rohr, J.R. & Tooker, J.F. (2014) Neonicotinoid insecticide travels through a soil food chain, disrupting biological control of non-target pests and decreasing soya bean yield. *Journal of Applied Ecology,* **52,** 250-260.

Drescher, W. & Geusen-Pfister, H. (1991) Comparative testing of the oral toxicity of acephate, dimethoate and methomyl to honeybees, bumblebees and syrphidae. *VI International Symposium on Pollination 288*, pp. 133-138. Acta Horticulturae.

Duchateau, M.J. & Velthuis, H. (1988) Development and reproductive strategies in *Bombus terrestris* colonies. *Behaviour,* **107,** 186-207.

Duchateau, M.J. & Velthuis, H.H.W. (1989) Ovarian development and egg-laying in workers of *Bombus terrestris*. *Entomologia Experimentalis Et Applicata,* **51,** 199-213.

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

EFSA Panel on Plant Protection Products and their Residues (PPR) (2013) Scientific opinion on the science behind the development of a risk assessment of plant protection products on bees (*Apis mellifera*, *Bombus* spp. and solitary bees). *EFSA Journal,* **11,** 3295.

Eilers, E.J., Kremen, C., Greenleaf, S.S., Garber, A.K. & Klein, A.-M. (2011) Contribution of pollinator-mediated crops to nutrients in the human food supply. *PLoS ONE,* **6**, e21363.

Eiri, D.M. & Nieh, J.C. (2012) A nicotinic acetylcholine receptor agonist affects honey bee sucrose responsiveness and decreases waggle dancing. *Journal of Experimental Biology,* **215,** 2022-2029.

Elston, C., Thompson, H.M. & Walters, K.F.A. (2013) Sub-lethal effects of thiamethoxam, a neonicotinoid pesticide, and propiconazole, a dmi fungicide, on colony initiation in bumblebee (*Bombus terrestris*) micro-colonies. *Apidologie,* **44,** 563-574.

Enayati, A.A., Ranson, H. & Hemingway, J. (2005) Insect glutathione transferases and insecticide resistance. *Insect Molecular Biology,* **14,** 3-8.

EPA (2014) Guidance for assessing pesticide risks to bees. Office of Pesticide Programs, United States Environmental Protection Agency, Washington.

EU (2013) Regulation (EC) no. 1107/2009 of the European parliament and of the Council concerning the placing of plant protection products on the market and repealing council directives 79/117/EEC and 91/414/EEC. *1107/2009*.

European Commission (2002) Guidance Document on Terrestrial Ecotoxicology Under Council Directive 91/414/EEC.

FAO (2015) Pesticide use in active ingredient on arable land and permanent crops (tonnes per 1000 ha) by country. FAO

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

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

FERA (2012) Pesticide Usage Survey Statistics. The Food and Environment Research Agency, York. Retrieved from https://secure.fera.defra.gov.uk/pusstats on 28/2/13

Field, A. (2009) *Discovering statistics using ibm spss statistics,* 3rd edn. Sage, London.

Fischer, J., Müller, T., Spatz, A.-K., Greggers, U., Grüenewald, B. & Menzel, R. (2014) Neonicotinoids interfere with specific components of navigation in honeybees. *PLoS ONE,* **9**, e91364.

Fishel, F.M. (2007) Pesticide use trends in the U.S.: Global comparison. Institute of Food and Agricultural Sciences / United States Department of Agriculture. Pl-143.

Fitzpatrick, Ú., Murray, T.E., Paxton, R.J., Breen, J., Cotton, D., Santorum, V. & Brown, M.J.F. (2007) Rarity and decline in bumblebees - a test of causes and correlates in the Irish fauna. *Biological Conservation,* **136,** 185-194.

Foley, J.A., DeFries, R., Asner, G.P., Barford, C., Bonan, G., Carpenter, S.R., Chapin, F.S., Coe, M.T., Daily, G.C., Gibbs, H.K., Helkowski, J.H., Holloway, T., Howard, E.A., Kucharik, C.J., Monfreda, C., Patz, J.A., Prentice, C., Ramankutty, N. & Snyder, P.K. (2005) Global consequences of land use. *Science,* **309,** 570-574.

Forsgren, E., Olofsson, T.C., Vásquez, A. & Fries, I. (2010) Novel lactic acid bacteria inhibiting *Paenibacillus larvae* in honey bee larvae. *Apidologie,* **41,** 99-108.

Fraune, S. & Bosch, T.C. (2010) Why bacteria matter in animal development and evolution. *BioEssays,* **32,** 571-580.

Fukuto, T.R. (1990) Mechanism of action of organophosphorus and carbamate insecticides. *Environmental Health Perspectives,* **87,** 245-254.

Fürst, M.A., McMahon, D.P., Osborne, J.L., Paxton, R.J. & Brown, M.J.F. (2014) Disease associations between honeybees and bumblebees as a threat to wild pollinators. *Nature,* **506,** 364-366.

Gallai, N., Salles, J.M., Settele, J. & Vaissière, B.E. (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological Economics,* **68,** 810-821.

Garibaldi, L.A., Steffan-Dewenter, I., Winfree, R., Aizen, M.A., Bommarco, R., Cunningham, S.A., Kremen, C., Carvalheiro, L.G., Harder, L.D., Afik, O., Bartomeus, I., Benjamin, F., Boreux, V., Cariveau, D., Chacoff, N.P., Dudenhöffer, J.H., Freitas, B.M., Ghazoul, J., Greenleaf, S., Hipólito, J., Holzschuh, A., Howlett, B., Isaacs, R., Javorek, S.K., Kennedy, C.M., Krewenka, K.M., Krishnan, S., Mandelik, Y., Mayfield, M.M., Motzke, I., Munyuli, T., Nault, B.A., Otieno, M., Petersen, J., Pisanty, G., Potts, S.G., Rader, R., Ricketts, T.H., Rundlöf, M., Seymour, C.L., Schüepp, C., Szentgyörgyi, H., Taki, H., Tscharntke, T., Vergara, C.H., Viana, B.F., Wanger, T.C., Westphal, C., Williams, N. & Klein, A.M. (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science,* **339,** 1608-1611.

Garthwaite, D.G., Barker, I., Parrish, G., Smith, L., Chippindale, C. & Pietravalle, S. (2010) Arable crops in the United Kingdom 2010. *Pesticide Usage Survey Reports,* DEFRA, **235.**

Garthwaite, D.G., Hudson, S., Barker, I., Parrish, G., Smith, L., Pietravalle, S. (2012a) Arable crops in the United Kingdom. *Pesticide Usage Survey Reports*. DEFRA. **250.**

Garthwaite, D.G., Hudson, S., Barker, I., Parrish, G., Smith, L., Pietravalle, S. (2012b) Soft fruit in the United Kingdom 2012. *Pesticide Usage Survey Reports*. DEFRA. **251.**

Gavrilescu, M. (2005) Fate of pesticides in the environment and its bioremediation. *Engineering in Life Sciences,* **5,** 497-526.

Genersch, E. (2010) American foulbrood in honeybees and its causative agent, *Paenibacillus larvae*. *Journal of Invertebrate Pathology,* **103,** S10-S19.

Genersch, E. & Aubert, M. (2010) Emerging and re-emerging viruses of the honey bee (*Apis mellifera* L.). *Veterinary research,* **41,** 54.

Genersch, E., Yue, C., Fries, I. & de Miranda, J.R. (2006) Detection of deformed wing virus, a honey bee viral pathogen, in bumble bees (*Bombus terrestris* and *Bombus pascuorum*) with wing deformities. *Journal of Invertebrate Pathology,* **91,** 61-63.

Gevao, B., Semple, K.T. & Jones, K.C. (2000) Bound pesticide residues in soils: a review. *Environmental Pollution,* **108,** 3-14.

Ghazoul, J. (2005a) Buzziness as usual? Questioning the global pollination crisis. *Trends in Ecology & Evolution,* **20,** 367-373.

Ghazoul, J. (2005b) Response to Steffan-Dewenter *et al*.: Questioning the global pollination crisis. *Trends in Ecology & Evolution,* **20,** 652-653.

Gibbons, D., Morrissey, C. & Mineau, P. (2014) A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. *Environmental Science and Pollution Research,* **22,** 103-118.

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

Gill, R.J., Ramos-Rodriguez, O. & Raine, N.E. (2012) Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature,* **491,** 105-108.

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

Gilliam, M. (1997) Identification and roles of non-pathogenic microflora associated with honey bees. *FEMS Microbiology Letters,* **155,** 1-10.

Girolami, V., Mazzon, L., Squartini, A., Mori, N., Marzaro, M., Di Bernardo, A., Greatti, M., Giorio, C. & Tapparo, A. (2009) Translocation of neonicotinoid insecticides from coated seeds to seedling guttation drops: A novel way of intoxication for bees. *Journal of Economic Entomology,* **102,** 1808-1815.

Godfray, H.C.J., Blacquiere, T., Field, L.M., Hails, R.S., Petrokofsky, G., Potts, S.G., Raine, N.E., Vanbergen, A.J. & McLean, A.R. (2014) A restatement of the natural science evidence

base concerning neonicotinoid insecticides and insect pollinators. *Proceedings of the Royal Society B: Biological Sciences,* **281:20140558**.

Goka, K. (2010) Introduction to the special feature for ecological risk assessment of introduced bumblebees: Status of the European bumblebee, *Bombus terrestris*, in Japan as a beneficial pollinator and an invasive alien species. *Applied Entomology and Zoology,* **45,** 1-6.

Goulson, D. (2013) Review: An overview of the environmental risks posed by neonicotinoid insecticides. *Journal of Applied Ecology,* **50,** 977-987.

Goulson, D. (2015) Neonicotinoids impact bumblebee colony fitness in the field; a reanalysis of the uk's food & environment research agency 2012 experiment. *PeerJ,* **3:e854**.

Goulson, D., Nicholls, E., Botías, C. & Rotheray, E.L. (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science***,** Doi:10.1126/science.1255957.

Goulson, D., Peat, J., Stout, J.C., Tucker, J., Darvill, B., Derwent, L.C. & Hughes, W.O.H. (2002) Can alloethism in workers of the bumblebee, *Bombus terrestris*, be explained in terms of foraging efficiency? *Animal Behaviour,* **64,** 123-130.

Gradish, A., Scott-Dupree, C. & Cutler, G. (2012) Susceptibility of *Megachile rotundata* to insecticides used in wild blueberry production in atlantic canada. *Journal of Pest Science,* **85,** 133-140.

Graystock, P., Goulson, D. & Hughes, W.O. (2014) The relationship between managed bees and the prevalence of parasites in bumblebees. *PeerJ,* **2:e522**.

Graystock, P., Yates, K., Evison, S.E.F., Darvill, B., Goulson, D. & Hughes, W.O.H. (2013) The trojan hives: Pollinator pathogens, imported and distributed in bumblebee colonies. *Journal of Applied Ecology,* **50,** 1207-1215.

Greig-Smith, P.W., Thompson, H.M., Hardy, A.R., Bew, M.H., Findlay, E. & Stevenson, J.H. (1994) Incidents of poisoning of honeybees (*Apis mellifera)* by agricultural pesticides in Great Britain 1981-1991. *Crop Protection,* **13,** 567-581.

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

Gross, M. (2014) Systemic pesticide concerns extend beyond the bees. *Current Biology,* **24,** R717-R720.

Hallmann, C.A., Foppen, R.P., van Turnhout, C.A., de Kroon, H. & Jongejans, E. (2014) Declines in insectivorous birds are associated with high neonicotinoid concentrations. *Nature,* **511,** 341-352.

Hanley, M.E., Franco, M., Dean, C.E., Franklin, E.L., Harris, H.R., Haynes, A.G., Rapson, S.R., Rowse, G., Thomas, K.C., Waterhouse, B.R. & Knight, M.E. (2011) Increased bumblebee abundance along the margins of a mass flowering crop: evidence for pollinator spill-over. *Oikos,* **120,** 1618-1624.

