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The neonicotinoid thiacloprid causes transcriptional alteration of genes associated with mitochondria at environmental concentrations in honey bees☆

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ABSTRACT

Thiacloprid is widely used in agriculture and may affect pollinators. However, its molecular effects are poorly known. Here, we report the global gene expression profile in the brain of honey bee foragers assessed by RNA-sequencing. Bees were exposed for 72 h to nominal concentrations of 25 and 250 ng/bee via sucrose solution. Determined residue concentrations by LC-MS/MS were 0.59 and 5.49 ng/bee, respectively. Thiacloprid exposure led to 5 and 71 differentially expressed genes (DEGs), respectively. Nuclear genes encoding mitochondrial ribosomal proteins and enzymes involved in oxidative phosphorylation, as well as metabolism enzymes and transporters were altered at 5.49 ng/bee. Kyoto Encylopedia of Genes and Genomes (KEGG) analysis revealed that mitochondrial ribosome proteins, mitochondrial oxidative phosphorylation, pyrimidine, nicotinate and nicotinamide metabolism and additional metabolic pathways were altered. Among 21 genes assessed by RT-qPCR, the transcript of *farnesol dehydrogenase* involved in juvenile hormone III synthesis was significantly down-regulated. Transcripts of *cyp6a14-like* and *apolipophorin-II like protein*, *cytochrome oxidase* (*cox17*) and the non-coding RNA (LOC102654625) were significantly up-regulated at 5.49 ng/bee. Our findings indicate that thiacloprid causes transcriptional changes of genes prominently associated with mitochondria, particularly oxidative phosphorylation. This highlight potential effects of this neonicotinoid on energy metabolism, which may compromise bee foraging and thriving populations at environmentally relevant concentrations.

1. Introduction

The documented decline of insects (Hallmann et al., 2017) and arthropods biomass and diversity (Seibold et al., 2019) are of major concern, which also holds for bees (Lee et al., 2015; Ollerton et al., 2014). Bee decline compromises pollination (Cameron et al., 2011; Goulson et al., 2015; Grab et al., 2019) and has many causes (Goulson et al., 2015) including loss of plant biodiversity associated with intensified farming, impacts of pathogens and pesticides (Sanchez-Bayo and Goka, 2014).

Insects are exposed to numerous agricultural pesticides (David et al., 2016) including neonicotinoids (Mullin et al., 2010; Sanchez-Bayo and Goka, 2014). Direct exposure of bees may cause acute toxicity but also chronic effects to the nervous system (Decourtye et al., 2004), immune system (Di Prisco et al., 2013) or on energy allocation (Christen et al., 2019a). Their neurotoxicity may compromise memory and orientation as demonstrated for thiacloprid that reduced homing success of honey bees (Tison et al., 2016). Neonicotinoid insecticides may also harm bee populations (Henry et al., 2012; Rundlöf et al., 2015; Tsvetkov et al., 2017).

The neonicotinoid thiacloprid has now been banned in the European Union as the highly toxic imidacloprid, thiamethoxam, clothianidin in outdoor applications due to their high risks for bees. However, it still finds application indoors (greenhouses).

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Thiacloprid binds to and activates acetylcholine receptors in the nervous system of bees (Manjon et al., 2018). The relatively lower toxicity of thiacloprid compared to the highly toxic neonicotinoids is suggested to be based on ready metabolism by cytochrome P450 dependent monooxygenases (cyp). Thiacloprid is detoxified in honey and bumble bees by cyp9q3, and to a lesser extent, by cyp9q2 (Iwasa et al., 2004; Manjon et al., 2018). Efficient metabolism may also convey tolerance to this insecticide as shown in the solitary bee *Osmia bicornis*, where cyp9bu is important for thiacloprid detoxification (Beadle et al., 2019). Over-expression of cyp enzyme transcripts were found in thiacloprid-treated honey bees (Alptekin et al., 2016) or insects resistant to this insecticide (Karunker et al., 2008).

Thiacloprid induces sublethal effects in bees including altered behaviour in the field that affected foraging, homing success and navigation (Fischer et al., 2014; Tison et al., 2016). Furthermore, adverse effects to the immune system (Brandt et al., 2016) were demonstrated and colony development of bumble bees was impacted under field conditions (Ellis et al., 2017).

Currently, the molecular effects of thiacloprid are poorly known. Metabolomic analyses in the head of thiacloprid treated bees fed with 120 ng/bee revealed 115 metabolites, which belonged to a wide range of pathways associated with oxidative stress and detoxification (Shi et al., 2018). Investigation into molecular effects of highly toxic neonicotinoids in the brain of honey bees by RNA-sequencing showed that metabolic and other pathways were affected at environmentally relevant concentrations (Christen et al., 2018a). Furthermore, clothianidin and imidacloprid affected biological processes including mitochondrial function in bumble bees (Colgan et al., 2019).

Sequencing of the whole genome of honey bees (Wallberg et al., 2014) allows now to perform better global transcriptome analyses by RNA-sequencing. The aim of our study was to evaluate molecular effects and the modes of action of thiacloprid in the brain of honey bees by means of RNA-sequencing and quantitative RT-PCR and thereby providing novel insight into the mechanisms mediating thiacloprid toxicity. Our current study provides several advancements in current knowledge including employment of the honey bee genome to identify specifically altered genes in bees and determination of actual exposure concentration in exposed bees.

2. Materials and methods

2.1. Experimental design

A description of chemicals used and the experimental details are given in the supplementary material (SM). Briefly, honey bees (*Apis mellifera carnica*) were collected from a hive at an uncontaminated rural location at the entrance from outside of the hive. Forager bees entering the hive with pollen pellets were not specifically identified, thus, foragers and non-flying workers with different ages were sampled. Sample collection and transported to the laboratory were as fast as possible to avoid stress reactions.

In the laboratory, after a 24 h adaptation, the foragers were randomly distributed and exposed in groups of 10 bees in PET bottles using five replicates per exposure via sucrose solution for 72 h as previously (Christen et al., 2016) with slight modifications, as described in the SM. Exposure groups consisted of a solvent control, in which bees received sucrose solution containing 0.1% DMSO (solvent control bees) and two thiacloprid exposure groups, in which bees received thiacloprid concentrations of 25 ng/bee (low concentration) and 250 ng/bee (high concentration). Nominal concentrations of 25 and 250 ng/bee were achieved by feeding 10 bees with 1 mL of a 250 or 2500 ng/mL sucrose solution (assuming the weight of bee of 0.1 g). The lower exposure concentration was similar as in nectar, and the higher concentration about five times above highest reported concentrations (Ellis et al., 2017).

