Thiamethoxam exposure in honey bees deregulates short ORF gene expression in the brain and compromises the immune response

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Abstract

Maximizing crop yields heavily relies on the use of agrochemicals to control insect pests. One of the most widely used insecticides are neonicotinoids. Here, we analysed the impact of sub-lethal chronic long-term exposure to the neonicotinoid Thiamethoxam on gene expression and alternative splicing in brains of Africanized honey bees *Apis mellifera*. Our results reveal a small number of differentially regulated genes showing a concentration dependent response to two low doses of chronic, 10-day Thiamethoxam exposure. Unexpectedly, most of these genes have no annotated function, but encode short Open Reading Frames (sORFs), a characteristic feature of anti-microbial peptides. Likewise, we find that Thiamethoxam exposure sensitizes bees to infection by non-pathogenic bacteria *Bacillus badius* and *Ochrobactrum anthropi*. Moreover, infection with estimated single *Serratia marcescens* kills bees arguing that *Varroa* mites may essentially contribute to colony collapse by penetrating the cuticle to spread this pathogen. Our results implicate an altered immune response to Thiamethoxam exposure compromising the immune response leading to a decline in bee populations.

INTRODUCTION

The western honey bee *Apis mellifera* is valued as a highly beneficial species for the human society for producing honey, but most importantly for its critical role in the provision of managed pollination services that contribute to increased yields of many food crops to sustaining global food security. Globally, crop productivity is also enhanced by continuous use of pesticides, often on highly simplified agro-ecosystems. There is a need to find a balance between the necessity of pesticide application and the unintended effects this has on pollinating insects, including honey bees, where agrochemicals are one of the factors implicated in contributing to their declining health and abundance [1–4].

Compared to organophosphate pesticides, neonicotinoids are relatively safe for vertebrates and have been one of the most important protection agents against insect pests outside the EU [5,6]. Neonicotinoids are applied to seeds and will systemically spread to the leaves of growing plants to kill leave eating pests. Applying neonicotinoids to plant seeds dramatically reduces side effects from spraying, but still retains toxic burden for pollinators as neonicotinoids are also found in pollen and nectar [6].

Neonicotinoids act as agonists, competing with the neurotransmitter acetylcholine in binding to nicotinic acetylcholine receptors (nAChR) [7–9]. The increased toxicity for insects is thought to be caused by the characteristically high nAChR density within insect nervous systems [reviewed in [10]]. Uptake of sublethal levels of some types of neonicotinoids affects the honey bee brain, in that foraging behaviour, navigation, communication, learning and memory is impaired [reviewed in [11]] [12–14].

Understanding the risk for honey bees requires detailed knowledge of cellular and molecular effects that result from the exposure to an insecticide in order to mitigate negative effects or refine the target specificity towards pest species.

Changes in gene expression and processing of RNAs, including alternative splicing, are one of the options available to an organism to respond to environmental perturbations [15,16]. Sub-lethal exposure of xenobiotics can induce modulation of splicing reactions [17–19].

Neonicotinoid exposure has been linked to a decline in bee health including a reduction of immune competence [20]; [21–23]. Insects do not have antibodies and rely on the innate immune system to fight microbial infections. The best insight on insect immunity stems from studies in the fruit fly *Drosophila melanogaster,* where cellular and humoral immune responses have been identified [24,25]. The cellular response is mediated by three types of hematopoetic cell lineages [24,26,27], while the humoral immune system can be split into Toll and Imd pathways [reviewed in [28]]. The Toll-pathway is triggered following an immune challenge by Gram-positive bacteria and fungi, ultimately leading to expression of antimicrobial peptides (AMPs) that are then secreted from the fat body into the hemolymph [24,29,30]. In contrast, the Immune deficiency (Imd) pathway leads to expression of a different set of AMPs after a Gram-negative bacterial infection activates pattern recognition receptors and a complex intracellular signaling cascade [24,25,29]. AMPs are short peptides of 10-100 amino acids and are characterised by an evolutionary dynamism among insect species [31].

We have previously shown that worker-bee larvae in colonies contaminated with the neonicotinoid imidacloprid, have altered expression of genes belonging to lipid-carbohydrate-mitochondrial metabolic networks [32]. We have further demonstrated that sub-lethal levels of Thiamethoxam, which is another member of the

neonicotinoid-class of insecticides, can cause impairment in the midgut and brain of the Africanized *Apis mellifera* (AHB), as well as contribute to a reduction in honey bee lifespan [33–36].

