

**Effects of neonicotinoid pesticide exposure on bee health:  
Molecular, physiological and behavioural investigations**

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## Abstract

Neonicotinoid exposure has been recognised as potentially impacting upon bee health, but whether realistic exposure scenarios are driving declines in bee health is not known. This thesis contributes new insights and perspectives to this research field investigating the use of molecular, physiological and behavioural endpoints as potential ecotoxicological markers for pesticide risk assessment. The thesis presents experimental data for dietary exposures of the European honey bee, *Apis mellifera*, and the buff-tailed bumble bee, *Bombus terrestris*, to one of two neonicotinoid pesticides, imidacloprid and thiamethoxam.

The first part of this thesis explores impacts of chronic dietary exposures to neonicotinoid pesticides on bee immunocompetence- the ability to mount an immune response- using an artificial challenge to invoke an immune response in adult workers. Levels of phenoloxidase, an enzyme involved in melanisation and part of the bee's defence system, were largely constitutive and resilient to exposure in honey bees and bumble bees. In honey bees, transient transcriptional changes in antimicrobial effector genes were observed following neonicotinoid exposure, but the physiological antimicrobial response was unaffected. In bumble bees, the induced antimicrobial response was impaired following neonicotinoid exposure, but only when exposed to concentrations likely higher than realistic environmental exposure scenarios.

The next phase of this thesis investigates whether transcriptional, physiological and behavioural endpoints associated with the functioning of the honey bee hypopharyngeal gland were altered by imidacloprid exposure. Imidacloprid exposure led to transcriptional changes in foraging genes (associated with the control of temporal polyethism) and major royal jelly proteins (fed to developing larvae by nurse workers) and enzymatic changes in glucose oxidase (an enzyme involved in social immunity), which I hypothesise are linked with hypopharyngeal gland development. Despite these laboratory observations, no behavioural effects were observed in a field setting, monitored using Radio Frequency Identification transponders.

Lastly, using RNA-Sequencing to investigate changes across the honey bee transcriptome, this thesis identified a suite of genes that were differentially

expressed in adult workers in response to immune challenge and/or dietary neonicotinoid exposure. Wounding and bacterial-like infection led to upregulation of known immune genes, including a peptidoglycan recognition protein and antimicrobial effectors. Chronic exposure to thiamethoxam and imidacloprid led to downregulation of genes associated with several metabolic pathways, such as oxidative phosphorylation, pyruvate- and purine- metabolic pathways, as well as ribosomal activity. Some of these genes identified provide candidates for further study to elucidate functional effects mechanisms and better understand health outcomes, as well as potential new biomarkers for use in pesticide risk assessment.

This thesis presents novel findings and offers opportunities for future research that will be of interest to a wide audience, including risk assessors and policy makers, as well as the broader biological community, including ecotoxicologists, insect physiologists and molecular biologists.

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*Figure 6.8.* (Page 244) Summary of GO terms enriched across the 163 DEGs in the Naïve 24 h Control vs Thiamethoxam subset, at an FDR of 0.05.

## **Author's declaration**

Unless otherwise stated, the author was responsible for all data collection and analysis. The use of the first person plural (i.e 'we' as opposed to 'I') reflects the contributions of the supervisors in providing advice in experimental design, data analysis and interpretation, and of the numerous apprentices, intern students and technicians at Fera in providing laboratory and field support during data collection.

## Abbreviations

°C - Degrees Celcius

µg - Micrograms

µgL<sup>-1</sup> - Micrograms per litre

µl - Microlitres

AMP - Antimicrobial peptide

AmPPO - *Apis mellifera* prophenoloxidase gene

ANCOVA - Analysis of Covariance

ANOVA - Analysis of Variance

BLAST - Basic Local Alignment Search Tool

BLASTX - BLAST search of a protein database using a translated nucleotide query

BQCV - Black queen cell virus

CCSS - Centre for Chemical Safety and Stewardship

cDNA - Complementary deoxyribonucleic acid

CO<sub>2</sub> - Carbon dioxide

COLOSS - Prevention of Honey Bee Colony Losses

CPV - Chronic paralysis virus

CRD- Chemicals Regulation Directorate

C<sub>t</sub> - Cycle threshold

d - Days

DEG - Differentially expressed gene

df - Degrees of freedom

DNA - Deoxyribonucleic acid

dNTP - Deoxyribose nucleoside triphosphate

Dscam - Down syndrome cell adhesion molecule

DWV - Deformed wing virus

EFSA - European Food Safety Authority

EU - European Union

FDR - False discovery rate

Fera - The Food and Environment Research Agency

g - Grams

GO - Gene ontology

GOX - Glucose oxidase

h – Hours

I $\kappa$ B - I kappa B

Imd - Immune deficiency

IMI - Imidacloprid

IPM - Integrated pest management

JAK/STAT - Janus kinase/ Signal transduction and transcription

JNK - c-Jun N-terminal kinases

KEGG - Kyoto Encyclopedia of Genes and Genomes

LD<sub>50</sub> - Lethal dose, 50 %

L-Dopa - L-3,4-dihydroxyphenylalanine

LLR - Leucine-rich repeats

LN - Natural logarithm

Log - Base<sup>-10</sup> logarithm

LPS - Lipopolysaccharides

M - Gene stability measure

mg - Milligrams

mgml<sup>-1</sup> - Milligrams per millilitre

mm - Millimetres

mM - Micromolar

MRJP - Major royal jelly protein

mRNA - Messenger ribonucleic acid

MTU - Molecular Technology Unit

n - Sample size

NBU - National Bee Unit

NCBI - National Center for Biotechnology Information

ng - Nanograms

nM - Nanomolar

PBS - Phosphate buffer solution

PCR - Polymerase chain reaction

PGRP - Peptidoglycan recognition protein

PIC - Post immune challenge

PO - Phenoloxidase

ppb - Parts per billion

ppm - Parts per million

PPOact - Prophenoloxidase-activating enzyme

PPP - Plant protection product

proPO - Prophenoloxidase

qPCR - Quantitative real-time polymerase chain reaction

RFID - Radio frequency identification

RH - Relative humidity

RNA - Ribonucleic acid

RNAi - Ribonucleic acid interference

RNA-Seq - Ribonucleic acid sequencing

RPM - Revolutions per minute

rRNA - Ribosomal ribonucleic acid

SBV - Sacbrood Virus

SD - Standard deviation

SE - Standard error

TBLASTX - BLAST search of a translated nucleotide database using a translated nucleotide query

Tak1 - TGFbeta Activated Kinase 1

THC - Total haemocyte count

$T_m$  - Melting temperature

TMX- Thiamethoxam

UK - United Kingdom

USA - United States of America

V - Pairwise variation

$V_{max}$  - Maximum reaction rate

w/v - Weight to volume

$\chi^2$  - Chi-square

## 1. General Introduction

Bees have long been of ecological, economic and cultural importance, and their value to both natural ecosystems and human societies remains just as vital today. However, there is widespread concern over declines in wild bee populations and losses of managed colonies. Anthropogenic activities are likely impacting on bee health, including habitat loss and fragmentation, the introduction of alien species (including plants, animals and microorganisms), the spread of pathogens and parasites, and pesticide application. The application of one particular class of pesticides, the neonicotinoids, has increased rapidly in the UK since introduction to the agrochemical market in the 1990s. These neonicotinoid pesticides have been of particular recent concern to bee health due to the potential exposure of bees through residues in nectar and pollen of flowering plants, but whether realistic exposure scenarios are driving declines in bee health remains unknown. Increasing debate over the impacts of pesticide exposure on bee health has questioned whether the current risk assessment schemes for plant protection products are appropriate.

Managed honey bees and wild bumble bee species are amongst the most important insect pollinators in the UK, contributing to the pollination of both wild flowering plants and commercial agricultural and horticultural crops. This chapter introduces these key pollinators and reviews our current understanding of the impacts of neonicotinoid exposure and other threats on bee health. It highlights key knowledge gaps in this research field and puts in context the aims and objectives of this thesis, which are summarised at the end of the chapter.

I first provide an overview of the biology of honey bees and bumble bees, focusing on the study species of this thesis, and recognise their importance as ecosystem service providers. I then introduce the current system in the UK for the risk assessment of plant protection products on bees. This thesis primarily focuses on the role of pathogenic infection and pesticide exposure as threats to bee health, and these are discussed in context in a separate paper within this introductory chapter, which has been accepted for publication.

### 1.1. *Biology of bees*

Here I briefly review bee natural history to highlight that there are many levels of organisation and potential targets for effects of pesticide exposure and therefore many ecotoxicological endpoints for study.

Today there are approximately 25,000 known bee species (of which 253 are known from the UK) (Goulson, 2010). The earliest bees probably diverged from the predatory sphecoid wasps during the Cretaceous period, 100-130 million years ago, diversifying away from a diet of insect prey to specialise on nectar and pollen (Winston, 1987). The appearance of the first bees coincided with the appearance of flowering plants (angiosperms) as the dominant vegetation. The earliest bees were almost certainly solitary species, but sociality amongst the bees probably evolved around 80 million years ago (Winston, 1987).

#### 1.1.1. *The honey bee, Apis mellifera*

The Western or European honey bee, *Apis mellifera* L., most likely originated in eastern tropical Africa during the Tertiary period, before spreading to Northern Europe and Asia (Winston, 1987, Whitfield et al., 2006). Although non-native to the Americas, it was first imported to North America by European colonists in the 17<sup>th</sup> century and its movement for beekeeping has resulted in a now worldwide distribution. Its vast geographic range has led to geographic differentiation into as many as 28 subspecies (races) (Engel, 1999), each with distinctive adaptations to different climates, habitats and flora. Although cross-fertile, these races show various local adaptations including brood cycles synchronised with localised floral phenology (Hepburn, 1998), enhanced foraging behaviours in desert areas (Alqarni, 2006) and migratory swarming in Africa (Nuru et al., 2002). Nevertheless, beekeeping has changed the range of these races too, and there is considerable hybridisation of races and selective breeding for certain traits.

The honey bee has a complex social organisation, in which approximately 60,000 individuals comprise a single colony. The colony has a perennial life cycle, which is briefly outlined here. There is typically a single queen in the colony, who is the only female to lay eggs. She will lay throughout the spring and summer (as many as 1500 eggs a day in peak production), only ceasing laying in late autumn and over winter, and she may live for several years (with

an average productive life span of 2-3 years). The majority of eggs will be fertilised by releasing several sperm from the spermatheca as she lays, developing into female workers (or potentially new queens). Each egg is laid one per cell of a wax brood comb, using its yolk as a food source as it develops. Larvae develop after three days and are fed by nurse worker bees with royal jelly, honey and pollen (bee bread), before the cells are capped with wax (typically after about six days for worker larvae). Within these capped cells, larvae develop into pupae, and after about 12 days newly developed adult bees emerge by chewing their way out. Unfertilised eggs are typically laid in larger cells and develop into haploid male drones (the largest individuals in the colony). Drones are normally fed by the workers, although they can also feed themselves from sources within the hive, but they do not forage for their own nectar or pollen. They are generally only found in late spring and summer, and may number several hundred in the colony during this time. Although not all drones will fertilise a virgin queen, this is their sole function (after which they die instantly). Virgin queens will attract drones during mating flights by releasing pheromones and may mate with 7-15 drones. New (virgin) queens are raised by workers i) to supersede the mother queen if she begins to fail and decreases production of her 'queen substance' pheromone, ii) as an 'emergency' if the mother queen is killed or lost from the colony, and iii) in preparation for swarming. In the latter case, eggs are raised in larger 'queen' cells in gaps in the wax brood comb, or along the bottom of the comb frames. Supersedure and emergency queens are typically raised by modifying worker cells so that they hang vertically from the comb surface. Caste development of fertilised eggs is thought to depend on the nutrition supplied to the larvae by the nurse workers (Kamakura, 2011).

Whilst honey bee and bumble bee colonies both consist of three castes of bees (queen, worker and drone), honey bees also show a further level of caste differentiation known as temporal polyethism. Female workers within the honey bee colony may specialise on different sets of tasks, and these tasks may change as an individual worker ages. In this way, younger workers may typically perform tasks within the hive, acting as nurses in brood care duties, whilst older workers may typically perform tasks outside the hive, acting as foragers and in colony defence duties (Calderone, 1998, Johnson, 2010).

### 1.1.2. The bumble bee, *Bombus terrestris*

Classified within the same subfamily as the honey bees (*Apinae*), the bumble bees comprise a separate genus (*Bombus*). The first bumble bees most likely originated 30-40 million years ago, in the mountains of central Asia, coinciding with a period of cooler temperatures that would have favoured the adaptation of larger, furrer bees (Goulson, 2010). Today, there are about 250 bumble bee species (Williams, 1998). Their natural distribution spans Europe, Asia, North and South America, and otherwise their distribution in the southern hemisphere (including Australia, New Zealand) results only from recent deliberate introductions by humans (e.g. Hopkins, 1914).

27 bumble bee species are found naturally in the UK, and amongst the most common of these is the buff-tailed bumble bee, *Bombus terrestris* L., which is widely distributed across England, Wales, Northern Ireland and southern and central Scotland (BWARS, 2014). It is also widespread in Europe, and middle and northern latitudes of Asia, and is the most widely used bumble bee species for commercial glasshouse pollination (Velthuis & Van Doorn, 2006).

Like most bumble bee species, *B. terrestris* colonies have an annual cycle, and at their largest may comprise up to 350 individuals (Goulson, 2010). They are often described as 'primitively eusocial', as their social organisation is simpler than that of the honey bee. Young mated queens at the end of the colony cycle are the only individuals to overwinter, hibernating until the following spring when they found a new colony. Queens emerge from hibernation in spring (typically March in the UK, depending on weather conditions) and search for suitable nest sites in cavities such as deserted small rodent nests, dead logs, dead tussocks of grass or the base of thick hedges. On finding a suitable nest site, the queen will exude wax and use this wax to form a honey pot, into which she regurgitates nectar to form a store of honey. She will also forage to collect pollen and return this to the nest where she will moisten it with nectar to form a ball of 'bee bread'. Feeding on pollen stimulates the queen's ovaries to produce eggs, and she will lay these in batches of about 4-16 on the ball of bee bread, then covering them in a layer of wax. The queen will brood these eggs at 30 °C, feeding on the honey pot close by, and leaving the eggs for only short periods if she needs to leave the nest to forage for more nectar and pollen. Eggs hatch after 4-5 days, and the larvae will feed on the bee bread pollen, pupating after

three weeks and these pupae then emerging as adult workers another two weeks later. These workers may spend their first few days as adults producing wax to build new egg cells, and then they begin to forage. By collecting pollen and returning it to the nest, this enables the queen to spend almost all her time in the nest, laying eggs to increase the colony size. When the new queen mated prior to hibernation in the autumn of the previous year, she stored the sperm of her mate in the spermatheca. Throughout the laying season, the queen lays fertilised eggs by releasing sperm from the spermatheca, in turn producing female workers. It is only at the end of the colony life cycle (in late spring/early summer) that the queen lays unfertilised eggs which develop into males. At this stage she will not lay any more eggs that will develop into female workers, but will only lay a few fertilised eggs to develop into new queens. The development of queens from these fertilised eggs is stimulated by the loss of production of a pheromone by the queen. The developing larvae and pupae will feed considerably more than developing workers, and consequently will be larger in size. At this late stage of the colony cycle, some remaining workers may also produce unfertilised eggs, developing into males. Males feed on honey stores in the nest for a few days when they first emerge as adults and then permanently leave the nest, foraging for themselves and flying in circuits depositing a sex-pheromone on objects such as tree trunks and rocks to attract new queens for mating. New queens emerge about a week later than the new males, and unlike these males they will return to the nest to shelter (although they do forage for themselves). When ready to mate, she will leave the nest and use the pheromone cues of the male to find a suitable mate, usually only mating with a single male whilst resting on the ground or vegetation. Whilst the mother queen, workers and drones all die in autumn, the newly mated queens stock their honey stomach (by feeding on nectar) and build their fat bodies (by feeding on pollen and nectar) before finding a suitable hibernation site in the ground to spend the winter.

## 1.2. Bees as providers of ecosystem services

Many wild plant communities and agricultural and horticultural crops rely on animal pollinators (Wilcock and Neiland, 2002, Kearns et al., 1998), including several species of honey bees, bumble bees, stingless bees and solitary bees (Klein et al., 2007). Natural pollination services as a whole have been estimated at £430 million a year in the UK (UK National Ecosystem Assessment, 2011), whilst wild bees have been valued at over \$3 billion a year to US crop production, contributing over \$750 million to soybean production alone (Losey and Vaughan, 2006). Where wild bee populations do not visit agricultural fields, managed populations of *A. mellifera* provide essential pollination services, increasing the edible yield of 40 % of the world's 115 leading crop species (Pesticide Action Network, 2008). In the UK alone, honey bee pollination services are estimated at a monetary value of £200 million a year (National Audit Office, 2009), and estimates for the role of honey bees in US crop production are as high as \$15 billion annually (Morse and Calderone, 2000). Bumble bees are also reared commercially for the pollination of greenhouse crops, such as tomatoes, peppers and strawberries (Velthuis and van Doorn, 2006). Whilst precise figures are unknown, global trade in bumble bee colonies has been estimated at over one million nests per year (Goulson, 2010), and Japan alone imports about 40,000 bumble bee colonies annually (Asada and Ono, 2000). Furthermore, the importance of pollinators for the maintenance of natural plant diversity should not be underestimated. For example, in a study in Britain and the Netherlands, Biesmeijer et al. (2006) found a parallel decline between pollinators and outcrossing insect-pollinated plants relative to other plant species. Increased bee diversity is associated with increased pollination success (Klein et al., 2003). These figures briefly highlight the importance of both honey bees and bumble bees to the pollination of worldwide crops and human food production, and the success of wild flowering plants. This demonstrates the need to understand declines in bee health to minimise the long term consequences of bee population declines to ecological, agricultural and horticultural systems.

### *1.3. Risk assessment of plant protection products on bees*

Here I give a brief synopsis of the current procedures for the authorisation of plant protection products (PPPs) in the UK and Europe. This introduction serves to put into context the study of pesticide effects on non-target organisms, including beneficial pollinators such as bees, and the application of these scientific studies in the ecotoxicological risk assessment of PPPs.

As an EU Member State, the UK must authorise the placing of commercial PPPs on the market, as well as their use and control within the European community, under Regulation (EC) No 1107/2009 of the European Parliament. This is regulated nationally in the UK by the Chemicals Regulation Directorate (CRD), and internationally by the European Food Safety Authority (EFSA). The Regulation sets out an approval criteria for active substances and PPPs containing these actives, which must be met prior to the authorisation of a product to meet specific protection goals that are set out with the aim that any effects of PPP exposure are within a defined level of 'acceptable harm'. Amongst these criteria includes an assessment of the risks of exposure and the effects of this exposure on bees, following a set of test guidelines based on scientific opinion (e.g. OECD, 1998). Given the scientific and public concern that bee health may be at particular threat from pesticide exposure, a new guidance document was published by EFSA in 2013, following a review of the scientific opinion on the risk assessment of PPPs on bees by EFSA in 2012. To summarise, this new guidance document proposed several new areas of assessment to be completed as part of a bee risk assessment, including a requirement to assess risks to bumble bees and solitary bees as well as honey bees, a requirement to include chronic oral, larval, accumulation potential and hypopharyngeal gland assessments in the lower tier screening assessment, and a requirement to assess the exposure from water consumption (such as guttation fluid, surface water and puddles) and the risk from metabolites. Several other recommendations were included in this guidance, including a priority for research to determine the most appropriate sublethal endpoints for use in future risk assessment, and the development of ecotoxicological biomarkers to enable prediction of potential sublethal effects (e.g. Badiou-Beneteau et al., 2012, Carvalho et al., 2013).

#### *1.4. Interactive effects of pesticide exposure and pathogen infection on bee health – a critical analysis*

Further introduction and background to support the aims of this thesis are discussed in the paper presented below, which was written by the primary author and formed a significant part of the research for this thesis. This paper was accepted for publication in *Biological Reviews* in June 2015 (Collison et al., 2015).

##### *1.4.1. Abstract*

Bees are fundamentally important for pollination services and declines in populations could have significant economic and environmental implications. Pesticide exposure and pathogen infection are recognised as potential stressors impacting upon bee populations and recently there has been a surge in research on pesticide–disease interactions to reflect environmentally realistic scenarios better. We critically analyse the findings on pesticide–disease interactions, including effects on the survival, pathogen loads and immunity of bees, and assess the suitability of various endpoints to inform our mechanistic understanding of these interactions. We show that pesticide exposure and pathogen infection have not yet been found to interact to affect worker survival under field-realistic scenarios. Colony-level implications of pesticide effects on *Nosema* infections, viral loads and honey bee immunity remain unclear as these effects have been observed in a laboratory setting only using a small range of pesticide exposures, generally exceeding those likely to occur in the natural environment, and assessing a highly selected series of immune-related endpoints. Future research priorities include the need for a better understanding of pesticide effects on the antimicrobial peptide (AMP) component of an individual's immune response and on social defence behaviours. Interactions between pesticide exposure and bacterial and fungal infections have yet to be addressed. The paucity of studies in non-*Apis* bee species is a further major knowledge gap.

##### *1.4.2. Introduction*

There is widespread concern over losses of managed honey bees and declines in wild bee populations (e.g. Biesmeijer et al., 2006; Goulson et al., 2008; Neumann & Carreck, 2010; vanEngelsdorp et al., 2008) due mainly to the

potential impact on pollination of crops and wild plants (see Potts et al., 2010). Potential stressors impacting on bee health include habitat loss and fragmentation, the introduction of alien species (including plants, animals and microorganisms), climate change, the spread of pathogens and parasites, and pesticide application (Potts et al., 2010). To manage bee declines better, it is important to understand how these multiple stressors could impact bee health, and whether the importance of different stressors varies with bee species, geographic location and time. Over the past 30 years there has been a steady accumulation of studies investigating the individual stressors that could cause bee declines, but investigation of different stressors in combination has only recently gained momentum (Figure 1.1.). The study of stressors in combination is important because this approach more likely reflects the complexity of realistic ecological scenarios and may reveal otherwise unforeseen sublethal effects and synergies among stressors. Combinations of stressors that have been studied have included infection with multiple pathogens (Martin et al., 2012; Yang & Cox-Foster, 2005) and exposure to chemical (pesticide) mixtures (Johnson et al., 2013; Johnson et al., 2009). Here, we focus on the effects of pesticide exposure in combination with pathogen infection. In a *Web of Science* search of the literature (conducted in 2014) using the key words 'disease', 'pesticides' and 'bees', only 20 of the 196 papers identified were on non-*Apis* bee species [16 on bumble bees (*Bombus* spp.) and four on solitary bees (e.g. *Andrena*, *Megachile* and *Osmia* spp.]. Our review is therefore necessarily focused primarily on honey bees (*Apis* spp.), but we also include studies of non-*Apis* bee species where they are available.

The effect of a stressor can be acutely lethal (increasing the rate of mortality) or sublethal, modifying performance, for example in aspects of growth, fecundity, immunity, longevity or behaviour (Desneux et al., 2007). There is an increasing body of evidence that some pesticide applications have sublethal effects that may impact on bee health, including inhibiting colony growth and queen production (Whitehorn et al., 2012), altering foraging behaviour (Decourtye et al., 2004; Mommaerts et al., 2010; Schneider et al., 2012), impairing learning ability (Decourtye et al., 2004) and reducing fecundity (Laycock et al., 2012; Mommaerts et al., 2010; Tasei et al., 2000). Whilst sublethal effects may not cause immediate population declines through increased bee mortality rates,

they may decrease the long-term survival and growth of colonies and thereby affect the success of bee populations. In this review we focus both on the lethal effects of simultaneous pesticide exposure and pathogen infection, and sublethal effects of pesticide exposure on the ability of bees to defend against pathogenic or parasitic attack, or 'immunocompetence'. Previous published reviews highlight a lack of studies investigating the effect of interactions between pesticide and disease exposure in bees (James & Xu, 2012). In response, there has been a recent proliferation in the investigation of pesticide–pathogen interactions to address this knowledge gap, with 25% of the total number of publications on the topic over the last 20 years published in 2013 alone (Figure 1.1.A).

This review aims to provide a synthesis and critical analysis of findings regarding pesticide–disease interactions in bees to identify trends and discrepancies in the data and to highlight gaps in our understanding. It is hoped that this will focus future research, contributing to our understanding of the underlying mechanisms driving pesticide–disease interactions, and be of value in the assessment of potential sublethal ecotoxicological endpoints for risk assessment. We first provide a brief overview of the pathogens and pesticides of most current concern for bees to set the required context for our analysis on the effects of pesticide–pathogen interactions on bee health.

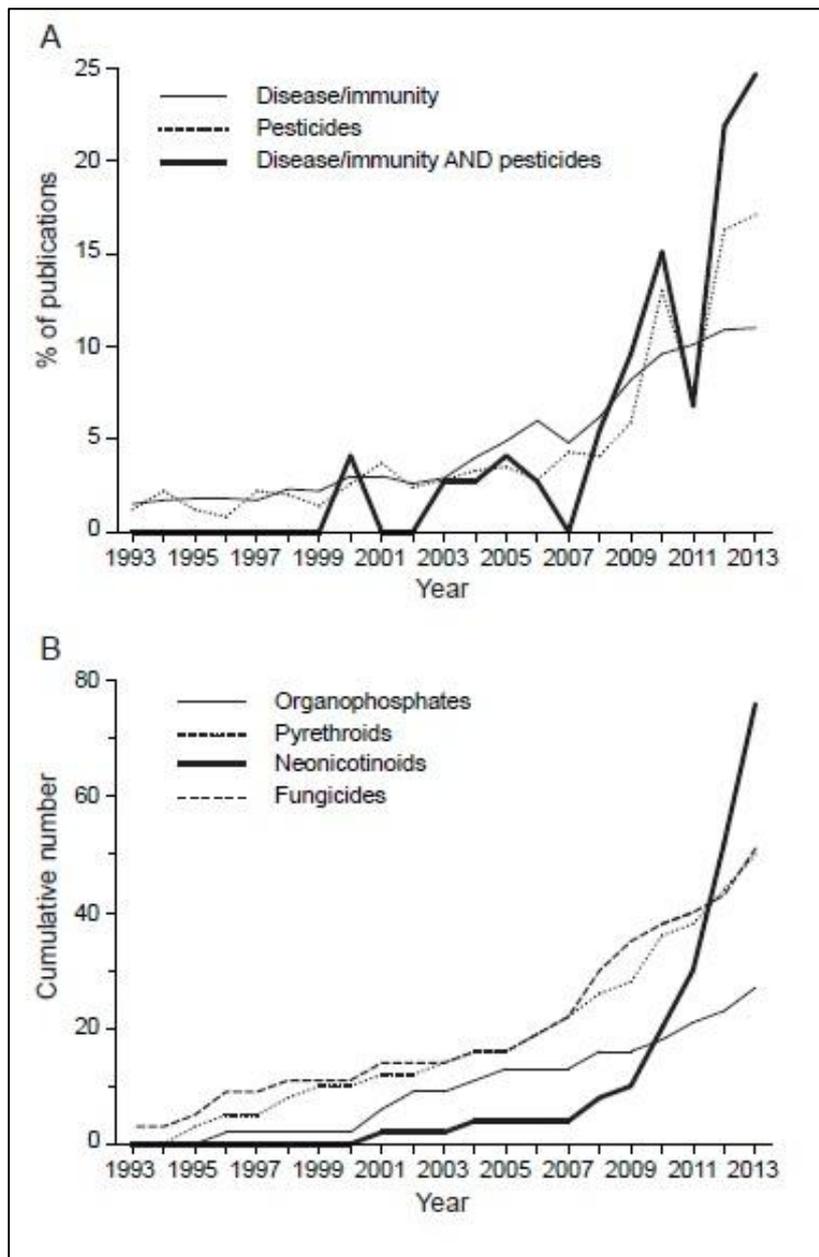


Figure 1.1. (A) Frequency of publications on each topic published each year as a percentage of the total number published in the last 20 years (1993–2013). (B) Cumulative number of publications on bees and pesticides in the last 20 years (1993–2013). In both cases, data are based on a *Web of Science* key word search in December 2013 using the key words ‘bee’ and those described in the key to each plot.

#### 1.4.2.1. Bee pathogens and parasites

Honey bees and bumble bees are susceptible to a wide range of pathogens and parasites, which can infect all stages of the bee life cycle. Severe infections can kill or weaken the colony in wild bumble bees (e.g. Schmid-Hempel & Schmid-Hempel, 1988) and domesticated honey bees (e.g. Genersch, 2010) causing significant economic impact to beekeepers (e.g. Botias et al., 2013; Zaghoul et al., 2005). The most common of these pathogens and parasites and their modes of action are summarised in Table 1.1., and they include bacterial, fungal and viral pathogens (with examples that include *Paenibacillus larvae*, *Ascophaera apis* and Deformed Wing Virus, respectively), microsporidia pathogens (most notably *Nosema* spp.), and ectoparasitic mites, the main example being the *Varroa* mite (*Varroa destructor*).

*Varroa destructor* has spread rapidly amongst honey bee populations across the world, and now has a near-global distribution (with the notable exception of Australia) (Oldroyd, 1999). Coupled with its close-association with the transmission of viruses, *Varroa destructor* is often considered the most important pest of *Apis mellifera* and has been linked to the loss of millions of colonies worldwide (Martin, 2001; Martin et al., 2012).

Two bacteria, *Paenibacillus larvae* and *Melissococcus plutonius*, are the infective agents behind American and European foulbrood disease, respectively. These are the only formalised bacterial diseases of honey bee larvae, but are found worldwide and considered among the primary threats to honey bee colony losses (Evans & Schwarz, 2011).

Whilst *Nosema* spp. are typically ubiquitous in honey bee colonies, its role in recent colony losses remains unclear, and its impact may result from interactions with other stressors, rather than as a sole key factor (see review by Evans & Schwarz, 2011).

However, in contrast to their relative prevalence and impact in honey bee colonies, amongst the most important pathogens and parasites of bumble bees are the trypanosome protozoa, parasitic flies and tracheal mites (Shykoff & Schmid-Hempel, 1991; Schmid-Hempel & Schmid-Hempel, 1998; Whitehorn et al., 2011).

**Table 1.1.** Summary of major pathogens and parasites of honey bees (*Apis* spp.) and bumble bees (*Bombus* spp.).

Species (common name)	Taxonomic affiliation	Mode of action	Affects <i>Apis</i>	Affects <i>Bombus</i>	References
<i>Varroa destructor</i> ( <i>Varroa</i> )	Mite (Acari: Arachnida)	Ectoparasite of pupae and adults. Strong associations with the transmission of several bee viruses	✓	✗	Martin (2001), Sammataro et al. (2000)
( <i>Nosema</i> spp.), including <i>N. apis</i> , <i>N. ceranae</i> and <i>N. bombi</i>	Microsporidian	Germinates in bee gut, leading to dysentery. Use host resources and suppress immune response	✓	✓	Coffey (2007), Otti & Schmid-Hempel (2007)
<i>Melissococcus plutonius</i> (European Foulbrood, EFB)	Bacterium	Multiplies in the larval gut, generally not lethal but stresses the colony	✓	✗	Forsgren (2010)
<i>Paenibacillus larvae</i> (American Foulbrood, AFB)	Bacterium	Multiplies in the larval gut, often leading to death of developing larvae	✓	✗	Hansen & Brodsgaard (1999)
<i>Ascospaera apis</i> (Chalkbrood)	Fungus	Infects the larval gut, leading to death of developing larvae	✓	✗	Aronstein & Murray (2010)
Several viruses, including Deformed Wing Virus (DWV), Black Queen Cell Virus (BQCV) and Acute Bee Paralysis Virus (ABPV)	Virus	Intracellular parasites, affecting various life stages. Often lead to deformities in developing bees, such as deformed wings	✓	✓	Graystock et al. (2013), Martin et al. (2012)
( <i>Crithidia</i> spp), including <i>C. bombi</i> , <i>C. expoeki</i> , <i>C. mellificae</i>	Trypanosome protozoa	Gut parasite, transmitted via contaminated faeces	✓	✓	Brown et al. (2003), Ravoet et al. (2013), Shykoff & Schmid-Hempel (1991), Whitehorn et al. (2011)
<i>Locustacarus buchneri</i>	Mite (Acari: Arachnida)	Tracheal mite, overwinters in hibernating queens	✗	✓	Otterstatter & Whidden (2004), Whitehorn et al. (2011)
Conopid and phorid flies	Fly (Diptera)	Parasite, laying eggs in bee abdomen	✓	✓	Core et al. (2012), Schmid-Hempel & Schmid-Hempel (1988)

#### 1.4.2.2. *Pesticides of current concern in bee research*

##### 1.4.2.2.1. *Chemicals and mode of toxicity*

Crops are protected from insect pests by the application of insecticides and three classes dominate current usage: organophosphates, pyrethroids and neonicotinoids (Koehler & Triebkorn, 2013). These three classes are all neurotoxic to insects, causing neurons to excite, with organophosphates inhibiting acetylcholinesterase, pyrethroids prolonging the opening of voltage-gated sodium channels, and neonicotinoids acting as nicotinic acetylcholine receptor (nAChR) agonists (reviewed by Belzunces et al., 2012). Over the last 5–10 years there has been a considerable increase in the number of publications investigating effects of these pesticides on wildlife (Koehler & Triebkorn, 2013). In line with this, the number of studies investigating effects of the three main insecticide classes (organophosphates, pyrethroids and neonicotinoids) specifically on bees has also increased with the greatest attention on the neonicotinoids. For example, in a *Web of Science* search of the literature (conducted in 2014) using the key words ‘bees’ and ‘organophosphates’, ‘pyrethroids’ or ‘neonicotinoids’, three, 12 and six publications were identified between 2006 and 2009 for organophosphates, pyrethroids and neonicotinoids, respectively, whilst 11, 22 and 66 were identified between 2010 and 2013 (Figure 1.1.B). Fungicides are also widely used and may be harmful to bees, mainly by their interactions with other pesticides, for example by inhibiting the metabolism, detoxification and excretion of insecticides (e.g. Johnson et al., 2013; Pilling et al., 1995; Schmuck et al., 2003).

##### 1.4.2.2.2. *Modes of exposure*

Pyrethroids, organophosphates and fungicides are usually applied to crops *via* sprays, so bees can be exposed by direct contact either during application or from residues present on plant surfaces, which can also be ingested in nectar and pollen. Neonicotinoids are applied as sprays, as well as *via* soil and topical plant treatments (e.g. leaf or root dipping), but most commonly they are applied as seed dressings. Irrespective of the mode of delivery, neonicotinoids are taken up by the plant and circulated systemically to the leaves, shoots and

flowers as they grow. In this way target insects, including wireworms, cutworms, aphids and leafhoppers, are exposed to the insecticide in their diet as they feed on the plant tissue, including seeds, roots, stems and sap. Beneficial pollinating insects, including honey bees and bumble bees, consume neonicotinoids at trace levels in the nectar and pollen of the treated crop plants (e.g. Pohorecka et al., 2012). Managed honey bee colonies are also exposed to acaricide pesticides, which are frequently applied directly to the hives by beekeepers to control for mite infestations. Acaricide treatments include plant-derived products (e.g. thymol), organic acids (e.g. formic acid) and synthetic pesticides, including formamidine, pyrazole, organophosphates and pyrethroid insecticides (Johnson et al., 2010), some of which are considered in this review.

#### *1.4.2.2.3. Spatial extent of exposure and evidence of contamination levels*

Here, we evaluate the spatial extent of pesticide application and bee exposure principally in the context of a European (and specifically UK) landscape, but we also include comparisons on usage with other countries. We note also that other developed countries generally follow similar agricultural practices. A quarter of the UK land area is arable (WorldBank, 2014), and 85% of this is sprayed with pesticides (Twining & Simpson, 2009). A small proportion (3.3%) of UK agricultural land (pasture and arable) is classified under organic management (DEFRA, 2013), similarly to that occurring in other developed European nations including France (3.6% in 2012) and Germany (5.8% in 2012) (Eurostat, 2015). Organic management does not however exclude this land from the use of pesticides as definitions of 'organic' vary, and most allow use of 'organic insecticides' which may pose risk to bees. The top pesticide classes, ranked on their 2012 usage on UK arable land (Garthwaite et al., 2013), are: fungicides (50% of treated pesticide area) > pyrethroids (6.3%) > neonicotinoids (1.7%) > carbamates (0.7%) > organophosphates (0.2%). By contrast, a tentative ranking based on the residues detected in honey bee colonies at given sites in North America (Mullin et al., 2010; Wu et al., 2012) and France (Chauzat et al., 2011; Lambert et al., 2013) shows: acaricide insecticides (pyrethroids and organophosphates) > fungicides > organophosphates > pyrethroids > carbamates and neonicotinoids. It is a shortcoming that the

application and residue data are collated from different geographical regions, but this tentative evaluation shows that the ranking of spatial exposure is not represented consistently in residues detected in honey bee colonies. The top-ranked pesticides detected as residues in colonies were typically acaricide insecticides and fungicides, consistent with the frequent direct application of acaricides to hives to control mites and the dominance of fungicides in UK pesticide usage. Pyrethroid residues were typically found in < 15% of hive samples, but nevertheless cypermethrin was amongst the top ten residues found in French wax samples, and deltamethrin in honey and bee samples (Chauzat et al., 2011). Despite their relatively minor share of the market, organophosphate residues, including chlorpyrifos, triphenylphosphate and azinphos-methyl, were more commonly found in hive samples than pyrethroids, neonicotinoids and carbamates. Overall neonicotinoid residues were found in < 1–5 % of samples analysed from across the USA and France, with the exception of one study in which imidacloprid and its metabolite 6-chloronicotinic acid were found in 20–40% of honey and pollen samples (Chauzat et al., 2011). Although both the use of neonicotinoids and the amounts of neonicotinoid residues found in apiaries are relatively low compared to the other major pesticide classes, their effects on bees have recently received greater relative interest (Figure 1.1.B), perhaps due to the very significant increase in their use since their introduction in the 1990s and their systemic nature. In the UK, the use of neonicotinoids has risen from a national application rate of 3 tonnes per year in 1994 to nearly 80 tonnes in 2011 (reviewed by Goulson, 2013). In 2008, neonicotinoids represented 24% of the global agrochemical market and they are registered for use in 120 countries (Jeschke et al., 2011). Growing concern over the sublethal effects of neonicotinoids on pollinator populations led the European Commission (2013) to introduce a two-year restriction on the use of three neonicotinoid pesticides (imidacloprid, clothianidin and thiamethoxam) on bee-attractive crops in Europe until further research can clarify their effects on pollinators.

### *1.4.3. Interactive effects of pesticide exposure and pathogen infection on bee health*

Here we address the following three key research questions that relate to the interactions of pesticides and pathogen infections on bee health: (1) do pesticide exposure and pathogen infection increase pathogen load? (2) Do pesticide exposure and pathogen infection reduce bee immunocompetence? (3) Do pesticide exposure and pathogen infection interact to reduce bee survival?

Each question focuses on a different endpoint of biological interest, the underlying biological mechanisms and/or the ecological relevance of pesticide–pathogen interactions. With respect to each question, we assess evidence in support or otherwise, and briefly outline the suitability of each endpoint to inform our understanding of pesticide–pathogen interactions.

In our review, we use the term ‘interaction’ between two or more stressors to indicate a combined effect on bee survival that is greater than the sum of the effects of each stressor alone (Figure 1.2.), i.e. a ‘more-than-additive’ statistical effect. In the context of pesticide–pathogen interactions, we avoid the use of ‘synergism’ and ‘antagonism’ because these terms bring interpretive complications. The terms ‘synergistic/antagonistic’ are widely used to describe any statistical departure from additivity, but in toxicology they specifically denote an interaction between two or more chemical stressors (IUPAC, 2015) through a common biological mechanism (Solomon et al., 2008), and they should be avoided in a toxicological context when either the underlying biological mechanisms are unknown (Kupper & Hogan, 1978) or when the two stressors are known to cause their effects through independent biological mechanisms (Blot & Day, 1979). Because we refer to both a chemical and non-chemical (pathogenic) stressor and our understanding of the biological basis of the impact of multiple stressors is incomplete, we have chosen to refer to statistical non-additivity between stressors by the term ‘interaction’ because it refers to solely an observable pattern that is mechanistically neutral and we restrict it to signify exclusively a positive interaction increasing bee mortality, as depicted in Figure 1.2.

Most studies on pathogen load and immunocompetence have investigated possible mechanisms that could underlie pesticide–pathogen interactions on

bee survival and they have not directly investigated interactions between these stressors. Any toxicant that increases bee pathogen load or reduces bee immunocompetence is analogous to a potentiation because the toxicant exposure alone cannot affect pathogen load or immunocompetence in the absence of the pathogen. However, the term potentiation has been previously used restrictively to refer to toxicant–toxicant interactions (IUPAC, 2015), and it seems both anomalous and misleading to extend it to pathogen–toxicant interactions. Here we address whether a combined pesticide exposure and pathogen infection increases pathogen load and/or reduces immunocompetence as possible mechanisms leading to more-than-additive effects on bee survival, but avoid specifically referring to each mechanism itself as an interaction or potentiation.

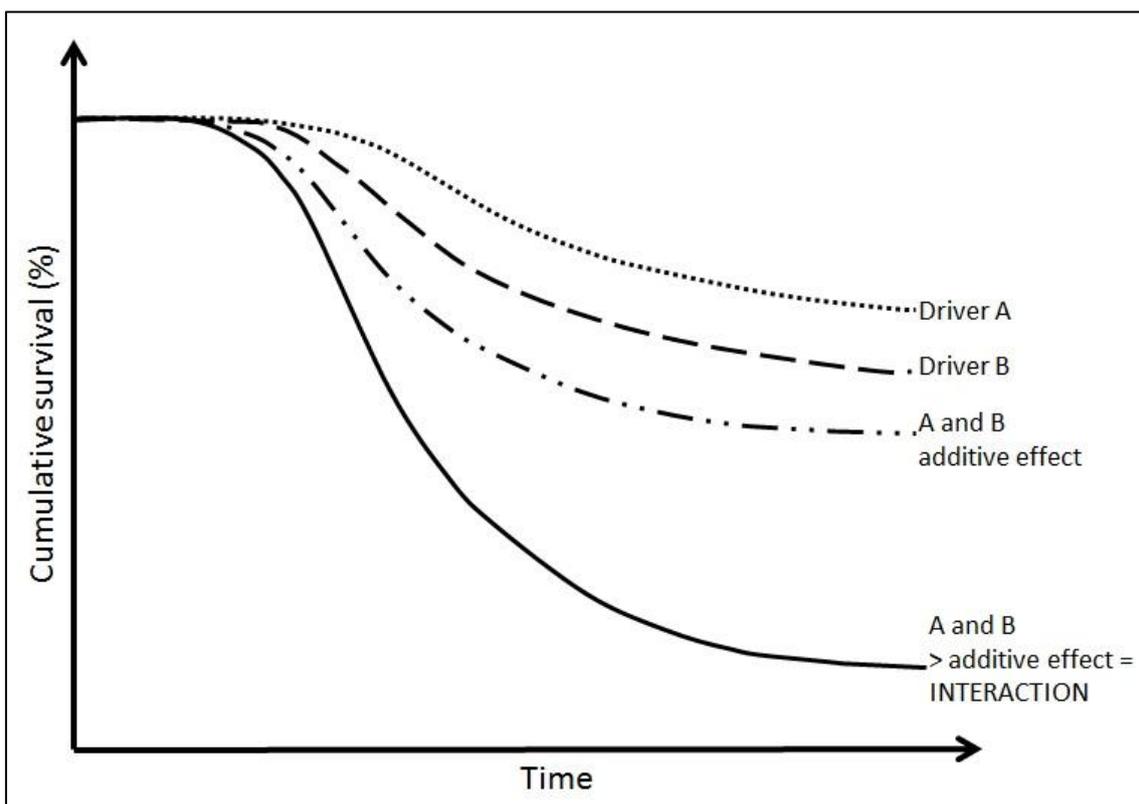


Figure 1.2. Hypothetical survival curve showing an interaction between stressors.

#### 1.4.3.1. Do pesticide exposure and pathogen infection increase pathogen load?

To date, this question has been investigated in the context of one of two pathogenic infections known to infect honey bees naturally (*Nosema* and viral infection), and one pathogen (*Crithidia bombi*) that naturally infects bumble bees, and we discuss each below.

Most work in this area has used a model system of honey bees infected with the microsporidian pathogen *Nosema* spp. Adult workers can be infected with *Nosema* spores by ingestion relatively easily in the laboratory. *Nosema* spore count in individual honey bees has been used as an endpoint in several studies to represent a measure of the proliferation of *Nosema* infection within the host. The studies conducted to date show both variable and contrasting effects of pesticide exposure on the *Nosema* spore count and the effects of pesticide exposure on *Nosema* load remain unclear (Figure 1.3.A). For example, thiacloprid exposure has been shown to increase *Nosema* spore count, whilst fipronil exposure reduces *Nosema* spore count (Vidau et al., 2011). The latter study revealed contrasting effects on *Nosema* spore count despite using concentrations of each pesticide equivalent to approximately 1/100 of the lethal dose, 50% (LD<sub>50</sub>). Altering the timing of fipronil exposure and *Nosema* infection also influenced the effects observed on *Nosema* spore counts (Aufauvre et al., 2012). In another study, several pollen diets from honey bee colonies were analysed for mixtures of pesticide residues and a 'relative risk' of *Nosema* infection was calculated based on relative *Nosema* loads in bees infected after consuming pollen contaminated with a specific pesticide in the laboratory. Whilst all of the neonicotinoids and organophosphates identified were associated with a reduced risk of *Nosema* infection, two acaricides, one herbicide, two cyclodiene insecticides, one pyrethroid insecticide and two fungicides were associated with a significantly increased *Nosema* load (Pettis et al., 2013). By contrast, a field study investigating five acaricide treatments found no effect of these on *Nosema ceranae* load (tested using a quantitative polymerase chain reaction (qPCR) approach) (Boncristiani et al., 2012). Honeybee colonies placed in close proximity to neonicotinoid-treated oilseed rape (*Brassica napus* L.) in the field did not show significantly altered levels of *Nosema* infection compared to colonies placed at other sites (Pohorecka et al.,

2012). However, in this work, no true controls were available as even the colonies that were situated in areas away from treated oilseed rape were also found to contain neonicotinoid residues.

Overall, there is no clear dose-dependent relationship between *Nosema* spore count and pesticide concentration based on the limited number of pesticides and concentrations tested (Figure 1.3.A; e.g. Pettis et al., 2012; Retschnig et al., 2014). In the only study attempting a laboratory–field comparison, honey bees originating from the same parent colonies showed context-dependent effects of imidacloprid exposure on *Nosema* spore counts (Pettis et al., 2012). Individual worker honey bees whose colonies had experienced imidacloprid exposure accumulated significantly increased spore counts after being fed syrup with pathogen spores in the laboratory. However, among the subset of experimental colonies that contracted *Nosema* under field conditions, average spore counts were, by contrast, inversely related to imidacloprid dosing (4.3, 2.9 and 0.5 million spores per bee in control, 5 ppb imidacloprid-, and 20 ppb imidacloprid-dosed colonies, respectively; Pettis et al., 2012). Furthermore, whilst it is easy to infect individual honey bees with *Nosema* spores, spore loads seem particularly variable even within treatments. Several studies have found low levels of spores within control groups (e.g. Alaux et al., 2010) and in some cases, experimental inoculation has not resulted in an increase in the proportion of bees infected compared to those naturally infected (Wu et al., 2012).

With no clear patterns across the data collectively, we argue that the use of spore counts has not provided a reliable measure of pesticide effects on honey bee colony health, nor has it yet proven to be a useful indicator of the mechanisms involved in any interactions between pesticide exposure and *Nosema* infection on bee survival.

Compared to *Nosema*, viral pathogens in honey bees may present a more tractable system. Quantitation of viral load is conveniently performed using molecular techniques, typically qPCR. To date, the effect of pesticide exposure on viral load has been investigated in relation to both acaricides and the neonicotinoid insecticides.

