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RESEARCH ARTICLE

RNA-seq reveals disruption of gene regulation when honey bees are caged and deprived of hive conditions

Mohamed Alburaki[1,*], Shahid Karim[2], Kurt Lamour[3], John Adamczyk[4] and Scott D. Stewart[5]

ABSTRACT

In this study, we present phenotypic and genetic data characterizing the impact of imidacloprid and caging stress on honey bee *Apis mellifera* physiological responses and regulation of 45 genes using targeted-RNA seq. The term 'caging stress' characterizes the effects of depriving honey bees of all hive aspects and conditions. Two cohorts of 1 day old sister bees were subjected to different conditions. One cohort was caged and fed different imidacloprid-tainted sugar solutions and the second was marked and introduced back to its natal hive. Physiological bee parameters and diet behavior were monitored daily for caged bees over several weeks. Bee samples from both cohorts were sampled weekly for RNA sequencing and oxidative stress analyses. Imidacloprid induced significant protein damage and post-ingestive aversion responses in caged bees, leading to lower tainted syrup consumption and higher water intake compared with the controls. No differentially expressed genes were observed among caged bees in regards to imidacloprid treatment. However, significant upregulation in antioxidant genes was recorded in caged bees as compared with hive bees, with overwhelming downregulation in all gene categories in caged bees at week 4. We identified two sets of genes that were constantly regulated in caged bees, including *Rsod* with unknown function in insects that could potentially characterize caging stress in honey bees.

KEY WORDS: Honey bee, RNA-seq, Cage stress, Imidacloprid, Gene regulation, Oxidative stress

INTRODUCTION

The honey bee *Apis mellifera* is an important eusocial insect pollinator (Giannini et al., 2015; Morandin et al., 2001; Sampson and Cane, 2000). Although the debate on the overall impact of pesticides on honey bee health is ongoing (Stokstad, 2017), honey bee exposure to agricultural pesticides, particularly the neonicotinoids, is one of many factors that contribute to bee population decline (Sanchez-Bayo and Goka, 2014; Tosi et al., 2017; Williamson et al., 2013; Woodcock et al., 2017). Measuring pesticide toxicity for bees under field conditions is difficult because of the complexity of honey bee biology and foraging behaviors (Alburaki et al., 2015, 2017b; Cutler and Scott-Dupree, 2007; Stewart et al., 2014). Therefore, cage experiments are commonly used to test bees (e.g. toxicological and behavioral assessments) under more controlled conditions (Alburaki et al., 2017a; Gregorc et al., 2017). Despite providing more controlled conditions, little is known about the physiological and molecular responses of bees to caging stress.

Honey bees live in highly organized colonies in which each worker performs a very specific function and changes tasks throughout her physiological development (Winston, 1987). Absence of the queen or any disruption in the pheromone communication inside the bee colony is considered fatal for the whole population. When bees are caged for experimental purposes, significant pheromone disruption occurs, altering bee social behavior (Grozinger et al., 2003). For example, absence of the queen mandibular pheromone delays honey bee behavioral maturation (Robinson et al., 1998), and its presence prevents the rearing of new queens (Winston et al., 1991) and inhibits worker ovary development (Hoover et al., 2003). The ability of worker bees to execute their appropriate tasks and communicate with their hive mates is crucial for colony wellbeing.

Gene regulation in honey bees is often studied in relation to pathogen infection (Gregorc et al., 2012), exposure to abiotic stressors (Alburaki et al., 2017b), and other physiological and genetic factors among honey bee castes and developmental phases (Evans and Wheeler, 1999). These efforts have led to a better understanding of honey bee gene regulation and the identification and annotation of an important number of antioxidant and immune genes evolved in honey bee response to various stressors (Corona and Robinson, 2006; Evans et al., 2006).

Imidacloprid, a broadly used neonicotinoid, is acutely toxic to bees and can impair honey bee performance at sublethal doses (Chakrabarti et al., 2015; Williamson et al., 2014). Imidacloprid has a high agonistic affinity with nicotinic acetylcholine receptors (nAChR), particularly in brain tissue (Decourtye et al., 2004). Bees are equipped with complex detoxification mechanisms, including networks of enzymatic antioxidants, to help reduce potential oxidative damage provoked by biotic and abiotic stressors, including pesticides (Corona and Robinson, 2006). Interestingly, caged bees administrated *ad libitum* sugar syrup containing 5–10 times their median lethal concentration (LD_{50}) of imidacloprid have shown the ability to survive for a relatively long period of time (4–7 weeks) (Alburaki et al., 2017a; Meikle et al., 2016). It is yet unclear whether those bees survive by minimizing their contaminated-syrup intake or have a metabolic capacity allowing them to quickly process the lethal molecules, or by the two scenarios together. It is also conceivable that the honey bee has the ability to sense the presence of those chemicals within the proposed syrup and refrains from feeding on such contaminated nutrients. It has been demonstrated that honey bees fed on neonicotinoids (thiamethoxam), 1 ng per bee for 12 days, significantly decreased their affinity for sucrose stimulation (Aliouane et al., 2009), which can partially explain the rejection of syrup with optimal sugar concentrations for bees. However, acetamiprid which is another neonicotinoid, orally

[1]USDA-ARS Bee Research Laboratory, Beltsville, MD 20705, USA. [2]The University of Southern Mississippi, Department of Cell and Molecular Biology Sciences, Hattiesburg, MS 39406, USA. [3]The University of Tennessee, Entomology and Plant Pathology Department, Knoxville, TN 37996, USA. [4]USDA-ARS Thad Cochran Horticulture Laboratory, Poplarville, MS 39470, USA. [5]The University of Tennessee, Department of Entomology and Plant Pathology, West Tennessee Research and Education Center, Jackson, TN 38301, USA.

