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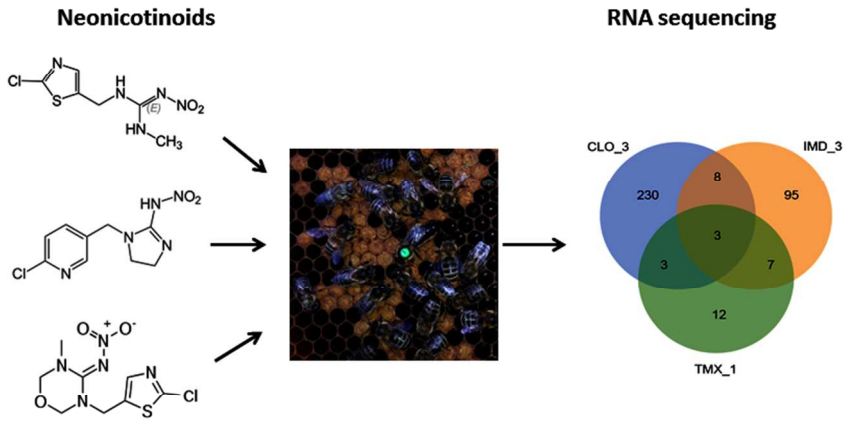

Bee transcriptomics

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Global transcriptomic effects of environmentally relevant concentrations of the neonicotinoids clothianidin, imidacloprid and thiamethoxam in the brain of honey bees (*Apis mellifera*)

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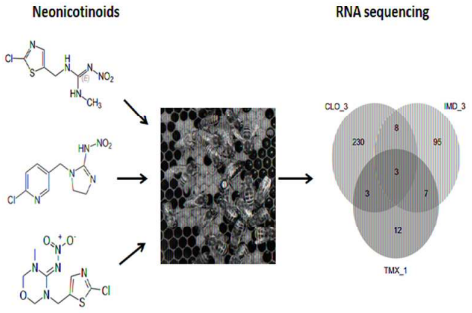
ABSTRACT

Neonicotinoids are implicated in the decline of honey bees but the molecular basis underlying adverse effects are poorly known. Here we describe global transcriptomic profiles in the brain of honey bee workers exposed for 48 h at one environmentally realistic and one sublethal concentration of 0.3 and 3.0 ng/bee clothianidin and imidacloprid, respectively, and 0.1 and 1.0 ng/bee thiamethoxam (1-30 ng/mL sucrose solution) by high-throughput RNA-sequencing. All neonicotinoids led to significant alteration (mainly down-regulation) of gene expression, generally with a concentration-dependent effect. Among many others, genes related to metabolism and detoxification were differently expressed. Gene ontology (GO) enrichment analysis of biological processes revealed catabolic carbohydrate metabolism (regulation of enzyme activities such as amylase), lipid metabolism and transport mechanisms as shared terms between all neonicotinoids at high concentrations. KEGG pathway analysis indicated that at least two neonicotinoids induced change in expression of various metabolic pathways: pentose phosphate pathways, starch and sucrose metabolism and sulfur metabolism, in which *glucose 1-dehydrogenase* and *alpha-amylase* were down-regulated and *3'(2'),5'-bisphosphate nucleotidase* up-regulated. RT-qPCR analysis confirmed the down-regulation of *major royal jelly proteins*, *hbg3* and *cyp9e2* found by RNA-seq. Our study highlights the comparative molecular effects of neonicotinoid exposure to bees. Further studies should link these effects with physiological outcomes.



TOC

INTRODUCTION

The global decline of bee populations is a multifactorial phenomenon driven by a combination of factors, mainly including parasites (e.g. *Varroa* mite), plant protection products (PPPs) and shortage of wild flowers (shrinking food sources).[1] The decline of bee populations may have significant negative impacts on plant pollination causing production losses in many domesticated crops. Nowadays, neonicotinoids are among frequently used PPPs in agriculture. Their persistence in the environment and accumulation in pollen and nectar leads to contamination of bees, pollen nectar and honey.[2,3] Rising concerns that the neonicotinoids clothianidin, imidacloprid and thiamethoxam might harm pollinators led to restrictions and partial ban on their use on crops attractive to bees in the European Union and Switzerland since 2013, while by the end of 2018 their use on outdoor crops will be totally banned.[4, 5]

Neonicotinoids are commonly applied as seed-coatings (seed dressing) to limit contact with insects and prevent losses. They are highly active neurotoxins specifically acting as agonists to acetylcholine receptors (AChRs). Particularly the nitro-substituted compounds, clothianidin, imidacloprid and thiamethoxam (metabolized to clothianidin in plants and insects)[6] show high acute toxicity with LD_{50} values in the range of a 1.11-81 ng/bee.[7,8 and 9] Sublethal concentrations were shown to induce negative pleiotrophic effects[10-18] including the immune system of bees.[19] Often, bees will be exposed to more than one PPP and combinatory effects between different PPPs may increase effects of neonicotinoids.[20-22]

Adverse effects of neonicotinoids have been demonstrated in laboratory experiments as well as in honey and wild bees under field conditions. Rape seed coated with clothianidin reduced wild bee density, solitary bee nesting, and bumblebee colony growth and reproduction.[23] Bumblebees exposed to clothianidin-treated weedy turf showed delayed weight gain and produced no new queens.[24] Sublethal thiacloprid exposure in the field impaired foraging behavior, navigation, homing success and social communication in honey bees.[25] Exposure to imidacloprid, clothianidin and fipronil led to a reduction of the proportion of active bees in the hive and reduced the efficiency of foraging flights.[10,12] Honey bee queens were also negatively affected by neonicotinoids, e.g. by reduced reproductive anatomy and physiology.[26] In large field experiments performed during crop flowering, neonicotinoids were found to harm bees in some countries (Hungary, U.K.) but not in another (Germany).[18] In Hungary, negative effects were associated with clothianidin exposure and resulted in smaller colonies in the next year, while wild bees suffered from reproduction effects affecting population establishment the next year.[18] Bee populations living on farmland and exposed to neonicotinoids may suffer from sublethal effects, including impaired homing ability, reduced survival and reproductive success that may result in population declines.[18] Thus, the European Union's food-safety agency concluded that some neonicotinoids pose a high risk to wild and honey bees (www.efsa.europa.eu).

In contrast to physiological effects including population relevant traits, such as colony size and survival, surprisingly little is known about molecular effects of neonicotinoids. We

found that neonicotinoids administered at environmentally relevant concentrations induce significant transcriptional effects in several key genes associated with neurotoxicity, memory formation, stress reaction, metabolism and life span, as well as regulation of immune system in laboratory and field exposures.[27,28] Chronic exposure to imidacloprid was also found to alter transcripts of genes related to immunity and metabolism,[29] sugar-metabolism [30] and other pathways in larvae and adults.[31,32]

Here we expand on this knowledge by evaluating molecular effects of restricted neonicotinoids, clothianidin, imidacloprid and thiamethoxam in the brain of adult honey bee workers by whole genome transcriptome sequencing at two sublethal concentrations. We focused on the brain as a target organ of neonicotinoids and to compare data with our previous study[27] Our aim is to better understand the molecular modes of actions of major neonicotinoids utilizing RNA-sequencing (RNA-seq) with targeted gene expression validation that could in turn frame future adverse outcome pathways (AOPs) for a better characterization of insecticides-bees interactions used in risk assessment. Besides deciphering compound-specific pathways, we tested whether these neonicotinoids have pathways in common or whether they alter a set of different gene transcripts. Our study reveals for the first time comparative effects on the global transcriptome of three neonicotinoids at environmentally relevant sublethal concentrations and help to better understand physiological adverse effects.

