Caste- and pesticide-specific effects of neonicotinoid pesticide exposure on gene expression in bumblebees

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Abstract

Social bees are important insect pollinators of wildflowers and agricultural crops, making their reported declines a global concern. A major factor implicated in these declines is the widespread use of neonicotinoid pesticides. Indeed, recent research has demonstrated that exposure to low doses of these neurotoxic pesticides impairs bee behaviors important for colony function and survival. However, our understanding of the molecular-genetic pathways that lead to such effects is limited, as is our knowledge of how effects may differ between colony members.

To understand what genes and pathways are affected by exposure of bumblebee workers and queens to neonicotinoid pesticides, we implemented a transcriptome-wide gene expression study. We chronically exposed *Bombus terrestris* colonies to either clothianidin or imidacloprid at field-realistic concentrations while controlling for factors including colony social environment and worker age. We reveal that genes involved in important biological processes including mitochondrial function are differentially expressed in response to neonicotinoid exposure. Additionally, clothianidin exposure had stronger effects on gene expression amplitude and alternative splicing than imidacloprid. Finally, exposure affected workers more strongly than queens. Our work demonstrates how RNA-Seq transcriptome profiling can provide detailed novel insight on the mechanisms mediating pesticide toxicity to a key insect pollinator.

Keywords: ecotoxicology, molecular diagnostics, neonicotinoid insecticides, nicotinic acetylcholine receptors, pollinator health, xenobiotics.

Introduction

Social bees are important pollinators crucial for maintaining biodiversity and ecosystem stability (Waser *et al.* 1996; Garibaldi *et al.* 2013). More than 85% of flowering plant species across the globe rely to some degree on animal pollination (Ollerton *et al.* 2011), and the agricultural industry values this pollination service at over €150 bn (Klein *et al.* 2007; Gallai *et al.* 2009; Garibaldi *et al.* 2014). Reported insect pollinator declines are thus of worldwide concern (Aizen & Harder 2009; Goulson *et al.* 2015; Gill *et al.* 2016; Potts *et al.* 2016). Factors implicated as contributors to such declines include habitat loss, climate change, pathogens and in particular agricultural intensification (Brown & Paxton 2009; Vanbergen 2013; Goulson *et al.* 2015). Indeed, agricultural intensification has led to the increased usage of pesticides which can have unintended effects on social bees (Desneux *et al.* 2007), with neonicotinoid pesticides having received particular attention (Henry *et al.* 2012; Whitehorn *et al.* 2012; Gill *et al.* 2012; Goulson 2013; Simon-Delso *et al.* 2015).

Neonicotinoids are a popular class of neuroactive insecticides as they efficiently kill insect pests while having significantly lower toxicity to vertebrates (Matsuda *et al.* 2001; Jeschke *et al.* 2011). Furthermore, these insecticides are systemic: they are readily absorbed by plants and translocated to all tissues (Elbert *et al.* 2008). A consequence of this, however, is that traces of neonicotinoids are detectable in the pollen and nectar of treated and contaminated flowering plants (Long & Krupke 2016; David *et al.* 2016) that bees feed on (Rortais *et al.* 2005). Neonicotinoids target nicotinic acetylcholine receptors (nAChRs) which they bind to and thus excite; this can result in paralysis, convulsions, and death (Matsuda *et al.* 2001). Controlled exposure experiments using honeybees and bumblebees have shown that exposure at comparable concentrations to those found in nectar and pollen can have sublethal effects on learning and memory (Stanley *et al.* 2015; Siviter *et al.* 2018), cognition and problem solving

(Williamson and Wright 2013; Samuelson et al. 2016; Baracchi et al. 2017), motor function (Williamson *et al.* 2014; Drummond *et al.* 2016), foraging performance (Henry *et al.* 2012; Gill & Raine 2014; Stanley *et al.* 2016), navigation abilities (Fischer *et al.* 2014) and the immune system (Di Prisco *et al.* 2013; Brandt *et al.* 2016, 2017). Despite the growing interest in the link between neonicotinoid exposure and toxicity to bees, we know little about the molecules and genes through which neonicotinoid action is mediated, or whether neonicotinoids may also affect "off-target" processes that are not mediated by nAChRs.

An additional consideration is that neonicotinoids differ in manners that are only beginning to be characterized. Clothianidin and imidacloprid differentially affect distinct subpopulations of Kenyon cells cultured from bumblebee brains, suggesting that pathways by which they act differ (Moffat *et al.* 2016). In line with this, genome-wide transcriptome profiling (RNA-Seq) of honeybee brains showed differences between pesticides, with clothianidin exposure resulting in greater transcriptional changes than imidacloprid or thiamethoxam, including for metabolic and detoxification genes (Christen *et al.* 2018). Similarly to its use for diagnosing and classifying human diseases (Byron *et al.* 2016), RNA-Seq can provide a holistic view of how pesticides affect genes underlying important processes, while also providing candidate genes for future functional studies.

Here, we aim to understand the impacts of neonicotinoid exposure on the bumblebee *Bombus terrestris,* a common wild Eurasian pollinator and the second-most economically important bee pollinator species worldwide, using transcriptome profiling. Using a tightly controlled experimental design, we provided whole colonies with untreated food, or with food treated with one of two common neonicotinoids, clothianidin and imidacloprid. We performed RNA-Seq gene expression profiling on heads of age-controlled worker bumblebees in addition to colony queens, from colonies kept under controlled environmental conditions. The head is likely the key center

for mediation of the detrimental effects of neonicotinoids on behavior and cognition because it contains important organs and tissues of the insect nervous system; in particular the brain, which contains an abundance of Kenyon cells, the neuronal cell type that neonicotinoids predominantly target within social bees (Palmer *et al.* 2013; Moffat *et al.* 2016). We exposed colonies for four days, a chronic exposure period after which neonicotinoid residues have previously been detected within the brains of exposed bumblebee workers (Moffat *et al.* 2015). We addressed the following questions: 1) Does neonicotinoid exposure lead to transcriptional changes in the head tissues of exposed bumblebees? 2) Do different neonicotinoids lead to different gene expression profiles? 3) Do workers and queens differ in their transcriptional response to neonicotinoids? Our work reveals pesticide- and caste-specific effects on gene expression amplitude and splicing, providing detailed novel insight on the mechanisms mediating pesticide toxicity to bumblebees.

