Gene expression in bumble bee larvae differs qualitatively between high and low concentration imidacloprid exposure levels

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Abstract

Neonicotinoid pesticides negatively affect important bumble bee traits, even at sublethal concentrations. Phenotypic responses to sublethal concentrations of the neonicotinoid imidacloprid have been studied, largely at individual adult and colony levels. Yet little is known about concentration-specific responses in developing larvae, particularly at the transcriptomic level. We hypothesize that relatively high (7.0 ppb) and low (0.7 ppb) field-realistic imidacloprid concentrations cause qualitative differences in gene expression of bumble bee larvae. To examine this hypothesis, worker-fed *Bombus impatiens* larvae were provisioned with either high or low imidacloprid treated pollen and sugar water, or untreated control food. Subsequent larval gene expression analysis revealed 678 genes differentially expressed in both the high and low imidacloprid larvae relative to controls, including genes involved in mitochondrial activity, development, and DNA replication. A set of 755 genes were differentially expressed only in the high imidacloprid concentration-exposed larvae, including starvation response and cuticle genes. Lastly, 191 genes were differentially expressed only in low imidacloprid concentration-exposed larvae, including genes associated with neural development and cell growth. Our findings show varying consequences of different neonicotinoid exposure concentrations within field-realistic ranges, and that even low concentrations affect the expression of genes that are fundamental to bumble bee health and development.

Introduction

The global decline of bumble bee populations has the potential to significantly reduce wild plant and agricultural crop pollination [1–3]. Multiple causes of these declines have been proposed [4,5], including exposure to pathogens [6–8] and pesticides [9–11]. In North America, populations of some bumble bee species (*Bombus* spp.) have declined precipitously since the late 1990s. This recent decline is linked to the co-occurrence of two potential causes: a significant increase in prevalence of the microsporidian pathogen *Nosema bombi* in declining bumble bee populations [6,12] and a major increase in the agricultural use of particular insecticides of concern, including neonicotinoids [13,14]. Teasing apart diverse stressors is necessary to understand causal factors of the relatively recent bumble bee declines in North America and threats to global bumble bee population health more broadly [4]. To do this, behavioral, physiological and molecular responses to stressors must be assessed at relevant life stages of development. We address this question by examining the effects of exposure to the neonicotinoid pesticide imidacloprid on both gene expression and feeding behavior in bumble bee larvae.

Neonicotinoid pesticides are used widely for agricultural pest control [15]. As systemic pesticides predominantly applied as seed coatings, neonicotinoids accumulate in all plant tissues during growth and development, including the nectar and pollen [16]. As a result, beneficial non-target insects such as pollinators can be exposed to their harmful effects [16]. Although field concentrations of some neonicotinoids, including imidacloprid, are usually lower than the lethal oral dose (LD_{50}) determined for bumble bees (20–40 ng per bee) [17], ongoing exposure to low concentrations (0.7–51 ppb [18]) cause

protracted sublethal effects [19–24]. Such effects include reduction of foraging efficiency [25,26], learning and short-term memory impairments [27], disruption of immune response [28], reduction of queen hibernation success [29], colony development [30–33] and reproduction [33]. Sublethal effects therefore have the potential to increase the risk of colony failure, particularly if additional sources of physiological stress, for instance infectious diseases, shortage of food or climatic conditions, occur concurrently with neonicotinoid exposure [30,34].

The majority of studies on the effects of neonicotinoid exposure on bumble bees have been performed at the colony, colony sub-section (microcolony) or individual adult levels. However, individual effects at the larval stage remain understudied, even though this stage of development may be particularly susceptible to pesticides, and the effects on larval development should be included in pesticide risk assessment studies [35]. In the solitary bee species *Osmia cornuta* and *O. bicornis*, larval exposure to the neonicotinoid thiacloprid increased developmental mortality and development time, and decreased pollen provision consumption and cocoon weight [36]. In addition to the effects that are apparent during larval development, exposure of larvae to neonicotinoids can have subsequent negative effects on adult bee traits, including morphology [37] and olfactory learning [38]. Similarly, larval exposure of *B. impatiens* to sublethal concentrations of Spinosad, a biopesticide that acts on the same receptor as neonicotinoids, negatively affected foraging efficiency in adults [39]. Analyzing the molecular responses of bumble bee larvae to different concentrations of neonicotinoids within field-realistic ranges is an important endeavor that will add to our understanding of the full range of effects that these pesticides can have on bumble bee health.

Gene expression (RNA-seq) studies have shown that exposure to sublethal neonicotinoid concentrations can affect gene expression in bees [40–45]. Molecular responses to exposure may vary depending on the context: for instance, research on *B. terrestris* found that clothianidin had a greater impact than imidacloprid on gene expression in head tissue, and the impact was greater in workers than in queens [42]. Another study of *B. terrestris* [40] documented major gene expression changes in the brains of imidacloprid exposed worker bees. Furthermore, the neonicotinoid clothianidin affected detoxification genes in a sex-specific manner in *B. impatiens* [45]. Comparing across these studies is difficult, because each of them used chronic exposures differing in both duration and dose. Additionally, each of these studies used a single sublethal concentration that is in the mid-high range of reported field-realistic concentrations (see Supplementary Table S1 for details). There are no comparative molecular data for bumble bees on the effects of neonicotinoids at the lower reported field-realistic concentrations, and it is important to include assessments of larval responses which are understudied. We hypothesize that different concentrations within sub-lethal field-realistic ranges of the neonicotinoid imidacloprid will differentially affect larval bumble bee gene expression. Exposing *B. impatiens* third instar larvae through food provisions, we use treatments of low (0.7 ppb) and relatively high (7.0 ppb) sublethal concentrations of imidacloprid to assess shared and concentration-specific gene expression responses in the larvae. These concentrations have been shown previously to have differential effects on adult bumble bee immunity [28]. We also

assess consumption of food provisions between the concentrations, as effects of pesticide exposure could result either from effects of direct exposure or indirect effects due to alterations of larval feeding by nursing workers or individual larval food consumption, as has been shown in *Apis* [37] and *Osmia* [36], respectively.