In our report, we give nominal concentrations and report effective exposure concentrations in parenthesis. Thiacloprid concentrations were assessed by trace analytical chemistry in solvent control bees and thiacloprid exposed bees after experimental exposure as described in the SM. The nominal concentrations were 1.47% and 14.7%, respectively, of the LD_{50} value of 1700 ng/bee (Sanchez-Bayo and Goka, 2014) and were selected based on reported residues in nectar and bees from thiacloprid treated fields (Ellis et al., 2017).

We exposed bees for 72 h to investigate effects of chronic exposure. Gene expression was assessed in the brain at 72 h. Of ten bees per flask, three bees were stored at −20 °C for RNA-extraction and the rest at −80 °C for chemical residue analysis and backup. Brains of three bees per bottle were dissected and pooled to obtain one RNA sample per replicate, which yielded five biological replicates per exposure group (total of 15 samples). RNA isolation is described in the SM. One bee per bottle was used for chemical residue analysis.

2.2. RNA-sequencing

Library preparation and sequencing was performed at Functional Genomics Center Zurich, ETH and University of Zürich, Switzerland. The library preparation was performed using the Illumina TruSeq stranded mRNA kit, which includes a poly-A enrichment step and is strand specific via dUTP incorporation during the second strand synthesis. The samples were barcoded with 15 different Illumina TruSeq index pairs. Barcoded libraries were sequenced on an Illumina NovaSeq6000 as single end, 100 bp reads (SE100bp). The libraries were then de-multiplexed based on their indices.

Quality checking of raw data and trimming, mapping of trimmed data, transcriptome assembly and transcript abundance estimation, analysis for differential expression and ratio of fold-change for differentially expressed genes (DEGs) between both thiacloprid treatments are described in the SM. The genome sequence and annotation of *Apis mellifera* assembly Amel_HAv3.1 (Wallberg et al., 2014) were downloaded from NCBI RefSeq (accession: GCF_003254395.2).

2.3. Gene ontology (GO) and KEGG analysis

GO information for all *A. mellifera* genes was retrieved from UniProt (uniprot.org) database. Exploiting this information a GO term enrichment analysis was performed for all DEGs from low and high exposures, respectively, using GOseq v. 1.34.1 (Young et al., 2010) (using method “Wallenius” for GO categories “GO:BP”, “GO:CC” and “GO:MF”). All genes with an assigned GO term were serving as the “background” genes and the DEGs with an assigned GO term as the set to be tested for category enrichment. Gene lengths were extracted using function “makeTxDbFromGFF” of Bioconductor package GenomicFeatures v.1.34.8 (Lawrence et al., 2013) from RefSeq annotation.

KEGG pathway analysis was performed using KOBAS 3.0 webserver (Xie et al., 2011). NCBI gene symbols of the DEGs were provided for both treatments individually to the KOBAS webserver. The analysis was performed for *A. mellifera* KEGG pathways.

2.4. Reverse transcription, and quantitative RT-qPCR

1000 ng RNA was reverse transcribed and qPCR based on SYBR green fluorescence (SYBR green PCR master mix; Roche) performed as previously described (Christen et al., 2016). To validate RNA-

sequencing data, DEGs from the low and high exposures were selected based on their significant changes, including non-coding RNA (ncRNA). Additional genes involved in mitochondrial oxidative phosphorylation, cyp enzymes, and genes differentially expressed by other neonicotinoids were determined. Primer sequences are given in the SM in Table S1 and the stability of three normalization genes used for quantitation is shown in Figure S1. Alterations in mRNA abundance in exposed bees were compared against solvent control (0.1% DMSO) bees.

2.5. Chemical residue analysis of bees

In addition to molecular analyses, we also assessed the body burdens (residue concentrations) of thiacloprid in experimental bees at 72 h. Residue concentrations of thiacloprid were determined in whole bee samples in solvent control bees and bees of both exposure concentrations based on a previously described procedure (David et al., 2015) with minor modifications. Details of the method including quality assessment is described in the SM and in Tables S2 and S3.

2.6. Data processing and statistical analysis

For graphical illustration and statistical analysis of RNA-sequencing data, programs described above and for qPCR data, graphPad Prism 8.00 (GraphPad Software, La Jolla, CA, USA) was used. Differences between solvent controls and treatments were assessed as follows. RT-qPCR data were first tested for Gaussian distribution using Kolmogorov-Smirnov test, and if appropriate, analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. Otherwise, the non-parametric Kruskal-Wallis test was used. qPCR results are given as means ± standard deviation.

3. Results

3.1. Thiacloprid concentrations in exposed bees

Trace chemical analysis of whole bees revealed no thiacloprid contamination in solvent controls, as shown in the SM, Table S4. In the low exposure of nominal 25 ng/bee, mean concentrations (±S.D.) were 0.59 ± 0.23 ng/bee (or 4.84 ± 2.25 ng/g) and in the high exposure of nominal 250 ng/bee it was 5.49 ± 1.80 ng/bee (or 43.87 ± 14.71 ng/g) (Table S4). Thus, real concentrations were 2.2−2.4% of nominal levels that were consumed via sucrose feeding. The significantly lower than expected body levels are most probably based on a combination of low absorption, ready metabolism and excretion of thiacloprid by the bees. Metabolism is probably the most important reason, as thiacloprid is readily metabolized in honey bees, particularly by the cytochrome P450 isoform cyp9q3 (Manjon et al., 2018). In our report, we provide nominal concentrations, as well as residue concentrations in parenthesis. Actual residue concentrations in exposed bees are very rarely assessed in experiments with bees.

3.2. Quality of RNA-sequencing data and trimming of raw sequencing data

Sequencing with Illumina NovaSeq6000 (100 bp length, single-end) resulted in a sufficient sequencing depth for all 15 samples (details see Supplementary Table S5). Of the high concentration treatment (250 ng/bee or measured 5.49 ng thiacloprid/bee), sample 1 initially contained a very high ratio of reads mapping to ribosomal RNA (rRNA) (39% versus about 1.5% for the other samples) as well as a deviating GC content (46% versus about 37%). For this reason, the sample was re-sequenced and these data taken. All samples exhibited a comparable GC content and rRNA read content (Table S5).

3.3. Mapping and transcript abundance estimation

Mapping the quality filtered data against the *A. mellifera* RefSeq genome assembly with HISAT (Kim et al., 2019) resulted in a high mapping rate from about 96 to 98% (percentage of sequence reads) for all samples (Table S5). Based on a comparison between the RefSeq genome annotation and a reference-based StringTie (Kovaka et al., 2019) transcriptome assembly, we took the RefSeq annotation for the transcript abundance estimation. While the transcriptome assembly led to an increased annotation-coverage (about 21% increase in genomic loci, 9% in exon numbers), a majority of the novel loci and exons did not have a strong support by the Illumina data and were of rater spurious nature. For this reason, we relied on the RefSeq annotation which is supported by experimental and homologous evidence and went through a rigorous annotation process (https://www.ncbi.nlm.nih.gov/genome/annotation_prok/).