Materials and Methods

Controlling colony size and worker age during colony rearing

We obtained 12 *Bombus terrestris audax* colonies containing a median of 56 workers (mean: 51.0; Standard Error (SE): 6.62, range: 15-93) from a commercial supplier (Agralan, UK). Each colony was randomly assigned to one of two identical controlled environment rooms maintained at 20°C and 60% humidity under constant red light illumination. Each colony was provided with *ad libitum* sucrose solution (40% w/w prepared using distilled water) and honeybee-collected pollen (Agralan, UK) three times per week (Monday 2 g, Wednesday 2 g, Friday 3 g). It is relevant to note that this pollen lacks an organic certification, thus it may contain trace amounts of xenobiotics, such as neonicotinoids or other insecticides. Therefore, we consider our experimental colonies to have been exposed to higher doses of the two pesticides in comparison to our control colonies.

Six days (144 hours) before starting the experimental treatment, we removed and tagged up to four newly eclosed workers per colony with a numbered Opalith tag (Abelo, UK). Once tagged, we placed them back into the colony. We also standardized the size of each colony by removing workers so that each colony contained the colony queen and a median of 20 workers (mean: 19.7; SE: 0.41; range: 15-21). For this, we marked each untagged worker in the colony with a white, non-toxic pen (Uniball Uni Posca). This enabled subsequent differentiation between old workers and newly eclosed workers. To maintain the number of workers in the colony constant, we removed marked (*i.e.*, older) workers when unmarked (*i.e.,* younger) workers eclosed, and immediately marked the new workers with the white pen.

Preparation of sucrose solutions containing neonicotinoid pesticides

We prepared stock solutions of each pesticide by dissolving either analytical grade clothianidin or imidacloprid (Sigma Aldrich, UK) in acetone to a concentration of 1.0×10^{-3} g/ml. We serially diluted the stock solution using 40% sucrose solution to produce a 1.0×10^{-6} g/ml working solution, which was stored in the dark at 4ºC for a maximum of four days. The working solution was then further diluted with 40% sucrose solution to produce a final concentration of 7.5×10^{-9} g/ml. We prepared solutions no more than 1 hour before providing them to the bumblebee colonies. As the mass of 1 liter of 40% sucrose is 1,160 g and contained 7.5 × 10-6 g of pesticide^{-6} g of pesticide, each sucrose solution contained 6.47 parts per billion (ppb) of pesticide, which is within the range that bees are considered to be exposed to within the field (Supp. Table S1).

Exposure of colonies to neonicotinoid-laced sucrose

We randomly assigned each colony to one of the three treatment groups: control (n = 4), clothianidin (n = 4) or imidacloprid (n = 4). For the purpose of measuring changes in worker gene

144 expression in response to neonicotinoid exposure, we only used workers age-controlled to 10
145 days post-eclosion (Supp. Fig. S1). At the start of day six, we removed the initial sucrose
146 feeders and any remaining pollen. We provided each colony with its allocated treatment and 2 g
147 pollen; we replaced the food of each colony after 24 and 48 hours, and we ended the
148 experiment after 96 hours of exposure. At the end of the experiment, we transferred the 10-day
149 old Opalith tagged workers and the colony queen into individual 2 ml and 5 ml Eppendorf tubes,
150 respectively, snap froze them in liquid nitrogen and then stored the tubes at -80°C.

151 ***RNA extractions, library preparations and high throughput sequencing***

152 We extracted RNA from the colony queen and from one worker per colony from 12 colonies
153 (n = 24 individuals). For this, we removed bumblebee-containing cryotubes from -80°C storage
154 and kept them on dry ice. Using sterilized forceps, we transferred each bumblebee from the
155 housing cryotube onto a sterilized 5 ml petri dish that had been chilled on ice. Using a new
156 sterile blade for each sample, we removed the head and transferred it into a new 2 ml
157 homogenization tube containing 150 µl of Tri reagent (Sigma, UK). The contents of each tube
158 were then frozen on dry ice and returned to -80°C storage. For total RNA extraction, each
159 individual sample was removed from storage and kept on ice. To each tube, we added 0.2 g
160 zirconium silicate (ZS) beads (Sigmund Lindner GmbH, Germany) and homogenized each
161 sample using a FastPrep-96 high throughput homogenizer using two cycles of 45 seconds at
162 1200 rpm. After homogenization, each sample was visually examined to ensure thorough
163 sample disruption. We added 850 µl of Tri reagent to each tube and incubated at room
164 temperature for 5 minutes to allow for complete dissociation of nucleoprotein complexes. We
165 isolated total RNA using chloroform following the manufacturer's recommendations. We
166 precipitated total RNA using isopropanol and performed a wash using molecular-grade ethanol.
167 To remove potential phenol and ethanol contamination, we further purified the extracted RNA for

each individual using the RNeasy MiniPrep kit (Qiagen, UK). Finally, we removed residual DNA using RNase-free DNase I (Qiagen, UK). We quantified total RNA using a Qubit RNA Broad-Range (BR) Assay kit (Invitrogen, UK).

We prepared sequencing libraries (n = 24) using the Illumina TruSeq stranded mRNA library preparation kit. For each library, we used a starting concentration of 1.5 µg of total RNA. We purified each library using AMPure XL beads (Beckman Coulter, UK) and quantified library size using a TapeStation 2200 (Agilent, UK). Using equal concentrations of each library, we created a single pooled library. We sequenced the pooled library on Illumina NextSeq 500 and HiSeq 4000 generating ~129.72 million reads of 76bp and ~314.6 million reads of 50bp. We thus obtained a mean of 18.51 million reads per sample (min: 9.84 million; max: 23.89 million reads per sample) (Supp. Table S2).

Quality assessment of Illumina RNA-Seq reads

We assessed the quality of raw reads using two primary measures. First, we initially assessed sequence quality using FastQC (v.0.11.3; Andrews 2010) to identify potential adapter contamination and base qualities. Subsequently, we aligned raw reads against the *Bombus terrestris* reference genome assembly (GCF_000214255.1; Sadd *et al.* 2015) using HISAT2 (v.2.1.0; Kim *et al.* 2015). We calculated mapping statistics for the resulting alignment files using qualimap (v.2.2.1; García-Alcalde *et al.* 2012) and visualized the output summaries using multiQC (v.0.7; Ewels *et al.* 2016). A summary of raw sequence quality and alignment statistics is provided in Supp. File S1. For each sample, >88% of reads mapped uniquely to the *B. terrestris* genome; all RNA-Seq libraries were of high quality and retained for analysis.