MATERIALS AND METHODS

Chemicals. Clothianidin, imidacloprid and thiamethoxam (purities of all > 99%) were purchased from Sigma–Aldrich (Buchs, Switzerland). Stock solutions for each compound were prepared in DMSO and diluted into sucrose-solution (0.1% DMSO).

Laboratory exposure experiments. Foraging adult worker honey bees (*Apis mellifera carnica*) of mixed age were collected from frames from one outdoor colony in June 2017 located in a rural site with no agricultural activity and pesticide use in the Black Forest (Germany, GPS: N 47.7667, E 7.8333). All of the bees used in the experiment were from the same colony. It had evidence of *Varroa destructor* infestation and was treated with formic acid (August 2016) and oxalic acid (December 2016). Honey bees were collected in small cylindrical plastic containers, cold anaesthetised at 4°C for 60 minutes, and transferred into 16.5 × 11 × 6.5 cm^3 PET bottles with small holes in the lid for gas exchange. A larger hole of 2 mm served for holding an Eppendorf micro-centrifuge tube filled with 2 mL of a 20% sucrose solution containing the individual neonicotinoids or 0.1 % DMSO as solvent control or pure sucrose solution as control. For each concentration, five replicates (five bottles) were employed. Concentrations of neonicotinoids were selected on the basis of our previous

study.[27] A summary of used concentrations expressed as ng/bee and as ng/ml sucrose solution is shown in Table S1 (supplementary information).

Concentrations of neonicotinoids were selected on the basis of environmentally realistic levels in nectar based on our previous study[27], which were well below LD_{50} values. Used concentrations were 0.3 and 3.0 ng/bee for clothianidin and imidacloprid, respectively, while for thiamethoxam used concentrations were 0.1 and 1.0 ng/bee (Table S1; supplementary information). Due to the lower LD_{50} value of thiamethoxam, used concentrations were lower compared to the other two neonicotinoids to be able to compare expression profiles between the different treatments. No compound related mortality occurred. Ten randomly chosen adult worker bees were placed into each bottle and placed in an incubator (28°C, 60% humidity). Bees were fed *ad libitum* with sucrose solutions containing the neonicotinoids for 48 h. After 24 h, the 2 mL sucrose solution was removed, the amount of sucrose solution taken up by the bees assessed, and replaced by a new solution. The average amount of sucrose solution was 100 µL per bee throughout all exposure experiments. Control, solvent control and bees exposed to the neonicotinoids were removed after 48 h, in case of clothianidin after 24 h and 48 h, and stored at -80°C until further analysis. A graphic overview of the experimental design is shown in Figure S1 (supplementary information).

RNA isolation, RNA-sequencing, Reverse Transcription and RT-qPCR

Raw reads have been submitted to the NCBI Sequence Read Archive (SRA) under accession number SRP140405. A detailed description of RNA isolation, RNA sequencing, reverse transcription, quantitative qPCR and selection of reference genes can be found in the supplementary material (Table S2).

RNA-sequencing Data Analysis. *De novo* Assembly and Functional Annotations

The quality of each library was assessed using the FastQC program (www.bioinformatics.babraham.ac.uk/projects/fastqc). To filter raw sequence reads, we used Trimmomatic [33] to remove Illumina adapters, bases at the start and end of a read below a quality threshold of 4, and low-quality segments from the end of a read using a 4 bp sliding window and a threshold for average quality of 20. Trimmed reads shorter than 30 bp were not used for further analyses. The filtered and trimmed reads were de novo assembled using the short read program Trinity v.2.4.0.[34] The trinity software combined reads of certain lengths of overlap to form longer fragments, so called contigs. Then, the reads were mapped back to these contigs until no more ends extended. The obtained longest sequences were defined as unigenes after removing any redundancy. To identify open reading frames (ORFs) of unigenes within our de novo assembly, we used TransDecoder.[34] The ORFs were functionally annotated

by homology search (e-value ≤ 1e−5) to known sequence data (BLAST+/SwissProt), and leveraging various annotation databases (GO/KEGG databases) using Trinotate.[35]

Analyses of Differentially Expressed Unigenes

RNA-seq by Expectation Maximization (RSEM) was used to estimate the abundance of reads by allocating the reads to transcripts of our de novo assembly.[36] To study transcriptional effects of the neonicotinoids relative to the solvent control (0.1% DMSO), differentially expressed unigenes between pairwise comparisons of the neonicotinoid samples and solvent control and between solvent control and sucrose solution were analysed with edgeR.[37] As selection criteria for unigenes to be differentially expressed between neonicotinoids treated bees and bees of the solvent, we set a cut-off of > 2 for fold changes and an FDR corrected p-value of < 0.05. We assessed the significance of the observed changes in reads per kilobase of target transcript length per million reads mapped (RPKM) for single-end sequences, and corrected *p*-values for multiple testing using the false discovery rate (FDR). To account for differences in total cellular RNA production across all samples, an additional TMM (trimmed mean of M-values) scaling normalization was performed using edgeR.[37] Differentially expressed unigenes met our requirements with FDR < 0.05 and an at least two-fold expression difference. Unigenes that were expressed in < 50% of the replicates of each exposure were not used for further analyses. Using GO annotations, differentially expressed unigenes were classified into three ontologies including biological process, cellular component and molecular function. Significantly enriched GO terms in comparison with the transcriptome background were identified with a Fisher's exact test ($p < 0.01$) using Goseq.[38] Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation was carried out to obtain pathway annotations for the differentially expressed unigenes. Pathways were identified using *Apis mellifera* (Ame) as reference organism.

Data Processing and Statistical Analysis

Heat maps of expressional changes were designed by importing analysed qPCR data into MEV 4.9 (Multi Experiment Viewer) software. Differences between treatments were assessed by one-way ANOVA followed by a Bonferroni`s multiple comparison test to compare treatment means with respective controls. qPCR results are given as means ± standard error of means. Differences were considered statistically significant with one asterisk at $p < 0.05$, two asterisks at $p < 0.01$, and three asterisks at $p < 0.001$.

RESULTS

Differentially Expressed Unigenes after Neonicotinoid Exposure

Genomic transcription profiles of neonicotinoids at two exposure concentrations (each in five replicates) were assessed upon 48 h exposure by RNA-seq (Figure S2). In total, 358'110 transcripts and 197'861 coding regions, i.e. unigenes, with a GC content of 34.2% were identified. The median contig length was 499 bp and the average contig length 1102 bp (with an N50 of 2312 bp). Using this *de novo* assembly to estimate the abundance of our reads, we found 27'311 and 26'578 unigenes to be expressed in bees treated with 0.3 ng/bee and 3.0 ng/bee clothianidin, 26'363 and 26'897 unigenes in bees treated with 0.3 ng/bee and 3.0 ng/bee imidacloprid, 27'085 and 26'859 unigenes in bees treated with 0.1 ng/bee and 1.0 ng/bee thiamethoxam, respectively, and 27'079 unigenes in bees treated with the solvent control.