Haydak, M.H. (1970) Honey bee nutrition. *Annual Review of Entomology,* **15,** 143 - 156.

Hegland, S.J., Nielsen, A., Lázaro, A., Bjerknes, A.L. & Totland, Ø. (2009) How does climate warming affect plant-pollinator interactions? *Ecology letters,* **12,** 184-195.

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

Henry, M., Bertrand, C., Le Féon, V., Requier, F., Odoux, J.-F., Aupinel, P., Bretagnolle, V. & Decourtye, A. (2014) Pesticide risk assessment in free-ranging bees is weather and landscape dependent. *Nature Communications,* **5,** 4359.

Heywood, V.H. (1995) *Global biodiversity assessment. United nations environment programme*. Cambridge University Press, Cambridge.

Higes, M., García-Palencia, P., Martín-Hernández, R. & Meana, A. (2007) Experimental infection of *Apis mellifera* honeybees with *Nosema ceranae* (*Microsporidia*). *Journal of Invertebrate Pathology,* **94,** 211-217.

Higes, M., Martin-Hernandez, R., Botias, C., Bailon, E.G., Gonzalez-Porto, A.V., Barrios, L., del Nozal, M.J., Bernal, J.L., Jimenez, J.J., Palencia, P.G. & Meana, A. (2008) How natural infection by *Nosema ceranae* causes honeybee colony collapse. *Environmental Microbiology,* **10,** 2659-2669.

Hladik, M.L., Kolpin, D.W. & Kuivila, K.M. (2014) Widespread occurrence of neonicotinoid insecticides in streams in a high corn and soybean producing region, USA. *Environmental Pollution,* **193,** 189-196.

Hoehn, P., Tscharntke, T., Tylianakis, J.M. & Steffan-Dewenter, I. (2008) Functional group diversity of bee pollinators increases crop yield. *Proceedings of the Royal Society B-Biological Sciences,* **275,** 2283-2291.

Højsgaard, S. & Halekoh, U. (2014) doBy: Groupwise statistics, lsmeans, linear contrasts, utilities.

Holm, S.N. (1972) Weight and life length of hibernating bumble bee queens (Hymenoptera: Bombidae) under controlled conditions. *Entomologica Scandinavica,* **3,** 313-320.

Hothorn, T., Bretz, F. & Westfall, P. (2008) Simultaneous inference in general parametric models. *Biometrical Journal,* **50,** 346-363.

Howard, D.C., Watkins, J.W., Clarke, R.T., Barnett, C.L. & Stark, G.J. (2003) Estimating the extent and change in broad habitats in great britain. *Journal of Environmental Management,* **67,** 219-227.

Huseth, A.S., Lindholm, J., Groves, C.L. & Groves, R.L. (2014) Variable concentration of soil-applied insecticides in potato over time: Implications for management of *Leptinotarsa decemlineata*. *Pest Management Science,* **70,** 1863-1871.

Imhoof, B. & Schmid-Hempel, P. (1998) Patterns of local adaptation of a protozoan parasite to its bumblebee host. *Oikos,* **82,** 59-65.

Inoue, M.N., Yokoyama, J. & Washitani, I. (2008) Displacement of Japanese native bumblebees by the recently introduced *Bombus terrestris* (L.)(Hymenoptera: Apidae). *Journal of Insect Conservation,* **12,** 135-146.

Ippolito, A., Kattwinkel, M., Rasmussen, J.J., Schäfer, R.B., Fornaroli, R. & Liess, M. (2015) Modeling global distribution of agricultural insecticides in surface waters. *Environmental Pollution,* **198,** 54-60.

IUCN (2014) The IUCN red list of threatened species. Version 2014.2013.

James, R. & Xu, J. (2012) Mechanisms by which pesticides affect insect immunity. *Journal of Invertebrate Pathology,* **109,** 175-182.

Jänsch, S., Frampton, G.K., Römbke, J., Van den Brink, P.J. & Scott-Fordsmand, J.J. (2006) Effects of pesticides on soil invertebrates in model ecosystem and field studies: A review and comparison with laboratory toxicity data. *Environmental Toxicology and Chemistry,* **25,** 2490-2501.

Johnsen, K., Jacobsen, C.S., Torsvik, V. & Sørensen, J. (2001) Pesticide effects on bacterial diversity in agricultural soils – a review. *Biology and Fertility of Soils,* **33,** 443-453.

Johnson, J.B. & Omland, K.S. (2004) Model selection in ecology and evolution. *Trends in Ecology & Evolution,* **19,** 101-108.

Johnson, R.M., Ellis, M.D., Mullin, C.A. & Frazier, M. (2010) Pesticides and honey bee toxicity - USA. *Apidologie,* **41,** 312-331.

Jones, A., Harrington, P. & Turnbull, G. (2014) Neonicotinoid concentrations in arable soils after seed treatment applications in preceding years. *Pest Management Science,* **70,** 1780-1784.

Jones, C.M. (2014) Bumble bees and their parasites across european communities: *Sphaerularia bombi* in native and non-native hosts. PhD, Royal Holloway University.

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

Kapustjanskij, A., Streinzer, M., Paulus, H.F. & Spaethe, J. (2007) Bigger is better: implications of body size for flight ability under different light conditions and the evolution of alloethism in bumblebees. *Functional Ecology,* **21,** 1130–1136.

Karazafiris, E., Tananaki, C., Menkissoglu-Spiroudi, U. & Thrasyvoulou, A. (2008) Residue distribution of the acaricide coumaphos in honey following application of a new slow-release formulation. *Pest Management Science,* **64,** 165-171.

Kelly, D.W., Poulin, R., Tompkins, D.M. & Townsend, C.R. (2010) Synergistic effects of glyphosate formulation and parasite infection on fish malformations and survival. *Journal of Applied Ecology,* **47,** 498-504.

Kiesecker, J.M. (2002) Synergism between trematode infection and pesticide exposure: A link to amphibian limb deformities in nature? *Proceedings of the National Academy of Sciences of the United States of America,* **99,** 9900-9904.

Killer, J., Votavová, A., Valterová, I., Vlková, E., Rada, V. & Hroncová, Z. (2014) *Lactobacillus bombi* sp. nov., from the digestive tract of laboratory-reared bumblebee queens (*Bombus terrestris*). *International Journal of Systematic and Evolutionary Microbiology,* **64,** 2611-2617.

Kissinger, C.N., Cameron, S.A., Thorp, R.W., White, B. & Solter, L.F. (2011) Survey of bumble bee (*Bombus*) pathogens and parasites in Illinois and selected areas of Northern California and Southern Oregon. *Journal of Invertebrate Pathology,* **107,** 220-224.

Klaenhammer, T.R. (1988) Bacteriocins of lactic acid bacteria. *Biochimie,* **70,** 337-349.

Klee, J., Besana, A.M., Genersch, E., Gisder, S., Nanetti, A., Tam, D.Q., Chinh, T.X., Puerta, F., Ruz, J.M., Kryger, P., Message, D., Hatjina, F., Korpela, S., Fries, I. & Paxton, R.J. (2007) Widespread dispersal of the microsporidian *Nosema ceranae*, an emergent pathogen of the western honey bee, *Apis mellifera*. *Journal of Invertebrate Pathology,* **96,** 1-10.

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

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

Knight, M.E., Osborne, J.L., Sanderson, R.A., Hale, R.J., Martin, A.P. & Goulson, D. (2009) Bumblebee nest density and the scale of available forage in arable landscapes. *Insect Conservation and Diversity,* **2,** 116-124.

Koch, H., Cisarovsky, G. & Schmid-Hempel, P. (2012) Ecological effects on gut bacterial communities in wild bumblebee colonies. *Journal of Animal Ecology,* **81,** 1202-1210.

Koch, H. & Schmid-Hempel, P. (2011a) Bacterial communities in central european bumblebees: low diversity and high specificity. *Microbial Ecology,* **62,** 121-133.

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

Koch, H. & Schmid-Hempel, P. (2012) Gut microbiota instead of host genotype drive the specificity in the interaction of a natural host-parasite system. *Ecology letters,* **15,** 1095-1103.

Korner, P. & Schmid-Hempel, P. (2003) Effects of sperm on female longevity in the bumble-bee *Bombus terrestris* L. *Proceedings of the Royal Society B-Biological Sciences,* **270,** S227-S229.

Kosior, A., Celary, W., Olejniczak, P., Fijał, J., Król, W., Solarz, W. & Płonka, P. (2007) The decline of the bumble bees and cuckoo bees (Hymenoptera : Apidae : Bombini) of Western and Central Europe. *Oryx,* **41,** 79-88.

Kralj, J. & Fuchs, S. (2010) *Nosema* sp influences flight behavior of infected honey bee (*Apis mellifera*) foragers. *Apidologie,* **41,** 21-28.

Kremen, C., Williams, N.M. & Thorp, R.W. (2002) Crop pollination from native bees at risk from agricultural intensification. *Proceedings of the National Academy of Sciences,* **99,** 16812-16816.

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

Laycock, I., Cotterell, K.C., O'Shea-Wheller, T.A. & Cresswell, J.E. (2013) Effects of the neonicotinoid pesticide thiamethoxam at field-realistic levels on microcolonies of *Bombus terrestris* worker bumble bees. *Ecotoxicology and Environmental Safety,* **100,** 153-158.

Laycock, I. & Cresswell, J.E. (2013) Repression and recuperation of brood production in *Bombus terrestris* bumble bees exposed to a pulse of the neonicotinoid pesticide imidacloprid. *PLoS ONE,* **8**, e79872.

Laycock, I., Lenthall, K.M., Barratt, A.T. & Cresswell, J.E. (2012) Effects of imidacloprid, a neonicotinoid pesticide, on reproduction in worker bumble bees (*Bombus terrestris*). *Ecotoxicology,* **21,** 1937-1945.

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

Lopez-Vaamonde, C., Raine, N.E., Koning, J.W., Brown, R.M., Pereboom, J.J.M., Ings, T.C., Ramos-Rodriguez, O., Jordan, W.C. & Bourke, A.F.G. (2009) Lifetime reproductive success and longevity of queens in an annual social insect. *Journal of Evolutionary Biology,* **22,** 983-996.

Macfarlane, R. & Gurr, L. (1995) Distribution of bumble bees in New Zealand. *New Zealand Entomologist,* **18,** 29-36.

Martin, S.J., Highfield, A.C., Brettell, L., Villalobos, E.M., Budge, G.E., Powell, M., Nikaido, S. & Schroeder, D.C. (2012) Global honey bee viral landscape altered by a parasitic mite. *Science,* **336,** 1304-1306.

Martinson, V.G., Danforth, B.N., Minckley, R.L., Rueppell, O., Tingek, S. & Moran, N.A. (2011) A simple and distinctive microbiota associated with honey bees and bumble bees. *Molecular Ecology,* **20,** 619-628.

May, R.M. (1988) How many species are there on earth? *Science,* **241,** 1441-1449.

Mayack, C. & Naug, D. (2009) Energetic stress in the honeybee *Apis mellifera* from *Nosema ceranae* infection. *Journal of Invertebrate Pathology,* **100,** 185-188.

McMahon, D.P., Fürst, M.A., Caspar, J., Theodorou, P., Brown, M.J.F. & Paxton, R.J. (2015) A sting in the spit: Widespread cross-infection of multiple RNA viruses across wild and managed bees. *Journal of Animal Ecology*, DOI:10.1111/1365/2656.12345

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

Meeus, I., de Miranda, J.R., de Graaf, D.C., Wäckers, F. & Smagghe, G. (2014) Effect of oral infection with Kashmir bee virus and Israeli acute paralysis virus on bumblebee (*Bombus terrestris*) reproductive success. *Journal of Invertebrate Pathology,* **121,** 64-69.

Memmott, J., Craze, P.G., Waser, N.M. & Price, M.V. (2007) Global warming and the disruption of plant–pollinator interactions. *Ecology letters,* **10,** 710-717.