Acaricides are typically used to control *Varroa* mites that are an important pest of honey bee colonies on a global scale (with the notable exception of

Australia). The close association between viral infection and parasitism by the *Varroa* mite may be related to mites acting as vectors for viral transmission, as well as *Varroa*-mediated suppression of the immune response (Yang & Cox-Foster, 2005). Hence, acaricide exposure aims to reduce the viral load of *Varroa*-associated viruses indirectly by reducing the load of *Varroa* mites. The pyrethroid acaricide tau-fluvalinate reduces *Varroa* numbers in honey bee colonies in the field, but it leads to increased loads of Deformed Wing Virus (DWV) and, to a lesser extent, Black Queen Cell Virus (BQCV) and Sacbrood virus (SBV) (Locke et al., 2012). However, loads of BQCV and SBV were highly variable over time, with and without acaricide treatments, making it difficult to draw firm conclusions about these infections. In a field experiment, five different acaricide treatments (applied in the absence of *Varroa*) had no effects on the loads of the pathogens tested, including DWV, BQCV and SBV (Boncristiani et al., 2012). Hence, the effect of acaricide exposure on viral load remains uncertain.

Across the few studies to investigate the effect of neonicotinoid exposure on viral load, exposures at a concentration  $\geq 1$  ppb generally led to increased viral loads in honey bees (Figure 1.3.A; Di Prisco et al., 2013; Doublet et al., 2015). Whilst the studies of acaricides were performed on colonies under field conditions, the effect of neonicotinoid exposure on viral load has been investigated only in a laboratory setting in the absence of *Varroa* mites, allowing for differentiation between effects of parasitism by *Varroa* and pesticide exposure. Given that there is a positive correlation between *Varroa* and viral loads (Francis et al., 2013) and *Varroa* is endemic to honey bee populations in many parts of the world, further work is needed to investigate the combined effects of neonicotinoid exposure and parasitism by *Varroa* on viral loads in a field setting, as well as to understand how changes in viral load affect colony health.

Pesticide exposure has not been observed to increase pathogenic infection in bumble bees, but this has been investigated only in relation to laboratory infection with the trypanosome gut parasite *Crithidia bombi*. Susceptibility of worker bumble bees to *C. bombi* and the intensity of *C. bombi* infection within individual bees was not affected by exposure to the neonicotinoids

thiamethoxam and clothianidin or the pyrethroid  $\lambda$ -cyhalothrin (Baron et al., 2014; Fauser-Misslin et al., 2014).

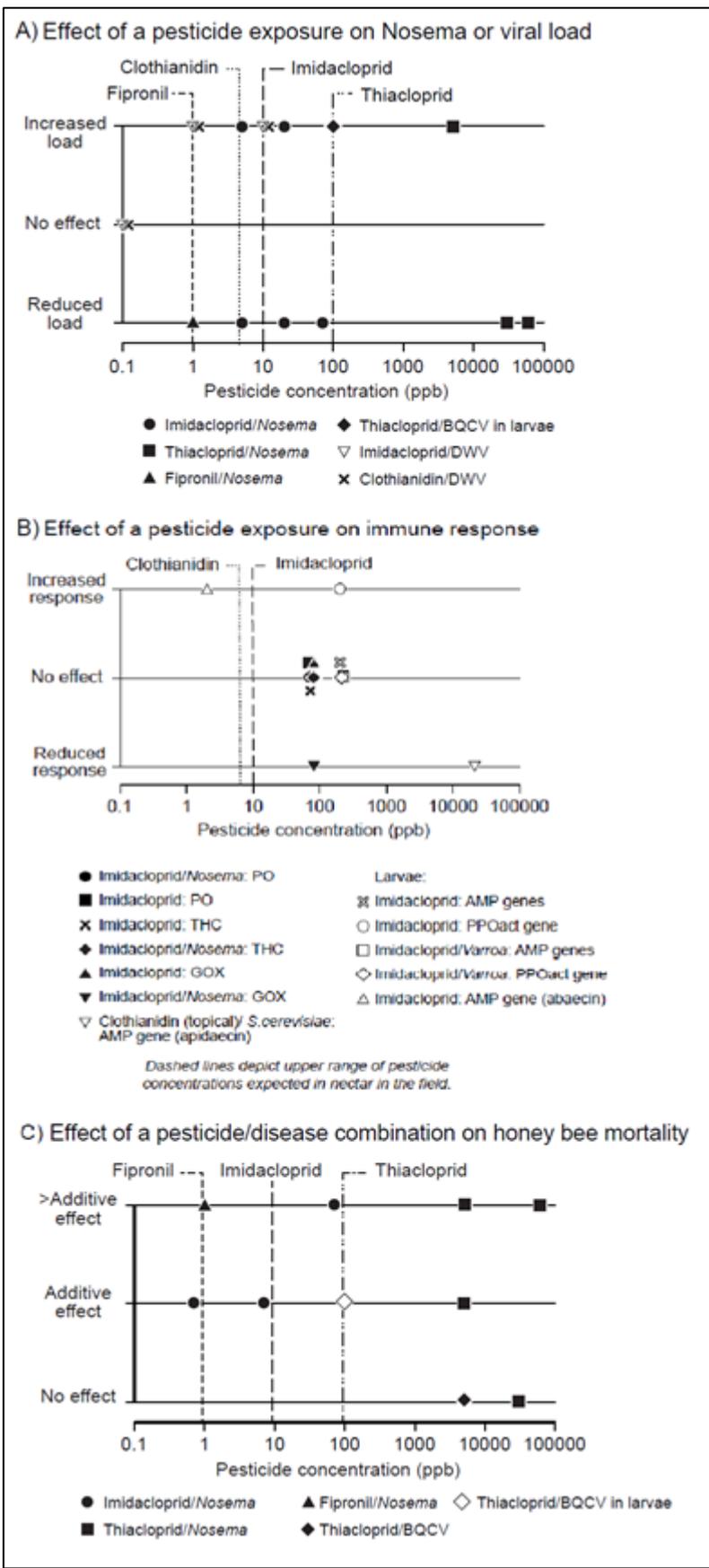


Figure 1.3. See legend overleaf.

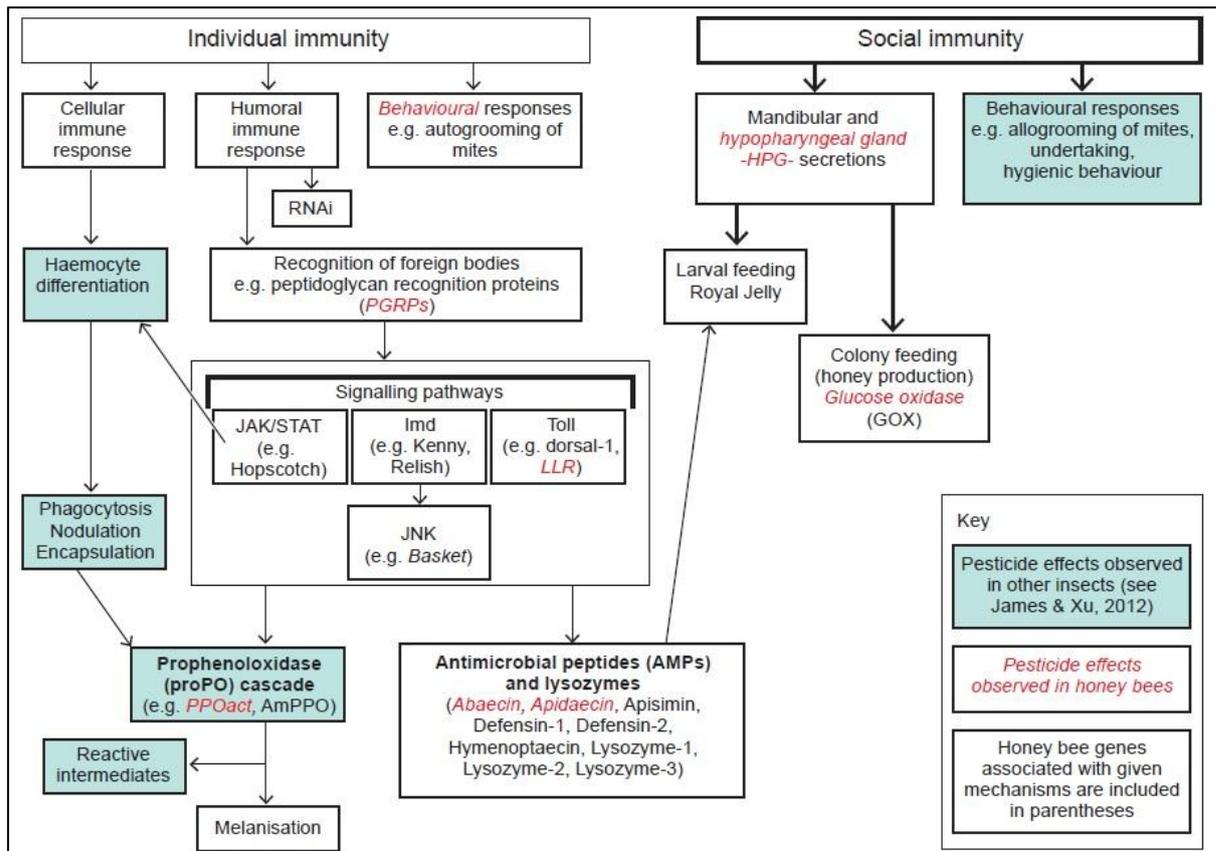
*Figure 1.3.* (previous page). Scatterplots of observed effects of neonicotinoids and fipronil on (A) bee pathogen load, (B) bee immunity and (C) bee mortality, based on data from Alaux et al. (2010), Aufauvre et al. (2012), Di Prisco et al. (2013), Doublet et al. (2015), Gregorc et al. (2012), Pettis et al. (2012), Retschnig et al. (2014) and Vidau et al. (2011). The observed effects at each pesticide concentration are based on the significance levels as defined within each publication. Different shapes used for each data point are used to show the different pesticide/pathogen combinations and/or endpoints used in each publication. The upper range of pesticide concentrations expected in nectar in the field, as shown by the vertical dashed lines, are approximations based on residue analysis from previous studies: fipronil (1 ppb) (Chauzat et al., 2011), clothianidin (5 ppb) (Cutler & Scott-Dupree, 2007; Pohorecka et al., 2012), imidacloprid (10 ppb) (Blacquiere et al., 2012; Pohorecka et al., 2012), thiacloprid (100 ppb) (Skerl et al., 2009; Pohorecka et al., 2012). AMP, antimicrobial peptides; BCQV, Black Queen Cell Virus; DWV, Deformed Wing Virus; GOX, glucose oxidase; PPOact, prophenoloxidase-activating enzyme, PO, phenoloxidase; THC, total haemocyte count.

#### *1.4.3.2. Do pesticide exposure and pathogenic infection reduce bee immunocompetence?*

Immunocompetence is the ability to mount an immune response (Wilson-Rich et al., 2009). If pesticide exposure leads to reduced immunocompetence, this could lead to increased susceptibility to pathogenic infection, potentially impacting on individual and colony survival. The individual bee's immune response is comprised of cellular responses such as phagocytosis and encapsulation, and humoral responses *via* both the prophenoloxidase cascade (leading to melanisation) and antimicrobial effectors (Figure 1.4.). Whilst not an immune response *per se*, individual bees may also respond to pathogenic or parasitic attack through changes in their behaviour, for example, by grooming themselves to remove ectoparasitic mites (Danka & Villa, 2003). In addition to these individual responses, social immunity in eusocial bee species such as

honey bees also plays a key role in defence against pathogens and parasites. These social defences may explain the dearth of individual immune genes found in the honey bee compared to other insects, such as fruit flies and mosquitoes (Evans et al., 2006). For example, gland secretions, such as glucose oxidase, are thought to act as a form of social immunity by sterilising colony honey supplies (White et al., 1963). Social behaviours, such as grooming of colony members, also play an important role in the defence of honey bee colonies against pathogenic and parasitic attack (see review by Cremer et al., 2007).

Here, we assess whether these various components of the bee immune response are responsive to pesticide exposure as measured by enzymatic (physiological) and/or transcriptomic (molecular) endpoints. This assessment solely addresses honey bee immunocompetence as there has been no reported investigation of non-*Apis* species.



**Figure 1.4.** Schematic of the honey bee immune response, including identification of those components shown to be affected by pesticides in other insect species and/or specifically in honey bees. AmPPO, *Apis mellifera* prophenoloxidase gene; Imd, immune deficiency; JAK/STAT, Janus kinase/Signal transduction and transcription; JNK, c-Jun N-terminal kinases; LLR, leucine-rich repeats; PPOact, prophenoloxidase-activating enzyme; RNAi, Ribonucleic acid interference.

#### *1.4.3.2.1. Individual immunity*

Haemocytes provide the cellular defence and are involved in the recognition of foreign surfaces, phagocytosis of small microbial targets, and the nodulation and encapsulation of larger bacteria and parasites. Production of haemocytes is often coupled with melanin production, catalysed by phenoloxidase enzymes, to seal wounding sites and suffocate or starve parasites by blocking nutrient absorption (reviewed by Gillespie et al., 1997; Kanost & Gorman, 2008). As yet, pesticide exposure has not reportedly affected the cellular and prophenoloxidase components of the bee immune response at a physiological level (Alaux et al., 2010). The expression of several gene candidates involved in the functioning of honey bee haemocytes and/or the phenoloxidase cascade has been altered by pesticide exposure (Table 1.2.), but typically in the absence of pathogenic infection and the biological and ecological relevance of these transcriptional changes remains unclear. Several pesticides, such as insect growth regulators, have been observed to affect components of the cellular immune response and prophenoloxidase cascade in other insect species, such as moths (James & Xu, 2012; Figure 1.4.), but bees are not at current risk of exposure to many of these pesticide types. Further research is needed to understand whether the effects observed in the expression of some immune genes in bees (Table 1.2.) relate to changes in haemocyte function and have significance for the phenoloxidase system of the immune response.

The second major component of the individual immune response involves the induction of antimicrobial peptides (AMPs). Several AMP genes have been observed to increase in expression following challenges with bacteria (Evans, 2004, 2006), fungi (Bull et al., 2012; Evans, 2006) and microsporidia (Antunez et al., 2009) and increased AMP protein levels have been observed when bees were artificially infected with bacteria (e.g. Gatschenberger et al., 2012; Laughton et al., 2011; Randolt et al., 2008). In bumble bees, AMP genes were also upregulated following challenge with trypanosomal gut parasites (Riddell et al., 2009; Riddell et al., 2011). AMPs are synthesised primarily by the fat body, and also by haemocytes, the Malpighian tubules and midgut (Gillespie et al., 1997), which are also major sites of pesticide detoxification (e.g. Hodgson, 1983; Mao et al., 2011). Potentially, there may be trade-offs in the allocation of resources for pesticide detoxification and the AMP immune response when

bees are simultaneously exposed to a pesticide and pathogenic infection. Additionally, exposure to imidacloprid has been found to degenerate the honey bee Malpighian tubules (Rossi et al., 2013), which may compromise AMP production. However, there has been no previous investigation of the effects of pesticide exposure on AMP activity in any insect species at a physiological level. Furthermore, most studies have observed no changes in honey bee AMP gene expression following pesticide exposure (e.g. Figure 1.3.B; Table 1.2.), in a wide range of contexts including field studies with five different acaricide treatments (Boncristiani et al., 2012), laboratory studies in larvae exposed to acaricides, fungicides or neonicotinoids (Gregorc et al., 2012), and in adult workers topically exposed to the organophosphate chlorpyrifos (Di Prisco et al., 2013). Despite these findings, there is some evidence to support the hypothesis that pesticide exposure alters the AMP component of the honey bee immune response at a molecular level (Figure 1.3.B; Table 1.2.). Dietary exposure to the neonicotinoid, imidacloprid, led to increased transcription of the gene coding for the AMP abaecin in larvae (Derecka et al., 2013), whilst topical exposure to another neonicotinoid, clothianidin, led to reduced transcription of the gene coding for the AMP apidaecin in adult workers (Di Prisco et al., 2013). Furthermore, exposure to the pyrethroid acaricide flumethrin led to increased expression of the AMP hymenoptaecin in nurse bees (Garrido et al., 2013). The few studies to observe pesticide effects on AMP gene expression however, did not include exposure to a natural pathogenic infection. Furthermore, those studies typically only tested and/or observed effects in a single AMP gene, but nine antimicrobial effector genes have been identified in the honey bee (Evans et al., 2006). There are therefore insufficient data to assess whether AMP gene expression in general is affected in a dose-dependent manner, or indeed whether the observed effects are repeatable (Figure 1.3.B). Furthermore, it remains unclear whether expression of a single AMP gene provides a relevant representation of the antimicrobial effector repertoire as a whole, and/or whether specific AMPs have greater biological relevance to colony strength than others. For example, colonies with larvae exhibiting higher transcript levels of the AMP abaecin were found to have lower infection with the natural bacterial pathogen, *Paenibacillus larvae*, but this correlation with lower infection rates was not observed for the AMP defensin (Evans & Pettis, 2005). More studies comparing molecular changes with protein levels at the individual and colony

level are needed to demonstrate the biological relevance of changes in AMP levels to colony strength and, ultimately, population survival.

Various other proteins are integral to the individual immune response to specific pathogens and they too could provide potential insights into the mechanistic understanding of pesticide–disease interactions. For example, recognition of pathogen surface molecules is a fundamental step in combating pathogens, leading to activation or upregulation of immune effector proteins (Evans et al., 2006; Figure 1.4.).  $\beta$ -glucan recognition proteins ( $\beta$ GRPs), galectins and fibrinogen-related proteins, Toll and Toll-like receptor (TLR) genes and peptidoglycan recognition proteins (PGRPs) have all been identified in the honey bee genome (Evans et al., 2006). The expression of a peptidoglycan recognition protein (PGRP-LC) has been observed to increase following neonicotinoid exposure (Derecka et al., 2013; Table 1.2.), and this component of immunocompetence also may warrant further investigation.

Overall, there is some evidence that genes associated with individual immunity are differentially expressed following pesticide exposure, but the functional significance of these changes remains unclear. Furthermore, there has been a lack of investigation of the effects of pesticide exposure on individual immunity in the presence of pathogenic infection, so the combined effects of these two stressors on individual immunity is not well understood.

**Table 1.2.** Summary of effects of pesticide exposure on expression of immune-related genes.

Gene	Expected role of gene	Pesticide(s)	Observed effect on gene expression	Reference
PPOact	Involved in phenoloxidase cascade/melanisation	Imidacloprid, myclobutanil, chlorothalonil	↑	Gregorc et al. (2012)
		Chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine	----	
Dscam	Haemocyte-specific loss of Dscam impairs phagocytosis of bacteria in fruit flies (Watson et al., 2005) *	Thymol, tau-fluvalinate	↓	Boncrisiani et al. (2012)
		Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine, formic acid	----	Boncrisiani et al. (2012), Gregorc et al. (2012)
Basket	Involved in JNK pathway [feedback to melanisation and antimicrobial effectors (Evans et al., 2006)]	Thymol, tau-fluvalinate	↓	Boncrisiani et al. (2012)
		Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine, formic acid	----	Boncrisiani et al. (2012), Gregorc et al. (2012)
Vitellogenin	Many functions, including as a zinc carrier to maintain haemocyte function (Amdam et al., 2004) and in reducing oxidative stress (Seehuus et al., 2006)	Thymol, tau-fluvalinate	↓	Boncrisiani et al. (2012)
		Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine, formic acid, flumethrin	----	Boncrisiani et al. (2012), Garrido et al. (2013), Gregorc et al. (2012)
Hymenoptaecin	Antimicrobial effector	Flumethrin (pyrethroid acaricide)	↑	Garrido et al. (2013)
		Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine, flumethrin	----	Garrido et al. (2013), Gregorc et al. (2012)
Abaecin	Antimicrobial effector	Imidacloprid (neonicotinoid insecticide)	↑	Derecka et al. (2013)
		Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine, flumethrin	----	Garrido et al. (2013), Gregorc et al. (2012)
Apidaecin	Antimicrobial effector	Clothianidin (neonicotinoid insecticide)	↓	Di Prisco et al. (2013)
		Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine	----	Di Prisco et al. (2013), Gregorc et al. (2012)
PGRP-LC	Peptidoglycan recognition protein	Imidacloprid (neonicotinoid insecticide)	↑	Derecka et al. (2013)
		Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine	----	Gregorc et al. (2012)
Defensin2	Antimicrobial effector	Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine, formic acid, flumethrin	----	Boncrisiani et al. (2012), Garrido et al. (2013), Gregorc et al. (2012)
Cactus	IκB transcription factor	Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine, formic acid	----	Boncrisiani et al. (2012), Gregorc et al. (2012)
Tab; Domeless	Tab Tak1- binding protein; Domeless JAK/STAT signalling pathway	Thymol, tau-fluvalinate, coumaphos, formic acid, amitraz	----	Boncrisiani et al. (2012)
Other immune genes, including Eater, Defensin1, Dorsal-1, Hopscotch, PGRPSC	Involved in several immune pathways	Imidacloprid, myclobutanil, chlorothalonil, chlorpyrifos, amitraz, fluvalinate, coumaphos, glyphosate, simazine	----	Gregorc et al. (2012)

\*But note, bacterial infection previously not shown to affect Dscam expression in honey bees (Evans et al., 2006).

↑= increase in expression. ↓=decrease in expression. ---- = no effect on expression. Significance values associated with these effects are defined in the appropriate references.

Dscam, Down syndrome cell adhesion molecule; I $\kappa$ B, I kappa B; JAK/STAT, Janus kinase/ Signal transduction and transcription; JNK, c-Jun N-terminal kinases; PGRP-LC, peptidoglycan recognition protein LC; PGRPSC, peptidoglycan recognition protein SC; PPOact, prophenoloxidase-activating enzyme; Tak1, TGFbeta Activated Kinase 1.

#### 1.4.3.2.2. *Social immunity*

Glucose oxidase is secreted from the honey bee hypopharyngeal gland (Ohashi et al., 1999), and is involved in the conversion of nectar to honey, catalysing the production of gluconic acid and hydrogen peroxide. Together, gluconic acid and hydrogen peroxide give honey antimicrobial properties, so sterilising brood food and colony honey supplies, and acting as a form of social immunity (White et al., 1963). Environmentally realistic concentrations of imidacloprid have been found to affect the development of the honey bee hypopharyngeal gland (Heylen et al., 2011; Skerl & Gregorc, 2010; Hatjina et al., 2013). However, only a single study to date has considered the effects of pesticide exposure on honey bee social immunity using the level of glucose oxidase activity as an endpoint. In that study, imidacloprid exposure reduced glucose oxidase activity when coupled with *Nosema* infection (Alaux et al., 2010). Further work is needed to elucidate the link between the effects of pesticide exposure on hypopharyngeal gland development and gland secretions, including glucose oxidase, and the consequences of these effects for colony health.

#### 1.4.3.2.3. *Behavioural defences as a form of individual and social immunity*

Given that behavioural defences have received significant attention in the literature for their role in honey bee defence against disease, it also seems relevant here briefly to assess the effect of pesticide exposure on behavioural endpoints relevant to social immunity. In termites and beetle larvae, exposure to imidacloprid is known to reduce grooming behaviours, leading to increased susceptibility to infection and parasitism (Boucias et al., 1996; Koppenhofer et al., 2000; Neves & Alves, 2000). Honey bees groom both themselves (Danka &

Villa, 2003) and other colony members (Khongphinitbunjong et al., 2012) to remove mites, as well as perform other social defence behaviours such as the uncapping and removal of infected pupae and adult corpses (e.g. Ibrahim & Spivak, 2006; Trumbo et al., 1997) and the collection of antimicrobial plant resins to form propolis for nest building (Simone et al., 2009). Neonicotinoid exposure has been shown to affect foraging behaviour of honey bees in a dose-dependent manner when exposed as single oral treatments (Bortolotti et al., 2003; Henry et al., 2012; Schneider et al., 2012; Yang et al., 2008). However, there has been no previous investigation of effects of pesticide exposure on social defence behaviours in honey bees or other bee species. Social defence behaviours are ecologically relevant at the colony level because colonies bred for hygienic behaviour have demonstrated resistance to American Foulbrood and greater honey production than non-hygienic colonies (Spivak & Reuter, 2001). We therefore argue that it seems appropriate to prioritise further investigation of pesticide effects on social defence behaviours that are known to alter colony functioning. Established behavioural assays could be easily adapted to provide a simple, cost-effective and biologically relevant endpoint for future investigations.

#### *1.4.3.2.4. Summary*

Pesticide exposure and pathogenic infection, when investigated separately, may alter expression of genes associated with the individual immune response, but there is no evidence that pesticide exposure reduces immunocompetence following natural pathogen infection in the context of honey bee individual immunity at a physiological level, or individual or social defence behaviours. Pesticide exposure coupled with pathogenic infection has been shown to reduce social immunity in the laboratory, but the ecological relevance of this finding to honey bee colonies remains unclear.

#### *1.4.3.3. Do pesticide exposure and pathogenic infection interact to reduce bee survival?*

To date, this question has been investigated in the context of one of two pathogenic infections known naturally to infect honey bees (*Nosema* and viral

infection), and one pathogen (*C. bombi*) that naturally infects bumble bees; we discuss each below.

The available laboratory data show that *Nosema* infection interacts with exposure to a sublethal concentration of phenylpyrazole (fipronil) or neonicotinoid (imidacloprid or thiacloprid) to reduce honey bee worker survival (Figure 1.3.C; Alaux et al., 2010; Aufauvre et al., 2012; Doublet et al., 2015; Retschnig et al., 2014; Vidau et al., 2011). However, the interaction has only been observed in laboratory studies in which pesticide exposures far exceed environmentally realistic scenarios in the field, with the exception of the two studies on fipronil (Aufauvre et al., 2012; Vidau et al., 2011; Figure 1.3.C). Two field experiments using field-realistic exposures have, by contrast, found no interaction (Pettis et al., 2012; Wu et al., 2012).

Additive effects reducing honey bee survival have been observed in the laboratory between pesticide exposure and viral infection, including between the pyrethroid cypermethrin and Chronic Paralysis Virus (CPV) (Bendahou et al., 1997), and the neonicotinoid thiacloprid and BQCV (Doublet et al., 2015), but no interaction has been observed (Figure 1.3.C).

An artificial challenge with *Escherichia coli* lipopolysaccharides (LPS) interacted with oral exposure to the natural plant toxin nicotine to reduce honey bee survival (Koehler et al., 2012). Nicotine and the neonicotinoid pesticides have a similar mode of action on the insect nicotinic acetylcholine receptors and LPS has been used in several studies to elicit a bacterial-like immune response (e.g. Laughton et al., 2011). We hypothesise that neonicotinoid exposure in combination with natural bacterial infections could lead to similar effects on honey bee survival, but this has not been tested yet.

Lastly, there was no observed interaction between neonicotinoid exposure and *C. bombi* infection on the production of bumble bee workers, gynes and males. However, queen longevity was lower when bumble bee colonies were exposed in the laboratory to both neonicotinoid exposure and *C. bombi* infection (Fauser-Misslin et al., 2014).

#### 1.4.4. Conclusions

(1) We demonstrate that through a combination of laboratory and field studies understanding is progressing on the extent to which bee populations are impacted in realistic scenarios of pesticide exposure and natural pathogenic infection. Laboratory studies are serving to isolate the possible underlying mechanisms of effects and to establish the direction of future work by elucidating potential pesticides and pathogens of concern to bee health and recognising informative endpoints. Laboratory studies allow also the opportunity to test pesticide effects against true no-pesticide controls, which is often not possible in field experiments due to the increasingly ubiquitous nature of residues in field colonies, as highlighted by Pohorecka et al. (2012). Field trials, however, establish ecological relevance for the laboratory-based findings, and we argue that field studies should increase if we are to close some of the very significant knowledge gaps in this research area.

(2) Effects of pesticide exposure and pathogenic infection on bee health vary among studies (Figure 1.3.), often making it difficult to draw conclusions on interactive or dose-dependent effects. Whilst some of this variation may result from different exposure and infection regimes, differences in the developmental stages of insects studied, and differences in approaches adopted, we also show that some endpoints, namely *Nosema* and viral pathogen loads, appear to be inherently variable. It is possible that both genetic and seasonal variation in the experimental bees may account for some of the differences seen among studies and in their susceptibility to pesticide exposure and pathogen infection. However, it remains unclear whether the variation within and among studies is methodological or inherent. The methodologies have not been explicitly contrasted herein, but features we suspect could lead to variation include the mode and timing of pesticide exposure and pathogenic infection, such as contact *versus* oral exposures/infections and the initial levels of infection administered. Similarly, it was not possible herein explicitly to contrast the genetic background of bees within each study, which could have a bearing on the results reported. In the studies reviewed investigating *Nosema* pathogen load various different honey bee races were used, including *A. mellifera carnica*, *A. mellifera ligustica*, *A. mellifera mellifera* and Buckfast hybrids (Alaux et al., 2010; Doublet et al., 2015; Vidau et al., 2011). To understand better the extent of methodological and/or natural variation in some of the endpoints, future

studies may benefit from an increasingly standardised approach. A recent publication (Dietemann et al., 2013) contains specific chapters for toxicology studies (Medrzycki et al., 2013), molecular studies (Evans et al., 2013) and study of specific honey bee pathogens (e.g. Fries et al., 2013) and this may help to standardise approaches in honey bee research methods. Standardisation of terms to describe the interactions between pesticide exposure and pathogen infection on bee health will harmonise interpretations of the same interactions, such as additive *versus* more-than-additive effects.

(3) Some areas for understanding pesticide–pathogen interaction in bees have received relatively little attention. Further investigation of pesticide effects on bee social defence behaviours and the antimicrobial peptide (AMP) component of the individual immune response are important, as these endpoints in particular have shown the potential to respond to pesticide exposure and to impact bee health at the colony level. We would also encourage further investigation to elucidate colony-level effects on social immunity due to pesticide-induced changes in hypopharyngeal gland development.

(4) To date, there has been no investigation of interactions between pesticide exposure and infection with bacterial or fungal pathogens, despite their prevalence amongst the most common causal agents of bee disease (Table 1.1.), and we highlight this as a knowledge gap worthy of future investigation. Upregulation of AMPs is thought to be important in response to bacterial infection (Evans et al., 2006; Korner & Schmid-Hempel, 2004), so we propose that our mechanistic understanding of any potential interactions between pesticide exposure and bacterial infection could benefit from the integrated investigation of the AMP component of the immune response. Furthermore, the most prevalent bacterial and fungal infections, including foulbrood and chalkbrood diseases, are symptomatic in honey bee larvae (Table 1.1.), so investigations on larval survival, pathogen load and immunocompetence may provide useful insights.

(5) Our review highlights the lack of assessment of non-*Apis* bee species. Other bee species have considerable roles in providing pollination services and increased bee diversity is associated with increased pollination success (Klein et al., 2003). In fact, native bee species alone are capable of pollinating

intensively farmed crops in the absence of managed honey bees (Winfree et al., 2007). Consideration of *Bombus* and other wild bee species in the investigation of interactions between pesticide exposure and pathogenic infection would be extremely valuable because susceptibility to specific pathogens and parasites, and risk of exposure and sensitivity to specific pesticides, differs among species (Cresswell et al., 2012).

### 1.5. Thesis aims

Having identified several knowledge gaps in our understanding of pesticide exposure as a potential stressor impacting upon bee health, the central aim of this thesis is to investigate sublethal effects of neonicotinoid exposure on honey bees and bumble bees, and to establish the relevance of different sublethal endpoints as appropriate ecotoxicological markers for risk assessment. In particular, this thesis focuses on the study of neonicotinoid exposure in combination with immune stressors, as a combination of stressors more likely reflects realistic ecological scenarios. This thesis addresses the following specific aims:

- 1) To establish whether exposure to two neonicotinoids, imidacloprid and thiamethoxam, impacts on immune-related gene expression and enzymatic activity in adult workers of the honey bee, *Apis mellifera*. This work will investigate effects on two key components of the insect immune response, the phenoloxidase cascade and antimicrobial effectors. It will better our understanding of the relationships between the temporal dynamics of transcriptional and physiological changes in the immune response, providing a foundation to understanding the potential regulatory value of gene expression bioassays as indicators of pesticide effects on honey bee health.
- 2) To establish whether exposure to the neonicotinoid, imidacloprid, impacts on the immune response of adult workers of the bumble bee, *Bombus terrestris*. This work will investigate effects on two key components of the insect immune response, the phenoloxidase cascade and antimicrobial effectors, providing new insights into the value of these physiological endpoints in the pesticide risk assessment of sublethal effects on non-*Apis* species.
- 3) To establish whether exposure to the neonicotinoid, imidacloprid, impacts on gene expression, enzymatic activity and flight behaviour associated with the development of the hypopharyngeal glands and their roles in temporal polyethism, nutrition and social immunity in the honey bee, *Apis mellifera*. This work aims also to improve our understanding of the social immune response to infection, and investigates a novel approach using Radio Frequency Identification (RFID) technology to monitor life-long behaviours of honey bees following chronic pesticide exposures.

4) To identify the genes that are differentially expressed across the transcriptome of adult workers of the honey bee, *Apis mellifera*, following immune challenge, neonicotinoid exposure or a combination of both stressors. This work will investigate effects of two neonicotinoids, imidacloprid and thiamethoxam, and aims to elucidate potential functions and molecular pathways that are altered by immune and chemical stressors, and reveal candidate genes of potential value as biomarkers for risk assessment.

Collectively, this work uses an artificial immune challenge *via* an injection with lipopolysaccharides (LPS) from *E.coli* to aim to mimic a bacterial-like infection. This approach standardises the invoking of an immune response across individual bees and excludes the dynamic behaviour of a real pathogen. The data presented in this thesis therefore does not aim to unfold exact biological mechanisms associated with specific natural infections, but rather provides an understanding of the general dynamics of pesticide-pathogen interactions using a well-established method for insect immune studies.

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## **2. Methods development and assay validations**

A variety of physiological and molecular assays have been used in the study of bees as informative endpoints for bee health (e.g. Laughton and Siva-Jothy, 2011, Alaux et al., 2010, Evans, 2006). Subsequent work described in this

thesis aimed to use a selection of assays to measure relative physiological activity and gene expression in bees in response to immune and chemical stressors, to investigate different components of bee physiology and behaviour and assess their suitability as appropriate ecotoxicological markers for risk assessment. Here I describe in detail and critically discuss the development, optimisation and validation of these assays, which formed a significant part of the research for this thesis, to provide relevant context for understanding their application to the hypotheses addressed and to help support a critical evaluation of the data obtained and interpretation of those data.

### *2.1. Method of immune elicitation*

An artificial immune challenge *via* an injection with lipopolysaccharides (LPS) from *Escherichia coli* was used as an immune elicitor, as described later in the relevant methods sections of the thesis. This approach was adopted to standardise the stimulus used to invoke a response in individual bees and to minimise the treatment variation inherent in using a real pathogen, which can be logistically difficult to control. Nevertheless, this artificial immune elicitor has relevance to the modes of action of the major pathogens and parasites of the two model bee species used. For example, the injection procedure involves a wounding of the bee's pleural membrane, which is comparable to the wounding likely to occur during the parasitism of honey bees by, for example, *Varroa* mites and the parasitism of bumble bees by, for example, conopid flies. These wounding events during parasitic attack are unlikely to be sterile, and therefore exposure to pathogens, such as bacteria, is likely. Furthermore, two bacterial infections, *Paenibacillus larvae* and *Melissococcus plutonius*, are considered major threats to honey bee colony losses (see Chapter 1).

### *2.2. Measurement of phenoloxidase activity*

Measurement of the phenoloxidase system of the honey bee immune response has previously been described using a spectrophotometry assay (Laughton and Siva-Jothy, 2011). This measures the conversion of a colourless test substrate, L-Dopa (3,4-dihydroxy-L-phenylalanine) to dopachrome (red-brown in colour), which is catalysed by the phenoloxidase (PO) enzymes contained in a haemolymph sample. This PO is present in insect haemolymph as an inactive precursor enzyme, prophenoloxidase (proPO), and the combination of proPO

and PO in a sample can be measured by first activating any proPO in the sample to PO, using a chemical activator,  $\alpha$ -chymotrypsin (Laughton and Siva-Jothy, 2011). However, inherent difficulties are thought to arise in quantification of haemolymph enzyme activity since methods of haemolymph collection often involve wounding of the individual, which itself can activate or upregulate enzyme activity. Several methods have been used in the literature to collect haemolymph samples for PO analysis in bumble bees and honey bees, including homogenisation of the thorax in a buffer solution (Korner and Schmid-Hempel, 2004), collection of neat haemolymph (Korner and Schmid-Hempel, 2004) and a perfusion bleed in which a buffer is flushed through the thorax and abdomen (Laughton et al., 2011). Here, pilot studies were set up to compare the measurement of proPO and PO in samples collected using different methods. This aimed, firstly, to establish the most suitable method to minimise activation of proPO during sample collection, and secondly, to establish whether a method of haemolymph collection could be used to measure both PO activity and antimicrobial activity (see section 2.2.) from the same individual bee. The different sampling methods could not be performed on the same individual bees, but by collecting samples from different bees using the different techniques we aimed to identify any obvious differences in the suitability of each technique, despite likely variation in the PO activity of individual bees. Phenoloxidase (PO) and total potential phenoloxidase response (total proPO/PO) could be measured for each individual bee. Three collection methods were investigated. First, the thorax was homogenised by hand in a buffer solution. Second, neat haemolymph was collected with a pipette by cutting a small slit in the abdominal tergites and placing gentle pressure to the thorax to release a bubble of haemolymph through this slit. Third, a perfusion bleed method was adopted, whereby a microsyringe and needle were used to insert a buffer solution between the head and thorax, and this solution was flushed through the body and collected along with the haemolymph.

### *2.2.1. Thorax homogenisation*

There was a trend towards higher measures of PO activity in samples collected using a thorax homogenisation approach compared with collection of neat haemolymph or *via* a perfusion bleed. It is possible that this resulted from the presence of cuticular PO, since PO also has an important role in melanin

biosynthesis used in cuticle hardening (sclerotization) and pigmentation of the insect exoskeleton (Sugumaran, 2002). To avoid this potential source of PO activity in an assay intended to inform on immune function, the use of a thorax homogenisation method was ruled out.

### *2.2.2. Neat haemolymph collection*

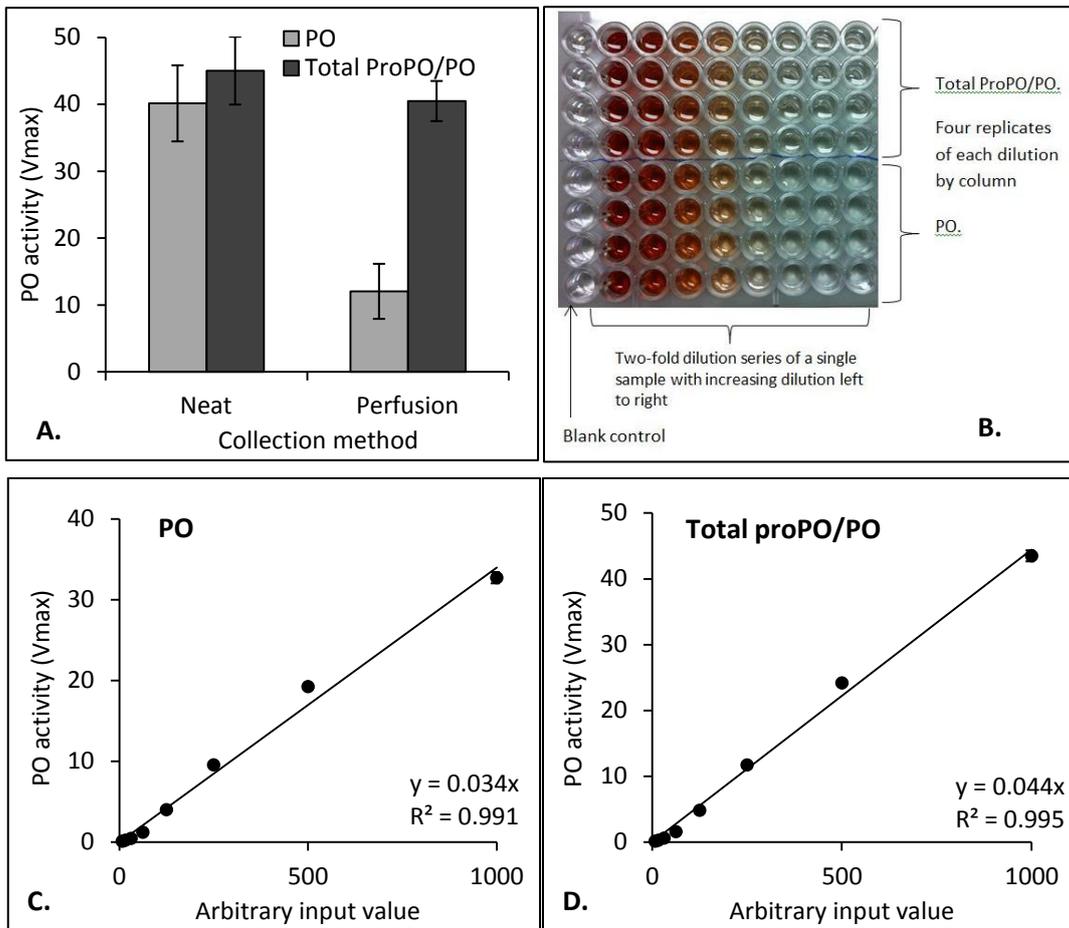
In neat haemolymph samples, similar levels of total proPO/PO and PO activity were measured (Figure 2.1.A.). This was contrary to expectation that measures of total proPO/PO activity would be considerably higher than those of PO only, as the former would include measurement of inactive proPO titres, as was indeed found in samples collected using the thorax homogenisation or perfusion bleed approaches. It is assumed that the much higher activity measured in the PO assay following neat haemolymph collection reflects a significant activation of proPO titres during the wounding caused by the process of neat haemolymph collection. Here it is likely that most of the proPO was activated to PO, and hence the two assays were essentially measuring the same overall total proPO/PO response (the collection technique itself acting as an equivalent proPO activator to the addition of  $\alpha$ -chymotrypsin).

### *2.2.3. Perfusion bleed approach*

Activity was higher in the total proPO/PO assay compared to PO alone, demonstrating that there was significant titre of proPO in the sample not activated to PO during the sample collection. Despite the much lower  $V_{\max}$  values in the PO assay, these were significantly greater than those observed in control wells in which samples were replaced by a buffer solution. It is recognised that even these (lower) PO titres could result from proPO activation during sample collection, but this is a limitation of any collection method that cannot be fully excluded. Nevertheless, this thesis aims only to compare relative activity levels between samples, not directly quantify natural PO titres. Hence, any observed differences in PO between experimental treatments can be assumed to be true differences as the level of proPO activation during sample collection should be consistent across samples.

It was concluded that the results of this pilot work, in conjunction with the standardised protocol outlined by Laughton and Siva-Jothy (2011), provided sufficient evidence that the perfusion bleed approach would be the most appropriate collection method for measurement of PO activity in bees. Perfusion bleeds allowed distinction between the measurement of PO titres only and total proPO/PO titres, and reduced the risk of measuring cuticular PO, that in turn, could lead to overestimation of the PO component of the immune response.

To validate the assay as an appropriate measure of relative differences in PO activity between samples, a two-fold dilution series of a single pooled haemolymph sample was prepared in sodium cacodylate (NaCac) buffer, and PO and total proPO/PO subsequently measured. Each measure was taken using four technical replicates (to confirm the repeatability of the method). As expected, there was a significant linear relationship between the sample input and both the measured PO activity (Figure 2.1.C; Linear regression:  $R^2 = 0.991$ ,  $P < 0.001$ ) and the measured total proPO/PO activity (Figure 2.1.D; Linear regression:  $R^2 = 0.996$ ,  $P < 0.001$ ). This was also evident visually from the respective colour changes of each dilution, with the most concentrated samples leading to a greater conversion of the colourless L-Dopa substrate to the dark red dopachrome (Figure 2.1.B). From this pilot work, it was concluded that the protocols used were appropriate and they were adopted for future work to investigate the phenoloxidase activity in bees subjected to immune and chemical stressors, as described later in this thesis.



*Figure 2.1.* Measurement of phenoloxidase activity. A. Comparison of neat haemolymph and perfusion bleed sampling collection methods for both the PO and total proPO/PO assays (Mean  $\pm$  SE). B. Photograph of the dilution series plate following spectrophotometry. C. Dilution series measuring PO activity of a single sample (Mean of four replicates  $\pm$  SE). D. Dilution series measuring total proPO/PO activity of a single sample (Mean of four replicates  $\pm$  SE). Trend lines show linear lines of best fit.

### 2.3. Measurement of antimicrobial activity

Antimicrobial peptide (AMP) activity has previously been quantified using an established assay, measuring the inhibition of bacterial or fungal growth on an agar plate containing wells of haemolymph samples (e.g. Moret and Schmid-Hempel, 2000, Rees et al., 1997, Mallon et al., 2003, Gatschenberger et al., 2012). In each case, these growth inhibition assays assume a concentration related increase in inhibition with higher levels of antimicrobial activity. The sensitivity of antimicrobial activity assays, however, may be highly dependent on both the growth medium and the bacterial strains used (Casteels et al., 1990, Taormina et al., 2001) and previous studies have used several bacterial species to measure AMP activity in bumble bees and honey bees. These bacterial species have been typically used as they can be readily cultured in the laboratory and they are sensitive to bee antimicrobial proteins, and they include *Arthrobacter globiformis* (Moret and Schmid-Hempel, 2000, Korner and Schmid-Hempel, 2004, Mallon et al., 2003, Laughton et al., 2011), *Escherichia coli* (Rees et al., 1997, Gatschenberger et al., 2012, Randolt et al., 2008), *Micrococcus flavus* (Randolt et al., 2008, Gatschenberger et al., 2012), *Micrococcus luteus* (Rees et al., 1997) and *Staphylococcus aureus* (Stow et al., 2007, McCleskey and Melampy, 1939). For work measuring antimicrobial responses in bees in this thesis, we aimed to adopt previously described protocols to ensure a reliable culture of bacterial strains that are sensitive to bee antimicrobial activity. Given that pilot work described earlier concluded that perfusion bleeds were the most appropriate technique of haemolymph collection for measuring PO activity, in some further pilot work we investigated whether perfusion bleed samples could also be used to measure AMP activity. If this were the case, this would allow direct comparison of phenoloxidase and antimicrobial activity from individual bees. Further work also tested alternative sample collection methods, as previously described in the literature for AMP measurement, including thorax homogenisation and neat haemolymph collection. All of this methods development work used samples from bees collected 24-72 hours post injection with an artificial immune challenge with bacterial lipopolysaccharides (LPS); it is well recognised that minimal antimicrobial activity is typically observed in the absence of an immune

challenge and this LPS approach is well documented in the literature (e.g. Laughton et al., 2011).

### 2.3.1. Measurement of antimicrobial activity in honey bees

#### 2.3.1.1. Bacterial growth inhibition assays

Despite following protocols previously described in the literature, whether using neat haemolymph, homogenised thoraces or perfusion bleeds, I did not find that honey bee samples inhibited the growth of *A. globiformis*, *E.coli*, or *S.aureus*. A few small zones of inhibition were seen against *M.luteus* growth using neat haemolymph samples, but these were seen in a very few samples, and this showed that the assay had too low a sensitivity to be reliable or informative. Expecting that the abdomen may contain more antimicrobial peptides, given that the fat body and midgut are the principle sites of AMP synthesis (Gillespie et al., 1997), samples collected *via* an homogenisation of the abdomen were also tested, but again no inhibition was observed. Despite the lack of inhibition, the majority of neat haemolymph samples were observed to darken the surrounding agar, demonstrating a melanisation response to the bacteria (as associated with the phenoloxidase cascade). This melanisation was not thought to affect the capability of any antimicrobial peptides to inhibit the bacterial growth; previous studies that have observed inhibition and also melanisation found no effect of melanin presence on the inhibition assay (Haine, unpublished data, cited in Laughton et al., 2011). Nevertheless, here melanisation confirmed that the haemolymph samples were diffusing into the inoculated agar.