Due to high lipophilicity, neonicotinoids were dissolved in 0.1% DMSO (solvent control), which led to differential expression of eight unigenes compared to sucrose alone (Table S3). Among these, unigenes were homologous to *apidaecins type 14*, *ecdysone-induced protein 75 (E75*), *histone H2A.Z-specific chaperone CHZ1-like*, *hymenoptaecin*, *RNA-binding protein 4.1-like*, *hbg3 gene for alpha-glucosidase*, and *neurofilament heavy polypeptide* (Table S4). To eliminate potential solvent control effects, differential gene expression values induced by neonicotinoids represent relative changes to the solvent control response.

Among differentially expressed unigenes, low concentrations of neonicotinoids led to an alteration of six to 26 unigenes, while high concentrations led to an alteration of 25 to 244 unigenes, indicating a concentration related effect (Figure 1A-C, Table S3). Furthermore, in almost all treatments (except for 0.1 ng/bee thiamethoxam), more unigenes were down- (two to 234) than up-regulated (four to 36).

In bees treated with 0.3 ng/bee clothianidin, 18 unigenes were differentially expressed; four unigenes were up- and 14 unigenes were down-regulated (Table S3). Among up-regulated unigenes were *opsin-1* and a protein kinase, while among the 14 down-regulated unigenes were mainly metabolic enzymes such as *glucose dehydrogenase*, *peroxidase* and *clavesin-1* (Table S4). The largest number of alterations occurred in bees treated with 3 ng/bee clothianidin, with 244 unigenes being differentially expressed, ten up- and 234 down-regulated (Table S3). Among the up-regulated unigenes were the immune system related genes *hymenoptaecin* and *apidaecin*, *cytochrome P450 4C1*, and *3'(2'),5'-bisphosphate nucleotidase 1*, while the down-regulated unigenes were related to embryonic development (*royal jelly proteins*), (glucose) metabolism, immune system reaction, and transcription factors, amongst others (Table S4). Among the two unigenes overlapping between both concentrations was *peroxidase* (Figure 1A).

In bees treated with 0.3 ng/bee imidacloprid, 26 unigenes were differentially expressed; seven unigenes were up- and 19 unigenes down-regulated (Table S3). Among up-regulated unigenes were *hymenoptaecin, rhodopsin, synaptogamin-14*, an *ATPase subunit* and one

protein kinase, while among down-regulated unigenes were *major royal jelly protein-3*, metabolism related and other enzymes (*alpha-glucosidase hbg3*, *alpha-amylase related protein*, *ubiquitin hydrolase*, *nucleotide phosphodiesterase*) (Table S4). In bees treated with 3 ng/bee imidacloprid, 113 unigenes were differentially expressed; 36 unigenes were up- and 77 unigenes were down-regulated (Table S3). Among up-regulated unigenes were immune system related genes (*hymenoptaecin, apidaecin*), metabolism enzymes (*amylase, glucose metabolism hbg3, inositol-3-phosphate synthase*, *α-glycosyltransferase, apyrase, esterase, aminolevulir, mitochondrial fatty acid beta-oxidation, 5-aminolevulinate synthase*), enzymes (*proton transporting ATPase, protein kinase C, D helicase*), genes for transport (*creatinine*), and ecdysteroid signalling (Table S4). Many of the down-regulated unigenes annotated to genes involved glucose metabolism and to odorant binding proteins, such as odorant binding protein 2 and 6 (*Obp2, Obp6, Obp69a* and *Obp72*). Between both imidacloprid concentrations, eleven unigenes overlapped and annotated to genes such as *hbg3 (coding for alpha-glucosidase)*, *calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase 1*, an *alpha-amylase-related protein*, *alpha-amylase 4N*, *hymenoptaecin*, and *ubiquitin carboxyl-terminal hydrolase 47* (Figure 1B).

The lowest number of alterations occurred in bees treated with 0.1 ng/bee thiamethoxam, with only six unigenes being differentially expressed; four unigenes were up- and two unigenes were down-regulated (Table S3). Among the four up-regulated unigenes annotations were to *basic-leucine zipper transcription factor A*, *hbg3*, *rhodopsin* and *3'(2'),5'-bisphosphate nucleotidase 1* (Table S4). Down-regulated unigenes were uncharacterized or had no functional annotation. In bees treated with 1 ng/bee thiamethoxam, 25 unigenes were differentially expressed; four unigenes were up- and 21 down-regulated (Table S3). Up-regulated unigenes were homologous to *3'(2'),5'-bisphosphate nucleotidase 1*, *junctophilin 1 like*, *opsin-1*, *protein turtle*, and down-regulated unigenes to genes mainly involved in glucose metabolism (*alpha-glucosidase hbg3, alpha-amylase and glucose dehydrogenase*), *CCAAT/enhancer-binding protein b* and *odorant binding proteins* (i.e. *general odorant binding protein 69a and 72*), among others (Table S4). Between both thiamethoxam concentrations, two out of three overlapping unigenes belonged to the genes *3'(2'),5'-bisphosphate nucleotidase 1* and *rhodopsin* (Figure 1C). The lower numbers of thiamethoxam-induced alterations may partly be due to the lower exposure concentrations compared to the other neonicotinoids. Therefore, thiamethoxam had less effect on gene expression compared to clothianidin and imidacloprid.

The comparison of all neonicotinoids shows that one unigene, annotated to *rhodopsin*, is shared among low concentrations of all three neonicotinoids (Figure 1D). Among high concentrations, three unigenes were shared, annotating to *hbg3*, *CCAAT/enhancer binding protein b* and *glucose dehydrogenase* (Figure 1E). Between clothianidin and imidacloprid,

eight unigenes were shared, annotated to *ras-related protein Rab-3*, *acyl-CoA delta(11) desaturase*, and *hymenoptaecin* (Figure 1E). Among clothianidin and thiamethoxam, three unigenes were shared, of which one belonged to *3'(2'), 5'-bisphosphate nucleotidase 1* (Figure 1E). Among imidacloprid and thiamethoxam, seven unigenes were shared, which mainly belonged to the genes *odorant binding protein 2 (Obp2)*, *odorant binding protein 6 (Obp6)*, *general odorant-binding protein*, *alpha-amylase 4N*, *alpha-amylase-related protein*, *ankyrin repeat* and *SOCS box protein 18*, and *scavenger receptor class B member 1* (Figure 1E).

Functional Analysis of Differentially Expressed Genes (GO Term Analysis)

To address the potential functional relevance of the differentially expressed unigenes, we conducted a functional annotation of the predicted gene sequences in the *de novo* assembly by GO enrichment analysis. Significantly ($p < 0.01$) enriched GO terms were classified in the three categories biological processes, molecular functions and cellular components (Figures 2A-C).

