Original article

Effects of neonicotinoid exposure on molecular and physiological indicators of honey bee immunocompetence

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Abstract – Bee declines have been associated with various stressors including pesticides and pathogens. We separately exposed immune-challenged adult worker honey bees (*Apis mellifera* L.) to two neonicotinoid pesticides, thiamethoxam (10 ppb) and imidacloprid (102 ppb), by dietary delivery. We found that whereas neonicotinoid exposure weakly affected transcriptional responses of antimicrobial genes, it did not detectably affect the physiological antimicrobial response as measured by a lytic clearance assay of haemolymph. Our findings add to the evidence that transcriptional responses in immune-related genes are not yet reliable indicators of pesticide impacts on bee health, which suggests caution in their future use as biomarkers in pesticide risk assessment.

imidacloprid / thiamethoxam / immunocompetence / antimicrobial peptide / gene expression

1. INTRODUCTION

There is widespread concern over losses of managed honey bees *(Apis mellifera* L.) but appropriate responses are unclear because a complex of multiple stressors is likely to be responsible for the observed decline in bee health (Potts et al. 2010). Therefore, recent studies have been conducted in an effort to investigate the potential interactions among stressors and their effects on various biological systems of honey bees. In particular, there is growing interest in the interactive effects of pesticide exposure and pathogen infection in bees (reviewed by Collison et al. 2016). The European Food Safety Authority (EFSA) Panel on Plant Protection Products and their Residues (PPR) recently recommended that further work is required to identify the molecular basis of interactions between exposure to pesticides and the range of diseases in the honey bee (EFSA 2012). Further, EFSA recommended the development of physiological and molecular biomarkers as diagnostic tools that could be valuable in improving risk assessment procedures for determining the effects of pesticides on bees. To better understand the mechanisms underlying disease-pesticide interactions, recent studies have been conducted in an effort to investigate the effects of pesticide exposure on bee immunocompetence (the ability to mount an immune response), with particular focus on the neonicotinoid pesticides, which have until recently been widely used in the protection of bee-attractive crops such as oilseed rape (*Brassica napus* L.).

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). The expression of some genes

related to these immune processes has been found to change following neonicotinoid exposure, but with no apparent consistency. For example, dietary exposure to imidacloprid led to increased transcription of the antimicrobial effector abaecin in larval honey bees in one study (Derecka et al. 2013), but not in another (Gregorc et al. 2012). In adult honey bees, by contrast, neonicotinoid exposure reduced transcription in a different antimicrobial effector, apidaecin (Di Prisco et al. 2013). Neonicotinoid exposure also causes detectable effects at the physiological level (Brandt et al. 2016), but again with little apparent consistency (Alaux et al. 2010a). Overall, therefore, it remains unclear whether neonicotinoid-induced changes in the transcription of immune genes consistently indicate the levels of immunocompetence that are realised at the physiological level. Previously, few studies have been conducted to test for these corresponding effects at both the molecular and the physiological levels using both a common exposure scenario and common immune-related endpoints. Consequently, the extent to which the different components of the immune response react in concert to neonicotinoid exposure has been unclear. Potentially, some of the non-correspondence between gene expression and physiological responses may have arisen because the studies to date have collected measurements at only a single sampling point in time, but insect immune studies have demonstrated the importance of temporality in the response to immune challenge (e.g. Gätschenberger et al. 2013; Korner and Schmid-Hempel 2004; Riddell et al. 2011). In summary, there is a need to investigate how neonicotinoid pesticides affect immunocompetence in honey bees and whether such an impact can be reliably detected by examination of molecular responses.

To begin to address this need, we investigated the effect of dietary neonicotinoids on the antimicrobial peptide (AMP) component of the honey bee immune response. We carried out a series of laboratory experiments investigating temporal correspondences between changes in gene expression and measures of physiological AMP activity following exposure to either imidacloprid or thiamethoxam. Besides testing the impact of these neonicotinoids on physiological indicators of immunocompetence, our study enabled us to evaluate gene expression as a biomarker of pesticide effects on bee health.