Difficulty in the setup of inhibition assays to measure antimicrobial activity has been recognised widely previously by other research groups. Haine et al. (2008) reported that attempts to measure antimicrobial activity in mealworm beetle haemolymph failed with use of *E.coli* or *Bacillus subtilis*. Others have also found difficulties in repeatedly measuring antimicrobial activity in bees against *A.globiformis* (A. Laughton, S. Rustage *personal communication*). In my pilot work I found no inhibition despite efforts to increase the sensitivity of the live bacterial cultures to enhance antimicrobial activity, using low nutrient agar and low concentrations of bacterial inoculants.

### 2.3.1.2. Lytic clearance assays

As an alternative to an inhibition assay against the growth of a live bacterial culture, studies of antimicrobial activity in other insects have used a clearance assay against a turbid suspension of lyophilised bacterial cells (e.g. Cytrynska et al., 2007, Cotter et al., 2008, Kurtz et al., 2000). In this case, these lytic clearance assays assume a concentration related increase in clearance with higher levels of antimicrobial activity, as antimicrobial proteins in the haemolymph sample lyse the peptidoglycan bacterial cell walls (an antimicrobial action typical of lysozymes (Gillespie et al., 1997)). Lyophilised *M.luteus* cells (*Micrococcus lysodeikticus*) are typically used for this assay. Whilst this has not been documented previously for bees, in our trials a few observations of inhibition against live *M.luteus* with honey bee neat haemolymph provided some support for testing the potential of this alternative assay. Here a pilot study, following the protocols of Cotter et al. (2008) and Kurtz et al. (2000) aimed to test whether honey bee antimicrobial activity could be measured using a lytic clearance assay.

Firstly, agar plates were set up containing a suspension of 5 mgml<sup>-1</sup> *M. lysodeikticus* and neat haemolymph samples from LPS-injected honey bees were found to give very faint zones of clearance (~3 mm diameter). Much clearer and larger zones were observed using lysozyme standards from chicken egg white. In an attempt to increase the sensitivity of the assay, a series of agar plates were set up containing a suspension of *M. lysodeikticus* across a dilution series from 5-0.05 mgml<sup>-1</sup>, and haemolymph samples were added to each plate. Across this dilution series, weaker suspensions of lyophilised cells successfully allowed for observation of clearer and larger clearance zones from aliquots of the same haemolymph sample. It was concluded that using a suspension of *M. lysodeikticus* in an agar assay at a concentration of 0.2 mgml<sup>-1</sup> was the most appropriate concentration for reliable measurement of antimicrobial activity from honey bee neat haemolymph samples.

To identify whether clearer or larger zones of clearance could be measured if the samples were left in wells of the agar plate for a longer time period, zones were measured 24, 48, 72 and 96 hours after the setup of the clearance assay, with measurements made blind to the previous day's results. There was a

significant difference in the presence of zones across the four time measurements, with a general trend that more zones were observed when the assay was left for a longer period of time (Friedman rank sum test:  $\chi^2_{df=3} = 10.3$ ,  $P = 0.017$ ). Nevertheless, of the samples from which zones were consistently measured at all four recording points, there was no significant difference in the diameter of these zones when measured at each point in time (Repeated measures ANOVA:  $F_{3,348} = 2.45$ ,  $P = 0.063$ ). A few plates were observed to have very small colonies of bacterial growth by 96 hours post setup, possibly due to the viability of some lyophilised bacterial cells (Antheuni, 1973) or other contamination. It was concluded that future use of the assay would measure lytic zones at 72 hours post injection, to allow for a sufficient length of time for even weak samples to clear the lyophilised cells, whilst minimising the risk of contamination by bacterial growth on the plates.

Lastly, lytic zones of clearance were not observed using perfusion bleed samples. From this we concluded that for appropriate measurement of both phenoloxidase activity and antimicrobial activity separate individual bees were required for each measurement (using perfusion bleeds and neat haemolymph, respectively).

To validate the lytic clearance assay as an appropriate measure of relative differences in antimicrobial activity between samples, a dilution series of a standard lysozyme solution was measured. Each measure was taken using two technical replicates to confirm the repeatability of the method. As expected, there was a significant relationship between log lysozyme concentration and the diameter of clearance zone measured against the *M. lysodeikticus* suspension (Figure 2.2.). It was concluded that the protocols used were appropriate for future work to investigate the antimicrobial activity in honey bees subjected to immune and chemical stressors, as described later in this thesis.

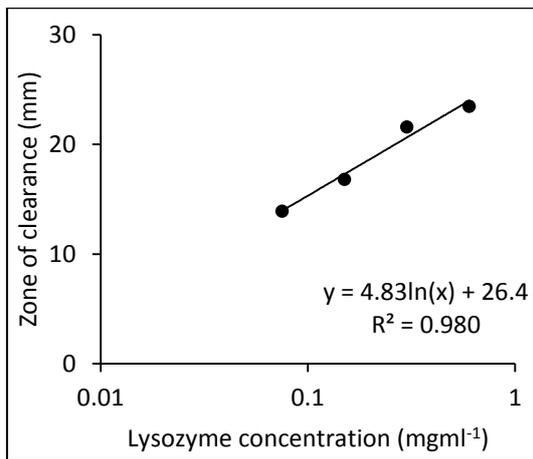


Figure 2.2. Dilution series measuring antimicrobial activity of a lysozyme standard from chicken egg white against the clearance of *M. lysodeikticus* (Mean of two replicates  $\pm$  SE). Trend line shows line of best fit.

### 2.3.2. Measurement of antimicrobial activity in bumble bees

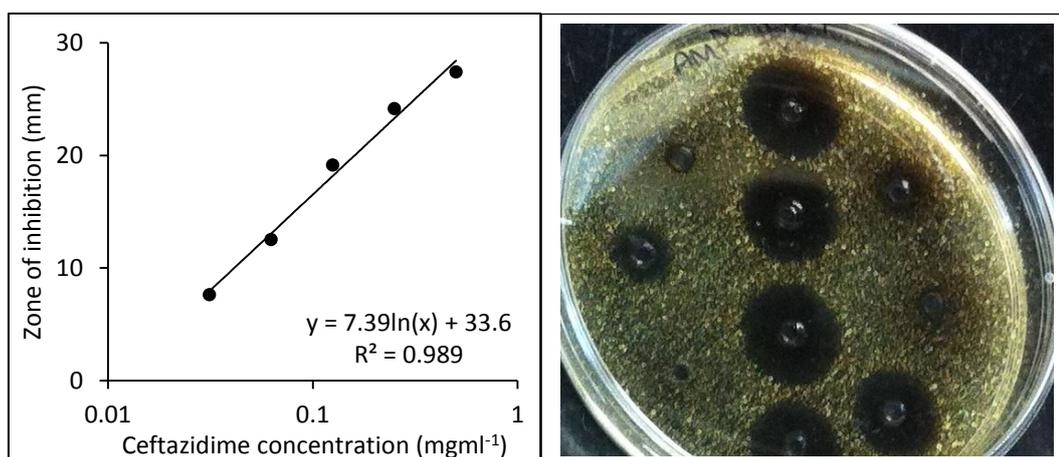
#### 2.3.2.1. Bacterial growth inhibition assays

Testing whether bumble bee neat haemolymph samples inhibited the growth of live bacterial cultures, I found small zones of inhibition (< 3 mm) of *A.globiformis* growth from LPS-injected bumble bees, but no inhibition of *E.coli*, or *S.aureus*. Furthermore, much clearer larger inhibition zones were observed using an inhibition assay with *M.luteus* (typically zones up to 8 mm diameter in samples collected 24 hours post LPS injection; Figure 2.3.). It was concluded that using a growth inhibition assay against *M.luteus* was appropriate for reliable measurement of antimicrobial activity from bumble bee neat haemolymph samples.

To validate the growth inhibition assay as an appropriate measure of relative differences in antimicrobial activity between samples, a dilution series of a standard antibiotic (ceftazidime) solution was measured. Each measure was taken using two technical replicates to confirm the repeatability of the method. As expected, there was a significant relationship between log ceftazidime concentration and the diameter of inhibition zone measured against growth of *M.luteus* (Figure 2.3.; Linear regression:  $R^2=0.989$ ,  $P<0.001$ ). It was concluded that the protocols used were appropriate for future work to investigate the

antimicrobial activity in bumble bees subjected to immune and chemical stressors, as described later in this thesis.

It is unclear as to why the best methods differed between honey bees and bumble bees, but it is likely that the repertoire of antimicrobial peptides differs between the species and hence the inhibitory action of these antimicrobial peptides on the growth of the test bacteria also differs.



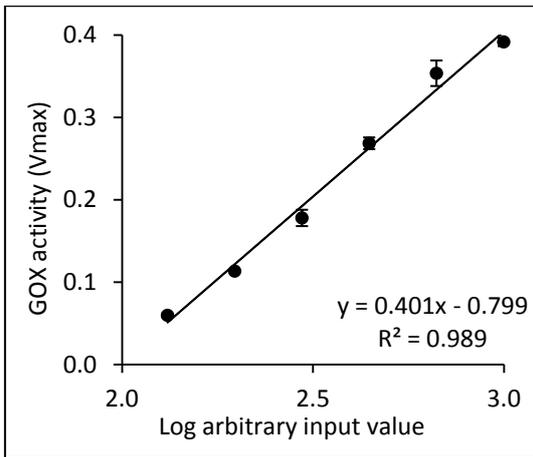
*Figure 2.3.* Left: Dilution series measuring antimicrobial activity of an antibiotic standard against the inhibition of *M. luteus*. (Mean of two replicates  $\pm$  SE). Trend line shows line of best fit. Right: Photograph of a growth inhibition plate, demonstrating zones of inhibition of varying diameter.

#### 2.4. Measurement of glucose oxidase activity

Glucose oxidase (GOX) activity has previously been quantified using a spectrophotometry assay (White et al., 1963, Alaux et al., 2010). This involves GOX catalysing the conversion of  $\beta$ -D-glucose in the reaction mixture to gluconic acid and hydrogen peroxide. This hydrogen peroxide subsequently reacts with a colourless substrate, o-dianisidine, in the presence of a peroxidase enzyme, to oxidise the substrate to a coloured product. Hence the greater the colour change the greater the initial concentration of GOX in the sample. Since GOX is secreted from the hypopharyngeal glands in the honey bee head, activity can be measured most easily from a homogenised head sample. My approach was to use methods previously described in the literature to measure glucose oxidase activity, replicating the protocol of Alaux et al.

(2010) as far as possible and given the laboratory facilities available to me. For this assay, I used a saturated solution of o-dianisidine, as the described concentration of 3 mM (= 0.7 mgml<sup>-1</sup>) was not found to be soluble in water at room temperature. All bee heads were homogenised in 200 µl PBS, centrifuged and the supernatant used in the assay. This volume of buffer presented the minimum volume that could be used (to maximise the concentration of GOX in the sample) since testing smaller volumes it was difficult to homogenise the head and subsequently collect enough debris-free supernatant for analysis of up to three replicates of each sample. Initial pilots allowed reactions to proceed for a three hour period in the spectrophotometer. Reaction curves typically demonstrated that the linear phase of the reaction was recorded within the first 80 minutes, with the slope of change in absorbance over time plateauing by 90-100 minutes. Since I was interested in the maximum linear phase of the reaction, it was concluded that allowing each reaction to proceed for 105 minutes (1 hour 45 minutes) would be sufficient to measure this phase of all reactions. This is in line with the protocol of Alaux et al., in which reactions were measured for 1 hour 30 minutes.

To validate the assay as an appropriate measure of relative differences in glucose oxidase activity between samples, a dilution series of a single pooled sample of homogenised bee heads was prepared in PBS, and GOX activity subsequently measured. Each measure was taken using two technical replicates to confirm the repeatability of the method. There was a significant linear relationship between the log sample input and GOX activity (Figure 2.4.; Linear regression:  $R^2 = 0.989$ ,  $P < 0.001$ ), demonstrating, as expected, that a greater  $V_{max}$  value corresponded to greater GOX activity. It was concluded that the protocols used were appropriate for future work to investigate the glucose oxidase activity in bees subjected to immune and chemical stressors, as described later in this thesis.



*Figure 2.4.* Dilution series measuring GOX activity of a single sample (Mean of two replicates  $\pm$  SE). Trend line shows line of best fit.

## 2.5. Gene expression assays using qPCR

### 2.5.1. Primer design and optimisation

Real-time quantitative PCR (qPCR) is a well-established research tool allowing a relatively cheap, quick and reliable method to study differential gene expression of specific genes of interest. For the majority of work presented in this thesis, honey bee genes were selected for investigation based on *a priori* knowledge of their candidate function and their regulation in response to immune or chemical stressors in previous studies. In these cases, primer sets applied in previous qPCR studies were used here, and they are described in the appropriate methods sections.

An optimisation study was carried out to investigate the most appropriate concentrations of forward and reverse primers to maximise the yield of amplified DNA for the *GOX* gene. Based on the manufacturer's recommendations for primer concentration optimisation using the SYBR Green PCR Master Mix, an optimisation matrix was used to test nine conditions, with each combination of three primer concentrations: 100 nM, 300 nM and 900 nM. For all conditions tested, threshold cycle ( $C_t$ ) values were undetermined for controls with no template included, confirming the absence of nonspecific amplification. An optimum mastermix containing both primers at a concentration of 900nM was found, providing greater amplification than any other conditions tested. In contrast, primer concentrations of 100 nM, consistent with those used by Yang and Cox-Foster (2005), were found to be the least efficient, giving the highest threshold cycle and hence the lowest amplification of all conditions tested. It was concluded that all future work would use both forward and reverse primers at a concentration of 900 nM in the *GOX* qPCR assay.

A new qPCR assay was established to measure expression of the honey bee *ALDH1L2* gene. *ALDH1L2* primers were designed using the Primer 3 software (Untergasser et al., 2012). Primer concentration was optimised, as for the *GOX* assay, using an optimisation matrix for each combination of three primer concentrations: 100 nM, 300 nM and 900 nM. For all conditions tested, threshold cycle ( $C_t$ ) values were undetermined for controls with no template included, and an optimum mastermix containing both primers at a concentration of 900 nM was found. Details of the *ALDH1L2* primers are described below:

*Forward primer:* GGGCCACAGAACCATAAAGC; Length 20 bases;  $T_m$  59.18 °C, GC content 55 %. No internal secondary structure.

*Reverse primer:* CGCCACGTTCCACAAATTTC; Length 20 bases;  $T_m$  58.60 °C, GC content 50 %. No internal secondary structure.

*Product size:* 58 bases

### *2.5.2. Preparation of standard PCR (calibration) curves*

To validate each PCR assay and to prepare standard curves for later calibration of input quantities, a dilution series of a single pooled cDNA sample was prepared and the  $C_t$  values measured by qPCR for each gene assay. Thirteen dilutions were chosen with the aim that expression levels of subsequent samples would fall within the limits of the standard curve. An arbitrary input quantity was assigned to each dilution, which was relative to the dilution concentration. This input quantity was transformed by  $\log_{10}$ , and then plotted against the  $C_t$  value and the slope, intercept and  $R^2$  value calculated (Figure 2.5.). The PCR efficiency,  $E$ , of each primer set was also calculated using the formula  $E = 10^{-1/\text{slope}} - 1$  and PCR efficiency was typically within the recommended 90 - 110 %.

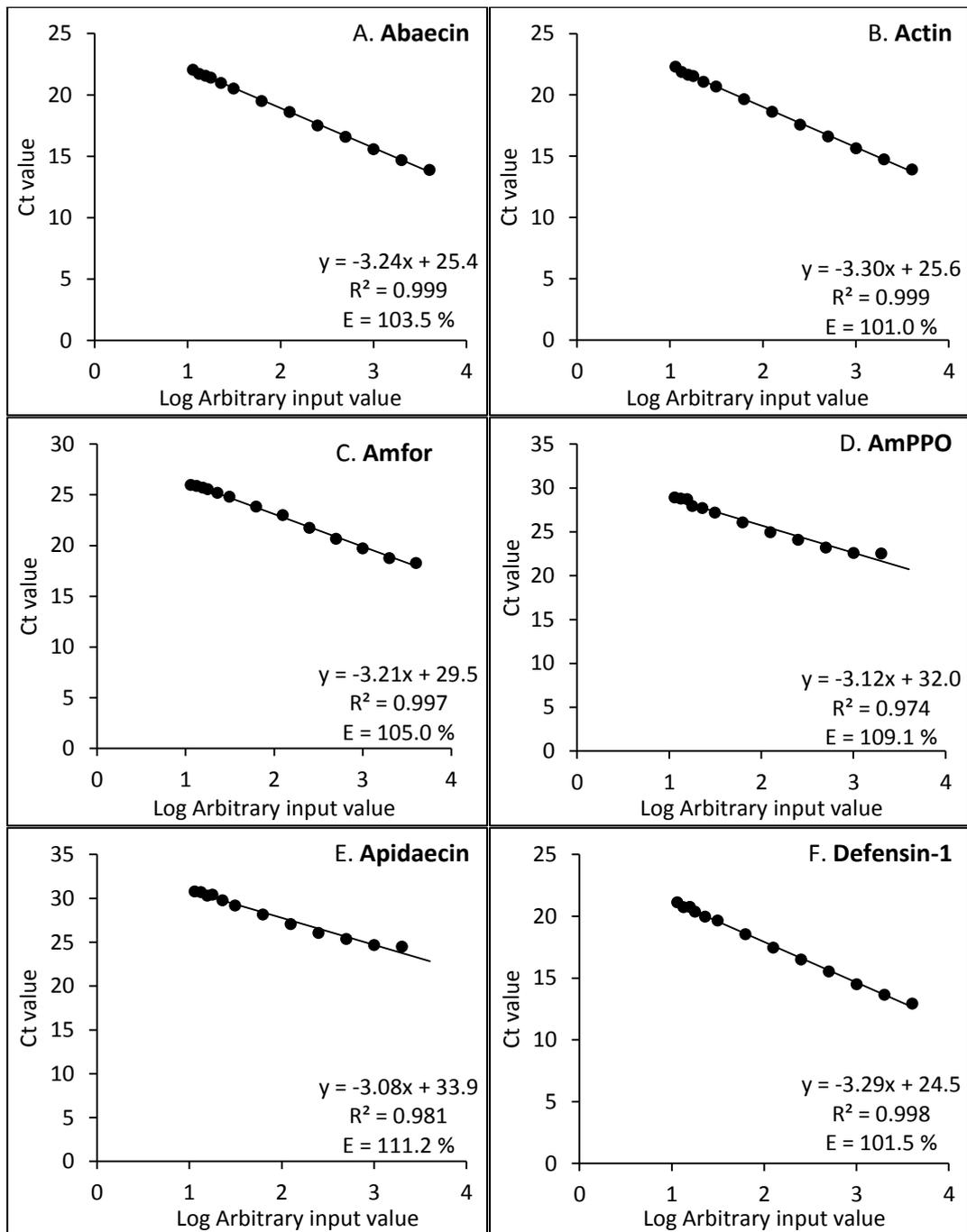


Figure 2.5 (NB. continued overleaf). Calibration curves for each qPCR assay. Trend lines show lines of best fit.

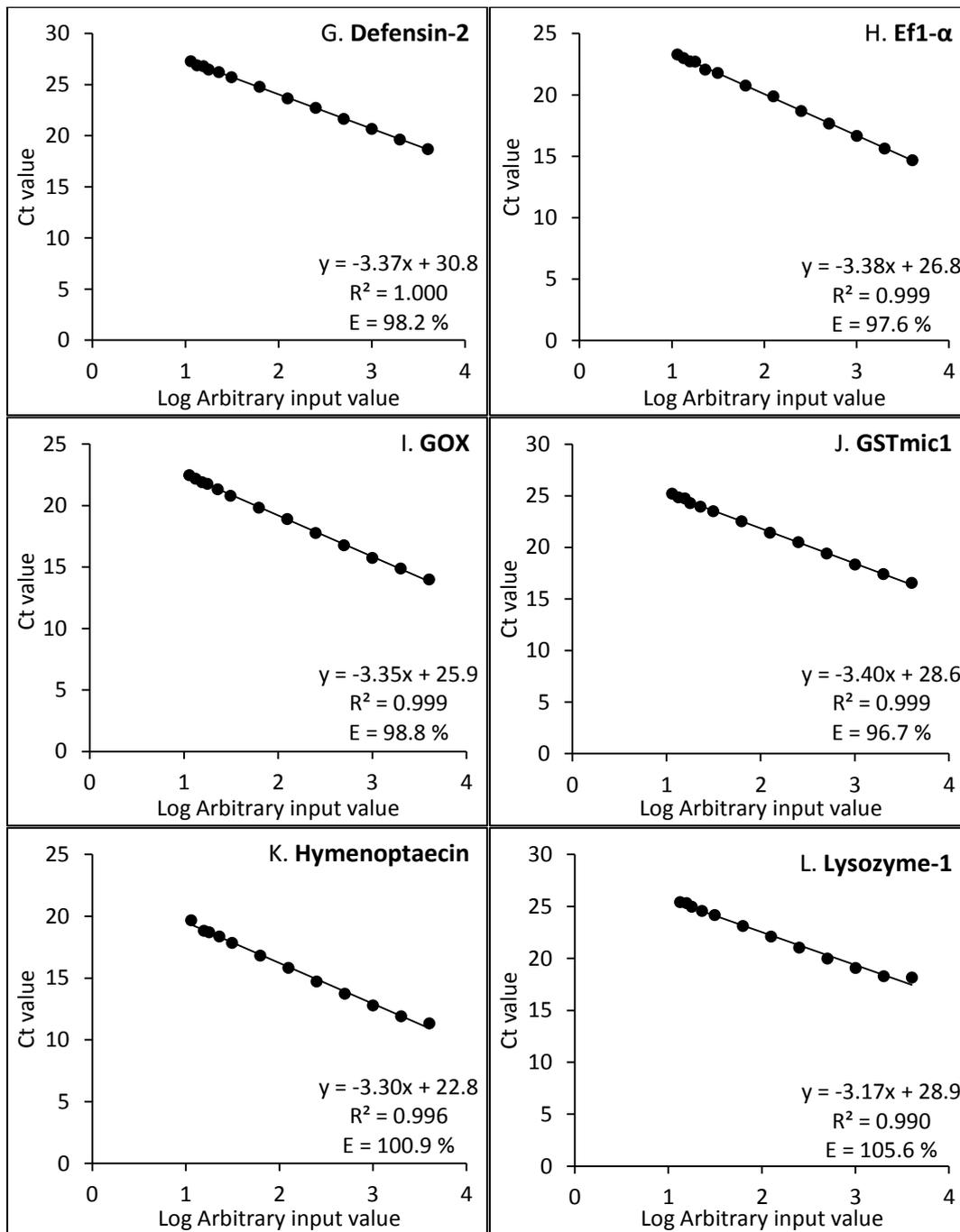


Figure 2.5 (NB. continued overleaf). Calibration curves for each qPCR assay. Trend lines show lines of best fit.

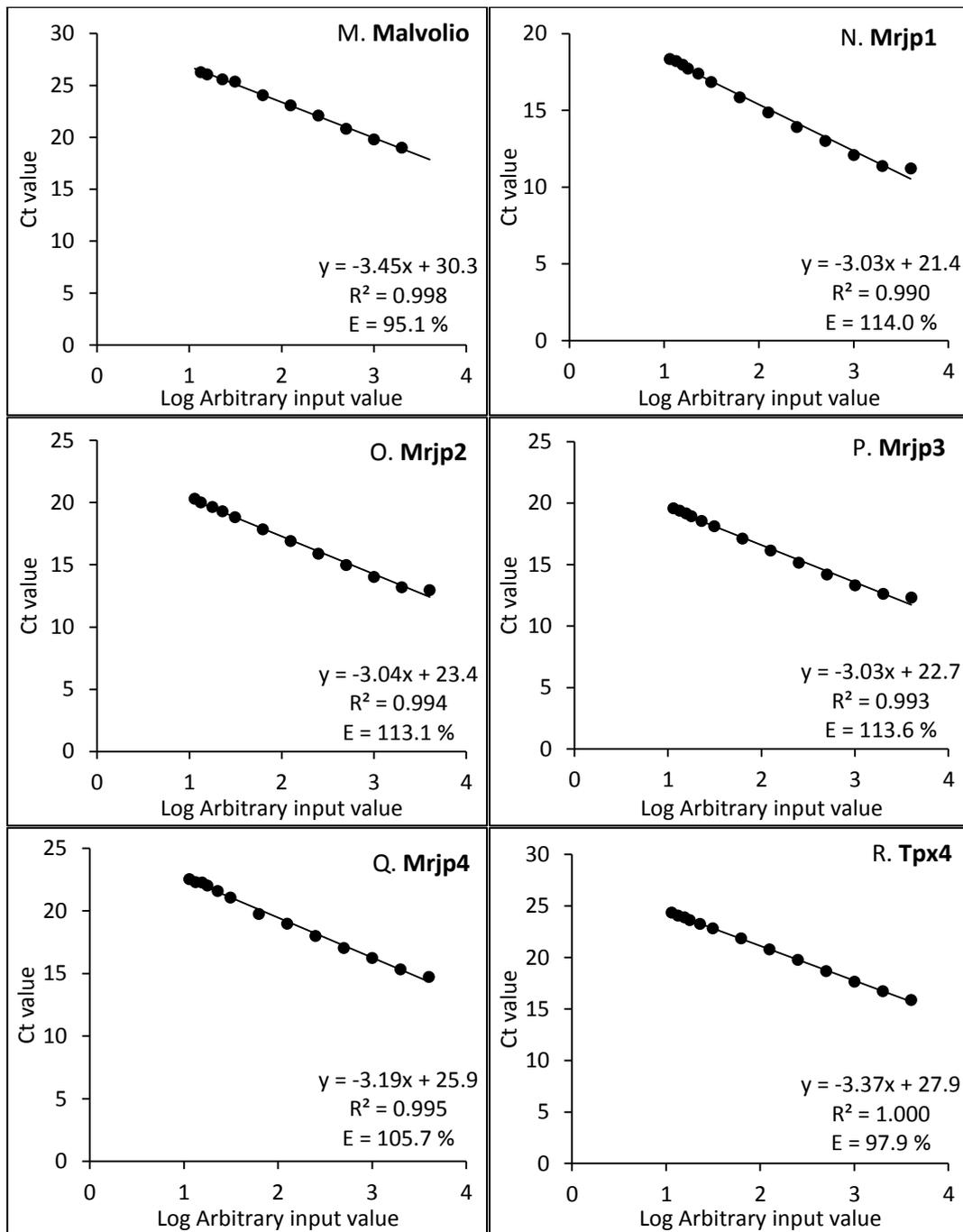


Figure 2.5 (NB. continued overleaf). Calibration curves for each qPCR assay. Trend lines show lines of best fit.

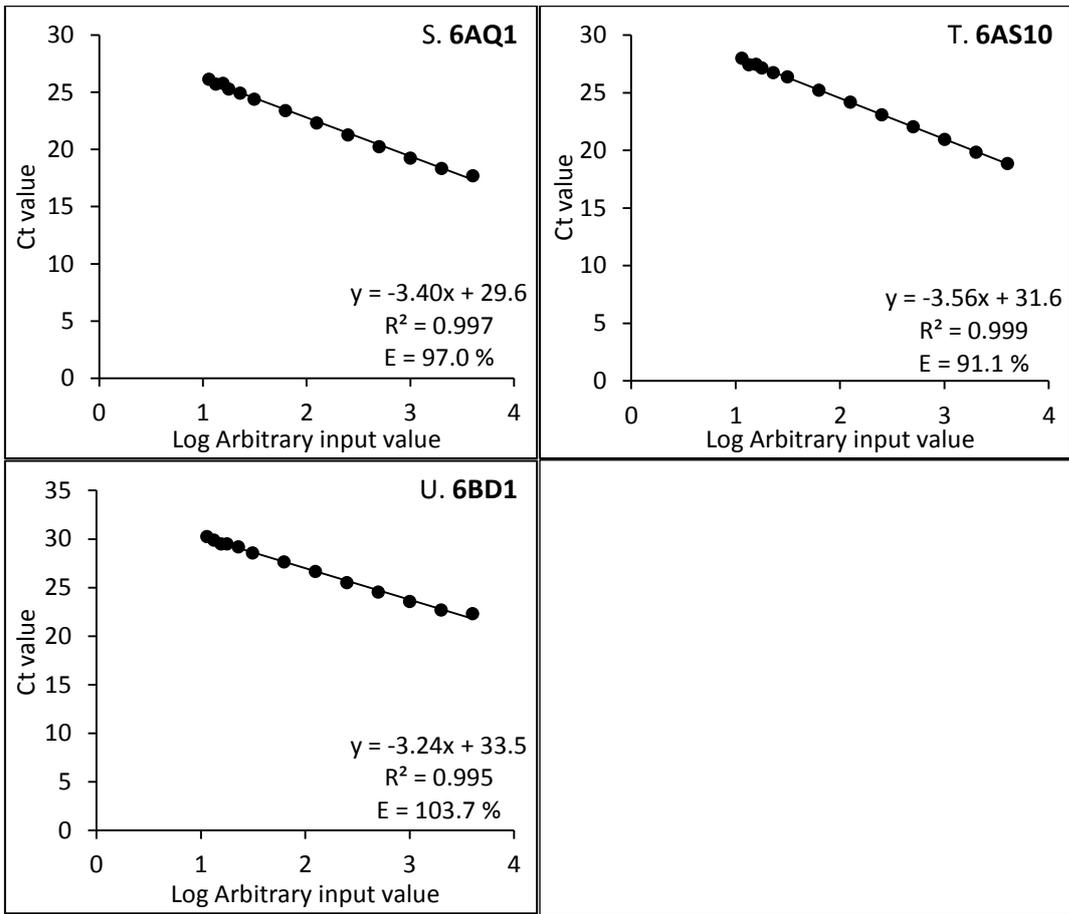


Figure 2.5. Calibration curves for each qPCR assay. Trend lines show lines of best fit.

### *2.5.3. Selection of reference genes for normalisation*

To reliably compare gene expression levels between samples it is essential to normalise the data to appropriate reference genes to control for variations between samples, such as the amount of starting material and differences in overall transcriptional activity. Reference genes should show similar levels of expression throughout an organism's development and should not vary in response to experimental treatments. Several reference genes have been validated as suitable for use in normalisation of qPCR data in honey bees (Lourenco et al., 2008). Nevertheless, in any given study it is appropriate to validate the reference genes to confirm their presumed stability of expression within the experimental treatments of the study (Andersen et al., 2004). Genes tested in each of the studies in this thesis were evaluated to identify those most stably expressed across the experimental treatments. Furthermore, it has been recognised that geometric averaging of multiple reference genes may provide a more reliable normalisation factor than a single gene (Vandesompele et al., 2002) and hence combinations of reference genes were evaluated to select the most suitable combination as a normalisation factor.

Data were analysed using two established approaches for reference gene selection. Firstly, the methods of Vandesompele et al. (2002) were followed to calculate a gene stability measure (M). Whilst further discussion of this approach will refer to the 'GeNorm' algorithm, I did not have access to the GeNorm software itself, and hence the analysis was performed manually on Excel following the GeNorm protocol described in Vandesompele et al. (2002) and outlined here. Secondly, NormFinder software (NormFinder Excel Add-In v0.953) (Andersen et al., 2004) was used to calculate an alternative measure of gene stability. Since different selection methods use different criteria it is recognised that the selection of reference genes may depend on the method used. Nevertheless, it is expected that best candidate reference genes will be appropriately identified in both established methods, as previous comparisons analysing samples using more than one method have found similar measures of gene expression stability between selection methods (Lourenco et al., 2008). The two approaches were included in the validation as a comparison of the two methods to select appropriate reference genes and the results of both methods were taken into consideration in the final selection of reference genes.

### 2.5.3.1. *GeNorm algorithm (Vandesompele et al., 2002)*

For each assay, the input quantities of all experimental samples (transformed from  $C_t$  values using the relative standard curve method) were placed in order of highest to lowest input quantity. Each sample input quantity was then divided by the highest input quantity to give a value between 0 and 1. This ratio will be referred to as the 'expression value'. For each gene, the expression ratio was calculated systematically compared to all other genes (for example, for every sample the abaecin expression value was divided by the actin expression value, by the ef1- $\alpha$  expression value and so on for all genes). The GeNorm approach relies on the principle that two ideal reference genes will show identical expression ratios across all samples. Each expression ratio was then transformed using the natural logarithm (LN) (to give a symmetrical distribution of the data around zero so that a given ratio and inverse ratio have equal absolute values, but opposite signs). For each gene, the pairwise variation in expression ratio,  $V$ , was then calculated as the standard deviation of the LN-transformed expression ratios of each sample. In this way, the pairwise variation  $V$  is independent of differences in abundance between genes and equally affected by outlying or extreme ratios caused by up- or down- regulation or low or high overall expression. By calculating the average pairwise variation across the candidate reference gene combinations, this gives a gene stability measure,  $M$ , whereby the gene with the highest  $M$  value is the least stably expressed. The least stably expressed gene was eliminated as a candidate reference gene and the analysis repeated, now excluding this gene. Stepwise analyses were performed, each time eliminating the least stable gene until left with a combination of the two most stably expressed genes across the samples.

### 2.5.3.2. *NormFinder algorithm (Andersen et al., 2004)*

As with the GeNorm approach, the expression value was calculated as the ratio of the highest input quantity to the sample input value, and this expression value was used as the input data for the NormFinder software. Like GeNorm, the NormFinder algorithm estimates the overall variation in expression of each candidate reference gene, but in addition NormFinder estimates the variation in expression between the treatment groups in the sample set. Using both the intra- and inter-group variation, a stability value is calculated, with lower stability

values representing more stably expressed genes. To enable the NormFinder software to generate this stability value, each combination of injection treatment, pesticide treatment and sampling time point was first identified as a separate treatment group in the input data.

#### *2.5.3.3. Results and Discussion*

This work initially used the dataset from the qPCR work outlined in Chapter 3. The results of the two analyses are summarised in Figure 2.6. Both methods found actin to be the most stably expressed gene. Actin has previously been validated as a suitable reference gene for normalisation in honey bee gene expression studies (Lourenco et al., 2008). Lourenco et al. (2008) also validated three other reference genes in the honey bee (ribosomal protein 49, elongation factor 1-alpha, tbp-association factor), of which elongation factor 1-alpha (ef1- $\alpha$ ) was included as a candidate reference gene also in this study. The NormFinder algorithm found ef1- $\alpha$  to be the third most stably expressed gene, whilst it was the fifth most stably expressed in the GeNorm output. However, there was little difference in stability value between actin and ef1- $\alpha$  in both outputs, as depicted by the relatively flat line between the two genes in Figure 2.6. These results support those of Lourenco et al. (2008) that actin and ef1- $\alpha$  have a generally uniform expression across honey bee samples and hence are suitable reference genes. Of the genes tested in this study, the three P450 detoxification enzymes (6AS10, 6AQ1 and 6BD1) also showed low variation in expression between samples. In particular, 6AQ1 was the third most stably expressed gene according to the GeNorm algorithm and the second most stably expressed according to NormFinder. Similarly, 6AS10 was the second most stably expressed according to GeNorm and the fourth most stably expressed according to NormFinder. 6BD1 seemed to be less suitable based on the NormFinder results. Based on these findings, four genes (actin, ef1- $\alpha$ , 6AS10 and 6AQ1) were considered as the most suitable candidate reference genes for normalisation of the data set.

In a further stage of analysis, combinations of multiple reference genes were evaluated to determine a suitable number of genes and the most suitable combination of genes for geometric averaging. The use of more than one reference gene is considered to be a more accurate and reliable approach to

normalisation than using a single gene and the minimum number of genes to use in this average may depend on the variation within the sample set. A geometric mean is favoured to an arithmetic mean as it is expected to better control for possible outliers and differences in transcript levels between different genes (Vandesompele et al., 2002).

Firstly, the NormFinder software identified defensin-1 and actin as the best combination of two genes (note, defensin-1 was ranked as the fifth most stably expressed gene according to NormFinder). In contrast, defensin-1 was found to be less stable according to GeNorm (Figure 2.6). It is recognised that the validity of the NormFinder approach requires firstly that the sample set contains at least 8 samples per treatment group, and furthermore the candidate genes in the analysis should have no prior expectation of differences between treatments (Andersen et al., 2004). Here, the sample set only allowed 3 samples per treatment group, and all the genes tested were included in the analysis, although not all were expected to show uniform expression. Despite the lack of conformity to the specified requirements, the NormFinder results generally support those of GeNorm, and reflect prior expectations, such as the high stability of actin and ef1- $\alpha$  (Lourenco et al., 2008). However, defensin-1 was initially included in the study for its hypothesised upregulation in response to injection treatment (for example, bacterial infection has previously been found to affect its expression (Evans, 2006)). Given this hypothesis and given that the accuracy of the analysis may be limited by the number of samples per treatment group, it seemed appropriate to take a cautious approach and avoid the use of defensin-1 within the normalisation factor of this study.

Secondly, using the GeNorm methods of Vandesompele et al. (2002), normalisation factors were calculated for different combinations of the four most stable genes identified above (actin, ef1- $\alpha$ , 6AS10 and 6AQ1). Using a systematic approach to include one to four reference genes, the pairwise variation,  $V$ , was calculated between normalisation factors with the sequential addition of a reference gene. A large variation, defined by a cut off value of 0.15, reflects that inclusion of an additional gene has a significant effect and hence this additional gene should be included in the normalisation factor to improve its reliability. Results of this analysis are summarised in Table 2.1. Since actin was the most stable gene according to both GeNorm and

NormFinder, the effect of a second gene in addition to actin was tested. Addition of any of the three genes significantly affected the pairwise variation, demonstrating that use of at least two genes in the normalisation factor was more appropriate than use of actin as a single reference gene. Overall, addition of a third gene was also shown to improve the normalisation factor compared to two genes. This supports the recommendation by Vandesompele et al. (2002) that a minimum of three reference genes should be used in the calculation of a normalisation factor. Addition of a fourth gene (in the case of all combinations analysed) was found to have no significant effect on the normalisation factor. On consideration of the different combinations tested, overall the addition of 6AQ1 as a third reference gene did not have a significant effect on the normalisation factor compared to using only two genes. It was concluded that a geometric average of three reference genes (actin, 6AS10 and ef1- $\alpha$ ) would be used as the normalisation factor in this sample set. This final conclusion was made given that i) actin and 6AS10 were the most stable genes according to GeNorm, ii) ef1- $\alpha$  was more stable than 6AS10 according to NormFinder, iii) ef1- $\alpha$  has previously been validated as a suitable reference gene in honey bees (Lourenco et al., 2008), and iv) the addition of ef1- $\alpha$  as a third gene overall was shown to have a significant effect on the normalisation factor (Table 2.1.).

Based on the data presented above, and that of Lourenco et al. (2008), actin and ef1- $\alpha$  were selected as appropriate candidate reference genes in a second gene expression study outlined in Chapter 5. Here, only two genes were used for normalisation due to the relatively few genes investigated in the study. Using the GeNorm algorithm (as above), the stability of these two genes was confirmed across the sample set, confirming their suitability as reference genes in this study (Figure 2.7.).

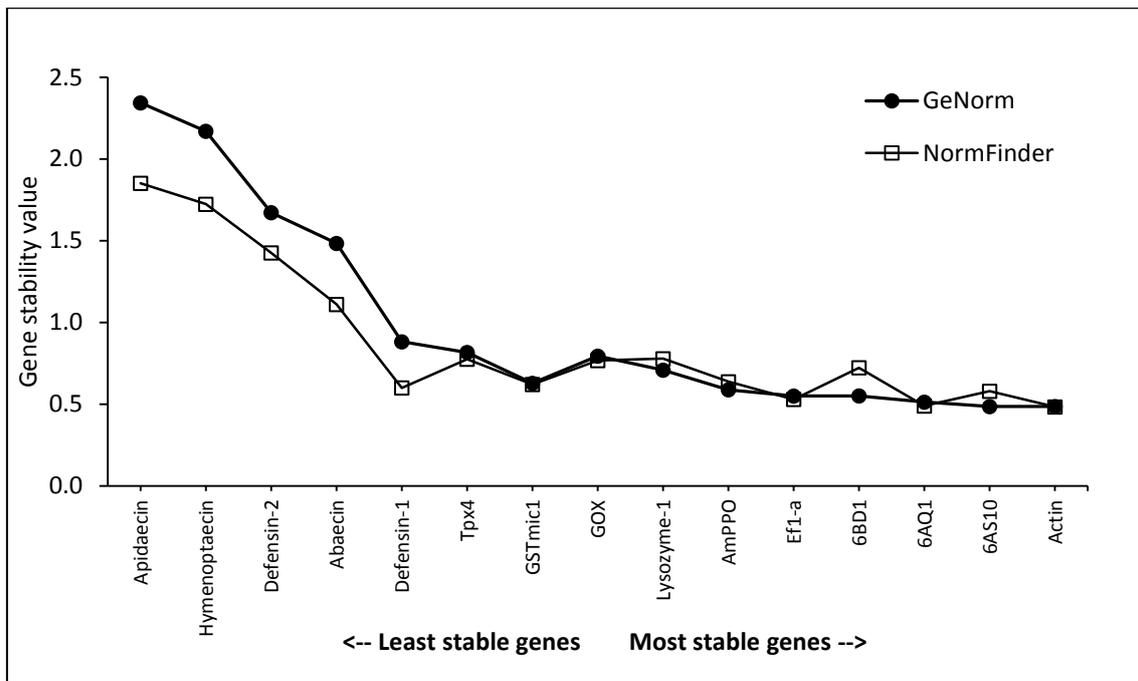
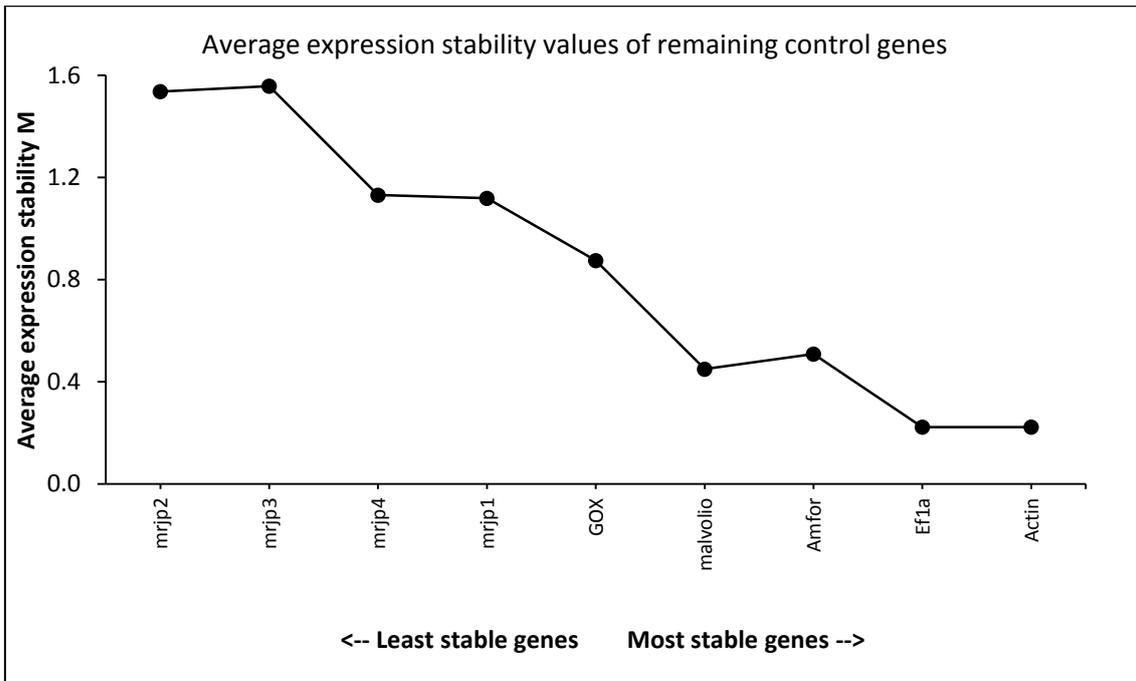


Figure 2.6. Gene stability values of each of the 15 genes in the qPCR study of Chapter 3, analysed using the GeNorm algorithm (black circles) and NormFinder algorithm (white squares). Genes are ordered along the x-axis from highest to lowest stability values from the GeNorm output, from least to most stable left to right. Whilst the NormFinder output showed a very similar trend (as shown by the shapes of the two curves), note that the order of stability was not identical between the two algorithms.

*Table 2.1.* Summary of pairwise variations between normalisation factors with the sequential addition of a reference gene, based on methods of Vandesompele et al. (2002). \*\*\* = significant effect of inclusion of gene (i.e.  $V > 0.15$ )

Normalisation factor A	Normalisation factor A+1	Pairwise variation, V	Equivalent sequential ranking of algorithm:
Actin	Actin + 6AS10	0.24***	GeNorm
Actin	Actin + 6AQ1	0.27***	NormFinder
Actin	Actin + Ef1- $\alpha$	0.30***	na
Actin + 6AS10	Actin + 6AS10 + 6AQ1	0.15	GeNorm
Actin + 6AQ1	Actin + 6AQ1 + Ef1- $\alpha$	0.16***	NormFinder
Actin + 6AQ1	Actin + 6AQ1 + 6AS10	0.14	na
Actin + 6AS10	Actin + 6AS10 + Ef1- $\alpha$	0.18***	na
Actin + Ef1- $\alpha$	Actin + Ef1- $\alpha$ + 6AS10	0.16***	na
Actin + Ef1- $\alpha$	Actin + Ef1- $\alpha$ + 6AQ1	0.15	na
Actin + 6AS10 + 6AQ1	Actin + 6AS10 + 6AQ1 + Ef1- $\alpha$	0.12	na
Actin + 6AS10 + 6AQ1	Actin + 6AS10 + 6AQ1 + 6BD1	0.12	GeNorm
Actin + 6AQ1 + Ef1- $\alpha$	Actin + 6AQ1 + Ef1- $\alpha$ + 6AS10	0.11	NormFinder
Actin + 6AS10 + Ef1- $\alpha$	Actin + 6AQ1 + Ef1- $\alpha$ + 6AS10	0.10	na



*Figure 2.7.* Gene stability values of each of the 9 genes in the qPCR study of Chapter 5, analysed using the GeNorm algorithm. Genes are ordered along the x-axis from highest to lowest stability values from the GeNorm output, from least to most stable left to right.

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### **3. Resilience of honey bee immunocompetence to chronic neonicotinoid exposure**

#### *3.1. Abstract*

Honey bees are of global economic and ecological importance due to their role in the pollination of crops and wild flowering plants. Concern over declines in bee health and colony losses has been associated with exposure to multiple stressors, including to neonicotinoid pesticides and pathogenic infection. Recent studies have found that neonicotinoid exposure alters the expression of immune-related genes, but the biological relevance of these transcriptional changes on the physiological immune functioning of individual bees has not been established. Here we show that chronic oral exposure to either 102 ppb imidacloprid or 10 ppb thiamethoxam caused transcriptional changes in antimicrobial effector genes, but the physiological antimicrobial response, measured by a lytic clearance assay, was unaffected, suggesting no functional adverse outcome. Levels of an enzyme involved in melanisation in the immune response, phenoloxidase, were largely constitutive and resilient to neonicotinoid exposure. This work suggests that transcriptional responses in a series of immune-related genes do not necessarily translate into functionally significant impacts on bee health and our data indicate a need for caution in their use as biomarkers in pesticide risk assessment.