Identifying pesticide exposure effects on gene expression amplitude

We quantified transcript abundance for each sample by pseudoaligning reads (kallisto; v.0.44.1; Bray *et al.* 2016; run parameters: --single -l 300 -s 20) to predicted transcripts from the *B. terrestris* genome (Ensembl release v.40). To facilitate reanalysis of these data, we provide raw estimated counts for all samples in Supp. Table S3. Estimated counts were summarised per gene using tximport (v.1.6.0; with countsFromAbundance = "no"; Soneson *et al.* 2015) and imported into DESeq2 (v.1.14.1; Love *et al.* 2014). We created a DESeq2 object containing the entire dataset. We used DESeq2 Wald tests to identify genes that were differentially expressed between each pesticide treatment and the control colonies (Benjamini-Hochberg adjusted $p < 0.05$). As an additional measure of confidence, repeated our analyses using gene-level counts generated by the HISAT2-HTSeq pipeline as input to DESeq2. We find broad overlap between the two analyses, thus strengthening our results. Indeed, overall trends are identical, and we find very high overlap in the genes and processes identified. More detailed information on the comparison between analyses are provided in the Supplemental Information.

Deleted: we identified >80% of these statistically significant genes to be also identified as statistically significant by with DESeq2 when

Deleted: . Similar to the kallisto approach, the use of HISAT2-based actual gene-level counts by DESeq2 identified caste- and pesticide-specific changes in bumblebee gene expression. Using the HISAT2-based approach, we identified greater amplitude changes in expression in workers in comparison to queens. In addition, for both castes, clothianidin exposure resulted in greater gene expression changes than imidacloprid. Additional

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Identifying pesticide exposure effects on alternative splicing

We aligned raw reads against the *B. terrestris* genome (Ensembl release v.40) using the splice aware aligner HISAT2 (v.2.1.0; Kim *et al.* 2015) and obtained read counts for each exon using HTSeq (v.0.9.1; with --stranded = “reverse”; Anders *et al.* 2015). To facilitate reanalysis of these data we provide exon-level counts in Supp. Table S4. We created DEXSeq objects and analyzed differential exon usage for each pesticide treatment in comparison to control individuals using DEXSeq informed by the Ensembl GTF file (v.1.20.2; Reyes *et al.* 2013).

Gene Ontology enrichment analysis

For each gene, we identified the *Drosophila melanogaster* ortholog from Ensembl Metazoa Biomart (Kinsella *et al.* 2011) and used its Gene Ontology (GO) annotations because little

functional information exists for *B. terrestris*. To test whether any Gene Ontology terms were overrepresented among the most highly differentially expressed genes in response to pesticide exposure, we sorted all *B. terrestris* genes by raw *p*-value (because of edge effects associated with adjusted *p*-values) and performed a rank-based test for each GO term. For this, we used Kolmogorov-Smirnov tests in topGO (v.2.26.0; with the "weight01" algorithm and nodeSize = 100; Alexa 2016).

Results

Clothianidin exposure leads to differential gene expression in worker and in queen bumblebees

We determined that 55 genes are significantly differentially expressed in workers in response to clothianidin exposure compared to workers fed on the control diet (Benjamini Hochberg (BH) adjusted $p < 0.05$, Fig. 1(a); Supp. Table S5). Among these genes, 31 (62%) were more highly expressed after exposure; this pattern was non-significant (binomial test $p = 0.4$). Several of the differentially expressed genes are involved in key biological processes, and orthologs to some of the genes have been shown to be differentially expressed in other species exposed to pesticides (see Discussion). In particular, three of the 55 genes identified were among the 244 genes differentially expressed in the brains of honeybee workers exposed to clothianidin (Christen *et al.* 2018), suggesting that certain similar biological processes may be affected across species. Two of these genes, *mab-21* (LOC100646781), a putative developmental gene, and *proton-coupled amino acid transporter-like protein pathetic* (LOC100643972), a putative solute transporter gene, had reduced expression in response to exposure in both experiments. Intriguingly, however, *glucose dehydrogenase* (LOC100648192) was more highly expressed in response to clothianidin in our bumblebees but had reduced expression after exposure in

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honeybees (Christen *et al.* 2018), also indicating that a single pesticide can have opposing effects on different species.

We also investigated whether clothianidin exposure affected expression profiles of colony queens. Seventeen genes were significantly differentially expressed (BH adjusted $p < 0.05$, Fig. 1(b); Supp. Table S5), and unlike in workers, we found a strong pattern of increased expression: only one of these genes had lower expression after exposure (binomial test $p < 10^{-3}$). Among the more highly expressed genes in the clothianidin-exposed queens were genes coding for a putative neurohormone receptor, *tachykinin-like peptides receptor 86C* (LOC100647109), a developmental gene, *protein yellow* (LOC100647223) and two putative odorant binding proteins (LOC100643514; LOC100650345).

Strikingly there was almost no overlap between the lists of genes differentially expressed in the two castes, suggesting that the phenotypic effects and susceptibility to exposure differs between castes. The one gene that was differentially expressed in both castes in response to clothianidin is LOC100650345, which contains an odorant binding protein A10 domain (IPR005055), suggesting it may play a role in the transport or perception of semiochemicals.

Clothianidin exposure leads to alternative splicing in worker and in queen bumblebees

Clothianidin exposure resulted in the significant alternative splicing of 45 genes in exposed workers (BH adjusted $p < 0.05$, Supp. Fig. S2). Two genes (LOC100646880; LOC100651821) were both differentially expressed and alternatively spliced after clothianidin exposure (Fig. 1(a)). By comparison, in queens, we identified no evidence of alternative splicing in response to clothianidin exposure.

Imidacloprid exposure induced weaker transcriptional changes than clothianidin

We also investigated changes in gene expression in response to imidacloprid exposure. Intriguingly, we found no differences in gene expression amplitude between exposed and control queens, but eight genes were alternatively spliced. Thus in queens imidacloprid exposure affects half as many genes as clothianidin and through a different mechanism.

In workers, only one gene was differentially expressed (LOC100644101), and a single different gene was alternatively spliced in response to imidacloprid exposure (LOC100649110). Interestingly, these two genes had similar expression patterns in terms of differential expression and alternative splicing, respectively, in clothianidin-exposed workers, highlighting a potential generic molecular response to neonicotinoid exposure in *B. terrestris* workers. LOC100644101 codes for a protein with homologs throughout Hymenoptera but no known functional domains. In comparison, LOC100649110 codes for a protein with a predicted cytochrome P450, E-class, group 1 functional domain (IPR002401), suggestive of a role in the metabolism of exogenous substances or endogenous physiologically-active compounds.