We investigated the effects of two neonicotinoid insecticides that have been widely used in crop protection: imidacloprid and thiamethoxam. In Europe and North America, imidacloprid has been replaced in normal agricultural practice by other neonicotinoids, such as thiamethoxam. Imidacloprid is nevertheless of interest because it has been the most commonly tested subject in ecotoxicological investigations of neonicotinoid impacts on bees. For our investigation, we tested the effect of a dietary concentration (102 parts per billion, ppb) that is much higher than environmentally realistic levels (< 10 ppb), because we wanted to improve the chance of detecting the response to intoxication. By contrast, in our experiment on thiamethoxam, we used a dietary dose (10 ppb) that exposed bees to environmentally realistic residue levels (e.g 11 ppb in nectar observed by Stoner and Eitzer 2012) as any findings made relative to this dose may have greater ecological relevance.

Each study was designed as a fully factorial experiment in which two levels of the pesticide (absent, present) were combined with three levels of immune challenge (see below). Our approach principally aimed to both elucidate the effects of pesticide exposure on immune responses and to test the correspondence of molecular and physiological immune endpoints. We did not set out to establish dose-dependent effects or to determine threshold levels of toxicity (e.g. NOECs).

In order to stimulate the immune system of honey bees experimentally, we employed an artificial immune challenge via an injection with lipopolysaccharides (LPS) from *Escherichia coli* to mimic a bacterial-like infection. This approach was adopted to standardise the stimulus and to avoid the 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 understand better the fundamental relationships between the temporal dynamics of transcriptional and physiological changes using a well-established method for insect immune studies.

2. MATERIALS AND METHODS

We exposed newly emerged adult worker honey bees to either un-dosed control or neonicotinoid-spiked sucrose feeders and imposed an immune challenge after 5 days of experimental feeding. While maintaining bees on the same dietary treatment, we collected samples for 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 h post immune challenge (PIC) or a longer time course of between 24 and 168 h (1–7 d) PIC. Samples were analysed either for expression levels of six AMP genes or for levels of antimicrobial activity at a physiological (enzymatic) level (Table I, see also supplementary material for an overall setup of treatment and sampling combinations).

Across all experiments, individual cages (i.e. and all bees within each cage) were randomly assigned to a specific sampling time point and sample type at the start of the experiment (with a total of three cages per treatment/time point combination). No repeated measures were taken, and all cages contained the same number of bees (10) throughout the experimental period, unless mortality occurred. Six bees within a cage were pooled for immune measurements (as discussed below) and each cage was treated as a biological replicate (giving a total of three replicates for each treatment/time point combination).

2.1. Honey bee provenance and husbandry

All honey bees were from colonies of British hybrid bees maintained on the home apiary at the Food and Environment Research Agency, Sand Hutton, York, UK. Visual inspections of these colonies showed no symptoms of disease or parasitic infection, but baseline levels of pathogens cannot be ruled out. Further confirmation of pathogen levels (e.g. via PCR) in tested bees or their colonies was not performed. Brood frames were collected from colonies between April and September (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 Ltd., London, UK). Each cage was designed to allow the bees ad libitum access to a sucrose solution (50% w/v) through a punctured 1.5-mL microcentrifuge tube. Cages were maintained in the environmental chamber throughout the experimental period (34 °C, 60% relative humidity, constant darkness). The five separate experiments described in Table I were each set up on a separate occasion between August 2013 and June 2014 (no winter bees were used) and each used workers derived from a single honey bee colony.

2.2. Neonicotinoid exposure

All of our experiments used chronic dietary exposures to sublethal concentrations of one neonicotinoid, either imidacloprid or thiamethoxam. Stock solutions of neonicotinoids were made in < 0.1% acetone, and used to create the appropriate concentrations. In the imidacloprid studies, the sucrose feeder syrup was spiked with 125 μg/L (= 102 ppb) imidacloprid (Fluka Analytical 37,894, Sigma-Aldrich, UK). In the thiamethoxam studies, the sucrose was spiked with 12 μg/L (= 10 ppb) thiamethoxam (Fluka Analytical 37,924, Sigma-Aldrich, UK). Analytical verification of test concentrations was not conducted. All cages were designed to allow bees to feed ad libitum from the sucrose feeder throughout the experiment. No other food was provided. Control sucrose contained < 0.1% acetone, equivalent to the volume of solvent used 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.

