Apidologie **SUPPLEMENTARY MATERIAL**

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

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Further details on methods and materials

Experimental setup

The overall setup was achieved in a series of five individual experiments, as outlined in Table 1. This provided data for the following combinations (below) of pesticide exposure/immune challenge, for analysis of either gene expression or physiological antimicrobial activity, and from samples collected across either a short time course (2-48 h post immune challenge, PIC) or long time course (24-168 h PIC). It is noted that some sampling times overlap across the two time courses, but we refer to ‘short’ or ‘long’ to distinguish the two regimes.

9 combinations of Pesticide exposure and Immune challenge:

1	Control-fed	x	Naïve
2	Control-fed	x	Ringers
3	Control-fed	x	LPS
4	IMI-fed	x	Naïve
5	IMI-fed	x	Ringers
6	IMI-fed	x	LPS
7	TMX-fed	x	Naïve
8	TMX-fed	x	Ringers
9	TMX-fed	x	LPS

IMI = imidacloprid; TMX = thiamethoxam
Please see main methods section for further details of pesticide exposure and immune challenge

For each of these 9 combinations, 20 sampling regimes (time point and measurement type):

i	0 h (‘short’)	x	Gene expression
ii	2 h (‘short’)	x	Gene expression
iii	4 h (‘short’)	x	Gene expression
iv	8 h (‘short’)	x	Gene expression
v	24 h (‘short’)	x	Gene expression
vi	48 h (‘short’)	x	Gene expression
vii	0 h (‘long’)	x	Gene expression
viii	24 h (‘long’)	x	Gene expression
ix	72 h (‘long’)	x	Gene expression
x	120 h (‘long’)	x	Gene expression
xi	168 h (‘long’)	x	Gene expression
xii	0 h (‘short’)	x	Antimicrobial activity
xiii	8 h (‘short’)	x	Antimicrobial activity
xiv	24 h (‘short’)	x	Antimicrobial activity
xv	48 h (‘short’)	x	Antimicrobial activity
xvi	0 h (‘long’)	x	Antimicrobial activity
xvii	24 h (‘long’)	x	Antimicrobial activity
xviii	72 h (‘long’)	x	Antimicrobial activity
xix	120 h (‘long’)	x	Antimicrobial activity
xx	168 h (‘long’)	x	Antimicrobial activity

9 x 20 = 180 combinations of pesticide x immune challenge x sampling time point x measurement type

3 cages of 10 bees per combination (with the cage acting as biological replicate; 3 replicates per combination)

As these combinations were achieved across several experiments (Table I), in all experiments a control-fed group (combinations 1-3) was always present and data analysis was only conducted between samples taken within a single experiment (i.e. data were compared to the control group in the given experiment). Due to the limitations of this setup, patterns of effects were compared, but no statistical comparison could be made, between experiments.

Immune challenge

To immobilise workers for injection treatments, individual cages in which honey bees were housed were placed in a freezer (-20 °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 µL of solution through the pleural membrane between the tergites (dorsal side) of the abdomen.

Measurement of gene expression

Total RNA was extracted using the *mir*Vana 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). Six AMP genes (see Table S1) were chosen for testing based on previous studies that found that these genes were strongly upregulated in response to bacterial infection (Evans et al. 2006). Oligonucleotide primers, used to amplify genes (Table S1), 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 ≤ 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-α 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); and NormFinder (Andersen et al. 2004). 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.

Data analysis

Where necessary, data were first transformed to meet the assumptions for parametric testing. Variation among treatments was analysed by factorial ANOVA with injection treatment, pesticide treatment and time post injection as fixed effects. This analysis was focussed only on 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. However, data presented in the graphs includes comparison to the naïve unchallenged controls, to demonstrate that responses were seen also to the Ringer's injection. 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 $P \leq 0.025$, accounting for a Bonferroni correction. For all other experiments, a significant effect was defined by $P \leq 0.05$.