Principal component analysis (PCA) of all 15 samples based on normalized read count data of all genes show that no markedly distinct clustering of the three groups occurred (Figure S2). However, there is a clear trend from "High" treatment towards "Control". The samples "High_1" and "Control_5" seem to be slight outliers. However, since the overall variation is comparably high, this seems of no concern. The high variability of samples within the same group suggests that the effect of thiacloprid in the applied concentrations is not very strong compared to other factors like sequencing noise and variation between individual bees.

The clustered heatmaps on normalized read count data of the low and high exposure show two major groups which separate the control and the high concentration samples (Figure S3). Exceptions are "Control_5" which clusters with the "high" samples and "High_1" which represent slight outliers. Samples of group "low" are split between the two groups. This suggests that effects of thiacloprid in the applied concentrations are not very strong compared to other factors. The two outliers seem not to be of concern, since the overall variation between the samples is rather high compared to the variation between the treatments.

3.4. Differentially expressed genes

Gene expression analysis with DESeq2 (Love et al., 2014) resulted in five significant DEGs, of which 3 DEGs were up-regulated and 2 down-regulated for the low exposure, and 71 DEGs, of which 39 DEGs were up-regulated and 32 DEGs down-regulated (Table 1, S6) for the high exposure (Fig. 1A). None of the DEGs showed significant alterations at both concentrations, thus, there was no overlap between the low and high exposures. Results of the principal component analysis (PCA) (Figure S2) and a clustered heatmap (Figure S3) based on normalized count data indicated that samples High_1 and Control_5 had slightly different characteristics, which can be explained by the re-sequencing of sample High_1 (batch effect and lager sample size) and the overall variance in the PCA and heatmap analysis. In addition, parameters like mapping rate and GC content looked normal for the two divergent samples (Table S7). Heatmap clustering without samples High_1 and Control_5 did not lead to an improved overall clustering of the remaining samples (data not shown). For these reasons we did not exclude the two samples but kept all 15 samples in the gene expression analysis.

Two additional heatmaps were generated based on the genes with lowest p-values regarding differential expression for the low and high exposure consisting of 50 and 100 genes, respectively. The

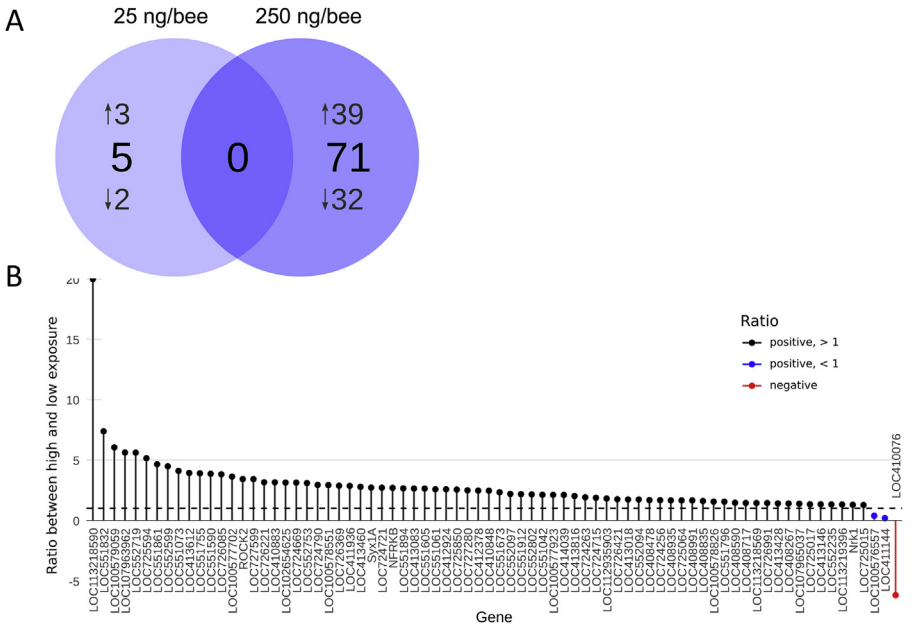

Fig. 1. Numbers of differentially expressed genes (DEGs) in brain of *A. mellifera* exposed to thiacloprid and concentration effect. **A,** number of DEGs altered in brain. Exposure to 25 ng thiacloprid/bee (measured concentration of 0.59 ng/bee) resulted in 5 differentially expressed genes (left circle, 3 DEGs up-regulated, 2 down-regulated) and exposure to 250 ng thiacloprid/bee (measured concentration of 5.49 ng/bee) in 71 differentially expressed genes (right circle, 39 DEGs up-regulated, 32 down-regulated). There is no intersect between the differentially expressed genes of both treatments. **B,** concentration effect in expression of all 76 DEGs between thiacloprid treatments. The y-axis shows the ratio between the fold-change in the 250 ng/bee treatment in relation to the fold-change in 25 ng/bee treatment (details of calculation given in methods). Genes on the x-axis are sorted according to their fold-change ratio. The 73 black bars indicate an increased differential expression of the gene with increased concentration of thiacloprid. The two blue bars show a decreased differential expression in the high versus low concentration but still with the same directionality (down-regulation). The red bar shows the single gene where the directionality of regulation changes from low thiacloprid concentration to high concentration. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

result showed distinct clusters for the control versus low exposure (Figure S4) and control versus high exposure (Fig. 2). Thus, all replicates of both thiacloprid exposures were distinctly different from the control replicates, indicating the very high quality of the data.

In order to show and confirm the effect of different thiacloprid concentrations, we compared the fold-change values of low and high exposures for all DEGs (Fig. 1B). This analysis also served as a further quality control for the expression analysis. The majority of genes (73 out of 76) show a clear concentration related effect with an increased fold-change relative to the control from low to high exposures. Just two genes show a decreased fold-change for high exposure (LOC100576557 and LOC411144) and only one gene (LOC410076) a fold-change in the opposite direction. The concentration related effect is confirmed as the high thiacloprid concentration caused higher amplitudes of expressional changes of almost all DEGs than the low concentration.

