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

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Running title: Thiamethoxam exposure, short ORFs and bee immunity

Key Words: Neonicotinoid, *Apis mellifera*, anti-microbial peptides,

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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 are neonicotinoids that interfere with signalling of the neurotransmitter acetylcholine, but these can also disrupt crop-pollination services provided by bees. Here, we analysed whether chronic low dose long-term exposure to the neonicotinoid thiamethoxam alters gene expression and alternative splicing in brains of Africanized honey bees, *Apis mellifera,* as adaptation to altered neuronal signalling. We find differentially regulated genes that show concentration-dependent responses to thiamethoxam, but no changes in alternative splicing. Most differentially expressed genes have no annotated function but encode short Open Reading Frames (sORFs), a characteristic feature of anti-microbial peptides. As this suggested that immune responses may be compromised by thiamethoxam exposure, we tested the impact of thiamethoxam on bee immunity by injecting bacteria. We show that intrinsically sub-lethal thiamethoxam exposure makes bees more vulnerable to normally non-pathogenic bacteria. Our findings imply a synergistic mechanism for the observed bee population declines that concern agriculturists, conservation ecologists and the public.

43 **INTRODUCTION**

44 The western honey bee *Apis mellifera* is highly beneficial for human societies.
45 Besides honey production, this semi-domesticated insect plays a critical role in
46 sustaining global food security through the provision of managed pollination
47 services that contribute to increased yields of many crops. Globally, crop
48 productivity is also enhanced by the application of pesticides, including
49 insecticides. Agrochemicals are, however, among the key factors implicated in
50 contributing to declining bee health and abundance and there is a need to find
51 a balance between the necessity of insecticide applications and the unintended
52 effects to non-target species [1–6].

53 Compared to organophosphate pesticides, neonicotinoids are relatively safe for
54 vertebrates and have been one of the most important classes of insecticides
55 for the last three decades [7]. Neonicotinoids can be applied to seeds and they
56 then spread systemically within growing plants, killing leaf-eating pests. This
57 reduces non-target effects compared to spraying but still retains toxic burden
58 for pollinators as neonicotinoids continue to be found in wildflowers adjacent to
59 treated fields [8,9].

60 Neonicotinoids act as agonists, competing with the neurotransmitter
61 acetylcholine in binding to nicotinic acetylcholine receptors (nAChR) [10–12]. The
62 increased toxicity for insects is thought to be caused by the characteristically
63 high nAChR density within insect nervous systems [reviewed in [13]]. Detailed
64 knowledge of cellular and molecular effects of insecticide exposure is required
65 to mitigate negative effects or refine target specificity. Changes in gene
66 expression and processing of RNAs, including alternative splicing, are among
67 the mechanisms available to an organism to adapt to environmental

68 perturbations [14,15]. Sub-lethal exposure to xenobiotics, such as pesticides, can
69 alter alternative splicing [16–18]. Since many ion channel genes and other
70 important neuronal genes undergo extensive alternative splicing, they are
71 prime targets for changes induced by xenobiotics [17,19]. Moreover, sub-lethal
72 uptake of some neonicotinoids affects the honey bee brain, impairing foraging
73 behaviour, flight, navigation, communication, learning and memory [20–25].
74 Neonicotinoid exposure has also been linked to a decline in bee health,
75 including a reduction of immune competence [26–28]. Insects do not have
76 antibodies and rely on the innate immune system to fight microbial infections;
77 both cellular and humoral responses have been identified [29,30]. The cellular
78 response leading to phagocytosis or encapsulation of pathogens is mediated
79 by three types of haematopoietic cell lineages [29,31,32]. The humoral immune
80 response is activated by Toll and Imd pathways [reviewed in [33]], leading to the
81 expression of short (10-100 amino acids) antimicrobial peptides (AMPs) which
82 are highly diverse among insect species [34]. The Toll-pathway is triggered by
83 Gram-positive bacteria and fungi, ultimately leading to expression of AMPs via
84 the NF_{kappa}-B transcription factor Dif, that are then secreted from the fat body
85 into the haemolymph [29,35,36]. The Immune deficiency (Imd) pathway leads to
86 expression of a different set of AMPs via the NF_{kappa}-B transcription factor relish
87 in response to peptidoglycans from primarily Gram-negative bacterial through
88 the activation of pattern recognition receptors and a complex intracellular
89 signalling cascade [29,30,35]. In addition to the fatbody, AMP expression activated
90 by the Imd pathway has also been detected in glial cells in the brain [37–40].
91 We have previously shown that worker-bee larvae in colonies contaminated
92 with the neonicotinoid imidacloprid, have altered expression of genes belonging

93 to lipid-carbohydrate-mitochondrial metabolic networks [41]. We have further
94 demonstrated that sub-lethal exposure to thiamethoxam, another
95 neonicotinoid, can cause impairment in the midgut and brain of the Africanized
96 *Apis mellifera*, as well as contribute to a reduction in honey bee lifespan [9,42–44].
97 In this study, we analysed the effects of chronic, sub-lethal, thiamethoxam
98 exposure on genome-wide gene expression and alternative splicing in the
99 brains of honey bee workers. We found 52 differentially regulated genes
100 showing a concentration dependent response to thiamethoxam exposure. Most
101 of these genes have no annotated function but the vast majority are
102 characterized by encoding short Open Reading Frames (sORFs), half of which
103 are predicted to encode antimicrobial peptides. This result suggested that
104 immune responses may be compromised by thiamethoxam exposure. Indeed,
105 we found that thiamethoxam-exposed bees that were also infected with bacteria
106 had greatly decreased viability compared to infected but chemically unexposed
107 bees. Overall, our results suggest that thiamethoxam makes bees vulnerable
108 to bacterial infection by compromising immune responses.
109