Menzel, R. & Müller, U. (1996) Learning and memory in honeybees: From behavior to neural substrates. *Annual Review of Neuroscience,* **19,** 379-404.

Meyer, W.B. & Turner, B.L. (1992) Human population growth and global land-use/cover change. *Annual Review of Ecology and Systematics***, 23**, 39-61.

Michener, C.D. (2000) *The bees of the world*. JHU Press, Baltimore.

Mills, M. (2011) *Introducing survival and event history analysis*. SAGE Pulications Ltd, London.

Moffat, C., Pacheco, J.G., Sharp, S., Samson, A.J., Bollan, K.A., Huang, J., Buckland, S.T. & Connolly, C.N. (2015) Chronic exposure to neonicotinoids increases neuronal

vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*). *The FASEB Journal***,** fj. 14-267179.

Mommaerts, V., Reynders, S., Boulet, J., Besard, L., Sterk, G. & Smagghe, G. (2010) Risk assessment for side-effects of neonicotinoids against bumblebees with and without impairing foraging behavior. *Ecotoxicology,* **19,** 207-215.

Mora, C., Tittensor, D.P., Adl, S., Simpson, A.G.B. & Worm, B. (2011) How many species are there on earth and in the ocean? *PLoS Biology,* **9,** e1001127.

Moran, N.A., Hansen, A.K., Powell, J.E. & Sabree, Z.L. (2012) Distinctive gut microbiota of honey bees assessed using deep sampling from individual worker bees. *PLoS ONE,* **7,** e36393.

Moret, Y. & Schmid-Hempel, P. (2004) Social life-history response to individual immune challenge of workers of *Bombus terrestris* L.: A possible new cooperative phenomenon. *Ecology Letters,* **7,** 146-152.

Müller, C.B. & Schmid-Hempel, P. (1992) Variation in life-history pattern in relation to worker mortality in the bumblebee, *Bombus lucorum*. *Functional Ecology,* **6,** 48-56.

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

Murray, T.E., Coffey, M.F., Kehoe, E. & Horgan, F.G. (2013) Pathogen prevalence in commercially reared bumble bees and evidence of spillover in conspecific populations. *Biological Conservation,* **159,** 269-276.

Mutinelli, F. (1996) Practical application of antibacterial drugs for the control of honey bee diseases. *Apiacta* **38**,149-155.

Navarro, A., Tauler, R., Lacorte, S. & Barceló, D. (2010) Occurrence and transport of pesticides and alkylphenols in water samples along the Ebro River Basin. *Journal of Hydrology,* **383,** 18-29.

Nieto, A., Roberts, S.P.M., Kemp, J., Rasmont, P., Kuhlmann, M., García Criado, M., Biesmeijer, J.C., Bogusch, P., Dathe, H.H., De la Rúa, P., De Meulemeester, T., Dehon, M., Dewulf, A., Ortiz-Sánchez, F.J., Lhomme, P., Pauly, A., Potts, S.G., Praz, C., Quaranta, M., Radchenko, V.G., Scheuchl, E., Smit, J., Straka, J., Terzo, M., Tomozii, B., Window, J. & Michez, D. (2014) European Red List of bees. Luxembourg: Publication Office of the European Union.

Oldroyd, B.P. (1999) Coevolution while you wait: *Varroa jacobsoni*, a new parasite of western honeybees, *Trends in Ecology & Evolution*, **14**, 312-315.

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

Ollerton, J., Winfree, R. & Tarrant, S. (2011) How many flowering plants are pollinated by animals? *Oikos,* **120,** 321-326.

Olofsson, T.C. & Vásquez, A. (2008) Detection and identification of a novel lactic acid bacterial flora within the honey stomach of the honeybee *Apis mellifera*. *Current Microbiology,* **57,** 356-363.

Omar, S. & Abdel-Sater, M. (2001) Microbial populations and enzyme activities in soil treated with pesticides. *Water, Air, and Soil Pollution,* **127,** 49-63.

Osborne, J.L., Williams, I.H. & Corbet, S.A. (1991) Bees, pollination and habitat change in the European community. *Bee World,* **72,** 99-116.

Otterstatter, M.C., Gegear, R.J., Colla, S.R. & Thomson, J.D. (2005) Effects of parasitic mites and protozoa on the flower constancy and foraging rate of bumble bees. *Behavioral Ecology and Sociobiology,* **58,** 383-389.

Otterstatter, M.C. & Thomson, J.D. (2008) Does pathogen spillover from commercially reared bumble bees threaten wild pollinators? *PLoS ONE,* **3**, e2771

Otti, O. & Schmid-Hempel, P. (2007) *Nosema bombi*: A pollinator parasite with detrimental fitness effects. *Journal of Invertebrate Pathology,* **96,** 118-124.

Otti, O. & Schmid-Hempel, P. (2008) A field experiment on the effect of *Nosema bombi* in colonies of the bumblebee *Bombus terrestris*. *Ecological Entomology,* **33,** 577-582.

Ouwehand, A.C., Salminen, S. & Isolauri, E. (2002) Probiotics: An overview of beneficial effects. *Antonie Van Leeuwenhoek,* **82,** 279-289.

Palmer, M.J., Moffat, C., Saranzewa, N., Harvey, J., Wright, G.A. & Connolly, C.N. (2013) Cholinergic pesticides cause mushroom body neuronal inactivation in honeybees. *Nature Communications,* **4,** 1634.

Paxton, R.J., Klee, J., Korpela, S. & Fries, I. (2007) *Nosema ceranae* has infected *Apis mellifera* in Europe since at least 1998 and may be more virulent than *Nosema apis*. *Apidologie,* **38,** 558-565.

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

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

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

Pilling, E.D., Bromley-Challenor, K.A.C., Walker, C.H. & Jepson, P.C. (1995) Mechanism of synergism between the pyrethroid insecticide λ-cyhalothrin and the imidazole fungicide prochloraz, in the honeybee (*Apis-mellifera* L). *Pesticide Biochemistry and Physiology,* **51,** 1-11.

Pilling, E.D. & Jepson, P.C. (1993) Synergism between ebi fungicides and a pyrethroid insecticide in the honeybee (*apis mellifera*). *Pesticide Science,* **39,** 293-297.

Pimentel, D. & Lehman, H. (1993) *The pesticide question: Environment, economics, and ethics*. Chapman and Hall, New York.

Pimm, S.L. & Raven, P. (2000) Biodiversity: Extinction by numbers. *Nature,* **403,** 843-845.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. & R Core Team (2015) Linear and nonlinear mixed effects models.

Pisa, L.W., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Downs, C.A., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., McField, M., Morrissey, C.A., Noome, D.A., Settele, J., Simon-Delso, N., Stark, J.D., Van der Sluijs, J.P., Van Dyck, H. & Wiemers, M. (2014) Effects of neonicotinoids and fipronil on non-target invertebrates. *Environmental Science and Pollution Research,* **22,** 68-102.

Pistorius, J., Bischoff, G., Heimbach, U. & Stähler, M. (2009) Bee poisoning incidents in Germany in spring 2008 caused by abrasion of active substance from treated seeds during sowing of maize. *Julius-Kühn-Archiv***, 423,** 118-126.

Pohorecka, K., Skubida, P., Miszczak, A., Semkiw, P., Sikorski, P., Zagibajło, K., Teper, D., Kołtowski, Z., Skubida, M., Zdańska, D. & Bober, A. (2012) Residues of neonicotinoid insecticides in bee collected plant materials from oilseed rape crops and their effect on bee colonies. *Journal of Apicultural Science,* **56,** 115-134.

Poinar, G.O. & Van Der Laan, P. (1972) Morphology and life history of *Sphaerularia bombi*. *Nematologica,* **18,** 239-252.

Porter, R.D. & Wiemeyer, S.N. (1969) Dieldrin and DDT: Effects on sparrow hawk eggshells and reproduction. *Science,* **165,** 199-200.

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

Potts, S.G., Roberts, S.P.M., Dean, R., Marris, G., Brown, M.A., Jones, R., Neumann, P. & Settele, J. (2010b) Declines of managed honey bees and beekeepers in Europe. *Journal of Apicultural Research,* **49,** 15-22.

Purwar, J. & Sachan, G. (2006) Synergistic effect of entomogenous fungi on some insecticides against bihar hairy caterpillar *Spilarctia obliqua* (Lepidoptera: Arctiidae). *Microbiological Research,* **161,** 38-42.

Ramakrishnan, R., Suiter, D.R., Nakatsu, C.H., Humber, R.A. & Bennett, G.W. (1999) Imidacloprid-enhanced *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) susceptibility to the entomopathogen metarhizium anisopliae. *Journal of Economic Entomology,* **92,** 1125-1132.

Rands, M. (1985) Pesticide use on cereals and the survival of grey partridge chicks: A field experiment. *Journal of Applied Ecology***,** 49-54.

Rani, M.S., Lakshmi, K.V., Devi, P.S., Madhuri, R.J., Aruna, S., Jyothi, K., Narasimha, G. & Venkateswarlu, K. (2008) Isolation and characterization of a chlorpyrifos-degrading bacterium from agricultural soil and its growth response. *African Journal of Microbiology Research,* **2,** 026-031.

Ratcliffe, D.A. (1967) Decrease in eggshell weight in certain birds of prey. *Nature*, **215,** 208-210

Rath, W. (1999) Co-adaptation of *Apis cerana* Fabr. and *Varroa jacobsoni* Oud. *Apidologie,* **30**, 97-110.

Ravoet, J., De Smet, L., Meeus, I., Smagghe, G., Wenseleers, T. & de Graaf, D.C. (2014) Widespread occurrence of honey bee pathogens in solitary bees. *Journal of Invertebrate Pathology,* **122,** 55-58.

Regan, E.C., Santini, L., Ingwall-King, L., Hoffmann, M., Rondinini, C., Symes, A., Taylor, J. & Butchart, S.H. (2015) Global trends in the status of bird and mammal pollinators. *Conservation Letters*, DOI:10.1111/conl.12162

Roulston, T.H. & Goodell, K. (2011) The role of resources and risks in regulating wild bee populations. *Annual Review of Entomology,* **56,** 293-312.

RStudio (2012) Rstudio: Integrated development environment for R [computer software]. Boston, MA.

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

Ruiz-González, M.X., Bryden, J., Moret, Y., Reber-Funk, C., Schmid-Hempel, P. & Brown, M.J.F. (2012) Dynamic transmission, host quality, and population structure in a multihost parasite of bumblebees. *Evolution,* **66,** 3053-3066.

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

Rutrecht, S.T. & Brown, M.J.F. (2008b) Within colony dynamics of *Nosema bombi* infections: disease establishment, epidemiology and potential vertical transmission. *Apidologie,* **39,** 504-514.

Rutrecht, S.T. & Brown, M.J.F. (2009) Differential virulence in a multiple-host parasite of bumble bees: resolving the paradox of parasite survival? *Oikos,* **118,** 941-949.

Rutrecht, S.T., Klee, J. & Brown, M.J.F. (2007) Horizontal transmission success of *Nosema bombi* to its adult bumble bee hosts: effects of dosage, spore source and host age. *Parasitology,* **134,** 1719-1726.

Sakwińska, O. (2002) Response to fish kairomone in *Daphnia galeata* life history traits relies on shift to earlier instar at maturation. *Oecologia,* **131,** 409-417.

Samson-Robert, O., Labrie, G., Chagnon, M. & Fournier, V. (2014) Neonicotinoid-contaminated puddles of water represent a risk of intoxication for honey bees. *PLoS ONE,* **9,** e108443.

Secretariat of the Convention on Biological Diversity (2015) Convention on biological diversity. URL: http://www.cbd.int/history/. Access date: 28/02/2015

Schmid-Hempel, P. (1998) Parasites in *social insects*. Princeton University Press, Princeton, New Jersey.

Schmid-Hempel, P. & Loosli, R. (1998) A contribution to the knowledge of *Nosema* infections in bumble bees, *Bombus* spp. *Apidologie,* **29,** 525-535.