Supplementary Tables

Table S1 Oligonucleotide primers used in qPCR assays

Gene	Gene category/ pathway	Forward primer (5' → 3')	Reverse primer (5' → 3')	Reference
Abaecin	Antimicrobial peptide	CAGCATTCGCATACGTACCA	GACCAGGAAACGTTGGAAAC	Morimoto et al. 2011
Apidaecin	Antimicrobial peptide	TAGTCGCGGTATTTGGGAAT	TTTCACGTGCTTCATATTCTTCA	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	CGGAATTGGAACCTGAGGATAC	CCTTGAATGACAATGGATCCTCTT	Designed in house
Lysozyme-1	Antimicrobial peptide	GAACACACGGTTGGTCACTG	ATTTCCAACCATCGTTTTCG	Evans et al. 2006
6AS10	P450 detoxification - reference	GGGGTACCTGGACCCAAGCCA	GCCAGAACACGCACGTTTCGC	Morimoto et al. 2011
Actin	Structural protein-reference	TGCCAACACTGTCCTTTCTG	AGAATTGACCCACCAATCCA	Lourenco et al. 2008
Elongation factor 1-alpha (ef1-alpha)	Protein synthesis-reference	GGAGATGCTGCCATCGTTAT	CAGCAGCGTCCTTGAAAGTT	Lourenco et al. 2008

Table S2 Effects of neonicotinoid exposure on sucrose consumption- Results of Wilcoxon Rank Sum tests

Results presented here represent the four days of feeding prior to immune challenge only and test the comparison in sucrose consumption between neonicotinoid- and control sucrose-fed bees.

Experiment (Pesticide)	W	P
A (Imidacloprid)	1874	**0.009**
B (Imidacloprid)	1011	**<0.001**
D (Imidacloprid)	686	**0.031**
A (Thiamethoxam)	1880	**0.008**
C (Thiamethoxam)	537	0.177
E (Thiamethoxam)	606	0.382