110 **MATERIALS AND METHODS**

111 We assessed whether the neonicotinoid thiamethoxam alters gene expression
112 in the brain of Africanized honey bees, *Apis mellifera,* after long-term exposure
113 to field-relevant concentrations.
114

115 **Thiamethoxam exposures**

116 Genetically unrelated bees from three unrelated hives from different locations
117 in the apiary of the Biosciences Institute*, UNESP*, Rio Claro-SP (Brazil). Brood

118 frames were removed from three different colonies and kept for 24 hours in
119 biochemical oxygen demand (BOD) at 34° C and relative humidity of 80 ± 10%.
120 The newly emerged bees (aged 0 to 24h) were marked with special non-toxic
121 pen (Posca pen) and returned to their respective colonies until they were
122 collected after 15 days. All experiments followed the recommendations of the
123 guidelines for xenobiotic assessments on bees (OECD, 1998a, 1998b).
124• Thereafter, bees were kept in groups of 20 individuals in small cages (12
125 cages/treatment) within an incubator at 32° C. Bees were fed *ad libitum* with
126 sugar solution (1:1 water and inverted sugar) from pierced 2 ml Eppendorf
127 tubes for the control group (dataset A, Supplemental Data 1) and, for toxin
128 exposure treatments, thiamethoxam (Sigma) was added at 2 ng/ml (low dose,
129 LD, dataset B, Supplementary Data 1) or at 50 ng/ml (high dose, HD, dataset
130 C, Supplementary Data 1). Sugar-feeders were re-filled before they were empty
131 to guarantee continuous consumption. The tested concentrations were chosen
132 based on the range of realistic field concentrations found in pollen and nectar
133 [9,45–48]. Assuming that caged worker bees consume about 22 µl of 50% sugar
134 per day [49] the bees would have ingested 0.44 ng and 11 ng after ten days for
135 the low and high dose exposure, respectively. After ten days, bees were cold-
136 anaesthetised and their brains were dissected for RNA extraction.
137

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

140 Total RNA was extracted from ten dissected brains per sample and three
141 replicates were done for the control, the low and the high dose treatment
142 (dataset A, Supplemental Data 1). To extract total RNA, brains were cracked in

143 liquid nitrogen with a pestle and then homogenized in 50 µl of Tri-reagent
144 (SIGMA). Then the volume was increased to 500 µl and proceeded following
145 the manufacturer's instructions. Total RNA was then stored in 70% ethanol and
146 transported at ambient temperature from Brazil to the UK.
147 Total RNA was treated with DNase I (Ambion) and stranded libraries for Illumina
148 sequencing were prepared after poly(A) selection from total RNA (1 µg) with
149 the TruSeq stranded mRNA kit (Illumina) using random primers for reverse
150 transcription according to the manufacturer's instructions. Pooled indexed
151 libraries were sequenced on an Illumina HiSeq2500 to yield 28–41 million
152 paired-end 125 bp reads for three control, three low dose and three high dose
153 samples. After demultiplexing, sequence reads were aligned to the *Apis*
154 *mellifera* genome (Amel-4.5-scaffolds) using Tophat2.0.6 [50]. Differential gene
155 expression was determined by Cufflinks-Cuffdiff and the FDR-correction for
156 multiple tests to raw P values, with $p < 0.05$ considered significant [51]. Illumina
157 sequencing and differential gene expression analysis was carried out by
158 Fasteris (Switzerland). Sequencing data are deposited in GEO under
159 GSE132858
160 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE132858).
161 Alternative splicing was analysed by rMATS [52] and manually inspected by
162 uploading bam files into the Integrated Genome Viewer [53] and comparing read
163 frequency. Comparison of gene lists was made with Venny 2.1
164 (https://bioinfogp.cnb.csic.es/tools/venny/). Protein sequences from
165 differentially expressed genes of bees were obtained from ensemble
166 (http://metazoa.ensembl.org/Apis_mellifera/Info/Index) and blasted against
167 *Drosophila* annotated proteins using flybase (http://flybase.org) to assign gene

168 functions. Proteins with no assigned functions were scanned for motifs using
169 the Interpro server (https://www.ebi.ac.uk/interpro/) [54]. Short ORFs were
170 analysed for antimicrobial peptide prediction using the following server:
171 https://www.dveltri.com/ascan/v2/ascan.html [55].
172
173 **Reverse transcription quantitative polymerase chain reaction (RT-qPCR)**
174 Reverse transcription was carried out with Superscript II (Invitrogen) as
175 previously described [56]. PCR was performed as described and PCR products
176 were analysed on ethidium bromide stained 3% agarose gels [19]. Neuronal
177 genes used as reference for gene expression comparison were bee *erect wing*
178 (*ewg*, *GB44653)* and *Amyloid Precursor Protein-like* (*Appl, GB48454*), based
179 on expression analysis in *Drosophila* [57,58]. To validate cDNAs obtained from
180 RT, the following primers were used to amplify bee *ewg* with AM ewgG F
181 (CCTGATGGTACCGTATCAATTATTCAAGTTG) and AM ewgH R
182 (CCGTGTCCATCTTCTCCTGTGAGAATGATTTG), bee *Appl* with AM Appl F
183 (GCGCGATTCCAGGAAACTGTTGCTGCTC) and AM Appl R2
184 (CTGCTGTCCAGCAGATGTTTGTAATGAG). Additional primers were
185 GB45995 (GTTGCATTTTTACGCGTACAGTTACACGACAG) and GB45995 R
186 (GGGAAATCCCCGGGAAGAGAGCAACTGGAG), GB45995 F
187 (GTTGCATTTTTACGCGTACAGTTACACGACAG) and GB45995 R
188 (GGGAAATCCCCGGGAAGAGAGCAACTGGAG), and GB47479 F
189 (GGGCTATTTGCTATCTAAGTGATCCTCC) and GB47479 R
190 (GGGTTTAGGAGTTTTCGTTTTAGCTGCTG) and PCR amplifications were
191 done by 30 sec initial denaturation at 94º C, and then 40 cycles in total with 30
192 sec at 94º C, annealing at 48º C with 30 sec extension at 72º C for 2 cycles,

