Thiamethoxam exposure deregulates short ORF gene expression in the honey bee and compromises immune response to bacteria

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Abstract

Maximizing crop yields relies on the use of agrochemicals to control insect pests. One of the most widely used classes of insecticides is the neonicotinoids but these can also disrupt crop-pollination services provided by bees. Here, we analysed the impact of chronic exposure to the neonicotinoid thiamethoxam on gene expression and alternative splicing in brains of Africanized honey bees, *Apis mellifera*. We find differentially regulated genes that show concentration-dependent responses to thiamethoxam. Most have no annotated function but encode short Open Reading Frames (sORFs), a characteristic feature of anti-microbial peptides. We show that intrinsically sub-lethal thiamethoxam exposure makes bees more vulnerable to pathogenic bacteria. Our findings imply a synergistic mechanism for the observed bee population declines that concern agriculturists, conservation ecologists and the public.

INTRODUCTION

The western honey bee *Apis mellifera* is highly beneficial for human societies. Besides honey production, this semi-domesticated insect plays a critical role in sustaining global food security through the provision of managed pollination services that contribute to increased yields of many crops. Globally, crop productivity is also enhanced by the application of pesticides, including insecticides. Agrochemicals are, however, among the key factors implicated in contributing to declining bee health and abundance and there is a need to find a balance between the necessity of insecticide applications and their unintended non-target effects [1–5].

Compared to organophosphate pesticides, neonicotinoids are relatively safe for vertebrates and have been one of the most important insecticides for the last three decades [6]. Neonicotinoids can be applied to seeds and they then spread systemically within growing plants, killing leaf-eating pests. This reduces non-target effects compared to spraying but still retains toxic burden for pollinators as neonicotinoids continue to be found in wildflowers adjacent to treated fields [7].

Neonicotinoids act as agonists, competing with the neurotransmitter acetylcholine in binding to nicotinic acetylcholine receptors (nAChR) [8–10]. The increased toxicity for insects is thought to be caused by the characteristically high nAChR density within insect nervous systems [reviewed in [11]]. Detailed knowledge of cellular and molecular effects of insecticide exposure is required to mitigate negative effects or refine target specificity. Changes in gene expression and processing of RNAs, including alternative splicing, are among mechanisms available to an organism to adapt to environmental perturbations

[12,13]. Sub-lethal exposure to xenobiotics, such as pesticides, can alter alternative splicing [14–16]. Since many ion channels and other important neuronal genes undergo extensive alternative splicing, they are prime targets for changes induced by xenobiotics [15,17]. Moreover, sub-lethal uptake of some neonicotinoids affects the honey bee brain, impairing foraging behaviour, navigation, communication, learning and memory [reviewed in [18–21]].

Neonicotinoid exposure has also been linked to a decline in bee health, including a reduction of immune competence [22–24]. Insects do not have antibodies and rely on the innate immune system to fight microbial infections; both cellular and humoral responses have been identified [25,26]. The cellular response leading to phagocytosis or encapsulation of pathogens is mediated by three types of haematopoietic cell lineages [25,27,28], while the humoral immune response is activated by Toll and Imd pathways [reviewed in [29]], leading to the expression of short (10-100 amino acids) antimicrobial peptides (AMPs) which are highly diverse among insect species [30]. The Toll-pathway is triggered by Gram-positive bacteria and fungi, ultimately leading to expression of AMPs that are then secreted from the fat body into the haemolymph [25,31,32]. The Immune deficiency (Imd) pathway leads to expression of a different set of AMPs after Gram-negative bacterial infection activates pattern recognition receptors and a complex intracellular signalling cascade [25,26,31].

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 [33]. We have further demonstrated that sub-lethal exposure to thiamethoxam, another neonicotinoid, can cause impairment in the midgut and brain of the

Africanized *Apis mellifera*, as well as contribute to a reduction in honey bee lifespan [34–37].

In this study, we analysed the effects of chronic, sub-lethal, thiamethoxam exposure on genome-wide gene expression and alternative splicing in the brains of honey bee workers. We found 52 differentially regulated genes showing a concentration dependent response to thiamethoxam exposure. Most of these genes have no annotated function but the vast majority are characterized by encoding short Open Reading Frames (sORFs), half of which are predicted to encode antimicrobial peptides. As this suggested that immune responses may be compromised by thiamethoxam exposure, we tested found that thiamethoxam-exposed bees that were also infected with bacteria had greatly decreased viability compared to infected but chemically unexposed bees. Overall, our results suggest that thiamethoxam makes bees vulnerable to bacterial infection by compromising immune responses.