### 3.2. Introduction

Currently, there is widespread concern over losses of managed honey bees and declines in wild bee populations (e.g. vanEngelsdorp et al., 2008, Neumann and Carreck, 2010, Goulson et al., 2008, Biesmeijer et al., 2006) due to the potential impact on the pollination of crops and wild flowering plants (Potts et al., 2010). Multiple stressors are likely contributing to declines in bee health, including habitat loss and fragmentation, the introduction of alien species (including plants, animals and microorganisms), climate change, the spread of pathogens and parasites, and pesticide application (Potts et al., 2010). Recent studies therefore have begun to investigate the biological mechanisms underlying interactions between multiple stressors, and there is evidence that exposure to neonicotinoid pesticides can impair the honey bee immune response, which could increase susceptibility to pathogenic infection and parasitic attack (e.g. Alaux et al., 2010). The immune response in individual honey bees consists primarily of cellular responses, such as phagocytosis and encapsulation, and humoral responses *via* the prophenoloxidase cascade (leading to melanisation) and antimicrobial effectors (Evans et al., 2006). Expression of some genes related to these immune processes alters following neonicotinoid exposure (Derecka et al., 2013, Di Prisco et al., 2013, Gregorc et al., 2012), although not all these studies used environmentally relevant exposure scenarios. It is unclear whether transcriptional changes in genes of immune function in individual bees are indicative of a threat to the viability of the colony as a whole. One previous study found no effect of exposure to one neonicotinoid, imidacloprid, on the cellular and phenoloxidase (PO) components of the bee immune response at a physiological level (Alaux et al., 2010), but it is unclear whether this result applies to all compounds in the neonicotinoid family and to other immune responses. For example, as yet there has been no investigation of neonicotinoid effects on antimicrobial peptide (AMP) enzyme activity, which means that it remains unclear whether neonicotinoid-induced suppression of AMP gene expression has functional significance by resulting in reduced protein levels. To broaden our understanding of the biological relevance of transcriptional changes in immune-related genes, we therefore carried out a series of

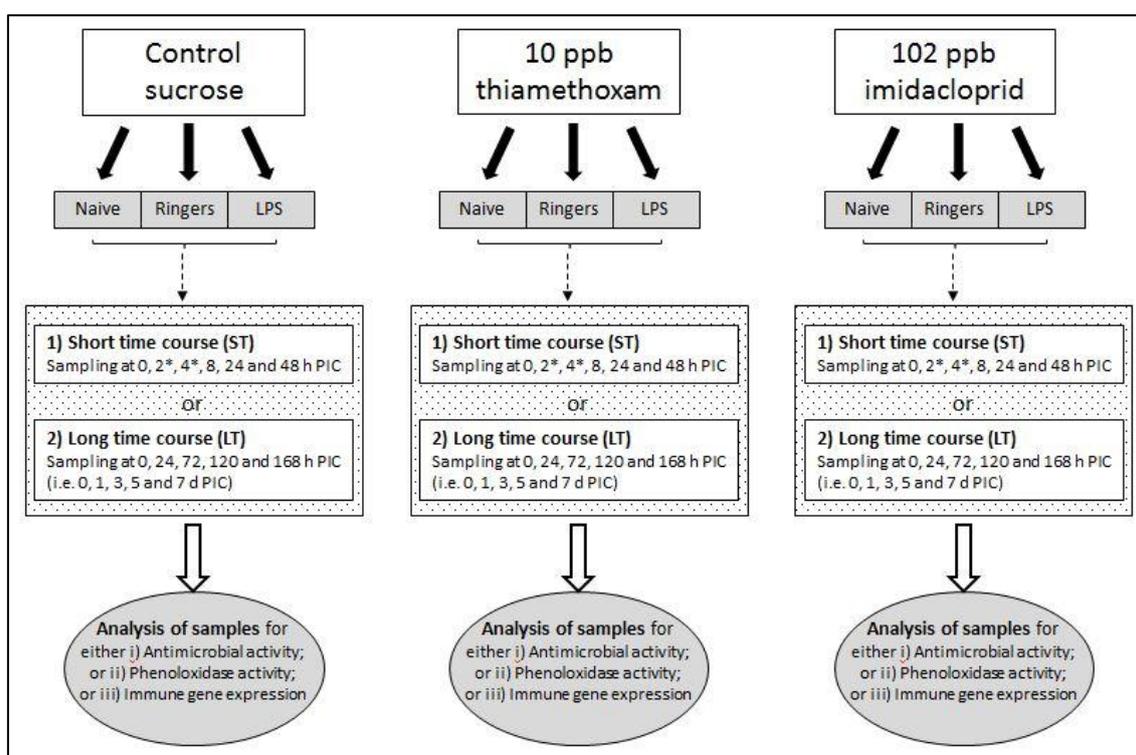
laboratory exposure experiments investigating relationships between changes in gene expression and measures of physiological honey bee immunity.

In our investigation, we aimed to establish whether exposure to neonicotinoids, including environmentally relevant exposure regimes, altered immune-related gene expression and whether these changes impacted on enzyme activity. Our study aimed to provide a foundation for understanding the potential regulatory value of gene expression bioassays as indicators of pesticide effects on bee health. Bees were exposed to thiamethoxam at 10 parts per billion (ppb), representative of field relevant residues in nectar (e.g mean  $\pm$ SD residues in squash of  $11\pm 6$  ppb; Stoner and Eitzer, 2012), and to imidacloprid at 102 ppb, which is at least ten-fold higher than typical residues in most crop systems (typically 1-10 ppb; Blacquiere et al., 2012), although residues as high as 101 ppb have been found in pollen from transplant-dripped pumpkin crops (Dively and Kamel, 2012). The study was designed as a factorial experiment in which only one dose of each compound was tested. This approach aimed to elucidate possible toxicological effects of pesticide exposure to test the biological relevance of immune endpoints, which could inform the direction of future work, and so we did not aim to establish dose-dependent effects or threshold levels to test for ecological relevance.

In our study, an artificial immune challenge *via* an injection with lipopolysaccharides (LPS) from *Escherichia coli* was used to mimic a bacterial-like infection and elicit an immune response. This approach was adopted to standardise the stimulus used to invoke a response in individual bees and to minimise the treatment variation inherent in using a real pathogen, which can be logistically difficult to control. The present study therefore does not aim to investigate the biological mechanisms associated with any specific natural infection, but instead seeks to better understand fundamental relationships between the temporal dynamics of transcriptional and physiological changes using a well-established method for insect immune studies.

### 3.3. Methods

We exposed newly emerged workers to either undosed control or neonicotinoid-spiked sucrose feeders and imposed an immune challenge after five days of experimental feeding. While maintaining bees on the same dietary treatment, we collected samples for various immune-related assays over the remainder of the experimental period. In separate experiments, sample collection followed either a short time course of between 2 and 48 hours post immune challenge (PIC), or a longer time course of between 24 and 168 hours (1-7 days) PIC (Figure 3.1.; Table 3.1.).



*Figure 3.1.* Schematic of overall experimental setup. Note, this overall setup was achieved in a series of individual experiments, as outlined in Table 3.1. For each pesticide exposure (top white boxes), bees received one of three immune challenges (middle grey boxes). For each pesticide/immune combination, samples were collected across either a short time course or long time course (depending on the individual experiments). \*Sampling at 2 and 4 h post immune challenge (PIC) was taken only in the qPCR experiments. Samples were then analysed to measure one of three immune endpoints (bottom grey ellipses).

Table 3.1. Overview of the test substances, sampling time course and endpoints measured in each of six experiments. Each experiment was performed with newly emerged honey bees from a single colony, but each experiment used a different colony.

	10 ppb TMX	102 ppb IMI	Short time course	Long time course	PO activity	AMP activity	Gene expression (qPCR)
Experiment 1	X		X			X	
Experiment 2		X	X			X	
Experiment 3		X	X		X		
Experiment 4	X	X	X				X
Experiment 5	X			X		X	X
Experiment 6		X		X		X	X

### 3.3.1. Honey bee provenance and husbandry

All honey bees were from *Apis mellifera* L. colonies of British hybrid bees maintained on the home apiary at the Food and Environment Research Agency, Sand Hutton, York, UK. Brood frames were collected from colonies between April and September of any given year (2013, 2014) and placed in an environmental chamber (34 °C, 60 % relative humidity, constant darkness; Sanyo Versatile Environmental Test Chamber). Workers were collected from these frames as they emerged and batches of 10 workers were housed in modified plastic cages (FK-RD8 clear PET containers; Ambican UK Ltd). Each cage allowed *ad libitum* access to a sucrose solution (50 % w/v) through a modified 1.5 ml microcentrifuge tube. Cages were maintained in the environmental chamber throughout the experimental period (34 °C, 60 % relative humidity, constant darkness). Six separate experiments were set up

between August 2013 and June 2014, each using workers derived from a single honey bee colony (Table 3.1.).

### 3.3.2. Neonicotinoid exposure

All studies used chronic dietary exposures to sublethal concentrations of neonicotinoid comprising either imidacloprid or thiamethoxam. Stock solutions of neonicotinoids were made up in acetone, and used to create the appropriate concentrations. In the imidacloprid studies, sucrose was spiked with  $125 \mu\text{gL}^{-1}$  (= 102 ppb) imidacloprid (Fluka Analytical 37894, Sigma-Aldrich, UK). In the thiamethoxam studies, treated sucrose was spiked with  $12 \mu\text{gL}^{-1}$  (= 10 ppb) thiamethoxam (Fluka Analytical 37924, Sigma-Aldrich, UK). All cages allowed *ad libitum* access to the sucrose feeder throughout the experiment and no other food was provided. Control sucrose contained a relevant volume of acetone equivalent to the solvent in the neonicotinoid treatments. To quantify feeding rates, the feeder of each cage was weighed every other day and corrected for evaporation using reference feeders kept in empty cages. To quantify mortality rates, dead bees were recorded and removed every other day.

### 3.3.3. Immune challenge

To immobilise workers for injection treatments, individual cages in which honey bees were housed were placed in a freezer ( $-20 \text{ }^{\circ}\text{C}$ ) for approximately two to five minutes until bees were torpid. All injections were performed using a fine needle on a Hamilton syringe with a repeating dispenser. Honey bees were injected with  $2 \mu\text{l}$  of solution through the pleural membrane between the tergites (dorsal side) of the abdomen. Bees received one of three injection treatments, with all workers within a cage subjected to the same treatment. In the 'naïve' treatment, bees were ice-immobilised but received no further treatment, which served as the unchallenged control. The 'Ringers' treatment group were injected with Insect Ringers solution (1:1:1 156 mM NaCl : 3 mM KCl : 2 mM  $\text{CaCl}_2$ ) to control for any effects of the injection process itself. The 'LPS' treatment group were injected with  $0.5 \text{ mgml}^{-1}$  LPS (Sigma L2755-Lipopolysaccharides from *Escherichia coli* 0128:B12, Sigma-Aldrich, UK) dissolved in Insect Ringers solution. This LPS concentration has been widely

used previously to elicit an immune response in honey bees without any acute effects on survival (e.g. Alaux et al., 2012, Koehler et al., 2012, Laughton et al., 2011).

There were no evident effects of pesticide or injection treatments on honey bee behaviour and activity. No treatment affected mortality, with the exception that Ringers injection increased mortality in Experiment 5 (Kruskal-Wallis:  $\chi^2_{2df} = 7.57$ ,  $P = 0.023$ ). In the short time course experiments 2-7 % of bees died, whilst 8-21 % of bees died in the longer time course experiments. The low mortality across treatments confirmed that the pesticide concentrations and immune challenges used were sublethal to worker honey bees.

#### *3.3.4. Measurement of gene expression*

At appropriate time points, bees were placed directly into a freezer at -80 °C and stored until RNA extraction. Total RNA was extracted from homogenates of six bees (entire bodies) from a single cage. For each feeding/injection treatment combination and time point, separate homogenates from three cages were used as biological replicates for real-time quantitative PCR (qPCR) analysis. Pooling of six bees per replicate homogenate served to minimise between-bee variation in gene expression profiles. Total RNA was extracted using the *mirVana* miRNA Isolation Kit (Ambion AM1561; Life technologies, UK). RNA quantity of each sample was measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, UK) and samples were subsequently standardised to 2 µg for cDNA synthesis. cDNA was synthesised using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814; Life technologies, UK) and a C1000 Thermal Cycler (Bio-Rad Laboratories, UK) under the following cycling conditions: 10 minutes at 25 °C, 120 minutes at 37 °C, 5 minutes at 85 °C, stored at 4 °C.

qPCR was performed using the SYBR Green PCR Master Mix (Applied Biosystems 4309155, Life technologies, UK). Oligonucleotide primers, used to amplify genes (Table 3.2.), were used at a concentration of 1400 nM. Each

sample was run in duplicate, with treatments randomly assigned across plates. The PCR reactions were carried out on 96-well plates in a ViiA 7 Real-Time PCR System (Applied Biosystems; Life technologies, UK) under standard cycling conditions (1 cycle of 2 minutes at 50 °C, 1 cycle of 10 minutes at 95 °C and 40 cycles of 15 seconds at 95 °C and 1 minute at 60 °C).

The threshold cycle ( $C_t$ ) value for each sample was calculated using the arithmetic mean of the two replicates.  $C_t$  values were used only if the standard deviation of the two replicates was  $\leq 0.5$ . If the standard deviation exceeded 0.5, the assay was repeated for the given sample.  $C_t$  values were transformed into input quantity values using the relative standard curve method (Larionov et al., 2005). Input quantities were normalised using the geometric mean of Actin, Ef1- $\alpha$  and 6AS10 as the normalisation factor. These genes were identified as the most stably expressed genes using two established approaches for reference gene selection: GeNorm (Vandesompele et al., 2002); NormFinder (Andersen et al., 2004) (see chapter 2.4.3.). Normalised input quantities were used for statistical analysis. The mean fold-change in expression was calculated for each injection/pesticide/time point compared to the mean expression of the control-fed/naïve/zero-hours-exposure group.

**Table 3.2.** Oligonucleotide primers used in qPCR assays.

<b>Gene</b>	<b>Gene category/ pathway</b>	<b>Forward primer (5' → 3')</b>	<b>Reverse primer (5' → 3')</b>	<b>Reference</b>
Abaecin	Antimicrobial peptide	CAGCATTTCGCATACGTACCA	GACCAGGAAACGTTGGAAAC	Morimoto et al. (2011)
Apidaecin	Antimicrobial peptide	TAGTCGCGGTATTTGGGAAT	TTTCACGTGCTTCATATTCTTC A	Evans et al. (2006)
Defensin-1	Antimicrobial peptide	TGCGCTGCTAACTGTCTCAG	AATGGCACTTAACCGAAACG	Evans et al. (2006)
Defensin-2	Antimicrobial peptide	GCAACTACCGCCTTTACGTC	GGGTAACGTGCGACGTTTTA	Evans et al. (2006)
Hymenoptaecin	Antimicrobial peptide	CGGAATTGGAACCTGAGGAT AC	CCTGAATGACAATGGATCCT CTT	Designed in house
Lysozyme-1	Antimicrobial peptide	GAACACACGGTTGGTCACTG	ATTCCAACCATCGTTTTCG	Evans et al. (2006)
AmPPO	Phenoloxidase immune cascade	AGATGGCATGCATTTGTTGA	CCACGCTCGTCTTCTTAGG	Evans et al. (2006)
6AS10	P450 detoxification-reference	GGGGTACCTGGACCCAAGCC A	GCCAGAACACGCACGTTTCG C	Morimoto et al. (2011)
Actin	Structural protein-reference	TGCCAACACTGTCTTTCTG	AGAATTGACCCACCAATCCA	Lourenco et al. (2008)
Elongation factor 1-alpha (ef1-alpha)	Protein synthesis-reference	GGAGATGCTGCCATCGTTAT	CAGCAGCGTCCTTGAAAGTT	Lourenco et al. (2008)

### 3.3.5. Measurement of antimicrobial activity

Haemolymph was collected from bees that had first been immobilised on ice. A small slit was made in the abdominal tergites using sterile dissecting scissors and gentle pressure to the thorax resulted in a bubble of haemolymph exiting this slit. 2-10  $\mu\text{l}$  of this neat haemolymph was collected with a pipette and immediately stored at  $-20\text{ }^{\circ}\text{C}$  for later analysis. Activity was measured from six individual bees from a single cage, and the cage mean was used as the biological replicate for statistical analysis. Three cages were measured for each feeding/injection treatment combination and at each time point.

Antimicrobial activity was measured using a lysozyme clearance assay (Cotter et al., 2008) in which antimicrobial proteins in the haemolymph lyse the peptidoglycan cell walls of the test bacteria to produce a clear zone in a bacterial suspension, which is a proxy for relative antimicrobial activity (a larger zone indicates greater antimicrobial activity). Phosphate buffer solution (PBS) containing 1% agar was inoculated with  $0.2\text{ mgml}^{-1}$  lyophilised *Micrococcus lystatei* (M3770; Sigma-Aldrich, UK). 8 ml of this suspension was poured immediately into a 9 cm-diameter petri dish and left to set. Ten wells (approximately 2 mm diameter) were bored on each plate. Each haemolymph sample was thawed on ice and a 2  $\mu\text{l}$  aliquot was added per well. Plates were incubated at  $27\text{ }^{\circ}\text{C}$  for 72 hours, and the diameters of clearance zones were measured using digital callipers.

Note, here we do not explicitly distinguish between lysozymes and other antimicrobial proteins, as in this context we consider lysozymes as a subgroup of antimicrobial proteins based on sequence similarity, which overall all play a role in antimicrobial activity (Gillespie et al., 1997).

### 3.3.6. Measurement of phenoloxidase activity

A perfusion-bleed method was used to collect haemolymph samples, whereby a microsyringe and needle were used to insert 300  $\mu\text{l}$  sodium cacodylate (NaCac) between the head and thorax. This solution was flushed through the body and collected along with the haemolymph in a microcentrifuge tube, which was

immediately snap-frozen in liquid nitrogen to release prophenoloxidase or phenoloxidase enzymes from haemocytes, before being stored at -20 °C for later analysis.

Spectrophotometric assays were used to measure activity of: phenoloxidase (PO); total prophenoloxidase and phenoloxidase (total proPO/PO) activity (Laughton and Siva-Jothy, 2011). Haemolymph samples were centrifuged (9600 RPM, 10 minutes, 4 °C) and kept on ice. For the PO assay, 5 µl of PBS was added to the bottom of each well in a 96-well plate. For the total proPO/PO assay, 5 µl of  $\alpha$ -chymotrypsin (5 mgml<sup>-1</sup>; Sigma C4129, Sigma-Aldrich, UK) was added to the bottom of each well. A 10 µl aliquot of a sample (supernatant) was added to each well, the plate was then shaken and left at room temperature for five minutes to allow the  $\alpha$ -chymotrypsin to activate any proPO in the sample. The plate was then placed back on ice and 175 µl L-Dopa mastermix [containing 20 µl L-Dopa solution (saturated 11 mgml<sup>-1</sup>, then filtered; Acros Organics 167530050, Fisher Scientific, UK), 20 µl PBS, 135 µl water] was added to each well. The plate was shaken and the reaction allowed to proceed at 25 °C in a spectrophotometer (ThermoMax microplate reader, Molecular Devices, UK). Absorbance was measured at 490 nm every 15 seconds (shaking the plate between reads) for 1 hour 15 minutes (= 4500 seconds). A blank was used containing 10 µl NaCac in place of sample, but otherwise contained the same reaction mixture. Each sample was measured in duplicate for each assay and treatments were randomly assigned across plates to minimise between plate variation. Softmax Pro v4.3 software (Molecular Devices, UK) was used to calculate enzyme activity measured as the maximum linear rate of substrate conversion ( $V_{max}$ ), using 100  $V_{max}$  points, across the 4500 s reaction period.

Activity was measured from six individual bees from a single cage, and the cage mean was used as the biological replicate for statistical analysis. Three cages were measured for each feeding/injection treatment combination and at each time point.

### 3.3.7. Data analysis

Where necessary, data were first transformed to meet the assumptions for parametric testing. Variation among treatments was analysed by ANOVA with injection treatment, pesticide treatment and time post injection as fixed effects. Where ANOVA results were significant, pairwise differences were tested by Tukey's HSD.

### 3.4. Results

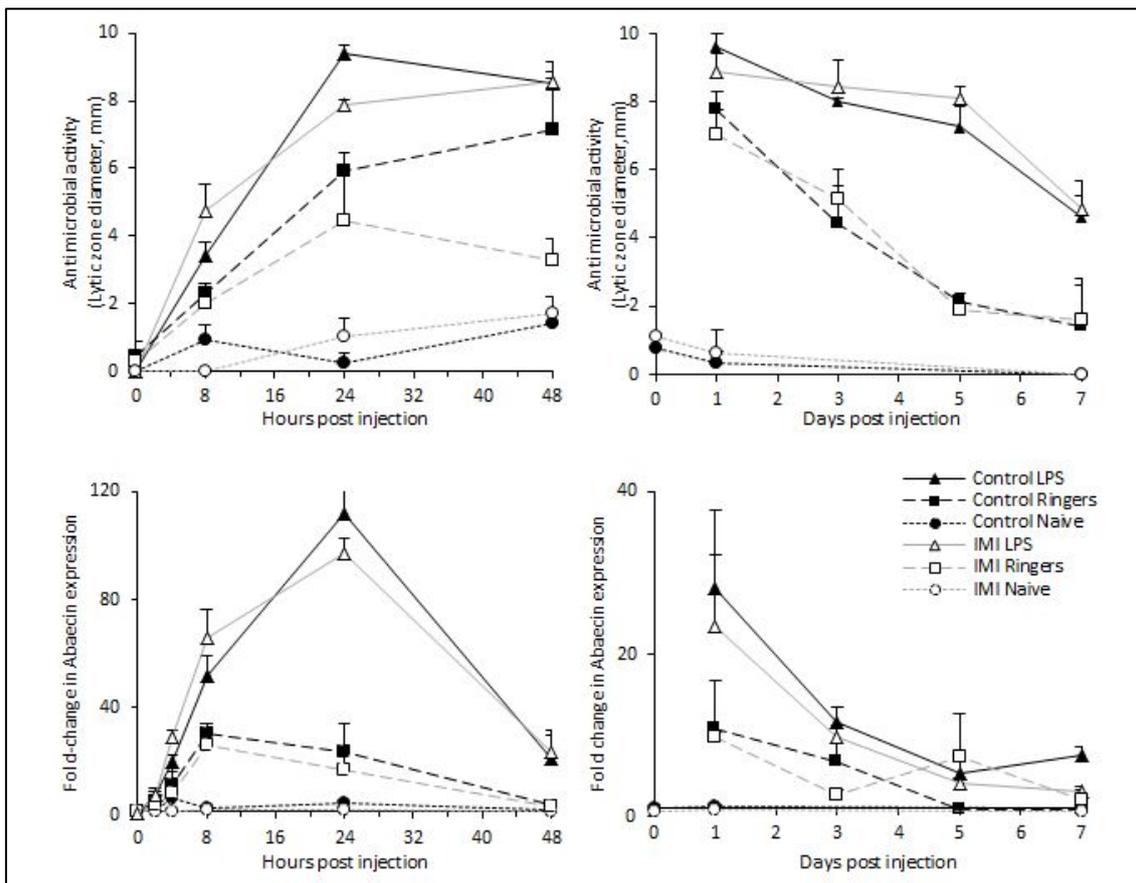
#### 3.4.1. Antimicrobial effector gene expression and enzymatic activity

In response to injection, four antimicrobial effector genes (abaecin, apidaecin and defensin-1 and hymenoptaecin) were significantly upregulated irrespective of whether the challenge contained LPS, but the challenge did not alter expression of defensin-2 and lysozyme-1. A similar temporal pattern of expression was observed for all four genes, despite between-gene differences in the magnitude of fold-changes in expression. Increased expression was typically observed as early as two hours PIC, increasing to peak expression at between eight and 24 hours PIC, after which the response showed a gradual decline over the following six days (Figures 3.2. and 3.3.; see also supporting information).

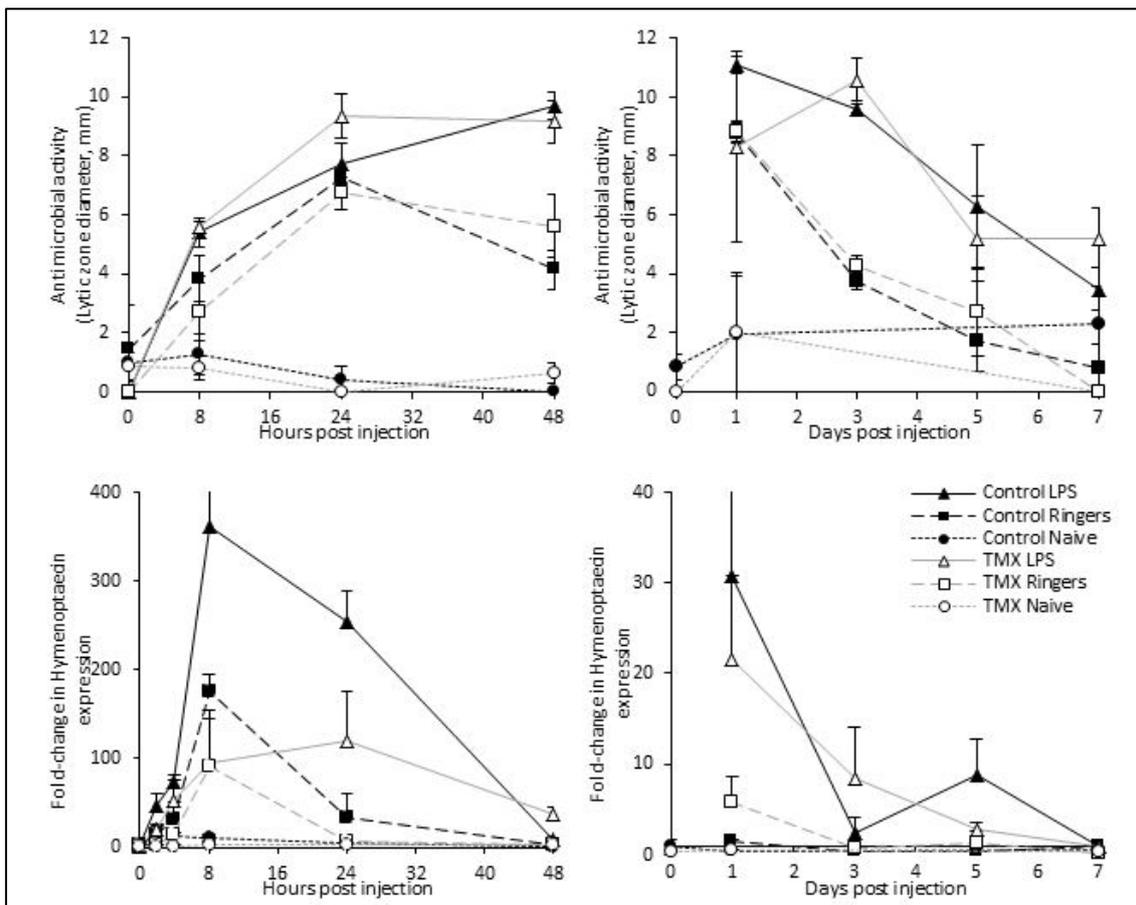
Antimicrobial activity at the protein/enzyme level typically succeeded upregulated gene expression following immune challenge. Specifically, a peak in activity was typically seen between 24 and 72 hours PIC, followed by a gradual decline over the days following this. At both the molecular and protein/enzyme levels Ringers injection induced a response compared with the naïve-control treatment and these responses were greater for LPS injection compared with the Ringers injection alone (Tukey's HSD tests:  $P \leq 0.05$ ).

Dietary pesticide affected AMP gene expression occasionally, and activity at the protein/enzyme level was apparently unaffected. Specifically, imidacloprid caused a reduction in abaecin titres, but only in naïve-control bees, and only in the short time course study (Experiment 4; Figure 3.2.; Pesticide\*Injection:  $F_{2,82}$

= 4.701,  $P = 0.012$ ). Dietary imidacloprid exposure also reduced defensin-1 titres over the short time course exposure (Experiment 4; Pesticide:  $F_{1,82} = 11.223$ ,  $P = 0.001$ ), but there were no detectable effects of imidacloprid on the expression of hymenoptaecin, apidaecin, defensin-2 or lysozyme-1 (see supporting information: Figures 3.5., 3.6.). In bees exposed to thiamethoxam, defensin-1 expression was suppressed (Experiment 4; Pesticide:  $F_{1,81} = 18.388$ ,  $P \leq 0.001$ ; supporting information: Figure 3.8.), whilst apidaecin expression was increased 8-24 hours PIC (Pesticide\*Time interaction  $F_{5,81} = 5.867$ ,  $P \leq 0.001$ ; supporting information: Figure 3.7.). This upregulation, however, was not seen at the equivalent 24 hour time point in the longer time course study (Experiment 5). Thiamethoxam exposure suppressed hymenoptaecin expression only in bees injected with Ringers and LPS, and within the first 24 hours post injection only (Pesticide\*Injection\*Time:  $F_{10,71} = 4.298$ ,  $P \leq 0.001$ ). Again this effect was not detectable at the equivalent 24 hour time point in the longer time course study (Figure 3.3.). The effects of thiamethoxam on abaecin expression were transient, and reduced titres in naïve bees only at the 4 hour sampling (Pesticide\*Injection\*Time:  $F_{10,71} = 2.664$ ,  $P = 0.008$ ).



*Figure 3.2.* Antimicrobial response to artificial immune challenge over time in control-fed (black) and imidacloprid-exposed (white/grey) bees. Top plots: Physiological AMP activity (as measured by a lytic clearance assay). Bottom plots: Expression of the AMP gene, abaecin. Note other AMP genes (see text 3.4.1.; supporting information) showed a similar temporal pattern. Plots on the left hand side show responses for a period of 2-48 hours post injection and plots on the right hand side for the responses of 1-7 days post injection. In all plots, symbols indicate sample means and error bars depict 1 SE.



*Figure 3.3.* Antimicrobial response to artificial immune challenge over time in control-fed (black) and thiamethoxam-exposed (white/grey) bees. Top plots: Physiological AMP activity (as measured by a lytic clearance assay). Bottom plots: Expression of the AMP gene, hymenoptaecin. Note other AMP genes (see text 3.4.1.; supporting information) showed a similar temporal pattern. Plots on the left hand side show responses for a period of 2-48 hours post injection and plots on the right hand side for the responses of 1-7 days post injection. In all plots, symbols indicate sample means and error bars depict 1 SE.

### *3.4.2. Phenoloxidase gene expression and enzymatic activity*

Total proPO/PO activity was higher than PO activity, confirming that the method of haemolymph collection itself had not lead to activation of all the proPO precursor enzymes. Immune challenge had no detectable effect on either AmPPO gene expression or total proPO/PO activity. However, LPS-injection increased PO activity whereas injection alone did not (Experiment 3; Injection:  $F_{2,53} = 5.308$   $P = 0.008$ ). The phenoloxidase cascade was largely unresponsive to treatments, and maintained a relatively stable level of activity throughout the experiments indicative of a constitutive role (Figure 3.4.). The exception was for total proPO/PO activity that was lower in imidacloprid-exposed bees (Pesticide:  $F_{1,53} = 5.527$ ,  $P = 0.022$ ).

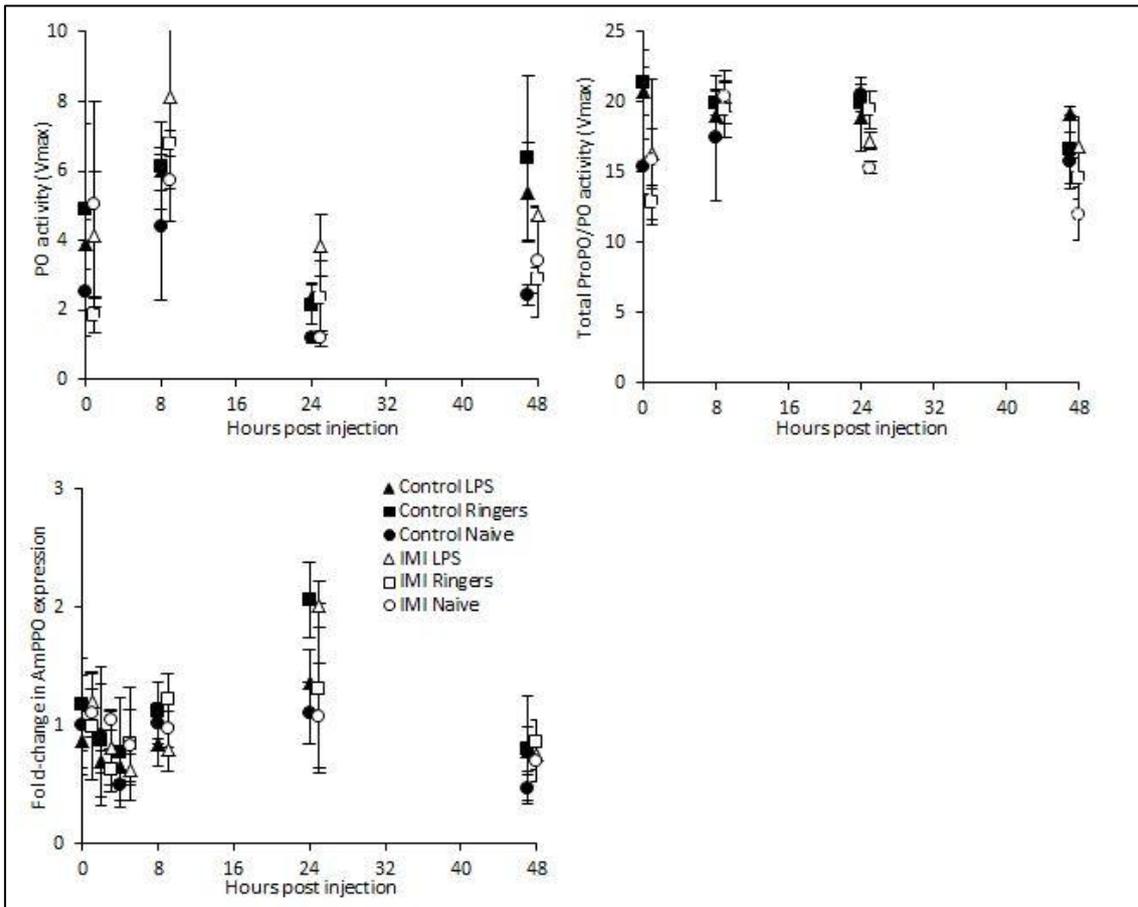


Figure 3.4. Phenoloxidase response to artificial immune challenge over time in control-fed (black) and imidacloprid-exposed (white) bees. Top plots: Physiological activity; Left- Active (functional) PO response, Right- Inactive (potential) proPO response. Bottom plot: Expression of the proPO gene, AmPPO.

### 3.4.3. Sucrose consumption

Consumption of imidacloprid-spiked sucrose was significantly lower than control sucrose, with the exception of the PO study in which no significant differences were observed. In general there were no significant differences between consumption by bees feeding on control and thiamethoxam-spiked sucrose. The exception to this was for bees in Experiment 4 where there was significantly reduced feeding in the thiamethoxam treatment (Wilcoxon Rank Sum:  $W = 1880$ ,  $P = 0.008$ ). Even when reduced feeding was observed in neonicotinoid-treated bees, bees ingested significant amounts of sucrose and consumed realistic doses of neonicotinoid (e.g. in Experiment 4, mean daily sucrose consumption in the four days prior to injection treatment for control-fed =  $46 \pm 3$  mg bee<sup>-1</sup>; for imidacloprid-fed =  $38 \pm 4$  mg bee<sup>-1</sup>; for thiamethoxam-fed bees =  $36 \pm 2$  mg bee<sup>-1</sup>). There was no evidence that injection treatment altered sucrose consumption.

### 3.5. Discussion

Four antimicrobial effector genes showed a clear upregulation in response to the artificial immune challenge. The lack of observed response of lysozyme-1 is consistent with most of the previous studies of bacterial infection in honey bees (e.g. Yang and Cox-Foster, 2005, Evans, 2006). This may reflect the proposition that lysozymes act by hydrolysing the peptidoglycan cell wall (Gillespie et al., 1997) and so are more effective against Gram-positive bacteria, which have a thicker peptidoglycan layer. Therefore, it is perhaps not surprising that lysozyme-1 was not found to be upregulated in response to the LPS molecules from Gram-negative *E.coli* in the present study. The response to immune challenge differed between the two defensin genes tested (defensin-1 and defensin-2), which is consistent with previous studies (Evans, 2006) and known differences in the regulation of the two genes (Klaudiny et al., 2005).

The transcriptional changes in AMP genes in response to immune challenge that occurred in our study have probable functional significance because they

were accompanied by increases in antimicrobial activity at the protein/enzyme level. Wounding by injection alone induced an antimicrobial response, albeit to a lesser extent than injection with bacterial LPS, which is consistent with previous studies (Evans et al., 2006, Yang and Cox-Foster, 2005, Laughton et al., 2011). This wound-response to injection is probably biologically relevant because, for example, parasitic flies cause similar puncture wounds by inserting their ovipositors into the honey bee abdomen (e.g. Core et al., 2012). It is likely this AMP response to wounding allows pre-emptive induction of the immune system because most natural cases of wounding will inevitably be non-sterile (Erler et al., 2011).

The phenoloxidase system was largely constitutive. Expression of the AmPPO gene was not altered following injection and this was supported by a lack of any change in the total proPO/PO activity. These results are comparable with previous studies, in which buffer or *E.coli* injection induced no transcriptional changes in AmPPO (Evans et al., 2006), and buffer or LPS injection induced no physiological changes in total proPO/PO activity in adult foragers (Laughton et al., 2011). In other studies, however, similar challenges have led to lower AmPPO expression (Yang and Cox-Foster, 2005) and, in newly emerged workers, lower total proPO/PO activity (Laughton et al., 2011). Here, PO activity was significantly higher following LPS injection, but our findings remain difficult to interpret as there was no clear temporal pattern in the phenoloxidase response (Figure 3.4.). Korner and Schmid-Hempel (2004) reported a significant effect of LPS injection on PO activity in worker bumble bees, but they too recognised that the temporal dynamics of the response were complex and difficult to interpret.

The effects of neonicotinoid exposure on transcriptional responses in the antimicrobial component of honey bee immunity were weak and inconsistent. Moreover, neonicotinoid exposure did not affect the physiological activity of the antimicrobial response, measured by a lytic clearance assay. This suggests that induced transcriptional changes in the individual AMP genes due to neonicotinoid exposure may not actually impinge on bee health. This is perhaps because the insecticide evokes balancing trade-offs in its effects on the

expression of multiple antimicrobial genes as, for example, when thiamethoxam was found to suppress hymenoptaecin expression but increase apidaecin expression. Where transcriptional changes were observed these differed between the two neonicotinoid compounds. Generally expression was altered in a greater number of genes tested following exposure to 10 ppb thiamethoxam than to the much higher exposure to 102 ppb imidacloprid, but even in the case of thiamethoxam the results were inconsistent. Nevertheless we highlight that effects may differ between compounds despite their similar chemical properties and mode of action as nicotinic acetylcholine receptor agonists. There has been recent interest to identify suitable molecular biomarkers for pesticide risk assessment on bees (EFSA, 2012). However, our findings do not support the use in risk assessment of the immune-related molecular biomarkers tested here as there is currently no evidence for the functional significance of pesticide-induced transcriptional changes in AMP genes, principally because we have shown that enzymatic/protein-level components of honey bee antimicrobial activity are resilient to neonicotinoid exposure. The patterns of gene expression, however, may be useful simply as indicators of neonicotinoid exposure.

Our results show that exposure to imidacloprid impaired the potential for immune response by honey bees, as measured by the precursor proPO. This may be important for honey bee immune functioning if exposure to imidacloprid disrupts the prophenoloxidase cascade, including the release of reactive intermediates and the melanisation response, for example impairing the ability to heal wounding sites and/or encapsulate bacterial and fungal spores. Nevertheless, levels of the active functional enzyme, PO, were not found to be affected by the exposure to imidacloprid. As in other studies (e.g. Laughton et al., 2011, Roberts and Hughes, 2014), levels of both proPO and PO were highly variable in time, which makes it difficult to interpret the biological significance of imidacloprid's effects on total proPO/PO activity given the apparent variable nature of the temporal response (Figure 3.4.). The overall effects of imidacloprid on measures of the prophenoloxidase cascade have been sometimes inconsistent among studies, but this may result in part from the use of different proPO/PO- related endpoints. For example, in a previous study (Gregorc et al.,

2012), transcription of the prophenoloxidase-activating gene (PPOact) was increased when honey bee larvae were exposed to imidacloprid, but at a physiological level, in contrast, PO activity was unaffected by imidacloprid exposure in adult workers (Alaux et al., 2010). Overall, it appears that phenoloxidase markers likely do not provide a reliable and informative measure of pesticide effects on honey bee immunity at this time due to the uncertainty in their interpretation and biological relevance to natural infections. For example, proPO activity has been observed to be negatively correlated with *Nosema* spore load, but showed no correlation with individual bee survival (Roberts and Hughes, 2014), and in a separate study imidacloprid exposure was found to reduce spore load despite no observed effect on PO activity (Alaux et al., 2010).

Our study begins to improve our understanding of the general relationships between transcriptional and physiological changes that underlie immune responses in honey bees. It is acknowledged that this work was conducted as a series of separate experiments on individual honey bee colonies and our findings must be interpreted in the context of this experimental setup. Our work begins to unravel the potential for differences in the response of different immune endpoints to pesticide exposure, but it is unclear from our findings whether this could result also from between-colony variation in pesticide sensitivity. Further work would benefit from studies designed to directly compare transcriptional and physiological changes of honey bees within a single colony, as well as to investigate these same measures in a number of colonies within and between seasons to increase the power of analyses to extrapolate findings to honey bee colonies as a whole. Further studies should establish also whether the apparent resilience of the immune response is observed using natural honey bee pathogens. For example, several AMP genes have been upregulated following infection with honey bee bacterial, fungal and microsporidian pathogens (Evans, 2004, Evans, 2006, Antunez et al., 2009), as well as in bumble bees infected with trypanosomal gut parasites (Riddell et al., 2009, Riddell et al., 2011). These may be suitable systems in which further investigations should measure both transcriptional and physiological antimicrobial responses, both in the absence and presence of pesticide

exposure, as it is only by understanding functional responses that we can truly assess impacts on bee health.

### *3.5.1. Conclusions*

Whilst these findings are yet to be confirmed in field colonies, we show no functionally significant impacts on honey bee immunity under laboratory conditions for neonicotinoid exposures that are likely to exceed most field relevant exposures. We are tentative in generalising our findings to bees under field conditions, however, because our experimental exposures were conducted for periods of up to 11 days whereas realistic exposures may be for longer periods. Furthermore we are tentative in generalising our findings to honey bee immunity as a whole, as we only tested a subset of endpoints and there are many other genes and enzymes, belonging to several pathways, in the honey bee immune system. In summary, our experiments showed an overall clear temporal pattern of AMP gene expression and physiological activity in response to a bacterial-like infection. This costly response could impact on bee health if it reduces the available resources for allocation to other needs. Transient transcriptional changes in AMP genes following neonicotinoid exposure were not found to be functionally significant, providing new evidence that this component of honey bee immunity may be more resilient to neonicotinoid exposure than previously thought based on molecular studies only. Responses to immune challenge were less marked in the PO system than those in the AMP system, which is likely to indicate a constitutive role of phenoloxidases in honey bee immunity. Exposure to imidacloprid impaired the potential for immune response by honey bees, as measured by the precursor proPO, whilst functional levels of active PO enzyme were apparently unaffected. The biological significance of imidacloprid's effects on total proPO/PO activity remains unclear given the apparent variable nature of the temporal response, but the investigation of neonicotinoid effects on the prophenoloxidase cascade warrants further work given its role in the encapsulation of pathogenic cells and melanisation of wounding sites.

### *3.5.2. Implications*

This new evidence is useful to regulators pursuing potential sublethal biomarkers for bee health. Based on our results, we argue that gene expression bioassays, for those genes studied here, may not be reliable indicators of pesticide effects on honey bee immune functioning at a physiological (and biologically relevant) level, although they may be useful in determining whether exposure has occurred.

### *3.6. Acknowledgements*

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### 3.8. Supporting information

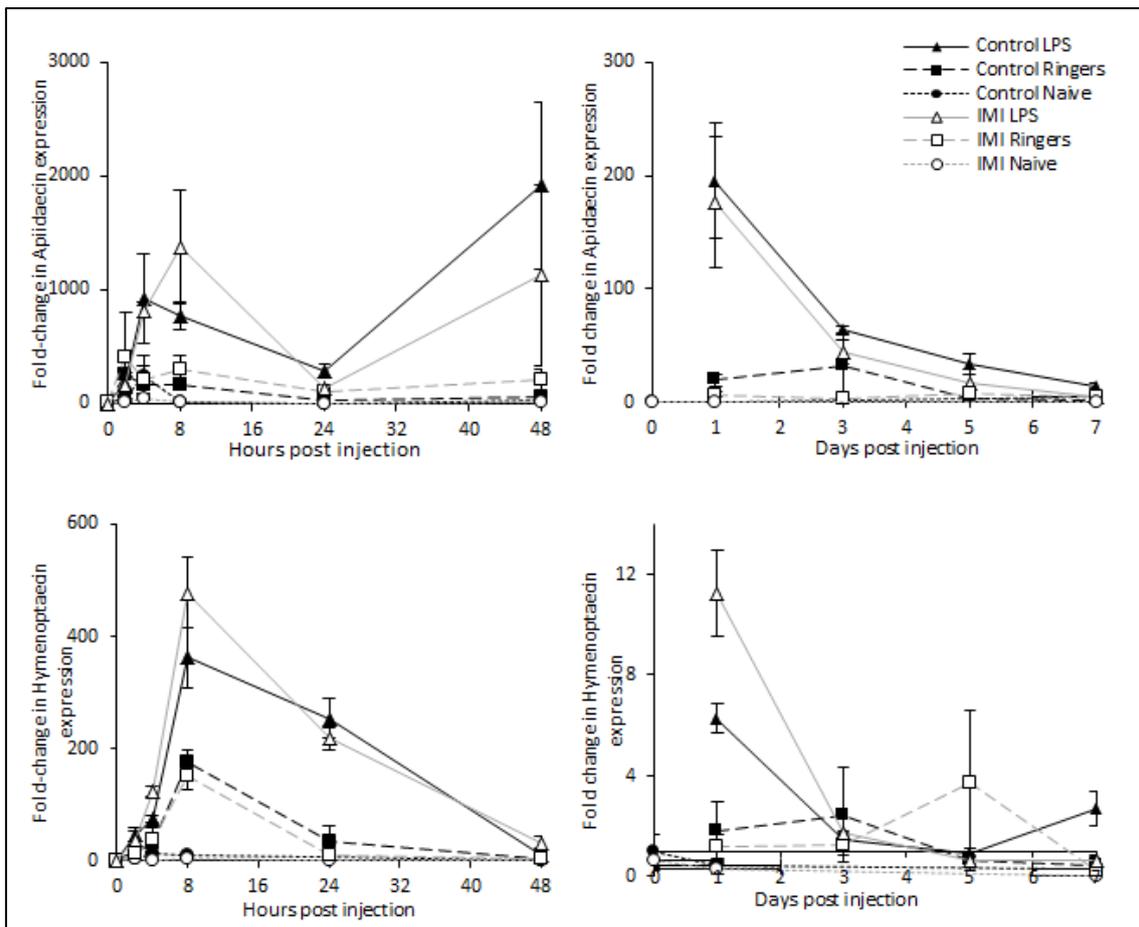
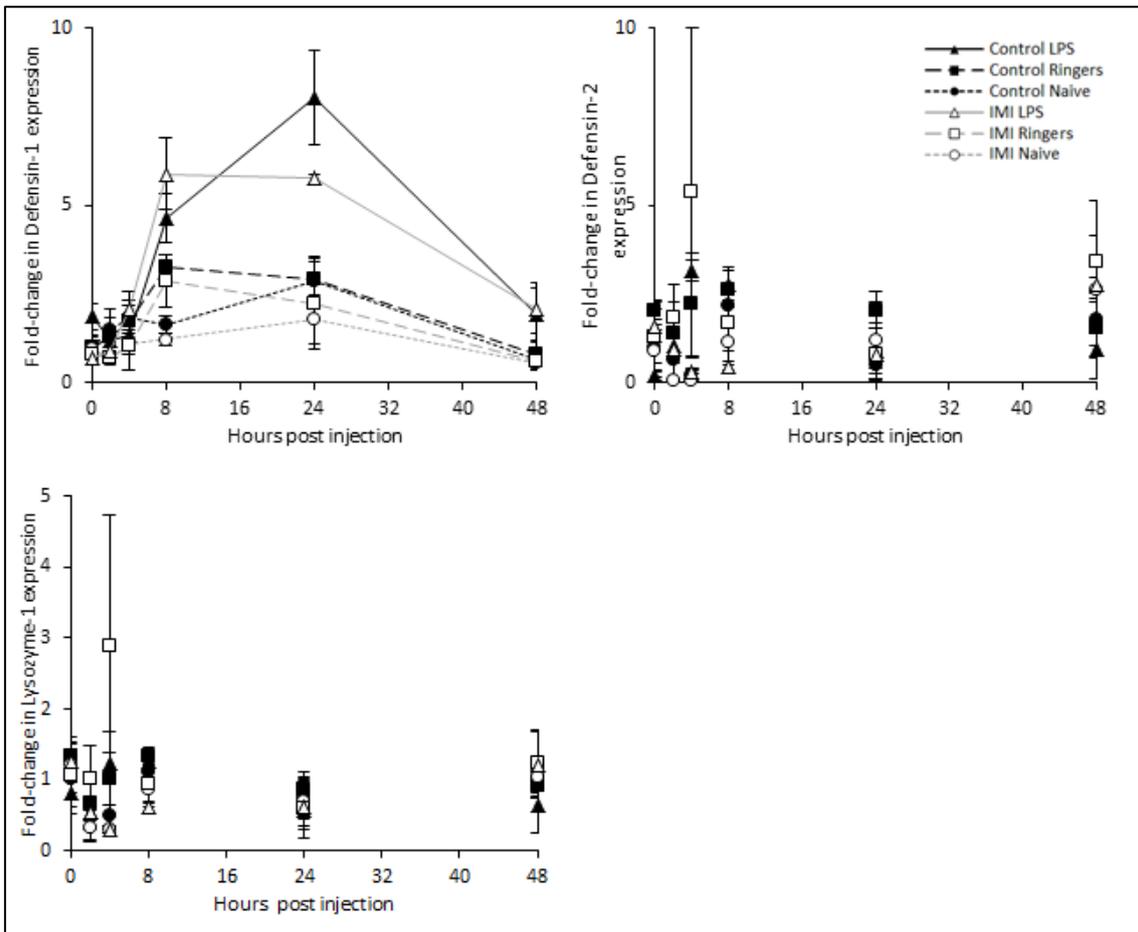


Figure 3.5. Antimicrobial response to artificial immune challenge over time in control-fed (black) and imidacloprid-exposed (white/grey) bees. Top plots: Expression of the AMP gene, apidaecin. Bottom plots: Expression of the AMP gene, hymenoptaecin. Plots on the left hand side show responses for a period of 2-48 hours post injection and plots on the right hand side for the responses of 1-7 days post injection. In all plots, symbols indicate sample means and error bars depict 1 SE.