Materials And Methods

Bumble bee source colonies. With the permission of the ParkLands Foundation (http://www.parklandsfoundation.org), we collected wild queens of *B. impatiens* from the Mackinaw River watershed (Lexington, IL, USA) during spring 2018. Successfully reared colonies were maintained following the methods for colony rearing described in [46]. Briefly, the colonies were fed sugar water (50% inverted sucrose weight/volume) *ad libitum* and honey bee pollen (Brushy Mountain Bee Farms, Moravian Falls, NC, USA) three times per week. Following the establishment of microcolonies (explained below), the pollen provided was honey bee collected pollen from CC Pollen Co. (https://www.beepollen.com, Phoenix, AZ, USA), which is collected in high desert habitat away from agricultural or residential areas and is deemed pesticide-free [47].

Microcolony design and imidacloprid treatments. Four laboratory-reared *B. impatiens* colonies (C01, C02, C03 and C04) served as sources for the microcolonies. From each source colony, three microcolonies were established for each treatment: control, 0.7 ppb imidacloprid and 7.0 ppb imidacloprid (total number of microcolonies: n = 12). Each microcolony was housed in a plastic box (17 cm L x 12 cm W x 10 cm H) and comprised five workers and a brood clump with seven third-instar larvae (mean ± SE = 7.000 ± 0.103). Microcolonies were provisioned *ad libitum* with sugar water from of a 15 mL plastic centrifuge tube with holes in the base for feeding and pollen dough in a small petri dish. The third-instar developmental stage was determined visually by similarity of the relative size of larvae within a cluster, relative to references of other instars. Microcolonies were initially provisioned with untreated pollen and sugar water and allowed to acclimate for 48 h after their establishment. After acclimation, the microcolonies were given their respective imidacloprid-treated provisions (control, 0.7 ppb imidacloprid and 7.0 ppb imidacloprid; see the section below). 48h after imidacloprid treatment initiation, three third-instar larvae per microcolony were flash frozen in liquid nitrogen and stored at -80°C for later RNA-seq analysis. We chose a 48h imidacloprid exposure period because previous studies in bees have shown significant gene expression changes at this time in response to neonicotinoids [48] and environmentally relevant levels of other insecticides [49].

Imidacloprid concentrations and preparation. Imidacloprid was provided to microcolonies at 0.7 ppb (low) and 7.0 ppb (high) concentrations through provisioned sugar water and pollen dough. The concentrations were chosen based on reported concentrations that bumble bees are often exposed to in the field [28]. Concentrations up to 1,000 ppb have been detected in pollen and nectar [16], but levels between < 1 and 15 ppb are typical [20,21,50,51]. Imidacloprid (Millipore Sigma, 37894) stock solutions (10,000 ppb) were prepared in ultrapure water, and diluted for experimental treatment immediately prior to use. Pollen

dough was made by mixing sugar water and ground honey bee pollen at a ratio of 1:3.2 (v/w). Control, untreated sugar water and pollen provisions were prepared in the same way, but without the addition of imidacloprid.

Food consumption measurement. To monitor feeding during the experimental imidacloprid exposure to ensure consumption of contaminated resources was taking place and to assess if this was affected by treatment, sugar water and pollen dough consumption per microcolony were measured. Sugar water consumption per microcolony was measured as the sugar water volume difference between the start and the end of the 48h treatment period. Pollen dough remnants from all microcolonies were dried at 55°C for 48h and weighed individually. Because dry weight of each pollen pellet could not be assessed prior to provisioning, pollen consumption was estimated as the mass difference between the dried pollen remnant and the mean dry weight of 10 consistently and identically made intact pollen dough provision standards. Both sugar water and pollen consumption were standardized by the number of days of the treatment and by the number of bumble bee adults and larvae in the microcolony. We tested for statistically significant differences in sugar water and pollen consumption between treatments with a Kruskal-Wallis rank sum tests.

RNA-seq analysis. We extracted RNA from individual larvae following the E.Z.N.A. Total RNA Kit I (Omega Bio-tek) protocol and with DNase I (Omega Bio-tek). After assessing the RNA quality with an agarose gel (1% w/v), we pooled three larval RNA samples per microcolony, resulting in a final RNA yield of 1 µg per pooled sample. A total of four 0.7 ppb imidacloprid, four 7.0 ppb imidacloprid and four control replicates yielded 12 pooled (three larvae per pool) samples. Pooled RNA samples from a microcolony were treated with poly-A tail selection and sequenced using Illumina technology (HiSeq4000, W.M. Keck Center for Comparative and Functional Genomics, Roy J. Carver Biotechnology Center, University of Illinois Urbana-Champaign), which yielded a total of 386,462,102 single-end reads from the 12 RNA libraries, with an average number of reads of 32,205,175 (minimum-maximum values: 27,142,118 – 39,048,533). The raw reads are available in the SRA repository (NCBI), with accession IDs SRR20446816-SRR20446827.