Supplementary Figures

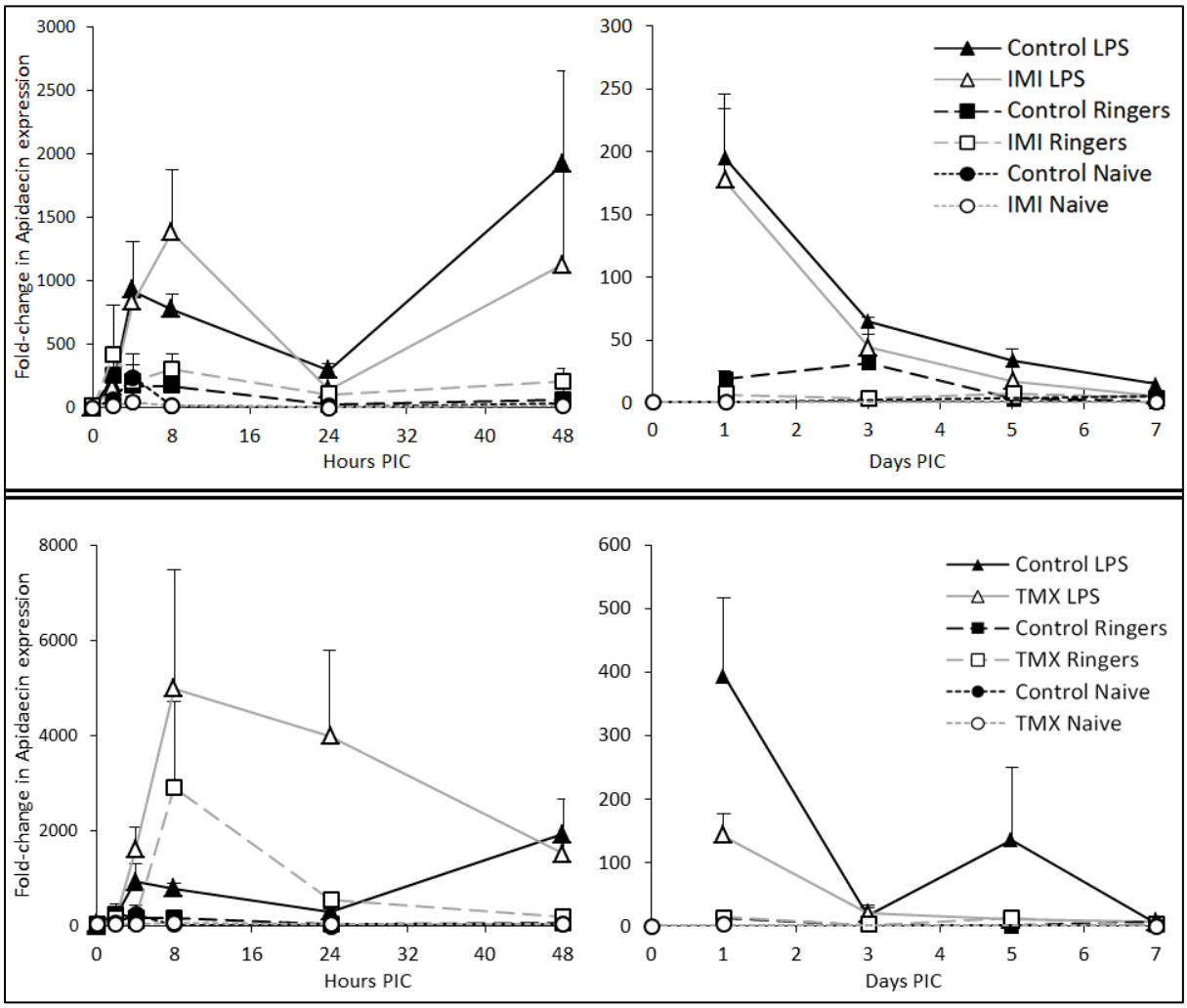

Fig. S1 Gene expression of the AMP gene, apidaecin, in response to artificial immune challenge over time in control-fed (black shapes) and neonicotinoid-exposed (white shapes) bees. Top plots: Results for exposure to 125 µg/L imidacloprid [Experiments A (left) and D (right)]. Bottom plots: Results for 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. Note, the y-axis scales differ between plots. In all plots, symbols indicate sample means and error bars depict 1 SE. Only upper error bars are shown to simplify the figures