The low thiacloprid concentration led to transcriptional alteration of genes encoding metabolism enzymes, such as *UDP-glucuronosyltransferase 2B15*, *cytochrome P450 6a14* and *cytochrome P450 6a14-like* and to *transcription initiation factor TFIID* as well as an uncharacterized gene (Table S6). Exposure to the high thiacloprid concentration caused transcriptional alteration of genes encoding diverse biological functions. Table 1 shows the top 27 DEGs with false discovery rate (FDR) <0.03 and Table S6 lists all DEGs. Prominent alterations occurred for nuclear genes associated with mitochondrial function, including a number of ribosomal proteins, mitochondrial enzymes and enzymes associated with oxidative phosphorylation, including *NADH dehydrogenase*, *COX assembly mitochondrial protein homolog* and *ATP synthase subunit e*. Additionally, enzymes such as *farnesol dehydrogenase* and kinases, as well as transporters (*high-affinity choline transporter 1*, *monocarboxylate transporter 3*) were altered among the 71 DEGs. Thus, while in the low thiacloprid concentration, transcripts of phase I and phase II enzymes , which are involved in xenobiotic metabolism, were altered, the high concentration mostly affected transcripts of genes encoding mitochondrial enzymes and proteins. Additionally, transcripts of other enzymes, particularly *farnesol dehydrogenase*, showed significant alterations. Furthermore, an uncharacterized ncRNA (LOC102654625) was induced, which is characterized by 252−379 bases and 10 exons on chromosome LG8 (Tables S7, S8). All DEGs are listed in Table S7 for the low, and in Table S8 for the high concentration.

3.5. Gene ontology enrichment and KEGG pathway analysis

Gene ontology (GO) enrichment analysis with GOSeq resulted in six significantly enriched GO terms for the low (Table S9) and 18 GO terms for high exposure (Table S10) (non-FDR corrected p-value < 0.05). Enriched GO terms in the low thiacloprid concentration were mainly associated with molecular functions related to cytochrome P450 monooxygenases. GO terms enriched in the high exposure consist of eleven GO terms assigned to molecular function (mainly enzymes), five to biological processes and two to cellular compartments.

Analysis of enriched KEGG pathways for the high exposure showed enrichment for pathways associated with mitochondrial function, including oxidative phosphorylation, mitochondrial ribosome, mitochondrial metabolism and arginine biosynthesis, as well as metabolic pathways, including pyrimidine metabolism, nicotinate and nicotinamide metabolism to name the most prominent pathways based on the number of DEGs involved. Table 2 lists the top six KEGG pathways and Table S12 lists all pathways. Figure S8 shows the DEGs located in the mitochondrion involved in

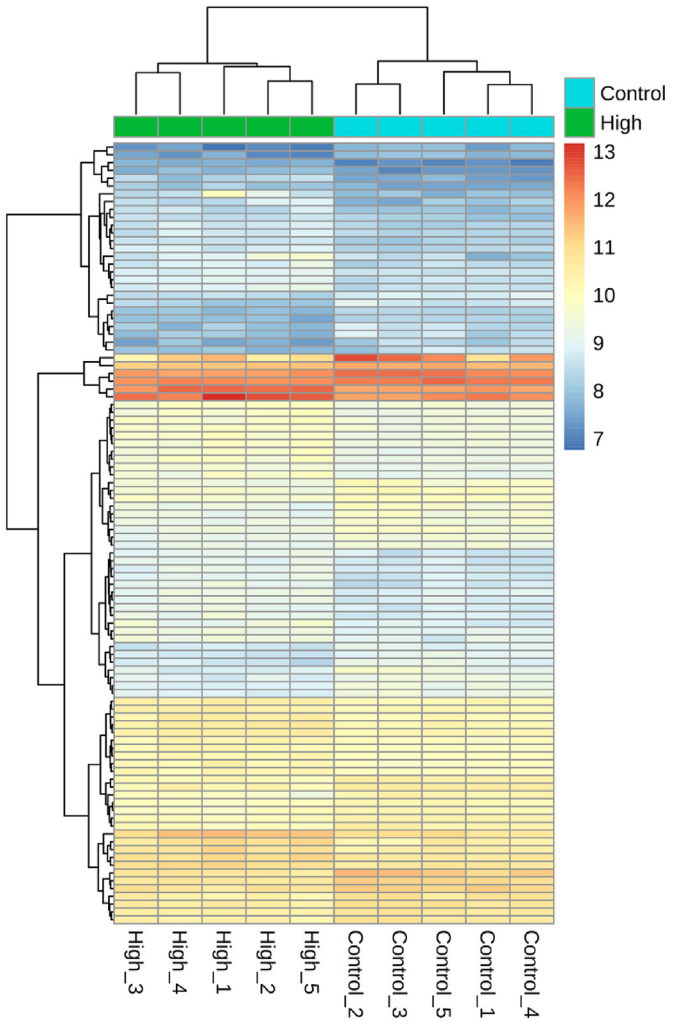

Fig. 2. Clustered heatmap for high exposure based on normalized read count data of 100 genes with lowest p-value. On the left the genes are clustered according to read counts per sample. On top a clustering of the Control and High samples according to counts per gene is shown. Key: control: blue, high concentration of 250 ng/bee (measured concentration of 5.49 ng/bee): green. The colored bar provides a reference for the normalized read count data (blue: low read count, red: high read count). Two major clusters can be observed clearly separating the Control and High samples. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

oxidative phosphorylation. Transcripts of DEGs associated with oxidative phosphorylation are highlighted. The pathway which was enriched in the low exposure is a transcription initiation factor subunit (Table S10).

4. Validation by quantitative RT-qPCR

To validate RNA-sequencing data, we selected DEGs with highest alterations in the low and high thiacloprid exposures for reverse transcription quantitative PCR (RT-qPCR). To evaluate concentration-related effects, we assessed 21 transcripts at both concentrations. The gene selection was based on the magnitude of alteration in RNA-sequencing and included different enzymes and proteins and the ncRNA, as well as on the specificity of the primer design. Additionally, genes encoding cytochrome P450 enzymes involved in thiacloprid metabolism (*cyp9q2*, *cyp9q3*) and genes encoding enzymes involved in oxidative phosphorylation were assessed. Transcripts of genes altered by other neonicotinoids that encode proteins involved in endocrine activity and immune system regulation (Christen et al., 2018a) were also assessed. The quantification of 21 transcripts was based on three normalization (housekeeping) genes that showed stable expression in controls and exposed bees (Figure S1).