Here, we analysed the effects of chronic sub-lethal Thiamethoxam exposure on gene expression and alternative splicing in the brain of worker honey bees. Our results reveal a small number of 52 differentially regulated genes showing a concentration dependent response to two low doses of chronic, 10-day Thiamethoxam exposure. Unexpectedly, most of these genes have no annotated function, but the vast majority are characterized by encoding short Open Reading Frames (sORFs). Half of these genes are predicted to encode antimicrobial peptides suggesting a compromised immune response after Thiamethoxam exposure. Indeed, challenge of Thiamethoxam exposed bees by infection with bacteria dramatically decreased viability. Hence, our results suggest that Thiamethoxam sensitizes bees to bacterial infection by compromising an immune response mediated by anti-microbial peptides.

MATERIALS AND METHODS

Toxicity assays

For chronic exposure to the neonicotinoid Thiamethoxam (Sigma), Africanized *Apis mellifera* bees were marked with a colour pen at eclosion and collected 15 days later from the hives in the apiary of the Biosciences Institute, UNESP, Rio Claro-SP (Brazil). Bees were then kept in small cages in an incubator at 32° C at relative humidity of 70 % for a 10 day duration of Thiamethoxam exposure. For each experimental group bees were collected from three different hives and kept in groups of 20 individuals. Bees were fed *ad libidum* with a solution containing water and inverted sugar (1:1) for the control and for toxin exposure Thiamethoxam was added

at field relevant concentrations of 2 ng/ml and 5 ng/ml, respectively. Ten days after the start of the exposure, bees were cold-anaesthetised, brains were dissected and RNA was extracted.

RNA extraction, Illumina sequencing and analysis of differential gene expression

Total RNA was extracted from five dissected brains per sample by cracking the brain in liquid nitrogen, brief homogenization with a pistil in 50 µl of Tri-reagent (SIGMA) before the volume was increased to 500 µl and proceeded according to the manufacturer's instructions. Total RNA was then stored in 70% ethanol and shipped at ambient temperature from Brazil to the UK.

Total RNA was treated with DNase I (Ambion) and stranded libraries for Illumina sequencing were prepared after poly(A) selection from total RNA (1 µg) with the TruSeq stranded mRNA kit (Illumina) using random primers for reverse transcription according to the manufacturer's instructions. Pooled indexed libraries were sequenced on an Illumina HiSeq2500 to yield 28–41 million paired-end 125 bp reads for three controls, three low and three high Thiamethoxam exposed brain samples. After demultiplexing, sequence reads were aligned to the *Apis mellifera* genome (Amel-4.5-scaffolds) using Tophat2.0.6 [37]. Differential gene expression was determined by Cufflinks-Cuffdiff and the FDR-correction for multiple tests to raw P values with $q < 0.05$ considered significant38. Illumina sequencing and differential gene expression analysis was done by Fasteris (Switzerland). Sequencing data is deposited in GEO under GSE132858.

Alternative splicing was analysed by rMATS [38] and manually inspected by uploading bam files into the Integrated Genome Viewer [39] and comparing read frequency.

Comparison of gene lists was done with Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/). Protein sequences from differentially expressed genes of bees were obtained from ensemble (http://metazoa.ensembl.org/Apis_mellifera/Info/Index) and blasted against *Drosophila* annotated proteins using flybase (http://flybase.org) to assign gene functions. Proteins with no assigned functions were scanned for motifs using the Interpro server (https://www.ebi.ac.uk/interpro/, [40]. Short ORFs were analysed for antimicrobial peptide prediction using the following server: https://www.dveltri.com/ascan/v2/ascan.html [41].