Schmid-Hempel, R., Eckhardt, M., Goulson, D., Heinzmann, D., Lange, C., Plischuk, S., Escudero, L.R., Salathé, R., Scriven, J.J. & Schmid-Hempel, P. (2014) The invasion of southern South America by imported bumblebees and associated parasites. *Journal of Animal Ecology,* **83,** 823-837.

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

Scholer, J. & Krischik, V. (2014) Chronic exposure of imidacloprid and clothianidin reduce queen survival, foraging, and nectar storing in colonies of *Bombus impatiens*. *PLoS ONE,* **9**, e91573

Scott-Dupree, C.D., Conroy, L. & Harris, C.R. (2009) Impact of currently used or potentially useful insecticides for canola agroecosystems on Bombus impatiens (Hymenoptera: Apidae), *Megachile rotundata* (Hymentoptera: Megachilidae), and *Osmia lignaria* (Hymenoptera: Megachilidae). *Journal of Economic Entomology,* **102,** 177-182.

Shykoff, J.A. & Schmid-Hempel, P. (1991a) Genetic relatedness and eusociality- parasite-mediated selection on the genetic composition of groups. *Behavioral Ecology and Sociobiology,* **28,** 371-376.

Shykoff, J.A. & Schmid-Hempel, P. (1991b) Incidence and effects of 4 parasites in natural-populations of bumble bees in Switzerland. *Apidologie,* **22,** 117-125.

Shykoff, J.A. & Schmid-Hempel, P. (1991c) Parasites delay worker reproduction in bumblebees: consequences for eusociality. *Behavioral Ecology,* **2,** 242-248.

Singh, R., Levitt, A.L., Rajotte, E.G., Holmes, E.C., Ostiguy, N., vanEngelsdorp, D., Lipkin, W.I., dePamphilis, C.W., Toth, A.L. & Cox-Foster, D.L. (2010) RNA viruses in hymenopteran pollinators: evidence of inter-taxa virus transmission via pollen and potential impact on non-*Apis* Hymenopteran species. *PLoS ONE,* **5**, e14357.

Soderlund, D.M. & Bloomquist, J.R. (1989) Neurotoxic actions of pyrethroid insecticides. *Annual Review of Entomology,* **34,** 77-96.

Spaethe, J., Brockmann, A., Halbig, C. & Tautz, J. (2007) Size determines antennal sensitivity and behavioral threshold to odors in bumblebee workers. *Naturwissenschaften,* **94,** 733-739.

Spaethe, J. & Chittka, L. (2003) Interindividual variation of eye optics and single object resolution in bumblebees. *Journal of Experimental Biology,* **206,** 3447-3453.

Spaethe, J. & Weidenmüller, A. (2002) Size variation and foraging rate in bumblebees (*Bombus terrestris*). *Insectes Sociaux,* **49,** 142-146.

Stanley, D., Smith, K. & Raine, N.E. (In Prep) Exposure to acetylcholinesterase inhibitors alters the physiology and motor function of honeybees.

Stanley, D.A., Gunning, D. & Stout, J.C. (2013) Pollinators and pollination of oilseed rape crops (*Brassica napus* L.) in Ireland: ecological and economic incentives for pollinator conservation. *Journal of Insect Conservation,* **17,** 1181-1189.

Steffan-Dewenter, I., Potts, S.G. & Packer, L. (2005) Pollinator diversity and crop pollination services are at risk. *Trends in Ecology & Evolution,* **20,** 651-652.

Stevenson, J.H. (1978) The acute toxicity of unformolated pesticides to worker honey bees (*Apis mellifera* L.). *Plant Pathology,* **27,** 38-40.

Stevenson, J.H. & Racey, P.A. (1966) Toxicity of insecticides to bumblebees. *Rothamsted Report***,** 176.

Stewart, S.D., Lorenz, G.M., Catchot, A.L., Gore, J., Cook, D., Skinner, J., Mueller, T.C., Johnson, D.R., Zawislak, J. & Barber, J. (2014) Potential exposure of pollinators to neonicotinoid insecticides from the use of insecticide seed treatments in the Mid-Southern United States. *Environmental Science & Technology,* **48,** 9762-9769.

Stout, J.C. & Morales, C.L. (2009) Ecological impacts of invasive alien species on bees. *Apidologie,* **40,** 388-409.

Suchail, S., Guez, D. & Belzunces, L.P. (2000) Characteristics of imidacloprid toxicity in two *Apis mellifera* subspecies. *Environmental Toxicology and Chemistry,* **19,** 1901-1905.

Sutcliffe, G.H. & Plowright, R.C. (1988) The effects of food-supply on adult size in the bumble bee *Bombus terricola* Kirby (Hymenoptera, Apidae). *Canadian Entomologist,* **120,** 1051-1058.

Syngenta Crop Protection, UK (2011) Hallmark with zeon technology, product label.

Tasei, J.N., Capou, J. & Michaud, D. (1977) Effect of some pesticides on a solitary bee (*Megachile-pacifica*-Panz) - (Hymenoptera, Megachilidae). *Apidologie,* **8,** 111-127.

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

R Core Team (2014) R: A language and environment for statistical computing. Foundation for Statistical Computing, Vienna, Austria.

Therneau, T. (2014) A package for survival analysis in s. R package version 2.37-7.

Thomas, C.D., Cameron, A., Green, R.E., Bakkenes, M., Beaumont, L.J., Collingham, Y.C., Erasmus, B.F., De Siqueira, M.F., Grainger, A., Hannah, L., Hughes, L., Huntley, B., van Jaarsveld, A.S., Midgley, G.F., Miles, L., Ortega-Huerta, M.A., Townsend Peterson, A., Phillips, O.L. & Williams, S.E. (2004) Extinction risk from climate change. *Nature,* **427,** 145-148.

Thomas, L. (1997) Retrospective power analysis. *Conservation Biology,* **11,** 276-280.

Thompson, H.M, Harrington, P., Wilkins, S., Pietravalle, S., Sweet, D. & Jones, A. (2013) Effects of neonicotinoid seed treatments on bumble bee colonies under field conditions. Food and Environment Research Agency, York.

Thompson, H.M. (2001) Assessing the exposure and toxicity of pesticides to bumblebees (*Bombus* sp.). *Apidologie,* **32,** 305-321.

Thompson, H.M. (2010) Risk assessment for honey bees and pesticides—recent developments and 'new issues'. *Pest Management Science,* **66,** 1157-1162.

Thompson, H.M. & Hunt, L.V. (1999) Extrapolating from honeybees to bumblebees in pesticide risk assessment. *Ecotoxicology,* **8,** 147-166.

Thompson, H.M., Wilkins, S., Harkin, S., Milner, S. & Walters, K.F. (2014) Neonicotinoids and bumblebees (*Bombus terrestris*): Effects on nectar consumption in individual workers. *Pest Management Science*, DOI: 10.1002/ps.3868.

Thomson, D. (2004) Competitive interactions between the invasive European honey bee and native bumble bees. *Ecology,* **85,** 458-470.

Thornhill, J.A., Jones, J.T. & Kusel, J.R. (1986) Increased oviposition and growth in immature *Biomphalaria glabrata* after exposure to *Schistosoma mansoni*. *Parasitology,* **93,** 443-450.

Tilman, D., Cassman, K.G., Matson, P.A., Naylor, R. & Polasky, S. (2002) Agricultural sustainability and intensive production practices. *Nature,* **418,** 671-677.

Tilman, D., Fargione, J., Wolff, B., D'Antonio, C., Dobson, A., Howarth, R., Schindler, D., Schlesinger, W.H., Simberloff, D. & Swackhamer, D. (2001) Forecasting agriculturally driven global environmental change. *Science,* **292,** 281-284.

Tomizawa, M. & Casida, J.E. (2003) Selective toxicity of neonicotinoids attributable to specificity of insect and mammalian nicotinic receptors. *Annual Review of Entomology,* **48,** 339-364.

Tuell, J.K. & Isaacs, R. (2010) Community and species-specific responses of wild bees to insect pest control programs applied to a pollinator-dependent crop. *Journal of Economic Entomology,* **103,** 668-675.

Ulrich, Y., Sadd, B.M. & Schmid-Hempel, P. (2011) Strain filtering and transmission of a mixed infection in a social insect. *Journal of Evolutionary Biology,* **24,** 354-362.

Valdovinos-Núñez, G.R., Quezada-Euán, J.J.G., Ancona-Xiu, P., Moo-Valle, H., Carmona, A. & Sánchez, E.R. (2009) Comparative toxicity of pesticides to stingless bees (Hymenoptera: Apidae: Meliponini). *Journal of Economic Entomology,* **102,** 1737-1742.

Vanbergen, A.J., Baude, M., Biesmeijer, J.C., Britton, N.F., Brown, M.J.F., Brown, M., Bryden, J., Budge, G.E., Bull, J.C., Carvel, C., Challinor, A.J., Connolly, C.N., Evans, D.J., Feil, E.J., Garratt, M.P., Greco, M.K., Heard, M.S., Jansen, V.A.A., Keeling, M.J., Kunis, W.E., Marris, G.C., Memmott, J., Murray, J.T., Nicolson, S.W., Osborne, J.L., Paxton, R.J., Pirk, C.W.W., Polce, C., Potts, S.G., Priest, N.K., Raine, N.E., Roberts, S., Ryabov, E.V., Shafir, S., Shirley, M.D.F., Simpson, S.J., Stevenson, P.C., Stone, G.N., Termansen, M., Wright, G.A. & Insect Pollinators, I. (2013) Threats to an ecosystem service: pressures on pollinators. *Frontiers in Ecology and the Environment,* **11,** 251-259.

vanEngelsdorp, D., Hayes, J.J., Underwood, R.M., Caron, D. & Pettis, J. (2011) A survey of managed honey bee colony losses in the USA, fall 2009 to winter 2010. *Journal of Apicultural Research,* **50,** 1-10.

vanEngelsdorp, D. & Meixner, M.D. (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. *Journal of Invertebrate Pathology,* **103,** S80-S95.

Vásquez, A., Forsgren, E., Fries, I., Paxton, R.J., Flaberg, E., Szekely, L. & Olofsson, T.C. (2012) Symbionts as major modulators of insect health: Lactic acid bacteria and honeybees. *PLoS ONE,* **7,** e33188.

Vásquez, A., Olofsson, T.C. & Sammataro, D. (2009) A scientific note on the lactic acid bacterial flora in honeybees in the USA – a comparison with bees from Sweden. *Apidologie,* **40,** 26-28.

Vidau, C., Diogon, M., Aufauvre, J., Fontbonne, R., Viguès, B., Brunet, J.-L., Texier, C., Biron, D.G., Blot, N., El Alaoui, H., Belzunces, L.P. & Delbac, F. (2011) Exposure to sublethal doses of fipronil and thiacloprid highly increases mortality of honeybees previously infected by *Nosema ceranae*. *PLoS ONE,* **6,** e21550.

Vitousek, P.M., Mooney, H.A., Lubchenco, J. & Melillo, J.M. (1997) Human domination of earth's ecosystems. *Science,* **277,** 494-499.

Wallner, K. (2009) Sprayed and seed dressed pesticides in pollen, nectar and honey of oilseed rape. *Julius Kühn Archive,* **423**, 152-153.

Webster, J., Bowles, R. & Williams, N. (1999) Estimating the economic benefits of alternative pesticide usage scenarios: Wheat production in the United Kingdom. *Crop Protection,* **18,** 83-89.

Westerkamp, C. & Gottsberger, G. (2000) Diversity pays in crop pollination. *Crop Science,* **40,** 1209-1222.

Westphal, C., Steffan-Dewenter, I. & Tscharntke, T. (2003) Mass flowering crops enhance pollinator densities at a landscape scale. *Ecology Letters,* **6,** 961-965.

Whitehorn, P.R., O'Connor, S., Wackers, F.L. & Goulson, D. (2012) Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science,* **336,** 351-352.