Discussion

Understanding the sublethal effects of pesticide exposure on beneficial organisms such as insect pollinators is important in order to assess the risks posed by pesticides. Focusing on the molecular-genetic level, we carried out genome-wide mRNA-sequencing of the heads of bumblebees chronically exposed for four days to one of two widely used neonicotinoid insecticides, clothianidin and imidacloprid. We reveal three major novel trends: i) head tissues of bumblebee workers and queens exhibit significant changes in gene expression amplitude and alternative splicing due to clothianidin or imidacloprid exposure; ii) clothianidin had stronger effects than imidacloprid on gene expression; iii) the worker and queen castes intriguingly

295 differed in their response to neonicotinoid exposure, with both neonicotinoids leading to greater
296 transcriptional changes in workers than in queens. Our results provide high-resolution insight
297 into the molecular-genetic pathways by which neonicotinoids affect colony members. Some of
298 these effects likely occur downstream of the nACh receptors that neonicotinoids target.
299 However, some of the effects we see could be due to interactions between the pesticide and
300 "off-target" receptors or other cellular components within the head or other body parts of the
301 exposed bumblebees.

302 We are wary of providing detailed potential interpretations regarding individual genes or
303 pathways seen in a single study because most of what we know about bumblebee genes is
304 bioinformatically inferred rather than being demonstrated experimentally. However, clothianidin
305 and imidacloprid have been observed to cause mitochondrial depolarization in Kenyon cells of
306 social bee brains (Moffat *et al.* 2015, 2016). Differentially expressed genes associated with
307 mitochondrial function such as *alanine-glyoxylate aminotransferase* and *phosphoenolpyruvate*
308 *carboxykinase* are thus strong candidate genes mediating such effects. The second of these
309 genes also has increased expression in imidacloprid-exposed honeybee larvae (Derecka *et al.*
310 2013) and in dichlorodiphenyltrichloroethane (DDT)-exposed *Drosophila melanogaster* (King-
311 Jones *et al.* 2006), suggesting a general mechanism of response to toxins across taxa. Due to
312 the role of *phosphoenolpyruvate carboxykinase* in glycolysis and gluconeogenesis pathways,
313 differential expression of this gene has been suggested to be associated with changes in energy
314 use in response to a xenobiotic challenge (King-Jones *et al.* 2006), as well as starvation (Zinke
315 *et al.* 1999). Gene Ontology terms associated with carbohydrate and lipid metabolism were also
316 enriched in clothianidin-exposed workers and queens (Supp. Fig. S3; Supp. Table S6)
317 suggesting potential changes in energy usage.

Clothianidin and imidacloprid belong to the chemical group of N-nitroguanidines (Jeschke & Nauen 2008), and within species, the two pesticides are generally thought to have similar toxicities based on toxicity values such as LD_{50} in honeybee (Iwasa *et al.* 2004; Brandt *et al.* 2016) and the western chinch bug (Stamm *et al.* 2011). However, some studies report higher lethality of clothianidin than imidacloprid in the honeybee *Apis mellifera* (Laurino *et al.* 2013), the bumblebee *B. impatiens*, the alfalfa leafcutter bee *Megachile rotundata* and in the orchard mason bee *Osmia lignaria* (Scott-Dupree *et al.* 2009). Furthermore, clothianidin has been shown to depolarises bumblebee neural mitochondria more rapidly than imidacloprid (Moffat *et al.* 2015). Our study found that chronic clothianidin exposure affected gene expression much more strongly than imidacloprid. This further mirrors findings on the honeybee brains (Christen *et al.* 2018) and overall suggests that clothianidin indeed has stronger transcriptional effects than imidacloprid. There may be technical and biological reasons for why we found relatively few effects of imidacloprid. We used a low concentration (6.47 ppb) of both pesticides, considered to be within range foraging bees are exposed to in the field (Supp. Table S1), rather than the high doses often used to demonstrate strong effects. It is also possible that the sample size we used lacked the power to detect subtle, but potentially important effects, of imidacloprid on gene expression. Furthermore, we examined gene expression at a single time-point after four days of chronic exposure yet the chronic effects of exposure may differ between pesticides. Indeed, at an extreme level, the phenylpyrazole insecticide fipronil accumulates within honeybees, leading to strong effects over time (Holder *et al.* 2018). The study of the effects of long-term exposure of different pesticide classes on bumblebees, as well as associated gene expression, is required. Finally, it is plausible that the different neonicotinoids have disproportionate effects on gene expression on different sets or subsets of neurons. Detecting such particularly localized effects can be challenging because we obtained for each gene the average expression across all of the cells in the entire head.

A key trait of social bees such as honeybees and most bumblebees is that colonies include a queen and workers that differ in morphology and physiology and have complementary behaviors essential for colony fitness. Thus castes may differ in how they are affected by pesticide exposure. Our genome-wide transcriptome RNA-Seq profiling approach found that only one gene, LOC100650345, was differentially expressed in both workers and queens. We know little about this gene other than it has been observed to be expressed in queen hemolymph (Sadd *et al.* 2015), and carries an odorant binding domain, suggesting that it may play a role in the transportation of semiochemicals such as odors and pheromones in the hemolymph. Its role may be conserved as a homologous gene in the whitefly *Bemisia tabaci* is also upregulated after exposure to the neonicotinoid thiamethoxam (Liu *et al.* 2014, 2016). Several general functions (Gene Ontology terms) were shared by workers and queens, including oxidation-reduction process, glucose metabolic process and single-organism catabolic process (Supp. Fig. S3). However, there were also marked differences. In queens, differentially expressed genes included functions related to the determination of lifespan, lipid metabolic process and ion transport, while genes affected in workers included genes involved in regulation of developmental growth, neuron projection guidance and regulation of the Notch signaling pathway (Supp. Fig. S3). A previous study reported that expression of cytochrome P450 genes, a family of genes typically involved in chemical detoxification, is affected by imidacloprid in honeybees (Chaimanee *et al.* 2016). In line with this, two cytochrome P450 CYP9Q subfamily genes in bumblebees metabolize the neonicotinoid thiacloprid but not imidacloprid (Manjon *et al.* 2018). Intriguingly, we found no effect of neonicotinoid exposure on either of these genes, suggesting that the genes are also unable to metabolize clothianidin, or that they function on different timescales or tissues than our study focused on. However, three other putative cytochrome P450 genes, LOC100652170, LOC100649441 and LOC100648391, respectively members of the CYP4, CYP6 and CYP9 subfamilies, were differentially expressed after

368 clothianidin exposure (Fig. 1), while one CYP6 family member (LOC100649110) was
369 alternatively spliced in workers in response to both neonicotinoids, thus providing additional
370 candidates for future work investigating the defense of bees against neonicotinoid pesticides.
371 Members of these families have higher expression within the hypopharyngeal and mandibular
372 glands of honeybee foragers in comparison to nurses suggestive of a role in the metabolism of
373 xenobiotic and phytochemicals that foragers are exposed to during natural foraging trips
374 (Vannette *et al.* 2015).