193 then at 50º C with 30 sec extension at 72º C for 2 cycles and then at 52º C with
194 and 60 sec extension at 72º C for 36 cycles with a final extension of 2 min at
195 72º C.
196 RNA quality and quantity were assessed using an Agilent 6000Nano Kit. 250
197 ng of DNAse-treated RNA (the same samples used for deep seq) was reversed
198 transcribed using a SuperScript III Invitrogen kit at 55ºC, following
199 manufacturer's instructions. Primers for quantitative real-time PCR (RT-qPCR)
200 were designed by Primer Express software (exon/exon junctions included
201 except for GB41813). Amplicon sizes were assessed in 15% acrylamide gel,
202 PCR products were then sequenced and efficiency of every pair of primers was
203 included in the analysis.
204 Duplicate samples of cDNA were amplified in Real Time PCR with SybrGreen
205 chemistry under following conditions: denaturation 93ºC for 30 sec, annealing
206 60ºC for 30 sec, elongation 72ºC for 30sec. *GB41923* was amplified with
207 primers GB41923 F1 (CGCGTTGATCGTCATGATATTG) and GB41923 R1
208 (CTATAAGGAAATTTTGAGCCTTCGA), *GB40669* with primers GB40669 F1
209 (GGCCGGATATCGCTTCAAA) and GB40669 R1
210 (GTCTCTTTTATCTTTTCCTCGGAATTC), and *GB48969* with primers
211 GB48969 F1 (TTGCAGCCGTAGCAAAAGGTA), GB48969 R1
212 (ACCGATTTGAGCACCTTGGT) and *bubblegum* (*bgm, GB51680*) with primers
213 bgm F1 (CATGCACAAAGAGTACAAAAATTTCA) and bgm R1
214 (TGGTCCCAATTCTCCAGTAACA). Analysis of CT values was performed in
215 Light Cycler480 software and normalization and differential expression was
216 determined with the $2^{-\Delta\Delta Ct}$ method [59] by normalizing to the expression of *ewg*
217 *(GB44653)*, *Appl (GB48454)* and *actin* (*GB44311*) genes. *Actin* and *ewg* were

218 amplified with primers ewg Fw2 (CCGCGTCTCCTACAGCTCTT) and ewg Rv2
219 (TGTAAAACTGCCGTAGGATATTGG) and actF1
220 (TTCCCATCTATCGTCGGAAGA) and actR1 (TTTGTCCCATGCCAACCAT),
221 and *Appl* with primers AM appl F and AM appl R2, following [41,60].
222
223 **Bacterial infection assays**
224 Thiamethoxam-induced alteration of anti-microbial peptide gene expression
225 could disrupt bees' immune response. To evaluate the effect of thiamethoxam
226 on immunity, we adopted an assay procedure, initially developed for
227 *Drosophila*, which assesses how efficiently injected non-pathogenic bacteria
228 are cleared by anti-microbial peptides [61]. To assay clearance in bees we used
229 *Bacillus badius*, a normally non-pathogenic bacterium commonly found in the
230 environment and *Ochrobactrum anthropi*, which are Gram-positive and Gram-
231 negative members of the honey bee microbiome [62,63]. *O. anthropi* were isolated
232 from worker bee gut cultures by plating the gut content on LB agar plates
233 incubated at 30º C.
234 Bacterial species were identified by colony PCR and ribosomal 16S
235 sequencing: A colony was picked from an LB agar plate with a yellow tip and
236 placed into 10 µl TE in a PCR tube and heated to 94º C for 5 min. The PCR mix
237 was added adjusting the MgCl concentration to 1.5 mM. PCR was done for 20-
238 40 cycles with 54º C annealing for 40 sec and 1 min extension at 72º C. A 490
239 bp fragment of the ribosomal 16S gene was amplified with primers 16S F
240 (ACTGAGACACGGYCCAGACTCCTACGTC) and 16S R
241 (GCGTGGACTACCAGGGTATCTAATCC) and sequenced with primer 16S
242 Fseq (CTCCTACGGGAGGCAGCAGTRGGGTC). If sequences did not yield a