Whittington, R. & Winston, M.L. (2003) Effects of *Nosema bombi* and its treatment fumagillin on bumble bee (*Bombus occidentalis*) colonies. *Journal of Invertebrate Pathology,* **84,** 54-58.

Wickham, H. (2009) *ggplot2: Elegant graphics for data analysis*. Springer New York.

Williams, P., Colla, S. & Xie, Z. (2009) Bumblebee vulnerability: Common correlates of winners and losers across three continents. *Conservation Biology,* **23,** 931-940.

Williams, P.H. (1982) The distribution and decline of british bumble bees (*Bombus* Latr). *Journal of Apicultural Research,* **21,** 236-245.

Williams, P.H. (1986) Environmental-change and the distributions of british bumble bees (*Bombus* Latr). *Bee World,* **67,** 50-61.

Williams, P.H. & Jepsen, S. (2014) IUCN Bumblebee Specialist Group Report 2013. Natural History Museum, London.

Williams, P.H. & Osborne, J.L. (2009) Bumblebee vulnerability and conservation world-wide. *Apidologie,* **40,** 367-387.

Williamson, S.M., Moffat, C., Gomersall, M.A., Saranzewa, N., Connolly, C.N. & Wright, G.A. (2013) Exposure to acetylcholinesterase inhibitors alters the physiology and motor function of honeybees. *Frontiers in Physiology,* **4**:13

Williamson, S.M. & Wright, G.A. (2013) Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. *The Journal of Experimental Biology,* **216,** 1799-1807.

Willmer, P.G., Bataw, A.A.M. & Hughes, J.P. (1994) The superiority of bumblebee to honeybees as pollinators - insect visits to raspberry flowers. *Ecological Entomology,* **19,** 271-284.

Wolf, S., McMahon, D.P., Lim, K.S., Pull, C.D., Clark, S.J., Paxton, R.J. & Osborne, J.L. (2014) So near and yet so far: Harmonic radar reveals reduced homing ability of *Nosema* infected honeybees. *PLoS ONE,* **9,** e103989.

Wright, G.A., Mustard, J.A., Simcock, N.K., Ross-Taylor, A.A.R., McNicholas, L.D., Popescu, A. & Marion-Poll, F. (2010) Parallel reinforcement pathways for conditioned food aversions in the honeybee. *Current Biology,* **20,** 2234-2240.

Wu, J., Li, J.-L., Peng, W.-J. & Hu, F.-L. (2010) Sensitivities of three bumblebee species to four pesticides applied commonly in greenhouses in China. *Insect Science,* **17,** 67-72.

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

Yourth, C.P. & Schmid-Hempel, P. (2006) Serial passage of the parasite *Crithidia bombi* within a colony of its host, *Bombus terrestris*, reduces success in unrelated hosts. *Proceedings of the Royal Society B-Biological Sciences,* **273,** 655-65

Zuur, A., Hilbe, J.M. & Ieno, E.N. (2013) *A beginner's guide to GLM and GLMM with R*. Highland Statistics Ltd, Newburgh, UK.

Zuur, A., Ieno, E.N., Walker, N., Saveliev, A.A. & Smith, G.M. (2009) *Mixed effects models and extensions in ecology with R*. Springer.

Appendix 1

Pilot study to assess the foraging and larval feeding by workers provided with λ-cyhalothrin treated pollen.

Methods: Two micro-colonies consisting of similar amounts of brood and 10 workers, were taken from two source colonies (Koppert Biological Systems). These were set up in wooden boxes (24 x 14 x 10.5 cm), with ad libitum pollen and 50% ambrosia solution. All workers were uniquely marked on the thorax with numbered, coloured tags (Opalith tags; Christian Graze KG, Germany). Both micro-colonies were kept in a dark room, at 22 °C, and all observations took place under red light. Five days after the micro-colonies had been set up, each was provided with an equal amount of λ-cyhalothrin treated pollen (pollen was treated in the same way as for the main experiment). Three observation sessions were undertaken for each micro-colony, lasting for five hours in total per micro-colony. These were spread across a 35-hour period, which began immediately after treated pollen was provided to the micro-colonies. Each time a bee approached the pollen dish, behavioural observations were recorded. These included duration of time spent in the pollen, activity in the pollen (collecting, or walking over it), activities undertaken immediately after exposure to the pollen (walking, self-grooming, sitting on -, cleaning -, or feeding brood). After three days, any remaining treated pollen was removed from the micro-colonies, and replaced with *ad libitum* untreated pollen for one day. Following this, each micro-colony was assessed and adjusted (by removing larger larvae, or adding smaller larvae from the source colony) to ensure that similar amounts of brood were present compared to the start of the experiment. An equal amount of fresh untreated pollen was then provided to each micro-colony, and observations as described above were repeated.

Results: Bees from both micro-colonies visited, foraged on, and fed larvae with λ-cyhalothrin treated pollen, and this was observed immediately after pollen was provided, and across the following 24 hours (Table S1). Whilst bees from micro-colony 1 spent a similar amount of time foraging on treated and untreated pollen, and fed larvae a similar number of times during both experiments, micro-colony 2 appeared to show reduced activity in general during the second experiment, when untreated pollen was provided. Throughout the observation period of both experiments, no pollen storing, or rejection of pollen was observed

Table A1.1 Summary of observational data from two *B. terrestris* micro-colonies, after provision of λ-cyhalothrin treated and untreated pollen.

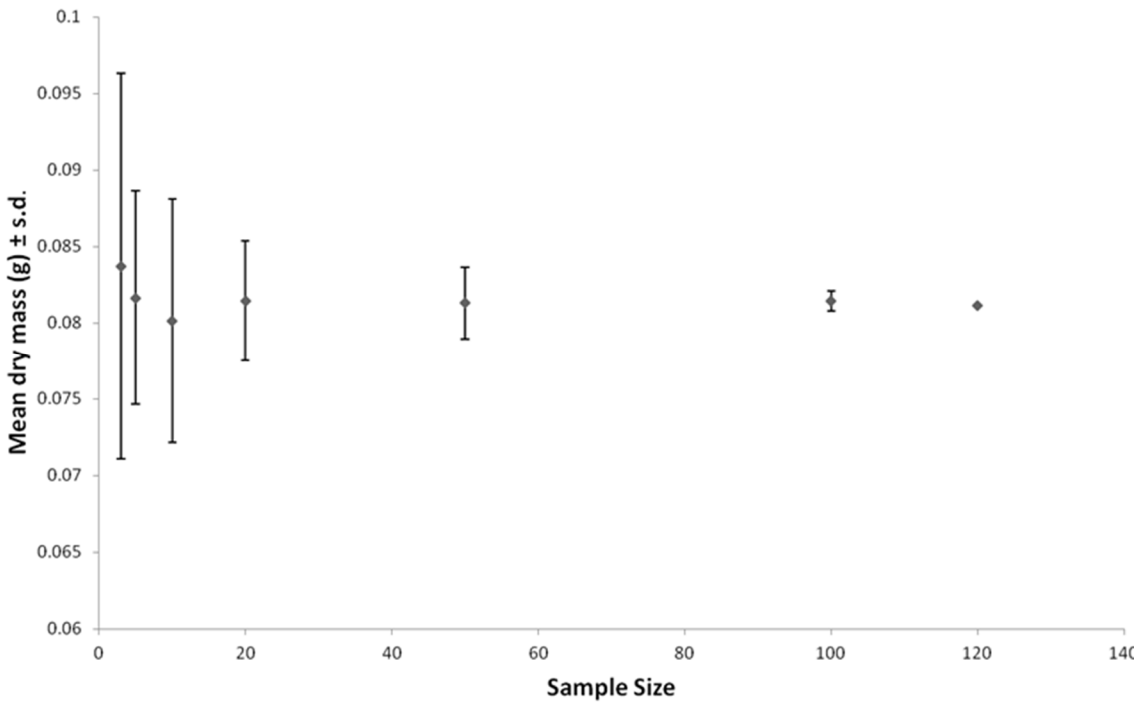
	Micro-colony 1		Micro-colony 2	
	Pesticide treated pollen	Untreated pollen	Pesticide treated pollen	Untreated pollen
Number of individual workers which visited the pollen	10	9	9	5
Number of visits to pollen (including walking across the dish)	31	30	39	19
Number visits to collect pollen (foraging only)	28	29	32	14
Total time at least one bee was in pollen (including all visits to pollen throughout observation period, hh:mm:ss)	01:40:44	01:34:05	02:00:40	00:42:30
Average time spent in pollen (hh:mm:ss)	00:03:28	00:03:15	00:03:33	00:02:50
Number of larval feeding events following pollen collection	9	8	6	0
Number of times an individual visited the brood immediately after pollen collection	24	18	26	13

Appendix 2

Explanation of subsampling procedure for measuring the average mass of workers

In order to measure mean worker mass in a standardised way across all colonies, we measured a sub-sample of 20 workers from each colony. To evaluate whether this sample size was sufficient to obtain a good estimate of mean mass, we weighed 120 workers from one colony, and randomly subsampled the data, using a range of sample sizes (Figure A2.1). Whilst very small sample sizes of three, five and ten workers show variation in the calculated mean mass, and large amounts of variance, the mean mass estimate with a sample size of 20 workers converges on the true mean and has lower variance.

Figure A2.1: Mean worker mass estimates from random sub-samples of the data. Each point represents the mean (± S.D.) of 50 iterations of randomly selected data points for each given sample size, subsampled from a total data set of 120 workers from a single colony. Error bars show the standard deviation.



Appendix 3

Power Analysis

In order to assess the power of our data to detect the impacts of pesticide exposure on colony development, we calculated the effect size for each variable (excluding total number of gynes, average gyne mass and total gyne mass which were bootstrapped in the original analysis), and the 95% confidence intervals for these effect sizes, as recommended by Thomas (1997). Figure A3.1 shows the percentage effects size and confidence intervals for all variables. Points which lie below the zero line indicate an overall negative impact of pesticide treatment, whilst those over the zero line indicate a positive effect. Of the variables measured, days until male production, mean dry mass of workers, and mean dry mass of males, have relatively small confidence intervals, which do not cross zero. This suggests that these results are reliable (although not significant, in the case of male production and mean male mass), and can be attributed to a real biological effect of the pesticide. Several of the variables measured have large confidence intervals that cross zero (e.g. number of worker deaths, queen longevity, days until competition point, total number of males and workers produced, total worker mass and sexual biomass). As the confidence intervals for these variables are large, we cannot have complete confidence in the non-significant result. Possibly a larger sample size would be required to fully assess the impacts and direction of effects of the pesticide exposure on these aspects of colony development.