MATERIALS AND METHODS

We assessed whether the neonicotinoid thiamethoxam alters gene expression in the brain of Africanized honey bees, *Apis mellifera,* after long-term exposure to field-relevant concentrations.

Thiamethoxam exposures

Bees from three unrelated hives in the apiary of the Biosciences Institute*,* *UNESP*, Rio Claro-SP (Brazil), were marked with a colour pen at eclosion 15 days later. They were then kept in groups of 20 individuals in small cages within an incubator at 32° C. Bees were fed *ad libitum* with sugar solution (1:1 water and inverted sugar) for the control treatment (sample A) and, for toxin exposure treatments, thiamethoxam (Sigma) was added at 2 ng/ml (low dose, LD, sample B) or at 5 ng/ml (high dose, HD, sample C). After ten days, bees were cold-anaesthetised and their brains were dissected for RNA extraction.

RNA extraction, Illumina sequencing, analysis of differential gene expression and splicing

Total RNA was extracted from ten dissected brains per sample by cracking the brain in liquid nitrogen, brief homogenization with a pestle in 50 µl of Tri-reagent (SIGMA) and then, following the manufacturer's instructions, the volume was increased to 500 µl. Total RNA was then stored in 70% ethanol and transported 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 control, three low dose and three high dose samples. After demultiplexing, sequence reads were aligned to the *Apis mellifera* genome (Amel-4.5-scaffolds) using Tophat2.0.6 [38]. Differential gene expression was determined by Cufflinks-Cuffdiff and the FDR-correction for multiple tests to raw P values with $p < 0.05$ was considered significant [39]. Illumina sequencing and differential gene expression analysis was carried out by Fasteris (Switzerland). Sequencing data are deposited in GEO under GSE132858 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132858).

Alternative splicing was analysed by rMATS [40] and manually inspected by uploading bam files into the Integrated Genome Viewer [41] and comparing read frequency. Comparison of gene lists was made 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/) [42]. Short ORFs were analysed for antimicrobial peptide prediction using the following server: https://www.dveltri.com/ascan/v2/ascan.html [43].

Alternative splicing has been suggested as a mechanism to adapt gene expression to environmental changes [15,44]. We analysed the RNA-seq data for

157 changes in alternative splicing but found no conclusive patterns
158 (Supplementary Information 1; Supplemental table 2).
159
160 **Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**
161 Reverse transcription was carried out with Superscript II (Invitrogen) as
162 previously described [45]. PCR was performed as described and PCR products
163 were analysed on ethidium bromide stained 3% agarose gels [17]. Neuronal
164 genes used as reference for gene expression comparison were bee *erect*
165 *wing* (*ewg*) and *Amyloid Precursor Protein-like* (*Appl*), based on expression
166 analysis in *Drosophila* [46]. To validate cDNAs obtained from RT, the following
167 primers were used to amplify bee *ewg* with AM ewgG F
168 (CCTGATGGTACCGTATCAATTATTCAAGTTG) and AM ewgH R
169 (CCGTGTCCATCTTCTCCTGTGAGAATGATTTG), bee *Appl* with AM Appl F
170 (GCGCGATTCCAGGAAACTGTTGCTGCTC) and AM Appl R2
171 (CTGCTGTCCAGCAGATGTTTGTAATGAG). Additional primers were
172 GB45995 (GTTGCATTTTTACGCGTACAGTTACACGACAG) and GB45995
173 R (GGGAAATCCCCGGGAAGAGAGCAACTGGAG), GB45995 F
174 (GTTGCATTTTTACGCGTACAGTTACACGACAG) and GB45995 R
175 (GGGAAATCCCCGGGAAGAGAGCAACTGGAG), and GB47479 F
176 (GGGCTATTTGCTATCTAAGTGATCCTCC) and GB47479 R
177 (GGGTTTAGGAGTTTTCGTTTTAGCTGCTG) and PCR amplifications were
178 done by 30 sec initial denaturation at 94º C, and then 40 cycles in total with
179 30 sec at 94º C, annealing at 48º C with 30 sec extension at 72º C for 2
180 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 an Agilent 6000Nano Kit. 250 ng of DNAse-treated RNA (the same samples used for deep seq) was reversed transcribed using a SuperScript III Invitrogen kit at 55^{o}C, following manufacturer's instructions. Primers for quantitative real-time PCR (RT-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 the analysis.