375 Multiple biological and technical reasons could explain differences between castes. First,
376 workers forage for food, care for brood, and build, maintain and defend the nest while the queen
377 lays batches of eggs daily. Additionally, queens live up to a year while *B. terrestris* workers live
378 two months on average (Alford 1975). Their behaviors and physiologies thus fundamentally
379 differ, and selection will over time have shaped response thresholds to external challenges in
380 caste-specific manners. Second, it is plausible that exposure differed between castes. Our study
381 was designed to prevent artefactual expression differences due to variation in colony size or the
382 absence of the queen: we maintained entire colonies. This did compromise, however, being able
383 to precisely control neonicotinoid dosage. Potential variation in exposure could come from
384 differences in feeding behaviors between and within castes, such as feeding directly from the
385 feeder or from nectar pots. Further sources of biological noise can come from inter-colony
386 variation. For example, colonies have baseline inherited differences in which alleles they carry,
387 in gene expression levels, in response thresholds for behaviors such as feeding rates, in
388 susceptibility to introduced compounds, and other biological differences. The effects of some
389 such differences are likely responsible for the variation in gene expression among the four
390 control colonies (Fig. 1). To account for such variation, future studies of ecologically relevant
391 organisms will benefit from strong replication at the appropriate (*e.g.,* colony) level. A final

source of biological noise comes from ages: we precisely controlled the ages of bumblebee workers to 10 days post-eclosion but were unable to determine the ages of queens because commercially supplied colonies come with no such information. An alternate explanation for finding differences between queens and workers may be technical, our study focuses on gene expression in heads. Indeed, heads include multiple tissues that differ in relative size between queens and workers (e.g., queens possess fully developed corpora allata while workers do not (Röseler & Röseler 1978)). Such allometric differences could affect our estimation of relative impacts on gene expression (Johnson *et al.* 2013). The use of alternative tissues, such as the digestive tract or malpighian tubules, key organs in xenobiotic metabolism, may provide additional insights into how castes respond to neonicotinoid exposure. Therefore, future expression studies will benefit from approaches targeting multiple specific tissues or cell types.

The majority of studies on the molecular effects of insecticides have focussed on the expression of their direct target sites, such as ligand- and voltage-gated ion channels, or on *a priori* candidate metabolic enzymes involved in detoxification of xenobiotic compounds. Whole transcriptome profiling studies such as ours have highlighted additional genes with altered expression in response to pesticide exposure. Some of the genes affected by clothianidin exposure in our study have also been affected by neonicotinoids or other pesticides in other studies and species. These include muscular genes such as *troponin* and *calponin* (Lewis *et al.* 2009; Wang *et al.* 2015; Kimura-Kuroda *et al.* 2016) and metabolic enzymes such as *glucose dehydrogenase* (Christen *et al.* 2018) and *hexosaminidase D* (Yang *et al.* 2008; Qi *et al.* 2018). At a different level, cellular transport genes such as the ABC transport family (Dermauw & Van Leeuwen 2014), one member of which was differentially expressed in our study, have been suggested to provide tolerance of neonicotinoids, such as imidacloprid, acetamiprid and thiacloprid, with greater mortality identified for neonicotinoid-exposed honeybee larvae treated

with an ABC inhibitor (Hawthorne & Dively 2011). Furthermore, G protein-coupled receptors, such as *tachykinin-like peptides receptor 86C*, which has increased expression in clothianidin-exposed queens in our study, have been identified as potential targets for the development of novel pesticides (Audsley & Down 2015). Further work will indicate to which extent the genes and pathways we have identified represent useful biomarkers of pesticide toxicity. Finally, we suggest that some of the other changes we identified in the expression of specific genes or pathways, such as genes under circadian control, may mediate phenotypic effects of pesticide exposure that remain to be fully characterized.

Conclusions

Our study represents an important step towards understanding the diversity of effects of chronic exposure to clothianidin and imidacloprid. In addition to identifying caste- and pesticide-specific effects, we provide lists of candidate genes for future research to improve our understanding of the impact of pesticides on bumblebee health. Our understanding of the significance of these genes and others will benefit from increased tissue profiling to identify tissue-specific responses, investigation of the effects of other pesticide compounds, and understanding of how effects of exposure change over time. Such detailed understanding can ultimately be helpful in classifying and quantifying the relative effects of pesticides on target pest species and beneficial species. Much like RNA-Seq has changed the way we diagnose and understand human disease (Byron *et al.* 2016), we thus expect it to become a valuable tool during the development as well as regulatory evaluation of novel pesticides.

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References

Alexa JR (2016) *topGO: Enrichment Analysis for Gene Ontology*.

Aizen MA, Harder LD (2009) The global stock of domesticated honey bees is growing slower than agricultural demand for pollination. *Current Biology: CB*, **19**, 915–918.

Alford DV (1975) *Bumblebees*. Davis-Poynter.

Anders S, Pyl PT, Huber W (2015) HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* , **31**, 166–169.

Andrews S (2010) *FastQC: a quality control tool for high throughput sequence data*.

Audsley N, Down RE (2015) G protein coupled receptors as targets for next generation pesticides. *Insect Biochemistry and Molecular Biology*, **67**, 27–37.

Baracchi D, Marples A, Jenkins AJ, Leitch AR, Chittka L (2017) Nicotine in floral nectar pharmacologically influences bumblebee learning of floral features. *Scientific Reports*, **7**, 1951.

Brandt A, Gorenflo A, Siede R, Meixner M, Büchler R (2016) The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.). *Journal of Insect Physiology*, **86**, 40–47.

Brandt A, Grikscheit K, Siede R *et al.* (2017) Immunosuppression in honeybee queens by the neonicotinoids thiacloprid and clothianidin. *Scientific Reports*, **7**, 4673.