243 single species, primers 16S F2 (GTGGACTACCAGGGTATCTAATCCTG) and
244 16S R2 (CCTACGGTTACCTTGTTACGACTTCAC) were used for amplification
245 of a 733 bp fragment, which was sequenced by 16S R2seq
246 (CCATGGTGTGACGGGCGGTGTGTAC).
247 Forager honey bees for infection assays were collected from colonies of the
248 Winterbourne Garden of the University of Birmingham (UK). They were kept
249 and injected with bacteria as we described previously [19]. Bacteria for injections
250 were freshly plated and grown overnight on LB plates. Then a single colony
251 was used to inoculate a 5 ml LB in a 50 ml Falcon tube and grown overnight to
252 saturation: 2 µl of this culture was then injected. Bacterial titres of cultures were
253 determined at the time of injections by plating 100 µl of 10^5, 10^6 and 10^7
254 dilutions in LB and counting the colonies the next day: this showed that 2-8 x
255 10^6 *B. badius* or *O. anthropi* were typically injected. In some treatments the
256 injected bacteria were diluted 10-fold (Fig. 3). Groups of 8 to 12 bees were
257 exposed to combinations of bacterial and thiamethoxam doses and survival
258 was assessed at 24 hours and 48 hours.
259 Data on survival were analysed using backwards stepwise logistic ANOVAs
260 adopting a logit-link function and assuming quasi-binomial distributed errors.
261 These analyses were carried out using GenStat v.19 (VSN International, Hemel
262 Hempstead). Statistical analysis of viability were done with GraphPad prism
263 using ANOVA followed by Tukey-Kramer post-hoc tests.
264

265 **RESULTS**

266 **Chronic thiamethoxam exposure effects gene expression**

267 After low (LD) and high dose (HD) exposure to thiamethoxam, there were 222
268 up- and 181 down-regulated genes for LD (Fig. 1a) and 233 up- and 114 down-
269 regulated genes for HD with a 1.5 fold difference in expression compared to the
270 control treatment (Fig. 1b; Supplemental Table 1). From these differentially
271 regulated genes, 37 were up-regulated and 15 were down-regulated in a dose-
272 sensitive manner (Fig. 1; Supplemental Table 1).

273 To validate these results from the Illumina sequencing, we performed RT-qPCR
274 for three of the dose-responsive genes: *GB41923*, a putative sodium-chloride
275 co-transporter, and *GB48969, GB40669,* two genes with unknown function. We
276 detected an expression difference for all three genes upon thiamethoxam
277 exposure (Supplemental Fig. 1). We also validated and confirmed differential
278 expression of *bubblegum*, encoding a very long-chain acyl-CoA synthetase,
279 which has been found to be down-regulated in honey bee larvae exposed to
280 the neonicotinoid imidacloprid [41] (Supplemental Fig. 1).

281 Alternative splicing has been suggested as a mechanism to adapt gene
282 expression to environmental changes [17,64]. We analysed the RNA-seq data for
283 changes in alternative splicing but found no conclusive patterns
284 (Supplementary Information 1; Supplemental data 2).

285

286 **Dose-responsive expression occurs mostly in genes encoding**
287 **uncharacterized ORFs**

288 Next, we categorized the genes dose-responsive to thiamethoxam according
289 to their functions, taking into account known functions of orthologues in

290 *Drosophila* and functions deduced from annotated protein domains retrieved by
291 BLAST analysis. Amongst the dose-sensitive genes that were up-regulated
292 (Fig. 1A), 14 % (5/37) were assigned roles in cellular signalling (with potential
293 links to altered neuronal function, such as olfactory and taste perception) and
294 as structural components (cytoskeleton), and 8 % (3/37) were assigned
295 functions in transcriptional regulation of gene expression (Fig. 2A). However,
296 59% (22/37) of dose-sensitive up-regulated genes and 73 % (11/15) of dose-
297 sensitive down-regulated genes had neither clear orthologues in *Drosophila* nor
298 any recognizable protein domains that would indicate a biological function (Fig.
299 2A), which is in contrast to about 20% of genes with unknown function in gene
300 expression studies in *Drosophila,* [65,66].
301
302 **Most thiamethoxam dose-responsive genes encode short proteins**
303 Many of the dose-responsive, differentially expressed genes with unknown
304 function coded for short ORFs. For thiamethoxam-induced differentially up- and
305 down-regulated genes, respectively 73 % (27/37) and 87 % (13/15) encode for
306 genes with ORFs of 250 amino acids or shorter (Fig. 2). Using a machine
307 learning algorithm [55], we predicted the 40 genes coding for peptides of ≤250
308 amino acids, 17 (43 %), the peptides have antimicrobial function (11/17 genes
309 were up-regulated and 6/17 were down-regulated, Fig. 2).
310
311 **Thiamethoxam makes worker bees more vulnerable to bacterial infection**
312 When saturated liquid cultures (2-8 Mio bacteria in 2 µl) of *B. badius* and *O.*
313 *anthropi* were injected into bees that were not exposed to thiamethoxam,
314 viability was little affected, indicating that the bee immune system usually clears