Duplicate samples of cDNA were amplified in Real Time PCR with SybrGreen chemistry under following conditions: denaturation 93^{o}C for 30 sec, annealing 60^{o}C for 30 sec, elongation 72^{o}C 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 [47] 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, following [33,48].

Bacterial infection assays

Thiamethoxam-induced alteration of anti-microbial peptide gene expression could disrupt bees' immune response. To evaluate the effect of thiamethoxam on immunity, we adopted an assay procedure, initially developed for *Drosophila*, which assesses how efficiently injected non-pathogenic bacteria are cleared by anti-microbial peptides [49]. To assay clearance in bees we used *Bacillus badius*, a non-pathogenic bacterium commonly found in the environment and *Ochrobactrum anthropi*, which are Gram-negative members of the honey bee microbiome [50,51]. *anthropi* were isolated from worker bee gut cultures by plating the gut content on LB agar plates incubated at 30º C.

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

231 amplification of a 733 bp fragment, which was sequenced by 16S R2seq
232 (CCATGGTGTGACGGGCGGTGTGTAC).
233 Forager honey bees for infection assays were collected from colonies of the
234 Winterbourne Garden of the University of Birmingham (UK). They were kept
235 and injected with bacteria as we described previously [17]. Bacteria for
236 injections were freshly plated and grown overnight on LB plates. Then a single
237 colony was used to inoculate a 5 ml LB in a 50 ml Falcon tube and grown
238 overnight to saturation: 2 µl of this culture was then injected. Bacterial titres of
239 cultures were determined at the time of injections by plating 100 µl of 10^5, 10^6
240 and 10^7 dilutions in LB and counting the colonies the next day: this showed
241 that 2-8 x 10^6 *B. badius* or *O. anthropi* were typically injected. In some
242 treatments the injected bacteria were diluted 10-fold (Fig. 3). Groups of 8 to
243 12 bees were exposed to combinations of bacterial and thiamethoxam doses
244 and survival was assessed at 24 hours and 48 hours.
245

RESULTS

Chronic thiamethoxam exposure effects gene expression

After low (LD) and high dose (HD) exposure to thiamethoxam, there were 222 up- and 181 down-regulated genes for LD (Fig. 1a) and 233 up- and 114 down-regulated genes for HD with a 1.5 fold difference in expression compared to the control treatment (Fig. 1b; Supplemental Table 1). From these differentially regulated genes, 37 were up-regulated and 15 were down-regulated in a dose-sensitive manner (Fig. 1; 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. We detected an expression difference for all three genes 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 to be down-regulated in honey bee larvae exposed to the neonicotinoid imidacloprid [33] (Supplemental Fig. 1).

Dose-responsive expression occurs mostly in genes encoding uncharacterized ORFs

Next, we categorized the genes dose-responsive to thiamethoxam according to their functions, taking into account known functions of orthologues in *Drosophila* and functions deduced from annotated protein domains retrieved by BLAST analysis. Amongst the dose-sensitive genes that were up-regulated (Fig. 1A), 14 % (5/37) were assigned roles in cellular signalling (with potential links to altered neuronal function, such as olfactory and taste perception) and

as structural components (cytoskeleton), and 8 % (3/37) were assigned functions in transcriptional regulation of gene expression (Fig. 2A). However, 59% (22/37) of dose-sensitive up-regulated genes and 73 % (11/15) of dose-sensitive down-regulated genes had neither clear orthologues in *Drosophila* nor any recognizable protein domains that would indicate a biological function (Fig. 2A), which is in contrast to about 20% of genes with unknown function in gene expression studies in *Drosophila,* [52,53].

Most thiamethoxam dose-responsive genes encode short proteins

Many of the dose-responsive, differentially expressed genes with unknown function coded for short ORFs. For thiamethoxam-induced differentially up- and down-regulated genes, respectively 73 % (27/37) and 87 % (13/15) encode for genes with ORFs of 250 amino acids or shorter (Fig. 2). Using a machine learning algorithm [43], we predicted the 40 genes coding for peptides of ≤250 amino acids, 17 (43 %), the peptides have antimicrobial function (11/17 genes were up-regulated and 6/17 were down-regulated, Fig. 2).