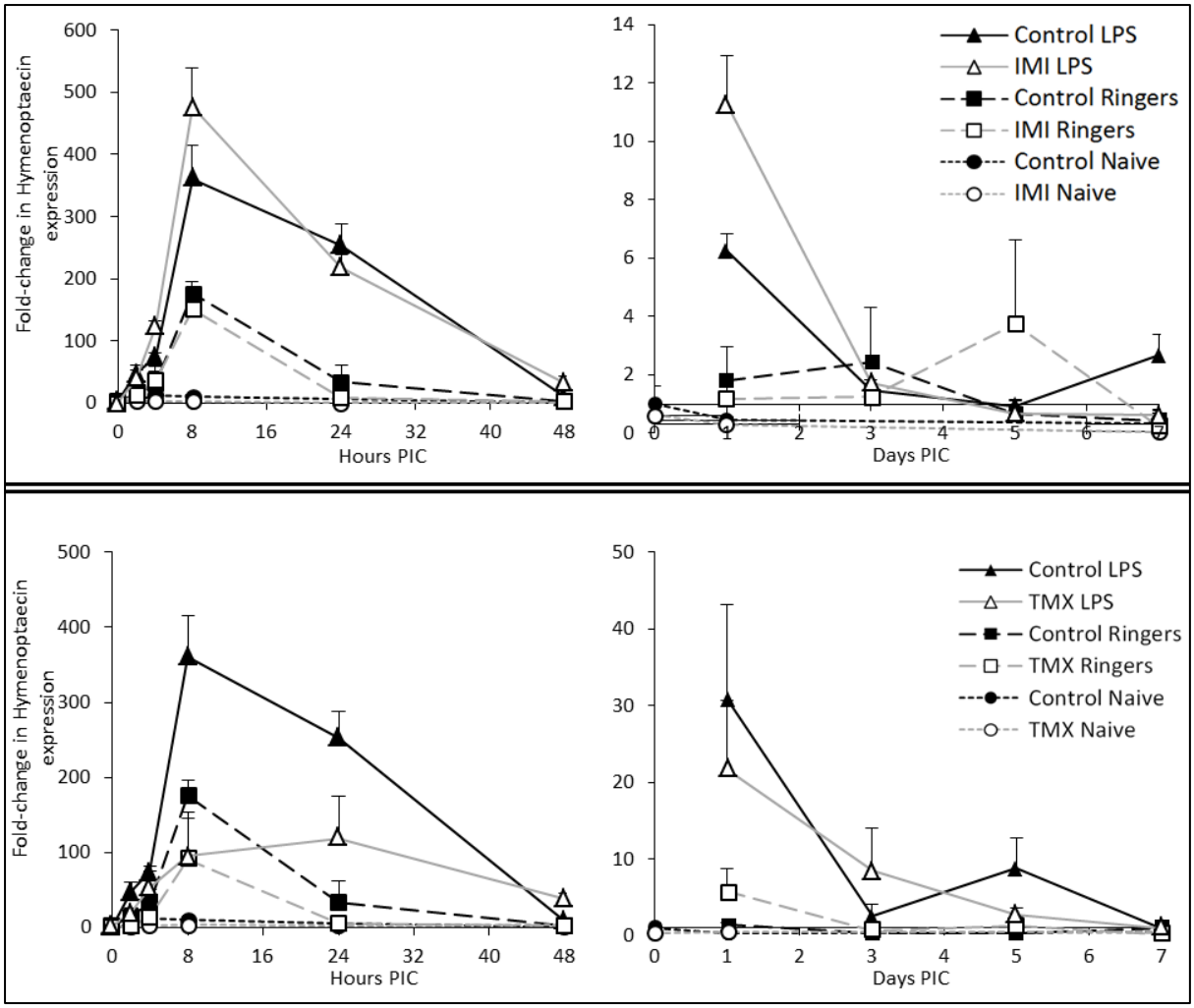

Fig. S2 Gene expression of the AMP gene, hymenoptaecin, in response to artificial immune challenge over time in control-fed (black shapes) and neonicotinoid-exposed (white shapes) bees. Top plots: Results for exposure to 125 µg/L imidacloprid [Experiments A (left) and D (right)]. Bottom plots: Results 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. Note, the y-axis scales differ between plots. In all plots, symbols indicate sample means and error bars depict 1 SE. Only upper error bars are shown to simplify the figures