Significant up-regulation occurred for *apolipophorin-III-like protein* and *cyp6a14-like* in the high thiacloprid exposure. Furthermore, the ncRNA showed a concentration-related induction. The *farnesol dehydrogenase* transcript was significantly down-regulated in the high exposure (Fig. 3). For both thiacloprid concentrations, significant alterations occurred for *cox17* encoding cytochrome *c* oxidase, a member of complex IV of the oxidative phosphorylation enzyme cascade (Figure S5). No significant transcriptional alterations occurred for genes encoding other enzymes involved in oxidative phosphorylation and enzymes involved in metabolism of thiacloprid, such as *cyp9q2* and *cyp9q3*. Furthermore, transcripts of genes associated with immune system regulation and endocrine function were not significantly altered (Figures S6, S7).

Fig. 4 shows that transcript levels determined by RNA-sequencing match well with those determined by RT-qPCR for most of the 17 transcripts, with the exception of *sensory neuron membrane protein 1* and *transcription activator MSS11* in the high exposure and *cox17* at both concentrations. This confirms the reliability of the RNA-sequencing data.

5. Discussion

Here, we present transcriptome data of thiacloprid derived from RNA-sequencing in bees. Our RNA-sequencing data provide an overall view on expressional alterations of genes in the brain of thiacloprid-exposed foragers. Prominent were transcriptional alterations of nuclear genes encoding structure and function of mitochondria, farnesyl dehydrogenase and metabolism enzymes. Key target of thiacloprid is the mitochondrion, particularly oxidative phosphorylation. This goes along with activation of acetylcholine receptors (AChRs) occurring in Kenyon cells in the mushroom body and other regions of the brain (Déglise et al., 2002; Moffat et al., 2016; Palmer et al., 2013) and associated neurotoxicity. These data lead to the hypothesis that thiacloprid affects mitochondrial structure and function, particularly oxidative phosphorylation.

Thiacloprid exhibited concentration-related effects as exposure to 25 ng/bee (measured 0.59 ng/bee) led to alteration of 5 transcripts, while 250 ng/bee (measured 5.49 ng/bee) significantly altered 71 transcripts (Figs. 1B, 2 and 3). Furthermore, RT-qPCR data showed a concentration-related induction of many transcripts, including *UDP-glucuronosyltransferase 2B15*. However, this did not occur for all transcripts, similarly as with other neonicotinoids and pesticides (Christen et al., 2018a, 2019b; Christen and Fent, 2017; Fent et al., 2020). Possible reason for the lack of classical concentration-response effects is the dynamic nature of the expressional responses in bees.

The most important transcriptional alterations occurred for genes associated with metabolism and mitochondrial related nuclear genes encoding proteins and enzymes involved in oxidative phosphorylation. Thus, mitochondria are key targets of thiacloprid as transcripts of genes encoding NADH dehydrogenase (complex I), cytochrome *c* oxidase (complex IV) and ATPase (complex V) were altered in the high exposure. KEGG analysis indicated oxidative phosphorylation as top enriched pathway, followed by mitochondrial ribosome. These effects are similar as with other compounds including phytochemicals (Mao et al., 2017), spinosad (Christen et al., 2019a) and fungicides (Mao et al., 2017).

Assuming that expressional alterations translate to the protein

Table 1
List of top 27 significantly DEGs (FDR < 0.03) at the high exposure of nominal 250 ng thiacloprid/bee (measured 5.49 ng/bee). Genes are sorted according to the FDR adjusted p-value. Key: Gene symbol: gene symbol as provided by NCBI, Description: description of the gene provided by NCBI, FC Log2: Log2-transformed fold-change of treatment versus control, FDR: false discovery rate-adjusted p-value.

Gene symbol	Description	FC Log2	FDR
LOC413428	uncharacterized LOC413428	−0.57	0.001191386
LOC412924	protein ROP	−0.38	0.003417541
LOC725594	PDZ and LIM domain protein Zasp	0.59	0.003417541
LOC727599	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial	0.48	0.008095836
LOC724790	39 S ribosomal protein L41, mitochondrial	0.45	0.010203727
LOC100579059	uncharacterized LOC100579059	0.78	0.010203727
LOC113219396	leucine-rich repeat serine/threonine-protein kinase 1	−0.45	0.013531597
LOC408935	high-affinity choline transporter 1	−0.45	0.013531597
LOC414039	probable small nuclear ribonucleoprotein Sm D2	0.56	0.013531597
LOC725015	monocarboxylate transporter 3	−0.46	0.014065809
LOC552235	histone-lysine N-methyltransferase E(z)	−0.39	0.014065809
LOC552719	mediator of RNA polymerase II transcription subunit 11	0.34	0.014065809
LOC551590	upstream stimulatory factor 1	0.43	0.014065809
LOC727280	uncharacterized LOC727280	0.42	0.014065809
LOC113218590	low molecular weight phosphotyrosine protein phosphatase-like	0.42	0.019141549
LOC102654625	uncharacterized LOC102654625	1.15	0.01981889
LOC724263	39 S ribosomal protein L51, mitochondrial	0.37	0.020458068
LOC551042	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6	0.39	0.021026193
LOC552753	uncharacterized LOC552753	0.48	0.021046975
LOC724669	peptidyl-prolyl cis-trans isomerase NIMA-interacting 4	0.53	0.021046975
LOC408267	syntaxin-binding protein 5	−0.29	0.021601314
LOC411936	prefoldin subunit 5	0.46	0.026049821
LOC413460	sorting nexin-13	−0.29	0.026215331
LOC552599	DNA-directed RNA polymerase II subunit RPB7	0.35	0.026309138
LOC408991	glutaminase kidney isoform, mitochondrial	−0.42	0.027481943
LOC726369	peptidyl-tRNA hydrolase 2, mitochondrial	0.57	0.028046439
LOC100578551	39 S ribosomal protein L34, mitochondrial	0.52	0.029644233

Table 2
KEGG pathway enrichment analysis of the DEGs for the high exposure of nominal 250 ng thiacloprid/bee (measured 5.49 ng/bee) exposure. Shown are the six top pathways. For every pathway all genes (column "Genes in pathway") and their function (column "Function of genes") are provided.