*Author for correspondence (mohamed.alburaki@usda.gov)

M.A., 0000-0003-4441-9391

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administrated to bees (0.1 µg per bee) increased their responsiveness to water (Aliouane et al., 2009).

This study was conducted on sister honey bees exposed to two different conditions: (1) honey bees were removed from their natal hive and placed in cages in the laboratory and (2) honey bees were labelled and kept in their natal hive. We addressed two major and interrelated hypotheses: (1) compared with an untreated control, sister honey bees caged under similar conditions and administrated *ad libitum* sugar syrup tainted with lethal and sublethal concentrations of imidacloprid would exhibit higher mortality and upregulation in their detoxification genes; and (2) depriving honey bees of hive conditions by caging them would trigger significant disruption of gene regulation compared with their sister-mates of the natal hive. This study provides new insights into how imidacloprid affects honey bee physiological responses and diet behavior as well as its implications for the regulation of the major bee antioxidant and immune genes. Furthermore, we characterized potential genes involved in the response to cage stress by studying the differentially expressed genes of caged bees and their sister-mates operating under natural hive conditions.

MATERIALS AND METHODS

Honey bee colony

One well-established honey bee colony, the ‘mother hive’, headed by a Carniolan queen (*Apis mellifera carnica* Pollman 1879) was used as the source of all worker samples of this study. A single hive was used as a source of worker bees in this study to minimize variability in hive conditions and genetic make-up of the target organisms. A total of eight capped worker brood frames ready to hatch were removed from this hive and placed in an incubator at 35°C with 70–80% relative humidity. The following day, several thousand 1 day old sister bees were collected into a sterile plastic box for this study.

Cage and hive experiment

Newly hatched worker bees collected in the plastic box were gently mixed and 500 of them were randomly picked and marked with a white dot on their dorsal tergite using a collective marking box (Alburaki et al., 2017a). These workers were placed in a new plastic box to allow the paint to fully dry, then these marked 1 day old bees were introduced back into their natal hive. Another set of ∼150, 1 day old bees were stored at −80°C and were designated as time 0 reference bees (Fig. 1). A third set of 1800, 1 day old bees were randomly divided into 12 groups (150 bees per group); each group was weighed separately and introduced into 12 separate cages. The cages used in this study (dimensions of 11.4×6.3×15.2 cm width: depth:height; Fig. S4) were specifically designed for feeding experiments and are fully described in Gregorc et al. (2018). The 12 bee cages were randomly assigned to four treatment groups (three treatments and one control) in triplicate (Fig. 1). Bees in each cage were provided with distilled water and 1:1 sugar syrup using 30 ml syringes, and 10 g of protein patty (Mann Lake Bee Pro Patties). Imidacloprid was administrated to bees through the sugar syrup at four different dosages (0, 5, 20 and 100 ppb; Fig. 1). Patty, water and syrup consumption and bee mortality were documented daily. Dead bees were collected daily from the cages and stored at −80°C. The protein patties, which were placed into rubber plugs, were weighed using a ±0.01 g sensitive scale and gently placed back in the cages. Both syrup and water consumption were recorded visually from syringes at 0.5 ml sensitivity.

Worker bee sampling

Worker bees were sampled at four time points: 25 workers were sampled weekly from each cage as well as 75 marked workers from the mother hive. Each cohort of workers was weighed and stored at −80°C for subsequent molecular analyses. In addition, dead bees collected daily from each cage during the experiment were counted and weighed at the end of the experiment. A representative set of dead bees from each treatment (0, 5, 20, 100 ppb imidacloprid) was sent for chemical pesticide residue analysis. Four neonicotinoid molecules were screened (imidacloprid, imidacloprid olfen, thiamethoxam and clothianidin) by liquid chromatography-mass spectrometry (LC-MS) (Barnett et al., 2007; Walorczyk and Gnusowski, 2009). Chemical analyses for pesticide residue detection were processed at the USDA National Scientific Laboratories (Gastonia, NC, USA).

Oxidative stress

Hydrogen peroxide and protein carbonyl content assays were conducted to assess potential honey bee physiological stress and protein damage resulting from exposure to imidacloprid and caging stress. The level of H_2O_2 was quantified in the hydrogen peroxide assay using the bee hemolymph of samples collected in week 1. Bees were individually crushed in 1.5 ml tubes with 300 µl ultra-sterilized water and centrifuged at 11,000 ***g*** for 3 min. In order to eliminate proteins, the supernatant containing the bee hemolymph was filtered through a 10 kDa filter and the assay was conducted using a BioVision kit (Milpitas, CA, USA) as per the manufacturer's instructions. The protein carbonyl content assay was carried out on samples from weeks 1 and 4. Proteins were solubilized from honey bee thorax in a protein extraction buffer consisting of 20 mmol l^{-1} Tris-HCl pH 8.0, 30 mmol l^{-1} NaCl and 10% glycerol. The tissues were crushed using a pestle and sonicated using a Bioruptor Pico (Diagenode) sonication device for 10 cycles of 30 s pulse and 30 s rest at 4°C. Homogenates were centrifuged at 5000 ***g*** for 10 min at 4°C and the supernatants were collected. Quantification was conducted using a kit from Sigma-Aldrich (St Louis, MO, USA) as per the manufacturer's protocol.

Brain RNA extraction

Bee brains were dissected under a binocular microscope in an RNase free, sterile work environment. Total RNA was extracted from a pool of 15 bee brains per sample following the TRIzol® Reagent protocol (Invitrogen) (Chomczynski, 1993) with some modifications (see Alburaki et al., 2017a). Briefly, dissected brains were added to 1 ml TRIzol with 5 mg of acid-washed glass beads and gently mixed for 2 min. Then, 200 µl of phenol–chloroform was added, and the total mixture was incubated at room temperature for 15 min followed by a centrifugation at 10,000 ***g*** for 15 min at 4°C. The integrity of the RNA was determined by using a Nanodrop (260 nm/280 nm absorbance) and RNA concentration was brought to ∼200 ng µl^{-1} and stored at −80°C.