Among the lists of differentially expressed unigenes, GO term annotations could be assigned to eight (44.4%) and 66 unigenes (27.1%) in the 0.3 ng/bee and 3 ng/bee clothianidin treatments, eight (30.8%) and 22 unigenes (19.5%) in the 0.3 ng/bee and 3 ng/bee imidacloprid, and two (33.3%) and nine unigenes (36.0%) in 0.1 ng/bee and 1 ng/bee thiamethoxam treatments, respectively. Possibly because of the small number of unigenes, GO categories were not significantly enriched after correction for an FDR of 5%.[39] This is a common result when the tested categories are not independent as in this case, where one gene can be associated with several GO terms.[40]

Ten biological processes were significantly overrepresented between the solvent control and control (Figure S3; Table S5), mainly belonging to immune and defense processes. Clothianidin exposures led to significantly overrepresented 40 biological processes, seven molecular functions and two cellular components in the low (Table S6), and 65 biological processes, 19 molecular functions and seven cellular components in the high concentration (Figure 2A; Table S7). The top enriched GO terms in low and high concentrations were N-acetylneuraminate catabolic process and protein refolding in biological processes, N-acetylneuraminate lyase activity and unfolded protein binding in molecular functions, and chorion and nucleosome in cellular components, respectively.

Imidacloprid led to significant overrepresentation of 14 biological processes and seven molecular functions in the low (Table S8), and two biological processes, eight molecular functions and two cellular components in the high concentration (Figure 2B, Table S9). The top enriched GO terms for low and high imidacloprid concentrations were male mating

behaviour and imaginal disc-derived wing vein specification in biological processes, and alpha-amylase and amylase activity in molecular functions, respectively.

Thiamethoxam led to significant overrepresentation of eight biological processes and three molecular functions in the low (Table S10), and 32 biological processes, 15 molecular functions and four cellular components in the high concentration (Figure 2C; Table S11). The top enriched GO terms for low and high concentrations were 3'-phosphoadenosine 5'-phosphosulfate metabolic process and carbohydrate metabolic process in biological processes, 3'(2'),5'-bisphosphate nucleotidase activity and alpha-amylase activity in molecular functions, respectively.

No enriched GO terms were shared among all neonicotinoids at low concentrations, while 15 enriched of biological processes were shared among high concentrations (Figure 3). Overall, the GO analyses revealed that differentially expressed mRNAs are significantly enriched for genes associated with regulation of enzyme activities (e.g. amylase activity), carbohydrate and lipid metabolisms, transport of lipids, and vitamin transport.

KEGG Pathway Analysis

To address the potential pathways, in which the differentially expressed unigenes were involved, we characterized them with KEGG annotations and pathways related to *A. mellifera*. They were assigned to six (33.3%) and 46 unigenes (18.9%) in the 0.3 ng/bee and 3 ng/bee clothianidin, six (23.1%) and 14 unigenes (12.4%) in the 0.3 ng/bee and 3 ng/bee imidacloprid, and one unigene (16.7%) and 7 unigenes (28.0%) in the 0.01 ng/bee and 1 ng/bee thiamethoxam treatment, respectively (Table 1).

Among the three neonicotinoids, KEGG pathways mainly belonged to metabolism, and involved enzymes were mainly down-regulated (Table 1). In high concentrations of all neonicotinoids, four pathways (metabolic, pentose phosphate, starch and sucrose metabolism, sulfur metabolism) were shared among at least two neonicotinoids, in which three unigenes (*glucose 1-dehydrogenase,* down-regulated*; 3'(2'), 5'-bisphosphate nucleotidase,* up-regulated*;* and *alpha-amylase,* down-regulated) were involved (Table 1).

RT-qPCR Analysis

For validation of RNA-seq data we performed RT-qPCR with ten selected unigenes that showed prominent alterations in the present study or gene transcripts, which showed an altered expression in response to neonicotinoids in our previous study.[27] Expression of the three reference (housekeeping) genes were unaltered after neonicotinoid exposure (Figure S3). For further analysis *actin* and *efl-*α were used as reference genes. In the high clothianidin exposure *major royal jelly protein 1-3* showed a concordant pattern of down-regulation in both, RNA-seq and RT-qPCR, as did *hbg-3* for clothianidin and imidacloprid (Figure 4).

352 *Biphosphate-nucleotidase*, *CAAT/enhancer binding protein beta*, and *cyp9e2* showed the
353 same tendency of transcriptional changes between RNA-seq and RT-qPCR, although
354 alterations were not significant in qPCR, except for *cyp9e2* at 3 ng/bee clothianidin.
355 Furthermore, the magnitude was lower with RT-qPCR (Figure 4). *Vitellogenin* and
356 acetylcholine receptors (*achrα1*, *achrα2*) showed no significant alterations (Figure 4, Figure
357 S5). These expression data indicate a correlation between RNA-seq and RT-qPCR data,
358 although they differed in the magnitude of alteration. In a case of imidacloprid and
359 thiamethoxam, alterations were not significant in qPCR, except for *hbg-3* in the 3 ng/bee
360 imidacloprid treatment.
361 Additionally, we analysed the expression of nine transcripts by RT-qPCR after a 24 h
362 exposure to clothianidin. A significant induction of *vitellogenin* (Figure S6) occurred, which was
363 lacking at 48 h (Figure 4), and is in line with previous data [27]. Additionally, *hbg-3* was down-
364 regulated at the high concentration (Figure S6).
365
366 **DISCUSSION**
367 The neonicotinoids analyzed in our study are implicated in adverse effects on the health and
368 performance of bees yet their molecular effects have so far been only little studied, e.g. by
369 targeted gene expression analysis,[22,27] microarrays [29] and RNA-seq.[30-32,41] Our present RNA-
370 seq study analyzing clothianidin, imidacloprid and thiamethoxam represents the first
371 comparative global transcription analysis of sublethal concentrations of neonicotinoids in bees.
372 We show that all neonicotinoids led to transcriptional alterations (mainly down-regulation) of
373 genes in the brain of bee workers, with concentration-relationships. Differentially expressed
374 unigenes were mostly distinct for each neonicotinoid treatment and concentration, however,
375 some functional annotations and pathways were shared among treatments. Prominent and
376 shared alterations in gene expression were mainly associated with carbohydrate and lipids
377 metabolism.
378 Carbohydrate- and lipid-metabolizing enzymes catalyze reactions that break down food
379 to release stored energy and synthesize an organism's primary energy stores .[42] In addition,
380 metabolic processes are important regulators of both caste determination and behavioral
381 development in honey bees. The down-regulation of genes for necessary enzymes for
382 glycolysis and lipids found in our study likely implies lower supply of carbohydrate resources
383 and lowered energy supply. Nutritional effects may not only occur in the brain but also in the
384 whole body of bees. Therefore, changes in genes related to metabolic processes may have
385 effects on the transition of nurse bees to foragers.[43]and additionally, likely has many adverse
386 implications on physiology and performance of bees, including duration of foraging, flight
387 performance and survival. Indeed, neonicotinoids were shown to impair foraging, delay return
388 flights and lead to loss of bees during foraging.[25]

Despite effecting similar pathways, GO term enrichment and KEGG pathway analyses showed distinct transcriptional patterns for each neonicotinoid treatment and concentration. For example, amylase and hydrolase activities were only shared by imidacloprid and thiamethoxam, whereas for clothianidin no such activity was significantly enriched. These distinct response patterns of the individual neonicotinoids, which are all antagonist to the nicotinic acetylcholine receptors, may be related to different binding strengths and sites in receptor binding.