*Figure 3.6.* Antimicrobial response to artificial immune challenge over time in control-fed (black) and imidacloprid-exposed (white/grey) bees. Clockwise from top-left plot: Expression of the AMP genes defensin-1, defensin-2 and lysozyme-1, for a period of 2-48 hours post injection. In all plots, symbols indicate sample means and error bars depict 1 SE.

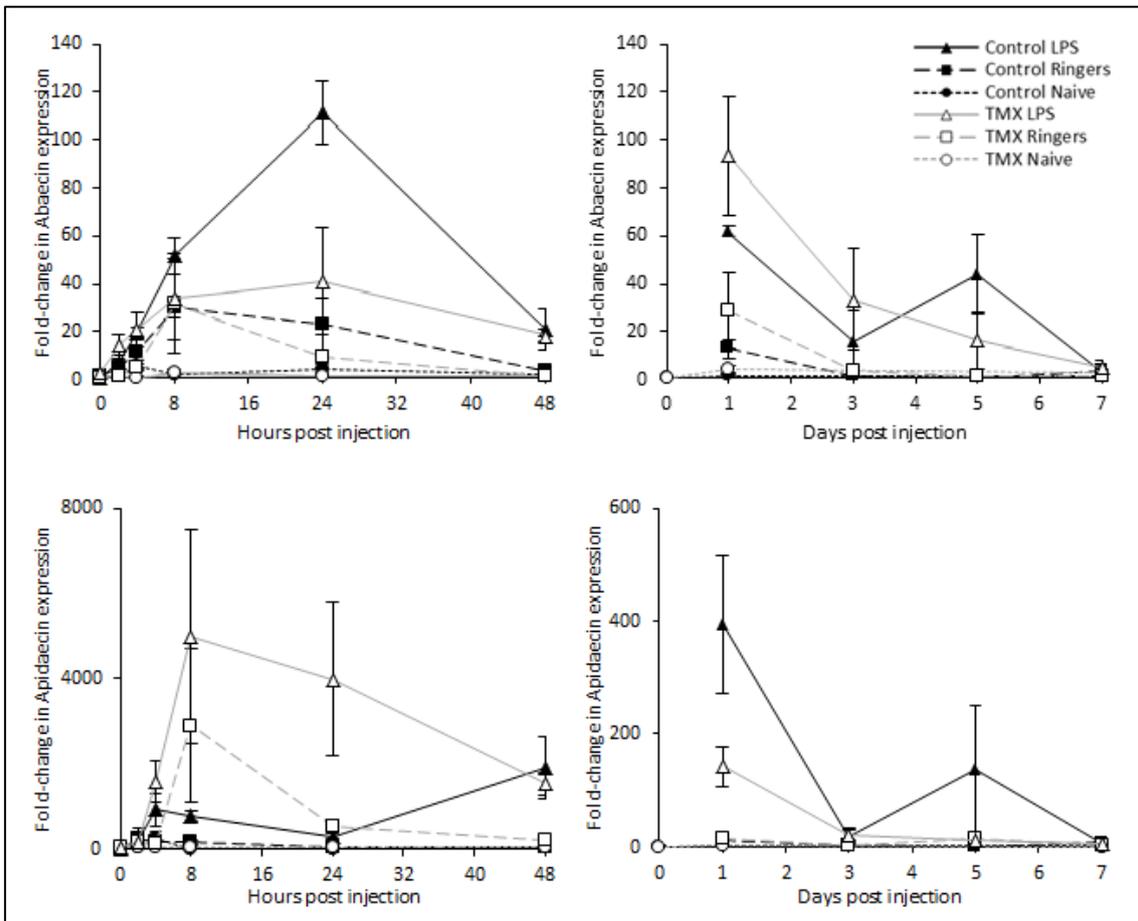


Figure 3.7. Antimicrobial response to artificial immune challenge over time in control-fed (black) and thiamethoxam-exposed (white/grey) bees. Top plots: Expression of the AMP gene, abaecin. Bottom plots: Expression of the AMP gene, apidaecin. Plots on the left hand side show responses for a period of 2-48 hours post injection and plots on the right hand side for the responses of 1-7 days post injection. In all plots, symbols indicate sample means and error bars depict 1 SE.

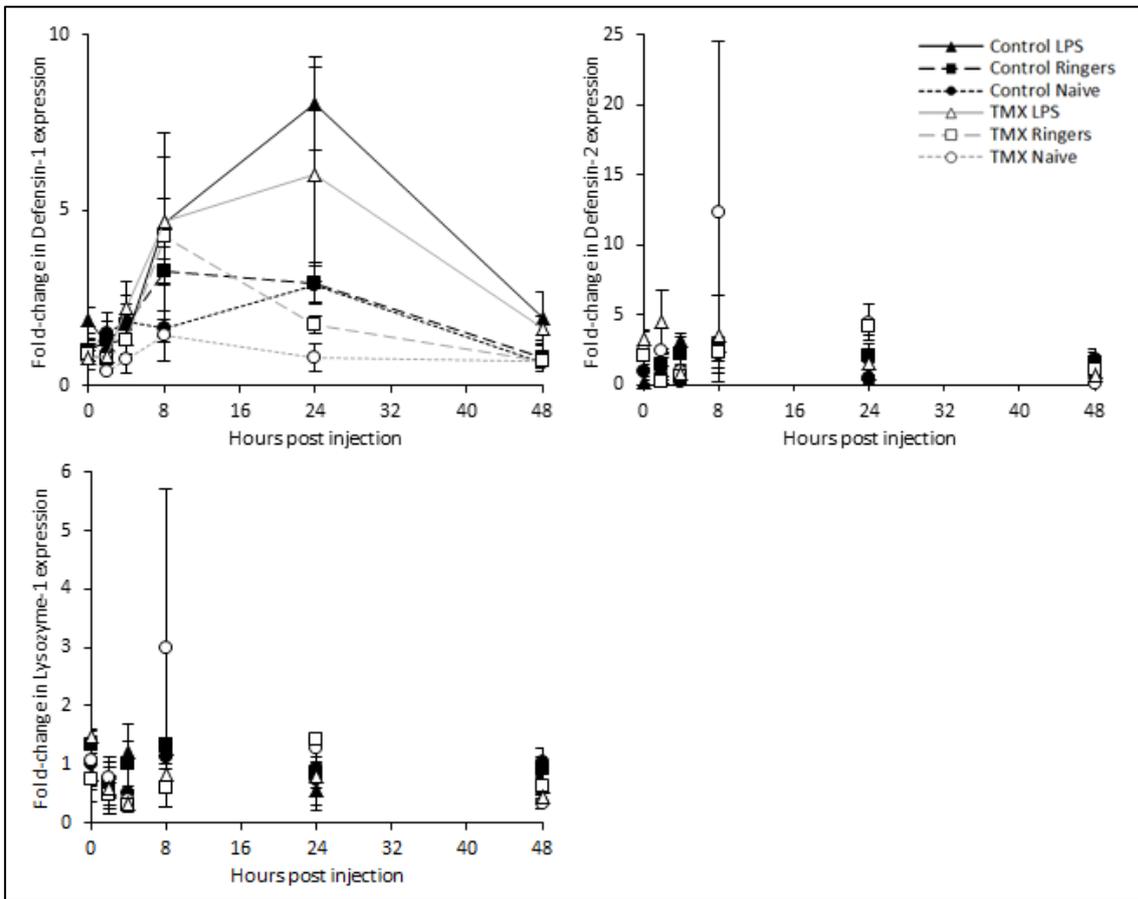


Figure 3.8. Antimicrobial response to artificial immune challenge over time in control-fed (black) and thiamethoxam-exposed (white/grey) bees. Clockwise from top-left plot: Expression of the AMP genes defensin-1, defensin-2 and lysozyme-1, for a period of 2-48 hours post injection. In all plots, symbols indicate sample means and error bars depict 1 SE.

## **4. The immune response of the bumble bee (*Bombus terrestris* L.) is resilient to dietary imidacloprid in field realistic exposures**

### *4.1. Abstract*

Multiple stressors, including pesticide application and the spread of pathogens and parasites, are likely contributing to declines in bee health. Recent recommendations for the risk assessment of plant protection products on bees have included the need for a better understanding of pesticide-disease interactions and the development of biomarkers to evaluate sublethal effects of pesticide exposure, such as immune system changes. Studies have begun to investigate the biological mechanisms underlying pesticide-disease interactions in honey bees, and there is some evidence that exposure to neonicotinoid pesticides impairs the honey bee immune response. However, there has been no investigation of neonicotinoid effects on the bumble bee immune response despite recognition that the sensitivity to neonicotinoids, and endpoints appropriate for risk assessment, could differ between bee species. We conducted a laboratory study to investigate the effects of a chronic exposure to a dietary neonicotinoid on the immune response of the bumble bee (*Bombus terrestris audax*). We tested whether imidacloprid exposure impaired the ability of workers to respond to an artificial immune challenge with bacterial lipopolysaccharides (LPS) by measuring two components of the insect immune response- phenoloxidase (PO) enzyme activity (associated with a melanisation response), and antimicrobial protein (AMP) activity. Neither immune challenge nor imidacloprid exposure was found to affect the apparently constitutive PO activity. Injection with LPS induced AMP activity and this was impaired in bees exposed to imidacloprid in sucrose solution at  $125 \mu\text{gL}^{-1}$ , but not at field relevant imidacloprid exposures. Our results begin to suggest that these two immune-related endpoints will have limited use in pesticide risk assessment in bumble bees, but further work should investigate whether pesticide exposures impair the immune response to natural pathogen infections.

#### 4.2. Introduction

Many wild plant communities and agricultural and horticultural crops rely on animal pollinators (Wilcock and Neiland, 2002, Kearns et al., 1998), including honey bees, bumble bees, stingless bees and solitary bees (Klein et al., 2007). In recent years, there has been increasing evidence of declines in populations of both managed honey bees (vanEngelsdorp et al., 2008) and wild bumble bees (Goulson et al., 2008, Williams and Osborne, 2009). Multiple stressors, including pesticide exposure and the spread of pathogens and parasites, are likely to be contributing to global declines in bee health (Potts et al., 2010). Bumble bees are susceptible to a wide range of pathogens and parasites, including microsporidia (Otti and Schmid-Hempel, 2007), viruses (Graystock et al., 2013), trypanosome protozoa (Shykoff and Schmid-Hempel, 1991), mites (Otterstatter and Whidden, 2004) and flies (Schmid-Hempel et al., 1990). The ability to mount an immune response against pathogen infection and parasite attack, or immunocompetence, is probably important to bee health (e.g. Brown et al., 2003), and to the sustainability of bee populations. Recent concerns have focussed on the impact of neonicotinoid pesticides on bumble bee health (e.g. Gill et al., 2012, Whitehorn et al., 2012, Laycock et al., 2012), which has contributed to the introduction of a two-year restriction on the use of three neonicotinoid pesticides by the European Commission (2013) until further research clarifies their effects on pollinators. Here, we tested the hypothesis that neonicotinoid exposure could harm bee health by affecting immunocompetence, which could increase the susceptibility of bees to infection and augment population declines.

Recent studies have found evidence that neonicotinoid exposure may interact with pathogen infection to reduce survival in worker honey bees (e.g. Vidau et al., 2011, Doublet et al., 2015, Retschnig et al., 2014), perhaps because neonicotinoids impair the honey bee immune response (e.g. Alaux et al., 2010, Di Prisco et al., 2013) and increase pathogen loads (e.g. Vidau et al., 2011, Pettis et al., 2012). However, sensitivity to neonicotinoids differs between honey bees (*Apis* spp.) and bumble bees (*Bombus* spp.) (Cresswell et al., 2012), and endpoints appropriate for pesticide risk assessment should therefore be

investigated carefully (European Food Safety Authority, EFSA, 2013, Thompson and Hunt, 1999). Despite this, only two studies to date have investigated neonicotinoid-pathogen interactions in bumble bees, and both were in relation to laboratory infection with the trypanosome gut parasite *Crithidia bombi*. Susceptibility of worker bumble bees to *C. bombi* and the intensity of *C. bombi* infection within individual bees were not affected by exposure to the neonicotinoids thiamethoxam and clothianidin (Baron et al., 2014, Fauser-Misslin et al., 2014). However, longevity of the foundress queen was reduced when bumble bee colonies were challenged with both neonicotinoid exposure and *C. bombi* infection (Fauser-Misslin et al., 2014). As yet, however, there has been no investigation of neonicotinoid effects on the bumble bee immune response at a molecular or physiological level, which could reveal the mechanistic basis of neonicotinoid effects on bumble bee health. Investigation of the molecular and physiological basis is timely, however, because the recent guidance document on the risk assessment of plant protection products on bees released by EFSA (2013) recommended the development of biomarkers to evaluate sublethal effects of pesticide exposure, such as immune system changes. We therefore report a laboratory study that was performed to investigate the effects of a chronic oral neonicotinoid exposure on the immune response of the bumble bee (*Bombus terrestris audax*).

The immune response in individual bumble bees is comprised primarily of cellular responses such as phagocytosis and encapsulation, and humoral responses *via* the phenoloxidase (PO) cascade (leading to melanisation) and antimicrobial protein (AMP) effectors (Hoffmann, 1995). Whilst bumble bees may also use some behavioural defences against parasitic attack (e.g. Hoffmann et al., 2008), they lack the complex social structure and many of the behavioural defences seen in honey bee colonies, so the innate immune response may be of greater importance to individual bumble bees compared to honey bees. Here, we tested whether imidacloprid exposure impaired the ability of workers to respond to an artificial immune challenge with *Escherichia coli* lipopolysaccharides (LPS) by measuring two components of the insect immune response: PO enzyme activity; and AMP activity. We used an artificial immune

challenge that mimics a bacterial-like infection, which allowed us to standardise the induction of an immune response across individual bees, avoiding the technical difficulties of controlling the titres of a live pathogen. In addition, it avoids the confounding responses of a live pathogen that may also respond to the neonicotinoid.

PO is present in insect haemolymph as an inactive precursor enzyme, prophenoloxidase (proPO), which can be activated to PO for subsequent production of melanin to isolate pathogens from access to host resources, as well as production of reactive intermediates toxic to pathogens (Wilson-Rich et al., 2009). We measured the total level of inactive proPO and active PO as an indicator of the potential immune response, and the level of active PO as an indicator of the functional PO present in the haemolymph at the given sampling time.

AMPs, including lysozymes, are typically synthesised by the fat body in response to detection of a pathogen within the insect haemolymph, although other tissues, including the Malpighian tubules and midgut may also play a role in AMP production (Gillespie et al., 1997). These AMPs may possess inhibitory properties against Gram-negative and Gram-positive bacteria and some fungi (Rees et al., 1997), and several bee AMP genes have been found also to be upregulated in response to trypanosome protozoa (Riddell et al., 2011) and microsporidian pathogens (Antunez et al., 2009).

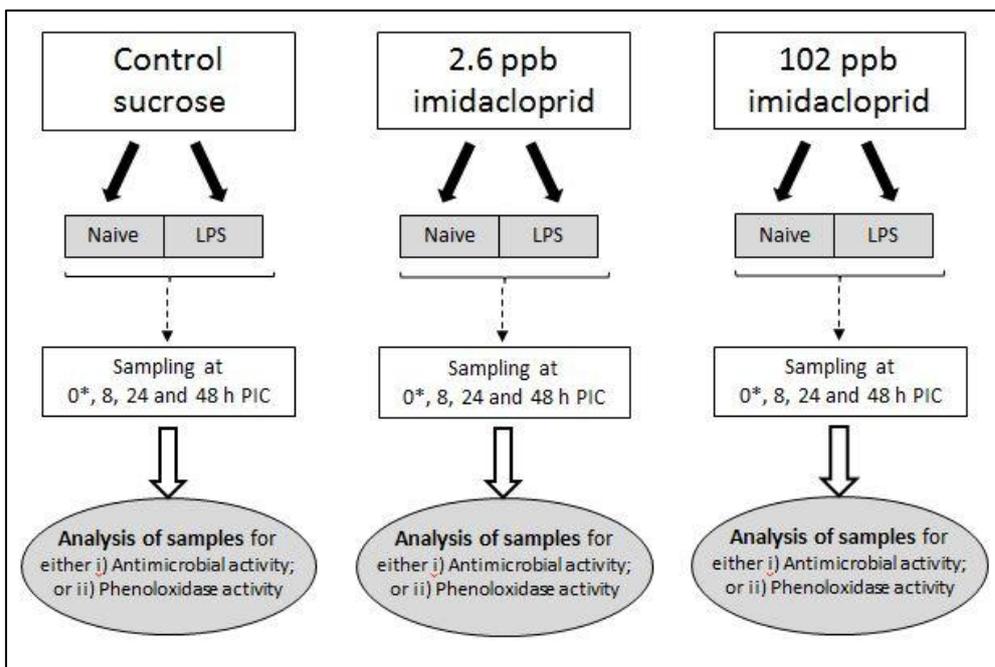
In summary, our study aimed to evaluate the effects of neonicotinoid exposure on bumble bee immunity, and to explore the potential value of two physiological immune endpoints for pesticide risk assessment in field-relevant scenarios.

#### *4.3. Materials and methods*

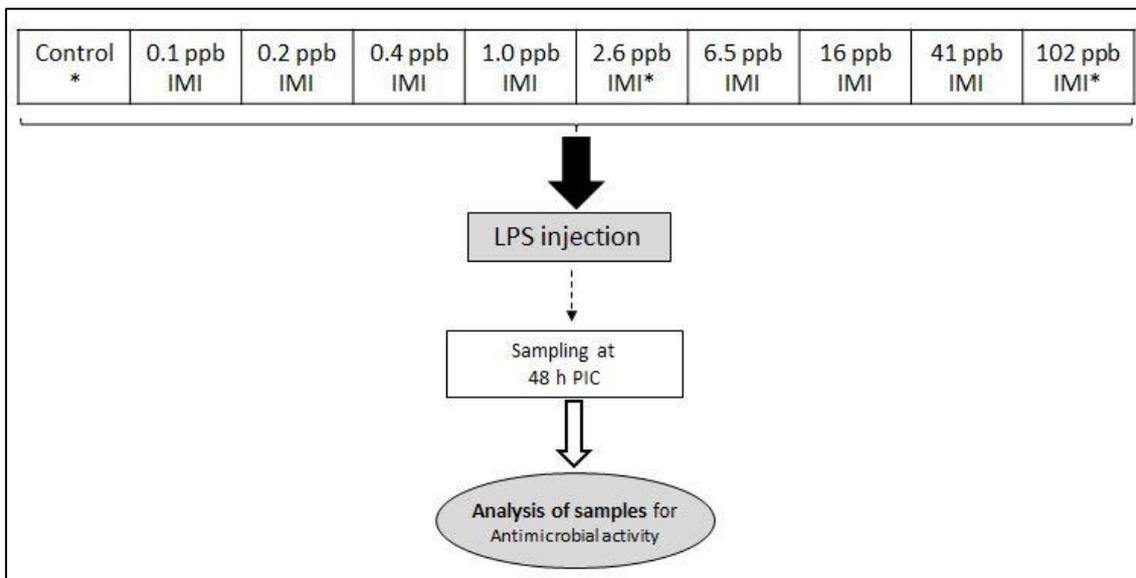
In two experiments, individually housed bumble bee workers were exposed to an imidacloprid-spiked sucrose feed or control sucrose for an initial period of four days. On day five, workers were injected with LPS as an artificial immune challenge, and then exposed continuously to the same imidacloprid or control

feeding treatment. Haemolymph samples were collected for measurement of immune responses at various intervals 'post immune challenge' (PIC) as described below.

In the 'time course' experiment, denoted 'TC', we characterized the development of the PIC immune response to select the optimum time for testing pesticide impacts. Specifically, we measured PO and AMP activity at intervals of 8, 24 and 48 hours (h) PIC after exposing bees to imidacloprid in dietary sucrose at either a 'low dose' of 2.6 parts per billion (ppb) or a 'high dose' of 102 ppb (Figure 4.1.). In the 'dose-response' experiment, denoted 'DR', we exposed bees to one of ten imidacloprid doses up to 102 ppb (see below), and subsequently measured AMP activity at 48 h PIC (Figure 4.2.).



*Figure 4.1.* Schematic of time course (TC) experimental setup. For each pesticide exposure (top white boxes), bees received one of two immune challenges (middle grey boxes). For each pesticide/immune combination, samples were collected across a short time course. \*Sampling at 0 h post immune challenge (PIC) was taken only from naïve samples. Samples were then analysed to measure one of two immune endpoints (bottom grey ellipses).



*Figure 4.2.* Schematic of dose-response (DR) experimental setup. For each pesticide exposure (top white boxes), bees received an immune challenge *via* injection with LPS molecules. \*Additionally in the control, 2.6 ppb and 102 ppb exposures, a manipulation-control group, denoted 'naïve', were ice-immobilised but received no further immune challenge. Samples were collected at 48 h post immune challenge (PIC) and subsequently analysed to measure antimicrobial activity.

#### 4.3.1. Bumble bee provenance and husbandry

Worker bumble bees (*Bombus terrestris audax*) were collected from domesticated colonies (Biobest Belgium N.V.) and housed in modified plastic cages (FK-RD8 clear PET containers; Ambican UK Ltd). Each cage allowed *ad libitum* access to a sucrose solution (50 % w/v) through a modified microcentrifuge tube (1.5 ml; Eppendorf UK Ltd) and was maintained in an environmental chamber throughout the experimental period (26 °C, 50 % relative humidity, constant darkness; Sanyo Versatile Environmental Test Chamber).

#### 4.3.2. Neonicotinoid exposure

Bees were exposed to an imidacloprid exposure between 0.1 ppb and 102 ppb in sucrose solution (50 % w/v). Treated sucrose was spiked with 0.08, 0.20, 0.51, 1.28, 3.20, 8.00, 20.00, 50.00 or 125.00  $\mu\text{g L}^{-1}$  imidacloprid (Fluka Analytical 37894) ( $\approx$  0.1, 0.2, 0.4, 1.0, 2.6, 6.5, 16.3, 40.7 and 101.7 ppb respectively). Treatment cages allowed *ad libitum* access to a single level of spiked sucrose throughout the experimental period, during which no other food source was available. Control bees received sucrose containing < 0.1 % acetone to control for the solvent in which original pesticide stocks were dissolved. The feeder of each cage was weighed every other day to allow an estimate of the mean daily sucrose consumption per bee to be calculated (accounting for any evaporation using feeders kept in empty cages).

#### 4.3.3. Immune challenge

Bumble bees were immobilised on ice for injection treatments, which were performed using a Hamilton syringe. Bumble bees were injected with 6  $\mu\text{l}$  of solution through the pleural membrane between the sternites (ventral side) of the abdomen. The 'LPS' treatment group were injected with 0.5  $\text{mgml}^{-1}$  LPS (Sigma L2755- Lipopolysaccharides from *Escherichia coli* 0128:B12) dissolved in Insect Ringers solution (1:1:1 156 mM NaCl : 3 mM KCl : 2 mM  $\text{CaCl}_2$ ). This LPS concentration has been widely used previously to elicit an immune response in bumble bees without any acute effects on survival (e.g. Korner and Schmid-Hempel, 2004, Moret and Schmid-Hempel, 2000). A manipulation-control group, denoted 'naïve', were ice-immobilised but received no further treatment.

In general < 5 % of bees died during the experimental period and these were distributed across imidacloprid and injection treatments, confirming that the pesticide concentrations and immune challenges used were sublethal to worker bumble bees.

#### 4.4.4. Measurement of PO activity

A 'perfusion-bleed' method was used to collect haemolymph samples from bees that had first been immobilised on ice. Specifically, a small slit was made in the

abdominal tergites using sterile dissecting scissors. A microsyringe was used to insert 300  $\mu\text{l}$  sodium cacodylate (NaCac) between the head and thorax and after this was flushed through the body it emerged through the slit in the abdomen, along with the haemolymph, and was collected in a microcentrifuge tube. Each sample was immediately snap frozen in liquid nitrogen to disrupt the haemocytes, releasing any proPO or PO enzymes present, and then stored at  $-20\text{ }^{\circ}\text{C}$  for later analysis.

Two spectrophotometry assays were used to measure: (i) PO activity; and (ii) total proPO and PO activity (based on Laughton and Siva-Jothy, 2011). Samples were centrifuged (9600 RPM, 10 minutes,  $4\text{ }^{\circ}\text{C}$ ) and kept on ice. For the PO assay, 5  $\mu\text{l}$  of phosphate buffer solution (PBS) were added to the bottom of each well in a 96-well plate. For the total proPO/PO assay, 5  $\mu\text{l}$  of  $\alpha$ -chymotrypsin ( $5\text{ mgml}^{-1}$ ; Sigma C4129) were added to the bottom of each well. 10  $\mu\text{l}$  of sample (supernatant) were added to each well, the plate was then shaken and left at room temperature for five minutes to allow the  $\alpha$ -chymotrypsin to activate any proPO in the sample. The plate was then placed back on ice and 175  $\mu\text{l}$  L-Dopa mastermix [containing 20  $\mu\text{l}$  L-Dopa solution (saturated  $11\text{ mgml}^{-1}$ , then filtered; Acros Organics 167530050, Fisher Scientific, UK), 20  $\mu\text{l}$  PBS, 135  $\mu\text{l}$  water] were added to each well. The plate was then shaken and the reaction allowed to proceed at  $25\text{ }^{\circ}\text{C}$  in a spectrophotometer (ThermoMax microplate reader, Molecular Devices). Absorbance was measured at 490 nm every 15 seconds (shaking between reads) for 1 hour 15 minutes (= 4500 seconds). A blank was used containing 10  $\mu\text{l}$  NaCac in place of sample, but otherwise contained the same reaction mixture. Each sample was measured in duplicate for each assay and treatments were assigned across plates to minimise between plate variation. Softmax Pro v4.3 software (Molecular Devices, UK) was used to calculate enzyme activity measured as the maximum linear rate of substrate conversion ( $V_{\text{max}}$ ), using 100  $V_{\text{max}}$  points, across the 4500 s reaction period.

#### *4.4.5. Measurement of AMP activity*

Haemolymph was collected from bees that had first been immobilised on ice. A small slit was made in the abdominal sternites using sterile dissecting scissors and gentle pressure to the thorax resulted in a bubble of haemolymph exiting this slit. 2-10  $\mu$ l of this neat haemolymph were collected with a pipette and immediately stored at -20 °C for later analysis.

AMP activity was measured using a bacterial inhibition assay (based on Korner and Schmid-Hempel, 2004). Here, antimicrobial proteins in the haemolymph inhibit the growth of the test bacteria, producing a clear zone in the bacterial culture that can be measured as an indicator of the relative antimicrobial activity of each sample (the greater the zone diameter the greater the antimicrobial activity). A fresh culture of *Micrococcus luteus* Schroeter was grown on Columbia Blood Agar. A single pure colony of *M.luteus* was placed into 10 ml nutrient broth and incubated overnight at 27 °C. 100  $\mu$ l of this overnight culture were used to inoculate 100 ml sterile broth medium (containing 1 % agar). 6 ml of inoculated medium were immediately poured into a 9 cm-diameter petri dish and left to set. Ten wells (approximately 2 mm diameter) were bored on each plate. Neat haemolymph samples were thawed on ice and a 2  $\mu$ l sample was added to each well. Plates were incubated at 27 °C for 24 h, and the diameter of the zones of inhibition then measured using digital callipers.

#### 4.4.6. Data analysis

In the DR experiment, the effect of imidacloprid was non-monotonic with dose so dosages were treated as levels of a categorical variable and effects were therefore tested using one-way ANOVA. Where ANOVA results were significant, pairwise differences between treatments were tested by Tukey's HSD.

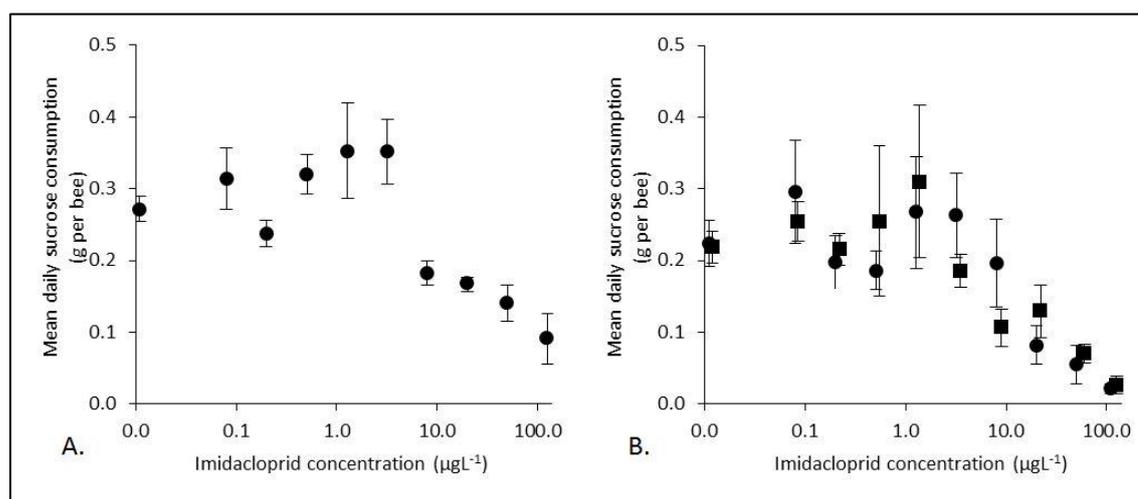
In the TC experiment, data did not meet the assumptions for parametric testing, so effects of injection, pesticide and time were tested using a Monte Carlo ANOVA implemented in R (R Core Team, 2013). Specifically, the data were pooled, randomly resampled and allocated among the treatments to recreate the dimensions of the original dataset, and then analysed by conventional factorial ANOVA with LPS challenge, neonicotinoid diet and duration of

exposure as fixed factors. For each randomization, the F-statistics were stored and 10,000 iterations of the resampling-ANOVA procedure were performed to produce the sampling distributions of the test statistics under the null hypotheses. These sampling distributions were then used to determine P values for the factor effects present in the observed data sets.

#### 4.4. Results

##### 4.4.1. Treatment effects on feeding rates

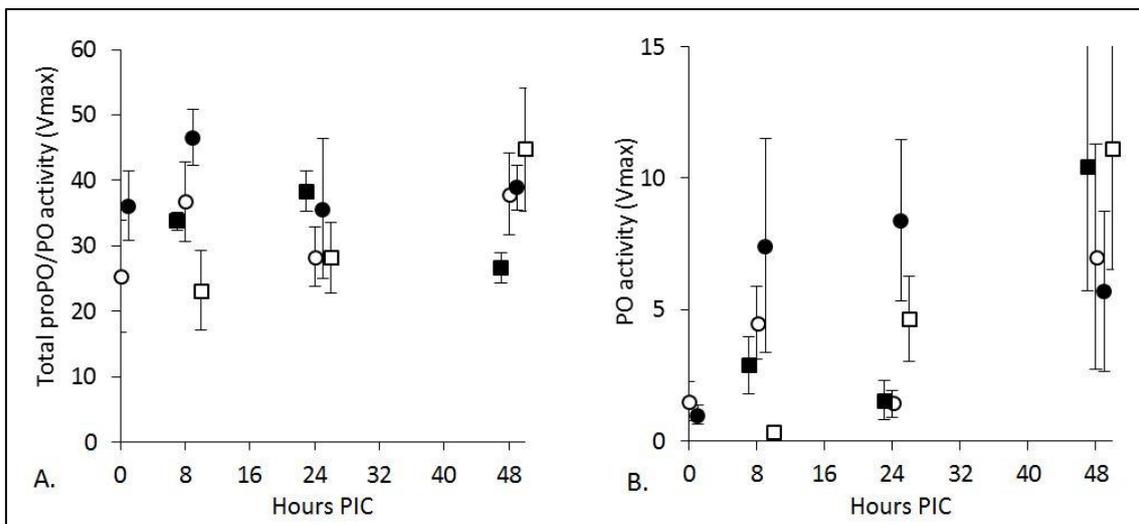
Dietary imidacloprid affected the bees' consumption of feeder sucrose (Expt TC One-way ANOVA:  $F_{2,193} = 42.3$ ,  $P \leq 0.001$ ; Expt DR Two-way ANOVA:  $F_{9,90} = 7.24$ ,  $P \leq 0.001$ ), but only at imidacloprid concentrations  $\geq 6.5$  ppb (Figure 4.3.). Injection treatment had no observable effect on feeding rate in the 48 h PIC (Figure 4.3.B). Despite the reduced feeding with increased imidacloprid concentration, bees exposed to the higher imidacloprid concentrations ingested larger amounts of imidacloprid (e.g. mean imidacloprid intake at 2.6 ppb =  $0.34 \text{ ng bee}^{-1} \text{ day}^{-1}$ ; at 102 ppb =  $4.1 \text{ ng bee}^{-1} \text{ day}^{-1}$ ).



*Figure 4.3.* Effects of dietary imidacloprid (x-axis,  $\mu\text{gL}^{-1}$ ) on daily consumption of feeder sucrose (y-axis,  $\text{g bee}^{-1} \text{ day}^{-1}$ ). A. Daily sucrose consumption for the four days prior to injection treatment in Expt TC. B. Daily sucrose consumption for the 48 h PIC in Expt TC. In both panels, symbols indicate sample means and error bars depict 1 SE. Some data points are adjusted slightly on the x-axis to reveal error bars (for exact imidacloprid concentrations see text). Squares indicate LPS-injected bees and circles indicate naïve manipulation controls.

#### 4.4.2. Time course of immune response to the simulated pathogen infection (TC experiment)

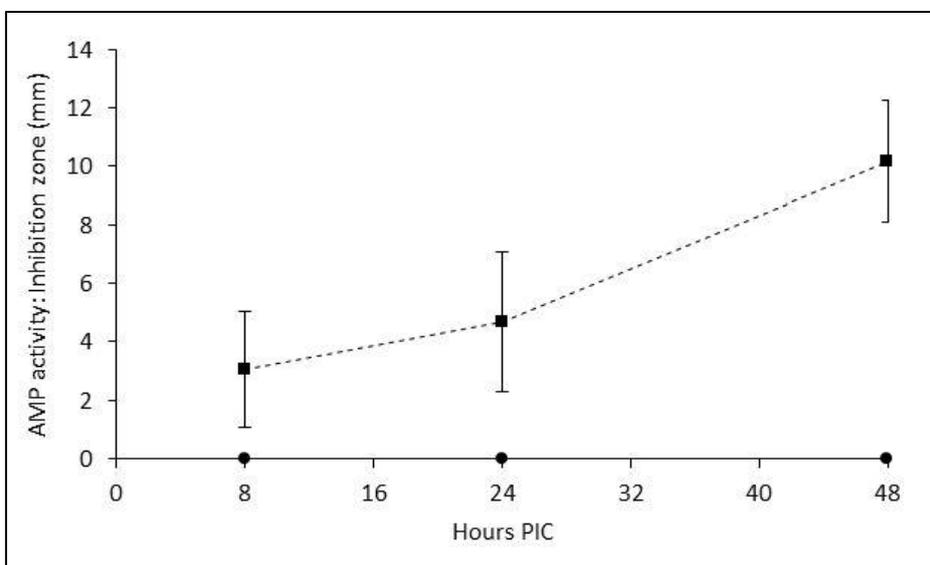
Neither injection nor dietary imidacloprid affected immune potential, measured by total proPO/PO activity, or functional PO activity within the timescale tested (Figure 4.4.; total proPO/PO ANOVA Injection  $F_{1,89} = 1.46$ , Imidacloprid  $F_{2,89} = 1.37$ ; PO Monte Carlo ANOVA Injection  $F_{1,89} = 0.081$ , Imidacloprid  $F_{2,89} = 0.009$ , P values > 0.05).



*Figure 4.4.* Measures of phenoloxidase activity (y-axis,  $V_{max}$ ) over time (x-axis, h PIC) in individual bumble bee workers. A. Total proPO/PO activity ('immune potential'); and B. Functional PO activity. Squares indicate LPS-injected bees and circles indicate naïve manipulation controls. Only data for control sucrose (open symbols) and 102 ppb imidacloprid (closed symbols) presented.  $n = 4$  to 6 bees per treatment/time point combination. In both panels, symbols indicate sample means and error bars depict 1 SE. Some data points are adjusted slightly on the x-axis to reveal error bars (for exact times PIC see text).

Across the 8 - 24 h PIC, immune challenge induced AMP activity, with a mean increase in the LPS-injected bees of 6.1 mm (SE = 1.4, n = 20). Activity generally increased with time PIC (Figure 4.5.; Linear regression,  $F_{1,21} = 5.83$ ,  $P = 0.025$ ,  $R^2 = 0.217$ ).

In the TC experiment, dietary imidacloprid had no significantly detectable effect on AMP activity (Monte Carlo ANOVA  $F_{2,60} = 0.045$ ,  $P > 0.05$ ), but this is probably because sample sizes were small. However, inspection suggests that bees exposed to the highest dose of imidacloprid ( $125 \mu\text{gL}^{-1}$ ;  $\approx 102 \text{ ppb}$ ) may have showed a weaker AMP activity in response to LPS challenge. i.e. at 48 h PIC mean zones of inhibition were 4.7 mm (SE = 2.4, n = 3) in bees fed  $125 \mu\text{gL}^{-1}$  imidacloprid vs. 12.5 mm (SE = 3.2, n = 3) in bees fed control sucrose.



*Figure 4.5.* AMP activity (y-axis, inhibition zone) over time (x-axis, h PIC) in individual bumble bee workers. Squares indicate LPS-injected bees and circles indicate naïve manipulation controls (naïve individuals showed no AMP activity in all cases). Symbols show mean activity in individual bees pooled from all three imidacloprid treatments and error bars depict 1 SE. n = 7 or 8 bees per injection/time combination.

#### 4.4.3. Dose response effects (DR experiment)

Based on TC (above), we selected 48 h PIC to investigate pesticide effects on AMP activity.

Dietary imidacloprid affected AMP activity in LPS-challenged bumble bees (One-way ANOVA:  $F_{10,50} = 4.04$ ,  $P \leq 0.001$ ; Figure 4.6.). However, the effects of dosage were not strictly monotonic (Figure 4.6.), although bees exposed to the highest dose of imidacloprid produced the lowest AMP activity PIC. Specifically, *post hoc* testing found significant pairwise differences in AMP activity only between  $0.08 \mu\text{gL}^{-1}$  vs.  $125 \mu\text{gL}^{-1}$  and  $0.08 \mu\text{gL}^{-1}$  vs.  $3.20 \mu\text{gL}^{-1}$ , and there were no significant pairwise differences between control-fed and imidacloprid-fed bees.

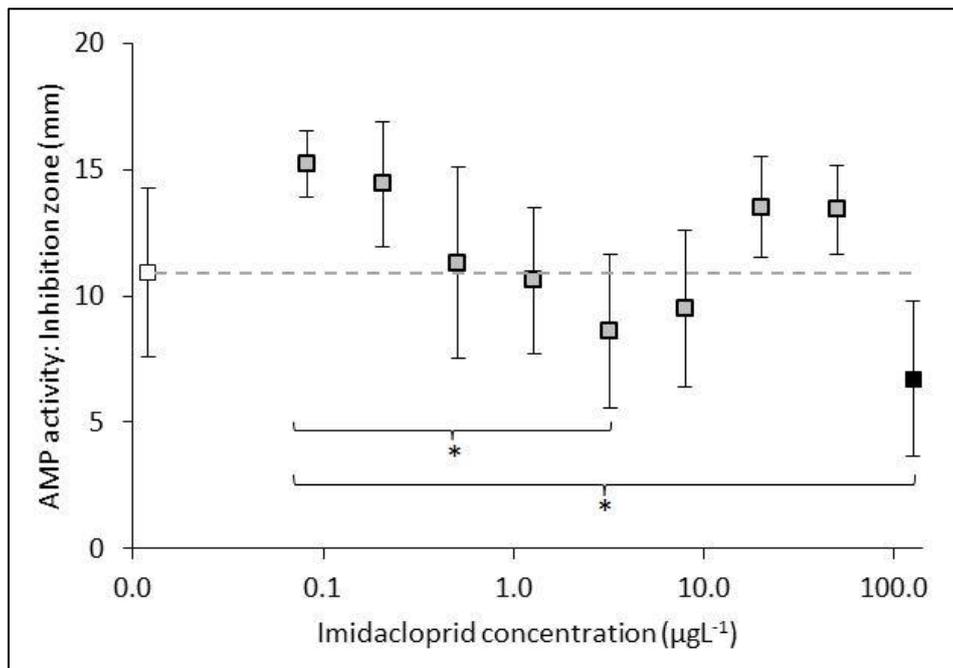


Figure 4.6. Effects of dietary imidacloprid (x-axis,  $\mu\text{gL}^{-1}$ ) on AMP activity (y-axis, inhibition zone) in individual bumble bee workers 48 h PIC following LPS injection. Data for naïve manipulation controls are not shown, as typically no AMP activity was detectable. Symbols indicate sample means and error bars depict 1 SE.  $n = 5$  bees per treatment. Some data points are adjusted slightly on the x-axis to reveal error bars (for exact imidacloprid concentrations see text). Open symbol indicates control sucrose and black closed symbol indicates 102 ppb). Dashed horizontal line indicates mean for control sucrose. \*Asterisks show *post hoc* testing significant pairwise differences in AMP activity between  $0.08 \mu\text{gL}^{-1}$  vs.  $125 \mu\text{gL}^{-1}$  and  $0.08 \mu\text{gL}^{-1}$  vs.  $3.20 \mu\text{gL}^{-1}$ .

#### 4.5. Discussion

Our results show that bumble bee immunocompetence is resilient to an exposure of dietary imidacloprid at concentrations up to 102 ppb even though individual bees exhibited toxic responses in the form of reduced feeding at dosages as low as 6.5 ppb, which is consistent with previous studies (Cresswell et al., 2012, Laycock et al., 2012). Our attribution of resilience is based on the following two observations.

First, our results suggest that the phenoloxidase system provides constitutive immunity and that its status is resilient to imidacloprid exposures as high as 102 ppb. Specifically, following an artificial immune challenge with bacterial LPS we found no increase in proPO or PO activity compared to baseline levels. Furthermore, we found no effect of imidacloprid exposure on total proPO/PO or PO activity whether the bees were challenged by LPS injection or not. These findings for the PO system are similar to a previous study of honey bees, in which a chronic exposure to imidacloprid had no effect on PO activity (Alaux et al., 2010). Although we found that the PO response was not inducible, previous studies have observed also that responses of the bee PO system to LPS injections are variable over time and difficult to interpret (e.g. Korner and Schmid-Hempel, 2004, Laughton et al., 2011).

Second, we show that AMP induction in bumble bees is resilient to field relevant imidacloprid exposures. In fact, we found no significant differences in AMP activity between control-fed and imidacloprid-fed bumble bees up to 102 ppb. Although residues as high as 101 ppb have been found in pollen from transplant-dripped neonicotinoid-treated pumpkin crops (Dively and Kamel, 2012), residues found in nectar and pollen in the field are typically much lower (1-10 ppb) (Blacquiere et al., 2012, Pohorecka et al., 2012). We found some evidence that imidacloprid exposure alters AMP activity, as AMP levels were found to be lower in bees exposed to 2.6 ppb and 102 ppb imidacloprid compared to 0.1 ppb imidacloprid in the DR experiment. In both experiments bees exposed to the highest dose of imidacloprid (102 ppb) produced the lowest AMP activity PIC, but the overall effects of dosage were not strictly monotonic. Our two experiments investigated effects on immunocompetence in individual bumble bees each from a single colony, using relatively small sample sizes. The experimental setup likely limits the power of our analyses to detect differences between treatment groups and our findings must be interpreted with caution when generalising to all bumble bee colonies in the field. Further studies are needed to better elucidate the potential effects on AMP activity observed at 2.6 and 102 ppb imidacloprid. Nevertheless, the weakened responses observed following exposure to 2.6 and 102 ppb imidacloprid offer

the first evidence in any insect species showing that an oral pesticide exposure may be capable of impairing the AMP component of the immune response. Based on our experiment, we can evaluate potential mechanisms underlying this apparent toxic effect as follows. Induction of an immune response is energetically costly (Moret and Schmid-Hempel, 2000) and dietary imidacloprid may have limited the resources available to induce the AMP system because it also reduced the feeding rates of individual bees. However, at imidacloprid concentrations between 6.5 and 41 ppb AMP levels were apparently sustained despite impaired feeding rates. Furthermore, there was no apparent increase in feeding rates in immune challenged bees to compensate for higher energy demands. We therefore conclude that limited energy resources alone do not appear to account for the variation in AMP induction. Instead, there may be trade-offs in the allocation of resources for pesticide detoxification and the AMP immune response when bumble bees are simultaneously exposed to a pesticide and pathogen infection. For example, exposure to imidacloprid degenerates the Malpighian tubules in honey bees (Rossi et al., 2013), and if this occurs also in bumble bees it could compromise AMP production. Other sites for potential trade-offs include the fat body, haemocytes and midgut, since these are all known to be involved in both AMP synthesis (Gillespie et al., 1997) and pesticide detoxification in honey bees (e.g. Mao et al., 2011, Hodgson, 1983).