2.3. Immune challenge

Bees received one of three injection treatments, with all workers within a cage subjected to the same treatment under ice-immobilisation (see supplementary material for further details). Bees in the 'LPS' treatment group were injected with a

Table I. Overview of the test substances, sampling time course and endpoints measured in each of five experiments. Each experiment was performed with newly emerged honey bees from a single colony. A different colony was used for each experiment. See supplementary material for an overall setup of treatment and sampling combinations

Experimental details		Experiment A	Experiment B	Experiment C	Experiment D	Experiment E
Pesticides tested in each experiment[a]	Control vs 125 µg/L IMI	√	√		√	
	Control vs 12 µg/L TMX	√		√		√
Samples taken across either 'short-' of 'long-' time course in each experiment	Short-time course (2–48 h)	√	√	√		
	Long-time course (24–168 h)				√	√
Endpoints measured in each experiment[b]	Gene expression (qPCR)	√			√	√
	AMP enzymatic activity		√	√	√	√
Total combinations tested (total no. of cages in parentheses)[c]		54 (162)	24 (72)	24 (72)	60 (180)	60 (180)

IMI imidacloprid; *TMX* thiamethoxam

[a] Where both compounds were tested in a single experiment, each compound was tested independently. i.e. cages of 10 bees were exposed to either IMI or TMX, but never to both. Where boxes are left blank, the pesticide of concern was not tested in the given experiment

[b] Where both gene expression and enzymatic activity were measured in a single experiment, each endpoint was tested independently. i.e. individual bees were used in samples for either gene expression or enzymatic activity, but never for both. Where boxes are left blank, the endpoint of concern was not tested in the given experiment

[c] Combinations of pesticide × immune challenge × sampling time point × measurement type. Please refer to supplementary material for overview of all combinations. Note, the total number of combinations across all experiments is greater than 180 because control groups were included in all experiments. In all experiments, three cages (replicates) of 10 bees were tested per combination

2-µL aliquot of 0.5 mg/mL LPS (Sigma L2755-Lipopolysaccharides from *E. coli* 0128:B12, Sigma-Aldrich, UK) dissolved in Insect Ringer's solution (1:1:1156 mM NaCl: 3 mM KCl: 2 mM $CaCl_2$). This same 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; Köhler et al. 2012; Laughton et al. 2011). Bees in the 'Ringer's' treatment group were injected with 2 µL Insect Ringer's solution to control for any effects of the injection process itself. In the 'naïve' treatment, bees were ice-immobilised but received no further treatment, which served as the unchallenged control.

2.4. Measurement of gene expression

At the designated time points, bees were placed directly into a freezer at − 80 °C and stored until RNA extraction. Total RNA was extracted from pooled 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. Following RNA extraction, cDNA was synthesised and the relative expression of six AMP genes (abaecin,

apidaecin, defensin-1, defensin-2, hymenoptaecin and lysozyme-1) was measured using qPCR (supplementary material).

2.5. 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. An aliquot of 2–10 μL of this haemolymph was collected with a pipette and immediately stored at – 20 °C for later analysis. The antimicrobial activity was separately measured in six individual bees from each 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 mg/mL lyophilised *Micrococcus lycodeikticus* (M3770; Sigma-Aldrich, UK). Eight millilitres of this suspension were 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-μL aliquot was added per well. Plates were incubated at 27 °C for 72 h, and the diameters of clearance zones were measured using digital callipers.

3. RESULTS

3.1. Survival

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

3.2. Gene expression

In response to LPS injection, four antimicrobial effector genes (abaecin, apidaecin and defensin-1 and hymenoptaecin) were significantly upregulated, but the immune challenge did not alter the expression of defensin-2 and lysozyme-1 (Table II). A similar temporal pattern of expression was observed for all four of the upregulated genes, despite between-gene differences in the magnitude of changes in expression. Increased expression was typically observed as early as 2 h PIC, increasing to peak expression at between 8 and 24 h PIC, after which the response showed a gradual decline over the following 6 days (Fig. 1; see also Fig. S1-S5).

Exposure to imidacloprid was not found to alter expression of any of the six AMP genes tested across the short- or long-time course experiments (Table II).

Exposure to thiamethoxam reduced the expression of abaecin dependent on the injection treatment, but this effect was not seen in the longer time course study (Table II). Reduced transcription of abaecin was most notable in response to thiamethoxam exposure 24 h post LPS injection (Fig. 1). Thiamethoxam exposure also had various effects on expression of apidaecin, hymenoptaecin and lysozyme-1, but these effects were dependent on the time of sampling, and only observed in the short-time course experiment (Table II). Here, increased transcription of apidaecin was observed following thiamethoxam exposure, most notably at 24 h post LPS injection (Fig. S1), whilst reduced transcription of hymenoptaecin was observed following thiamethoxam exposure between 8 and 24 h PIC (Fig. S2) and the effects of thiamethoxam on lysozyme-1 were transient (Fig. S5).