Our data on thiamethoxam exposure resemble in part those of Shi et al.[41], in which RNA-seq was applied to determine expressional changes in the whole body of bees exposed for ten days to 10 ng thiamethoxam/mL sucrose. Besides, only imidacloprid was previously investigated, while transcriptome data on clothianidin are presented here for the first time. Wu et al.[31] analyzed transcripts in heads of newly emerged bees after 4 days exposure of larvae (2 ng/larvae, 2 ng/mL syrup) to imidacloprid. Among 578 altered gene transcripts, 329 genes had known functions belonging to different pathways. In our study, exposure to 3 ng/bee changed the expression of only 113 transcripts. This discrepancy can be explained by the shorter exposure time in our study and by the exposure of adults in our study versus the exposure of more sensitive developing stages in the study by Wu et al. [31]. Adverse effects on locomotor activity upon exposure to imidacloprid (10 ng/mL sucrose solution for 8 days), down-regulation of muscle-related genes and up-regulation of ribosomal protein genes were observed in whole bees. [32] Imidacloprid also altered transcripts of genes related to immunity and metabolism as assessed by a microarray specifically designed for these pathways.[29] Similarly, in our study we observed effects on the expression of immune system genes and metabolism related genes. A more exhaustive RNA-seq study revealed 300 differentially expressed genes in worker bee larvae experimentally exposed for 15 days to 2 µg/L imidacloprid. [30]

In our study, the main gene clusters affected by the majority of the neonicotinoids belonged to enzyme activities (e.g. amylase activity), catabolic processes and lipids metabolism as indicated by the GO term enrichment analysis, and metabolic pathways, mainly glycolytic and carbohydrate metabolism but also sulfur metabolism as indicated by the KEGG analysis. As the brain has a high energy demand, negative effects on metabolic pathways can imply adverse effects on brain function and the whole organism. For instance, changes in nutritional physiology and in the expression of metabolic genes can influence the devision of labour.[44] Effects on metabolic pathways are in line with data upon imidacloprid exposure in larvae, in which differentially expressed genes were involved in lipid-carbohydrate-mitochondrial metabolic networks or belonged to cytochrome P450 enzymes and to genes of the glycolytic and sugar-metabolising pathways.[30] Additionally, genes involved in sulfur metabolism were up-regulated, potentially for the higher need of glutathione, which plays not

426 only a role in metabolism but the detoxification of reactive oxygen species. Therefore, the
427 need for glutathione for detoxification was likely behind the accelerated sulfur metabolism.
428 Down-regulation of necessary enzymes for glycolysis and lipids likely implies lower
429 supply of carbohydrate resources and lowered energy supply. Nutritional effects may not only
430 occur in the brain but also in the whole body of bees, hence possibly also affecting larval
431 feeding. It is not known whether the down-regulation of carbohydrate metabolism is a result of
432 lower energy demand in the brain due to neonicotinoid exposure or whether this is associated
433 with inhibitory action on acetylcholine receptors. Ultimately, down-regulation of carbohydrate
434 and lipids metabolism yields lower energy supply, which likely has many adverse implications
435 in physiology and performance of bees, including duration of foraging, flight performance and
436 survival. Indeed, neonicotinoids were shown to impair foraging, delay return flights and lead to
437 loss of bees during foraging.[25]
438 Our analysis shows that in the solvent control (sucrose solution and 0.1% DMSO) led
439 to enrichment of immune and defense processes in comparison with the control (sucrose
440 alone) indicating that the use of 0.1% DMSO, which was necessary to dissolve lipophilic
441 neoncotinoids, already initiated transcriptional alterations of selected genes. This unexpected
442 finding itself is interesting and not reported before. Thus, the here found effects of
443 neonicotinoids are superimposed on DMSO-induced changes. Further studies should test
444 whether lower concentrations than 0.1% DMSO or other non-toxic solvents may have lower
445 effects.
446 Our RT-qPCR validation confirmed the down-regulation of members of *royal jelly*
447 *proteins (mrjp)* by clothianidin, which also occurred after larval exposure to imidacloprid.[31]
448 These proteins are involved in brain function, possibly in learning ability and memory, and are
449 expressed in the mushroom.[45] Their down-regulation may be associated with demonstrated
450 adverse effects of neonicotinoids on memory and learning ability.[46] Because MRJPs are major
451 constituents in royal jelly, lower production may result in fewer queens and in nutrition
452 reduction in larvae. MRJPs are also a family of antimicrobial peptides.[47] Thus, down-regulation
453 of these multifunctional proteins may also have negative implications at the physiological level,
454 including brain and immune function, and nutrition.
455 We assessed gene expression levels of eight genes of toxicologically relevant
456 pathways, which have previously been shown to be transcriptionally altered by two to all
457 neonicotinoids, clothianidin, imidacloprid, and thiamethoxam.[27] Primers from the present study
458 were mapped against our *de novo* assembly to gain insight why these genes of interest were
459 not amongst the significantly differentially expressed genes in the present study. Although all
460 genes of interest were always transcribed, *nAChRα2, apidaecin, pka* and *vitellogenin* showed
461 no strong transcriptional alterations in any concentration at 48 h exposures (Table S12). While
462 *catalase* and *creb* were down-regulated (logFC ≅ 1) only with 3 ng/bee clothianidin, *nAChRα1*

was down-regulated in the high concentrations of all three neonicotinoids (logFC > 1). However, none of these genes reached the significant thresholds of logFC > 2 and FDR < 0.05 that we used in our study (Table S12), and therefore they do not appear in our lists of differentially expressed unigenes.

In our previous studies we found that imidacloprid and thiamethoxam induced *vitellogenin* and *AChRs* upon 48 h exposure,[22, 27,] while no significant alterations were found in our present RNA-seq data. Among possible reasons for this lack of concordance are variabilities in the sensitivity against neonicotinoids between bee colonies that differed from the previous studies, and therefore have a different genetic background. Variable sensitivity was also found for acute toxicity.[7] Additional reasons are technical ones. The signal of these genes may be reduced by stronger effects on other genes when whole transcriptome data are sequenced, or the choice of reference genes that can impact results of qPCRs, while in RNA-seq all genes serves as reference.[48] However, RT-qPCR data confirmed differential expression of *vitellogenin* for clothianidin exposure after 24 h, as found previously [27].

In our present study we found three gene transcripts altered by all neonicotinoids, i.e. *hbg3* gene for alpha-glucosidase*, CCAAT/enhancer-binding protein beta,* and *glucose dehydrogenase*. Forthcoming studies should evaluate, whether these or additional transcripts including *mrjp* may serve as biomarkers for sublethal neonicotinoid exposure, whether transcriptional alterations translate into protein levels in brain and other tissues and whether effects persist upon chronic exposures. Our data highlight molecular effects and affected pathways such as the metabolic network by these important agricultural insecticides. Our data will help in the hazard and risk assessment and complement data of neonicotinoid effects on the molecular level that ultimately may translate to important physiological effects.