We trimmed adapter sequences and bases with low quality from reads (Phred < 28) with Trimmomatic v0.38 [52], mapped the trimmed reads to the *B. impatiens* genome v2.2 [53] with STAR 2.7 [54], and then summarized the read counts from the genome's gene features with *htseq-count* [55] using the "union" method. Alignment of the reads to the *B. impatiens* genome resulted in 96.48% (96.00-96.90%) of reads aligned to the genome, with 84.57% (82.00–87.00%) of them uniquely aligned to a gene feature.

We monitored the clustering of samples with principal component analysis (PCA), using the expression values of the top 500 most expressed genes. We performed the differential gene expression analysis with *DESeq2* [56], using the treatment variable (control, 0.7 ppb imidacloprid and 7.0 ppb imidacloprid) as the factor, and tested the source colony effect. We considered a gene as differentially expressed (DEG) when its false discovery rate (FDR) was lower than 0.05 for treatment comparisons (control vs. 0.7 ppb imidacloprid, control vs. 7.0 ppb imidacloprid, and 0.7 ppb imidacloprid vs. 7.0 ppb imidacloprid). We generated Venn diagrams comparing the DEG sets from each comparison using the on-line tools from

http://www.interactivenn.net/ and tested whether the overlapping number of DEGs between different DEG sets was statistically greater than expected with Fisher's exact test, using the *phyper* command in R v3.6.1 [57].

We mapped Gene Ontology (GO) terms to the *B. impatiens* genes that were expressed in our analysis from the NCBI genome repository (April 10, 2020), and performed GO enrichment analysis of the terms for up-regulated and down-regulated DEG sets with *topGO* [58], focusing on the biological process ontology. We considered a GO term as enriched if the weighted Fisher's exact test p-value, corrected through FDR, was less than 0.05. We also calculated the logarithm of fold enrichment (logFE) by dividing the observed counts of a given GO term by its expected counts, then calculating the logarithm to the base 2.

Results

Bumble bees exposed to the high imidacloprid concentration treatment consumed less pollen. Microcolonies exposed to 7.0 ppb imidacloprid consumed less pollen than the 0.7 ppb imidacloprid and control treatments (Kruskal-Wallis test $\chi^2_2 = 10.632$, $p = 0.005$, Fig. 1a). There was no significant effect of imidacloprid treatment on sugar water consumption (Kruskal-Wallis test $\chi^2_2 = 1.167$, $p = 0.558$, Fig. 1b).

Both high and low concentrations of imidacloprid induced quantitative differences in gene expression. PCA of the 500 top expressed genes (Supplementary Fig. S1) and the heatmap with DEG expression profiles (Supplementary Fig. S2) showed high gene expression variability among the replicates of each treatment, but overall, significant differential gene patterns were still detected between the treatments. Relative to controls, we detected 869 differentially expressed genes in the 0.7 ppb imidacloprid treatment (550 up-regulated and 319 down-regulated), and 1,433 (760 up-regulated and 673 down-regulated) DEGs in the 7.0 ppb imidacloprid treatment (Fig. 2a,b, Supplementary Tables S2 and S3). We also found that 982 genes showed source colony effects, 335 of them being DEGs.

There is a shared set of differentially expressed genes across both concentrations. A shared set of 678 DEGs were affected by both high and low imidacloprid concentrations (Fig. 2b). Fisher's exact test indicated that this overlap was significantly higher than expected by chance ($p < 0.001$). All these DEGs were expressed in the same direction (up- or down-regulated) for both concentrations, with the exception of the uncharacterized protein LOC100747518, up-regulated in the high concentration and down-regulated in the low concentration. For both the high and low concentrations, the top up-regulated DEG was a gene homologous to *15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like* (LOC100740825, involved in the metabolism of prostaglandins and alcohol dehydrogenase activity in insects, Fig. 2c,d) and the top down-regulated DEG was the *NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3* (LOC100747652, part of the complex that transfers electrons to the respiratory chain in mitochondria, Fig. 2c,d). Among the top up-regulated overlapping DEGs were those associated with detoxification (three cytochrome P450s (*CYPs*) from the CYP9 family and one cytochrome b5), neural and anatomical development and hormone regulation (regulators of prostaglandin and juvenile hormone). Among the

downregulated overlapping DEGs were genes involved in DNA replication, DNA packaging (histone proteins) and proteolysis (mostly digestive enzymes).

Different imidacloprid treatments also show unique concentration-specific differential expression gene sets. We found 191 DEGs affected exclusively by the low concentration treatment (Fig. 2b), including CYPs (*CYP6a13* and a *CYP28d1*), venom proteins, cuticle developmental and neural developmental genes among the up-regulated DEGs; among the down-regulated DEGs were those associated with cell proliferation and histone proteins. In addition, 755 DEGs were associated exclusively with the high concentration treatment (Fig. 2b), including cuticle developmental genes, transport proteins, neuropeptide and neurotransmitter receptors, and the detoxification gene *CYP6a14* among the up-regulated DEGs; and DNA replication genes, cell cycle regulatory genes and digestive enzymes among the down-regulated DEGs. When testing for differential expression between low and high concentration treatments we detected 66 DEGs (Supplementary Table S4): 60 up-regulated in the high concentration compared to the low concentration, and six DEGs up-regulated in the low concentration compared to the high concentration (Supplementary Fig. S3a). Of these DEGs between low and high concentration treatments, 22 of them (Supplementary Fig. S3b) were genes not showing differences in expression when comparing either the low or high concentration treatments against the control.