Table II. ANOVA results for all five experiments. Data analysis was only conducted between samples within a single experiment, but presented below to show all comparisons/endpoints tested across the five experiments. Variation among treatments was analysed by factorial ANOVA with injection (immune challenge) treatment, pesticide treatment and time post immune challenge as fixed effects. 'Injection' refers to comparison between the 'Ringer's' and 'LPS' injection groups; this injection comparison was seen of most biological relevance as it reflected responses only to the bacterial molecules (albeit artificial), but not to the injection procedure itself. 'Pesticide' refers to comparison between control-fed and pesticide-fed bees (either imidacloprid or thiamethoxam, depending on the experiment)

	df	Abaecin F	Abaecin P	Apidaecin F	Apidaecin P	Hymenoptaecin F	Hymenoptaecin P	Defensin1 F	Defensin1 P
Short-time course (0–48 h) — Experiment A[a] — Imidacloprid									
Injection	1	106	**<0.001**	27.0	**<0.001**	176	**<0.001**	30.3	**<0.001**
Pesticide	1	1.21	0.278	0.617	0.436	0.051	0.823	4.62	0.036
Time	5	141	**<0.001**	34.3	**<0.001**	153	**<0.001**	28.6	**<0.001**
Injection*Pesticide	1	3.39	0.071	2.79	0.101	5.38	**0.024**	0.532	0.469
Pesticide*Time	5	0.319	0.899	0.068	0.997	1.41	0.235	0.868	0.509
Injection*Time	5	13.1	**<0.001**	3.73	**0.006**	20.3	**<0.001**	2.39	0.050
Residuals	53								
Short-time course (0–48 h) — Experiment A[a] — Thiamethoxam									
Injection	1	96.8	**<0.001**	31.3	**<0.001**	93.3	**<0.001**	20.6	**<0.001**
Pesticide	1	11.5	**0.001**	21.8	**<0.001**	47.8	**<0.001**	3.85	0.055
Time	5	81.6	**<0.001**	35.4	**<0.001**	87.0	**<0.001**	27.3	**<0.001**
Injection*Pesticide	1	10.7	**0.002**	0.615	0.436	3.31	0.075	0.012	0.915
Pesticide*Time	5	1.87	0.115	3.88	**0.005**	14.3	**<0.001**	1.28	0.287
Injection*Time	5	7.45	**<0.001**	3.12	**0.015**	16.1	**<0.001**	3.13	**0.015**
Residuals	52								
Long-time course (0–168 h) — Experiment D[b,c] — Imidacloprid									
Injection	1	25.4	**<0.001**	48.3	**<0.001**	14.6	**<0.001**	–	–
Pesticide	1	0.022	0.883	2.75	0.106	0.074	0.787	–	–
Time	3	16.0	**<0.001**	13.2	**<0.001**	9.41	**<0.001**	–	–
Injection*Pesticide	1	3.38	0.075	0.879	0.355	0.629	0.433	–	–
Pesticide*Time	3	1.18	0.334	0.279	0.840	1.23	0.315	–	–
Injection*Time	3	0.801	0.502	0.879	0.485	4.90	**0.006**	–	–
Residuals	34								

Table II (continued)

Long-time course (0–168 h)	Thiamethoxam	df	Abaecin F	Abaecin P	Apidaecin F	Apidaecin P	Hymenoptaecin F	Hymenoptaecin P	Defensin1 F	Defensin1 P
		Experiment E[b,c]								
	Injection	1	32.9	**<0.001**	32.2	**<0.001**	37.0	**<0.001**	–	–
	Pesticide	1	0.989	0.327	0.359	0.554	0.272	0.606	–	–
	Time	3	13.7	**<0.001**	15.8	**<0.001**	15.1	**<0.001**	–	–
	Injection*Pesticide	1	0.25	0.624	1.57	0.219	0.047	0.830	–	–
	Pesticide*Time	3	0.799	0.503	0.199	0.896	1.81	0.164	–	–
	Injection*Time	3	3.06	**0.042**	4.29	**0.012**	2.36	0.090	–	–
	Residuals	33								