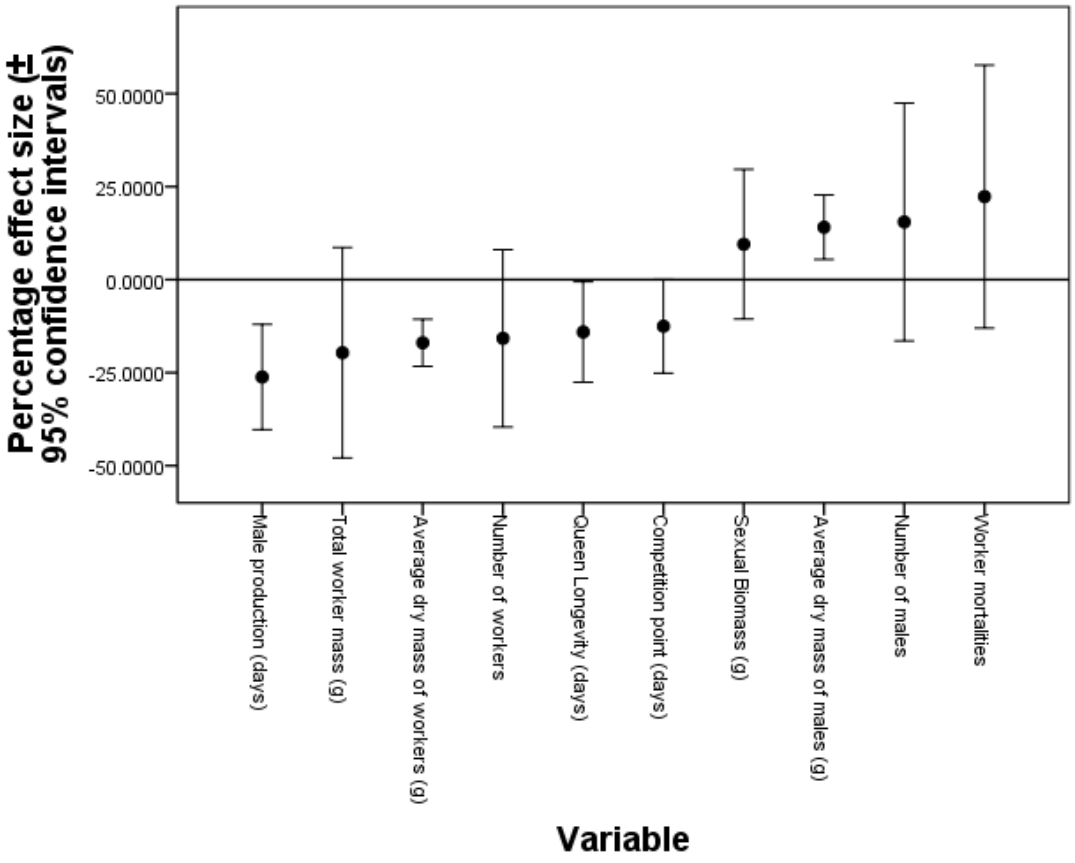


Figure A3.1: Percentage Effect Size (± 95% Confidence Intervals) of variables measured in λ-cyhalothrin treated, and control treated *B. terrestris* colonies. The % effect size was calculated from the raw effect size (mean of pesticide treated colonies minus the mean of control colonies).

Appendix 3 References

Thomas, L. (1997) Retrospective power analysis. *Conservation Biology,* **11,** 276-280.

Appendix 4

Model selection of candidate models for analyses in Chapter 3

Candidate models were constructed including each of the fixed factors individually and in biologically relevant combinations. These were compared with the null model (no fixed factors), and full model (all fixed factors). Two random factors, queen colony and male colony, were included in initial comparisons, but did not improve fit of any of the models, and so were not included here. Two way interactions between treatments were considered, but due to lack of coverage, three way interactions were not. Interactions between covariates and treatments were included if data visualisation indicated this may be useful. The AICc values were used (these were chosen over AIC values due to small sample sizes), and the optimal model (with the lowest AICc) was selected (highlighted in bold in each table below). When multiple models were within 2 AICc of the lowest (Delta ≤ 2), model averaging was undertaken (Johnson & Omland 2004).

Table A4.1: Candidate linear models for proportion of weight lost by queens during hibernation.

Fixed Factors	Loglik	AICc	Delta	Weight
Null	-694.672	1393.4	55.22	0
Hibernation	**-666.937**	**1340**	**1.81**	**0.173**
Parasite	-693.163	1392.4	54.26	0
Thorax	-694.668	1395.4	57.27	0
Hibernation + Parasite	**-664.995**	**1338.2**	**0**	**0.428**
Parasite + Thorax	-693.152	1394.5	56.31	0
Hibernation + Thorax	-666.749	1341.7	3.51	0.074
Hibernation * Parasite	-664.994	1340.3	2.09	0.15
Hibernation + Parasite + Thorax	**-664.844**	**1340**	**1.79**	**0.175**

Table A4.2: Candidate generalised linear models for queen survival during hibernation.

Fixed Factors	Loglik	AICc	Delta	Weight
Null	-68.499	141	12.02	0.001
Weight (pre-hibernation)	**-61.755**	**129.6**	**0.57**	**0.201**
Parasite	-67.838	141.8	12.73	0
Hibernation	-66.823	139.7	10.71	0.001
Weight + Parasite	**-61.368**	**130.9**	**1.84**	**0.106**
Weight + Hibernation	**-60.445**	**129**	**0**	**0.267**
Hibernation + Parasite	-66.253	140.6	11.62	0.001
Hibernation + Parasite + Weight	**-60.094**	**130.4**	**1.36**	**0.135**
Parasite * Weight	-60.839	131.9	2.85	0.064
Hibernation * Weight	**-59.597**	**129.4**	**0.37**	**0.222**
Hibernation * Parasite	-66.17	142.5	13.51	0

Table A4.3: Candidate generalised linear models for queen survival after hibernation**.**

Fixed Factors	Loglik	AICc	Delta	Weight
NULL	**-182.487**	**365**	**0**	**0.235**
Hibernation	**-182.418**	**366.9**	**1.88**	**0.092**
Pesticide	**-182.234**	**366.7**	**1.69**	**0.101**
Parasite	**-182.337**	**366.7**	**1.72**	**0.099**
Thorax	**-182.148**	**366.3**	**1.34**	**0.12**
Hiberantion + Pesticide	-182.252	368.6	3.58	0.039
Hibernation + Parasite	-182.252	368.6	3.58	0.039
Hiberantion * Pesticide	-181.986	370.1	5.1	0.018
Hibernation * Parasite	-181.74	369.6	4.61	0.023
Hibernation + Thorax	-182.103	368.3	3.28	0.045
Pesticide + Parasite	-182.168	368.4	3.42	0.043
Pesticide * Parasite	-182.148	370.4	5.43	0.016
Pesticide + Thorax	-181.937	367.9	2.95	0.054
Parasite + Thorax	-181.954	368	2.99	0.053
Hibernation + Pesticide + Parasite	-182.08	370.3	5.29	0.017
Hibernation + Pesticide + Parasite + Thorax	-181.889	372	6.98	0.007

Table A4.4: Candidate linear models for syrup consumption by queens during the two week pesticide exposure period (models including Hibernation, Pesticide, Parasite and Thorax only)

Fixed Factors	Loglik	AICc	Delta	Weight
null	-102.161	208.4	28.61	0
Hibernation	**-86.83**	**179.8**	**0**	**0.422**
Pesticide	-102.095	210.3	30.53	0
Parasite	-102.026	210.2	30.39	0
Thorax	-102.069	210.2	30.48	0
Pesticide + Hibernation	**-86.709**	**181.6**	**1.83**	**0.169**
Pesticide * Hibernation	-86.16	182.6	2.83	0.103
Pesticide + Parasite	-101.959	212.1	32.33	0
Pesticide * Parasite	-101.092	212.5	32.69	0
Hibernation + Parasite	-86.806	181.8	2.03	0.103
Hibernation * Parasite	-86.757	183.8	4.02	0.056
Pesticide + Hibernation + Parasite	-86.806	181.8	3.88	0.061
Pesticide + Hibernation + Parasite + Thorax	-86.155	184.7	4.93	0.036

Table A4.5: Candidate linear models for syrup consumption by queens during the two week pesticide exposure period (models including Weight loss and Thorax only)

Fixed Factors	Loglik	AICc	Delta	Weight
null	-102.161	208.4	14.3	0.001
Weight loss	**-93.984**	**194.1**	**0**	**0.716**
Thorax	-102.069	210.2	16.17	0
Thorax + Weight loss	**-93.874**	**195.9**	**1.86**	**0.283**

Table A4.6: Candidate binomial generalised linear models for presence or absence of egg laying by queens

Fixed Factors	Loglik	AICc	Delta	Weight
Null	-135.632	273.3	24.55	0
Hibernation	-123.924	251.9	3.18	0.061
Pesticide	-133.772	271.6	22.87	0
Parasite	-135.62	275.3	26.57	0
Thorax	-134.734	273.5	24.8	0
Hibernation + Pesticide	**-121.647**	**249.4**	**0.69**	**0.211**
Hibernation * Pesticide	-121.273	250.8	2.02	0.108
Hibernation + Parasite	-123.895	253.9	5.18	0.022
Hibernation * Parasite	-122.825	253.9	5.13	0.023
Hibernation + Thorax	**-122.166**	**250.5**	**1.73**	**0.126**
Pesticide + Parasite	-133.764	273.7	24.92	0
Pesticide * Parasite	-133.585	275.4	26.65	0
Pesticide + Thorax	-133.112	272.3	23.62	0
Parasite + Thorax	-134.7	275.5	26.79	0
Hibernation + Pesticide + Thorax	**-120.262**	**248.7**	**0**	**0.298**
Pesticide + Parasite + Thorax	-133.089	274.4	25.65	0
Hibernation + Parasite + Thorax	-122.16	252.5	3.8	0.045
Hibernation + Pesticide + Parasite + Thorax	-120.245	250.8	2.07	0.106

Table A4.7: Candidate Cox regression models for timing of egg laying by queens

Fixed Factors	Loglik	AICc	Delta	Weight
Null	-425.639	851.3	36.13	0
Hibernation	-433.319	868.6	17.53	0
P1 + P2	-433.473	871	19.86	0
Parasite	-443.8	889.6	38.49	0
Thorax	-442.973	888	36.84	0
Hibernation + P1 + P2	**-422.528**	**851.1**	**0**	**0.489**
Hibernation * P1 + Hibernation *P2	-421.762	853.7	2.57	0.136
Hibernation + Parasite	-433.275	870.6	19.46	0
Hibernation * Parasite	-432.172	870.4	19.29	0
Hibernation + Thorax	-431.853	867.7	16.62	0
P1 + P2 + Parasite	-433.375	872.8	21.69	0
P1 * Parasite + P2 * Parasite	-433.254	876.7	25.55	0
P1 + P2 + Thorax	-432.835	871.7	20.61	0
Parasite + Thorax	-442.832	889.7	38.58	0
Hibernation + P1 + P2 + Parasite	**-422.474**	**853.1**	**1.94**	**0.186**
Hibernation + P1 + P2 + Parasite + Thorax	**-421.426**	**853**	**1.89**	**0.19**

Table A4.8: Candidate binomial generalised linear models for presence or absence of developing oocytes in queens

Fixed Factors	Loglik	AICc	Delta	Weight
Null	-109.385	220.8	13.99	0
Hibernation	-102.668	209.4	2.6	0.094
Pesticide	-109.191	222.4	15.65	0
Parasite	-108.687	221.4	14.64	0
Thorax	-107.879	219.8	13.02	0.001
Hibernation + Pesticide	-102.421	211	4.17	0.043
Hibernation * Pesticide	-102.396	213	6.2	0.016
Hibernation + Parasite	-102.242	210.6	3.81	0.051
Hibernation * Parasite	-102.24	212.7	5.89	0.018
Hibernation + Thorax	**-100.337**	**206.8**	**0**	**0.345**
Pesticide + Parasite	-108.503	223.1	16.33	0
Pesticide * Parasite	-108.455	225.1	18.32	0
Pesticide + Thorax	-107.779	221.7	14.88	0
Parasite + Thorax	-107.031	220.2	13.39	0
Hibernation + Pesticide + Thorax	-100.183	208.6	1.78	0.142
Pesticide + Parasite + Thorax	-106.943	222.1	15.3	0
Hibernation + Parasite + Thorax	**-99.813**	**207.8**	**1.04**	**0.206**
Hibernation + Parasite + Pesticide +Thorax	-99.669	209.7	2.85	0.083

Table A4.9: Candidate binomial generalised linear models for presence or absence of adult offspring (including all queens)

Fixed Factors	Loglik	AICc	Delta	Weight
Null	**-104.63**	**211.3**	**0**	**0.15**
Hibernation	**-103.636**	**211.3**	**0.05**	**0.146**
Pesticide	**-104.14**	**212.3**	**1.06**	**0.088**
Parasite	**-104.577**	**213.2**	**1.94**	**0.057**
Thorax	**-103.996**	**212.1**	**0.77**	**0.102**
Hibernation + Pesticide	**-103.163**	**212.4**	**1.17**	**0.084**
Hibernation + Parasite	**-103.538**	**213.2**	**1.92**	**0.058**
Hibernation + Thorax	**-102.851**	**211.8**	**0.55**	**0.114**
Pesticide + Parasite	-104.092	214.3	3.03	0.033
Pesticide + Thorax	**-103.364**	**212.9**	**1.57**	**0.069**
Parasite + Thorax	-103.968	214.1	2.78	0.037
Hibernation + Pesticide + Parasite	-103.073	214.4	3.07	0.032
Hibernation + Pesticide + Parasite + Thorax	-102.155	214.6	3.34	0.028