GO term enrichment analysis shows that both imidacloprid concentrations down-regulate mitochondrial and DNA replication biological processes. Out of the 10,161 GO terms associated with the 9,045 genes from the *B. impatiens* genome expressed in our study, 23 GO terms were enriched in the DEG set (Fig. 3, Supplementary Table S5). The DEGs affected by the low 0.7 ppb imidacloprid concentration showed only cytoplasmic translation (GO:0002181) as enriched for the up-regulated DEGs, while seven GO terms were enriched in the down-regulated DEGs, related to mitochondrial activity (5/7) and DNA replication (2/7). Regarding the DEGs affected by the high 7.0 ppb imidacloprid concentration, the GO terms cellular response to starvation (GO:0009267), macroautophagy (GO:0016236) and fatty acid catabolic process (GO:0009062) were enriched in the up-regulated DEGs; while DEGs down-regulated by the high concentration were enriched for 19 GO terms, related to mitochondrial activity (9/19), DNA replication (5/19) and gene expression (5/19). The only enriched GO term in the DEGs from the comparison between the high and the low concentrations was chitin-based cuticle development (GO:0040003), enriched among the up-regulated DEGs.

Discussion

Our results demonstrate that both high (7.0 ppb) and low (0.7 ppb) sublethal field-realistic concentrations of imidacloprid affect the expression of genes that are important to bumble bee larval development and health. This adds perturbed larval molecular responses to the range of effects already documented for neonicotinoids in bumble bee adults [4]. It appears that the number of genes affected in our study is proportional to the imidacloprid concentration, which has also been found in honey bees [41]. Our combined result of reduced pollen consumption and increased expression of genes relating to starvation when exposed to our high field relevant imidacloprid concentration (7.0 ppb) indicate that indirect effects

of neonicotinoid exposure, such as altered feeding, may detrimentally impact the larval physiology. However, we also see that lower field-relevant concentrations of imidacloprid directly change the expression of regulatory and other important genes in larvae that have the potential to have lasting effects on individual and colony health.

Imidacloprid is metabolized by cytochrome P450s (CYP) in insects [59–61], but some of the resulting metabolites are still toxic [62,63], and could have side effects on bumble bee health even after degradation of the initial compound. Accordingly, we found *CYP6* and *CYP9* proteins up-regulated in both treatments (Fig. 4a): some gene copies of *CYP6* have been associated with neonicotinoid detoxification in *Drosophila* [59,64] and in the brown planthopper *Nilaparvata lugens* [65], and some gene copies of *CYP9* are involved in detoxification of acaricides in the honey bee [66]. There were also CYPs up-regulated in either the low (Fig. 4b) or high (Fig. 4c) imidacloprid treatments. This could suggest the existence of either a concentration-dependent regulation for either imidacloprid itself or its metabolites. Other cytochromes, such as *cyt b5* (which enhances CYP activity [67]) and *cyt c* (involved in cell respiration, apoptosis and detoxification [68]), were down-regulated by imidacloprid exposure. Other elements of the mitochondrial cell respiration pathway in addition to *cyt c* were also down-regulated by both concentrations of imidacloprid, such as *NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3*. This is consistent with the literature showing that imidacloprid disrupts mitochondrial activity in insects [69–71]. The inference from this is that no matter the imidacloprid concentration with the range used here, larvae exposed to this pesticide not only face additional energy investments producing CYPs and investing in costly detoxification processes [72,73], but also experience constrains on their ability to produce energy.

The effects of imidacloprid exposure have been studied more frequently in adults or at the whole colony level in bumble bees, but its effect during larval stages, and consequently for larval development and health, remain understudied. We detected that exposure to imidacloprid influenced the expression of genes involved in cell growth regulation and chromatin modification: several histone proteins (DNA packaging elements) were down-regulated under both the high and low concentration treatments, with a higher number of histone proteins down-regulated under the high concentration, also suggesting a concentration dependent response. Histone protein depletion induces cell cycle arrest [74], meaning that both concentrations may be delaying larval development. In fact, imidacloprid induces pupal ecdysis arrest in various Lepidoptera species [75], and these gene expression changes may interfere with bumble bee larval development too. This, combined with increased worker mortality could explain decreased colony growth under neonicotinoid exposure [30–33]. The fact that exposure to the high imidacloprid concentration also down-regulates genes related to DNA-replication and cell growth also supports this likely effect on larval development.

Knowing how neonicotinoids adversely affect learning and memory in bumble bees [76–78], it was not surprising to find neural activity-related genes affected by imidacloprid exposure. For instance, the transcription factor *GATA-binding factor C-like* protein (LOC100742684, homologous to a protein essential for development and axon guidance [79]) was up-regulated under both treatment concentrations, as well as

circadian rhythm proteins (such as *pigment-dispersing hormone peptides*, *protein quiver* or *retinol-binding protein pinta*). Among the DEGs exclusively up-regulated under the high imidacloprid concentration, we found several nervous system and neuron development genes (such as *neurogenin-1, rho GTPase-activating protein 100F, neurogenic locus notch homolog protein 1-like* and *neurofilament heavy polypeptide-like*) and neuropeptide and neurotransmitter receptors (such as *tachykinin-like peptides receptor 86C, neuropeptide CCHamide-1 receptor-like, glutamate receptor ionotropic kainate 2, 5-hydroxytryptamine receptor* or *pyrokinin-1 receptor*), potentially affecting both activity and development of the larval nervous system. The low concentration also up-regulated some nervous system developmental genes (such as *lachesin* and a paralog of the *GATA-binding factor C-like*, LOC100743387), but a smaller set. Altering the expression of these genes during larval stage may generate potentially permanent cognitive problems in the adults. This has been shown to occur in the Asian honey bee *Apis cerana*, where larvae exposed to imidacloprid can develop into adults with impaired olfactory learning ability [38], although no genetic effectors have been linked yet to this phenomenon.