315 the infection efficiently (Fig. 3, Table 1). In contrast, co-injection of normally
316 sub-lethal doses of thiamethoxam together with either *B. badius* or *O. anthropi*
317 resulted in bee death (Fig. 3, Table 1). In particular, at a dose of 1 μM
318 thiamethoxam (arrow in Fig 3) bees generally survived for 48 h. In contrast,
319 presence of either *B. badius* or *O. anthropi* together with thiamethoxam killed
320 almost all bees within 48 h.
321 Using data on each bacterial species separately, along with replicates without
322 bacterial exposure, showed that bee viability was negatively affected by
323 increasing doses of thiamethoxam and of bacteria (Table 1: 2-way logistic
324 ANOVAs). At 48 hours, a synergistic interaction between these main effects
325 was detected when the bacterial species was *B. badius*: bee viability declined
326 more rapidly in response to bacterial dose when bees were exposed to higher
327 doses of thiamethoxam (Table 1, Fig. 3).
328 To test for differences between the effects of the two bacterial species,
329 replicates without bacterial exposure were excluded and the effects of species,
330 non-zero bacterial dose, thiamethoxam and their interactions were explored
331 (Table 1: 3-way logistic ANOVAs). At 24 hours, bee viability declined with both
332 thiamethoxam dose and bacterial dose and was lower when the bacterium
333 applied was *O. anthropi*, without interactions (Table 1). At 48 hours, the same
334 dosage effects were found but the species effect was not significant (Table 1).
335 There was, however, a marginally non-significant interaction between bacterial
336 species and bacterial dose: as probability estimates from logistic analyses are
337 inexact, we explored the consequences of retaining this interaction in the
338 model. This generated a further significant interaction between thiamethoxam

339 dose and the species of bacteria applied ($F_{1,21}$ = 5.85, P=0.025): *O. anthropi*
340 affected bees more negatively than did *B. badius* as pesticide dose increased.
341 We conclude that thiamethoxam suppresses the abilities of bees to cope with
342 natural challenges of the immune system, which would normally not be fatal.
343

344 **DISCUSSION**

345 Our study addresses the effect of thiamethoxam on gene expression at field
346 relevant doses of thiamethoxam found in pollen and nectar. In particular we
347 chose for these experiments a low and a high dose, which is about 0.04 ng and
348 1.1 ng per bee per day for a 10 day chronic long-term exposure. A number of
349 studies have used thiamethoxam doses in the same range for analysing
350 changes in gene expression or observing an impact on behaviour following
351 short term exposure [18,20–24,67,68]. In fact, it was found that chronic low-dose long-
352 term thiamethoxam exposure altered bee activity, motor function and
353 movement to light [24], but to our knowledge no previous analysis of gene
354 expression under such conditions has been done.
355 A key finding of our analysis of honey bee transcriptomes is the highly enriched
356 fraction of dose-responsive, uncharacterised genes encoding short open
357 reading frames (sORFs). Such sORFs have only recently been recognized to
358 encode functional peptides [69–71], some of which play important roles during
359 development or have a function in the immune system [72,73]. Intriguingly, the
360 majority of the dose-responsive sORFs we have identified are predicted to
361 encode peptides with antimicrobial function (anti-microbial peptides, AMPs).
362 The sORFs we identified have not been reported in prior whole-transcriptome
363 evaluations of neonicotinoid exposure in bee brains despite using similar

364 concentrations of neonicotinoids [18,67,68]. A possible explanation for the different
365 gene set we obtained after thiamethoxan exposure is that we analysed changes
366 after long-term, low dose exposure, while prior studies used shorter exposures.
367 The finding that mostly sORFs, and among them many predicted AMPs are
368 differentially expressed points to the immune system for being dysregulated by
369 neonicotinoids. The best-characterized AMPs are expressed in the fat body
370 upon pathogen exposure, but it is now recognized that glial cells in the brain
371 also exert immune functions and express AMPs [37,39,40]. Accordingly, the sORFs
372 that we found to be differentially expressed upon chronic low dose
373 thiamethoxam exposure might form a basal immune defence, similar to the
374 antimicrobial environment present in saliva in vertebrates which many AMPs.
375 Glia are support cells of neurons and are protective in a number of ways.
376 Dysregulated AMP's in the brain have been linked to neurodegenerative
377 disease [37,38,40]. Hence, the dysregulation of sORFs in response to
378 thiamethoxam exposure could indicate disturbed communication between
379 these two cell types beyond immune functions. Likewise, antimicrobial peptides
380 have also been identified in having a role in learning and memory in *Drosophila*,
381 but the molecular mechanism how AMPs enhance learning and memory
382 remains unknown [74].
383 We noted that other studies found an overrepresentation of down-regulated
384 genes with known function in immune and defence processes upon exposure
385 to different types of neonicotinoids [18,26,67,68,75]. In contrast we did not find
386 differentially regulated immune genes including the known AMPs in the brain.
387 AMPs are highly expressed in the fat body. For the analysis of gene expression
388 in bee brains we dissected them from cold-anaesthetized bees and ensured

389 that all fat body surrounding the brain was removed. It is possible, that freezing
390 of brains prior to dissection [67] or residual fat body adhering to the brain
391 contributed considerably to a different profile of immune gene expression.
392 Immunosuppression has also observed been upon exposure to neonicotinoids
393 co-infecting bees with viruses of *Nosema, a* unicellular parasite of bees. In
394 particular, this affected the cellular response, but also expression of AMPs
395 [18,26,67,68,75]. Since we did not detect differential expression of known immune
396 genes, the bees in our study were not infected with these known pathogens,
397 but we cannot exclude an impact of other microbiota that are non-pathogenic
398 in healthy bees. Moreover, our analysis focused on gene expression in the
399 brain, where glial cells govern immune functions, which is a different humoral
400 response than mediated by fatbody cells secreting AMPs into the hemolymph.
401 Bees, including *Apis mellifera*, are characterised by their limited set of canonical
402 immune genes, compared to non-social insects, such as the fruit fly *D.*
403 *melanogaster* [76–78]. Currently, only six antimicrobial-peptide genes comprising
404 four gene families have been described in honey bees [78]. In contrast,
405 *Drosophila* has 20 antimicrobial-peptide genes comprising eight gene families
406 [29,78]. From these genes, only *defensin* is conserved between honey bees and
407 *Drosophila,* consistent with the idea that antimicrobial peptides evolve fast to
408 adapt to species-specific environmental conditions [34]. Given the low number of
409 known antimicrobial peptides in bees, it is conceivable that new (currently
410 uncharacterised) genes encoding antimicrobial peptides are evolving.
411 Consistent with this idea, some of the sORFs differentially regulated by
412 thiamethoxam are bee or hymenopteran specific.