KEGG pathway term	KEGG pathway ID	P-Value	FDR adjusted p-value	Genes in pathway	Function of genes
Oxidative phosphorylation	ame00190	0.012381781	0.207900749	LOC551861	ATP synthase subunit e, mitochondrial
				LOC551042	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6
				LOC727599	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial
Ribosome (mitochondrial)	ame03010	0.023077845	0.207900749	LOC100577702	39 S ribosomal protein L11, mitochondrial
				LOC552097	39 S ribosomal protein L14, mitochondrial
				LOC100578551	39 S ribosomal protein L34, mitochondrial
Pyrimidine metabolism	ame00240	0.057577873	0.207900749	LOC410076	probable uridine-cytidine kinase
				LOC552599	DNA-directed RNA polymerase II subunit RPB7
Arginine biosynthesis	ame00220	0.062081447	0.207900749	LOC408991	glutaminase kidney isoform, mitochondrial
Nicotinate and nicotinamide metabolism	ame00760	0.062081447	0.207900749	Nrk1	nicotinamide riboside kinase
Metabolic pathways	ame01100	0.074754818	0.207900749	LOC552599	DNA-directed RNA polymerase II subunit RPB7
				Nrk1	nicotinamide riboside kinase
				LOC551861	ATP synthase subunit e, mitochondrial
				LOC408991	glutaminase kidney isoform, mitochondrial
				LOC727599	NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial
				LOC410076	probable uridine-cytidine kinase
				LOC551042	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 6

level, thiacloprid affects metabolism and energy allocation in the brain by compromising mitochondrial ATP production. This will affect the physiological and behavioural performance of foragers, including reduced foraging and homing capacity. These molecular effects may be an underlying reason for the increase in homing time in return flights, previously reported when bees were exposed to 4.5 ppm (Tison et al., 2016). Thus, reduced energy allocation due to compromising mitochondria, may be an additional reason besides altered orientation and leaning behaviour, found after exposure to another neonicotinoid, clothianidin (Tison et al., 2019).

Adverse effects on energy allocation has also been reported by other pesticides including imidacloprid and fipronil in the brain of bees (Nicodemo et al., 2014) and flight muscles of bumble bees (Syromyatnikov et al., 2017). Alterations in energy metabolism and ATP production affect numerous biochemical processes in the brain that may translate to behavioural changes in flight performance and social behaviour (Li-Byarlay et al., 2014). The oxidative phosphorylation capacity in the head is higher in nurse bees than foragers (Cervoni et al., 2017). Thus, alteration of mitochondrial oxidative phosphorylation may also have adverse consequences for

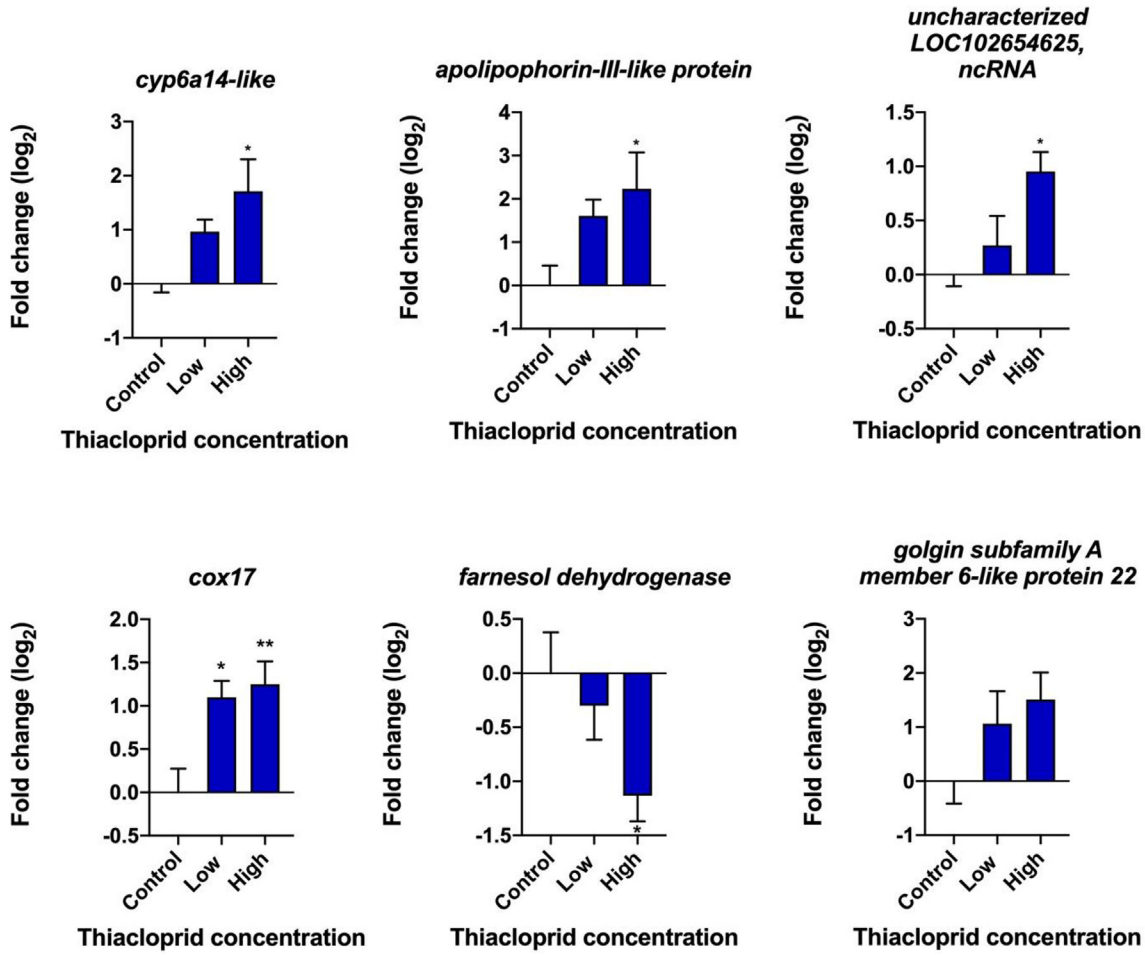

Fig. 3. Transcripts of significantly altered genes encoding *cytochrome P450 6a14-like* (*cyp6a14-like*), *apolipophorin-III-like protein*, non-coding RNA, *cytochrome oxidase* (*cox17*), *fanesol dehydrogenase* and *golgin subfamily A member 6-like protein 22* assessed by RT-qPCR. All transcripts with the exception of *cox17* were also found induced by RNA-sequencing. *Results of transcript levels in fold-changes (log2) are shown as means of five replicates ± standard deviation. Statistical significance is shown with * $p \leq 0.05$; ** $p \leq 0.01$.*

the social behaviour. Together, these molecular and biochemical alterations may be one of the reasons behind the compromised thriving of bee populations (Ellis et al., 2017).

RNA-sequencing and RT-qPCR demonstrated transcriptional down-regulation of *farnesol dehydrogenase*. Farnesol dehydrogenase catalyses the oxidation of farnesol to farnesal and plays a key role in juvenile hormone III biosynthesis (Mayoral et al., 2009). Synthesis takes place in the *Corpora allata* of the brain and oxidation of farnesol is the rate-limiting step. Down-regulation of this enzyme may reduce synthesis of juvenile hormone III that plays an important role in development and behavioural changes of bee worker castes. Consequently, reduced hormone synthesis may affect the endocrine regulation including transition of nurse and forager bees (Christen et al., 2018b).