AMP enzymatic activity

Short-time course (0–48 h)	Defensin2 F	Defensin2 P	Lysozyme1 F	Lysozyme1 P	df	F	P
					Experiment B[b]		
	2.42	0.126	2.68	0.107	1	18.2	**<0.001**
	0.497	0.484	1.04	0.313	1	2.93	0.096
	1.09	0.377	1.98	0.097	3	160	**<0.001**
	0.187	0.668	0.176	0.676	1	4.12	0.050
	1.28	0.287	0.876	0.504	3	1.49	0.234
	0.220	0.952	0.147	0.980	3	6.72	**0.001**
					35		

Short-time course (0–48 h)	Defensin2 F	Defensin2 P	Lysozyme1 F	Lysozyme1 P	df	F	P
					Experiment C[b]		
	0.489	0.487	0.368	0.547	1	27.8	**<0.001**
	1.67	0.202	8.60	**0.005**	1	0.013	0.908
	2.66	0.033	5.45	**<0.001**	3	88.6	**<0.001**
	4.70	0.035	1.62	0.209	1	0.952	0.336
	2.24	0.064	6.07	**<0.001**	3	0.811	0.496
	2.23	0.065	1.88	0.113	3	9.15	**<0.001**

Table II (continued)

	Defensin2		Lysozyme1		AMP enzymatic activity		
	F	P	F	P			
					35		
					df	F	P
Long-time course (0–168 h)	—	—	—	—	1	119	**<0.001**
	—	—	—	—	1	0.134	0.717
	—	—	—	—	3	42.1	**<0.001**
	—	—	—	—	1	0.105	0.748
	—	—	—	—	3	1.36	0.272
	—	—	—	—	3	6.99	**<0.001**
					34		
	F	P	F	P	df	F	P
Long-time course (0–168 h)	—	—	—	—	1	44.3	**<0.001**
	—	—	—	—	1	0.039	0.845
	—	—	—	—	3	35.0	**<0.001**
	—	—	—	—	1	0.088	0.769
	—	—	—	—	3	0.655	0.586
	—	—	—	—	3	3.39	**0.029**
					33		

[a] In Experiment A, where imidacloprid and thiamethoxam treatments were both included within the single experiment, the imidacloprid and thiamethoxam datasets were analysed separately as we were only interested in the differences between each pesticide and the control-fed bees. Since the control dataset was therefore used in both analyses, in Experiment A, a significant effect was defined by $P \leq 0.025$ (in bold), accounting for a Bonferroni correction

[b] Significant effect defined by $P \leq 0.05$ (in bold)

[c] Expression of the genes Defensin1, Defensin2 and Lysozyme1 was not measured in the long-time course experiments (experiments D and E)
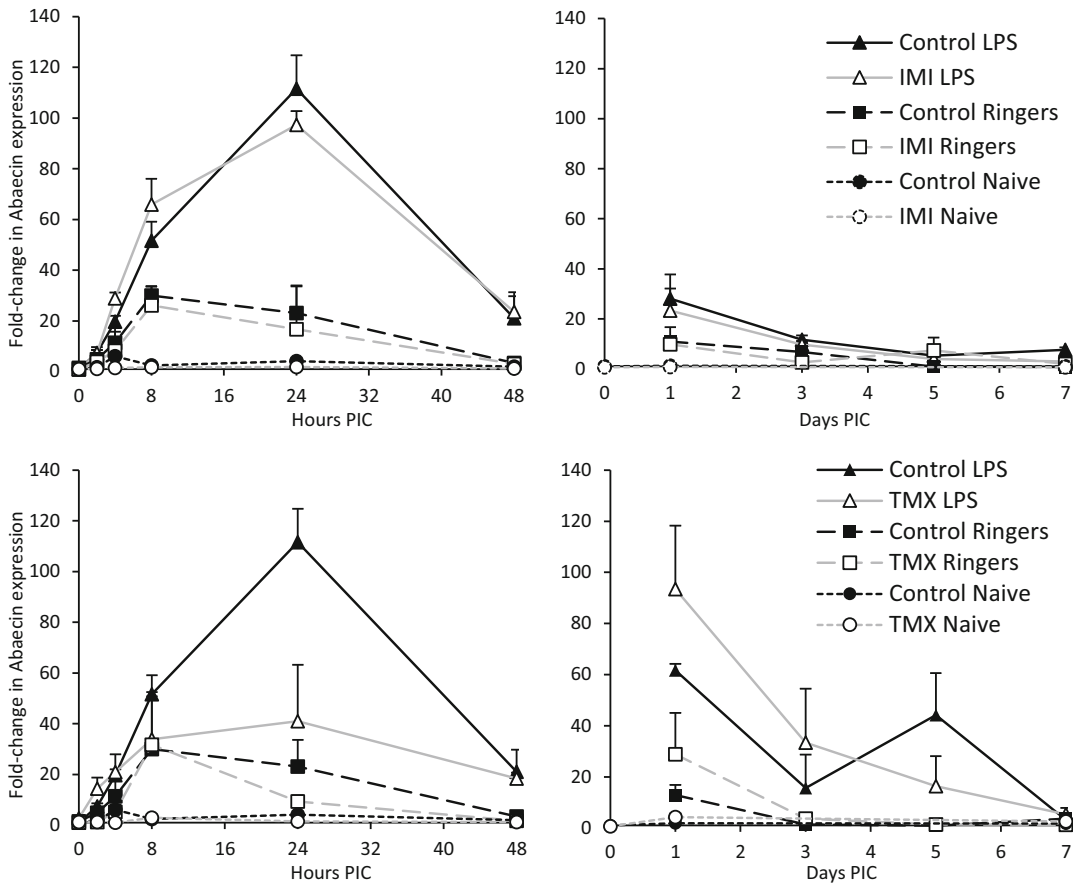
Figure 1. Gene expression of the AMP gene, abaecin, in response to artificial immune challenge over time in control-fed (black shapes) and neonicotinoid-exposed (white shapes) bees. Top plots: results for exposure to 125 µg/L imidacloprid [Experiments A (left) and D (right)]. Bottom plots: results for exposure to 12 µg/L thiamethoxam [Experiments A (left) and E (right)]. Plots on the left-hand side show responses for a period of 2–48 h post immune challenge (PIC) and plots on the right-hand side for a period of 1–7 d PIC. In all plots, shapes indicate sample means and error bars depict 1 SE. Only upper error bars are shown to simplify the figures.