Table A4.10: Candidate binomial generalised linear models for presence or absence of adult offspring (including only queens which laid eggs)

Fixed Factors	Loglik	AICc	Delta	Weight
Null	-61.684	125.4	6.9	0.013
Hibernation	-59.865	123.9	5.35	0.029
Pesticide	**-58.087**	**120.3**	**1.79**	**0.169**
Parasite	-61.549	127.2	8.72	0.005
Thorax	-81.593	127.3	8.81	0.005
Hibernation + Pesticide	**-56.119**	**118.5**	**0**	**0.415**
Hibernation + Parasite	-59.853	126	7.47	0.01
Hibernation + Thorax	-59.855	126	7.47	0.01
Pesticide + Parasite	-57.888	122.1	3.54	0.071
Pesticide + Thorax	-57.809	121.9	3.38	0.077
Parasite + Thorax	-61.495	129.3	10.75	0.002
Hibernation + Pesticide + Parasite	-56.09	120.7	2.14	0.143
Hibernation + Pesticide + Parasite + Thorax	-55.992	122.7	4.19	0.051

Appendix 5

Summary of life history traits of the four bumblebee species used in Chapter 4

Table A5.1: Life-history traits of the four focal bumblebee species used in this study. Worker foraging ranges indicate estimates of the maximum ranges observed in several studies. Sources: [1] Alford (1975), [2] Müller & Schmid-Hempel (1992), [3] Brian (1951; 1952), [4] Benton (2006), [5] Duchateau & Velthuis (1988), [6] Goulson *et al.* (2005), [7] Knight *et al.* (2005), [8] Chapman, Wang & Bourke (2003), [9] (Wolf & Moritz 2008), [10] (Osborne *et al.* 2008).

Species	**Queen Size (length mm) [1]**	**Queens foraging [1]**	**Nesting sites [1]**	**Workers foraging [1]**	**Larval feeding strategy [1]**	**Colony size**	**Worker tongue length (mm) [6]**	**Worker foraging range (m) [7,8,9,10]**
Bombus lucorum	18-21	March	Below-ground	Mid-April - July	Pollen storer	Medium (~100 workers) [2]	7.5 ± 0.5	No data available
Bombus pascuorum	15-18	April	Above ground - long tussocky grass	Late April - throughout summer	Pocket maker	Mid sized (100-200 workers) [3]	8.5 ± 0.6	312- 3200 [7, 8]
Bombus pratorum	15-17	Late Feb - March	Both below and above ground	Late March - late April	Pollen storer	Small (<100 workers) [4]	7.3 ± 0.4	674 [7]
Bombus terrestris	20-23	Late Feb - March	Below-ground	March - June	Pollen storer	Large (several hundred workers) [5]	7.6 ± 0.5	583 – 3900 [7,8,9,10]

Appendix 5 References

Alford, D.V. (1975) *Bumblebees*. Davis-Poynter Ltd, London.

Benton, T. (2006) *Bumblebees*. HarperCollins, London.

Brian, A. (1951) Brood development in *bombus agrorum* (Hym., Bombidae). *Entomologists Monthly Magazine,* **87,** 207-212.

Brian, A.D. (1952) Division of labour and foraging in B*ombus agrorum* Fabricius. *The Journal of Animal Ecology***,** 223-240.

Chapman, R., Wang, J. & Bourke, A. (2003) Genetic analysis of spatial foraging patterns and resource sharing in bumble bee pollinators. *Molecular Ecology,* **12,** 2801-2808.

Duchateau, M.J. & Velthuis, H. (1988) Development and reproductive strategies in *Bombus terrestris* colonies. *Behaviour,* **107,** 186-207.

Goulson, D., Hanley, M.E., Darvill, B., Ellis, J. & Knight, M.E. (2005) Causes of rarity in bumblebees. *Biological Conservation,* **122,** 1-8.

Knight, M.E., Martin, A.P., Bishop, S., Osborne, J.L., Hale, R.J., Sanderson, R.A. & Goulson, D. (2005) An interspecific comparison of foraging range and nest density of four bumblebee (*Bombus*) species. *Molecular Ecology,* **14,** 1811-1820.

Müller, C.B. & Schmid-Hempel, P. (1992) Correlates of reproductive success among field colonies of *Bombus lucorum*: The importance of growth and parasites. *Ecological Entomology,* **17,** 343-353.

Osborne, J.L., Martin, A.P., Carreck, N.L., Swain, J.L., Knight, M.E., Goulson, D., Hale, R.J. & Sanderson, R.A. (2008) Bumblebee flight distances in relation to the forage landscape. *Journal of Animal Ecology,* **77,** 406-415.

Wolf, S. & Moritz, R.F. (2008) Foraging distance in *Bombus terrestris* L. (Hymenoptera: Apidae). *Apidologie,* **39,** 419-427.

Appendix 6

Model selection of candidate models for analyses in Chapter 4

Candidate models were constructed including each of the fixed factors individually and in biologically relevant combinations. These were compared with the null model (no fixed factors), and full model (all fixed factors). Two way interactions between treatment and species were considered for linear models (Syrup consumption and Oocyte length), but not for generalised linear models (GLMs) due to small sample sizes for some groups. The AICc values were used (these were chosen over AIC values due to small sample sizes), and the optimal model (with the lowest AICc) was selected (highlighted in bold in each table below). When multiple models were within 2 AICc of the lowest (Delta ≤ 2), model averaging was undertaken (Johnson & Omland 2004). The results from final models can be found in Chapter 4.

Table A6.1: Candidate linear models for average daily syrup consumption (g/mm^3) by four species of bumblebee queen exposed to one of three thiamethoxam treatments.

Fixed Factors	Loglik	AICc	Delta	Weight
null	1200.994	-2397.9	108.96	0
Treatment	1203.634	-2399.1	107.81	0
Species	1254.68	-2499.1	7.81	0.013
Treatment + Species	**1260.062**	**-2505.6**	**1.28**	**0.34**
Treatment * Species	**1267.296**	**-2506.9**	**0**	**0.646**

Table A6.2: Candidate GLMs for the survival to four weeks of four species of bumblebee queen exposed to one of three thiamethoxam treatments.

Fixed Factors	Loglik	AICc	Delta	Weight
null	-83.19	168.4	6.13	0.036
Treatment	-83.074	172.3	9.99	0.005
Species	-82.571	173.3	11.05	0.003
SizeZ	**-79.107**	**162.3**	**0**	**0.774**
Treatment + SizeZ	-79.025	166.2	3.96	0.107
Treatment + Species	-82.439	177.3	14.99	0
Species + SizeZ	-78.459	167.2	4.92	0.066
Treatment + SizeZ + Species	-78.385	171.3	9.01	0.009

Table A6.3: Candidate Cox regression models for the survival to four weeks of four species of bumblebee queen exposed to one of three thiamethoxam treatments.

Fixed Factors	Loglik	AICc	Delta	Weight
null	**-1024.4**	**2048.8**	**0**	**0.547**
Treatment	-1024.38	2052.8	4.01	0.074
Species	**-1024.02**	**2050**	**1.25**	**0.292**
SizeZ	-1024.32	2054.8	5.96	0.028
Treatment + SizeZ	-1023.99	2054.1	5.3	0.039
Treatment + Species	-1023.92	2056	7.23	0.015
Species + SizeZ	-1024.3	2058.9	10.07	0.004
Treatment + SizeZ + Species	-1023.89	2060.2	11.37	0.002

Table A6.4: Candidate GLMs for the presence or absence of waxing behaviour by queens of four bumblebee species exposed to one of three thiamethoxam treatments

Fixed Factors	Loglik	AICc	Delta	Weight
null	-140.292	282.6	3.14	0.095
Treatment	-139.61	285.3	5.87	0.024
Species	**-135.633**	**279.5**	**0**	**0.456**
SizeZ	-140.263	284.6	5.12	0.035
Treatment + SizeZ	-139.597	287.4	7.93	0.009
Treatment + Species	-134.55	281.5	2.06	0.163
Species + SizeZ	-135.618	281.5	2.07	0.162
Treatment + SizeZ + Species	-134.549	283.7	4.2	0.056

Table A6.5: Candidate GLMs for the presence or absence of egg laying by queens of four bumblebee species exposed to one of three thiamethoxam treatments. A cox regression analysis of timing of egg laying gave the same outcome – data not shown.

Fixed Factors	Loglik	AICc	Delta	Weight
null	-93.318	188.7	2.4	0.132
Treatment	-93.035	192.2	5.93	0.023
Species	**-89.029**	**186.3**	**0**	**0.437**
SizeZ	-93.019	190.1	3.84	0.064
Treatment + SizeZ	-92.73	193.7	7.4	0.011
Treatment + Species	-88.683	189.8	3.54	0.075
Species + SizeZ	-88.653	187.6	1.35	0.222
Treatment + SizeZ + Species	-88.322	191.2	4.96	0.037

Table A6.6: Candidate linear models for the average length of oocytes of queens of four bumblebee species exposed to one of three thiamethoxam treatments.

Fixed Factors	Loglik	AICc	Delta	Weight
null	-281.767	567.6	5.8	0.031
Treatment	**-277.522**	**563.2**	**1.45**	**0.275**
Species	-281.767	573.8	12.05	0.001
SizeZ	-279.744	454.6	3.82	0.084
Treatment + SizeZ	**-275.743**	**561.8**	**0**	**0.569**
Treatment + Species	-273.144	574.2	12.45	0.001
Species + SizeZ	-279.737	571.9	10.12	0.004
Treatment + SizeZ + Species	-275.734	568.2	6.43	0.023
Treatment * Species	-273.144	574.2	12.45	0.001

Table A6.7: Candidate linear models for the average length of oocytes of queens of four bumblebee species exposed to one of three thiamethoxam treatments.

Following from the model selection shown in Table A6.6, 3 additional models including an interaction between treatment and syrup consumption were added (see Chapter 4 Results for further details).