Our study shows for the first time that a pesticide can cause expressional alterations of ncRNAs in bees. The induction of ncRNA (LOC102654625) is specific and did not occur with other neonicotinoids (Christen et al., 2018a). ncRNAs are involved in a wide range of regulatory functions, including RNA modification and protein synthesis. They represent a diverse class of transcripts that structurally resemble mRNAs but do not encode proteins. The function and potential targets of this ncRNA is unknown. However, the ncRNA may be involved in gene regulation or interference with a target mRNA and its translation. Generally, ncRNAs play an important role in worker bees as indicted by their differential expression in foragers and nurse bees (Liu et al., 2019). ncRNA expression was also shown to change according to age-dependent task transition (Tadano et al., 2009). Further investigations should reveal the function of this ncRNA and the implications of its expressional alteration by thiacloprid.

Genes encoding metabolism enzymes were also altered. KEGG pathway analysis showed that they encompass pyrimidine, purine, nicotinate and nicotinamide metabolism. This may result in altered metabolism of nucleotides used for nucleic acids, NADH and NADPH, and thus may contribute to malfunction of mitochondria and other enzymatic and nuclear processes.

Cytochrome P450 enzymes are responsible for metabolism of pesticides, of which enzymes of the cyp9q and cyp6 families play an important role (Mao et al., 2011; Iwasa et al., 2004; Johnson et al., 2006). Thiacloprid is mainly metabolized by cypq9q2 and cyp9q3 in honey bees (Manjon et al., 2018), and tau-fluvalinate and coumaphos are metabolized by cyp9q1, cyp9q2 and cyp9q3 (Mao et al., 2011). Often, the substrate or xenobiotics cause their induction, most prominently known for cyp1a (Bucheli and Fent, 1995). However, this was not the case for thiacloprid; transcripts of *cyp9q2* and *cyp9q3* did not show significant alterations (Fig. 3). Thus, thiacloprid did not affect its own metabolism by up-regulation of *cyp9q2* and *cyp9q3* transcripts. In contrast, other pesticides caused expressional alteration of *cyp9q1*, *cyp9q2* and *cyp9q3* in the brain, including chlorpyrifos, malathion, cypermethrin and

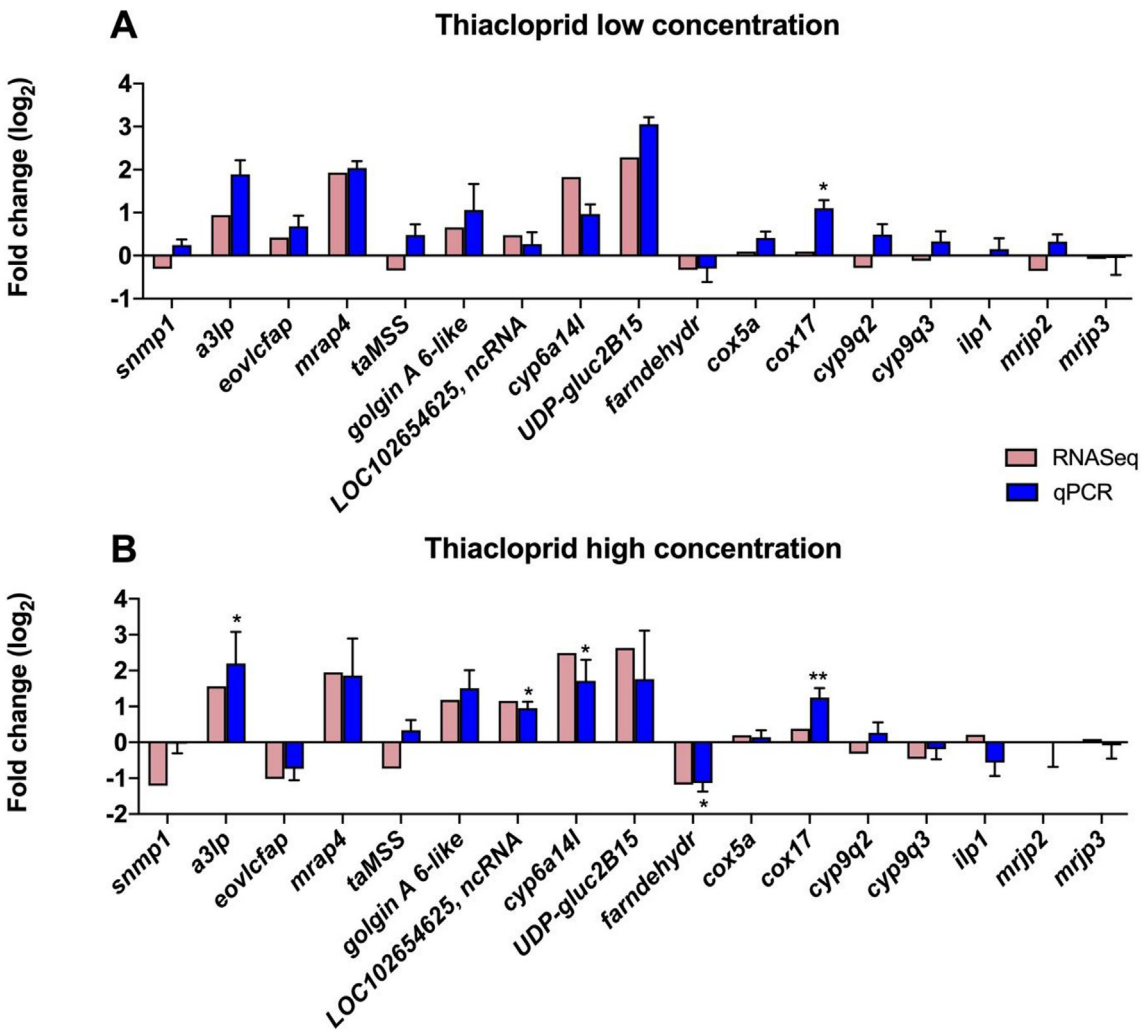

Fig. 4. Validation of RNA-sequencing data by RT-qPCR for the low (A, above) and high (B, below) thiacloprid exposure. Abundance of transcripts in the brain of bees encoding 17 genes for both exposures. Shown are abundance of genes as log2 fold changes determined by RNA-sequencing (red) and RT-qPCR (blue). Genes are listed below x-axis. Shown are results of five biological replicates per concentration and for RT-qPCR in fold-changes (log2) as means ± standard deviation. Significance is shown with * $p \leq 0.05$ and ** $p \leq 0.01$. *Abbreviations: snmp1, sensory neuron membrane protein 1*; *a3lp, apolipophorin−III−like-protein*; *eovlcfap, elongation of very long chain fatty acids protein*; *mrap4, multidrug resistance-associated protein 4*; *taMSS, transcription activator MSS11*; *golgin A6-like, globin subfamily A member 6-like protein 22*; nrRNA, non-coding RNA LOC102654625; *cyp6a14 l, cytochrome P450 6a14-like; UDP-gluc2B15, UDP-glucuronosyltransferase 2B15*; *farndehydr, farnesol dehydrogenase*; *cox5a, cytochrome c oxidase*; *cox17, cytochrome c oxidase*; *cyp9q2, cytochrome P450 9q2*; *cyp9q3, cytochrome P450 9q3*; *ilp1, insulin like protein 1*; *mrjp2, major royal jelly protein 2*; *mrjp3, major royal jelly protein 3*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