In our study, microcolonies treated with the high imidacloprid concentration consumed less pollen (Fig. 1). This could explain why we detected up-regulated DEGs associated to the GO term *cellular response to starvation* (GO:0009267) and down-regulated DEGs with digestive functions. These DEGs include *pigment-dispersing hormone peptides* (involved in digestion regulation [80]), *sestrin-1* (associated with starvation response [81]), *pyrokinin-1 receptor* (neural receptor involved in insulin production regulation [82]), *chymotrypsin-1* and *digestive cysteine proteinase 1* (both digestive enzymes). The reduction in pollen consumption in the high concentration treated microcolonies may have been caused by impaired feeding behavior of nurse workers exposed to this imidacloprid concentration, as seen in [83], but we cannot confirm this since we did not monitor worker feeding behavior in the microcolonies. Honey bee workers exposed to imidacloprid show impaired nursing ability, provoking starvation and development delay in larvae [84]. While neglect by attending workers is one possibility, these results on overall microcolony consumption and gene expression patterns could arise from changes in consumption by larvae themselves. Reduced consumption of pollen provisions following neonicotinoid exposure has been shown for larvae of the solitary bee *Osmia* [36]. Independent of the root cause, such indirect effects will compound existing direct effects on the developing larvae, and these may also contribute to some of the other gene expression patterns unique to the high 7.0 ppb imidacloprid treatment group.

We see a clear differentiation between gene expression profiles of larvae from the imidacloprid treatments, with many of the patterns that could be expected given previously documented effects. However, we see relatively high expression variation across samples from the same treatment (Supplementary Figs. S1, S2). This could mean that some genes that are affected in only a subset of the colonies are not identified as differentially expressed in our analysis, even though they would have important consequences for those individuals. Explanations for this inter-sample variation include, i) developmental stage differences in sampled larvae that generates noise in the gene expression background, ii) irregular larval feeding by nurses that induces differences in gene expression between

sampled larvae, or iii) real differences of response to a treatment by larvae from different colonies due to genetic variation. We validated our data comparing the DEG lists with those from three similar studies [40,42,45] (Supplementary Table S1), and we found that the overlap between the lists of DEGs from these three studies was greater than expected by chance (Supplementary Table S6). This suggests that our analysis uncovers important general gene expression responses to imidacloprid exposure in bumble bee larvae. Moreover, three genes were consistently differentially expressed in our study and at least another two studies [40,42]: the detoxification gene *cytochrome P450 6k1* (LOC100745845), the mitochondrial gene *phosphoenolpyruvate carboxykinase [GTP]* (LOC105680266) and the uncharacterized protein LOC100746060 (which contains a MYND finger domain). This set of DEGs might be part of a general molecular response to neonicotinoids in bumble bees, and could be used to track down gene expression signatures in similar studies or in field scenarios.

In conclusion, we demonstrate that low and high field-realistic sublethal concentrations of imidacloprid, differing by an order of magnitude, trigger molecular responses in bumble bee larvae that included genes involved in key biological processes, including detoxification, neural processes, and larval development. While a core set of differentially expressed genes is shared across the two imidacloprid exposure treatments, each treatment stimulates its own unique gene set, which is larger in the high concentration exposure treatment. While differential expression in the low concentration groups suggests direct effects of the imidacloprid exposure, some of the differences in the high concerntration group may come from stronger indirect effects due to disrupted feeding. Overall, our study reveals a molecular basis for the detrimental impact of neonicotinoid exposure on larval health, the exact nature of which may vary depending on exposure concentration.

Declarations

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Author contributions

R.M.B. performed RNA extraction, RNA-seq bioinformatic analysis and statistical analysis. A.C.C. and B.M.S. conducted queen sampling and colony rearing. A.C.C. and R.M.B. set the microcolonies and monitored microcolony food consumption. S.A.C., B.M.S. and R.M.B. conceived the experimental approach of the study. R.M.B. wrote the draft of the paper, which was then contributed to by all authors.

Competing interests

The authors declare no competing interests.

Data availability

The online version of this article has available supplementary information: **link**

The Illumina reads generated during the current study are available at the SRA repository (NCBI), under the BioProject PRJNA861317 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA861317), with accession numbers from SRR20446816 to SRR20446827.

The scripts used to analyze the data are available under request to the corresponding author.