Reverse transcription quantitative polymerase chain reaction (RT-qPCR)

Reverse transcription was done with Superscript II (Invitrogen) as previously described [42]. PCR was done as described and PCR products were analysed on ethidium bromide stained 3% agarose gels [43]. Neuronal genes used as reference for gene expression comparison were bee *erect wing* (*ewg*) and *Amyloid Precursor Protein-like* (*Appl*) based on expression analysis in *Drosophila* [44]. To validate cDNAs obtained from RT the following primers were used to amplify bee *ewg* with AM ewgG F (CCTGATGGTACCGTATCAATTATTCAAGTTG) and AM ewgH R (CCGTGTCCATCTTCTCCTGTGAGAATGATTTG), bee *Appl* with AM Appl F (GCGCGATTCCAGGAAACTGTTGCTGCTC) and AM Appl R2 (CTGCTGTCCAGCAGATGTTTGTAATGAG). Additional primers were GB45995 F (GTTGCATTTTTACGCGTACAGTTACACGACAG) and GB45995 R (GGGAAATCCCCGGGAAGAGAGCAACTGGAG), GB45995 F (GTTGCATTTTTACGCGTACAGTTACACGACAG) and GB45995 R (GGGAAATCCCCGGGAAGAGAGCAACTGGAG), and GB47479 F

(GGGCTATTTGCTATCTAAGTGATCCTCC) and GB47479 R (GGGTTTAGGAGTTTTCGTTTTAGCTGCTG) and PCR amplifications were done by 30 sec initial denaturation at 94º C, and then 40 cycles in total with 30 sec at 94º C, annealing at 48º C with 30 sec extension at 72º C for 2 cycles, then at 50º C with 30 sec extension at 72º C for 2 cycles and then at 52º C with and 60 sec extension at 72º C for 36 cycles with a final extension of 2 min at 72º C.

RNA quality and quantification was assessed using Agilent 6000Nano Kit. 250 ng of DNAse-treated RNA (the same samples used for deep seq) was reversed transcribed using SuperScript III Invitrogen kit in 55ºC, according to manufacturer's instructions. Primers for quantitative real-time PCR (qPCR) were designed by Primer Express software (exon/exon junctions included except for GB41813). Amplicon sizes were assessed in 15% acrylamide gel, PCR products were then sequenced and efficiency of every pair of primers was included in analysis.

Duplicate samples of cDNA was amplified in Real Time PCR with SybrGreen chemistry under following conditions: denaturation 93ºC for 30 sec, annealing 60ºC for 30 sec, elongation 72ºC for 30sec.

GB41923 was amplified with primers GB41923 F1 (CGCGTTGATCGTCATGATATTG) and GB41923 R1 (CTATAAGGAAATTTTGAGCCTTCGA), *GB40669* with primers GB40669 F1 (GGCCGGATATCGCTTCAAA) and GB40669 R1 (GTCTCTTTTATCTTTTCCTCGGAATTC), and *GB48969* with primers GB48969 F1 (TTGCAGCCGTAGCAAAAGGTA), GB48969 R1 (ACCGATTTGAGCACCTTGGT) and *bubblegum* (*bgm, GB51680*) with primers bgm F1 (CATGCACAAAGAGTACAAAAATTTCA) and bgm R1

(TGGTCCCAATTCTCCAGTAACA). Analysis of CT values was performed in Light Cycler480 software and normalization and differential expression was determined with the $2^{-\Delta\Delta Ct}$ method [45] by normalizing to the expression of *ewg*, *Appl* and *actin* genes. *actin* and *ewg* were amplified with primers ewg Fw2 (CCGCGTCTCCTACAGCTCTT) and ewg Rv2 (TGTAAAACTGCCGTAGGATATTGG) and actF1 (TTCCCATCTATCGTCGGAAGA) and actR1 (TTTGTCCCATGCCAACCAT), and Appl with primers AM appl F and AM appl R2, as described previously [32,46].

Bacterial infection assays

For infection assays, *Apis mellifera* from the University of Birmingham Winterbourne garden hives were used and injected with *Bacillus badius*, *Serratia marescens* strains SY13 and 274, *Serratia fonitcola* and *Ochrobactrum anthropi* as detailed below.*S. fonitcola* and *O. anthropi* were isolated from honey bee guts by plating the gut content on LB agar plates incubated at 30º C. Individual colonies were then grown up in LB media and glycerol stocks frozen. Bacterial species were identified by colony PCR and ribosomal 16S sequencing: A colony was picked from an LB agar plate with a yellow tip and placed into 10 µl TE in a PCR tube and heated to 94º C for 5 min. The PCR mix was added adjusting the MgCl concentration to 1.5 mM. PCR was done for 20-40 cycles with 54º C annealing for 40 sec and 1 min extension at 72º C. A 490 bp fragment of the ribosomal 16S gene was amplified with primers 16S F (ACTGAGACACGGYCCAGACTCCTACGTC) and 16S R (GCGTGGACTACCAGGGTATCTAATCC) and sequenced with primer 16S Fseq (CTCCTACGGGAGGCAGCAGTRGGGTC). If sequences did not yield a single species, primers 16S F2 (GTGGACTACCAGGGTATCTAATCCTG) and 16S R2