Bray NL, Pimentel H, Melsted P, Pachter L (2016) Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology*, **34**, 525–527.

Brown MJF, Paxton RJ (2009) The conservation of bees: a global perspective. *Apidologie*, **40**, 410–416.

Byron SA, Van Keuren-Jensen KR, Engelthaler DM, Carpten JD, Craig DW (2016) Translating RNA sequencing into clinical diagnostics: opportunities and challenges. *Nature Reviews. Genetics*, **17**, 257–271.

Chaimanee V, Evans JD, Chen Y, Jackson C, Pettis JS (2016) Sperm viability and gene expression in honey bee queens (*Apis mellifera*) following exposure to the neonicotinoid insecticide imidacloprid and the organophosphate acaricide coumaphos. *Journal of Insect Physiology*, **89**, 1–8.

Christen V, Schirrmann M, Frey JE, Fent K (2018) Global transcriptomic effects of

environmentally relevant concentrations of the neonicotinoids clothianidin, imidacloprid, and thiamethoxam in the brain of honey bees (*Apis mellifera*). *Environmental Science & Technology*, **52**, 7534–7544.
David A, Botías C, Abdul-Sada A *et al.* (2016) Widespread contamination of wildflower and bee-collected pollen with complex mixtures of neonicotinoids and fungicides commonly applied to crops. *Environment International*, **88**, 169–178.
Derecka K, Blythe MJ, Malla S *et al.* (2013) Transient exposure to low levels of insecticide affects metabolic networks of honeybee larvae. *PloS One*, **8**, e68191.
Dermauw W, Van Leeuwen T (2014) The ABC gene family in arthropods: comparative genomics and role in insecticide transport and resistance. *Insect Biochemistry and Molecular Biology*, **45**, 89–110.
Desneux N, Decourtye A, Delpuech J-M (2007) The sublethal effects of pesticides on beneficial arthropods. *Annual Review of Entomology*, **52**, 81–106.
Di Prisco G, Cavaliere V, Annoscia D *et al.* (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees. *Proceedings of the National Academy of Sciences of the United States of America*, **110**, 18466–18471.
Drummond J, Williamson SM, Fitchett AE, Wright GA, Judge SJ (2016) Spontaneous honeybee behaviour is altered by persistent organic pollutants. *Ecotoxicology*, **26**, 141–150.
Elbert A, Haas M, Springer B, Thielert W, Nauen R (2008) Applied aspects of neonicotinoid uses in crop protection. *Pest Management Science*, **64**, 1099–1105.
Ewels P, Magnusson M, Lundin S, Käller M (2016) MultiQC: summarize analysis results for multiple tools and samples in a single report. *Bioinformatics*, **32**, 3047–3048.
Fischer J, Müller T, Spatz A-K *et al.* (2014) Neonicotinoids interfere with specific components of navigation in honeybees. *PloS One*, **9**, e91364.
Gallai N, Salles J-M, Settele J, Vaissière BE (2009) Economic valuation of the vulnerability of world agriculture confronted with pollinator decline. *Ecological economics: The Journal of the International Society for Ecological Economics*, **68**, 810–821.
García-Alcalde F, Okonechnikov K, Carbonell J *et al.* (2012) Qualimap: evaluating next-generation sequencing alignment data. *Bioinformatics*, **28**, 2678–2679.
Garibaldi LA, Carvalheiro LG, Leonhardt SD *et al.* (2014) From research to action: enhancing crop yield through wild pollinators. *Frontiers in Ecology and the Environment*, **12**, 439–447.
Garibaldi LA, Steffan-Dewenter I, Winfree R *et al.* (2013) Wild pollinators enhance fruit set of crops regardless of honey bee abundance. *Science*, **339**, 1608–1611.
Gill RJ, Baldock KCR, Brown MJF *et al.* (2016) Chapter Four - Protecting an ecosystem service: Approaches to understanding and mitigating threats to wild insect pollinators. In: *Advances in Ecological Research* (eds Woodward G, Bohan DA), pp. 135–206. Academic Press.
Gill RJ, Raine NE (2014) Chronic impairment of bumblebee natural foraging behaviour induced by sublethal pesticide exposure. *Functional Ecology*, **28**, 1459–1471.
Gill RJ, Ramos-Rodriguez O, Raine NE (2012) Combined pesticide exposure severely affects individual- and colony-level traits in bees. *Nature*, **491**, 105–108.
Goulson D (2013) Review: An overview of the environmental risks posed by neonicotinoid insecticides (D Kleijn, Ed,). *The Journal of Applied Ecology*, **50**, 977–987.
Goulson D, Nicholls E, Botías C, Rotheray EL (2015) Bee declines driven by combined stress from parasites, pesticides, and lack of flowers. *Science*, **347**, 1255957.
Hawthorne DJ, Dively GP (2011) Killing them with kindness? In-hive medications may inhibit xenobiotic efflux transporters and endanger honey bees. *PloS One*, **6**, e26796.
Henry M, Béguin M, Requier F *et al.* (2012) A common pesticide decreases foraging success and survival in honey bees. *Science*, **336**, 348–350.
Holder PJ, Jones A, Tyler CR, Cresswell JE (2018) Fipronil pesticide as a suspect in historical