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Figures

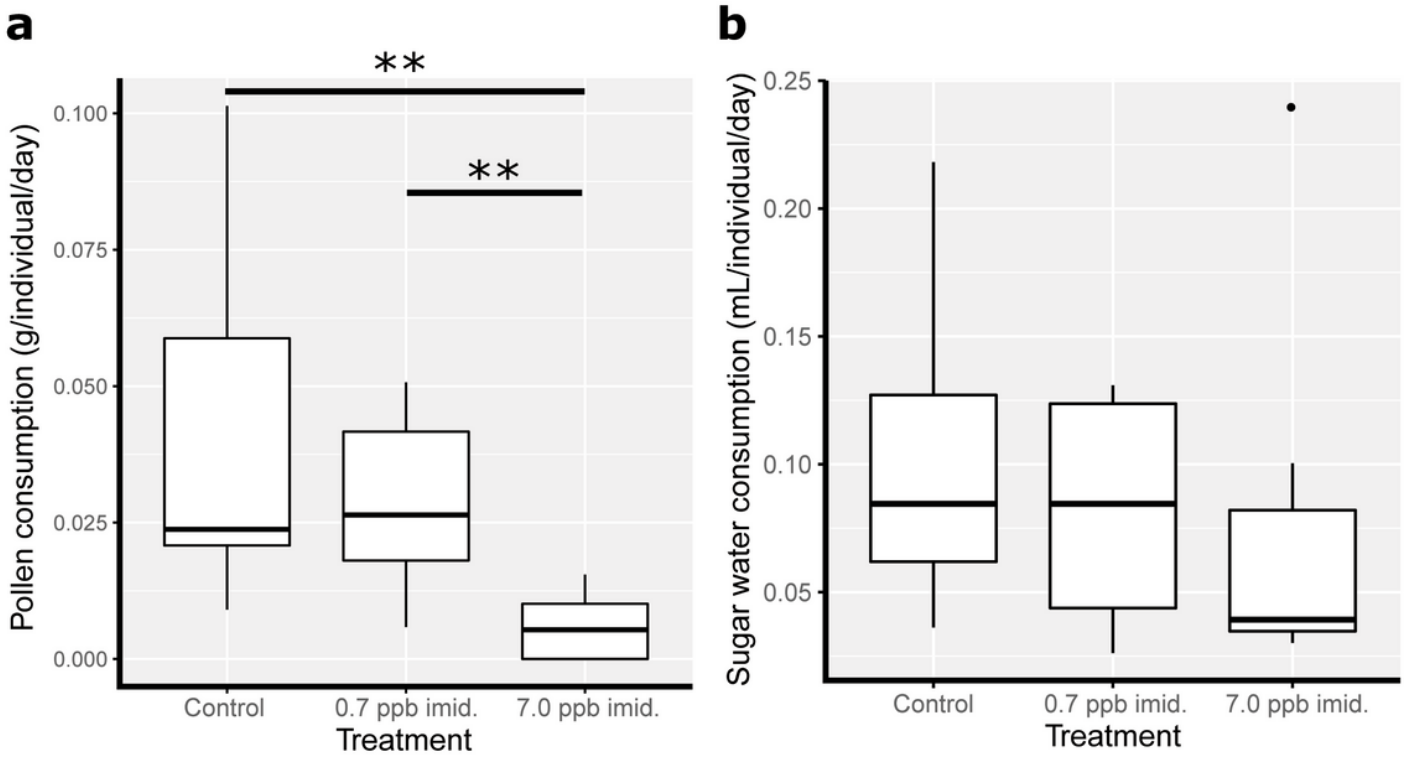

Figure 1

Pollen (**a**) and sugar water (**b**) consumption in *B. impatiens* microcolonies for control, 0.7 ppb imidacloprid and 7.0 ppb imidacloprid treatments. Horizontal bars between boxes indicate significant differences between treatments, asterisks indicate the Dunn's test significance values associated with the pairwise comparison (**: 0.01 > p > 0.001).