3.3. Antimicrobial activity

Antimicrobial activity at a physiological (enzymatic) level was significantly increased in response to LPS injection in a time-dependent manner during both the short- and long-time course experiments (Table II; Fig. 2). Ringer's injection also induced a response, but to a lesser extent than in bees injected with LPS (Fig. 2). A peak in activity occurred between 24 and 72 h PIC, followed by a gradual decline over the following days, and levels typically had not returned to the baseline levels by the last sampling point 7 d PIC (Fig. 2).

No effects of exposure to either imidacloprid or thiamethoxam were observed on antimicrobial activity in any of the experiments (Table II) and the temporal dynamics of the response to injection were virtually identical in bees fed with control sucrose and those exposed to the neonicotinoids (Fig. 2).

3.4. Sucrose consumption

Consumption of imidacloprid-spiked sucrose was significantly lower than the consumption of control sucrose in all experiments (Table S2). In general, there were no significant differences in
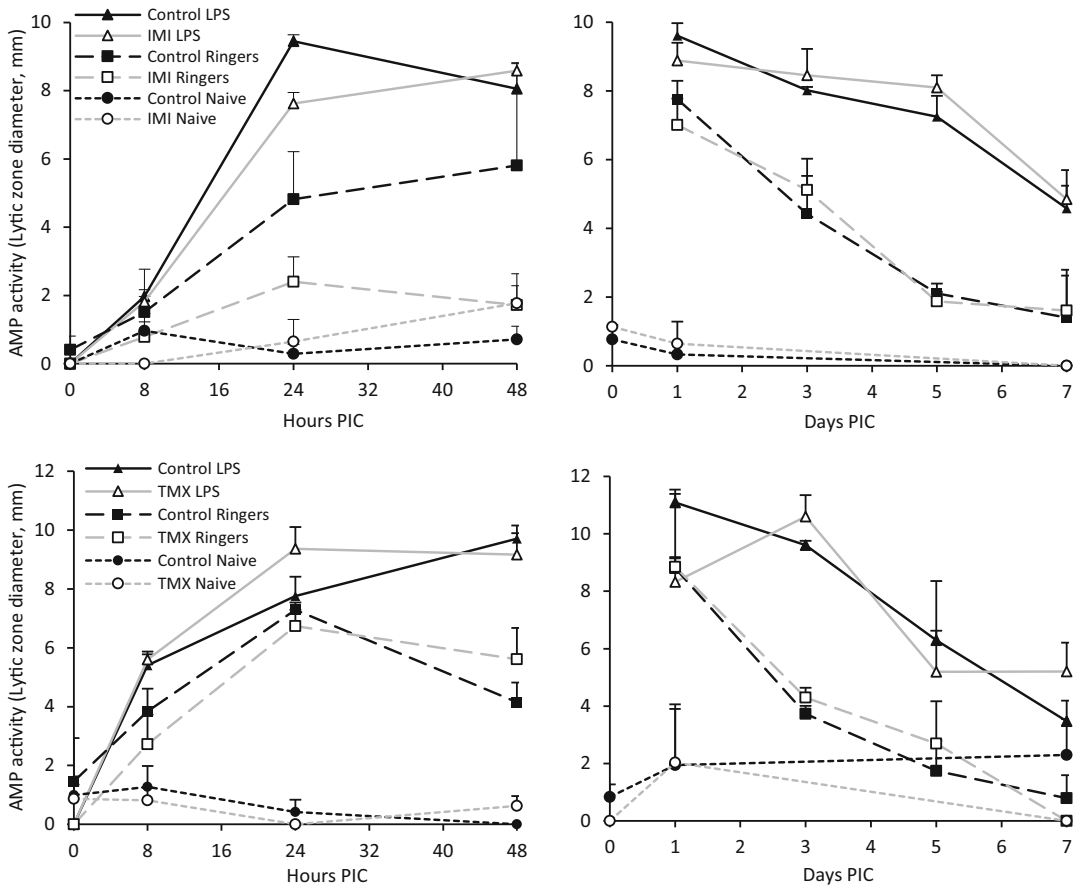
Figure 2. Physiological antimicrobial activity to artificial immune challenge over time in control-fed (black shapes) and neonicotinoid-exposed (white shapes) bees. Top plots: results for exposure to 125 µg/L imidacloprid [Experiments B (left) and D (right)]. Bottom plots: results for exposure to 12 µg/L thiamethoxam [Experiments C (left) and E (right)]. Plots on the left-hand side show responses for a period of 2–48 h post immune challenge (PIC) and plots on the right-hand side for a period of 1–7 d PIC. Note, the y-axis scales differ between plots. In all plots, shapes indicate sample means and error bars depict 1 SE. Only upper error bars are shown to simplify the figures.

consumption between bees feeding on control and thiamethoxam-spiked sucrose. The exception to this was for bees in Experiment A, where there was significantly reduced feeding in the thiamethoxam treatment (Table S2). Even when reduced feeding was observed in neonicotinoid-treated bees, bees ingested substantial amounts of sucrose (e.g. in Experiment A, mean daily sucrose consumption in the 4 days prior to immune challenge for control-fed: 46 ± 3 mg/bee; for imidacloprid-fed: 38 ± 4 mg/bee; for thiamethoxam-fed bees: 36 ± 2 mg/bee) and