Targeted RNA-seq

In total, 45 honey bee genes involved in various functions (detoxification, immune defense and chemosensory roles, physiological development and nervous system regulation) were studied (Table S1). Approximately 200 bp of each exon was processed using the online primer picking program Batch Primer 3 (https://probes.pw.usda.gov/batchprimer3/) under the default setting for general primers to select forward and reverse primers that produce amplicons of 60–70 bp in length (You et al., 2008). The resulting primers were then tested in a single multiplex mixture using bee genomic DNA as the template. The genomic DNA was extracted using a MagJET system according to the manufacturer's directions (ThermoFisher). All amplifications from genomic DNA or cDNA were accomplished by Floodlight Genomics (Floodlight Genomics, Knoxville, TN, USA) through their no-cost Educational Outreach

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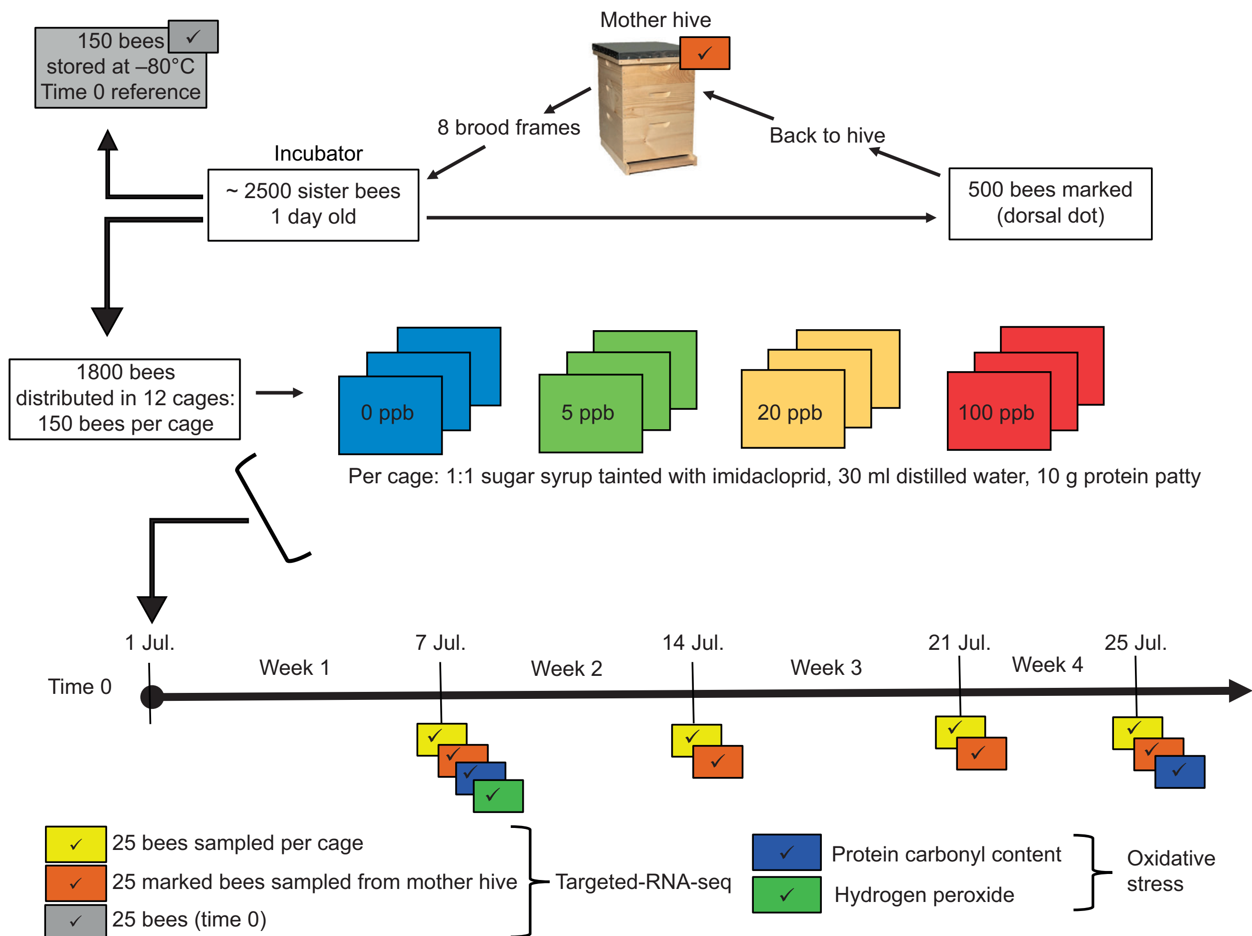


Fig. 1. Experimental design and procedures. One-day-old sister bees were hatched in an incubator (35°C, 70% relative humidity) and distributed across 12 cages (150 bees per cage). Three imidacloprid treatments (5, 20, 100 ppb) and one control (0 ppb) were established in triplicate. In addition, 150 bees were stored at –80°C at time 0 and 500 others were marked and put back in the hive. Caged bees were provided with sugar syrup, water and a protein patty. Imidacloprid was administrated to bees of the treatment groups via the sugar syrup. Four samplings (25 bees per cage or hive) were carried out (once a week) from both cages and the hive (marked bees).