(CCTACGGTTACCTTGTTACGACTTCAC) were used for amplification of a 733 bp fragment, which was sequenced by 16S R2seq (CCATGGTGTGACGGGCGGTGTGTAC).

Forager honey bees for infection assays were collected from colonies of the Winterbourne Garden of the University of Birmingham (UK) and kept as described [43]. Injections were done as described [43]. Bacteria for injections were freshly plated and grown overnight on LB plates. Then a single colony was used to inoculate a 5 ml LB in a 50 ml Falcon tube and grown over night to saturation and 2 µl of this culture was injected. Bacterial titres of cultures were determined at the time of injections by plating 100 µl of 10^5, 10^6 and 10^7 dilutions in LB and counting of colonies the next day. Typically 2-8 x 10^6 bacteria were injected for *B. badius* and *O. anthropi*. Statistics was done in GraphPad prism using Anova followed by Tukey-Kramer post hoc test.

RESULTS

Chronic Thiamethoxam exposure alters expression of a subset of genes in honey bee brains

Here, we assessed whether the neonicotinoid Thiamethoxam alters gene expression in the brain of Africanized honey bees *Apis mellifera* at field relevant concentrations after long-term exposure. For these experiments age-matched, control sugar-fed bees (sample A) were compared to bees exposed to 2 ng/ml (low dose, LD, sample B) and 5 ng/ml (high dose, HD, sample C) Thiamethoxam contained in the sugar solution given for feeding for 10 days. To enhance robustness against genetic variation, we chose worker honey bees from three unrelated hives.

Analysis of differential gene expression between LD exposure to Thiamethoxam and the control revealed 222 up- and 181 down-regulated genes, and between HD and the control revealed 233 up- and 114 down-regulated genes with a 1.5 fold difference in expression (Supplemental table 1). From this comparison, 37 were up-regulated and 15 were down-regulated upon Thiamethoxam exposure in a dose-responsive manner (Supplemental table 1).

To validate these results from the Illumina sequencing, we performed RT-qPCR for three of the dose-responsive genes (*GB41923*, a putative Sodium chloride co-transporter, and *GB48969, GB40669,* two genes with unknown function). For all three genes we detected an expression difference upon Thiamethoxam exposure (Supplemental Fig 1). We also validated and confirmed differential expression of *bubblegum*, encoding a very long-chain acyl-CoA synthetase, which has been found down-regulated in honey bee larvae exposed to the neonicotinoid imidaclorid (Derecka et al., 2013, Supplemental Fig 1).

Alternative splicing has been suggested as a mechanism to adapt gene expression to environmental changes [18,47]. Accordingly, we analysed the RNA-seq data for changes in alternative splicing (Supplemental table 2). Although this analysis revealed significant differences, upon manual inspection of sequencing traces using Integrated Genome Viewer (IGV) [39], we did not further consider this, because the analysis process mostly selected genes with complex splicing patterns that showed no obvious differences in the number of sequence reads over alternatively spliced gene sections in IGV (data not shown).

Dose-responsive expression following Thiamethoxam exposure occurs mostly in genes encoding uncharacterized ORFs

Next, we categorized the genes responsive to the dose of Thiamethoxam according to their functions, taking into account known functions for orthologues in *Drosophila* and functions deduced from annotated protein domains retrieved by BLAST analysis. This analysis revealed that 59 % (22 from 37) and 73 % (11 from 15) of up- or down-regulated genes, respectively, had neither clear orthologues in *Drosophila* nor any recognizable protein domains that would indicate a biological function (Fig 2A). Among up-regulated genes 14 % (5 from 37) were assigned roles in cellular signaling, which potentially could be linked to altered neuronal function, such as olfactory and taste perception, and as structural components (cytoskeleton), and 8 % (3 from 37) of genes were assigned functions in regulation of gene expression by transcriptional regulation (Fig 2A). The very high number of genes with unknown function is unexpected compared with *Drosophila* as in this organism the fraction of genes with unknown functions in gene expression studies is about 20 % [48,49].