consumed substantial doses of neonicotinoid (3.0 ± 0.3 ng imidacloprid/bee; 2.8 ± 0.2 ng thiamethoxam/bee). There was no evidence that injection treatment altered sucrose consumption (all experiments: ANOVA $P > 0.05$).

4. DISCUSSION

Four antimicrobial effector genes showed a clear upregulation in response to the artificial immune challenge. 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 lack of a detectable response by lysozyme-1 is consistent with most of the previous studies of bacterial infection in honey bees (e.g. Evans 2006; Yang and Cox-Foster 2005). 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 transcriptional changes in AMP genes in response to immune challenge that occurred in our study probably have 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; Laughton et al. 2011; Yang and Cox-Foster 2005). The wound response to injection that we observed may be similar to the response to other natural wounding events, such as puncturing by *Varroa* mites (Kanbar and Engels 2003) and parasitic flies (Core et al. 2012). It is likely that an increased response in transcription of AMP-related genes following wounding is adaptive by allowing the honey bee to pre-emptively induce its immune system before microbial infection, because most natural wounds will inevitably be non-sterile (Erler et al. 2011; Kanbar and Engels 2003).

The effects of neonicotinoid exposure on transcriptional responses in the antimicrobial component of honey bee immunity were weak and inconsistent. Where transcriptional changes were observed, they differed between the two neonicotinoid compounds. Generally, expression was altered in a greater number of the genes tested following exposure to thiamethoxam than to the much higher exposure to imidacloprid, but even in the case of thiamethoxam the results were inconsistent because effects differed between the short- and long-time course experiments, even at equivalent time points.