Program using an optimized Hi-plex approach (Nguyen-Dumont et al., 2013). Briefly, primers were mixed into a single multiplex mixture and amplified according to the parameters previously outlined for the Hi-plex approach. The resulting amplicons have a sample-specific barcode sequence incorporated during the PCR and all amplicons were pooled and a dual-index Illumina library constructed and quantified using a KAPA PCR-free kit according to the manufacturer's directions (Roche Sequencing and Life Sciences). Sequencing was accomplished on an Illumina HiSeq device running a 2×150 configuration (Novogene). The resulting sequences were mapped to the original target sequences using CLC Genomics Workbench version 9.5.2 (Qiagen) at default settings and the sequence coverage assessed manually to determine targets with no amplification.

cDNA synthesis and determination of relative gene expression

Total RNA was converted to cDNA using the Promega GoScript reverse transcription system (Madison, WI, USA) according to the manufacturer's directions. The resulting cDNA was used as a template for multiplex amplifications as described above. Each amplification was accomplished twice and an RNA template (no cDNA) reaction was included as a third reaction for each sample. The resulting amplicons were sequenced and mapped as described above. To normalize the data and determine the relative gene expression, the total number of reads mapping to each target was divided by the average sequence coverage of four housekeeping genes (*Actin*, *CaMKII*, *GAPDH* and *E2F*) (Scharlaken et al., 2008). The sequences of the gene-specific primers have been published in Scharlaken et al. (2008) and Alburaki et al. (2017a).

Statistical analysis

Statistical analyses and figure generation were conducted within the R environment (http://www.R-project.org/). Analysis of variance (ANOVA) was performed to study the difference between variables regarding the treatment at a 95% confidence level. Sequences were checked for their quality using FastQC software and normalized using four housekeeping genes as mentioned above. A few genes that were very weakly amplified were discarded from the dataset. Bioconductor package EdgeR (Robinson et al., 2010) version 3.6 was used in R environment version 3.4.3 for differential expression analyses of read counts arising from our RNA-seq data (Robinson and Oshlack, 2010). Simple list-based data objects (DGEList) were created using the function readDGE, and genes with very low counts were filtered not by direct count but with counts per million (cpm). RNA composition was normalized using the function CalcNormFactors by finding a set of scaling factors that minimize the log-fold change (logFC) between samples for most genes, and these scale factors were computed using a trimmed mean of M-values between each pair of samples (Robinson

and Oshlack, 2010). Heatmaps were carried out using the library ComplexHeatmap of the package bioclite Limma. Other plots such as multi-dimensional scaling (MDS), hierarchical clustering and differential gene expression (DEG) were generated using their appropriate and respected functions in EdgeR. DEG data were calculated using EdgeR's Fisher's exact test after sample normalization. False discovery rates (FDRs) in DGEExact object were calculated at three different P-cutoffs (0.05, 0.01, 0.001) and their values were retained for each gene found to be upregulated or downregulated at 5%.

RESULTS

Nutrient consumption

Average syrup intake per week showed no differences among the groups until week 4, when bees in the 100 ppb imidacloprid treatment significantly reduced their syrup consumption (P<0.05, Fig. 2). Similar results were obtained when accounting for overall syrup consumption (P<0.01, Fig. 2). Interestingly, all groups treated with imidacloprid consumed a significantly (P<0.01) higher quantity of water than the control treatment group (0 ppb, Fig. 2), while protein patty consumption did not differ among treatments (data not shown). The correlation coefficients of syrup consumption and imidacloprid concentration indicated significant negative correlations in all treatment categories, particularly for 100 ppb (R=−0.15, P<0.001), with the exception of the 5 ppb treatment, in which no correlation was found (Fig. 2).

Bee weight and mortality

The 1 day old sister bees used in cage and hive experiments had similar body mass at time 0, at week 3 and for overall mass (Fig. 3). However, significant differences between the mass of caged and hive bees were recorded at weeks 1, 2 and 4, with no differences among caged bees (Fig. 3). In the cage experiment, there were no significant differences in the total number of dead bees collected throughout the experiment (P=0.7) amongst the treatments. However, dead bees of the control treatment were significantly (P<0.05) greater in mass than those in all other treatment groups (Fig. 3). LC-MS pesticide residue analysis of the dead bees showed trace levels of imidacloprid and imidacloprid olfen only in bees of the 100 ppb treatment (Fig. 3).