Thiamethoxam makes worker bees more vulnerable to bacterial infection

When saturated liquid cultures (2-8 Mio bacteria in 2 µl) of *B. badius* and *O. antrhopi* were injected into bees that were not exposed to thiamethoxam, viability was affected significantly but not greatly, indicating that the bee immune system usually clears the infection efficiently (Fig. 3, Table 1). In contrast, injection of normally sub-lethal doses of thiamethoxam together with either *B. badius* and *O. antrhopi* frequently resulted in bee death (Fig. 3, Table

296 1). After 48 hours a positive interaction effect between thiamethoxam dose
297 and bacterial dose was detected (Table 1). We conclude that thiamethoxam
298 negatively affects bees' abilities to cope with natural immuno-challenges,
299 which would not normally prove fatal.

300 **DISCUSSION**

301 A key finding of our analysis of honey bee transcriptomes is the highly
302 enriched fraction of dose-responsive, uncharacterised genes encoding short
303 open reading frames (sORFs). Such sORFs have only recently been
304 recognized to encode functional peptides [54–56], some of which play important
305 roles during development [57] and the majority of the dose-responsive sORFs
306 we have identified are predicted to encode peptides with antimicrobial
307 function. The sORFs we identified have not been reported in prior whole-
308 transcriptome evaluations of neonicotinoid exposure in bee brains [16,58,59]. The
309 main methodological difference is that we analysed changes after long-term,
310 low dose exposure, while prior studies used shorter exposures. However, we
311 note that other studies infecting bees with viruses of *Nosema, a* unicellular
312 parasite of bees, found an overrepresentation of genes with known function in
313 immune and defence processes, where expression is altered upon exposure
314 to different types of neonicotinoids [16,22,58–60]. Since we did not detect
315 differential expression of known immune genes, the bees in our study were
316 not infected with these known pathogens, but we cannot exclude an impact of
317 other microbiota that are non-pathogenic in healthy bees.
318 Bees, including *Apis mellifera*, are characterised by their limited set of
319 canonical immune genes, compared to non-social insects, such as the fruit fly
320 *D. melanogaster* [61–63]. Currently, only six antimicrobial-peptide genes

321 comprising four gene families have been described in honey bees [63]. In
322 contrast, *Drosophila* has 20 antimicrobial-peptide genes comprising eight
323 gene families [25,63]. From these genes, only *defensin* is conserved between
324 honey bees and *Drosophila,* consistent with the idea that antimicrobial
325 peptides evolve fast to adapt to species-specific environmental conditions [30].
326 Given the low number of known antimicrobial peptides in bees, it is
327 conceivable that new (currently uncharacterised) genes encoding
328 antimicrobial peptides are evolving. Alternatively, the short ORFs that we
329 found to be differentially expressed upon chronic low dose thiamethoxam
330 exposure might form a basal immune defence, similar to the antimicrobial
331 environment present in saliva containing antimicrobial peptides in many
332 animal species. However, antimicrobial peptides have also been identified in
333 having a role in learning and memory in *Drosophila* [64].
334 Various agrochemicals have been shown to alter the gut microbiome [65,66]. Our
335 results are consistent with previous findings where neonicotinoid exposure
336 adversely affects insect immunity [66,67]. Specifically, we have shown that
337 immune challenges from what are normally non-pathogenic bacteria become
338 fatal to bees when combined with thiamethoxam exposure.
339 We note that pathogens can enter bee haemolymph through punctures
340 inflicted by *Varroa destructor* mites [51] and thus there may be considerable
341 mortality within hives infested with *Varroa* and also exposed to thiamethoxam.
342 In summary, the most prominent changes in gene expression upon long-term
343 low-dose thiamethoxam exposure identified mostly genes that encode short
344 ORFs, around half of which are predicted to code for antimicrobial peptides.
345 Furthermore, thiamethoxam exposure reduced the capacity of bees to

346 withstand microbial infection. Taken together, these findings imply that low
347 doses of neonicotinoids may be intrinsically sub-lethal to bees but can be
348 ultimately fatal via a weakened immune response to extrinsic pathogens. The
349 roles of the identified genes in the immune response of bees will need to be
350 identified to establish how bee immunity might be strengthened to resist
351 bacterial infections.
352
353 **Authors' contributions**
354 P.D., K.D. and D.S. performed the experiments, T.C.R, I.C.W.H., O.M., N.M.
355 analysed data, R.S. and M.S. supervised experiments and analysed data,
356 R.S. and M.S. wrote the manuscript with help from P.D., P.U. and I.C.W.H.
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559

Table 1. Effects of bacterial dose and thiamethoxam dose on bee viability.