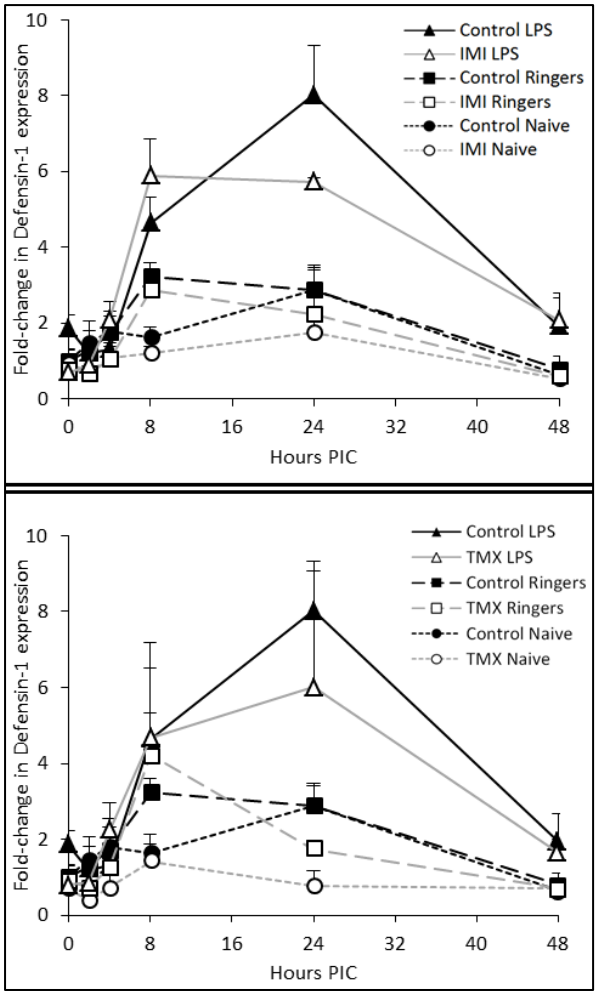

Fig. S3 Gene expression of the AMP gene, defensin-1, in response to artificial immune challenge over time in control-fed (black shapes) and neonicotinoid-exposed (white shapes) bees. Top plot: Results for exposure to 125 µg/L imidacloprid [Experiment A]. Bottom plot: Results for exposure to 12 µg/L thiamethoxam [Experiment A]. For this gene, expression was measured only for a period of 2-48 h post immune challenge. In both plots, symbols indicate sample means and error bars depict 1 SE. Only upper error bars are shown to simplify the figures

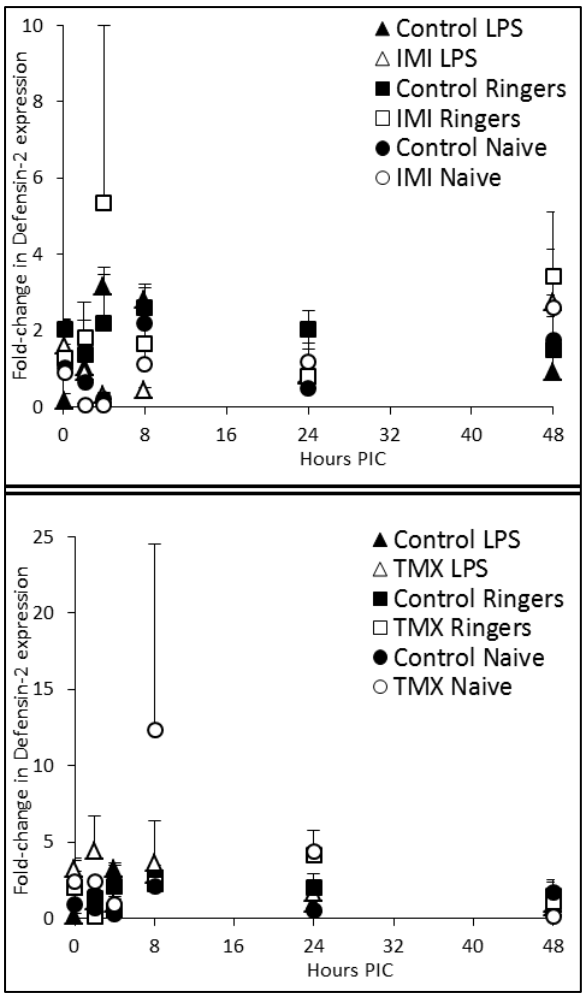

Fig. S4 Gene expression of the AMP gene, defensin-2, in response to artificial immune challenge over time in control-fed (black shapes) and neonicotinoid-exposed (white shapes) bees. Top plot: Results for exposure to 125 µg/L imidacloprid [Experiment A]. Bottom plot: Results for exposure to 12 µg/L thiamethoxam [Experiment A]. For this gene, expression was measured only for a period of 2-48 h post immune challenge. Lines are not shown between data points as no significant effect of immune challenge was observed and hence lines are not necessary to demonstrate the pattern of response. Note, the y-axis scales differ between plots. In both plots, symbols indicate sample means and error bars depict 1 SE. Only upper error bars are shown to simplify the figures