Fixed Factors	Loglik	AICc	Delta	Weight
null	-281.767	567.6	8.28	0.007
Treatment	-277.522	563.2	3.93	0.066
Species	-281.767	573.8	14.53	0
SizeZ	-279.744	565.6	6.29	0.02
Treatment + SizeZ	-275.743	561.8	2.48	0.136
Treatment + Species	-277.508	569.6	10.28	0.003
Species + SizeZ	-279.737	571.9	12.59	0.001
Treatment + SizeZ + Species	-275.734	568.2	8.91	0.005
Treatment * Species	-273.144	574.2	14.93	0
Treatment * Syrup	**-273.207**	**561**	**1.68**	**0.202**
Treatment * Syrup + SizeZ	**-271.281**	**559.3**	**0**	**0.468**
Treatment * Syrup + SizeZ + Species	-269.593	562.6	3.27	0.091

Appendix 7

Thiamethoxam consumption

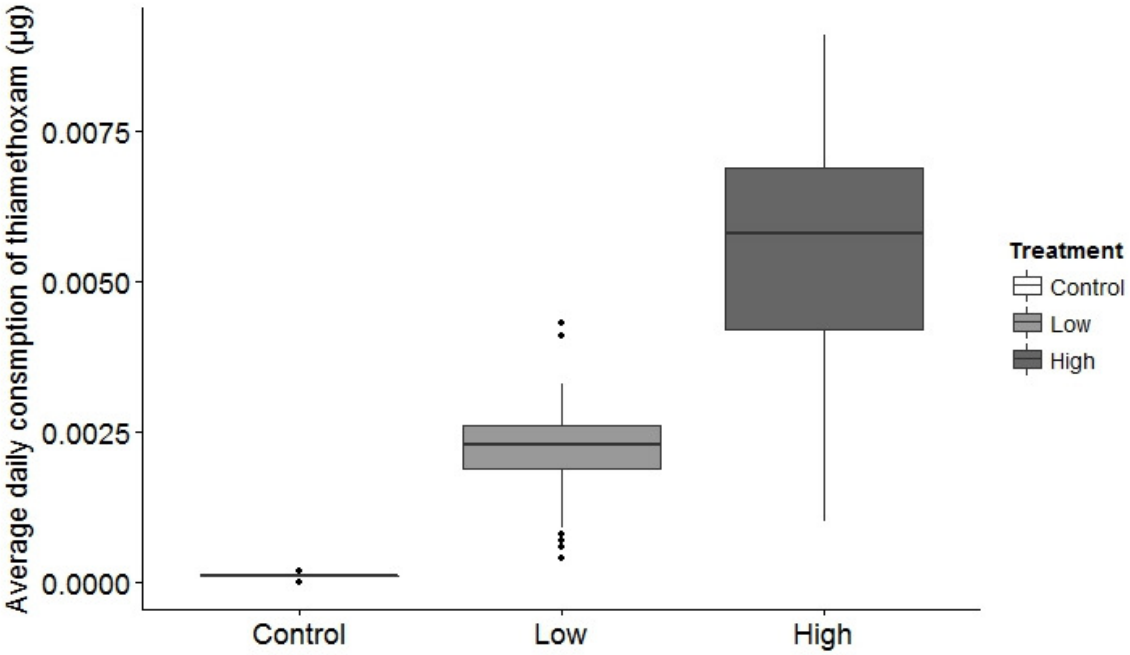


Figure 2: The average daily amount of thiamethoxam consumed by queens of four species of bumblebee queen. Values calculated from actual residue levels. Boxplots show the median (central line), interquartile range (box), range which lies within 1.5 time the interquartile range from the box (whiskers), and outliers (dots).

Appendix 8

Lactic Acid Bacteria ANOVA Results

ANOVA results for change in absorbance of each LAB phylotype when exposed to one of four pesticides; boscalid (a), coumaphos (b), λ-cyhalothrin (c), thiamethoxam (d), or a mixture of all four (e). Treatment (Control, Acetone, Low, Medium or High level of the pesticide (see Chapter 5, Table 5.1)), and plate were included as fixed factors. D.F. indicates degrees of freedom (effect, error), p-values highlighted in bold show significant effects ($p<0.05$). Results from Tukey's post hoc testing for differences between treatments are presented in Table 5.2.

Pesticide	LAB phylotype	Factor	F value	D.F.	p
a Boscalid	Bin2	Treatment	0.9351	4,41	4.53E-01
		Plate	9.5074	4,41	**1.56E-05**
	Bin4	Treatment	1.9971	4,41	1.12E-01
		Plate	7.3647	4,41	**1.00E-04**
	Bin7	Treatment	0.861	4,41	4.95E-01
		Plate	0.8879	4,41	4.80E-01
	Biut2	Treatment	2.2034	4,41	8.55E-02
		Plate	3.5068	4,41	**1.49E-02**
	Bma5	**Treatment**	**3.7326**	**4,41**	**1.11E-02**
		Plate	0.9077	4,41	4.68E-01
	Bma6	**Treatment**	**3.7537**	**4,41**	**1.08E-02**
		Plate	7.2991	4,41	**1.55E-04**
	Fhon13	**Treatment**	**11.8815**	**4,41**	**1.69E-06**
		Plate	0.9635	4,41	4.38E-01
	Fhon2	**Treatment**	**11.9022**	**4,41**	**1.65E-06**
		Plate	0.4743	4,41	7.54E-01
	Hma11	Treatment	2.1684	4,41	8.96E-02
		Plate	25.3112	4,41	**1.31E-10**
	Hma2	**Treatment**	**6.3038**	**4,41**	**4.76E-04**
		Plate	5.822	4,41	**8.34E-04**
	Hma3	Treatment	0.4399	4,41	7.79E-01
		Plate	8.0102	4,41	7.22E-05
	Hma8	Treatment	0.7429	4,41	5.68E-01
		Plate	4.3633	4,41	**4.96E-03**
	Hon2	**Treatment**	**32.3144**	**4,41**	**3.49E-12**
		Plate	7.4922	4,41	**1.26E-04**
	Mix	Treatment	1.0089	4,43	4.13E-01
		Plate	1.8455	4,43	1.38E-01

Pesticide	LAB phylotype	factor	F value	D.F.	p
b Coumaphos	Bin2	Treatment	1.4052	4,41	2.49E-01
		Plate	12.383	4,41	**1.08E-06**
	Bin4	Treatment	1.476	4,41	2.27E-01
		Plate	511.816	4,41	**2.00E-16**
	Bin7	**Treatment**	**4.2438**	**4,41**	**5.77E-03**
		Plate	33.525	4,41	**1.98E-12**
	Biut2	Treatment	0.3727	4,41	8.27E-01
		Plate	1.5804	4,41	1.98E-01
	Bma5	**Treatment**	**3.8762**	**4,41**	**9.24E-03**
		Plate	0.7516	4,41	5.63E-01
	Bma6	Treatment	1.2571	4,41	3.02E-01
		Plate	7.8123	4,41	**8.92E-05**
	Fhon13	Treatment	2.4602	4,41	6.04E-02
		Plate	4.3296	4,41	**5.10E-03**
	Fhon2	**Treatment**	**6.6033**	**4,41**	**3.37E-04**
		Plate	3752.956	4,41	**2.20E-16**
	Hma11	Treatment	0.2553	4,41	9.05E-01
		Plate	45.4225	4,41	**1.52E-14**
	Hma2	Treatment	0.6525	4,41	6.28E-01
		Plate	47.7597	4,41	**6.61E-15**
	Hma3	**Treatment**	**3.6546**	**4,41**	**1.23E-02**
		Plate	18.3506	4,41	**1.04E-08**
	Hma8	Treatment	1.792	4,41	1.49E-01
		Plate	16.072	4,41	**5.43E-08**
	Hon2	Treatment	2.0323	4,41	1.08E-01
		Plate	126.7846	4,41	**2.00E-16**
	Mix	Treatment	0.1964	4,41	9.39E-01
		Plate	4.1842	4,41	**6.22E-03**

Pesticide	LAB phylotype	factor	F value	D.F.	p
c λ-cyhalothrin	Bin2	Treatment	2.0102	4,41	1.11E-01
		Plate	8.5987	4,41	**3.91E-05**
	Bin4	Treatment	2.3361	4,41	7.14E-02
		Plate	2.2849	4,41	7.65E-02
	Bin7	**Treatment**	**3.1508**	**4,41**	**2.39E-02**
		Plate	3.5825	4,41	**1.35E-02**
	Biut2	Treatment	1.756	4,41	1.56E-01
		Plate	11.341	4,41	**2.74E-06**
	Bma5	Treatment	1.1853	4,41	3.32E-01
		Plate	18.4982	4,41	**9.34E-09**
	Bma6	**Treatment**	**5.6985**	**4,41**	**9.65E-04**
		Plate	7.2563	4,41	**1.63E-04**
	Fhon13	**Treatment**	**13.5257**	**4,41**	**4.09E-07**
		Plate	7.9283	4,41	**7.88E-05**
	Fhon2	**Treatment**	**26.0797**	**4,41**	**8.53E-11**
		Plate	4.2339	4,41	**5.84E-03**
	Hma11	Treatment	0.3332	4,41	8.54E-01
		Plate	23.8212	4,41	**3.10E-10**
	Hma2	Treatment	1.7927	4,41	1.49E-01
		Plate	1.0837	4,41	3.77E-01
	Hma3	**Treatment**	**8.1833**	**4,41**	**6.02E-05**
		Plate	14.6701	4,41	**1.61E-07**
	Hma8	Treatment	1.3984	4,41	2.52E-01
		Plate	7.5983	4,41	**1.12E-04**
	Hon2	Treatment	0.8798	4,41	4.85E-01
		Plate	8.8019	4,41	**3.17E-05**
	Mix	Treatment	0.5878	4,39	6.73E-01
		Plate	0.4432	4,39	7.77E-01

Pesticide	LAB phylotype	factor	F value	D.F.	p
d Thiamethoxam	Bin2	Treatment	0.514	4,40	7.26E-01
		Plate	0.5911	5,40	7.07E-01
	Bin4	Treatment	1.1671	4,40	3.40E-01
		Plate	7.582	5,40	**4.36E-05**
	Bin7	Treatment	0.7242	4,40	5.81E-01
		Plate	1.5174	5,40	2.06E-01
	Biut2	**Treatment**	**2.8014**	**4,40**	**3.85E-02**
		Plate	14.2497	5,40	**5.12E-08**
	Bma5	**Treatment**	**3.5137**	**4,40**	**1.50E-02**
		Plate	18.9676	5,40	**1.25E-09**
	Bma6	Treatment	0.816	4,40	5.23E-01
		Plate	1.1391	5,40	3.56E-01
	Fhon13	Treatment	1.3968	4,40	2.53E-01
		Plate	4.1757	5,40	**3.80E-03**
	Fhon2	Treatment	0.969	4,40	4.35E-01
		Plate	2.7721	5,40	**3.05E-02**
	Hma11	Treatment	0.3205	4,40	8.63E-01
		Plate	15.9329	5,40	**1.26E-08**
	Hma2	Treatment	0.993	4,40	4.23E-01
		Plate	1.5767	5,40	1.89E-01
	Hma3	Treatment	1.0295	4,40	4.06E-01
		Plate	26.4773	5,40	**1.04E-11**
	Hma8	**Treatment**	**3.0357**	**4,40**	**2.82E-02**
		Plate	16.1111	5,40	**1.09E-08**
	Hon2	Treatment	2.3941	4,40	6.65E-02
		Plate	5.9655	5,40	**3.27E-04**
	Mix	**Treatment**	**4.5719**	**4,20**	**8.74E-03**

Pesticide	LAB phylotype	factor	F value	D.F.	p
e Mix	**Bin2**	**Treatment**	**10.1849**	**2,24**	**6.27E-04**
		Plate	2.9921	3,24	5.08E-02
	Bin4	**Treatment**	**5.867**	**2,24**	**8.43E-03**
		Plate	6.1795	3,24	**2.90E-03**
	Bin7	Treatment	2.1717	2,24	1.36E-01
		Plate	3.2405	3,24	**3.98E-02**
	Biut2	Treatment	0.2281	2,24	7.98E-01
		Plate	1.9415	3,24	1.50E-01
	Bma5	Treatment	2.4722	2,24	1.06E-01
		Plate	3.0072	3,24	5.01E-02
	Bma6	Treatment	0.0701	2,24	9.33E-01
		Plate	0.2102	3,24	8.88E-01
	Fhon13	Treatment	1.0475	2,24	3.66E-01
		Plate	2.3695	3,24	9.57E-02
	Fhon2	Treatment	0.8971	2,24	4.21E-01
		Plate	1.5808	3,24	2.20E-01
	Hma11	Treatment	1.8214	2,24	1.84E-01
		Plate	42.2443	3,24	**9.88E-10**
	Hma2	Treatment	1.0113	2,24	3.79E-01
		Plate	8.0874	3,24	**6.77E-04**
	Hma3	Treatment	0.3118	2,24	7.35E-01
		Plate	43.1662	3,24	**7.96E-10**
	Hma8	Treatment	1.8227	2,24	1.83E-01
		Plate	21.9739	3,24	**4.57E-07**
	Hon2	**Treatment**	**7.9983**	**2,24**	**2.18E-03**
		Plate	9.6802	3,24	**2.26E-04**
	Mix	Treatment	2.6076	2,24	9.45E-02
		Plate	6.6763	3,24	**1.95E-03**