413 Various agrochemicals, have been shown to alter the gut microbiome [79,80]. Our
414 results are consistent with previous findings where neonicotinoid exposure
415 adversely affects insect immunity [80,81]. Specifically, we have shown that
416 immune challenges from what are normally non-pathogenic bacteria become
417 fatal to bees when combined with thiamethoxam exposure. How foreign
418 bacteria enter the body of bees appears to be crucial For example, Dickel and
419 colleagues found that oral ingestion of *Enterococcus faecalis* bacteria,
420 increases the survival neonicotinoid-exposed, caged worker bees [82].In our
421 study, the bacteria were injected between segments, which mimics punctures
422 sustained by attacks of the *Varroa* mite. We note that pathogens can enter bee
423 haemolymph through punctures inflicted by *Varroa destructor* mites [63] and thus
424 there may be considerable mortality within hives infested with *Varroa* and also
425 exposed to thiamethoxam.
426 In summary, the most prominent changes in gene expression upon long-term
427 low-dose thiamethoxam exposure identified mostly genes that encode short
428 ORFs, around half of which are predicted to code for antimicrobial peptides.
429 Furthermore, thiamethoxam exposure reduced the capacity of bees to
430 withstand microbial infection. Taken together, these findings imply that low
431 doses of neonicotinoids may be intrinsically sub-lethal to bees but can be
432 ultimately fatal via a weakened immune response to extrinsic pathogens. The
433 roles of the identified genes in the immune response of bees will need to be
434 identified to establish how bee immunity might be strengthened to resist
435 bacterial infections.
436
437 **Authors' contributions**

P.D., P.U. and K.D. performed the experiments, T.C.R, I.C.W.H., O.M., N.M. analysed data, R.S. and M.S. supervised experiments and analysed data, R.S. and M.S. wrote the manuscript with help from P.D., P.U. and I.C.W.H.

Acknowledgments

We thank N. Parker and the Winterbourne garden for bees, N. Parker for bee suits, V. Soller-Haussmann and K. Nallasivan for help with bee collections. G. Salmond for bacterial strains, D. Scocchia for culturing and genotyping bacteria, and L. Orsini, E. Davies and I. Haussmann for comments on the manuscript. For this work we acknowledge funding from the Foundation for Research Support of the State of São Paulo, FAPESP (2012 / 13370-8; 2014 / 23197-7), the Biotechnology and Biological Sciences Research Council (BBSRC), the Nottingham-Birmingham Fund, and the Sukran Sinan Memory Fund.

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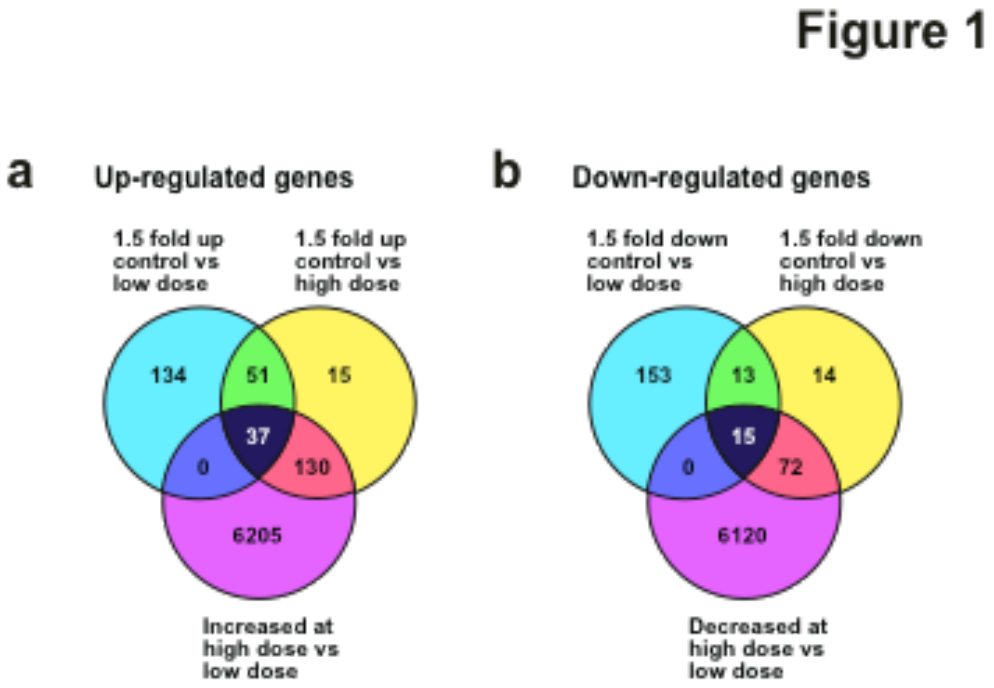
Table 1. Effects of thiamethoxam dose, bacterial dose and species on bee viability.