Most Thiamethoxam dose-responsive genes in the brain encode short proteins

When analyzing the set of dose-responsive, differentially expressed genes for the presence of annotated proteins and protein domains to assign functions, we noticed that many of the genes with unknown function coded for short ORFs. For Thiamethoxam induced differentially up- and down-regulated genes 73 % (27 from 37) and 87 % (13 form 15) encode for genes with ORFs of 250 aa or shorter, respectively (Fig 2B).

Since these short ORFs could encode anti-microbial peptides, we used a machine learning algorithm to predict whether they encode anti-microbial peptides [41]. From the 40 genes encoding peptides shorter than 250 aa, 43 % (17 genes) were predicted to

code for antimicrobial peptides, of which 52 % (11 genes) and 55% (6 genes) were from differentially up- and down-regulated genes, respectively (Fig 2B).

Thiamethoxam sensitizes worker bees to bacterial infection

Thiamethoxam-induced alteration of anti-microbial peptide gene expression could disturb the immune response. To test this hypothesis we adopted a procedure initially developed for *Drosophila,* where viability is monitored after injection of bacteria. In this assay, non-pathogenic bacteria are efficiently cleared by anti-microbial peptides with little impact on viability [50]. To test efficient clearance of non-pathogenic bacteria in bees, we used *Bacillus badius*, a non-pathogenic bacterium commonly found in the environment (Fig 3A). In addition, we isolated two bacterial species from worker bee gut cultures. Genotyping by sequencing variable fragments of the 16S ribosomal RNA gene identified them as *Serratia fonticola* and *Ochrobactrum anthropi,* both Gram-negative bacteria which have previously been identified in the honey bee microbiome [51,52].

When saturated liquid cultures (2-8 Mio bacteria in 2 µl) of *B. badius* and *O. antrhopi* were injected into bees, viability was not affected, indicating that the bee immune system is able to clear the infection efficiently (Fig 3A and B). Injection of *S. fonticola* at these concentrations was lethal. In contrast, injection of sub-lethal doses of Thiamethoxam together with either *B. badius* and *O. antrhopi* resulted in lethality (Fig 3A and B). Intriguingly, diluting either Thiamethoxam or injected bacteria 10 fold still dramatically decreased viability 48 h after injections.

We also assessed the effect of *Serratia marescens*, a known bacterial pathogen of bees [53]. Injection of *S. marescens* killed bees even at a very low bacterial load,

where we estimate that only one bacterium was present in the injected volume (Fig 4).

DISCUSSION

A key finding of our analysis of brain transcriptomes of worker honey bees is the highly enriched fraction of dose-responsive, uncharacterised genes encoding short open reading frames (sORFs). Strikingly, the majority of these dose-responsive sORFs are predicted to encode antimicrobial peptides. Rather than being non-coding RNAs, sORFs have only recently been recognized to encode functional peptides [54–56] some of which play important roles during development [57]. Differential regulation of sORFs was not reported in recent whole-transcriptome studies to evaluate the effects of neonicotinoid exposure in brains of honey bees or bumblebees [19,58,59]. Likewise, we did not find an overlap of the differentially expressed genes. The main difference to our study is that we analysed changes upon long-term low dose chronic exposure, while these studies used shorter term exposures. However, we note that other studies infecting bees with viruses of *Nosema* revealed an overrepresentation of genes with known function in immune and defence processes, where expression is altered upon exposure to different types of neonicotinoids [19,21,58–60]. Since we did not detect differential expression of known immune genes, the bees in our study were not infected with these known pathogens, but we cannot exclude an impact of other microbiota that are non-pathogenic in healthy bees.

Bees, including *Apis mellifera* are characterised by their limited set of canonical immune genes, compared to non-social insects, such as the fruit fly *D. melanogaster* [61–63]. Currently, only six antimicrobial-peptide genes comprising four gene families have been described in honey bees [63]. In contrast, *Drosophila* has 20 antimicrobial-

peptide genes comprising eight gene families [24,63]. From these genes, only defensin is conserved between honey bees and *Drosophila,* consistent with the idea that antimicrobial peptides evolve fast to adapt to species-specific environmental conditions [31]. Given the low number of known antimicrobial peptides in bees, it is conceivable that new, as yet uncharacterised genes encoding antimicrobial peptides are evolving. Alternatively, the short ORFs that we found to be differentially expressed upon chronic low dose Thiamethoxam exposure in the brain might form a basal immune defence in the brain, similar to the antimicrobial environment present in saliva containing many antimicrobial peptides in many animal species. However, antimicrobial peptides have also been identified in having role in learning and memory in *Drosophila* [64].