#### *4.5.1. Evaluation of the threat posed by dietary neonicotinoids to bee health*

Our findings begin to suggest that bumble bee immunocompetence is resilient to dietary neonicotinoids at field-relevant exposures, although there are several limitations to our study. First, we conducted imidacloprid exposures for periods of only 6 days, but bees may be exposed for longer periods in the wild. For example, oilseed rape (*Brassica napus* L.) blooms for several weeks (Pernal and Currie, 1998) so bumble bees foraging on treated crops could be exposed throughout their foraging life of 2 - 3 weeks (Rodd et al., 1980). Second, our study only measured AMP activity up to 48 h PIC, but the bumble bee AMP

response to LPS injection has been observed up to 14 days later (Korner and Schmid-Hempel, 2004). Consequently, it is possible that the resilience declines with time. Third, we did not measure colony-level responses, such as pathogen levels and the development of new bees and reproductive individuals, which are critical to population sustainability. Furthermore, it is acknowledged that our study investigated effects in individually housed bees in order to ensure each test individual was exposed to the imidacloprid-spiked sucrose, but in a colony setting bees will inevitably be in contact with other colony members. PO and AMP immune responses have been found to be dependent on the social context of bumble bees (Ruiz-Gonzalez et al., 2009) and therefore further understanding at the colony-level is important to better understand realistic scenarios of pathogen and pesticide exposure. In line with this, our laboratory study did not offer access to pollen feeding, but bumble bees deprived of a protein source have previously shown altered immune responses to infection (Brunner et al., 2014). It remains unclear whether the apparent resilience of the bumble bee immune response to dietary neonicotinoids can be generalised to both pollen-fed and socially-grouped bumble bees.

The biological relevance of the PO and AMP responses to natural bumble bee infections needs further investigation. For example, here we cannot verify whether the AMP response was induced directly by the bacterial LPS molecules or wounding during injection. The increase in AMP activity relative to naïve manipulation controls is likely, however, a response to a combination of the wounding *and* LPS, as previous studies in bumble bees and honey bees have demonstrated induced AMP activity in response to placebo injection, with a further elevated response to LPS (e.g. Korner and Schmid-Hempel, 2004). Here, a wounding-like response may have some biological relevance as wounding is likely to occur when bees are parasitised by mites and flies, which are both common parasites of bumble bees (Kissinger et al., 2011, Shykoff and Schmid-Hempel, 1991). Furthermore, the AMP response is probably a fundamental defence against a wide range of pathogens. There is molecular evidence that the AMP response is induced in bumble bees infected with the trypanosome gut parasite *C. bombi* (Riddell et al., 2011), although no induction has been

detected at the protein level (Brown et al., 2003). Similarly, there is molecular evidence for AMP induction in honey bees infected with natural bacterial, fungal and microsporidian pathogens (Evans, 2004, Evans, 2006, Antunez et al., 2009). Further work should establish whether realistic pesticide exposures impair the immune response to natural pathogen infections and evaluate interactions between pesticides and pathogens using endpoints relevant to population dynamics.

#### *4.5.2. Implications for regulatory risk assessment*

Overall, our data gives little support for the use of PO and AMP immune endpoints in individual bees as a basis for the risk assessment of sublethal effects of pesticides on bumble bees. PO activity appears to be a constitutive system for defending against infection that is unresponsive to dietary imidacloprid in field-relevant exposures. By contrast, AMP activity was clearly induced by a simulated pathogen attack, but it too was generally unaffected by field relevant exposures. Our observations of the resilience of the systems that underpin bumble bee immunocompetence under field-relevant exposures provisionally provide no support for the use of immune endpoints in pesticide risk assessment, at least when measured by physiological titres as biomarkers. However, our results do not rule out the possibility that pesticide-pathogen interactions may impact detrimentally on bumble bees when the effects are measured in other ways, such as by colony performance or on other ecologically relevant endpoints.

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## **5. Disruption of hypopharyngeal gland function in honey bees exposed to a dietary neonicotinoid (imidacloprid)**

### *5.1. Abstract*

The neonicotinoid imidacloprid impairs development of the honey bee (*Apis mellifera*) hypopharyngeal gland (HPG), but whether HPG function is correspondingly disrupted has been untested, which undermines the ecological relevance of HPG-related assays in pesticide risk assessment. To determine whether imidacloprid indeed disrupts HPG function, we therefore implemented laboratory and field studies, using enzymatic, molecular and behavioural endpoints. Specifically, we investigated whether dietary exposure affected temporal polyethism, and the enzyme systems that support larval nutrition (major royal jelly proteins, or MRJPs) and social immunity (glucose oxidase, or GOX). Dietary imidacloprid induced the upregulation of foraging genes, and we hypothesised a predisposition to precocious foraging. However, using Radio Frequency Identification (RFID) technology to track hive traffic in free-flying bees, we found no corresponding behavioural consequences at field-relevant exposures. We demonstrate for the first time that chronic imidacloprid exposure reduced the expression of genes for MRJPs, which could impact on the nutrition of larvae and queens. We found that GOX is a constitutive enzyme system but that imidacloprid exposure lowered levels of GOX in immune-challenged bees, which implicates disruption to the social immune response. Overall, we detected generalised disruption of HPG function following exposure to imidacloprid and we believe this provides new support for the ecological relevance of HPG-related assays in pesticide risk assessment. Some disruption (temporal polyethism) was not evidently ecologically consequential, highlighting the need to cross reference gene expression bioassays with ecologically relevant behaviour. Disruption to other functions (namely nutrition and social immunity) is likely to be important and requires further field testing.

## 5.2. Introduction

Declines in honey bee (*Apis mellifera* L.) health have been a focus of recent scientific and public concern due to the potential impact on the pollination of crops and wild flowering plants (Potts et al., 2010). Multiple stressors are likely contributing to declines in honey bee health, including habitat loss, pathogenic infection and pesticide application (Potts et al., 2010). One class of pesticides, the neonicotinoids, has been of particular concern due to the potential exposure of bees through residues in nectar and pollen of mass-flowering crops (e.g. Stoner and Eitzer, 2012). Several studies have reported sublethal effects of neonicotinoid exposure on honey bee physiology and behaviour, including a weakened immune response (Alaux et al., 2010), impaired learning ability (Decourtye et al., 2004) and decreased foraging success (Henry et al., 2012). Additionally, several studies have found that oral exposure to sublethal doses of a neonicotinoid pesticide, imidacloprid, reduced the size of honey bee hypopharyngeal glands (HPGs) (Heylen et al., 2011, Skerl and Gregorc, 2010, Hatjina et al., 2013). Disruption to the HPGs could have profound consequences for individual and colony function if these imidacloprid-induced changes alter normal HPG functioning, but these possible consequences have yet to be investigated. Here we consider three aspects of honey bee biology that are associated with HPG activity, and aim to address whether imidacloprid exposure impairs these processes. First we consider the HPGs' role in honey bee worker temporal polyethism (i.e. the age-dependent division of labour). Then we consider the secretory role of HPGs for the production of enzymes involved in larval nutrition (major royal jelly proteins, or MRJPs) and social immunity (glucose oxidase, or GOX). These mechanisms are introduced below, along with the endpoints used in this study to measure their relative activity in honey bees exposed to different pesticide and immune treatments.

We note that these hypothesised consequences of pesticide-induced physiological changes to HPGs explain the recommendation that HPG assessments be included in the European Union's new protocols for the risk assessment of plant protection products on bees (European Food Safety

Authority, EFSA, 2013). However, there is a lack of studies validating these hypotheses and testing their environmental relevance, without which we cannot assess the colony-level impacts of pesticide-induced changes in HPG development. Our study aims to remedy these gaps in ecotoxicological understanding, thereby improving the established basis for regulatory testing.

#### *5.2.1. The role of HPG development in worker temporal polyethism*

The tasks performed by an individual worker change throughout her life (e.g. from nurse to forager) in an age-dependent manner known as temporal polyethism, which ensures a division of labour in the colony. The HPG has been implicated as a controller of these transitions (Free, 1961). Nurse workers typically have larger HPGs, which primarily secrete MRJPs of nutritional function. As workers age and increasingly adopt roles in the processing of nectar, typically as middle-age (in-hive) and forager workers, their HPGs have been found to reduce in size, in turn reducing MRJP production whilst increasing production of carbohydrate-metabolising enzymes, including GOX of social immune function (Ohashi et al., 1999, Feng et al., 2009).

We hypothesised that an imidacloprid-induced reduction in HPG size could be associated with precocious foraging in worker honey bees, which could accelerate colony failure by reducing the population of nurses and in-hive workers (Henry et al., 2012) and increasing forager death rates (Perry et al. 2015). To test whether imidacloprid exposure led to precocious foraging, we used physiological and molecular endpoints as proxy measures of the transition of honey bee workers from roles in in-hive tasks to the onset of foraging and then also tested the relevance of the proxy endpoints using a behavioural assay in the field.

Specifically, we characterised the expression of two genes (*Amfor* and *malvolio*) in the honey bee brain, which are potentially valuable molecular markers for the onset of foraging behaviour (e.g. Alaux et al., 2012). Expression of *Amfor* is elevated in undertakers and foragers compared with nurse bees (Ben-Shahar et al., 2003, Ben-Shahar et al., 2002) and shows a peak in expression around the expected transition to foraging (Heylen et al., 2008). Upregulation of *malvolio*

has been associated also with foraging behaviour (Ben-Shahar et al., 2004). We tested whether *Amfor* and *malvolio* expression changed in an age-dependent manner, and whether it increased following chronic imidacloprid exposure, as an indicator of precocious foraging.

We aimed also to measure expression of MRJP and GOX genes in honey bee workers from 5-20 days old, to test whether their activity changed in an age-dependent manner as would be indicative of an age-dependent shift in nursing and foraging roles (*via* nutritional and social immune functions respectively). We then tested whether the phenology of these endpoints altered following a sustained exposure to dietary imidacloprid.

To confirm the ecological relevance of responses by the molecular endpoints, we conducted a field study to test whether the enzymatic and molecular responses measured in the laboratory studies corresponded with behavioural effects in free-flying workers in the field. Acute neonicotinoid exposures have been observed to alter the foraging behaviour of honey bee workers across short timescales (Bortolotti et al., 2003, Henry et al., 2012, Fischer et al., 2014, Schneider et al., 2012), but there has been no investigation of long term changes in individual's foraging activity. We therefore tested whether dietary exposure to imidacloprid altered the normal foraging behaviour of workers throughout their life using a novel approach that utilised Radio Frequency Identification (RFID) technology.

#### *5.2.2. Secretory role of HPGs in nutrition: Major royal jelly proteins*

MRJPs are typically secreted by nurse workers and fed to the developing brood and adult queen and they therefore play an important role in honey bee nutrition. These proteins are thought to account for 82 – 90 % of total larval jelly protein and they contain relatively high amounts of essential amino acids (Schmitzova et al., 1998). Furthermore, royal jelly is thought to play a critical role in regulating female caste differentiation of honey bee larvae into either workers or egg-laying queens (Kamakura, 2011).

Here, we aimed to test whether a chronic exposure to imidacloprid reduced expression of four MRJP genes, as an indicator of an altered nutritional function of the HPGs.

### 5.2.3. *Secretory role of HPGs in social immunity: Glucose oxidase*

GOX enzymes, along with other carbohydrate-metabolising enzymes secreted by the HPG (Ohashi et al., 1999), are involved in the processing of nectar to honey. Specifically, GOX catalyses the conversion of glucose to gluconic acid and hydrogen peroxide, which give honey antimicrobial properties, thereby sterilising brood food and colony honey supplies and so acting as a form of social immunity (White et al., 1963).

We hypothesised that imidacloprid-induced changes in HPG development could impair the production of GOX, with the potential to increase the susceptibility of the colony to pathogenic attack, but this is yet to be fully investigated. To date, a single study has observed reduced GOX activity only when honey bee workers were exposed to imidacloprid in combination with pathogenic infection (Alaux et al., 2010). Here, we aimed to test whether chronic imidacloprid exposure at environmentally relevant exposures reduced enzymatic activity of GOX both with and without an accompanying immune challenge, as an additional indicator of an altered function of the HPGs.

Fewer immune-related genes have been identified in the honey bee compared to other solitary insect species (Evans et al., 2006), which may reflect the importance of social mechanisms in the honey bee's defence against pathogenic attack. However, the role of social immunity has received relatively little attention in the literature compared to the immune responses of individual bees (e.g. Laughton et al., 2011) and so whilst GOX is recognised for its social immune role, there are few studies investigating the regulation of GOX activity. Indeed, it has been unclear whether individual bees respond to individual or colony infection by increasing GOX levels or whether, instead, GOX is a constitutive defence. In the one previous study to test this, no effect of *Nosema* infection on GOX activity was observed (Alaux et al., 2010). In our study we took the opportunity to test also whether GOX activity responded to an

individual artificial bacterial-like challenge with lipopolysaccharides (LPS) as an indicator of the regulation of social immunity.

### 5.3. Materials and Methods

Below, we describe laboratory experiments, denoted 'L', and field experiments, denoted 'F'. Different experiments of each type are distinguished by subscripts indicating the concentration of dietary neonicotinoid; for example, L<sub>102</sub> indicates the laboratory experiment where imidacloprid was in dietary sucrose at 102 parts per billion (ppb). Subsets of these experiments are denoted by acronyms indicating the endpoints tested in each study. In experiment L<sub>102-N</sub> several endpoints were measured; expression of genes for MRJPs, as well as genes for GOX and foraging, and enzymatic activity of GOX. F<sub>10-B</sub> indicates the field experiment in which behavioural endpoints were measured. L<sub>102-GOX</sub> and L<sub>0.1-102-GOX</sub> indicate the experiments in which enzymatic activity of GOX was measured only.

#### 5.3.1. Honey bee provenance and husbandry – general protocol

Honey bees (*Apis mellifera* L.) were obtained from colonies of British hybrid bees maintained at the Food and Environment Research Agency (Fera), York, UK. Brood frames were collected from colonies between April and September of any given year and placed in an environmental chamber (34 °C, 60 % relative humidity (RH), constant darkness; Versatile Environmental Test Chamber, Sanyo, UK). Workers were collected from these frames as they emerged and were housed in modified plastic cages (FK-RD8 clear PET containers, Ambican Ltd, UK) in an environmental chamber (34 °C, 60 % RH, constant darkness), with *ad libitum* access to a sucrose solution (50 % w/v) through a modified microcentrifuge tube (1.5 ml; Eppendorf Ltd, UK). In L<sub>102-N</sub> and F<sub>10-B</sub>, each cage also allowed *ad libitum* access to a pollen paste (Organic Spanish Bee Pollen, BodyMe, UK). Experiments were conducted between August 2013 and September 2014 and therefore workers for each experiment were derived from a different single honey bee colony.

### *5.3.2. Neonicotinoid exposure – general protocol*

In all studies, we exposed bees to sublethal pesticide concentrations as an oral exposure to simulate a field situation in which bees consume residues in the nectar and pollen of treated mass-blooming crops, such as oilseed rape. Stock solutions of imidacloprid were made up in acetone and used to dissolve known concentrations in sucrose solution (50% w/v). Treated sucrose solution was spiked with 0.08, 0.20, 0.51, 1.28, 3.20, 8.00, 20.00, 50.00 or 125.00  $\mu\text{gL}^{-1}$  imidacloprid (Fluka Analytical 37894, Sigma-Aldrich, UK) ( $\approx$  0.1, 0.2, 0.4, 1.0, 2.6, 6.5, 16.3, 40.7 and 101.7 ppb, respectively). Control cages contained sucrose with appropriate aliquots of acetone to control for the solvent in which pesticide stocks were dissolved. The feeder of each cage was weighed every other day and any dead bees were removed to allow calculation of feeding rates.

### *5.3.3. The role of HPG development in worker temporal polyethism*

#### *5.3.3.1. Laboratory endpoints as biomarkers for age-dependent tasks*

As below, L<sub>102-N</sub> measured enzymatic activity of GOX and expression of genes for *Amfor*, *malvolio*, GOX and MRJPs (Table 5.1.) in honey bee workers from 5 - 20 days old to test whether their activity changed in an age-dependent manner, as potential markers of the onset of foraging behaviour. For all endpoints, we tested whether a long term exposure to 102 ppb imidacloprid altered activity/expression compared to bees fed on control sucrose, as an indicator of impacts on temporal polyethism.

Table 5.1. Oligonucleotide primers used in qPCR assays in experiment L<sub>102-N</sub>.

Gene	Gene category/ pathway	Forward primer (5' → 3')	Reverse primer (5' → 3')	Concentration (nM)	Reference
<b>GOX</b>	Glucose oxidase- social immunity	GAGCGAGGTTTCGAATTG GA	GTCGTTCCCCCGAGATTC TT	900	Yang and Cox-Foster (2005)
<b>Amfor</b>	cGMP-dependent protein kinase (PKG)- Transition to foraging	AATATAACTTCCGGTGCA ACGTATT	CGTTTGGATCACGGAAGA AAG	300	Alaux et al. (2012)
<b>malvolio</b>	Manganese transport- Transition to foraging	CCTTGGTATAAAGATTAT GACAGGAATATG	CAAGAGCACTGTGAAGAT ACAAGTTATG	300	Alaux et al. (2012)
<b>mrjp1</b>	Major Royal Jelly Protein- larval feeding	TGACATACATTACGAAGG AGTCCA	ATCCGAAGAAGAGAACGC CA	300	Buttstedt et al. (2013)
<b>mrjp2</b>	Major Royal Jelly Protein- larval feeding	CGTCCAATACCAAGGATC CGAA	ACAAGTCCGACGAAGAG GAC	300	Buttstedt et al. (2013)
<b>mrjp3</b>	Major Royal Jelly Protein- larval feeding	TGGACAGATGGCGTGATA AGAC	GAGGTCCACCTTTGCCCT TT	300	Buttstedt et al. (2013)
<b>mrjp4</b>	Major Royal Jelly Protein- larval feeding	AGACAAAATATCGATGTC GTAGCTC	TGCCAGATTGTGGAACGT TTT	300	Buttstedt et al. (2013)
<b>Actin</b>	Structural protein- reference	TGCCAACACTGTCCTTTC TG	AGAATTGACCCACCAATC CA	1400	Lourenco et al. (2008)
<b>Ef1-α</b>	Protein synthesis- reference	GGAGATGCTGCCATCGTT AT	CAGCAGCGTCCTTGAAAG TT	1400	Lourenco et al. (2008)

### 5.3.3.2. *Behavioural endpoints in field colonies*

In F<sub>10-B</sub>, we tested whether the enzymatic and molecular responses measured in laboratory study L<sub>102-N</sub> corresponded with behavioural effects in a field setting. Newly emerged workers fitted with RFID transponders ('tags') were exposed for five days (d) in the laboratory to imidacloprid (10 ppb) to ensure dietary exposure to a concentration within a realistic range typical of residues found in nectar and pollen in the field. After this initial exposure, workers were introduced into hives in the field and their activity (departures and returns to the hive) was recorded over a period of six weeks to quantify the onset of foraging behaviour.

On emergence, workers were immobilised using CO<sub>2</sub>, for approximately 45 seconds, and an RFID tag (16k bit, dimensions 2.0 x 1.7 x 0.8 mm; mic3-TAG, Microsensus, Germany) was secured to the thorax with shellac adhesive (Figure 5.1.). Two cohorts were used across a one week interval, with workers in each cohort derived from a different honey bee colony. In each cohort, 400 bees were tagged and 200 were subsequently fed on control sucrose and 200 on an imidacloprid-spiked sucrose solution (10 ppb). After initial exposure, tagged bees were immobilised with CO<sub>2</sub> for approximately one minute and placed into queen cages for introduction into field colonies. The initial spell in the queen cage enables the workers to be accepted into the recipient hive without aggression. The queen cages were inserted between brood frames (Figure 5.1.) and their entrances were initially blocked by a fondant icing, which bees chewed through, resulting in their eventual release into the hive. Cohorts 1 and 2 were placed into colonies on 11<sup>th</sup> and 19<sup>th</sup> August 2014, respectively. In each cohort, 50 control-fed and 50 imidacloprid-fed bees were introduced to each of four colonies, which were adjacent to one another in the apiary at Fera, York, UK. None of these recipient colonies was the parent colony of the experimental bees. Each recipient colony was fitted with an RFID reader at the hive entrance through which every bee had to pass when leaving or entering the hive. These readers operated for six weeks until 26<sup>th</sup> September 2014.

To test whether the activity of tagged bees was artifactually affected by transfer to a new hive, we also introduced 20 foragers that were collected from the flight board of their parent colony as they returned home. These were immediately taken to the laboratory, immobilised with CO<sub>2</sub> and an RFID tag secured to the thorax. These foragers were then immediately returned to their original colony and released on the flight board (within 2 hours, h, of their capture) and their activity levels were also monitored by an entrance reader.

A 'read' was recorded when a tagged bee entered or left the colony. A bee was described as 'active' if it made a trip in which it left the colony and subsequently returned. 33 trips were excluded from the analysis as anomalies because the trip duration exceeded 5 h. Anomalous trips were distributed across all colonies, cohorts and treatments without apparent pattern and excluding them therefore does not bias our analysis. Similarly, intermittent technical failure among the RFID readers meant that some short periods (less than one day) went unrecorded, but these too were independent of treatment and introduced no bias.

55 % of the 800 young tagged bees recorded a read and 32 % were active (Figure 5.2.). We did not find that reintroduction affected the activity levels, because the proportion of active individuals was very similar among bees tagged as foragers and released into their natal hive (mean  $\pm$  SE = 27.5  $\pm$  8.3 %). The number of active bees varied among cohorts (Figure 5.2.) and given these differences in sample sizes between cohorts, further analyses of pesticide treatment differences were performed for each cohort separately.

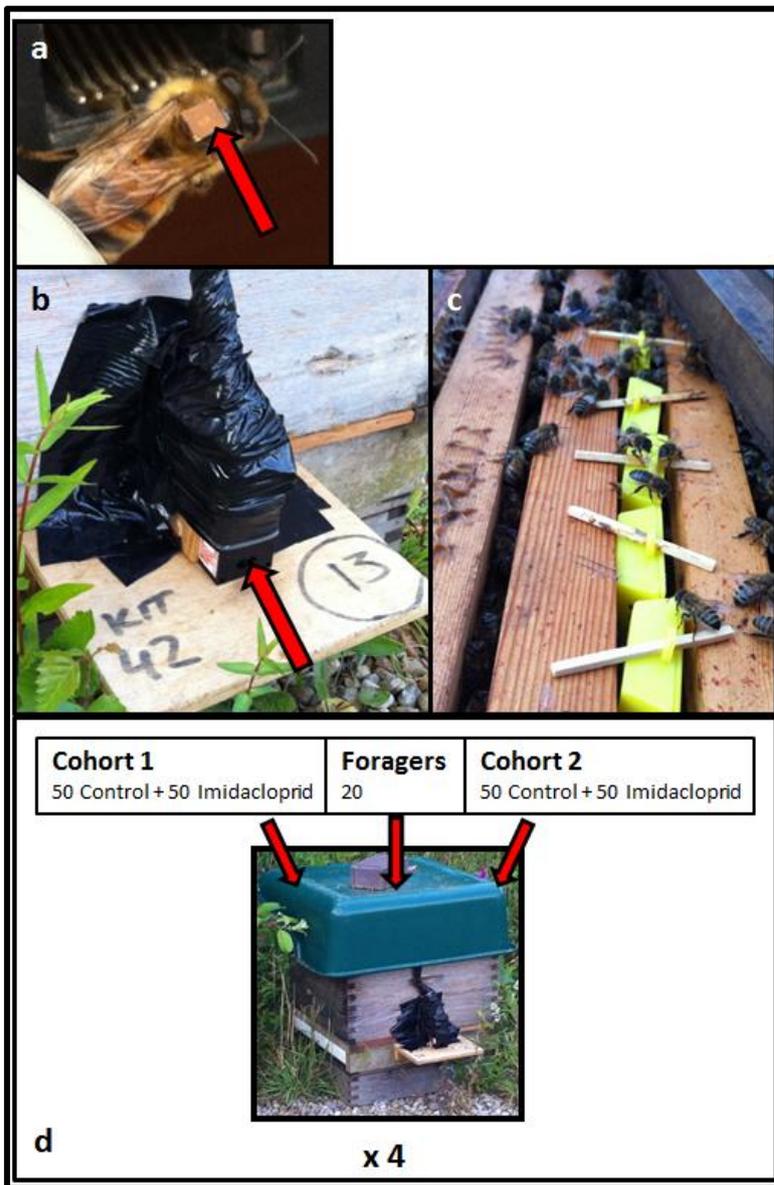


Figure 5.1. (a) Honey bee with RFID tag secured to thorax. (b) RFID reader on hive (arrow shows entrance). (c) Yellow queen cages placed between frames to introduce tagged bees. (d) Schematic of setup of Experiment F<sub>10-B</sub>.

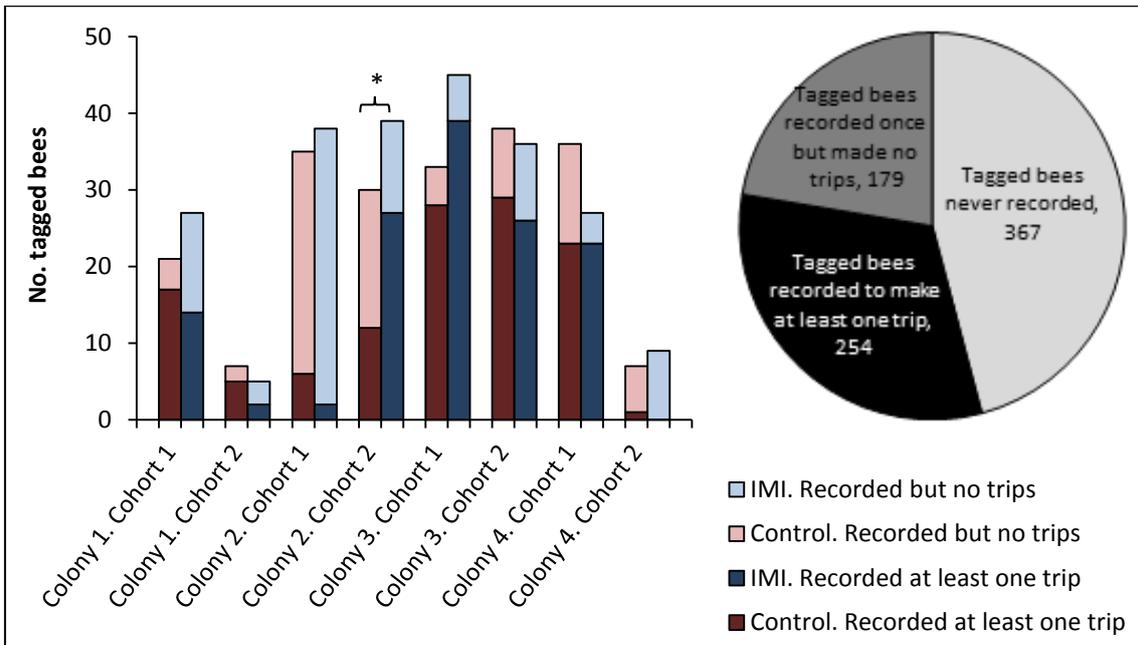


Figure 5.2. Proportion of the 800 tagged young bees that were ever recorded, and to make a return trip to the colony. NB. 50 control and 50 imidacloprid (IMI)-exposed bees were initially tagged in each colony/cohort.

#### 5.3.4. Secretory role of HPGs in nutrition: MRJPs

In L<sub>102-N</sub>, worker bees were housed 10 per cage and exposed to imidacloprid (102 ppb) for up to 20 d and the expression of four genes, *mrjp -1*, *-2*, *-3* and *-4*, was measured at sampling intervals of 5, 10, 15 or 20 d.

##### 5.3.4.1. Measurement of gene expression

At the appropriate sampling times, experimentally treated bees were placed directly into a -80 °C freezer until RNA extraction. Total RNA was extracted from six heads pooled from a single cage, with four cages (biological replicates) per treatment/age combination. Total RNA was extracted using the *mirVana* miRNA Isolation Kit (Ambion AM1561; Life technologies, UK) and RNA quantity was measured using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, UK). cDNA was synthesised from 2 µg RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems 4368814; Life technologies, UK) and a C1000 Thermal Cycler (Bio-Rad Laboratories, UK) under the following cycling conditions: 10 minutes 25 °C, 120 minutes 37 °C, 5 minutes 85 °C, stored at 4 °C.

Real-time quantitative PCR (qPCR) was performed using the SYBR Green PCR Master Mix (Applied Biosystems 4309155; Life technologies, UK). Oligonucleotide primers used to amplify genes are shown in Table 5.1. Each sample was run in duplicate, with treatments randomly assigned across plates. PCR reactions were carried out in a ViiA 7 Real-Time PCR System (Applied Biosystems; Life technologies, UK) under standard cycling conditions (2 minutes 50 °C, 10 minutes 95 °C and 40 cycles of 15 seconds 95 °C and 1 minute 60 °C).

The threshold cycle ( $C_t$ ) value for each sample was calculated only if the standard deviation of the two replicates was  $\leq 0.5$ , and transformed into an input quantity value using the relative standard curve method. Input quantities were normalised using the geometric mean of Actin and Ef1- $\alpha$ , which were identified as appropriate reference genes using GeNorm (Vandesompele et al., 2002). Normalised input quantities were used for statistical analysis.

### 5.3.5. Secretary role of HPGs in social immunity: GOX

In L<sub>102-GOX</sub> and L<sub>0.1-102-GOX</sub>, workers were housed six per cage and exposed to dietary imidacloprid for an initial period of four days, then subjected to an immune challenge on day five, and subsequently exposed as before for up to 48 h. L<sub>102-GOX</sub> tested the effects of a single concentration of imidacloprid (102 ppb) and an immune challenge, across a time course of 8 - 48 h post immune challenge (PIC). To determine whether the effects were also observed at field-relevant levels, in L<sub>0.1-102-GOX</sub> workers were exposed to one of ten imidacloprid concentrations (0.1, 0.2, 0.4, 1.0, 2.6, 6.5, 16.3, 40.7 and 101.7 ppb), and assays were conducted at a single time point, 24 h PIC. For both studies, GOX activity was measured from 4-6 individual bees from a single cage at the appropriate time points. The cage mean acted as the biological replicate for statistical analysis, with three replicates of each immune-challenge treatment, dosage and time point.

#### 5.3.5.1. Immune challenge

To immobilise workers for injection, bees were placed in a freezer (-20 °C) for two to five minutes until torpid. Injections were performed using a fine needle on a Hamilton syringe with a repeating dispenser. Bees were injected with 2 µl of solution through the pleural membrane between the tergites of the abdomen. Bees received one of three treatments, with all workers within a cage subjected to the same treatment. To invoke a bacterial-like immune response, 'LPS' bees were injected with 0.5 mgml<sup>-1</sup> lipopolysaccharides from *Escherichia coli* 0128:B12 (Sigma L2755, Sigma-Aldrich, UK) dissolved in Insect Ringers. 'Ringers' bees were injected with Insect Ringers solution (1:1:1 156 mM NaCl : 3 mM KCl : 2 mM CaCl<sub>2</sub>) to control for any effects of the injection process. 'Naïve' bees were ice-immobilised but received no other treatment (unchallenged control).

#### 5.3.5.2. Measurement of GOX enzymatic activity

Experimentally treated bees were ice-immobilised and decapitated using sterile dissecting scissors. Each head, which contained the HPG, was homogenised

by hand in 200  $\mu\text{l}$  phosphate buffer solution (PBS) using a micro-pestle, and centrifuged (9600 RPM, 10 minutes, 4  $^{\circ}\text{C}$ ). 100  $\mu\text{l}$  of supernatant was immediately stored at -20  $^{\circ}\text{C}$  for later analysis.

GOX activity was measured using a spectrophotometric assay (based on Alaux et al., 2010). Samples were thawed on ice and 20  $\mu\text{l}$  added to a 96-well plate. 180  $\mu\text{l}$  of reaction mixture [20  $\mu\text{l}$   $\beta$ -D-glucose (18  $\text{mgml}^{-1}$ ; Sigma G8270), 100  $\mu\text{l}$   $\text{dH}_2\text{O}$ , 50  $\mu\text{l}$  PBS, 10  $\mu\text{l}$  horseradish peroxidase (4.8  $\text{mgml}^{-1}$ ; Sigma P8125)] were placed in each well, the plate was shaken, then incubated at 37  $^{\circ}\text{C}$  for 10 minutes. The plate was placed back on ice and 20  $\mu\text{l}$  of o-dianisidine [saturated (0.7  $\text{mgml}^{-1}$ ) and filtered; Sigma D9143] added to each well. The plate was shaken and the reaction allowed to proceed at 37  $^{\circ}\text{C}$  in a spectrophotometer (ThermoMax microplate reader, Molecular Devices, UK). Absorbance was measured at 405 nm every 20 seconds for 1 hour 45 minutes. Each sample was measured in duplicate and treatments were assigned across plates to minimise between-plate variation. Softmax Pro v4.3 software (Molecular Devices, UK) was used to calculate enzyme activity measured as the maximum linear rate of substrate conversion ( $V_{\text{max}}$ ).

## 5.4. Results

### 5.4.1. Effect of imidacloprid on general health indicators during laboratory exposures

Our experimental honey bees exhibited good health, because across the experiments, only 31/2086 bees (1.5 %) died during the laboratory exposure period and the limited mortality was not associated with experimental treatment effects. Imidacloprid effects on feeding rates were inconsistent across experiments. Bees sometimes consumed less sucrose containing 102 ppb (L<sub>102-GOX</sub> : Wilcoxon Rank Sum:  $W = 1010.5$ ,  $P \leq 0.001$ ; and L<sub>102-N</sub> : Kruskal-Wallis rank sum tests: 5 – 9 d  $\chi^2_{df1} = 8.08$ ,  $P = 0.004$ , 10 – 14 d  $\chi^2_{df1} = 11.3$ ,  $P \leq 0.001$ ), but otherwise no treatment effects were observed.

### 5.4.2. The role of HPG development in worker temporal polyethism

#### 5.4.2.1. Laboratory endpoints as biomarkers for age-dependent tasks

The enzymatic activity of GOX did not vary with the age of the honey bee, nor did GOX gene expression. The youngest workers (5 days old) had higher transcripts of three MRJP genes (*mrjp -2*, *-3* and *-4*) compared with older workers (L<sub>102-N</sub>: ANOVAs *mrjp2*  $F_{3,21} = 8.81$ ,  $P \leq 0.001$ ; *mrjp3*  $F_{3,20} = 3.23$ ,  $P = 0.044$ ; *mrjp4*  $F_{3,23} = 4.75$ ,  $P = 0.010$ ) and there was a general pattern of reduced expression with increased worker age for all four MRJP genes investigated (Figures 5.3.E-H). Expression of *Amfor* was independent of worker age. Expression of *malvolio* was higher only among the oldest workers (20 days old) in imidacloprid-exposed bees (L<sub>102-N</sub>: Figure 5.3.D, ANOVA age\*pesticide  $F_{3,24} = 5.19$ ,  $P = 0.007$ ).

Imidacloprid exposure increased expression of *Amfor* (L<sub>102-N</sub>: Figure 5.3.C, ANOVA  $F_{1,24} = 29.2$ ,  $P \leq 0.001$ ), and exposure increased expression of *malvolio* in 20 day old workers only (L<sub>102-N</sub>: Figure 5.3.D, ANOVA age\*pesticide  $F_{3,24} = 5.19$ ,  $P = 0.007$ ).

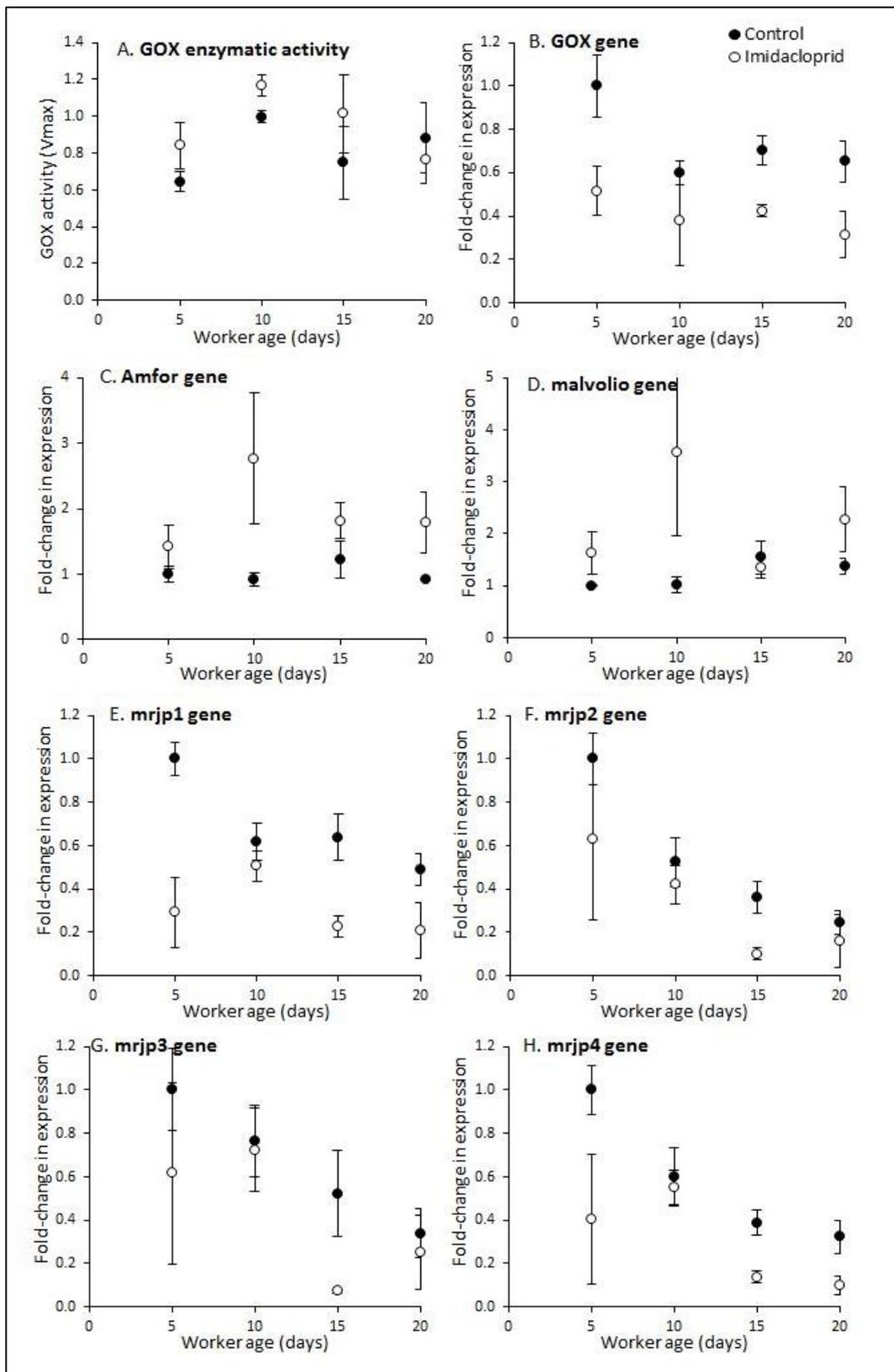


Figure 5.3. See figure legend overleaf.

*Figure 5.3.* A) Enzymatic GOX activity; B-H) Relative gene expression in honey bee workers exposed to  $125 \mu\text{gL}^{-1}$  (102 ppb) imidacloprid for 5-20 days post emergence (experiment L<sub>102-N</sub>). The mean fold-change in expression was calculated for each pesticide/age compared to the mean expression of the control-fed/5-day-old group. Data presented as Mean  $\pm$  SE (n = 4).

#### 5.4.2.2. Behavioural endpoints in field colonies

Imidacloprid exposure did not affect the number of reads recorded within each cohort ( $\chi^2$  tests  $P > 0.05$ ), or the proportion of active bees. The exception to this was the second cohort in Colony 2, in which fewer bees were active in the control group compared to imidacloprid-exposed bees ( $F_{10-B}$ : Figure 5.2., Binomial test  $\chi^2_{1df} = 4.77$ ,  $P = 0.029$ ).

In general, imidacloprid did not affect the age of first emergence from the colony (Figure 5.4.A). There were two exceptions to this that were each specific to a cohort and colony, where imidacloprid-exposed bees were significantly older than control bees when they first left the colony ( $F_{10-B}$ : Colony 2 Cohort 2: Welch's  $t_{61.7df} = -2.54$ ,  $P = 0.014$ ; Colony 3 Cohort 1: Welch's  $t_{63.3df} = -2.51$ ,  $P = 0.015$ ).

Imidacloprid did not affect the mean number of trips taken per active bee (Figure 5.4.B), with one exception in which imidacloprid-exposed bees took significantly more trips than control-fed bees ( $F_{10-B}$ : Colony 2 Cohort 2: Welch's  $t_{67.0df} = -2.32$ ,  $P = 0.023$ ).

Imidacloprid did not affect the mean duration of trips (Figure 5.4.C), with one exception in which imidacloprid-exposed bees took significantly longer trips than control-fed bees ( $F_{10-B}$ : Colony 1 Cohort 1: Welch's  $t_{22.9df} = -2.90$ ,  $P = 0.008$ ).

Imidacloprid exposure had no consistent effects on the temporal pattern of trips taken (Figure 5.5.).

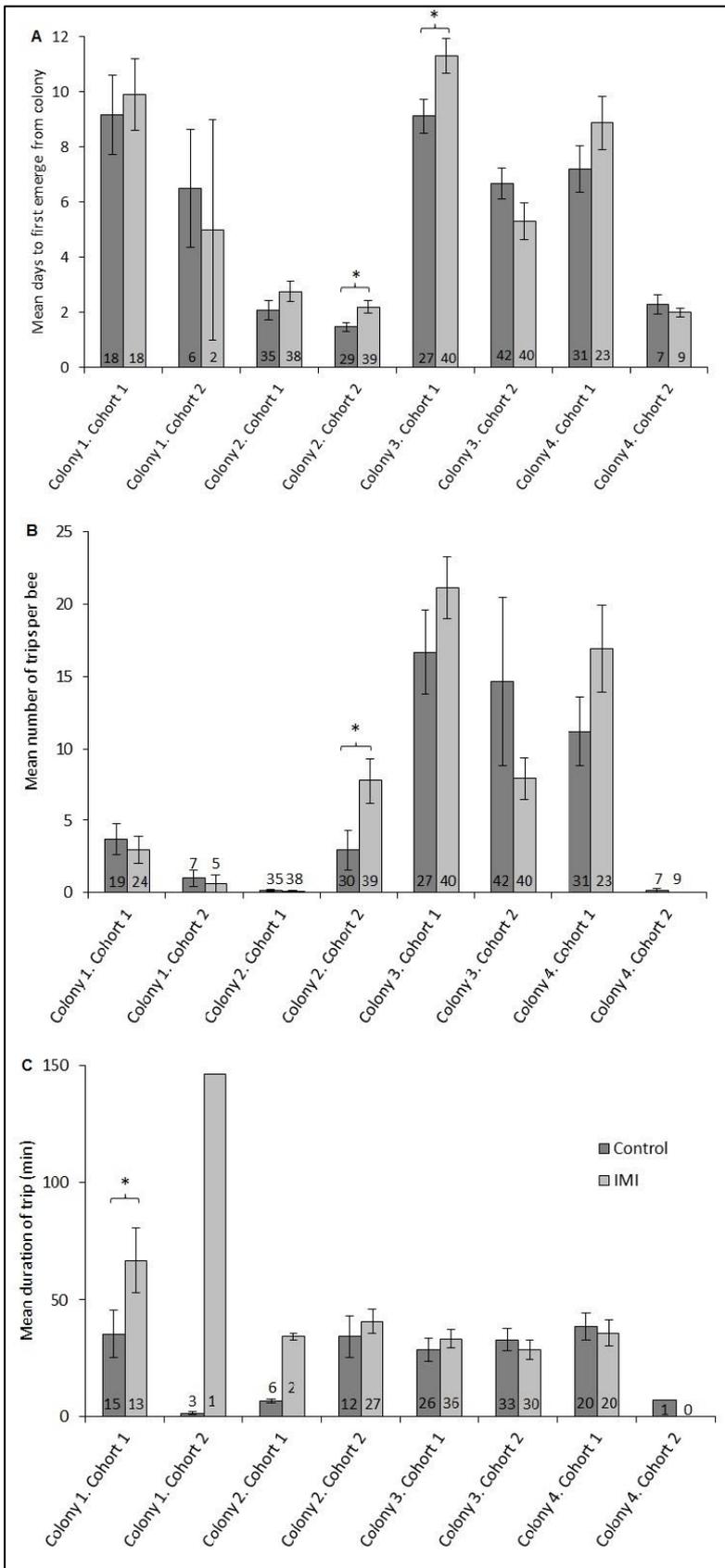


Figure 5.4. See figure legend overleaf.

*Figure 5.4.* A) Mean  $\pm$  SE number of days for tagged bees to first record to leave the colony following their introduction to the colony at 6 days old. B) Mean  $\pm$  SE number of trips recorded by each bee. C) Mean  $\pm$  SE duration of trips recorded by each bee. Numbers in each bar represent the number of bees recorded in each cohort (n). \* Asterisks show significant differences between control and imidacloprid groups.

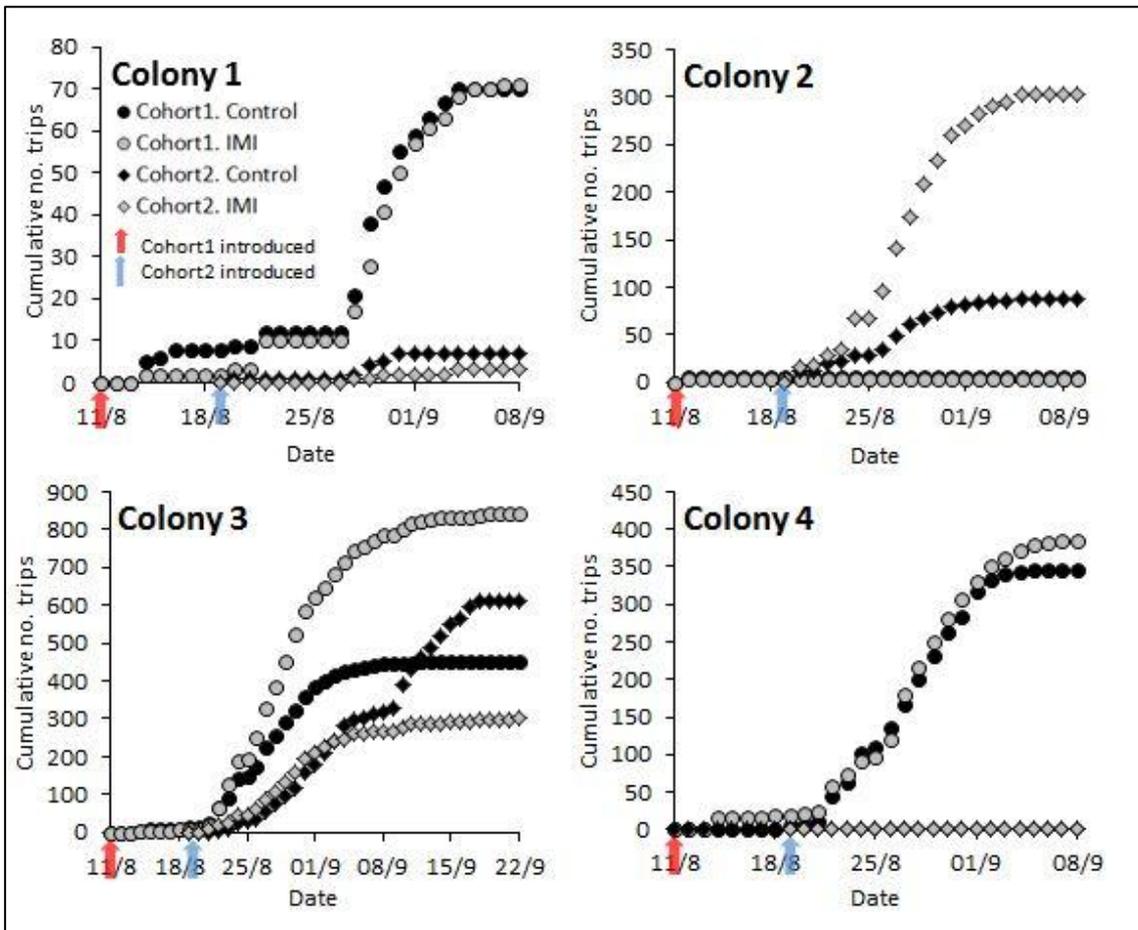


Figure 5.5. Cumulative number of trips recorded in each cohort over the 6+ weeks of the study.