	Survival after 24 hours				Survival after 48 hours			
Source	*d.f.*	Deviance	*F*	*P*	*d.f.*	Deviance	*F*	*P*
2-way logistic ANOVAs								
B. badius dose	1	60.69	59.26	<0.001	1	74.64	50.04	<0.001
Thiamethoxam dose	1	73.10	71.38	<0.001	1	49.92	33.47	<0.001
Bacteria × Thiamethoxam	1	0.55	0.54	0.472	1	36.79	24.66	<0.001
Residual	17	17.41			17	25.36		
Total	20	121.44			20	186.72		
O. anthropi dose	1	55.42	18.99	<0.001	1	76.28	1.76	0.003
Thiamethoxam dose	1	59.99	20.55	<0.001	1	44.51	11.12	<0.001
Bacteria × Thiamethoxam	1	0.09	0.03	0.865	1	7.06	19.06	0.199
Residual	20	58.38			20	80.04		
Total	23	156.51			23	191.16		
3-way logistic ANOVAs								
Bacterial species	1	15.41		0.021	1	6.21	0.25	0.622
Bacterial dose	1	32.67		0.002	1	14.87	6.28	0.021
Thiamethoxam dose	1	90.19		<0.001	1	85.56	36.12	<0.001
Bacterial species × Bacterial dose	1	3.01		0.281	1	8.73	3.68	0.069
Bacterial species × Thiamethoxam	1	0.13		0.204	1	6.21	2.62	0.121
Bacterial dose × Thiamethoxam	1	4.23		0.824	1	2.56	1.08	0.311
Residual	20	49.08			20	47.39		
Total	26	155.30			26	151.08		

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.



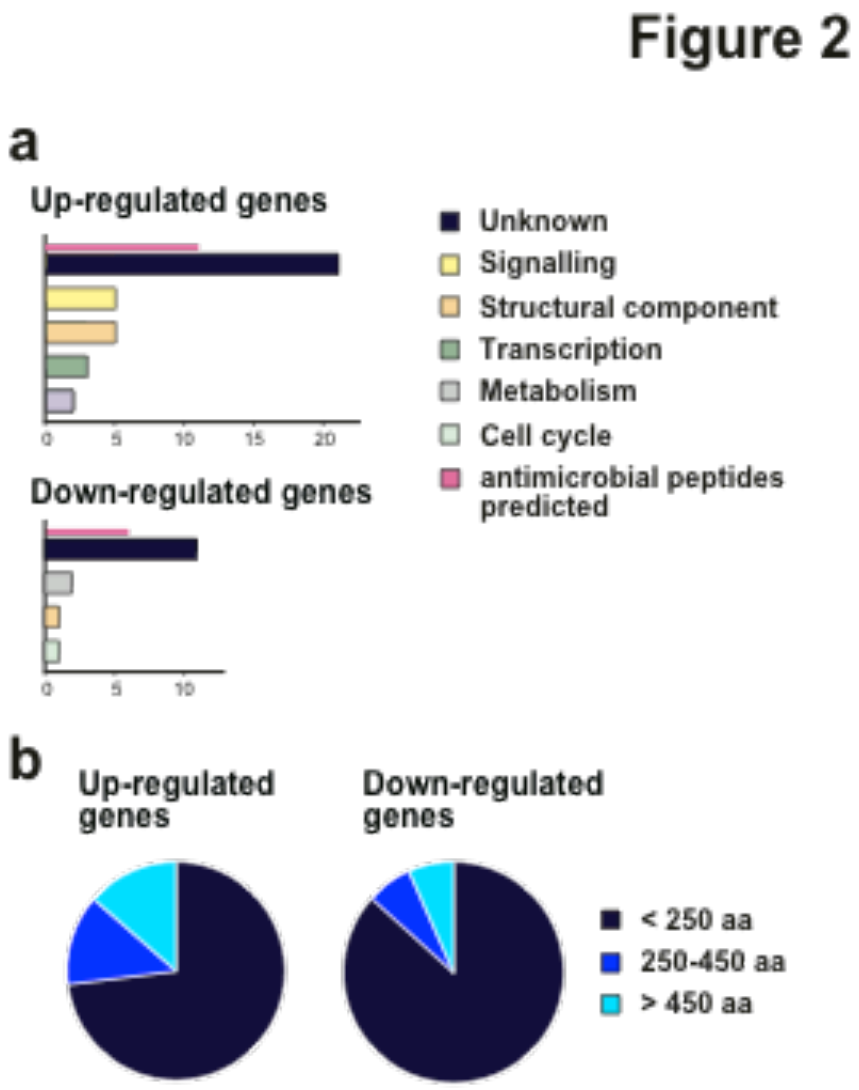


Figure 2: Classification of thiamethoxam induced differentially expressed genes according to function and size. (A) Numbers of genes are plotted according to functions for thiamethoxam induced differentially expressed genes with annotated functions for up- (top) and down-regulated (bottom) genes. (B)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