chlorantraniliprole (Christen and Fent, 2017), as well as the biopesticide spinosad (Christen et al., 2019a).

On the other hand, thiacloprid induced the transcripts *cyp6a14* and *cyp6a14-like*, which is in line with previously reported alteration of other isoenzymes, including *cyp6be1*, *cyp305d1*, *cyp6as5*, *cyp315a1*, *cyp301a1* and carboxyl/cholinesterase *cce8* (Alptekin et al., 2016). In our study, expression of the phase II enzyme *UDP-glucuronosyltransferase 2B15* was also induced at the low thiacloprid concentration. Thus, thiacloprid altered expression of phase I and phase II enzymes, which implies an interference with or alteration of xenobiotic metabolism. Previously, RNA-sequencing revealed that imidacloprid, clothianidin and thiamethoxam caused alterations of carbohydrate, lipids and sulfur metabolism, and down-regulation of *cyp9e2* and other pathways (Christen et al., 2018a). Members of the cyp4, cyp6 and cyp9 subfamilies were also differentially expressed after clothianidin exposure in bumble bees (Colgan et al., 2019) and resistance to imidacloprid was based on over-expression of *cyp6cm1* (Karunker et al., 2008). These findings suggest that thiacloprid differs from highly toxic neonicotinoids in the cyp induction profile elicited in the bees.

Our data show that the expression pattern induced by thiacloprid in the bees is different from that of highly toxic neonicotinoids clothianidin, imidacloprid and thiamethoxam, which altered expression of genes associated with carbohydrate and lipids metabolism among other pathways (Christen et al., 2018a). This signifies that agonistic binding to AChRs may result in different molecular effects patterns on the gene expression level, probably based on different binding affinities and binding sites on AChRs (Moffat et al., 2016). Thus, different neonicotinoids exhibit to some extent different gene expression profiles in exposed bees as previously found for the highly toxic neonicotinoids (Christen et al., 2018a). Other neurotoxins such as cypermethrin also differ in their expression profiles induced in exposed bees as it mainly affected muscular processes and lipids metabolism (Fent et al., 2020). Thus, the adverse outcome pathways of thiacloprid differs from that of highly toxic neonicotinoids.

A limitation of our study is the use of mixed age workers (foragers, non-flying bees), which increases the variability in responses

compared to age-controlled forager bees. In addition to experiments with age-controlled bees, it would further be favourable to use bees from different hives that have a different genetic background to confirm our data. Further recommended in future experiments is to perform analyses of the most prevalent pathogens in experimental bees. However, the latter is most probably not of influence as we did not detect immune- or pathogen-related responses. Bees used for controls and exposures were randomly chosen for the experiment, and thus, both were similar, also in this respect.

Chemical analysis of exposed bees showed that thiacloprid residues were only 2.2–2.4% of expected nominal sucrose concentrations. This low residue concentration in experimental bees can be explained by a combination low uptake, rapid metabolism and excretion by the bees, and to a minor extent, by sorption of thiacloprid to feeder tube walls. It is known that thiacloprid is readily metabolized, particularly by cyp9q3 (Manjon et al., 2018), which seems to be the main reason for the low body burdens. Our finding emphasizes that it is important to determine real exposure concentrations in ecotoxicological experiments with bees. Determination of real exposure concentrations should be established in bee research for better relying exposure to effect concentrations.

The residue data confirm that effects of thiacloprid occurred at environmentally relevant concentrations. In nectar, concentrations of 12–561 ng/mL (Ellis et al., 2017) and 0.44 ng/mL (Nicholls et al., 2018), and in pollen, between 0.22 and 77 ng/g (David et al., 2016; Ellis et al., 2017; Nicholls et al., 2018; Smodiš Škerl et al., 2009) were reported. The lower exposure concentration in our experiments was similar as in nectar, and the higher about five times above highest reported concentrations (Ellis et al., 2017). In bees from the field, thiacloprid body residues were 5–10 ng/g (Daniele et al., 2018) and in poisoned bees, they were between 3.3 and 174 ng/g (Kiljanek et al., 2016). Thus, in our study, the low exposure concentration of 0.59 ng/bee (4.84 ng/g) was similar as levels in bees from the field. The higher exposure concentration of 5.49 ng/bee (43.87 ng/g) was four times higher than the maximal reported residues. Thus, our exposure concentrations were environmentally realistic.

Together, our study indicates that thiacloprid alters expression of genes encoding proteins and enzymes related to mitochondria (structure and function), metabolism and an enzyme involved in juvenile hormone III synthesis at environmental concentrations. Thus, adverse outcome pathways of thiacloprid are altered mitochondrial function and energy allocation, and ultimately, compromised foraging activity and social behaviour. Our study provides novel insights into molecular mechanisms of thiacloprid toxicity. Forthcoming studies should focus on the link between molecular and physiological effects at environmentally realistic concentrations to corroborate our hypothesis that thiacloprid affects mitochondrial oxidative phosphorylation on the enzyme level, as well as on the function and implications of altered ncRNA.

Upload of raw and processed data to NCBI GEO database

The raw sequencing data, StringTie2 transcript abundance, the raw, unnormalized count data together with metadata information were uploaded to NCBI Gene Expression Omnibus (GEO) database under accession GSE143210.

Author statement

Karl Fent: Funding acquisition, Conceptualization, Methodology, Project management, Visualization, Supervision, Writing - original draft, Writing - review & editing; **Michael Schmid**: Methodology, Formal analysis (bioinformatics), Data curation, Visualization, Writing - review; **Timm Hettich**: Methodology, Chemical analysis, Data curation; **Simon Schmid**: Methodology, RT-qPCR, Data curation, Visualization

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115297.

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