Neonicotinoid exposure did not affect the physiological activity of the antimicrobial response in our study as measured by a lytic clearance assay, even when transcriptional changes in AMP gene expression were observed. Taken together, our findings therefore suggest that the transcriptional changes in AMP genes due to neonicotinoid exposure that we observed did not indicate a detrimental impact on bee health through suppressed immunocompetence, contrary to what has been proposed by others (e.g. Di Prisco et al. 2013). We tentatively speculate that differential gene expression may fail to cause a correspondingly altered physiological response. If the insecticide evokes various positive and negative changes in the expression of antimicrobial genes, overall, the system may be virtually left in balance. For example, we observed that thiamethoxam both suppressed hymenoptaecin expression and increased apidaecin expression.

Our findings have implications for the future development of molecular biomarkers of bee health under pesticide and pathogen exposures. There has been recent interest in identifying suitable molecular biomarkers for pesticide risk assessment on bees (EFSA 2012). However, our findings do not yet validate the use of the immune-related molecular biomarkers in risk assessment, principally because we found that enzymatic/protein-level components of honey bee antimicrobial activity were unaffected by neonicotinoid exposure despite the occurrence of some temporally coincident, pesticide-induced transcriptional changes in AMP genes. In summary, if the loci that we studied were to be used as molecular biomarkers, our findings imply that they would be overly sensitive indicators of alterations in the physiological capacities of honey bees to mount an immune response. However, it is unknown whether our findings for neonicotinoids are typical for other classes of chemical compounds.

We recognise that our findings must be considered with some caveats. First, we conducted a series of separate experiments on individual honey bee colonies and this may have some bearing on our conclusions. Specifically, while our findings certainly suggest the potential for differences in the response of different immune endpoints to pesticide exposure, some of these differences

could result also from between-colony variation in pesticide sensitivity. In the future, further work could compare transcriptional and physiological responses of honey bees both within a single colony as well as among a number of colonies, and also within and between seasons. Second, further research is needed to establish whether the resilience of the response to simulated immune-stimulus in pesticide-exposed honey bees also arises when they are challenged by their natural pathogens. We tentatively predict that our findings will generalise to natural pathogens because of the commonality of responses by several AMP genes under infection with a broad range of challenges, including honey bee bacterial, fungal and microsporidian pathogens (Antunez et al. 2009; Evans 2006). In the future, however, only investigations using natural pathogens can truly assess environmentally relevant impacts of pesticides on bee health. Finally, our laboratory findings are yet to be generalised to honey bee colonies under realistic in-hive conditions. Although we detected no functionally significant impacts on honey bee immunocompetence in laboratory exposures that probably exceed many environmentally relevant neonicotinoid exposures, we are also tentative in generalising our findings to bees under field-relevant conditions for three reasons. First, our experimental exposures were conducted for periods of up to 11 days, whereas realistic in-hive exposures may be for longer periods. Second, our laboratory setup did not provide access to a pollen supply and pollen nutrition has been shown to affect immunocompetence (Alaux et al. 2010b). Third, we tested only a subset of endpoints and there are many other functionally relevant genes and enzymes belonging to various pathways in the honey bee immune system.

5. CONCLUSIONS

In summary, our experiments showed an overall clear temporal pattern of AMP gene expression and physiological activity in response to a bacterial-like infection. Our principal finding is that the transcriptional changes in AMP genes following neonicotinoid exposure did not correspond with immune suppression at the physiological level. If this finding turns out to be general, it suggests that honey bee immunity may be more resilient to neonicotinoid exposure than previously thought based on molecular studies only, but this will need further investigation and, particularly, using honey bees maintained under environmentally realistic conditions. Our findings begin to enable regulators to better evaluate the potential of gene expression as a biomarker for bee health. For those genes studied here, bioassays based on gene expression are not yet reliable indicators of pesticide effects on honey bee immune functioning at a physiological level.

CONTRIBUTIONS

All authors conceived and designed experiments; EJC performed experiments and analysis; EJC wrote the paper and all authors participated in the revisions of it and approved the final manuscript.

Effets de l'exposition à un néonicotinoïde sur les indicateurs moléculaires et physiologiques de l'immunocompétence de l'abeille

Imidaclopride / thiaméthoxame / peptide antimicrobien / expression génique / *Apis mellifera*

Auswirkungen von Neonikotinoidexpositionen auf molekulare und physiologische Immunkompetenzindikatoren von Honigbienen

Imidacloprid / Thiamethoxam / Immunkompetenz / antimikrobielle Peptide / Genexpression

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