mass mortalities of honey bees. *Proceedings of the National Academy of Sciences of the United States of America*, **115**, 13033–13038.
Iwasa T, Motoyama N, Ambrose JT, Roe RM (2004) Mechanism for the differential toxicity of neonicotinoid insecticides in the honey bee, *Apis mellifera*. *Crop Protection* , **23**, 371–378.
Jeschke P, Nauen R (2008) Neonicotinoids-from zero to hero in insecticide chemistry. *Pest Management Science*, **64**, 1084–1098.
Jeschke P, Nauen R, Schindler M, Elbert A (2011) Overview of the status and global strategy for neonicotinoids. *Journal of Agricultural and Food Chemistry*, **59**, 2897–2908.
Johnson BR, Atallah J, Plachetzki DC (2013) The importance of tissue specificity for RNA-seq: highlighting the errors of composite structure extractions. *BMC Genomics*, **14**, 586.
Kim D, Langmead B, Salzberg SL (2015) HISAT: a fast spliced aligner with low memory requirements. *Nature Methods*, **12**, 357–360.
Kimura-Kuroda J, Nishito Y, Yanagisawa H *et al.* (2016) Neonicotinoid insecticides alter the gene expression profile of neuron-enriched cultures from neonatal rat cerebellum. *International Journal of Environmental Research and Public Health*, **13**.
King-Jones K, Horner MA, Lam G, Thummel CS (2006) The DHR96 nuclear receptor regulates xenobiotic responses in *Drosophila*. *Cell Metabolism*, **4**, 37–48.
Kinsella RJ, Kähäri A, Haider S *et al.* (2011) Ensembl BioMarts: a hub for data retrieval across taxonomic space. *Database: the Journal of Biological Databases and Curation*, **2011**, bar030.
Klein A-M, Vaissière BE, Cane JH *et al.* (2007) Importance of pollinators in changing landscapes for world crops. *Proceedings. Biological sciences / The Royal Society*, **274**, 303–313.
Laurino D, Manino A, Patetta A, Porporato M (2013) Toxicity of neonicotinoid insecticides on different honey bee genotypes. *Bulletin of Insectology*, **66**, 119–126.
Lewis JA, Szilagyi M, Gehman E, Dennis WE, Jackson DA (2009) Distinct patterns of gene and protein expression elicited by organophosphorus pesticides in *Caenorhabditis elegans*. *BMC Genomics*, **10**, 202.
Liu G, Ma H, Xie H *et al.* (2016) Biotype characterization, developmental profiling, insecticide response and binding property of *Bemisia tabaci* chemosensory proteins: Role of CSP in insect defense. *PloS One*, **11**, e0154706.
Liu GX, Xuan N, Chu D *et al.* (2014) Biotype expression and insecticide response of *Bemisia tabaci* chemosensory protein-1. *Archives of Insect Biochemistry and Physiology*, **85**, 137–151.
Long EY, Krupke CH (2016) Non-cultivated plants present a season-long route of pesticide exposure for honey bees. *Nature Communications*, **7**, 11629.
Love MI, Huber W, Anders S (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology*, **15**, 550.
Manjon C, Troczka BJ, Zaworra M *et al.* (2018) Unravelling the molecular determinants of bee sensitivity to neonicotinoid insecticides. *Current Biology: CB*, **28**, 1137–1143.e5.
Matsuda K, Buckingham SD, Kleier D *et al.* (2001) Neonicotinoids: insecticides acting on insect nicotinic acetylcholine receptors. *Trends in Pharmacological Sciences*, **22**, 573–580.
Moffat C, Buckland ST, Samson AJ *et al.* (2016) Neonicotinoids target distinct nicotinic acetylcholine receptors and neurons, leading to differential risks to bumblebees. *Scientific Reports*, **6**, 24764.
Moffat C, Pacheco JG, Sharp S *et al.* (2015) Chronic exposure to neonicotinoids increases neuronal vulnerability to mitochondrial dysfunction in the bumblebee (*Bombus terrestris*). *FASEB journal: official publication of the Federation of American Societies for Experimental Biology*, **29**, 2112–2119.
Ollerton J, Winfree R, Tarrant S (2011) How many flowering plants are pollinated by animals?

576 *Oikos* , **120**, 321–326.
577 Palmer MJ, Moffat C, Saranzewa N *et al.* (2013) Cholinergic pesticides cause mushroom body
578 neuronal inactivation in honeybees. *Nature Communications*, **4**, 1634.
579 Potts SG, Imperatriz-Fonseca V, Ngo HT *et al.* (2016) Safeguarding pollinators and their values
580 to human well-being. *Nature*, **540**, 220–229.
581 Qi S, Wang D, Zhu L *et al.* (2018) Effects of a novel neonicotinoid insecticide cycloxaprid on
582 earthworm, *Eisenia fetida*. *Environmental Science and Pollution Research International*, **25**,
583 14138–14147.
584 Reyes A, Anders S, Weatheritt RJ *et al.* (2013) Drift and conservation of differential exon usage
585 across tissues in primate species. *Proceedings of the National Academy of Sciences of the*
586 *United States of America*, **110**, 15377–15382.
587 Rortais A, Arnold G, Halm M-P, Touffet-Briens F (2005) Modes of honeybees exposure to
588 systemic insecticides: estimated amounts of contaminated pollen and nectar consumed by
589 different categories of bees. *Apidologie*, **36**, 71–83.
590 Röseler P-F, Röseler I (1978) Studies on the regulation of the juvenile hormone titre in
591 bumblebee workers, *Bombus terrestris*. *Journal of Insect Physiology*, **24**, 707–713.
592 Sadd BM, Barribeau SM, Bloch G *et al.* (2015) The genomes of two key bumblebee species with
593 primitive eusocial organization. *Genome Biology*, **16**, 76.
594 Samuelson EEW, Chen-Wishart ZP, Gill RJ, Leadbeater E (2016) Effect of acute pesticide
595 exposure on bee spatial working memory using an analogue of the radial-arm maze.
596 *Scientific Reports*, **6**, 38957.
597 Scott-Dupree CD, Conroy L, Harris CR (2009) Impact of currently used or potentially useful
598 insecticides for canola agroecosystems on *Bombus impatiens* (Hymenoptera: Apidae),
599 *Megachile rotundata* (Hymentoptera: Megachilidae), and *Osmia lignaria* (Hymenoptera:
600 Megachilidae). *Journal of Economic Entomology*, **102**, 177–182.
601 Simon-Delso N, Amaral-Rogers V, Belzunces LP *et al.* (2015) Systemic insecticides
602 (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. *Environmental*
603 *Science and Pollution Research International*, **22**, 5–34.
604 Siviter H, Koricheva J, Brown MJF, Leadbeater E (2018) Quantifying the impact of pesticides on
605 learning and memory in bees. *The Journal of Applied Ecology*.
606 Soneson C, Love MI, Robinson MD (2015) Differential analyses for RNA-seq: transcript-level
607 estimates improve gene-level inferences. *F1000Research*, **4**, 1521.
608 Stamm MD, Baxendale FP, Heng-Moss TM *et al.* (2011) Dose-response relationships of
609 clothianidin, imidacloprid, and thiamethoxam to *Blissus occiduus* (Hemiptera: Blissidae).
610 *Journal of economic entomology*, **104**, 205–210.
611 Stanley DA, Russell AL, Morrison SJ, Rogers C, Raine NE (2016) Investigating the impacts of
612 field-realistic exposure to a neonicotinoid pesticide on bumblebee foraging, homing ability
613 and colony growth. *The Journal of Applied Ecology*, **53**, 1440–1449.
614 Stanley DA, Smith KE, Raine NE (2015) Bumblebee learning and memory is impaired by chronic
615 exposure to a neonicotinoid pesticide. *Scientific Reports*, **5**, 16508.
616 Vanbergen AJ (2013) Threats to an ecosystem service: pressures on pollinators. *Frontiers in*
617 *Ecology and the Environment*.
618 Vannette RL, Mohamed A, Johnson BR (2015) Forager bees (*Apis mellifera*) highly express
619 immune and detoxification genes in tissues associated with nectar processing. *Scientific*
620 *Reports*, **5**, 16224.
621 Wang W, Lv Y, Fang F *et al.* (2015) Identification of proteins associated with pyrethroid
622 resistance by iTRAQ-based quantitative proteomic analysis in *Culex pipiens pallens*.
623 *Parasites & Vectors*, **8**, 95.
624 Waser NM, Chittka L, Price MV, Williams NM, Ollerton J (1996) Generalization in pollination