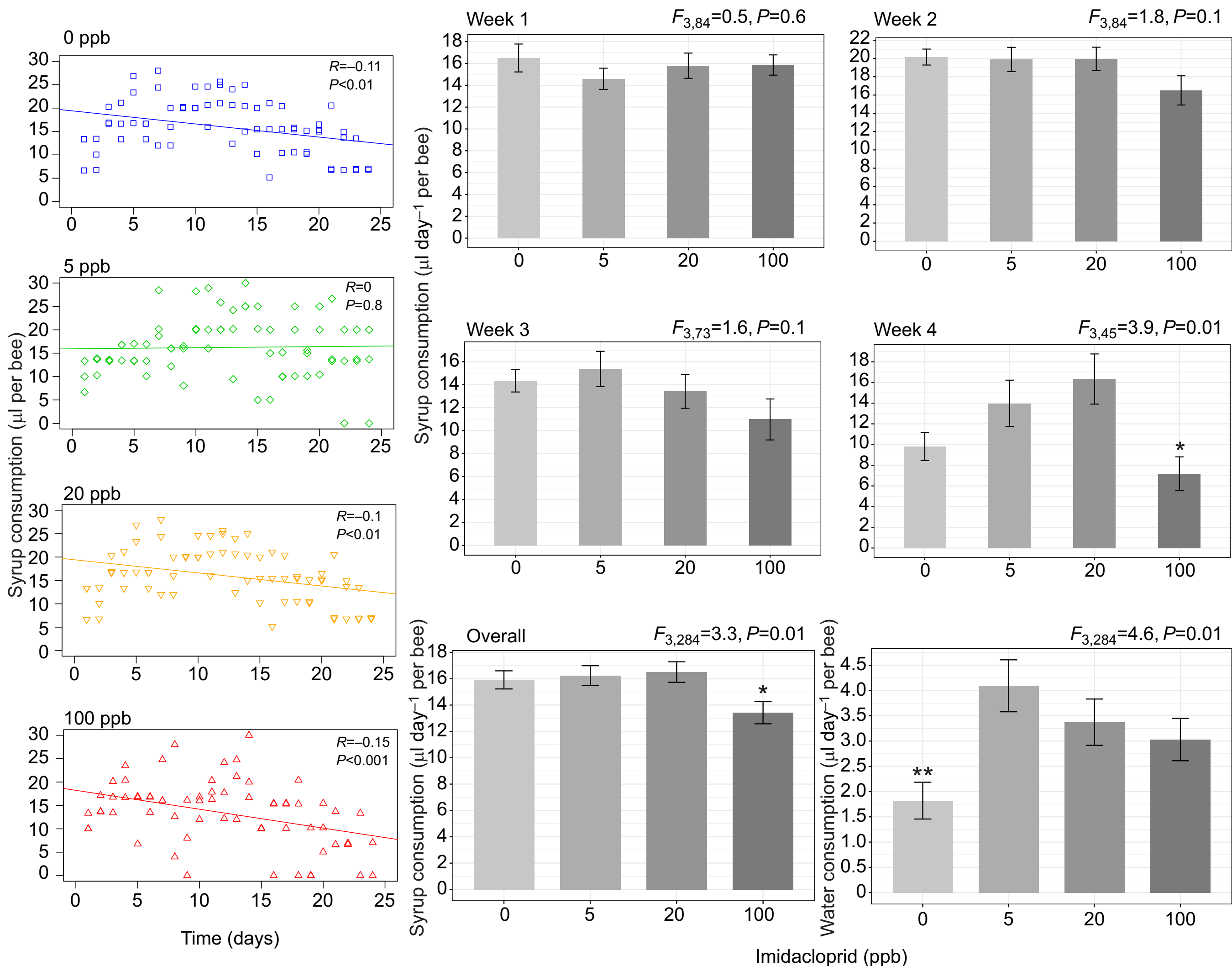
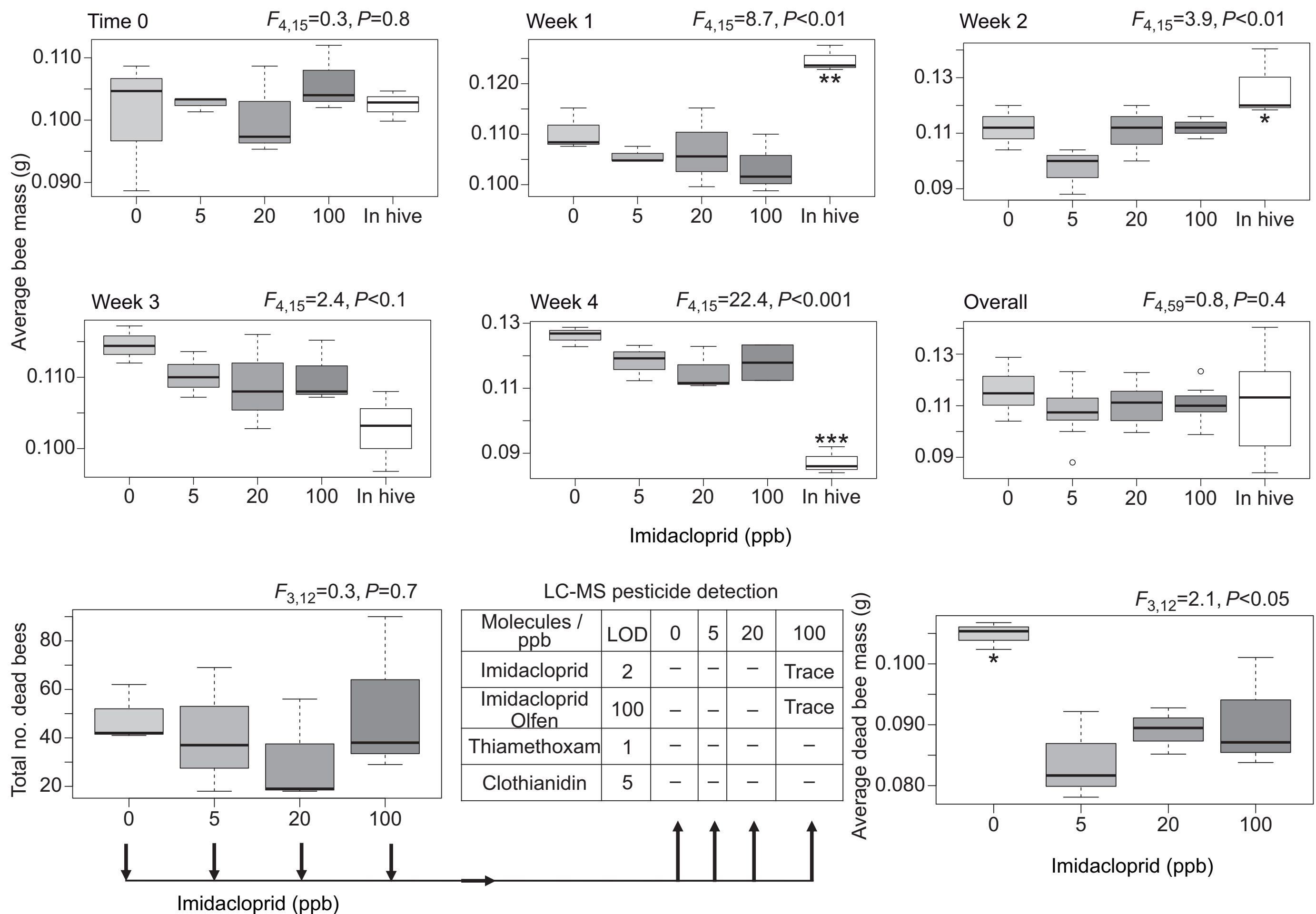


Fig. 2. Syrup consumption of treatment groups during the 24 day experiment. Left, scatterplots showing daily syrup consumption and Pearson correlation coefficient (R) are provided for each treatment category. Right, weekly and overall syrup intake and water intake calculated per treatment. ANOVA was conducted at a 95% confidence level and error bars represent the s.e.m. (*P<0.05; **P<0.01).