#### 5.4.3. Secretary role of HPGs in nutrition: MRJPs

Imidacloprid exposure lowered expression of *mrjp1* ( $L_{102-N}$ : Figure 5.3.E, ANOVA  $F_{1,24} = 11.2$ ,  $P = 0.003$ ) and *mrjp4* ( $L_{102-N}$ : Figure 5.3.H, ANOVA  $F_{1,23} = 4.40$ ,  $P = 0.047$ ). A similar trend was observed in the expression profiles of *mrjp2* and *mrjp3* in the 5- and 15- day old workers (Figures 5.3.F and 1G).

#### 5.4.4. Secretary role of HPGs in social immunity: GOX

Overall, imidacloprid exposure increased enzymatic GOX activity, but activity was significantly greater than control-fed bees only for 41 ppb and 102 ppb imidacloprid treatments ( $L_{GOX-102}$ : Figure 5.6., ANOVA pesticide  $F_{1,54} = 30.1$ ,  $P \leq 0.001$ ;  $L_{GOX-0.1-102}$ : Figure 5.7., ANCOVA pesticide concentration  $F_{1,85} = 27.6$ ,  $P \leq 0.001$ , Tukey HSD post hoc tests  $P \leq 0.05$  for pairwise comparisons of 0 - 41 ppb and 0 - 102 ppb only). In contrast, there were no significant effects of 102 ppb imidacloprid across an exposure of 5 - 20 d ( $L_{102-N}$ ), although there was a general trend towards increased GOX activity in imidacloprid-exposed bees (Figure 5.3.). Furthermore, in one case the increase in GOX activity following imidacloprid exposure was not observed when bees were simultaneously challenged with LPS-injection ( $L_{102-GOX}$ : Figure 5.6.; ANOVA injection\*pesticide  $F_{2,54} = 3.57$ ,  $P = 0.035$ ). There was a significant trend towards lower expression of the GOX gene in imidacloprid-exposed bees ( $L_{102-N}$ : Figure 5.3.B, ANOVA  $F_{1,23} = 4.08$ ,  $P = 0.055$ ).

Overall, immune challenge lowered GOX activity compared with naïve controls ( $L_{102-GOX}$ : Figure 5.6., ANOVA injection  $F_{2,54} = 8.90$ ,  $P \leq 0.001$ ;  $L_{0.1-102-GOX}$ : Figure 5.7., ANCOVA injection  $F_{2,85} = 6.48$ ,  $P = 0.002$ ). This effect was observed at 24 and 48 h PIC only ( $L_{102-GOX}$ : Figure 5.6., ANOVA injection\*time  $F_{6,54} = 4.65$ ,  $P \leq 0.001$ ). However, LPS-injection lowered GOX activity compared to naïve controls only when coupled with imidacloprid exposure ( $L_{102-GOX}$ : ANOVA injection\*pesticide  $F_{2,54} = 3.57$ ,  $P = 0.035$ ). In  $L_{0.1-102-GOX}$  there was no injection\*pesticide interaction, although the differences in GOX activity following injection treatment were not observed in control-fed bees (Figure 5.7.).

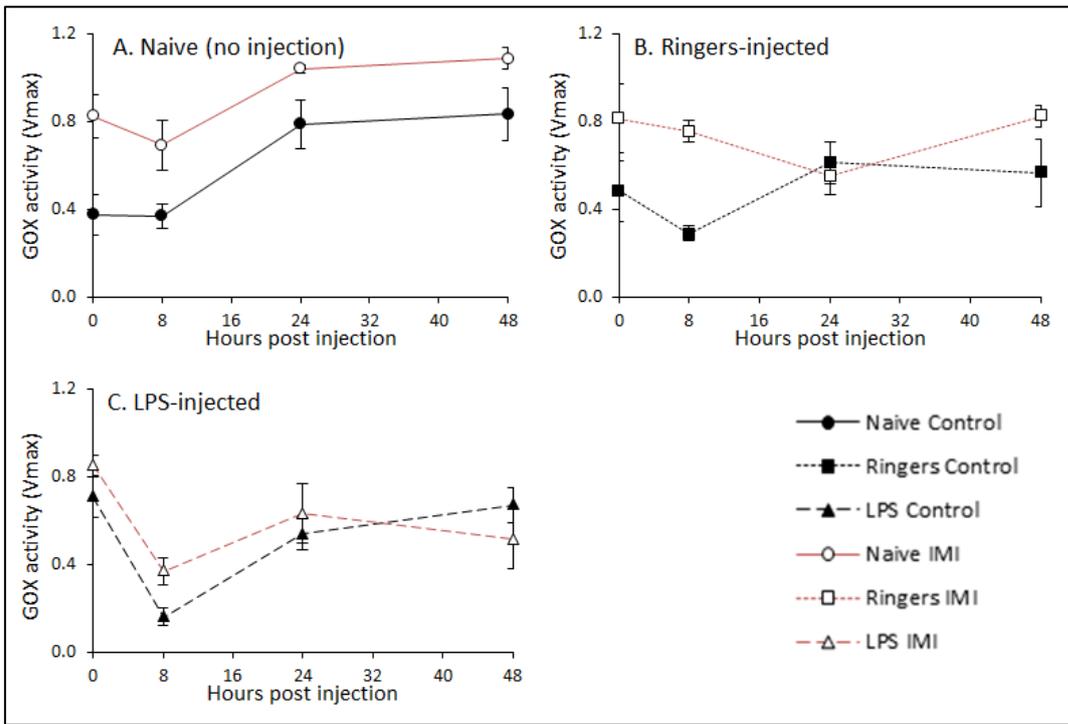


Figure 5.6. Enzymatic activity of GOX measured 0-48 hours PIC, following exposure to  $125 \mu\text{gL}^{-1}$  (102 ppb) imidacloprid (IMI) (experiment L<sub>102</sub>-GOX). Data presented as Mean  $\pm$  SE (n = 3).

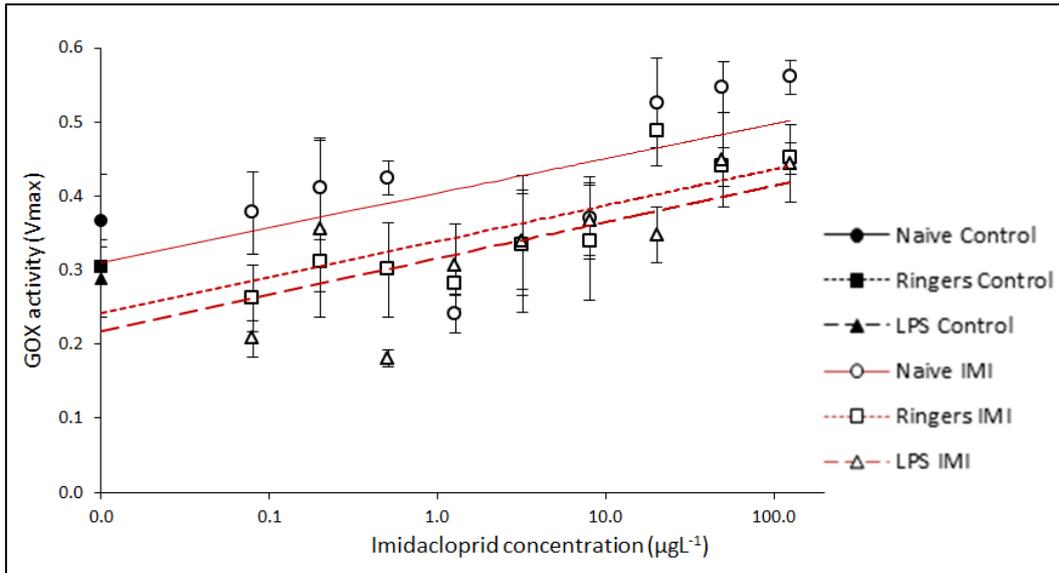


Figure 5.7. Enzymatic activity of GOX measured 24 hours PIC, following exposure to 0.08 - 125  $\mu\text{gL}^{-1}$  (0.1 – 102 ppb) imidacloprid (IMI) (experiment L<sub>0.1-102-GOX</sub>). Data presented as Mean  $\pm$  SE (n = 3), and linear lines of best fit.

### 5.5. Discussion

Our results show molecular evidence that imidacloprid exposure has potential to disrupt the control of temporal polyethism and induce precocious behavioural transitions. Specifically, we found that imidacloprid exposure increased expression of two foraging genes (*Amfor* and *malvolio*), which supports the proposition that it could cause precocious onset of foraging, perhaps as a result of an imidacloprid-induced reduction in HPG size. Similarly, we also found that imidacloprid exposure reduced the expression of MRJP genes and increased correspondingly the enzymatic activity of the GOX system. These latter effects appear to indicate that individual bees are exhibiting a precocious shift from nursing roles (providing MRJP nutrition to brood) to foraging (processing nectar to honey using GOX). However, we recognise here that differential expression was observed only following exposure to imidacloprid at a concentration of 102 ppb and in a laboratory setting in which bees were unable to undertake nursing

or foraging activities. These exposures are likely to be higher than those that workers would be exposed to in realistic natural scenarios. Residues found in nectar and pollen in the field are typically 1-10 ppb (Blacquiere et al., 2012), although residues as high as 101 ppb have been found in pollen from transplant-dripped pumpkin crops (Dively and Kamel, 2012).

Our results indicate that ecotoxicological responses in molecular endpoints may not be reflected in corresponding behavioural endpoints. For example, in our field study we found no corresponding behavioural effects in free-flying workers following a field relevant exposure, so we could not confirm the behavioural relevance of the enzymatic and molecular responses associated with temporal polyethism in the laboratory. Specifically, we found no consistent effects of chronic exposure to 10 ppb imidacloprid on foraging behaviour. Most importantly, exposure to imidacloprid failed to induce the precocious transition to foraging behaviour that had been strongly indicated by our molecular endpoints. Since our laboratory and field studies tested effects following exposure to 102 and 10 ppb imidacloprid respectively, we cannot verify whether the non-correspondence between molecular and behavioural endpoints is a result of a dose-dependent effect on temporal polyethism or whether the molecular endpoints are not useful indicators of ecologically relevant foraging behaviour. Nevertheless, within the limitations of the study our data offers promise that field relevant exposures may not disrupt foraging behaviour at the colony level.

The failure to detect precocious foraging may have arisen from shortcomings in our experimental field setup. For example, we were not able to account for the relatively low overall returns for tagged bees. This was not thought to be as a consequence of the tagging process causing mortality directly, nor the detachment of tags, as this did not occur during the laboratory exposure period. It is possible that introduced bees may have been rejected and killed by the recipient colony, however, newly emerged bees are typically accepted into foreign colonies as they lack the colony-specific odours (recognition pheromones) that are acquired through contact with the nesting material of a

particular colony (Breed et al., 2004). We introduced bees into the colonies as young as possible, whilst still enabling a chronic imidacloprid exposure period in the laboratory. We did so using a well-established beekeeping method to introduce new queens, using a fondant sugar screen to enable odour contact and exchange before any physical contact, enabling time for acceptance of the introduced bees by the recipient colony. It is also possible that the dearth of bees recorded resulted from a lack of technical reads rather than a lack of active bees. For example, whilst substantial efforts were made to ensure there was only a single access point to bees in and out of each hive (passing through the RFID reader) some bees were occasionally observed entering or leaving the hive through other small holes in the hive structure. In this case, bees may have been actively foraging, but their activity went unrecorded by the RFID reader. This occurrence may have been particularly prevalent during periods of high activity, such as warm weather conditions favourable for foraging, and hence the high traffic of bees in and out of the hive led to bees seeking alternative entrances to by-pass the limited flow offered by a single RFID reader at the main hive entrance. Whilst relatively small nucleus colonies were used in this study to minimise the build-up of hive traffic, future studies may benefit from the use of multiple RFID readers (and therefore entrances) per colony to better address this limitation. The relatively low activity levels recorded may have limited the statistical power of the study to detect major impacts on temporal polyethism had they existed, and further work is needed to truly establish the sample sizes necessary in RFID studies to reliably detect changes in foraging behaviour. Foraging behaviour is inherently variable, driven by multiple factors, including colony demands for food, genetic variability, hormonal regulation, and HPG development (Calderone, 1998; Johnson, 2010). Therefore, future studies would benefit from power analyses, taking into account the relative importance of the number of bees within colonies, the number of colonies and seasonal variations. Here too we made several assumptions in the interpretation of the data. Specifically, we assumed that the first emergence of a given bee from the colony represented a shift from in-hive (e.g. nursing or comb-building) tasks to out-of-hive (e.g. foraging) tasks, and that a trip represented a foraging trip. Nevertheless, this preliminary study presents the first insights into imidacloprid

effects on foraging behaviour throughout honey bee worker life, and our findings offer promise that field relevant exposures may not disrupt foraging behaviour. In the future, coupling the RFID approach adopted here with behavioural observations should enhance studies of this nature and better enable understanding as to whether imidacloprid and other pesticide exposures can lead to impacts on foraging and survivorship in bees in the natural environment.

We have demonstrated for the first time that chronic exposure to imidacloprid reduced expression of MRJP genes, but we did not test the production of MRJPs at the protein level and in field realistic scenarios. A reduced production of MRJPs by nurse workers has the potential to impact on larval and queen development by restricting their nutritional supply of proteins, including essential amino acids (Schmitzova et al., 1998), and altering the regulation of female caste differentiation (Kamakura, 2011). This in turn could have colony- and population- level implications if reduced development slows colony growth and queen production. Given the potential non-correspondence between molecular and physiological markers (see above), we therefore recommend that further work should prioritise the investigation of imidacloprid effects on the nutritional supply of proteins to developing larvae and subsequent effects on larval development in field realistic scenarios. Follow-up work to investigate MRJPs at the protein level could benefit from the use of highly specific antibodies against MRJPs, for use in western blotting (e.g. Shen et al., 2015).

Our results show also that, overall, dietary imidacloprid altered GOX activity, which implies that neonicotinoid exposure can cause disruption to the social immune response. However, the effects observed were not always consistent and were observed only when coupled with an immune challenge. This could be important in realistic field scenarios in which bees are likely exposed to multiple stressors, but we did not test the impacts of changes in GOX levels on the realised levels of in-hive social hygiene and on the susceptibility of the colony to pathogen infection under realistic conditions. Consequently, the ecological relevance of our findings remains to be fully demonstrated. In the absence of immune challenge dietary imidacloprid at concentrations exceeding 40 ppb caused increased levels of GOX, somewhat consistent with previous

observations that imidacloprid at concentrations as low as 0.5 ppb reduces HPG size (Heylen et al., 2011, Skerl and Gregorc, 2010, Hatjina et al., 2013) and in line with our hypothesis of precocious foraging. However, imidacloprid exposures as low as 0.1 ppb lowered GOX levels in immune-challenged bees, consistent with a previous study in which GOX activity was lowered in bees exposed to imidacloprid only when coupled with an immune challenge to *Nosema* (Alaux et al., 2010). We propose that there may be a trade-off in the allocation of a limited resource pool towards costly detoxification systems and the social immune response. There remains no evidence that individual bees induce GOX production from the HPGs in response to immune challenge, suggesting GOX acts as a constitutive social immune response, but further investigation is needed to better understand the role of GOX in social immunity.

Our experimental design did not elicit the full range of normal physiological age-dependent changes and it remains unclear whether the expression of the genes tested provides a useful indicator of disruption to the normal functioning of the HPG. Even in the confined conditions of the laboratory experiment we detected a reduction in expression of MRJP genes in older honey bee workers, demonstrating an innate underlying age-dependent regulation of MRJP expression, consistent with changes observed at the proteomic level (Feng et al., 2009). In contrast, we failed to detect an age-dependent increase in expression of GOX, *Amfor* and *malvolio* genes that normally occurs during the transition of worker honey bees from nursing to foraging roles in the wild (Ben-Shahar et al., 2003, Ben-Shahar et al., 2004, Ohashi et al., 1999), implying this differential expression is likely driven by more complex mechanisms than simply an age-dependent regulation. The imidacloprid-induced increase in enzymatic activity of GOX was not correlated with expression of the GOX gene, which was reduced following imidacloprid exposure, but it is possible that mRNA titres that were translated to GOX enzymes were not replenished. Given the inconsistencies in our findings, we welcome further work to elucidate the biological relevance of the molecular changes observed.

Overall, we have demonstrated that dietary exposure of honey bees to neonicotinoid pesticide is potentially capable of causing generalised disruption

of HPG function, which implies that imidacloprid-induced changes in HPG development can lead to detrimental effects on colony function. We believe this provides some overall support for the ecological relevance of HPG-related assays in pesticide risk assessment involving sublethal effects on honey bees. It is evident that further work is needed to test the field-relevance of some of our findings (namely imidacloprid-induced disruption to nutrition and social immunity), as in some cases (temporal polyethism) transcriptional responses that we observed did not translate into functionally significant impacts on bee health, highlighting the need to cross reference gene expression bioassays with ecologically relevant behaviour. Our work highlights the potential utility of new enzymatic, molecular and behavioural ecotoxicological endpoints for risk assessment of sublethal effects on honey bees, if further work can clarify their ecological relevance to colony-level impacts on bee health under more field realistic exposures.

### *5.6. Acknowledgements*

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## **6. Transcriptome sequencing analysis of the honey bee following exposure to immune and chemical stressors**

### *6.1. Abstract*

Pathogen infection and exposure to neonicotinoid pesticides have the potential to impact on bee health, but little is known about the molecular response to combinations of these stressors in adult honey bee workers. A new opportunity to understand these molecular mechanisms has been made possible by the sequencing of the honey bee genome and advances in transcriptome-sequencing technology. Using RNA-Sequencing, we identified a suite of genes that were differentially expressed in adult honey bee workers in response to immune stress, dietary neonicotinoid exposure (including field relevant concentrations) or a combination of both immune and chemical stressors. As expected, immune stress imposed by wounding and bacterial-like infection led to upregulation of genes with known immune function, including a peptidoglycan recognition protein and antimicrobial effectors. Transcriptional changes occurred also in genes not previously associated with immune stress, for example a laccase-1-like gene, LOC724890. Exposure to a dietary neonicotinoid, either thiamethoxam or imidacloprid, led to downregulation of genes associated with several metabolic pathways, such as oxidative phosphorylation, pathways associated with pyruvate and purine metabolism, and ribosomal activity, and to upregulation of three cytochrome P450 genes, including CYP6AS16P and CYP6AS15. The combined stress of dietary neonicotinoid and artificial infection dramatically changed the expression of immune-related genes that previously had responded to immune challenge alone. Similarly, transcriptomic responses to neonicotinoid exposure were altered with the additional stress of an immune challenge. However, the implications of these transcriptomic manifestations of an interaction between stressors are difficult both to interpret and to relate to bee health. Nevertheless, our study has identified some candidate genes for further study to better

understand health outcomes and for development as potential biomarkers for use in pesticide risk assessment.

## *6.2. Introduction*

The sequencing of the honey bee genome (Weinstock et al., 2006) and the increasing availability and affordability of next-generation sequencing technologies has provided a new opportunity to investigate the molecular mechanisms underlying key questions in the study of honey bees. Several studies have demonstrated the value of RNA-Sequencing (RNA-Seq) for identifying mRNA transcripts involved in honey bee responses to different diets (Mao et al., 2013, Wheeler and Robinson, 2014), to immune challenge (Ryabov et al., 2014, Nazzi et al., 2012, Cornman et al., 2013) and to chemical stressors, such as dietary pesticides (Derecka et al., 2013). However, the transcriptome-wide analysis of differential gene expression in response to a bacterial infection or neonicotinoid exposure previously has been investigated in honey bee larvae only (Derecka et al., 2013, Cornman et al., 2013). Furthermore, RNA-Seq studies have been applied to investigate differential expression in response to single stressors, rather than to a combination of both immune and chemical stressors. Whilst there is growing interest in understanding the effects of pathogen-pesticide interactions on bee health, most studies have approached investigations of these interactions by testing whether pesticide exposure alters bees' ability to mount an immune response (e.g. Alaux et al., 2010, Di Prisco et al., 2013, Garrido et al., 2013). There has been some, but far less, consideration of a second hypothesis in pathogen-pesticide interactions; immune stress alters bees' ability to respond to pesticide exposure. For example, Vidau et al. (2011) found no strong decrease in detoxification systems when bees were simultaneously exposed to immune and pesticide stressors. Here, we aimed to characterise the transcriptomic responses of adult honey bee workers when exposed to an immune challenge, a neonicotinoid exposure, or both stressors in combination. Furthermore, we aimed to assign functional annotations to the differentially expressed genes (DEGs) that we identified in order to elucidate potential functions and molecular pathways that were affected by these stressors. We investigated the combination of stressors by assessing

first whether the response to immune challenge was altered in the presence of dietary pesticide, and second whether the response to dietary pesticide was altered in the presence of immune challenge.

We used an unbiased RNA-Seq approach for this work for the following reasons. First, our earlier work using a qPCR approach found evidence for differential gene expression in adult workers exposed to a combination of immune and neonicotinoid stressors (Chapter 3), but these data were restricted to a very small set of targeted genes that had been selected for investigation based on an *a priori* interest in these genes. In the present study, an RNA-Seq approach allows investigation of transcriptional responses across the whole transcriptome because it is not limited to a subset of genes. Certainly, the alternative approach of a genomic tiling DNA microarray approach might have allowed measurement of expression levels of large numbers of genes simultaneously by using a set of overlapping oligonucleotide probes that represent a large subset of the genome at high resolution. However, we chose instead to use RNA-Seq over a microarray approach as this offers several advantages. Firstly, RNA-Seq is not limited to detecting transcripts that correspond to existing genomic sequence and also it can be used to map transcriptomes of large genomes at much lower costs with a smaller input of RNA than the alternative microarray approaches. Secondly, RNA-Seq has little, if any, background signal, and has no upper limit of quantification, which provides the advantage of much higher sensitivity for genes expressed at either low or high levels and hence a much larger dynamic range than DNA microarrays (Wang et al., 2009).

### *6.3. Materials and Methods*

We performed a laboratory experiment whereby newly emerged adult honey bee workers were exposed to a neonicotinoid-spiked sucrose feed for an initial period of 4 days, and then experienced an immune challenge the next day, i.e. five days post eclosion. The bees were fed continuously with the same neonicotinoid treatment throughout the experimental period until collection of

samples for RNA extraction, at either 8 or 24 hours (h) post immune challenge (PIC) (see Supporting Information: Figure 6.5.).

### 6.3.1. Honey bee provenance and husbandry

All honey bees were from a single *Apis mellifera* colony maintained in an apiary at the Food and Environment Research Agency, Sand Hutton, York, UK. Two brood frames were collected (September 2013) and placed in an environmental chamber (34°C, 60% relative humidity (RH), constant darkness) (Sanyo Versatile Environmental Test Chamber). Workers were collected from these frames as they emerged and housed in groups of 10 workers in plastic cages (modified 8 oz clear PET containers; FK-RD8 Ambican UK Ltd). The use of late-season workers gives added value to this study because in field scenarios, brood developing later in the season are likely to be exposed to the season's accumulation of agrochemicals, and the health of these workers is important for the over-wintering survival of the colony. Each cage allowed workers *ad libitum* access to a sucrose solution (50% w/v) through a modified microcentrifuge tube (1.5ml; Eppendorf UK Ltd) and all cages were maintained in an environmental chamber throughout the experimental period (34°C, 60% relative humidity (RH), constant darkness). For each feeding/injection treatment combination, three cages were used at the 8 h sampling point and two cages were used at the 24 h sampling point, giving three/two biological replicates for analysis respectively.

### 6.3.2. Neonicotinoid exposure

In order to simulate field-relevant exposures to residues in the nectar and pollen of neonicotinoid-treated crops, bees were exposed to sublethal concentrations of pesticides as a chronic oral exposure. Bees were exposed to either thiamethoxam at 10 parts per billion (ppb) (e.g mean  $\pm$  SD thiamethoxam residues in squash of  $11 \pm 6$  ppb; Stoner and Eitzer, 2012), or imidacloprid at 102 ppb, which is at least ten-fold higher than typical residues in the field (typically 1-10 ppb; Blacquiere et al., 2012), but which may represent a 'worst-case' scenario (residues as high as 101 ppb have been found in pollen from imidacloprid-treated transplant-dripped pumpkin crops; Dively and Kamel, 2012). Stock solutions of neonicotinoids were made up in acetone, and stocks

were subsequently used to create known concentrations in sucrose solution (50 % w/v). Treated sucrose was spiked with either 12  $\mu\text{gL}^{-1}$  (= 10 ppb) thiamethoxam (Fluka Analytical 37924) or 125  $\mu\text{gL}^{-1}$  (= 102ppb) imidacloprid (Fluka Analytical 37894). Treated sucrose replaced control sucrose in the cages, in which bees were allowed *ad libitum* access to the spiked-sucrose throughout the experimental period. Control cages contained sucrose with < 0.1 % acetone to control for the solvent in which pesticide stocks were dissolved. The feeder of each cage was weighed every other day and any dead bees were removed and recorded to allow the mean daily sucrose consumption per bee to be calculated. When calculating consumption rates, we accounted for evaporation using reference feeders kept in empty cages.

### 6.3.3. Immune challenge

In order to invoke a standardised immune response across individual bees, bees were injected with bacterial lipopolysaccharides (LPS) to mimic a bacterial-like infection because this artificial stimulus activates the immune response while excluding the dynamic behaviour of a real pathogen. To immobilise bees for injection, experimental cages were placed briefly in a freezer (-20 °C) for approximately two to five minutes or until bees were immobile. All injections were performed using a fine needle on a Hamilton syringe. Honey bees were injected with 2  $\mu\text{l}$  of solution through the pleural membrane between the tergites (dorsal side) of the abdomen. Bees received one of three immune treatments, with all workers within a cage subjected to the same treatment. The 'LPS' treatment group were injected with 0.5  $\text{mgml}^{-1}$  LPS (Sigma L2755- Lipopolysaccharides from *Escherichia coli* 0128:B12) dissolved in Insect Ringers solution. The 'Ringers' treatment group were injected with a placebo dose of Insect Ringers solution (1:1:1 156 mM NaCl: 3 mM KCl: 2 mM  $\text{CaCl}_2$ ) to control for any effects observed as a result of the injection process itself. 'Naïve' bees were ice-immobilised but received no further treatment as an unchallenged control.

### 6.3.4. RNA Extraction, Library Preparation, and Sequencing

At each sampling point, bees were frozen at -80 °C and stored until RNA extraction. Total RNA was extracted from pools of 6 whole bees from a single cage. Pooling of 6 bees per replicate cage served to minimise between-bee variation in gene expression profiles. Total RNA was extracted using the mirVana miRNA Isolation Kit (Ambion AM1561; Life technologies). 1 µg of total RNA was DNase-treated to remove any genomic DNA in the RNA extract using RNase-free DNase I (Fermentas; EN0521) according to the manufacturer's protocol. Prior to library preparation, 1 µg of DNA-free RNA was treated to remove rRNA using the Ribo-Zero Magnetic rRNA Removal Kit (Human/Mouse/Rat) Low Input (Epicentre Biotechnologies; MRZH11124) according to the manufacturer's protocol. This rRNA-depleted sample was purified using the Agencourt RNAClean XP Kit (Beckman Coulter; A63987). RNA-Seq libraries were subsequently prepared from 1 µg Ribo-Zero treated RNA using the ScriptSeq v2 RNA-Seq Library Preparation Kit (Epicentre Biotechnologies; SSV21124) according to the manufacturer's protocol. During library amplification, a unique Index was added to each library (ScriptSeq Index PCR Primers (All Sets) - Epicentre Biotechnologies; SSIP1234). On completion, the concentration of DNA in each individual sample library was quantified using a Qubit 2.0 Fluorometer (Life technologies). 15 libraries were pooled to give a pooled library containing equal concentrations of each individual library. The DNA concentration of the pooled library was subsequently quantified by Qubit, and the quality and size distribution of the library assessed using a 2200 TapeStation (Agilent technologies). The DNA concentration and average size of the library were then used to calculate the target template concentration for RNA-sequencing. Each pooled library was sequenced using a paired-end 100bp lane on the Illumina HiSeq 2500 system (sequencing was performed by staff at the University of Exeter's Sequencing Service).

#### *6.3.5. Validation of RNA-Seq data with qPCR*

The results of the RNA-Seq analysis (see below) identified DEGs of interest for further study, including an aldehyde dehydrogenase gene, ALDH1L2. This gene was found to be upregulated following immune challenge, and the data indicated a potential impairment of this response to immune challenge when

bees were simultaneously exposed to thiamethoxam (see Results). To further test this hypothesis, ALDH1L2 was selected for further investigation across a greater range of time points PIC (0 to 48 h) and changes in its expression were measured using qPCR, following the methods described in Chapter 3. Oligonucleotide primers were used to amplify genes at a concentration of 900 nM (ALDH1L2 Forward primer: GGGCCACAGAACCATAAAGC, ALDH1L2 Reverse primer: CGCCACGTTCCACAAATTTC).

#### 6.4. Data analysis

##### 6.4.1. Data processing for read counts

For each of the 45 sample libraries, the total number of raw sequencing reads obtained was on average 19699691 ( $\pm$  SE 2028338), ranging from 5962002 to 73572872. To remove error-prone regions of sequence, the original data reads were trimmed and filtered using *sickle* (Joshi and Fass, 2011), with a Phred-scaled quality threshold of 30 and a minimum length threshold of 50. Any reads shorter than this minimum length cutoff, and any reads left unpaired after trimming, were excluded from further analysis (excluding an average of 15 % of reads per library; see Supporting Information: Figure 6.6.).

Since initial analysis of two libraries recognised that > 50 % of reads did not align to the *A. mellifera* genome (see below), the following steps were included prior to *A. mellifera* alignment in attempt to account for some of the unmapped reads. The *Bowtie2* alignment tool (version 2.1.0; Langmead et al., 2009) was used to first align any reads to genomes of the bacteriophage PhiX174 and the bacteria *Escherichia coli* O157. *SAMtools* (Li et al., 2009) was then used to filter reads that did not map to either of these genomes and these remaining reads were used for subsequent analyses. Alignment to PhiX was included as a precaution since samples were spiked with a 1 % PhiX sequence as a quality control during sequencing, but these sequences should have been filtered out prior to receiving the sequencing files. Alignment to *E. coli* was included as a precaution in case of *E. coli* sequence contamination given that some bees were injected with LPS from *E. coli* as part of the experimental design. However, < 2 % of reads aligned to the PhiX and *E. coli* genomes (see Supporting Information:

Figure 6.6.), confirming that neither the PhiX spike, nor LPS injection, significantly contaminated reads.

In order to align reads to honey bee genes, the splice aware mapping tool, *Tophat* (version 2.0.11; Kim et al., 2013), was used to align reads to the *Apis mellifera* reference genome (assembly Amel\_4.5) (Honey Bee Genome Sequencing Consortium, 2014) and *SAMtools* (Li et al., 2009) was used to filter for paired aligned reads. Of the original raw reads, approximately 30 % aligned to the *A.mellifera* genome, of which two thirds were paired reads (black sections of Supporting Information: Figure 6.6.), and subsequently used in the analysis of read counts and differential expression. In general the percentage of sequenced reads that aligned to the *A. mellifera* genome was similar across samples, but over 50 % of reads remained unmapped (see Supporting Information: Figure 6.6.), with their identity unclear. It is possible that some of these reads could map to natural sources of infection in honey bees, such as bacterial and fungal spores that these experimental bees could have been exposed to as larvae in the field prior to their emergence in the laboratory. Furthermore, it is recognised that adult honey bee workers typically harbour a characteristic gut microbiota, including at least nine bacterial phylotypes (Martinson et al., 2011, Martinson et al., 2012, Moran et al., 2012). Many of these bacteria may have a mutualistic relationship with their bee host by aiding in the metabolism of carbohydrates and/or producing inhibitory compounds against pathogens (Martinson et al., 2011). In the present study RNA was extracted from whole bee samples so it is hypothesised that a large proportion of the sequences unaccounted for by the current analysis are associated with the gut microbiota. Further work could benefit from annotating the unmapped reads in these samples to elucidate their origin, as this too could provide new insights, particularly if treatment effects are present, such as a differential gut microbiota activity associated with immune/pesticide treatments. Nevertheless, there will be no further discussion of those unmapped reads here, and for the purposes of the current investigation it was assumed that the unmapped reads could be overlooked given that the percentage of raw sequence reads that were aligned to the reference honey bee genome was relatively consistent across

samples, and any differences in library size were accounted for by the normalisation step of the *DESeq2* pipeline below.

In order to count how many reads mapped to each honey bee gene, we used *HTSeq* (version 0.6.0; Anders et al., 2014).

#### 6.4.2. Statistical analysis for identification of differentially expressed genes

In order to analyse variation among treatments in gene expression, the raw counts of sequencing reads obtained from *HTSeq* were used as count values for the analysis of differential gene expression using the R package *DESeq2* (Version\_1.2.10; Anders and Huber, 2010). A gene was considered as differentially expressed when the  $\log_2$  fold change was  $\geq 1$  or  $\leq -1$  (i.e. at least a two-fold increase or decrease in expression) and when the false discovery rate (FDR)- adjusted P-value was  $\leq 0.05$ . DEGs were identified for contrasts of interest by first sub-setting the sample set (see below). This multiple testing approach was corrected for in the adjusted P value/ FDR estimation performed automatically in the *DESeq2* pipeline.

First, to analyse whether there was a transcriptional response to immune challenge, we identified DEGs between injection treatments in bees fed only on control sucrose (i.e. in the absence of any pesticide exposure) for each time PIC in turn. Pairwise comparisons (contrasts) identified genes that were differentially expressed i) following Ringers-injection compared to naïve unchallenged controls, ii) following LPS-injection compared to naïve controls, and iii) following LPS-injection compared to Ringers-injection.

Second, to analyse whether there was a transcriptional response to pesticide exposure, we identified DEGs between pesticide treatments in naïve bees (in the absence of an additional immune challenge). Here, we were interested in whether genes were differentially expressed in imidacloprid- or thiamethoxam-exposed bees compared to control-fed bees, so only two contrasts were tested.

Third, to analyse whether the transcriptional response to immune challenge was maintained when bees were also exposed to neonicotinoid treatment, we focussed our analysis on the Ringers vs LPS contrast 8 h PIC, to identify

whether the particular DEGs in this contrast varied for each pesticide treatment. This immune contrast was seen of most biological relevance as it reflected transcriptional responses only to the bacterial molecules (albeit artificial), but not to the injection procedure itself.

Lastly, to analyse whether the transcriptional response to pesticide exposure was maintained when bees were also exposed to immune challenge, we compared the DEGs in each control-imidacloprid or control-thiamethoxam contrast for each injection treatment group.

#### 6.4.3. Functional analysis of differentially expressed genes

In order to identify the molecular function and/or molecular pathways to which the DEGs are associated, we performed functional analyses. This involved first assigning each honey bee gene to known functions ('gene ontology, GO, annotation') and then assessing whether any functions were under- or over-represented in the DEGs compared to the honey bee genome as a whole ('GO enrichment analysis'). Honey bee genes were also assigned to known molecular pathways using the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway database.

We compared two annotation methods to best assign functions to each honey bee gene, as the honey bee genome remains relatively poorly annotated so the functional role of expressed genes remains poorly understood.

The first annotation method used a TBLASTX search to tentatively assign molecular function terms to each honey bee gene based on the GO terms associated with their *Drosophila melanogaster* fruit fly orthologues. Honey bee RNA-Seq studies have typically assigned functional terms to honey bee genes based on orthology to fruit fly genes (e.g. Cornman et al., 2013, Derecka et al., 2013), as the fly has been used as a model insect species in molecular studies and has a greater percentage of functionally annotated sequences. A TBLASTX search of all *A.mellifera* translated sequences was run against a list of *D.melanogaster* translated sequences. Annotation of honey bee genes with FlyBase IDs was taken only when TBLASTX matches had an E value  $\leq 1e^{-10}$ ,

matched across 90 % of the gene length, and had a one-to-one orthology relationship between bee and fly genes. However, it is well recognised that this approach poses limitations on the functional annotation of honey bee genes (e.g. Chan et al., 2011), not least because the evolutionary distance between *Drosophila* and *Apis* is relatively large ( $\approx$  300 million years). This likely explains the low number of honey bee genes (16 % of the 15304) we found to match to fly genes and the poor annotation of only 14 % of bee sequences that were assigned GO terms using this approach (see Supporting Information: Figure 6.7.).

To aim to assign GO terms to a greater proportion of honey bee genes, we used a second alternative annotation method in which genes were matched to protein orthologues of other species (not limited to *Drosophila*) and annotated with GO terms using BLAST2GO. We performed a BLASTX of all *A.mellifera* gene sequences against the non-redundant coding sequence database, *nr* (downloaded from NCBI on 5<sup>th</sup> June 2014). As expected, this gave a greater percentage of successful hits (54 %), and amongst the top species to which honey bee genes matched included other bee species (*Bombus* spp. and *Megachile* spp.), and other Hymenopteran social insects including ants (e.g. *Cerapachys biroi*, *Camponotus floridanus*) and wasps (e.g. *Nasonia vitripennis*). The hits obtained from this BLAST search were then mapped and annotated using Blast2GO (Conesa et al., 2005) and the InterPro annotation function to retrieve GO terms that were tentatively assigned to the *A.mellifera* gene sequences. Nevertheless, functional annotation was still highly restricted, with only 44 % of the 15304 bee gene sequences tentatively assigned GO terms, giving a reference set of 6779 sequences for subsequent GO term enrichment analysis (see Supporting Information: Figure 6.7.). This annotation is comparable with a previous annotation of honey bee protein sequences based on orthology to other species using BLAST2GO, in which 37 % of honey bee sequences were assigned GO terms (Chan et al., 2011). We encourage future studies to adopt this annotation approach in preference to a *Drosophila*-orthology based method as here we demonstrate the improved annotation this

approach offers, and hence further GO and pathway enrichment analysis was based on this second annotation method.

GO enrichment analysis was performed by comparing lists of DEGs to the background set of annotated hits using a Fisher's Exact Test and correction for multiple testing in Blast2GO. Enriched GO terms were reported at an FDR of 0.05.

Similarly, pathway annotation was performed by assigning honey bee genes with enzyme codes using Blast2GO, and pathway maps were retrieved from the KEGG database to list any pathways containing the enzyme code numbers of selected sequences.

## *6.5. Results*

We first characterised the transcriptomic responses of adult honey bee workers when exposed either to an immune challenge or a neonicotinoid exposure alone. We then assessed whether the response to immune challenge was altered in the presence of neonicotinoid exposure, and similarly whether the response to neonicotinoid exposure was altered in the presence of immune challenge.

### *6.5.1. Transcriptional response to immune challenge without neonicotinoid exposure*

Overall, a greater number of DEGS were found 8 h PIC compared to 24 h, with more genes upregulated in response to immune challenge than were downregulated (Figure 6.1.). Focussing on the 8 h dataset, five genes were upregulated irrespective of the specific immune challenge (i.e. whether injected with Ringers placebo or bacterial LPS), but LPS injection led to greater upregulation than Ringers injection alone (Figure 6.1.). These five upregulated genes included three well-known immune genes associated with the Toll pathway (Evans et al., 2006)- a peptidoglycan recognition protein (Pgrp-s2) and two antimicrobial effector proteins, apidaecin (Apid1) and hymenoptaecin (LOC406124). Additionally, the ETS homologous factor-like gene (LOC552797)

was recognised from GO annotation for its role in the regulation of transcription, whilst the laccase-1-like gene (LOC724890) is thought to be involved in copper ion binding and oxidoreductase activity.

Overall, 25 genes were upregulated and 15 genes were downregulated in LPS-injected bees compared to those challenged only with a Ringers placebo, and the top five up- and down- regulated genes (based on the largest fold-changes in expression) are summarised in Table 6.1., along with their associated GO annotations, but no enriched gene functions or pathways were identified.

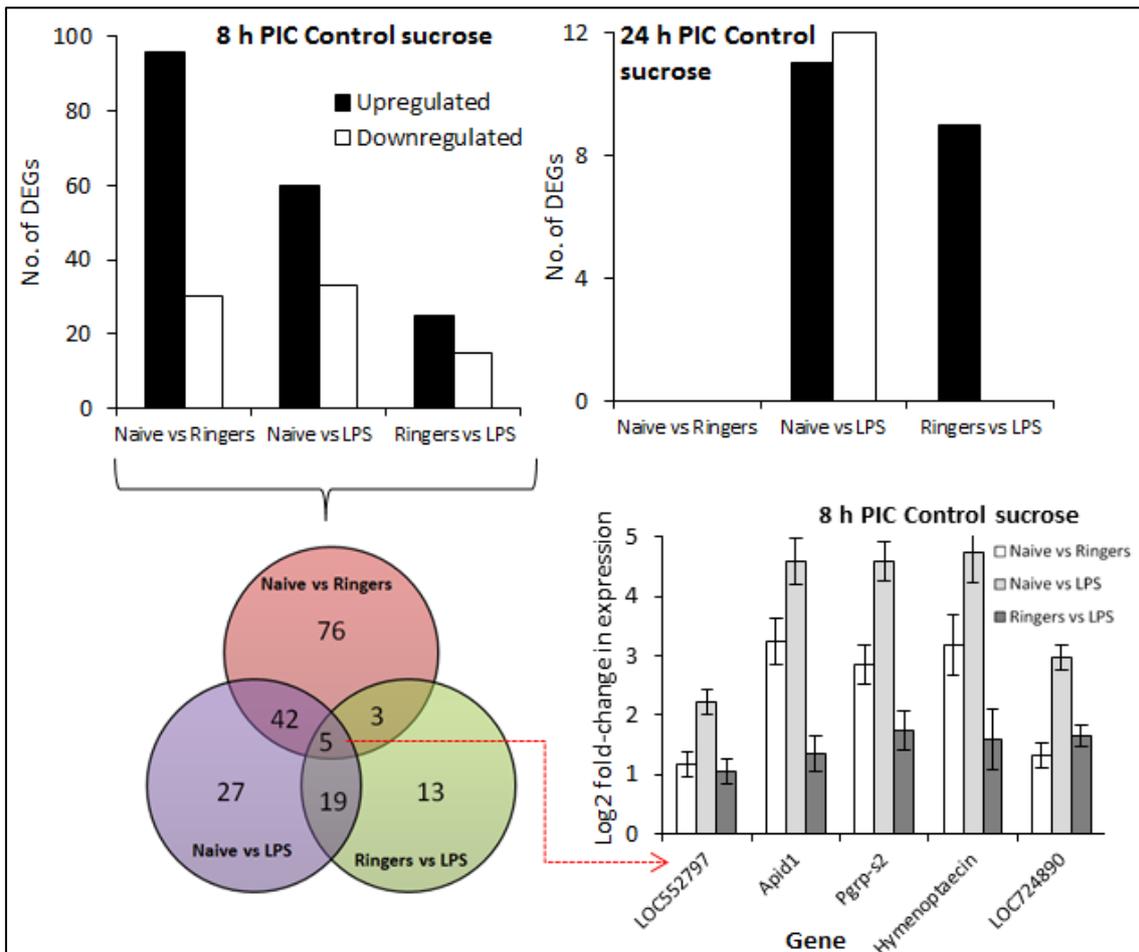


Figure 6.1. Top: Number of DEGs between pairwise comparisons of injection treatment in control-fed bees, 8 and 24 h PIC. e.g. the Naive vs Ringers comparison refers to no. of genes up- or down- regulated in Ringers-injected samples compared to naïve samples. Black bars = Upregulated genes, White bars = Downregulated genes. Bottom: Venn diagram to show the number of DEGs in the 8 h samples that overlap among the injection treatment comparisons. Bar chart to show the magnitude of expression between the three injection contrasts for each of the five DEGs that were shared between these contrasts in control-fed bees 8 hours PIC. Log2 fold-change mean  $\pm$  SE.

*Table 6.1. Summary of the top five upregulated and top five downregulated genes in the control-fed Ringers vs LPS injection contrast 8 h PIC.*

Gene	Beebase ID	NCBI Official Full Name	Annotated GO terms	Log2 fold change	SE of fold change
LOC414027	GB51005	n-alpha-acetyltransferase 20, NatB catalytic subunit-like	C:cytoskeleton	2.402168	0.795074
LOC410884	GB50722	phospholipase A1 member A-like	P: lipid metabolic process; F: catalytic activity	2.285008	0.692504
ALDH1L2	GB43892	aldehyde dehydrogenase 1 family, member L2	F: hydroxymethyl-, formyl- and related tranferase activity; P: biosynthetic process; P:oxidation-reduction process; F: oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor; F: methyltransferase activity	2.030762	0.180568
LOC100576592	GB50550	uncharacterised	none	1.997877	0.404135
LOC727401	GB43783	uncharacterised	none	1.866179	0.255861
GB40915	GB40915	uncharacterised	none	-2.24068	0.733396
LOC410870	GB49753	otopetrin-3-like	none	-1.89129	0.498082
LOC726283	GB46153	neuronal calcium sensor 2-like	calcium ion binding	-1.88963	0.64018
GB40683	GB40683	uncharacterised	P: transmembrane transport; F: transmembrane transporter activity; C: integral to membrane	-1.58177	0.536571
LOC726790	GB54723	uncharacterised	F: ATP binding; P: protein phosphorylation; F: protein serine/threonine kinase activity	-1.58072	0.481059

### 6.5.2. *Transcriptional response to immune challenge with neonicotinoid exposure*

The transcriptional response to LPS challenge was dependent on the feeding regime of the adult bees (Figure 6.2.). Inspection of fold-changes in expression suggests that the upregulation of three genes (Rel, LOC100577430 and ALDH1L2) may be weakened in thiamethoxam-exposed bees (Figure 6.2.). We tested this further only for the ALDH1L2 gene, using a qPCR approach. We thereby confirmed that ALDH1L2 was upregulated in response to immune challenge in a time-dependent manner (ANOVA; Injection\*Time  $F_{10,82} = 8.75$ ,  $P \leq 0.001$ ), but we could not verify that this response was detectably altered by neonicotinoid exposure (Figure 6.3.).

22 DEGs that were identified in response to LPS injection without neonicotinoid exposure were not identified when bees were simultaneously exposed to either of the two neonicotinoids (Figure 6.2. Venn Diagram). No enriched GO terms were identified for this set of 22 DEGs, but further inspection identified several potential genes of interest. This included the upregulated antimicrobial peptide genes apidaecin (Apid1) and hymenoptaecin (LOC406142), and the downregulated scavenger receptor class C gene (SCR-C), which was annotated with an 'immune response' GO term and has previously been found to be downregulated in honey bees infected with an entomopathogenic fungus (Bull et al., 2012). Similarly, we found genes that responded to immune challenge only when exposed to imidacloprid or thiamethoxam (Figure 6.2.), but no enriched GO terms were identified.