Figure 3

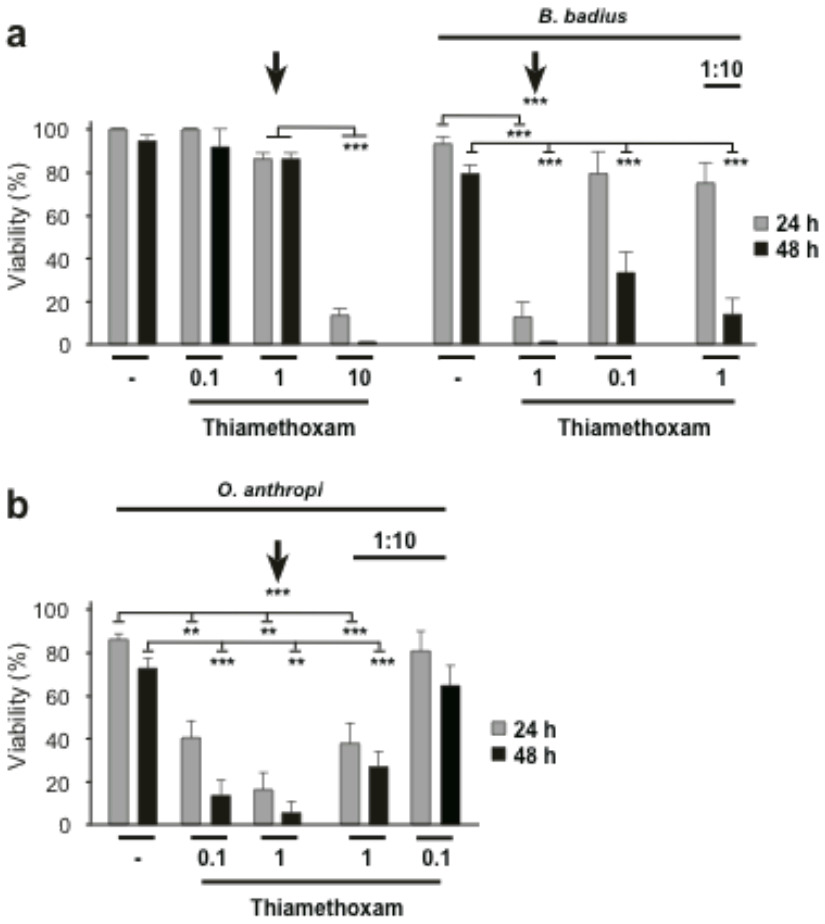


Figure 3: Thiamethoxam exposure makes bees vulnerable to infection by *B. badius* and *O. anthropi*. Viability of groups of 8-12 bees 24 h (grey bars) and 48 h (black bars) after injection with *B. badius* (A) or *O. anthropi* (B) (2-8 x 10^6 and diluted 1:10) alone or together with thiamethoxam (at a range of doses, µM) shown as means and standard errors of three replicates. Arrows indicate injection of a 1 µM Thiamethoxam solution with (A right and B) or without bacteria (A left). Statistical significance is indicated by ** ($p<0.01$) and *** ($p<0.001$).

SUPPLEMENTAL INFORMATION AND DISCUSSION

Analysis of alternative splicing

As alternative splicing has been suggested as a mechanism to adapt gene expression to environmental changes [17,64], we analysed the RNA-seq data for changes in alternative splicing. We found significant differences (Supplemental Table 2). Subsequent inspection of sequencing traces using Integrated Genome Viewer (IGV) [53] found that most of these genes had complex splicing patterns that showed no obvious differences in the number of sequence reads over alternatively spliced gene sections (data not shown); therefore, we did not explore these genes further in this study.

Not to find changes in alternative splicing in response to low dose long-term exposure was unexpected, but we also did not find changes upon acute high dose xenobiotic exposure in selected genes in our earlier study on bees [19]. In other insects, alternative splicing of the *Dscam* gene, which acts as a pattern recognition receptor in the immune response, can generate many different isoforms [83,84] and the splicing patterns can change upon bacterial infection [85]. Since *Dscam* alternative splicing is robust against perturbations of splicing factors [86,87], the absence of changes might indicate that a specific immune challenge is required to change its splicing pattern [85]. 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 [19,65,66]. In contrast to most other species, bees have only a single *elav* gene making it unlikely that the lack of splicing differences is due to redundancy

among close related ELAV RNA binding proteins [88]. Potentially, alternative splicing might be changed 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 [17].

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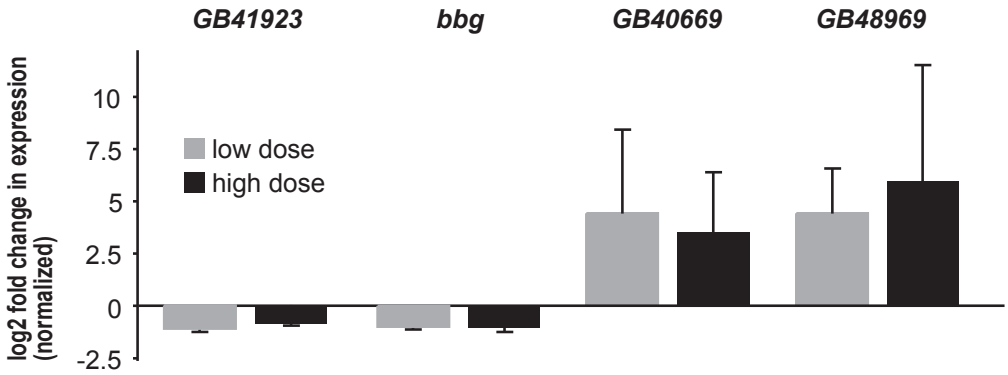
Supplemental Figure 1



Supplemental Figure legend

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