Molecules / ppb	LOD	0	5	20	100
Imidacloprid	2	–	–	–	Trace
Imidacloprid Olfen	100	–	–	–	Trace
Thiamethoxam	1	–	–	–	–
Clothianidin	5	–	–	–	–

Fig. 3. Average mass of bees at time 0 and sampled weekly from cages and hive for the different treatment groups. Average mass and total number of dead bees collected daily from cages are also shown. LC-MS screening for four neonicotinoid molecules conducted on the total number of dead bees collected for each treatment is displayed in the table with values of the limit of detection LOD. ANOVA was conducted at a 95% confidence level (*P<0.05; **P<0.01; ***P<0.001).

Honey bee oxidative stress

At week 1, caged bees of the control treatment (0 ppb) showed a significantly higher level of hydrogen peroxide (P<0.001) than all other groups including the hive bees (Fig. 4). Protein carbonyl content was significantly higher in caged bees given the highest concentration of imidacloprid (100 ppb; P<0.05) at week 1. However, the same assay applied on samples at week 4 showed higher carbonyl content in bees of the 5 ppb group (Fig. 4). Similar results were obtained for whole-bee body protein conducted on samples at week 4 (Fig. 4).

DEGs

In order to test the effect of both factors (imidacloprid and caging stress) on honey bee gene regulation, DEG analyses were carried out among caged bees only (imidacloprid versus control) and between caged and hive bees (caged versus hive). To gain an initial overview of the data, heatmaps for the whole dataset, including all samples, were generated. Major variation in color pattern was visually identifiable between the hive bee group and the rest of the samples (Fig. S1). We refined our analysis by running it on a weekly basis (Fig. 5). In the caged bees, imidacloprid showed no DEG at any time in the four studied weeks (Fig. S2). The only variation in gene regulation was found between caged and hive bees for both per-week (Fig. 5) and overall week data (Fig. S3). The biological coefficient of variance constantly distinguished two major groups of variables across the weeks with DEGs (in-hive bees versus caged bees, Fig. 5A–C). Heatmaps carried out weekly produced similar findings; genes with potentially different regulation are marked with asterisks in Fig. 5A–C. Based on their function (Table S1), antioxidant genes were the major DEGs in caged bees compared to hive bees in all weeks. At week 1, caged bees upregulated seven antioxidant genes and downregulated nine with no upregulation in the developmental and hormonal genes (Fig. 5A). Relatively similar regulation was observed in week 2 (Fig. 5B), with an increasing downregulation in all DEG categories up to week 4 (Fig. 5D). At week 4, upregulation of both immune defense and hormone genes depleted completely and a significant downregulation in all DEGs took place in caged bees. Gathering together the weekly DEG data in the caged bees, we identified constant upregulation of two antioxidant genes (*Rsod* and *Trx-1*) and downregulation of six others (*Trx-2*, *Nmdar1*, *Vg*, *CSP3*, *AChE-2* and *MsrA*; Table S2; Fig. 6) *Rsod* is a poorly known gene that encodes an uncharacterized protein of 1123 amino acids, while *Trx-1* is a gene encoding a major honey bee antioxidant of 136 amino acids and belongs to the thioredoxin family.

DISCUSSION

Honey bees are social insects, living together in highly organized colonies (Winston, 1987). Worker bees carry out different tasks