Various xenobiotics have been shown to alter the gut microbiome [65,66]. Our results are consistent with previous findings where neonicotinoid exposure adversely affects insect immunity [66,67]. The immune challenge by non-pathogenic bacteria was well-tolerated by worker bees, while the Thiamethoxam-exposed worker bees succumbed by the same bacterial challenge demonstrating that pesticide exposure weakens immunity of honey bees.

We note that pathogens can enter the hemolymph through punctures inflicted by *Varroa destructor* mites. In case of *Serratia marcescens*, our data indicate that very low numbers of bacteria are sufficient to kill bees of the worker caste. Likely, bees are confronted with other less infective pathogens, that normally are efficiently cleared by the immune response when transmitted by *Varroa* mites. However, it can be anticipated that if the immune response is compromised by Thiamethoxam exposure, such pathogens can lead to considerable mortality in a hive. The situation

is further aggravated as *Varroa* mites likely change the infected host upon its death leading to more rapid spreading of pathogens.

The *Down Syndrome Cell Adhesion Molecule* (*Dscam*) gene acts as a pattern recognition receptor in the immune response that through mutually exclusive alternative splicing can generate 36,012 different isoforms in *Drosophila* [68,69]. We did not find changes in *Dscam* alternative splicing in response to acute high dose xenobiotic exposure in bees [43], but also not to low dose long-term exposure (this study). Since *Dscam* alternative splicing is robust against perturbations of splicing factors [70,71], the absence of changes might indicate that a specific immune challenge is required to change its splicing pattern [72]. We previously also analysed alternative splicing in bee *elav* and *Xbp-1* genes as potential markers for defects in synapse formation and the stress response upon high dose acute Thiamethoxam exposure, but also low dose chronic exposure did not affect their splicing [43,48,49]. Potentially, alternative splicing might change only in a few cells in the brain eluding detection without single cell analysis or could in addition also be more subtle, requiring more replicates for detection of significantly altered alternative splicing changes [18].

Taken together, the most prominent changes in gene expression upon long-term chronic low dose Thiamethoxam exposure identified mostly genes with unknown function with the vast majority encoding short ORFs, of which about half are predicted antimicrobial peptides. Moreover, Thiamethoxam exposure sensitizes bees to infection. Future research will determine the role of these genes in the immune response and reveal how bee immunity can be strengthened to resist infections.

Authors' contribution

P.D., P.U., K.D. and D.S. performed the experiments, T.C.R, I.C.W.H., O.M., N.M. analyzed data, R.S. and M.S. supervised experiments and analyzed data, T.C.R, M.S. and R.S. conceptualised the Thiamethoxam exposure and RNA-Seq portion of the work; M.S. conceptualised the bacterial injection work; R.S. and M.S. wrote the manuscript with help from P.D. and P.U.

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Figure legends

Figure 1: Thiamethoxam induces differential expression in a subset of genes. Venn diagrams indicating the number of differentially expressed genes between control bees and bees exposed to a low dose (left) and high dose (right) of Thiamethoxam that were up- (A) or down-regulated (B). Genes that responded to the injected dose are indicated by a white number on dark background).

Figure 2: Classification of Thiamethoxam induced differentially expressed genes according to function and size.

(A) Numbers of genes are plotted according to functions for Thiamethoxam induced differentially expressed genes with annotated functions for up- (top) and down-regulated (bottom) genes.

(B) Chart pies indicating the fraction of Thiamethoxam induced differentially expressed genes according the ORF length for up- (left) and down-regulated (right) genes.

Figure 3: Thiamethoxam exposure sensitizes bees to infection by *B. badius* and *B. melitensis*. Viability of bees 24 h (grey bars) and 48 h (black bars) after injection with *B. badius* (A) and *B. melitensis* (B) alone or together with Thiamethoxam shown as mean from three experiments with the standard error. Arrows indicate injection of a 1 µM Thiamethoxam solution with (A right and B) or without bacteria (A left). Statistical significance is indicated by stars (** $p<0.01$ and *** $p<0.001$).