Data on survival were analysed using logistic analyses adopting a logit-link function and assuming quasi-binomial distributed errors. The two species of bacteria did not differ significantly in their effect on bee viability (24h: $F_{1,7}$=1.18, P=0.319; 48h: $F_{1,7}$=0.37, P=0.566) and bacterial dose was further analysed without species distinction. Bee viability, measured at either 24 hours or 48 hours, was negatively affected by increasing doses of thiamethoxam and of bacteria. At 48 hours after exposure, a synergistic interaction between these main effects was also detected: bee viability declined more rapidly in response to bacterial doses when bees were exposed to higher doses of thiamethoxam, Fig. 3). These analyses were carried out using GenStat v.19 (VSN International, Hemel Hempstead).

	Survival after 24 hours				Survival after 48 hours			
Source	*d.f.*	Deviance	*F*	*P*	*d.f.*	Deviance	*F*	*P*
Bacterial dose	1	73.56	27.61	<0.001	1	88.47	26.10	<0.001
Thiamethoxam dose	1	106.12	39.83	<0.001	1	70.87	20.91	<0.001
Bacteria × Thiamethoxam interaction	1	1.79	0.67	0.418	1	18.50	5.46	0.026
Residual	32	85.27			32	108.47		
Total	35	229.62			35	286.32		

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- (B) regulated. 37 genes were up-regulated and 15 genes were down-regulated in a dose-sensitive manner.

Figure 1

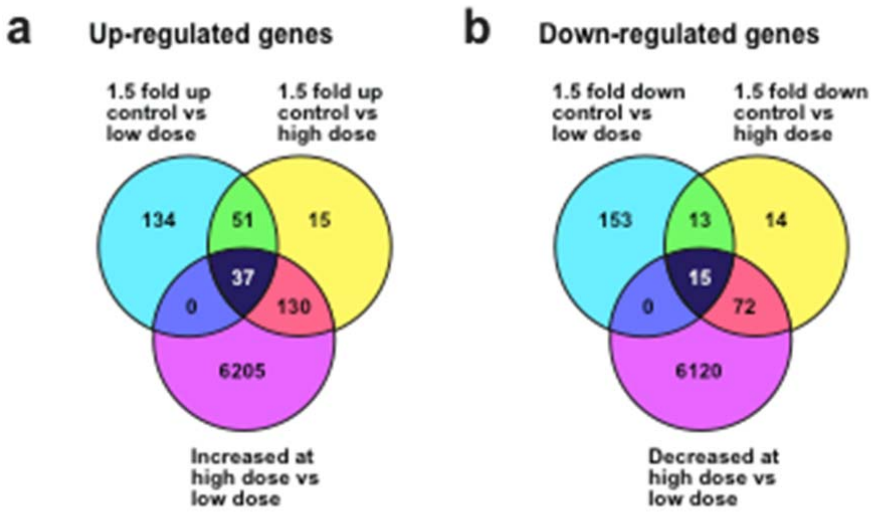
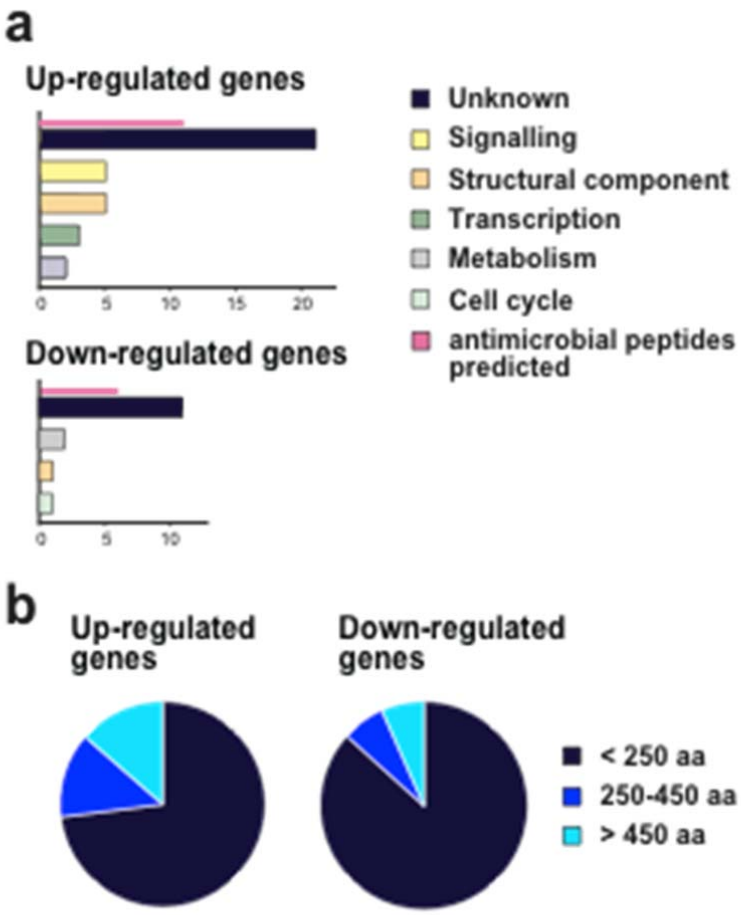