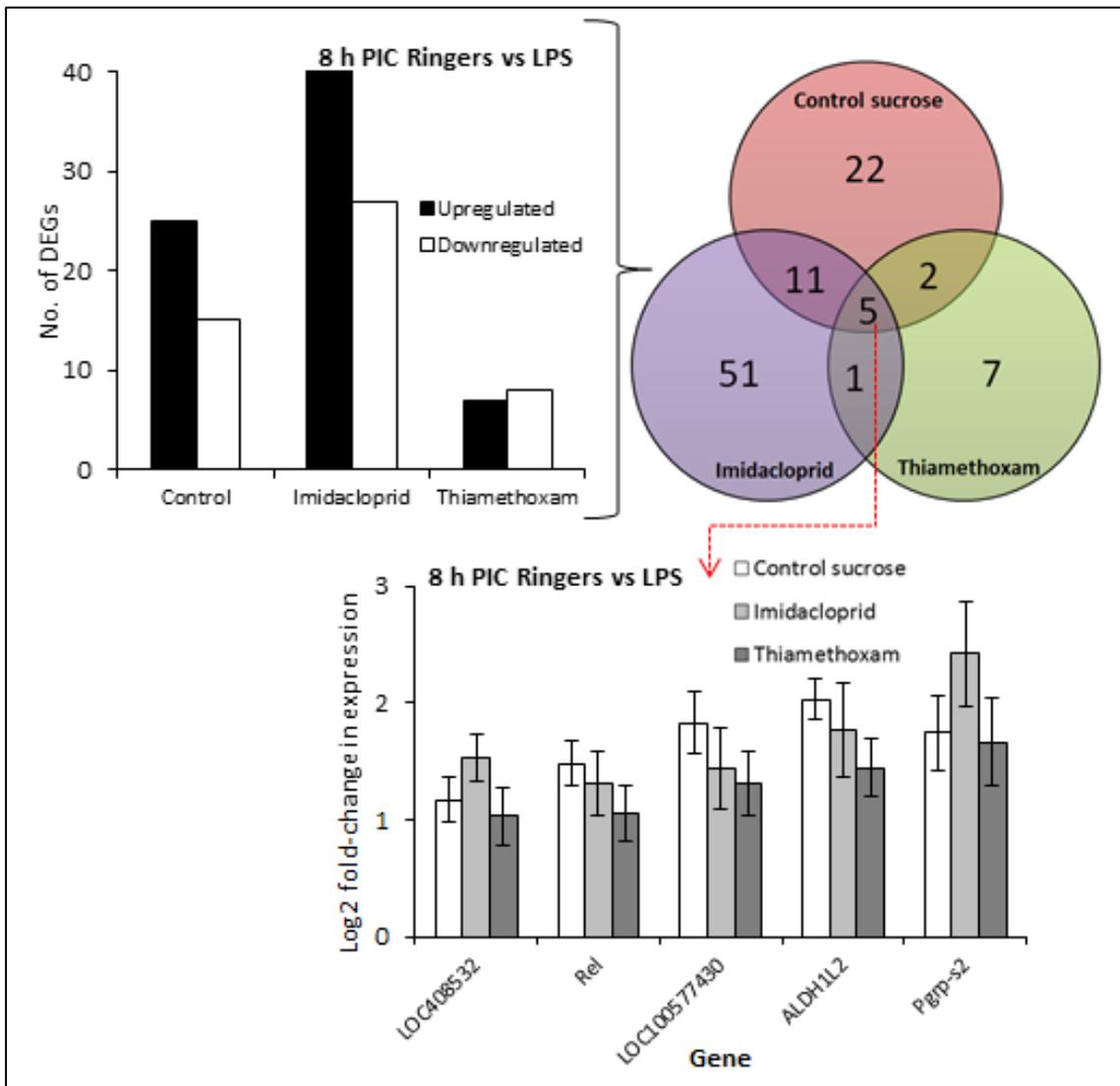


Figure 6.2. Top: Number of genes up- or down- regulated in LPS-injected samples compared to Ringers-injected samples 8 hours PIC in each pesticide group. Venn diagram shows the number of these DEGs that overlap among these contrasts in each pesticide treatment. Bottom: Bar chart to show the magnitude of expression following LPS injection compared to Ringers injection in each pesticide treatment, for each of the five DEGs that were shared between these contrasts. Log<sub>2</sub> fold-change mean  $\pm$  SE.

Table 6.2. Description of the five genes that were upregulated in LPS-injected bees compared to Ringers- injected bees in all three pesticide groups (i.e. the five genes that overlap in Figure 6.2.)

GeneID	BeeBase ID	NCBI Official full name	GO terms
LOC408532	GB50123	myophilin-like	none
Rel	GB40654	NF-kappaB transcription factor relish	F: protein binding; P: regulation of transcription, DNA-dependent; F: sequence-specific DNA binding transcription factor activity; C: nucleus
LOC100577430	GB43784	uncharacterised	none
ALDH1L2	GB43892	aldehyde dehydrogenase 1 family, member L2	F: hydroxymethyl-, formyl- and related transferase activity; P: biosynthetic process; P: oxidation-reduction process; F: oxidoreductase activity, acting on the aldehyde or oxo group of donors, NAD or NADP as acceptor; F: methyltransferase activity
Pgrp-s2	GB47805	peptidoglycan recognition protein S2	F: N-acetylmuramoyl-L-alanine amidase activity; P: peptidoglycan catabolic process; F: zinc ion binding

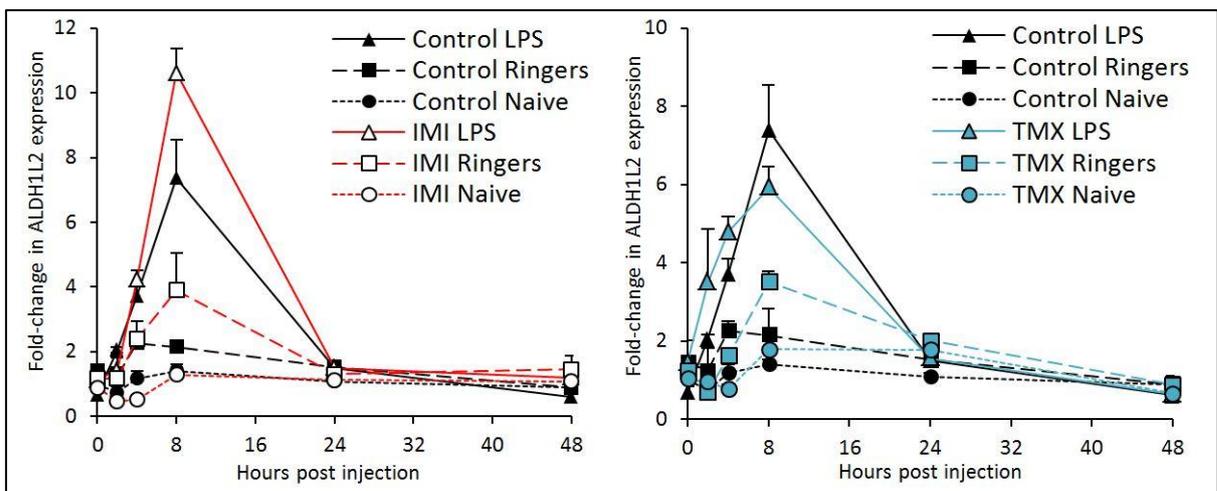


Figure 6.3. Relative expression of ALDH1L2 gene in response to immune challenge over a period of 2 - 48 h PIC. Mean  $\pm$  SE fold-changes are shown relative to expression in control-fed, naïve (unchallenged) bees at the 0 h time point. Left plot shows expression profile for control and imidacloprid-exposed bees. Right plot shows expression profile for control and thiamethoxam-exposed bees.

### *6.5.3. Transcriptional response to pesticide exposure without immune challenge*

Thiamethoxam exposure led to 31 DEGs in the 8 h samples and 164 DEGs in the 24 h samples. Further analysis of the differential expression to thiamethoxam was focussed on the 24 h samples. Among the 164 DEGs, 80 % were downregulated following thiamethoxam exposure. Genes found in the ribosome and mitochondrion were over-represented in this data set, including genes involved in maintaining the structure of the ribosome, the translation of proteins in the ribosome, and the catalysis of redox reactions (see Supporting Information: Figure 6.8.). Six genes were associated with a single pathway (oxidative phosphorylation), and several others were associated with other metabolic processes (Table 6.3.). Only a single gene (GstO1) was associated with drug metabolism pathways, and here this gene was downregulated in the thiamethoxam-exposed bees.

Imidacloprid caused a different pattern in differential expression. Overall, 27 genes were upregulated and five genes were downregulated in imidacloprid-exposed bees in the 8 h samples, whilst only 23 genes were downregulated in the 24 h samples, and there were no enriched gene functions. Nine genes had identifiable associations with several metabolic pathways, including pyruvate-, purine-, glycine-, serine-, threonine-, cysteine-, and propoanoate- metabolism, glycolysis/ gluconeogenesis and oxidative phosphorylation. Imidacloprid exposure led also to upregulation of two genes (CYP6AS16P and CYP6AS15) with known function as cytochrome P450s and another gene (LOC726222) associated with the P450 pathway, which has a known role in detoxification of xenobiotics.

**Table 6.3.** KEGG pathways associated with DEGs in the Control-Thiamethoxam contrast (naïve bees only, 24 h sample set).

KEGG Pathway	No. of DEGs associated with pathway	Enzyme	Enzyme ID	DEGs associated with pathway	Pathway ID
Carbon fixation pathways in prokaryotes	1	hydratase	ec:4.2.1.3	LOC408446	map00720
Oxidative phosphorylation	6	reductase (H <sup>+</sup> -translocating)	ec:1.6.5.3	LOC724827, Ndufs7	map00190
Oxidative phosphorylation	6	dehydrogenase	ec:1.6.99.3	GB55150	map00190
Oxidative phosphorylation	6	oxidase	ec:1.9.3.1	Cox5b, Cox6c, LOC552829	map00190
Drug metabolism - cytochrome P450	1	transferase	ec:2.5.1.18	GstO1	map00982
Citrate cycle (TCA cycle)	1	hydratase	ec:4.2.1.3	LOC408446	map00020
Metabolism of xenobiotics by cytochrome P450	1	transferase	ec:2.5.1.18	GstO1	map00980
Phenylalanine metabolism	1	lactoperoxidase	ec:1.11.1.7	LOC412774	map00360
Glycosaminoglycan degradation	1	hexosaminidase	ec:3.2.1.52	LOC726818	map00531
Alanine, aspartate and glutamate metabolism	1	carbamoyltransferase	ec:2.1.3.2	Cad	map00250
Aminobenzoate degradation	1	acetylphosphatase	ec:3.6.1.7	LOC724904	map00627
Glutathione metabolism	1	transferase	ec:2.5.1.18	GstO1	map00480
Pyruvate metabolism	1	acetylphosphatase	ec:3.6.1.7	LOC724904	map00620
Various types of N-glycan biosynthesis	1	hexosaminidase	ec:3.2.1.52	LOC726818	map00513
Other glycan degradation	1	hexosaminidase	ec:3.2.1.52	LOC726818	map00511
Purine metabolism	1	kinase	ec:2.7.4.6	awd	map00230
Glycine, serine and threonine metabolism	2	dehydrogenase	ec:1.1.99.1	GMCOX3, GMCOX12	map00260
Glycosphingolipid biosynthesis - ganglio series	1	hexosaminidase	ec:3.2.1.52	LOC726818	map00604
Glycosphingolipid biosynthesis - globo series	1	hexosaminidase	ec:3.2.1.52	LOC726818	map00603
Glyoxylate and dicarboxylate metabolism	1	hydratase	ec:4.2.1.3	LOC408446	map00630
Phenylpropanoid biosynthesis	1	lactoperoxidase	ec:1.11.1.7	LOC412774	map00940
Amino sugar and nucleotide sugar metabolism	1	hexosaminidase	ec:3.2.1.52	LOC726818	map00520
Pyrimidine metabolism	2	kinase	ec:2.7.4.6	awd	map00240
Pyrimidine metabolism	2	carbamoyltransferase	ec:2.1.3.2	Cad	map00240
Porphyrin and chlorophyll metabolism	1	ceruloplasmin	ec:1.16.3.1	fh	map00860

#### *6.5.4. Transcriptional response to pesticide exposure with immune challenge*

The transcriptional response to thiamethoxam exposure was dependent on the immune treatment of the adult bees (Figure 6.4.). Specifically, there was a trend towards fewer DEGs when thiamethoxam-exposed bees were immune challenged (Figure 6.4.).

The proportion of genes up- or down- regulated in response to thiamethoxam exposure was not consistent across immune treatments. For example, in naïve bees 80 % of the 164 DEGs were downregulated, compared to the 80 % of the 63 DEGs that were upregulated in Ringers-challenged bees. In the 24 h samples, only three DEGs were identified irrespective of the immune challenge received (GB48920 upregulated, GB47541 and LOC726228 downregulated), and immune challenged bees did not share the same enriched GO terms as those identified in naïve bees (see above; Supporting Information: Figure 6.8.).

The transcriptional response to imidacloprid exposure was also dependent on the immune treatment of the adult bees, but the pattern varied to that seen for thiamethoxam exposure. A larger number of DEGs were identified in the 8 h samples than the 24 h samples, with the majority of these in LPS-challenged bees (70 upregulated, 64 downregulated; Figure 6.4.), where almost all were expressed uniquely compared to naïve or Ringers-challenged bees, but no enriched functions were identified.

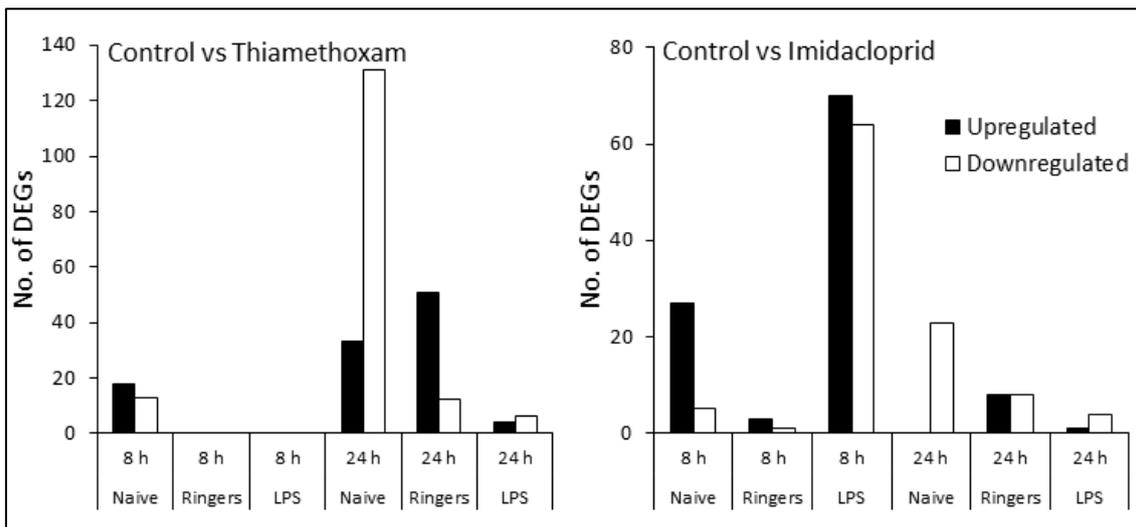


Figure 6.4. Number of up- or down-regulated genes in (left) thiamethoxam-exposed bees; and (right) in imidacloprid-exposed bees, for each injection treatment and time PIC.

### 6.6. Discussion

By using next generation sequencing technology, this study discovered differentially expressed genes (DEGs) and then identified their potential functions in adult honey bee workers exposed to an immune stressor, a dietary neonicotinoid stressor or a combination of both. Below, we discuss the functional implications of the DEGs detected for each stressor taken separately and we explore also the complex effects of the interactions between stressors. In particular, we pursue molecular evidence for the propositions that neonicotinoids affect the immunocompetence of honey bees, and that immune challenge affects the ability of honey bees to respond to neonicotinoid exposure.

Immune stress led to upregulation of genes with known immune function, including a peptidoglycan recognition protein, a NF- $\kappa$ B transcription factor and antimicrobial effectors. The response to LPS injection was typically stronger than to Ringers buffer alone. These findings support previous work targeting expression profiles of antimicrobial effector genes using qPCR (chapter 3), which begins to validate the use of the RNA-Seq approach to identify

functionally relevant DEGs. Different genes were expressed in response to a placebo injection challenge and an injection with bacterial LPS molecules, which demonstrates the importance of using relevant controls when using artificial methods to apply stressors. Additionally, the differential responses to wounding and bacterial infection highlight the possible importance of stressor combinations to individual bee health, which we discuss further below.

Transcriptional changes also occurred in genes that have not been previously associated with immune stressors, including an ETS homologous factor-like gene (LOC552797), a laccase-1-like gene (LOC724890), a myophillin-like gene (LOC408532) and the uncharacterised LOC100577430. We found also upregulation of an aldehyde dehydrogenase (ALDH1L2), corresponding with findings of a previous proteomic study, in which infection with the natural bacterium *Paenibacillus larvae* led to upregulation of an aldehyde dehydrogenase enzyme in honey bee larvae (Chan et al., 2009). These genes warrant further investigation to elucidate their role in responding to immune stressors.

The transcriptional response to immune challenge was dramatically changed when bees were also exposed to a dietary neonicotinoid, but the biological implications of this remain difficult to unravel. Specifically, 22 genes were identified that were differentially expressed in response to immune challenge alone, but others were additionally expressed when the immune stressor was coupled with dietary imidacloprid or thiamethoxam. Imidacloprid increased the number of DEGs in response to immune challenge, whereas thiamethoxam exposure had the opposite effect. These observations begin to suggest that the toxicants interact differently with bee metabolism despite their common neurotoxic mechanism. There was some indication that dietary thiamethoxam weakened the upregulation of Relish, which is a transcription factor in the NF- $\kappa$ B immune signalling pathway that has previously shown neonicotinoid sensitivity (Di Prisco et al., 2013). We suggest that further investigation of the neonicotinoid sensitivity of Relish could shed new light on the sublethal effects of insecticides on bee immunocompetence.

We found that a dietary neonicotinoid alone could alter the honey bee transcriptome, which begins to suggest that DEGs could be used as biomarkers of pesticide exposure. However, the pattern of gene expression differed across the two time points tested, highlighting the potential importance of the exposure period to effects on bee health.

Among the DEGs downregulated by dietary thiamethoxam, functional analysis found that several were associated with the translation of proteins in the ribosome, catalysis of redox reactions and metabolic pathways, including oxidative phosphorylation. Our results are similar to the only previous transcriptome-wide study of neonicotinoid exposure, which investigated imidacloprid (Derecka et al., 2013). Collectively, these results begin to suggest that neonicotinoids may have some common effects on the metabolism of honey bees. In bumble bees, neonicotinoid exposure can induce torpor (Cresswell et al., 2012) and honey bees may be showing a similar decrease in overall metabolic activity.

We found that dietary imidacloprid also altered genes associated with various metabolic pathways, including the upregulation of three genes involved in cytochrome P450 pathways. Our results are consistent with those of Derecka et al. (2013), who found nine cytochrome P450 genes that were upregulated in response to imidacloprid exposure in honey bee larvae. Cytochrome P450s are thought to be important in the detoxification of natural xenobiotics and synthetic insecticides in many insect species (Li et al., 2007), including pyrethroid and organophosphate metabolism in honey bees (Mao et al., 2011) and neonicotinoid resistance in *Drosophila* (Daborn et al., 2002). The 6AS subfamily of P450s is probably an important group of P450 genes in xenobiotic metabolism in honey bees (Mao et al., 2009), and it may be particularly important in the honey bee compared to other Hymenopteran insects. Our finding of the upregulation of two 6AS P450s in response to imidacloprid may provide new evidence in support of their role in the apparently rapid detoxification of neonicotinoids by honey bees (Cresswell et al., 2014).

The transcriptional response to neonicotinoid exposure was also altered when coupled with an immune challenge, but the interaction is difficult to interpret because the functional significance of the DEGs is largely obscure. Our study emphasises the need for further work to understand combinations of stressors, not least because our current ignorance confounds the development of biomarkers of pesticide exposure as bees are exposed to multiple stressors in many realistic scenarios.

The currently incomplete annotation of the honey bee genome has hindered the functional analyses of DEGs in our study. It is likely that given a greater percentage of GO-annotated sequences more enriched GO terms would have been identified. We therefore expect that the low number of enriched GO terms identified in the analysis is conservative for this reason. Similarly, the retrieval of KEGG pathways relies upon well annotated enzyme codes in the study set of genes, which also was not the case in our study. With this limitation to statistical tests of functional analysis, our assessment of DEGs was limited largely to a descriptive analysis, in which potential genes of interest were highlighted based on *a priori* interest in these genes, such as antimicrobial effector proteins, and/or recognition of their candidate functions where GO terms were assigned to individual genes. Given predicted improvements in gene ontology annotations, coupled with further bioinformatics expertise, we expect that future applications of the promising methodologies used in our study will provide further insights into the mechanisms by which pesticides and infections affect an economically and ecologically valuable pollinator, the honey bee.

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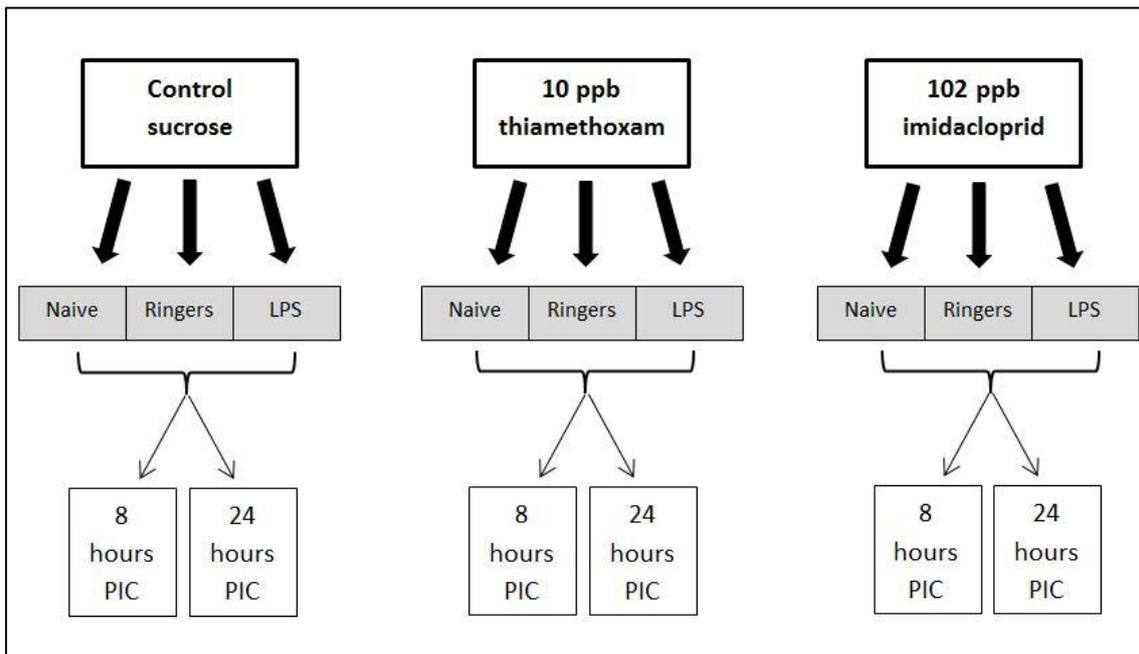
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## 6.9. Supporting information



*Figure 6.5.* Schematic of experimental design. For each pesticide exposure (top white boxes), bees received one of three immune challenges (middle grey boxes). For each pesticide/immune combination, samples were collected at either 8 or 24 h PIC.

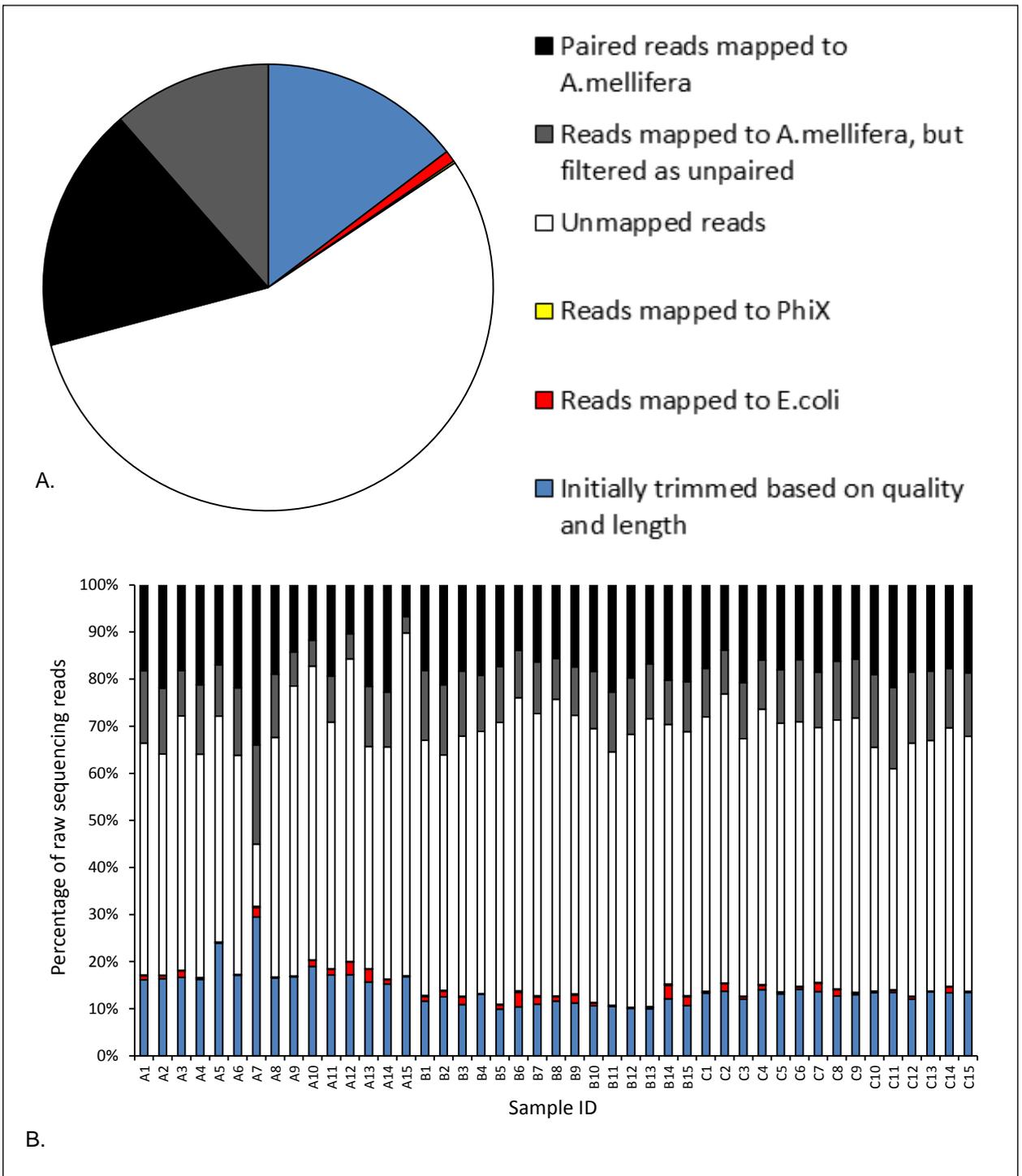


Figure 6.6. Percentage breakdown of raw sequencing reads following trimming and alignment pipeline. A. Pie chart depicting average of all reads across the 45 samples. B. Bar chart depicting breakdown for each sample in turn.

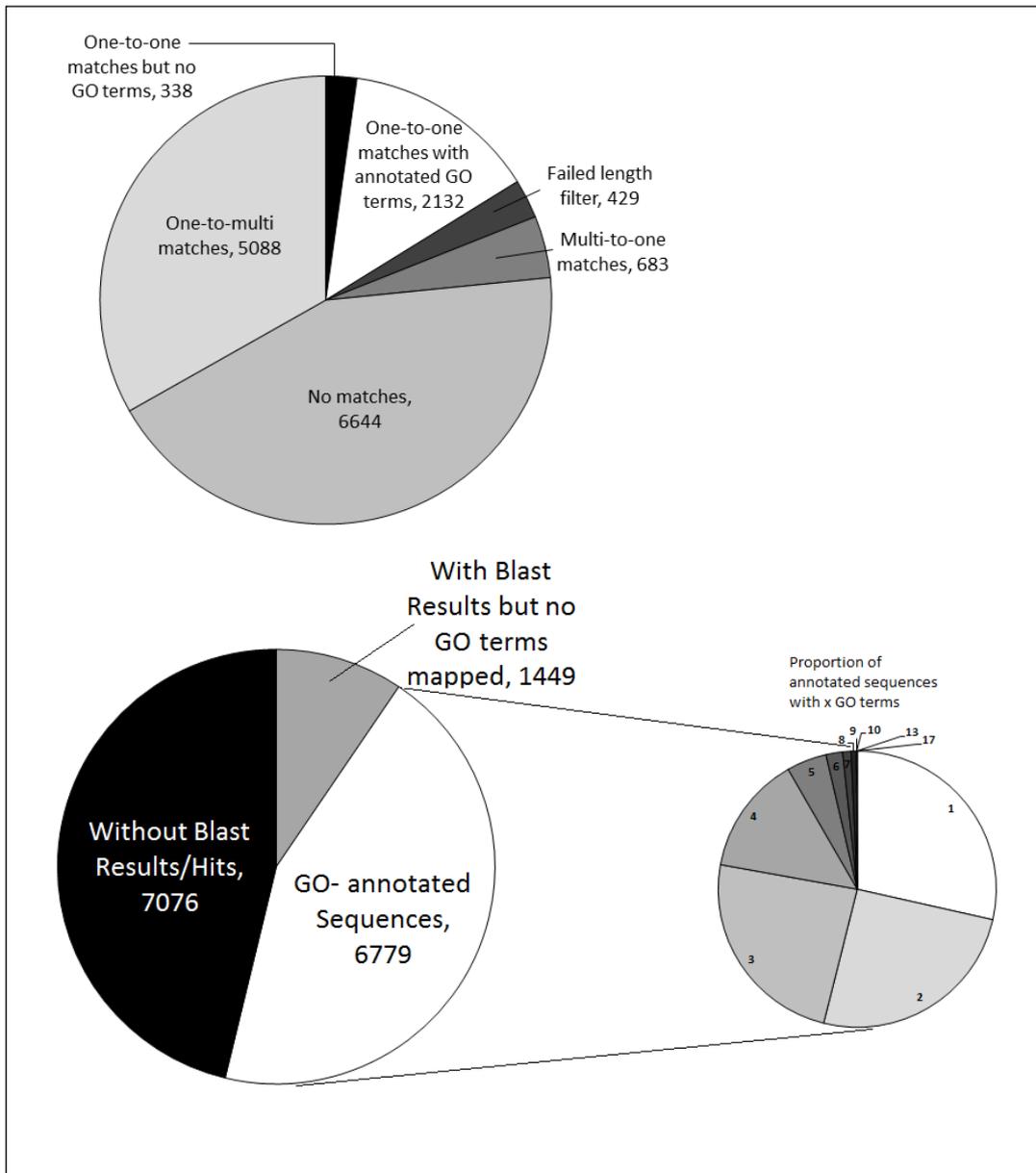


Figure 6.7. Pie chart representing the data distribution of *Apis mellifera* sequences following the functional analysis pipelines i) (Top chart) using a 1:1 homology TBLASTX against *Drosophila melanogaster* and annotation using GOrilla (Eden et al., 2009); and ii) (Bottom chart) using a BLASTX against the NCBI non-redundant coding sequence database and annotation using Blast2GO.

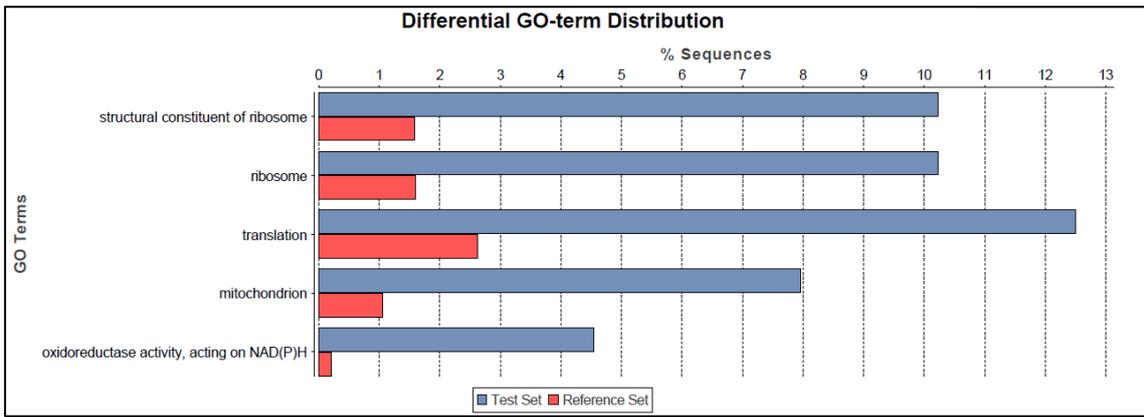


Figure 6.8. Summary of GO terms enriched across the 163 DEGs in the Naïve 24 h Control vs Thiamethoxam subset, at an FDR of 0.05.

## 7. General Discussion

### 7.1. Introduction and overview of main findings

In recent years there has been worldwide interest in understanding the stressors driving declines in bee health due to the environmental and economic implications of impacts on the pollination of crops and wild flowering plants (Potts et al., 2010). Two stressors implicated for their potential impact on bee health include pathogen infection and pesticide exposure, and in particular the neonicotinoid class of insecticides has been the focus of public and scientific concern. Neonicotinoid effects on honey bees and bumble bees have been well studied (Desneux et al., 2007, Goulson, 2013, Blacquiere et al., 2012), but there remains many uncertainties and knowledge gaps in the field. Recognition of a need for further research to address these uncertainties and knowledge gaps led to a two-year restriction on the use of three neonicotinoid pesticides on bee-attractive crops by the European Commission (2013).

Recent guidance recommended also an improved scheme for the risk assessment of plant protection products, including neonicotinoid pesticides, on bees (EFSA, 2013). An improved risk assessment includes the need for better understanding of the sublethal effects of pesticides on honey bees and bumble bees.

The central aim of this thesis therefore was to investigate sublethal effects of neonicotinoid exposure on honey bees and bumble bees, and to establish the relevance of different sublethal endpoints as appropriate ecotoxicological markers for risk assessment. In particular, this thesis focussed on the study of neonicotinoid exposure in combination with immune stressors, as a combination of stressors more likely reflects realistic ecological scenarios.

The first objective was to establish whether neonicotinoid exposure impacts on immune-related gene expression and enzymatic activity in adult workers of the honey bee, *Apis mellifera*. I found that exposures to imidacloprid and thiamethoxam impaired the transcriptional response of antimicrobial effector

genes to an artificial immune challenge, but no adverse effects were observed on antimicrobial enzyme activity or the phenoloxidase component of the insect immune response (Chapter 3).

Second, I established that exposure to the neonicotinoid, imidacloprid, impaired the physiological response of antimicrobial proteins to an artificial immune challenge in adult workers of the bumble bee, *Bombus terrestris*, but this adverse effect was not observed at realistic concentrations of neonicotinoid exposure. I found also no adverse effects on the phenoloxidase system (Chapter 4).

Third, I found impacts of imidacloprid exposure on gene expression and enzymatic activity associated with the development of the hypopharyngeal glands and their role in temporal polyethism, nutrition and social immunity in the honey bee, *A. mellifera*, but found no adverse effects on foraging behaviour at the colony level (Chapter 5).

Lastly, I identified transcriptome-wide transcriptional changes in adult honey bee workers following immune challenge and/or neonicotinoid exposure, elucidating potential gene functions and candidate ecotoxicological biomarkers (Chapter 6).

Here I discuss these findings and their implications in the broader context of the research field, and suggest areas where future work could provide valuable new insights and understanding.

## *7.2. Implications for pesticide risk assessment schemes*

The most recent guidance document on the risk assessment of plant protection products on bees (EFSA, 2013) suggested the implementation of a tiered risk assessment scheme in which a simple, cost-effective screening and ‘first tier’ scheme is followed by more complex higher tier semi-field and field studies. The scientific opinion on the science behind the development of this risk assessment scheme (EFSA, 2012) recommended that appropriate sublethal

effect studies should be incorporated into the first tier of testing and recognised a research priority to determine biologically relevant endpoints that could be easily measured and interpreted. Amongst the suggestions included the development of behavioural methodologies and potential biomarkers, including enzymatic/protein and molecular markers that could enable prediction of physiological sublethal effects, such as immune system changes. Biomarkers could be a valuable addition to the risk assessment scheme if they could be easily obtained from a small sample of individual bees and high-throughput assays could be carried out using routine laboratory techniques.

In any branch of toxicology it is recognised that biomarkers should meet several basic criteria if they are to be appropriate in measuring the extent of a toxic response, including that they should be quantitative, sensitive, non-invasive, specific, easily measurable; and should relate to biological mechanisms and work at realistic doses (Timbrell, 1998). The data available to assess these criteria in candidate bee biomarkers are insufficient to warrant their inclusion in current risk assessment schemes (EFSA, 2013).

It is hoped that data in this thesis contributes towards the assessment of several potential endpoints. Overall I found little support for the use of any given biomarker in this thesis, largely as any observed responses were typically at doses above realistic exposure scenarios. However, my work does support the need for inclusion of non-*Apis* bee species in risk assessment schemes as I found that responses to imidacloprid differed between honey bees and bumble bees, specifically in feeding rates and antimicrobial protein activity, providing further evidence that the sensitivity and the endpoints appropriate for risk assessment vary between bee species. This thesis also highlights the importance of verifying the functional biological relevance of endpoints, as contrary to expectation, transcriptional changes were not always found to relate to physiological changes in protein activity or behavioural modifications.

### *7.3. Pesticide effects on insect immunity*

Research into the interactions between multiple stressors impacting on bee health, including pathogen infection and pesticide exposure, has gained momentum only in the last five years or so and studies specifically investigating pesticide effects on bee immunity remain sparse. Furthermore, despite the significant increase in the use of neonicotinoids since their introduction in the 1990s, investigations of neonicotinoid effects on insect immunity in general are comparatively sparse compared to the effects of other pesticide classes (James and Xu, 2012). Neonicotinoids target receptors in the insect nervous system, but their effects on bees have been found to be manifold, including impairments of brood production (Laycock et al., 2012), foraging efficiency (Gill et al., 2012) and learning (Decourtye et al., 2004).

Investigations of neonicotinoid effects on the immune system seem appropriate given previously observed links between the insect nervous and immune systems (Mallon et al., 2003, Riddell and Mallon, 2006). In this respect this thesis provides valuable data to the limited field of knowledge. In particular, one major component of the insect immune response- the induction of antimicrobial protein activity- has not been previously studied in any insect species in respect to effects of any pesticide class, although some effects of neonicotinoid exposure on expression of bee AMP genes have been observed (e.g. Di Prisco et al., 2013). Here, as expected, an artificial wounding and/or bacterial-like challenge was consistently found to induce upregulation in expression of AMP genes and/or protein activity in honey bees and bumble bees. This thesis presents the first evidence showing that an oral imidacloprid exposure may impair the antimicrobial protein component of the immune response in bumble bees, although similar effects were not observed in honey bees. Over 100 insect antimicrobial proteins have been identified across several peptide classes (Hoffmann et al., 1996), and honey bees have been found to possess comparatively fewer antimicrobial proteins than other insect species, such as mosquitoes and flies (Evans et al., 2006). Further studies of the pesticide effects on the antimicrobial protein component of the insect immune response

could be valuable to a broader research field beyond bee ecotoxicology. In particular, an understanding of pesticide effects on the immune response of pest insect species could be valuable to improving their control, helping to safeguard agricultural crop production and control the transmission of insect-borne human disease through integrated pest management (IPM) systems. For example, the bacteria *Bacillus thuringiensis* (Bt) or Bt toxins can be used as biopesticides, but in some lepidopteran pests the induction of antimicrobial proteins or the phenoloxidase cascade in response to Bt infection can increase the tolerance of the pests to the biopesticide (Hwang and Kim, 2011, Rahman et al., 2011). If sublethal neonicotinoid concentrations were found to impair the immune response of these lepidopteran pests they could be used in combination with biopesticides to improve pest control by reducing pest resistance to Bt. In fact, Bt-toxins and imidacloprid are already used in some IPM systems (e.g. Bambawale et al., 2004). Insect pest management requires a balance between maintaining pest control whilst minimising the loss of beneficial insects, such as pollinators. New information could be gained through further investigation of the effects of different pesticides on the immune responses of both pest and beneficial insects to evaluate both the effectiveness and sustainability of IPM systems.

Here, I suggest three recommendations of work to include in any further investigation of pesticide effects on insect immunity. Firstly, future work would benefit from including measures of pesticide concentrations within the test animals. This will be informative in understanding the concentrations of pesticide leading to observed effects, as well as understanding specific tissues at risk of exposure. For example, in honey bees orally exposed to radio-labelled imidacloprid, higher imidacloprid concentrations were found in the midgut and rectum compared with the haemolymph (Suchail et al., 2004). This could be important in understanding the nature of a possible effect of oral imidacloprid exposure on the immune response as, for example, the midgut is a site of antimicrobial peptide synthesis. Whilst most studies have orally exposed bees to a pesticide *via* a sub-lethal chronic exposure, some have investigated effects of an acute topical (contact) exposure (e.g. Di Prisco et al., 2013). Different

routes of pesticide exposure potentially lead to differences in the toxicodynamics and the target tissues for the pesticide. It seems appropriate also to compare pesticide concentrations in bees exposed orally compared to those exposed topically to investigate this.

Secondly, as our understanding of the relevant tissues at risk and/or the roles of specific tissues improves, future work would benefit from testing protein activity and/or gene expression within the relevant tissues rather than composite structures, such as the whole bee or the whole head. Whilst the use of composite structures is a valuable starting point for initial studies with limited resources, this approach may confuse interpretation, for example if genes have variable patterns of expression in different tissues (Johnson et al., 2013). Within this thesis it is recognised that the observed effects of imidacloprid exposure on *mrjp*, *Amfor*, *malvolio* and *GOX* expression were observed only in the qPCR assays using mRNA extractions from bee heads (Chapter 5), but not in the RNA-Seq study using extractions from whole bees (Chapter 6), and it is possible that the different composite structures used in the studies influenced these results.

Lastly, when attempting to understand the mechanisms behind pesticide-disease interactions, most studies to date have approached this by testing the hypothesis that pesticide exposure leads to reduced immunocompetence. Pesticide exposure could potentially result in an increase of resource allocation towards detoxification systems in a trade-off through a reduction of resource allocation towards immune responses. There has been less attention directed towards testing the alternative hypothesis that pathogenic infection and/or parasitic attack could result in an increased allocation of resources towards immune responses in a trade-off through a reduction of resource allocation towards detoxification systems. Further consideration is warranted on the effects of pathogen infection on bee detoxification mechanisms.

#### *7.4. Questions raised*

Here I propose key areas of research that would address some of the questions raised in this thesis and improve our understanding of the implications of neonicotinoid exposure on bee health.

##### *7.4.1. Does field relevant neonicotinoid exposure affect bee nursing roles and larval development?*

Work in this thesis (Chapter 5) found the first evidence that an imidacloprid exposure likely at a concentration exceeding realistic concentrations in field residues impaired the gene expression of major royal jelly proteins, which are typically produced by nurse bees to feed developing larvae. A separate study of immune-related genes (Chapter 3), examining the relationship between transcriptional and physiological responses to imidacloprid exposure, found that changes at the gene level were not detected at the protein level. There, as yet, has been no investigation of the effects of neonicotinoid exposure on the production of major royal jelly proteins, nor of the implications of changes in protein production to the development of larvae. A study of this kind would benefit from investigation of several neonicotinoid pesticides, including field relevant exposure scenarios, and could provide valuable insights into the transgenerational effects of neonicotinoids on the developing brood as a result of exposure to adult workers. This work would be valuable to regulators and policy makers due to its relevance to the risk assessment of honey bee hypopharyngeal glands, and the potential impact of neonicotinoid exposure at the colony- and population- levels. Changes in larval development have the potential to impact colony sizes, and consequently colony production and overwintering success, as well as the production of queens, and consequently population success.

Further studies to improve our understanding of hypopharyngeal gland secretions and nursing roles in non-*Apis* bee species will enable a better evaluation of the endpoints appropriate for pesticide risk assessment in different bee species. For example, the role of the bumble bee hypopharyngeal gland

has been poorly studied in comparison to that in the honey bee, but it warrants further investigation given the observed effects of imidacloprid exposure on honey bee hypopharyngeal glands (Heylen *et al.*, 2010; Smodis Skerl and Gregorc, 2010; Hatjina *et al.*, 2013) and their recommended inclusion in pesticide risk assessments (EFSA, 2013). The bumble bee hypopharyngeal gland is known to be comparatively smaller than those of honey bees, and only a single major royal jelly protein (BtRJPL) has been identified, which may not have a nutritive role as in honey bees (Kupke *et al.*, 2012). Comparative glucose oxidase levels have been found also in honey bee and bumble bee honey (Dornhaus and Chittka, 2004), but there has been no investigation of the role of glucose oxidase, or neonicotinoid effects on hypopharyngeal gland secretions, in bumble bees. Furthermore, bumble bee workers are not thought to show the same temporal polyethism as honey bees, and bumble bees more typically maintain a given role in the colony throughout their life (Free, 1955). Unlike the uniformity in size of honey bee workers, bumble bee workers within a single nest can vary in mass by approximately 10-fold (Alford, 1975), and small bumble bees are more likely to be nurse workers than foragers (Goulson *et al.*, 2002). Further investigations of hypopharyngeal gland secretions and nursing roles in non-*Apis* bee species, and how they respond to neonicotinoid exposure, would provide valuable new insights to the research field.

#### *7.4.2. Does neonicotinoid exposure impair the immune response to natural pathogen infections and parasitic attack?*

This thesis (Chapters 3 and 4) presents the first investigations of pesticide effects on the physiological antimicrobial protein response of any insect species following an artificial immune challenge. However, these studies did not aim to investigate the biological mechanisms associated with any specific natural infection. Laboratory studies using artificial immune challenges remain valuable because this approach allows standardisation of the stimulus used to invoke an immune response, minimising the treatment variation inherent in using a real pathogen, which can be logistically difficult to control. This approach allows also the isolation of possible pesticide effects acting on the host versus the pathogen/parasite. For example, it has been found that exposure of the

parasitoid wasp, *Leptopilina bouleari*, to the insecticide chlorpyrifos, increased the encapsulation of its eggs by its *Drosophila* host (Delpuech and Tekinel-Ozalp, 1991 cited in Desneux et al., 2007).

Laboratory studies can establish the direction of future work by elucidating potential pesticides of concern to bee health and recognising informative endpoints. Work in this thesis has recognised the potential for imidacloprid to affect antimicrobial protein activity and demonstrated the utility of bacterial-clearance assays to measure pesticide effects on antimicrobial activity at the enzymatic/protein level. Further research would be valuable to establish the relationships between immune responses to natural pathogen infections and parasitic attack in honey bees and bumble bees exposed also to field relevant pesticide concentrations as this would reflect more realistic scenarios of potential stressors impacting bee health in the wild. In particular, upregulation of antimicrobial proteins is thought to be important in response to bacterial infection, as supported by the clear temporal response to bacterial lipopolysaccharides in this thesis. The main bacterial infections of bees are *Paenibacillus larvae* and *Melissococcus plutonius*, which are symptomatic in honey bee larvae. Established methods can be used to experimentally rear honey bee larvae in the laboratory in the presence of a pesticide exposure (Hendriksma et al., 2011, Aupinel et al., 2007), and a bacterial-clearance assay could be used also to measure antimicrobial protein activity in the haemolymph of larvae. Fungal, microsporidian, trypanosomal and mite parasites have been found also to upregulate expression of AMP genes at the molecular level (Evans, 2004, Evans, 2006, Antunez et al., 2009, Riddell et al., 2011, Khongphinitbunjong et al., 2015), so these too would be useful systems to investigate the effects of pesticide exposure on antimicrobial protein activity.

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