Figure 4: Injection of *Serratia marcescens* is highly toxic to bees. Viability of groups of 10 bees injected with medium (grey diamonds) or medium containing *S. marcescens* (black diamonds) according to the number of bacteria injected as determined from plating on LB agar plates (cfu, colony forming units) on a logarithmic scale.

Supplemental Figure 1: Validation of Thiamethoxam induced differentially expressed genes by RT-qPCR. Means with standard error from three experiments are represented log2 fold change in expression levels normalized to *ewg*, *Appl* and *actin* genes.

Figure 1

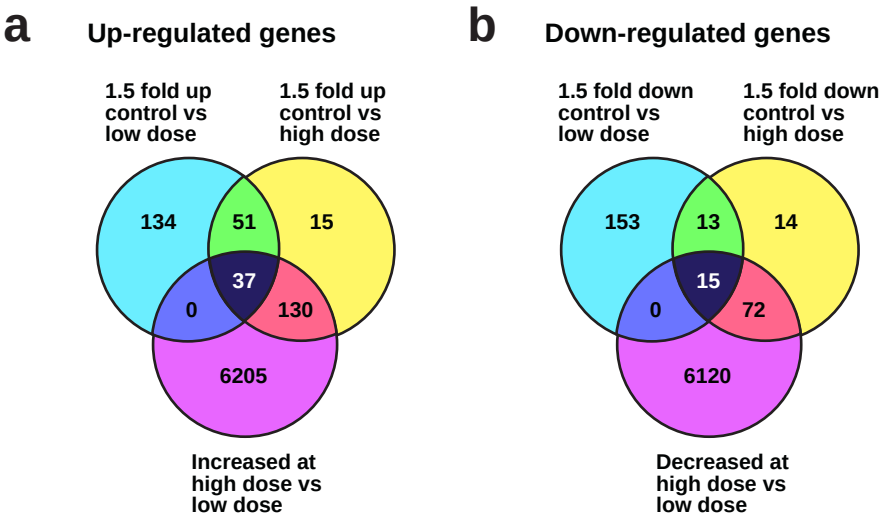


Figure 2

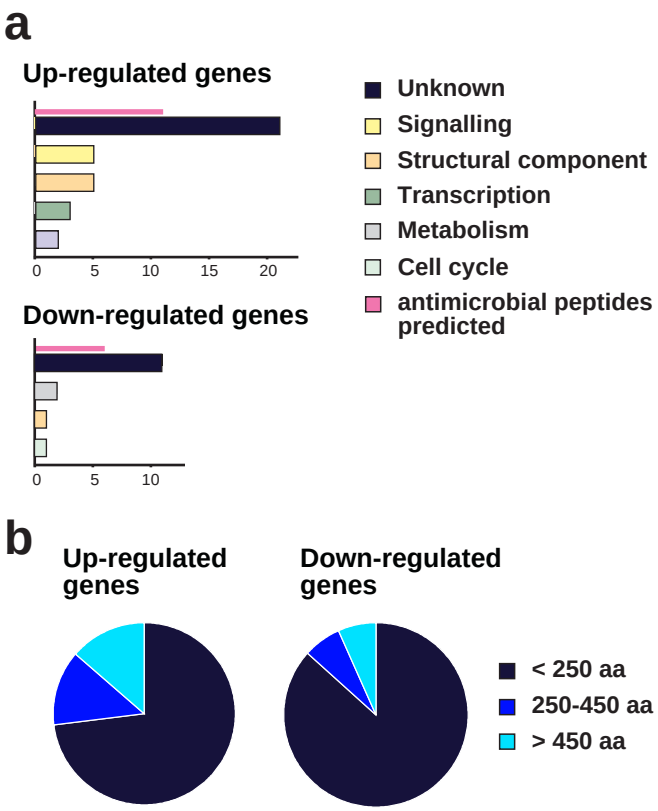


Figure 3

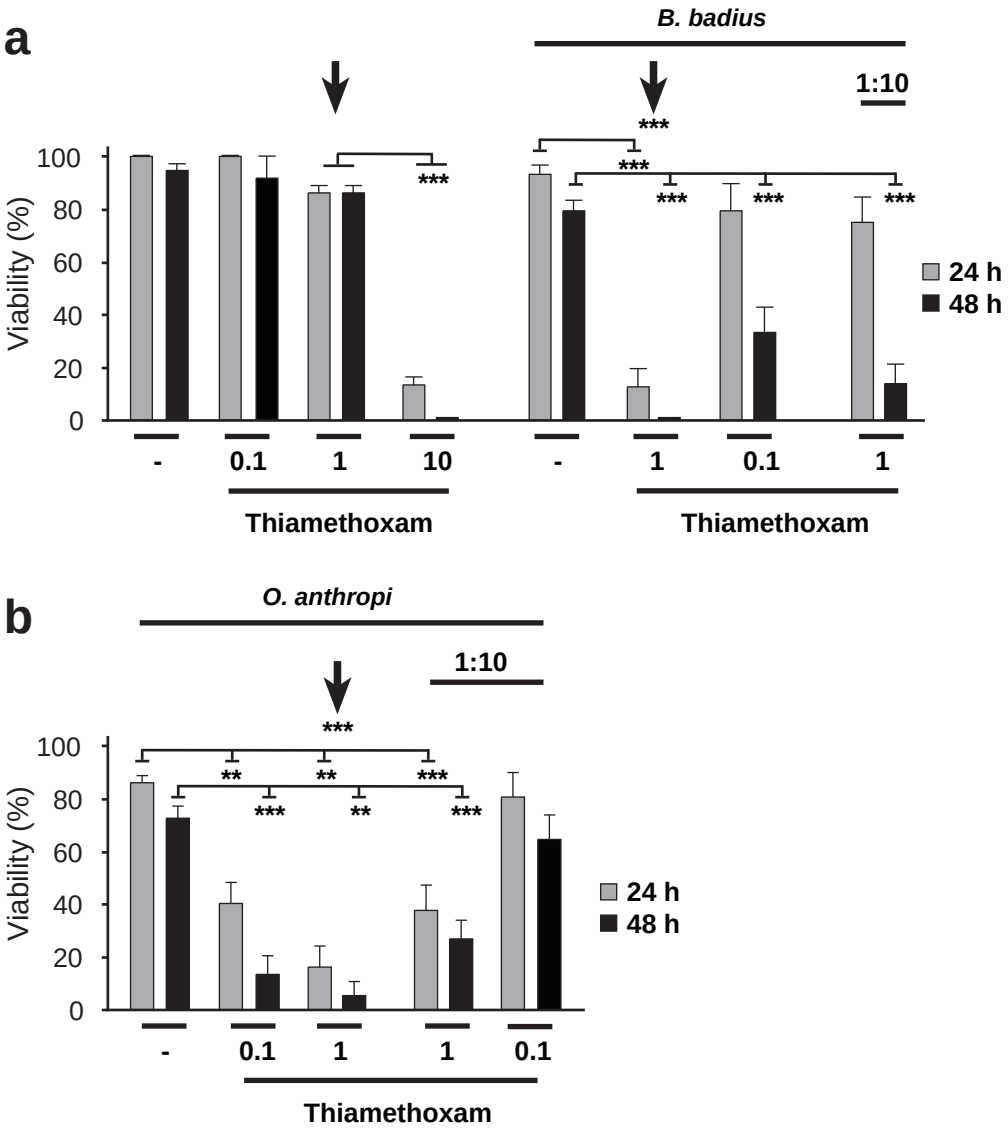
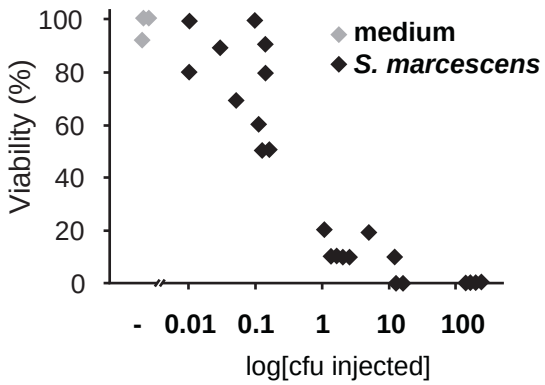


Figure 4



Supplemental Figure 1