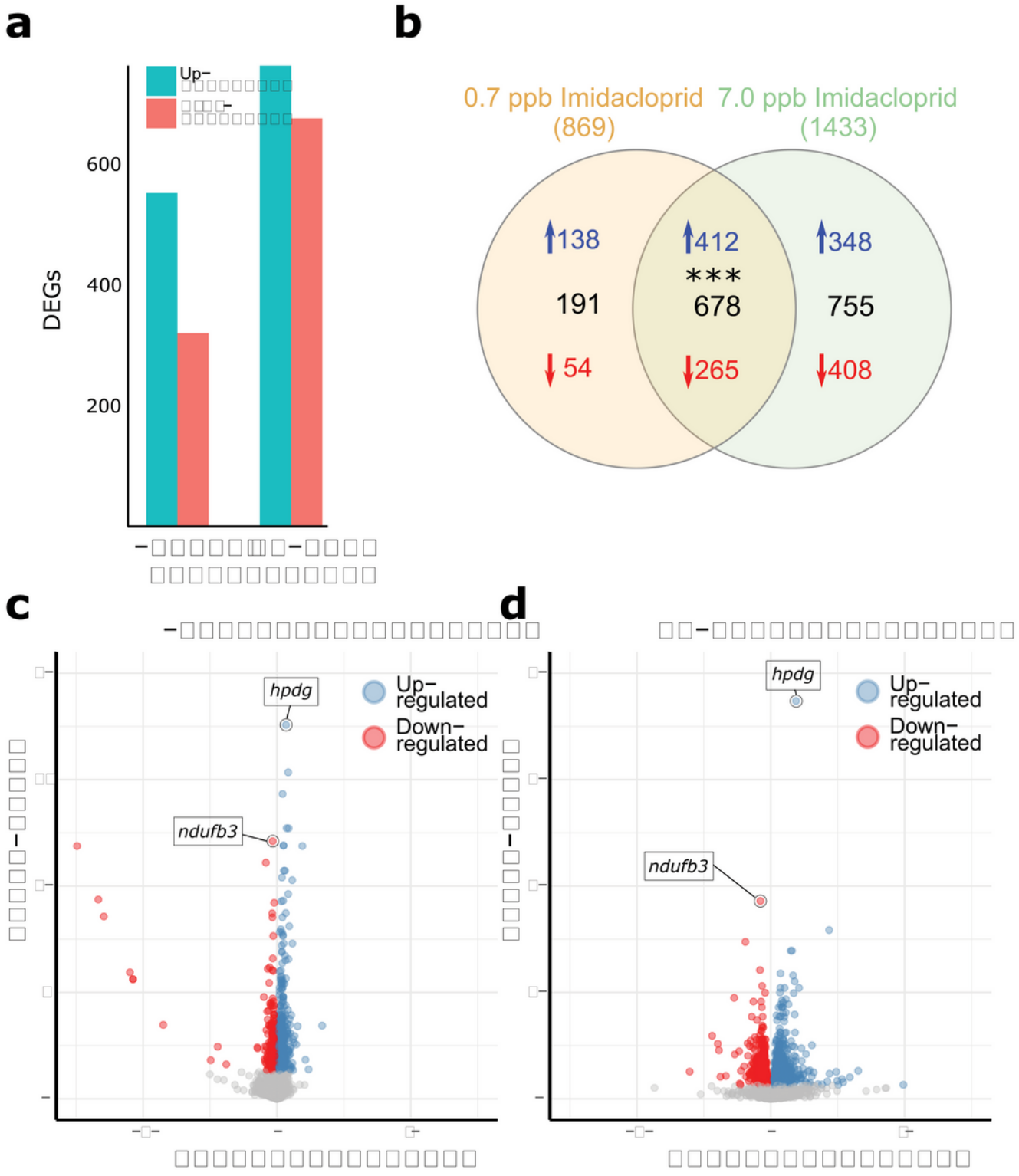

Figure 2

Differential gene expression induced by low and high imidacloprid concentrations. (**a**) Number of up-regulated and down-regulated differentially expressed genes in each treatment. (**b**) Venn diagram with the number of differentially expressed genes (DEGs) associated to the 0.7 and 7.0 ppb imidacloprid concentration treatments when compared to the control treatment. Numbers in parentheses below the imidacloprid concentration treatments are the total number of differentially expressed genes in that group

relative to controls. Black numbers indicate the total number of unique or shared DEGs, blue numbers with upward arrows indicate up-regulated genes and red numbers with downward arrows indicate down-regulated genes. Asterisks indicate the hypergeometric test significance (***: $p < 0.001$). Note that up-regulated (blue) and down-regulated (red) DEG values do not exactly match the total DEGs (black) since uncharacterized protein LOC100747518 was down-regulated by the low concentration and up-regulated by the high concentration. Volcano plots for the (**c**) control vs. 0.7 ppb imidacloprid comparison and for the (**d**) control vs. 7.0 ppb imidacloprid comparison: x-axis shows the logarithm to the base 2 of the fold change (FC), y-axis shows the negative logarithm to the base 10 of the false discovery rate (FDR) from DEGs, grey dots represent genes without differential expression (FDR > 0.05), red dots are down-regulated genes and blue dots are up-regulated genes. DEGs with the lowest FDR values are marked with a black circle, pointing to a box including the gene's short name (*hpdg*: 15-hydroxyprostaglandin dehydrogenase [NAD(+)]-like; *ndufb3*: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 3).