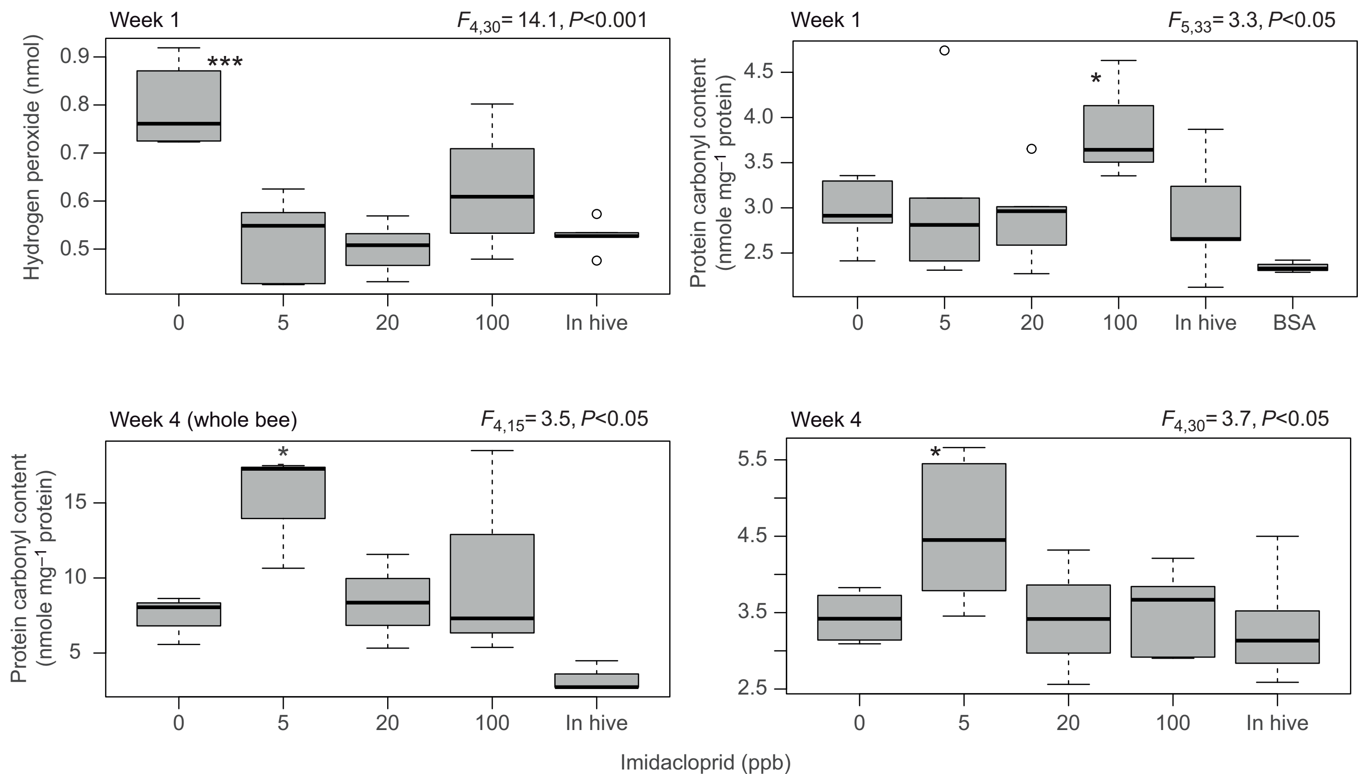


Fig. 4. Quantification of hydrogen peroxide and protein carbonyl content in caged and hive bees. The hydrogen peroxide assay was conducted on bees sampled at week 1 and the protein carbonyl assay was conducted on bees at week 1 and 4. Each boxplot represents an average of six biological replicates. Bovine serum albumin (BSA) data were not included in the statistical analysis. Error bars are quartiles and outliers; ANOVA levels of significance are *$P<0.05$ and ***$P<0.001$.

throughout their developmental cycle from nurses to foragers, a behavioral maturation that is mainly governed by gene regulation (Grozinger et al., 2003; Robinson, 2002). Cage experiments offer a more controllable environment and are widely used in honey bee behavioral and toxicological studies. Nevertheless, little is known about the physiological and gene regulation changes in bees when caged and deprived of hive conditions. In this study, we found that imidacloprid induced a slight effect on honey bee diet behavior; however, an overwhelming difference in gene regulation was observed in bees deprived of hive conditions. Cohorts of caged sister bees consumed the same amount of syrup during the first 3 weeks of the study, regardless of the imidacloprid dose (0, 5, 20, 100 ppb) in the syrup. There were negative correlations between syrup ingestion and time for all concentrations except 5 ppb. This correlation was more pronounced at the highest dose (100 ppb, $r=-0.15$, $P<0.001$; Fig. 2), and during week 4, bees consumed significantly less syrup in the 100 ppb imidacloprid group. Consequently, overall consumption was less for syrup at the 100 ppb dose ($P<0.05$). It is not clear whether bees sensed the presence of imidacloprid in the tainted syrup and avoided it (Meikle et al., 2016) or whether a post-ingestive aversion response previously described in other invertebrates occurred (Behmer et al., 2005). Bees exposed to imidacloprid showed similar survival to the control group ($P=0.7$; Fig. 4). Despite surviving the ingestion of imidacloprid, symptoms of insecticide toxicity such as increased grooming and ventilation, disorientation, slower activity and difficulty in flying (Williamson et al., 2014) were clearly observed in the treated bees. Although only traces of imidacloprid and its metabolite molecule (imidacloprid olfen) were detected in bees fed 100 ppb, imidacloprid did induce significant protein damage in honey bees at weeks 1 and 4 (Figs 3 and 4). Surprisingly, and presumably as a mechanism to minimize the effects of exposure to pesticides, treated bees consumed more water than the control group (Fig. 2). Dead bees in the control group weighed more than those exposed to imidacloprid ($P<0.05$; Fig. 4), but there were no differences in the mass of living bees (Fig. 3). Pairing these results together, it appears that the evaporation of water content from the bodies of dead bees explains this contradictory finding.

While caged bees showed no weekly differences in mass, hive bees exhibited completely different patterns (Fig. 3). The mass patterns of hive bees clearly reflect their physiological development from nurse to forager bees. Hive bees were significantly heavier than caged bees at week 1 as well as week 2 ($P<0.05$), until becoming equal in mass at week 3. When they switched to foraging behavior at week 4, hive bees lost significant mass ($P<0.001$) compared with the relatively inactive caged bees (Fig. 3). Overall, our data indicate that caging bees induces a profound alteration in their physiological development compared with their sister-mates operating under hive conditions.