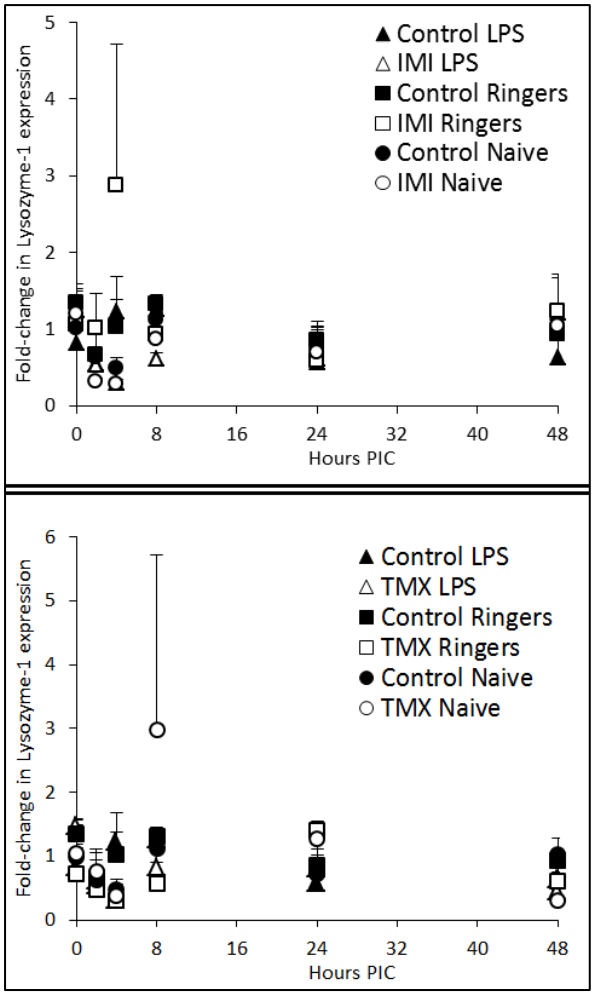

Fig. S5 Gene expression of the AMP gene, lysozyme-1, in response to artificial immune challenge over time in control-fed (black shapes) and neonicotinoid-exposed (white shapes) bees. Top plot: Results for exposure to 125 µg/L imidacloprid [Experiment A]. Bottom plot: Results for exposure to 12 µg/L thiamethoxam [Experiment A]. For this gene, expression was measured only for a period of 2-48 h post immune challenge. Lines are not shown between data points as no significant effect of immune challenge was observed and hence lines are not necessary to demonstrate the pattern of response. In both plots, symbols indicate sample means and error bars depict 1 SE. Only upper error bars are shown to simplify the figures

References for supplementary material

Andersen, C. L., Jensen, J. L., Orntoft, T. F. (2004). Normalization of real-time quantitative reverse transcription-PCR data: A model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. **64**, 5245-5250

Evans, J. D., Aronstein, K., Chen, Y. P., Hetru, C., Imler, J. L., Jiang, H., Kanost, M., Thompson, G. J., Zou, Z., Hultmark, D. (2006) Immune pathways and defence mechanisms in honey bees *Apis mellifera.* Insect Mol. Biol. **15**, 645-656

Larionov, A., Krause, A., Miller, W. (2005) A standard curve based method for relative real time PCR data processing. Bmc Bioinformatics **6**, 62

Lourenco, A. P., Mackert, A., Cristino, A. D. S., Paulino Simoes, Z. L. (2008) Validation of reference genes for gene expression studies in the honey bee, *Apis mellifera*, by quantitative real-time RT-PCR. Apidologie **39**, 372-385

Morimoto, T., Kojima, Y., Toki, T., Komeda, Y., Yoshiyama, M., Kimura, K., Nirasawa, K., Kadowaki, T. (2011) The habitat disruption induces immune-suppression and oxidative stress in honey bees. Ecol. Evol. **1**, 201-217

Vandesompele, J., de Preter, K., Pattyn, F., Poppe, B., van Roy, N., de Paepe, A., Speleman, F. (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. **3**(7), research0034