systems, and why it matters. *Ecology*, **77**, 1043–1060.
Whitehorn PR, O'Connor S, Wackers FL, Goulson D (2012) Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science*, **336**, 351–352.
Williamson SM, Willis SJ, Wright GA (2014) Exposure to neonicotinoids influences the motor function of adult worker honeybees. *Ecotoxicology*, **23**, 1409–1418.
Williamson SM, Wright GA (2013) Exposure to multiple cholinergic pesticides impairs olfactory learning and memory in honeybees. *The Journal of Experimental Biology*, **216**, 1799–1807.
Yang Q, Liu T, Liu F, Qu M, Qian X (2008) A novel β-N-acetyl-d-hexosaminidase from the insect *Ostrinia furnacalis* (Guenée). *The FEBS journal*, **275**, 5690–5702.
Zinke I, Kirchner C, Chao LC, Tetzlaff MT, Pankratz MJ (1999) Suppression of food intake and growth by amino acids in *Drosophila*: the role of pumpless, a fat body expressed gene with homology to vertebrate glycine cleavage system. *Development*, **126**, 5275–5284.

Author contributions

This study is part of the postdoctoral research of TJC under the supervision of YW. RJG, LC and YW conceived the idea; TJC, ANA, RJG and YW designed the experiment; ANA and ARR performed the exposure experiments; TJC and ES generated the RNA-Seq libraries; TJC, IKF and YW analysed the data; TJC, IKF, ANA, RJG and YW wrote the manuscript. All authors edited the manuscript and gave final approval for publication.

Data accessibility

Raw sequence data files are deposited in the NCBI short read archive (Accession ID: PRJNA508397). Scripts underpinning the analysis of differential expression, differential exon usage and gene ontology term enrichment are archived on Github (https://github.com/wurmlab/Bter_neonicotinoid_exposure_experiment). Raw sequence counts for each sample are provided in the supplemental information.

Tables

Table 1. Summary table for differentially expressed genes within neonicotinoid-exposed bumblebees. For each treatment, the number of genes with differential amplitude and differential splicing per caste are shown.

Treatment	Caste	Genes with differential amplitude	Genes with differential splicing
Clothianidin	Workers	55	45
	Queens	17	0
Imidacloprid	Workers	1	1
	Queens	0	8

Figures

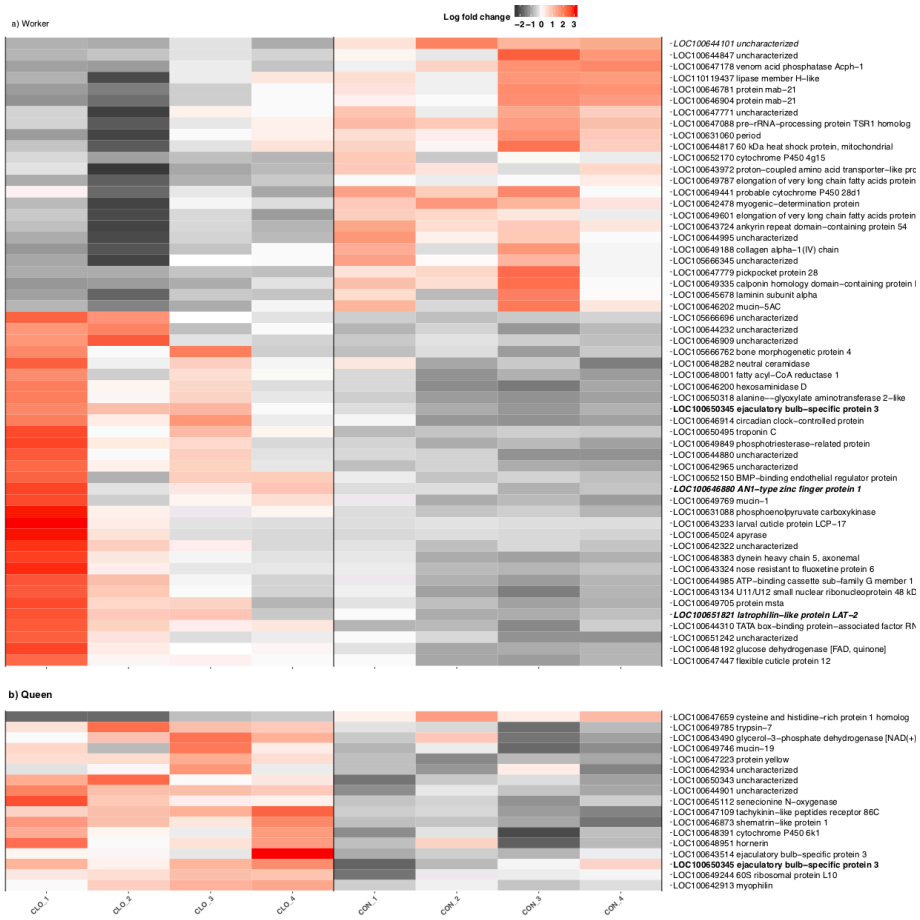

Fig. 1. Chronic clothianidin exposure leads to gene expression changes in bumblebee workers and queens. Heatmaps displaying genes differentially expressed in workers (A; n=55) and in queens (B; n=17) between clothianidin-exposed and control colonies. For each differentially expressed gene we show the log fold change for each biological replicate, as well as the gene identifier and NCBI's functional gene description. The single gene differentially expressed in both castes is indicated in bold. The single gene also differentially expressed in imidacloprid-exposed workers is indicated in italics. The two genes identified to be differentially expressed and alternatively spliced within clothianidin-exposed workers are indicated in bold and italics.