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Data accessibility

The datasets supporting the conclusions of this article are available in Sequence Read Archive SRA (SRP140405; https://www.ncbi.nlm.nih.gov/sra).

Associated content

Supporting information include exposure concentrations of used neonicotinoids (Table S1), sequences of used qPCR primers (Table S2), number of up- and down-regulated unigenes

(Table S3), list of differentially expressed unigenes (Table S4), enriched GO terms among differentially expressed unigenes (Tables S5-11), transcriptional alterations of selected genes from Christen et al (2016), experimental design of laboratory exposure experiment (Figure S1), MDS plot of the normalized expression data (Figure S2), enriched GO terms between control and solvent control (Figure S3), validation of reference genes (Figure S4), abundance of achra1, (Figure S5) and abundance of 9 different transcripts after 24 h exposure to clothianidin (Figure S6).

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Table 1: KEGG pathway analysis of the differentially expressed unigenes of *A. mellifera* treated with 0.3 ng/bee and 3 ng/bee clothianidin (CLO_0_3 and CLO_3), 0.3 ng/bee and 3 ng/bee imidacloprid (IMD_0_3 and IMD_3), and 0.1 ng/bee and 1 ng/bee thiamethoxam (TMX_0_1 TMX_1), respectively. Pathways and unigenes in bold font are shared among the high concentrations of the three neonicotinoids. Up- (↑) or down-regulation (↓) of unigenes is indicated by the respective arrows behind enzyme names.

Treatment	Pathways	Enzymes	Unigenes
CLO_0_3	Pentose phosphate pathway	glucose 1-dehydrogenase (FAD, quinone) ↓	DN96981_c0_g1
	Metabolic pathways	glucose 1-dehydrogenase (FAD, quinone) ↓	DN96981_c0_g1
CLO_3	Protein processing in endoplasmic reticulum	heat shock 70kDa protein 1/2/6/8 ↓	DN74940_c0_g1
		molecular chaperone HtpG ↓	DN74940_c0_g1
		crystallin, alpha B ↓	DN68606_c3_g2, DN59262_c6_g1, DN67466_c0_g1, DN74437_c0_g1, DN71360_c2_g1
	Longevity regulating pathway - multiple species	heat shock 70kDa protein 1/2/6/8 ↓	DN74940_c0_g1
		crystallin, alpha B ↓	DN68606_c3_g2, DN59262_c6_g1, DN67466_c0_g1, DN74437_c0_g1, DN71360_c2_g1
	Endocytosis	heat shock 70kDa protein 1/2/6/8 ↓	DN74940_c0_g1
		G protein-coupled receptor kinase ↓	DN73959_c0_g1
	Metabolic pathways	**glucose 1-dehydrogenase (FAD, quinone) ↓**	**DN75371_c1_g1**
		cysQ; 3'(2'), 5'-bisphosphate nucleotidase ↑	**DN45341_c0_g1**
	Proteasome	26S proteasome regulatory subunit N1 ↓	DN70964_c0_g1
	Pentose phosphate pathway	**glucose 1-dehydrogenase (FAD, quinone) ↓**	**DN75371_c1_g1**
	Spliceosome	heat shock 70kDa protein 1/2/6/8 ↓	DN74940_c0_g1
	MAPK signaling pathway - fly	dual oxidase ↓	DN75864_c1_g1
	Toll and Imd signaling pathway	dual oxidase ↓	DN75864_c1_g1
	Hippo signaling pathway - fly	homeobox protein homothorax ↓	DN65314_c0_g1
	Hedgehog signaling pathway - fly	G protein-coupled receptor kinase ↓	DN73959_c0_g1
	Sulfur metabolism	**3'(2'), 5'-bisphosphate nucleotidase ↑**	**DN45341_c0_g1**
IMD_0_3	Metabolic pathways	alpha-amylase ↓	DN74754_c11_g2
	Starch and sucrose metabolism	alpha-amylase ↓	DN74754_c11_g2
	Purine metabolism	calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase ↓	DN75806_c0_g2
IMD_3	**Metabolic pathways**	**glucose 1-dehydrogenase (FAD,**	**DN75371_c1_g1**

		quinone) ↓	
		5-aminolevulinate synthase ↑	**DN178528_c0_g1**
		alpha-amylase ↓	**DN74754_c11_g2**
	Pentose phosphate pathway	**glucose 1-dehydrogenase (FAD, quinone) ↓**	**DN75371_c1_g1**
	Purine metabolism	calcium/calmodulin-dependent 3',5'-cyclic nucleotide phosphodiesterase ↓	DN75806_c0_g2
	Glycine, serine and threonine metabolism	5-aminolevulinate synthase ↑	DN178528_c0_g1
	Porphyrin metabolism	5-aminolevulinate synthase ↑	DN178528_c0_g1
	Starch and sucrose metabolism	**alpha-amylase ↓**	**DN74754_c11_g2**
TMX_0_1	Sulfur metabolism	3'(2'), 5'-bisphosphate nucleotidase ↑	DN45341_c0_g1
	Metabolic pathways	3'(2'), 5'-bisphosphate nucleotidase ↑	DN45341_c0_g1
TMX_1	**Metabolic pathways**	**glucose 1-dehydrogenase (FAD, quinone) ↓**	**DN75371_c1_g1**
		3'(2'), 5'-bisphosphate nucleotidase ↑	**DN45341_c0_g1**
		alpha-amylase ↓	**DN74754_c11_g2**
	Pentose phosphate pathway	**glucose 1-dehydrogenase (FAD, quinone) ↓**	**DN75371_c1_g1**
	Starch and sucrose metabolism	**alpha-amylase ↓**	**DN74754_c11_g2**
	Sulfur metabolism	**3'(2'), 5'-bisphosphate nucleotidase ↑**	**DN45341_c0_g1**

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Figure legends:

Figure 1: Numbers of differentially expressed unigenes of *A. mellifera* treated with (A) 0.3 ng/bee and 3 ng/bee clothianidin (CLO_0_3 and CLO_3), (B) 0.3 ng/bee and 3 ng/bee imidacloprid (IMD_0_3 and IMD_3), and (C) 0.1 ng/bee and 1 ng/bee thiamethoxam (TMX_0_1 and TMX_1). Numbers of differentially expresses unigenes shared between (D) low concentrations and (E) high concentrations of the three neonicotinoids.

Figure 2: Enriched top five or all GO terms that were significantly overrepresented before multiple testing (Fisher's exact test; $p < 0.01$) of differentially expressed unigenes of *A. mellifera* treated with (A) 3 ng/bee clothianidin, (B) 3 ng/bee imidacloprid, (C) 1 ng/bee thiamethoxam. Biological processes are in blue, molecular functions in orange, and cellular components in green.

Figure 3: Enriched GO terms of biological processes that were significantly overrepresented before multiple testing (Fisher's exact test; $p < 0.01$) of differentially expressed unigenes of *A. mellifera* overlapping between high concentrations of the three neonicotinoids clothianidin (3 ng/bee, CLO 3), imidacloprid (3 ng/bee, IMD 3) and thiamethoxam (1 ng/bee, TMX1).