Figure 2



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592 **Figure 2: Classification of thiamethoxam induced differentially**
593 **expressed genes according to function and size.** (A) Numbers of genes
594 are plotted according to functions for thiamethoxam induced differentially
595 expressed genes with annotated functions for up- (top) and down-regulated
596 (bottom) genes. (B) Pie charts indicating the fraction of thiamethoxam induced
597 differentially expressed genes according the ORF length for up- (left) and
598 down- (right) regulated genes.

Figure 3

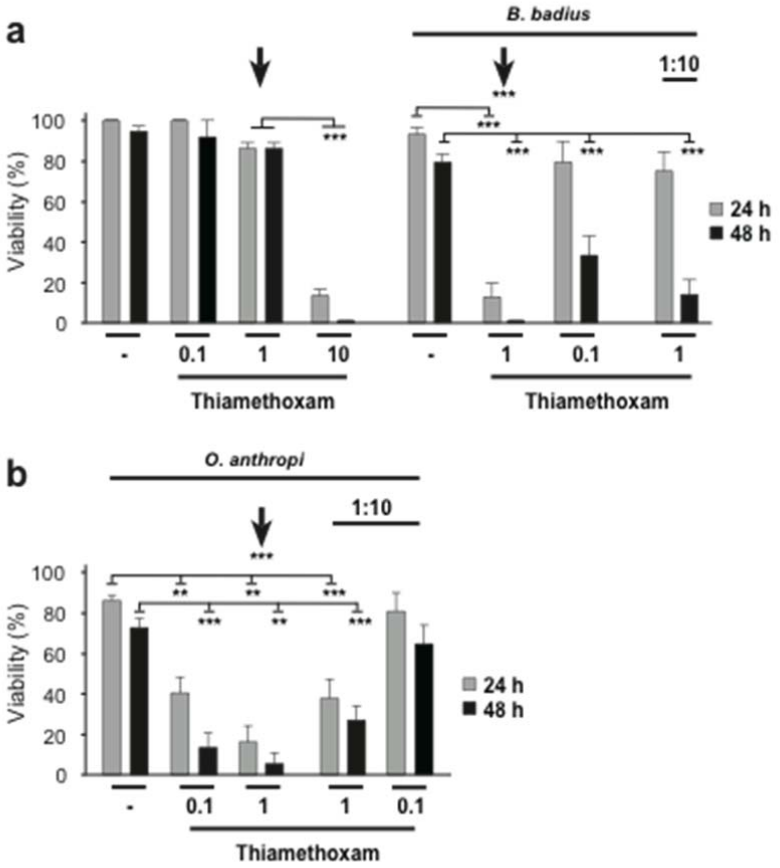

Figure 3: Thiamethoxam exposure makes bees vulnerable to infection by *B. badius* and *O. anthropi*. Viability of bees 24 h (grey bars) and 48 h (black bars) after injection with *B. badius* (A) or *O. anthropi* (B) (2-8 x 10^6 and diluted 1:10) alone or together with thiamethoxam (at a range of doses, µM) shown as means and standard errors. Arrows indicate injection of a 1 µM Thiamethoxam solution with (A right and B) or without bacteria (A left). Statistical analyses were carried out in GraphPad prism using ANOVA followed by Tukey-Kramer post-hoc tests. Statistical significance is indicated by ** ($p<0.01$) and *** ($p<0.001$).