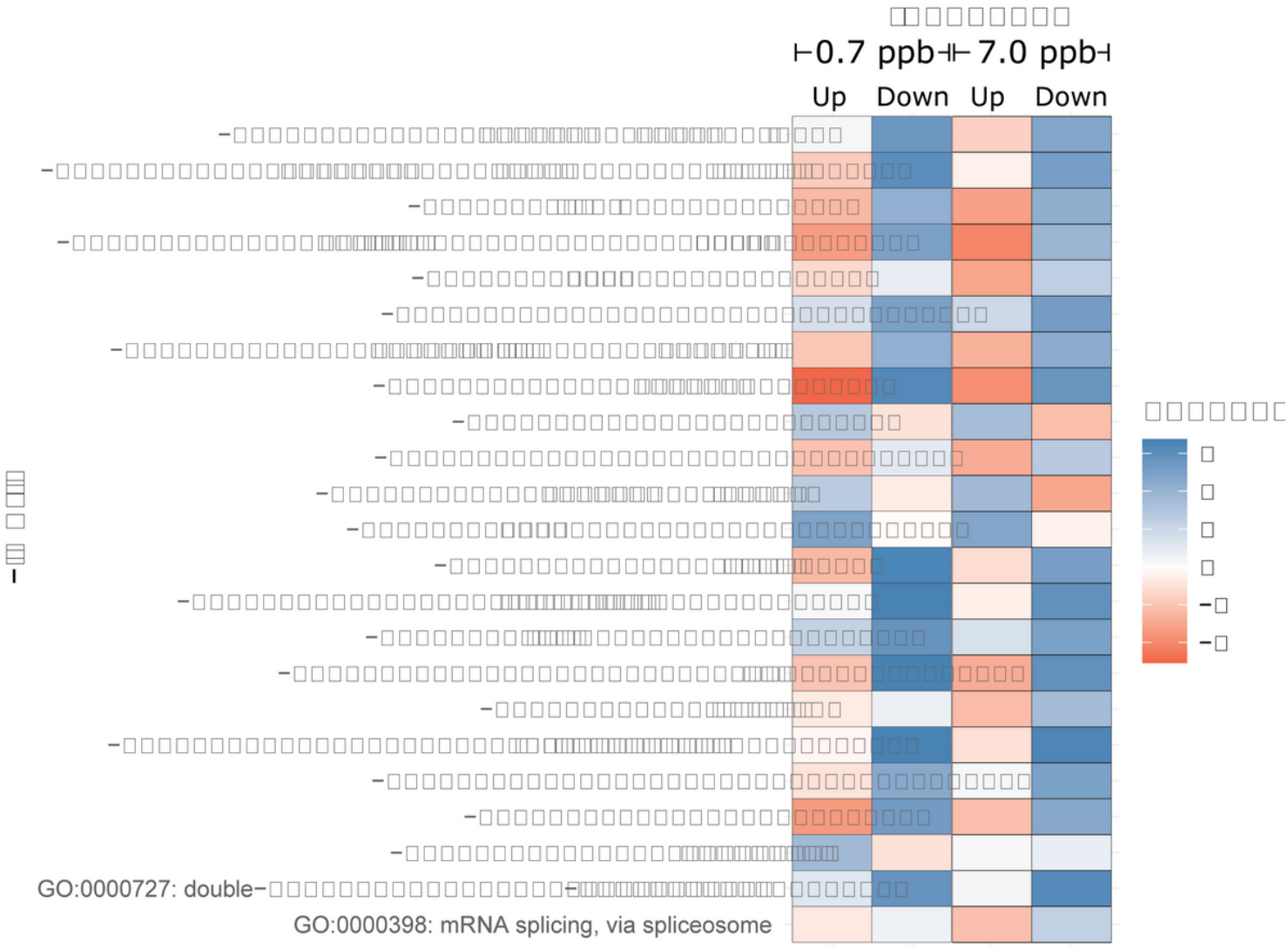

Figure 3

Gene ontology terms enriched in the differentially expressed gene sets. The enriched gene ontology (GO) term names are shown on the Y axis. Tile color show values for the logarithm to the base 2 of the fold enrichment of the GO term (i.e., observed count divided by expected count of a specific GO term in the sample). The x-axis shows the up- and down-regulated DEG sets from the low (0.7 ppb) and high (7.0 ppb) imidacloprid treatments. Red tiles represent under-represented GO terms, blue tiles represent enriched GO terms, according to the logarithm in base two of the fold enrichment.

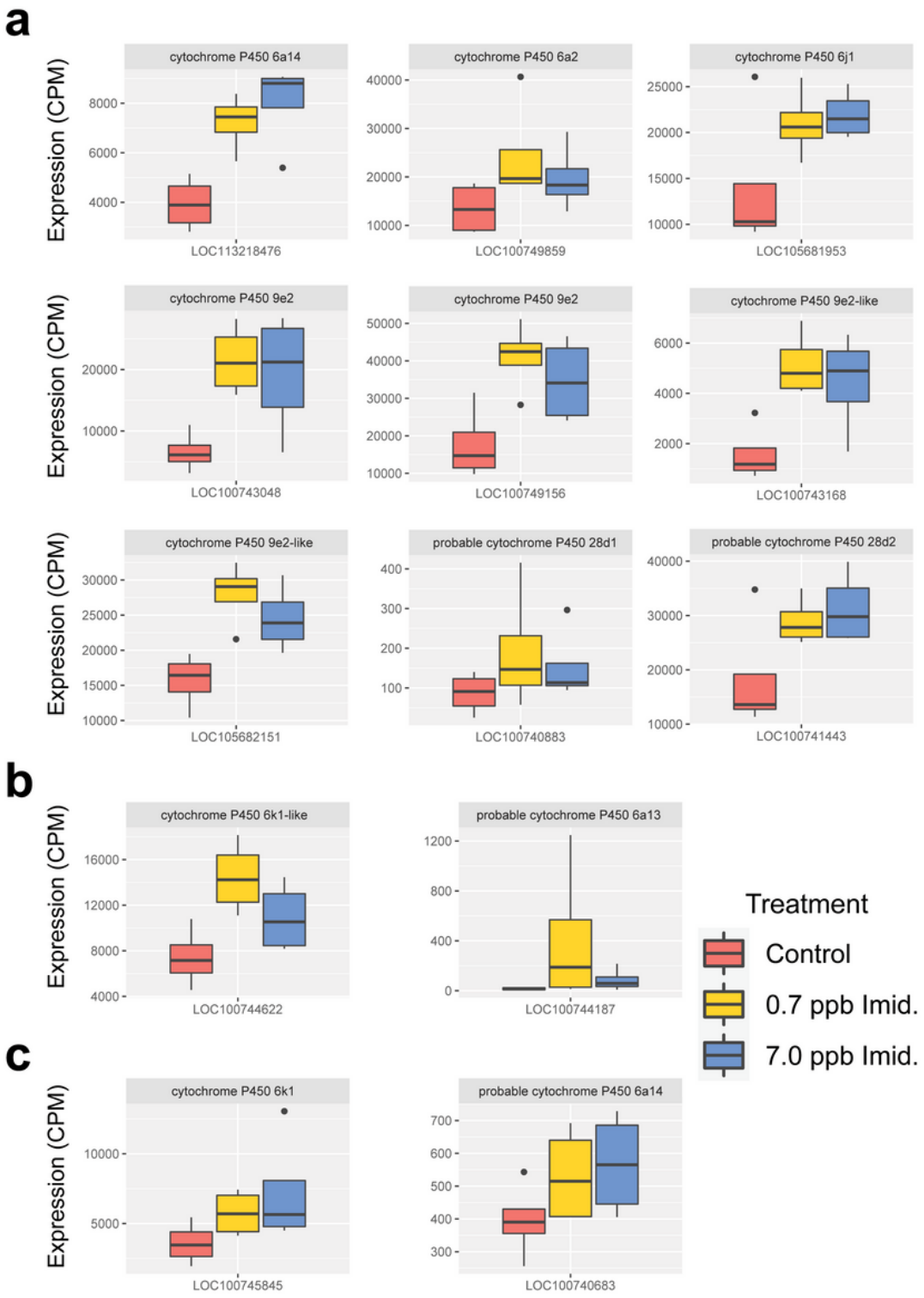

Figure 4

Differentially expressed cytochrome P450s in both 0.7 ppb and 7.0 ppb (**a**), only in the 0.7 ppb treatment (**b**), or only in the 7.0 ppb imidacloprid treatment (**c**), relative to controls. The boxplots show the expression values of cytochromes P450s (CYPs) in read counts per million mapped reads (CPMs, calculated through *DESeq2*), for control, 0.7 ppb imidacloprid and 7.0 ppb imidacloprid treatments. In

each boxplot, the title of the plot shows the name of the CYP, and the x-axis shows the unique gene identification number of the CYP.

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