Figure 4: Validation of RNA sequencing data by RT-qPCR. (A) Heat map of differentially expressed genes determined by RT-qPCR. (B) Abundance of transcripts of *mrjp1*, *mrjp2* and *mrjp3* (upper line), *hbg-3*, *biphosphate nucleotidase* and *CAAT/enhancer binding protein beta* (middle line) and *cyp9e2*, *achrα2* and *vitellogenin* (lower line) in the brain of honey bees following exposure to different concentrations of clothianidin (blue bars), imidacloprid (green bars) and thiamethoxam (yellow bars) for 48 h. Values above bars represent log2 (fold change) values determined by RNA sequencing for comparison. Shown are the qPCR results of five biological replicates per concentration. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

Figures

Figure 1

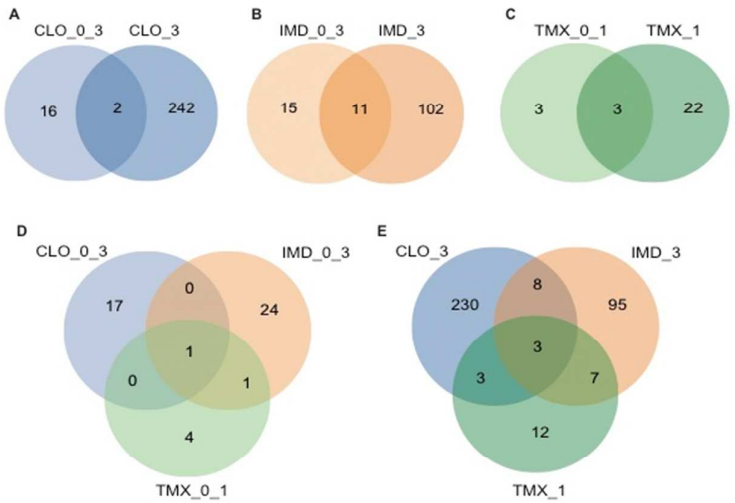
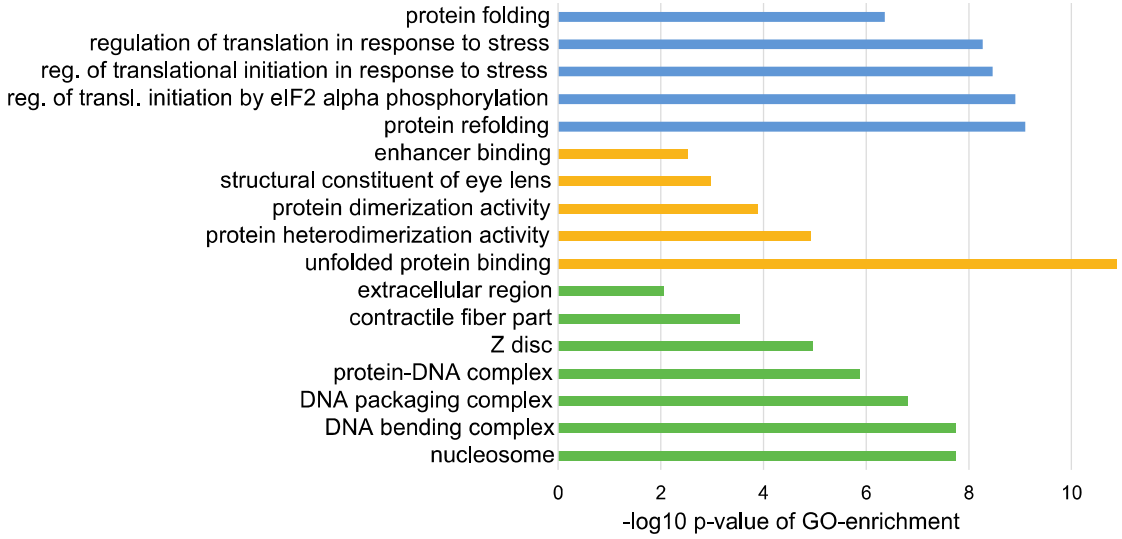
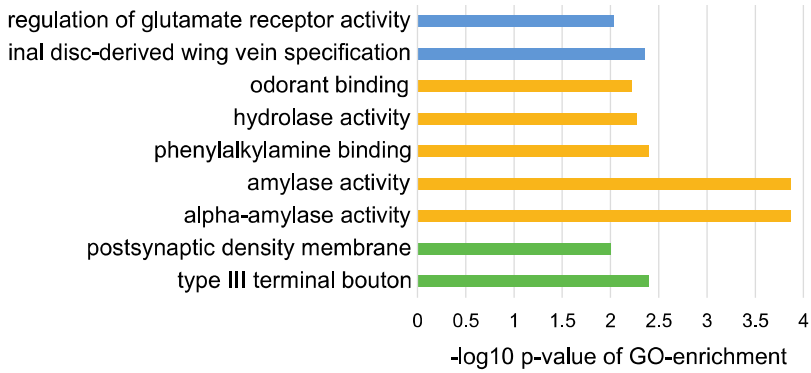


Figure 2

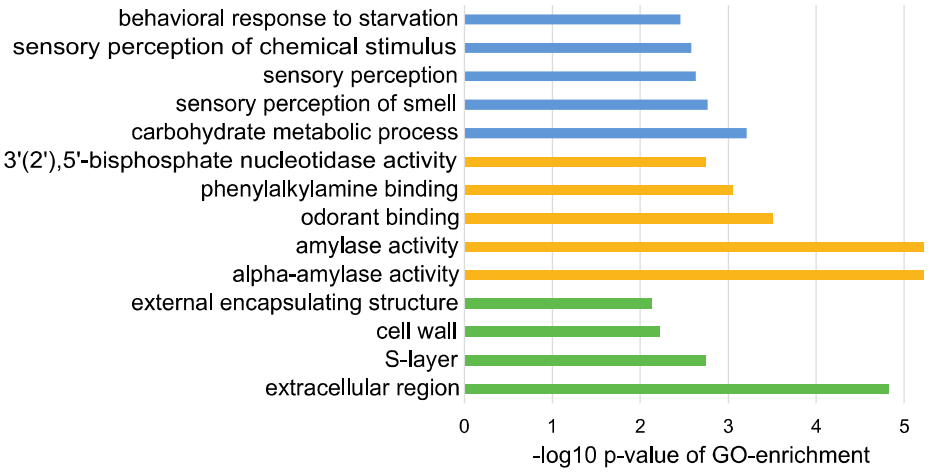
A


protein folding
regulation of translation in response to stress
reg. of translational initiation in response to stress
reg. of transl. initiation by eIF2 alpha phosphorylation
protein refolding
enhancer binding
structural constituent of eye lens
protein dimerization activity
protein heterodimerization activity
unfolded protein binding
extracellular region
contractile fiber part
Z disc
protein-DNA complex
DNA packaging complex
DNA bending complex
nucleosome
0
2
4
6
8
10
-log10 p-value of GO-enrichment

B

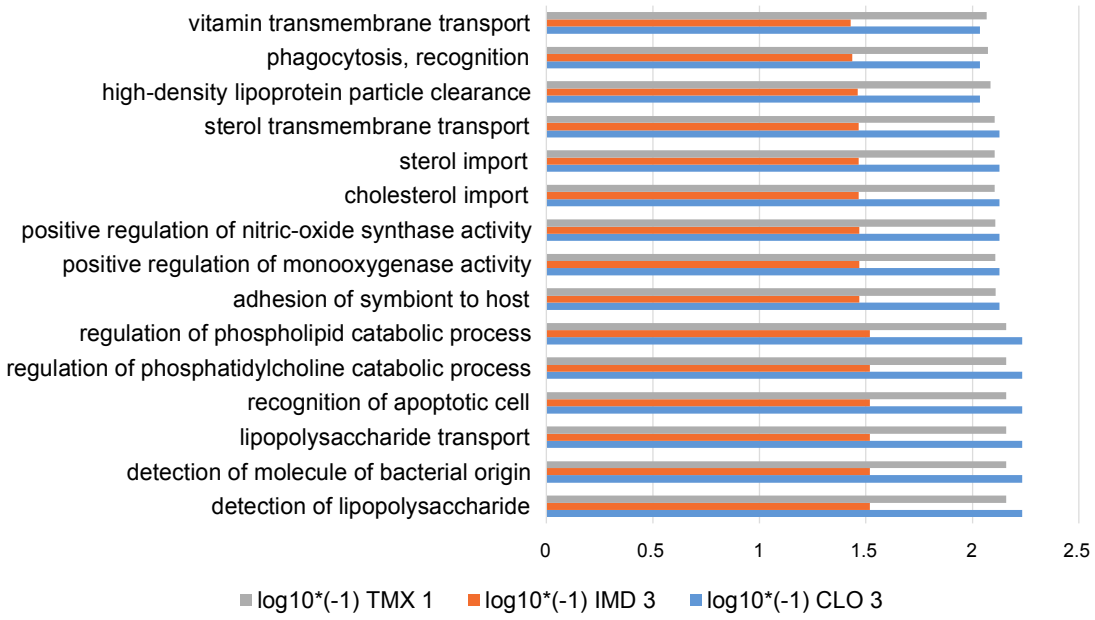

regulation of glutamate receptor activity
inal disc-derived wing vein specification
odorant binding
hydrolase activity
phenylalkylamine binding
amylase activity
alpha-amylase activity
postsynaptic density membrane
type III terminal bouton
0
0.5
1
1.5
2
2.5
3
3.5
4
-log10 p-value of GO-enrichment

C


behavioral response to starvation
sensory perception of chemical stimulus
sensory perception
sensory perception of smell
carbohydrate metabolic process
3'(2'),5'-bisphosphate nucleotidase activity
phenylalkylamine binding
odorant binding
amylase activity
alpha-amylase activity
external encapsulating structure
cell wall
S-layer
extracellular region
0
1
2
3
4
5
-log10 p-value of GO-enrichment

735

736 Figure 3



737

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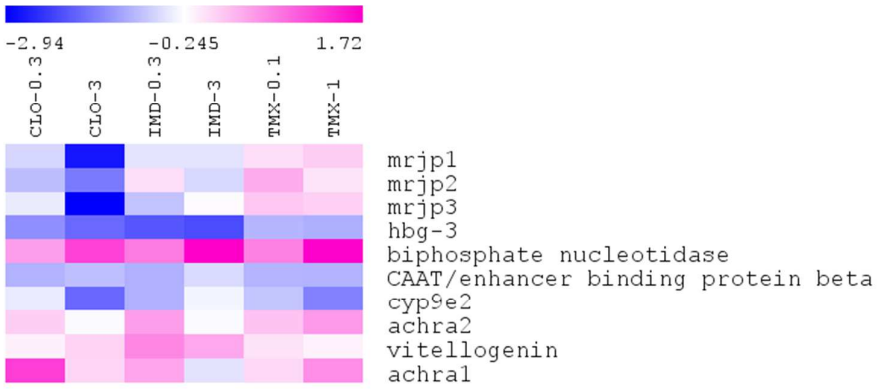
739 Figure 4

740 A:



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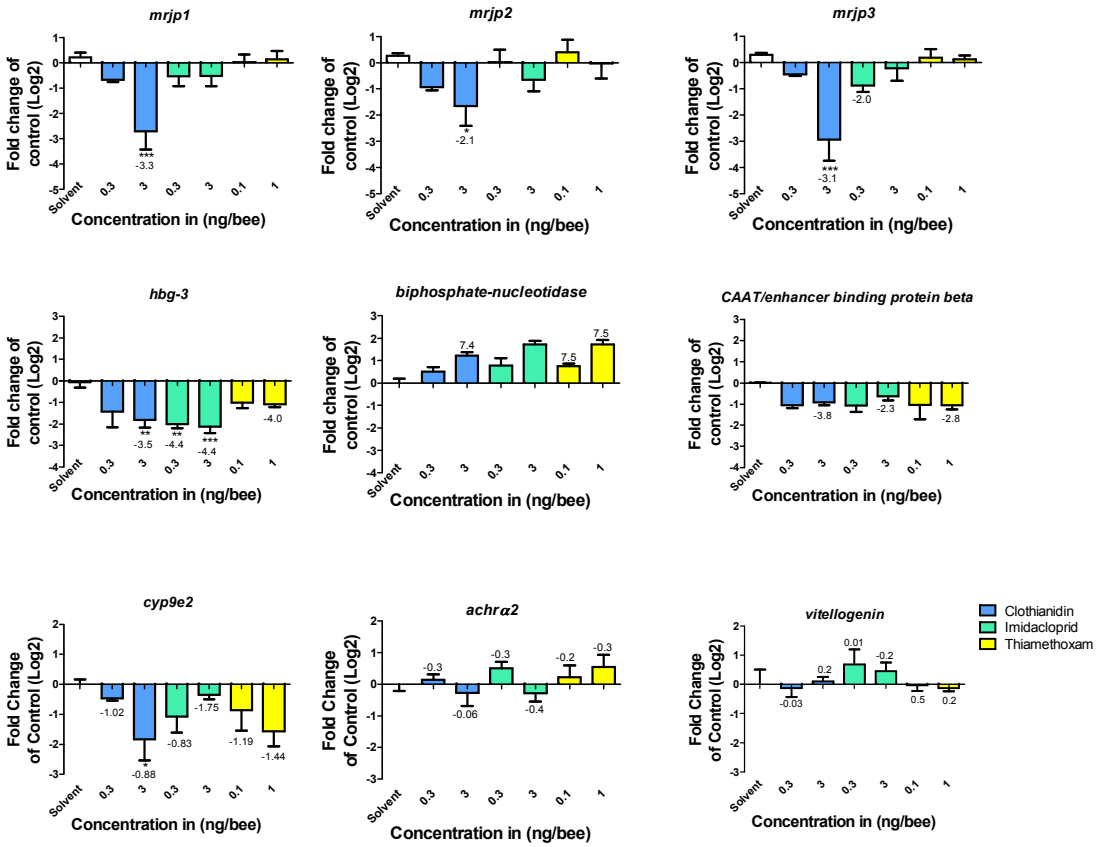
743 B:



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