The DEG analysis showed a clear divergence in gene regulation of both bee sets (caged versus hive). Based on the genes with the five highest FDR values found in our DEG study, gene upregulation is the predominant response during the first 2 weeks in caged bees followed by overwhelming downregulation at weeks 3 and 4 (Fig. 5C,D), which points to potential depletion in caged bees compared with that of bees in the hive. Upregulation of the gene group involved in the antioxidative process was recorded in caged bees as compared with hive bees at all times and particularly during the first 3 weeks (Fig. 5A–D). Some of those genes belong to the thioredoxin family, such as *Trx-1*, *Trx1-like2* and *Trxr-1*, as well as the glutaredoxin and glutathione families, all known to be involved

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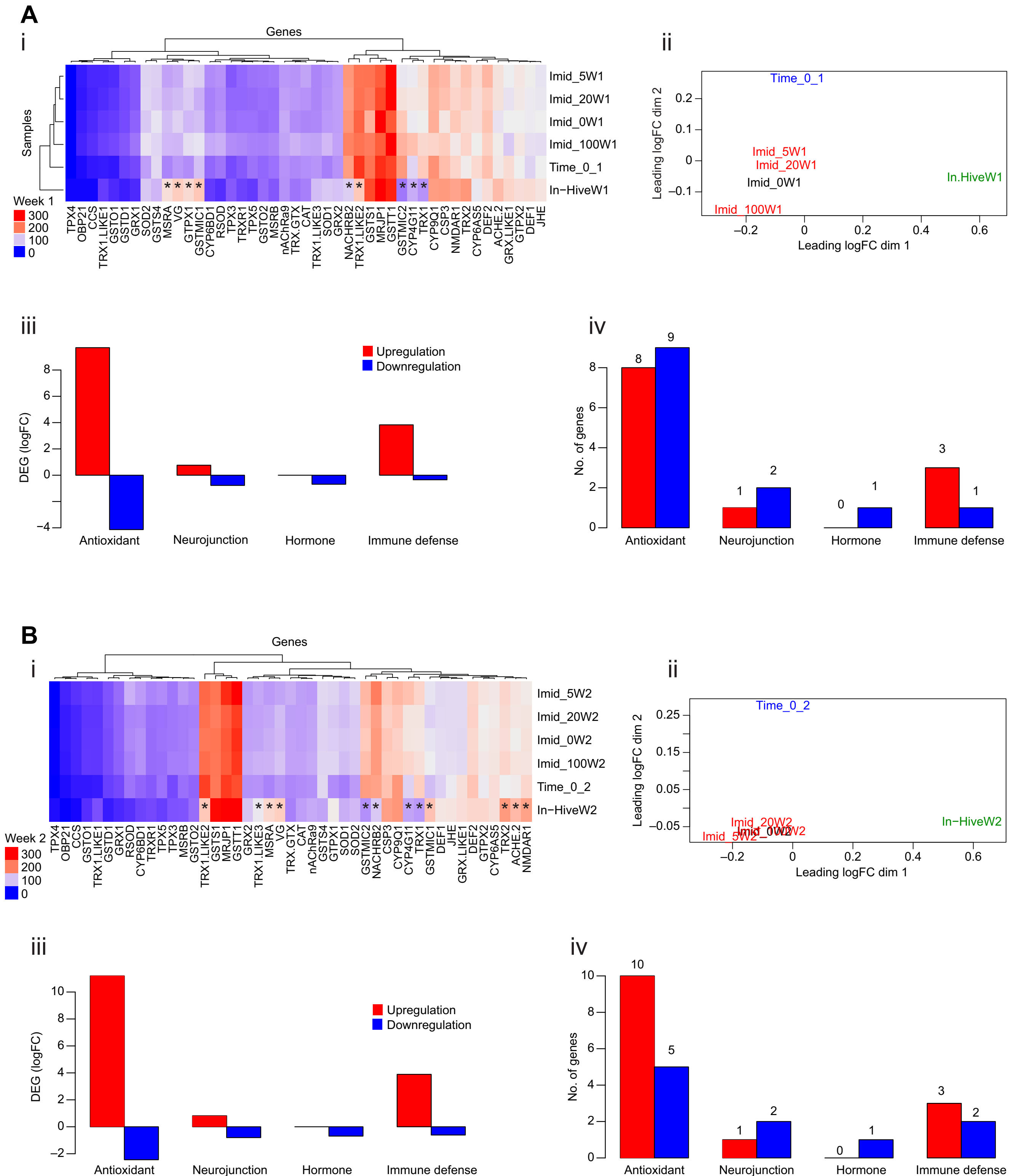

Fig. 5. Honey bee gene regulation. (A–D) Data for weeks 1–4, respectively. (i) Heatmap of all samples and target genes (45) using moderated log counts per million. Asterisks represent genes differentially expressed in hive bees. (ii) Multi-dimensional scaling (MDS) or biological coefficient of variance of the samples based on the log fold-change (logFC) between each pair of RNA samples. (iii,iv) Data sorted by gene function for differential gene expression studies (DEG; iii) and the number of genes regulated (iv) in caged bees compared with hive bees in week 1.

with *Apis cerana*'s response to oxidative stress (Meng et al., 2014). Fewer genes coding for other functions were found to be differentially expressed in caged bees, such as *Mrjp1*, *Fed1* and *Vg* (Table S2). Vitellogenin, a glycolipoprotein, which is often more abundant in nurse bees (Amdam et al., 2005; Ihle et al., 2009; Nelson et al., 2007), was among the six genes that were constantly downregulated (*Vg*, *CSP3*, *Trx2*, *Nmdar1*, *AChE-2*, *MsrA*) in caged bees throughout the 4 week experiment (Fig. 6). The honey bee

Journal of Experimental Biology

Fig. 5. Continued.

genome contains 21 genes encoding odorant-binding proteins (Obps; The Honeybee Genome Sequencing Consortium, 2006) which, beside chemosensory proteins (CSPs), mediate both bee perception and release of chemical stimuli and probably have other functions (Iovinella et al., 2011). Obp21 was only upregulated in caged bees at week 3, while *CSP3* was constantly downregulated, which suggest different functions of these genes. The constant downregulation of *AChE* suggests an absence of toxicity stress in caged bees as this gene was described in previous studies to be well linked to neonicotinoid toxicity (Alburaki et al., 2015; Badiou-Bénéteau et al., 2012). In this study, we identified two genes that were constantly upregulated in caged bees; *Rsod*, an

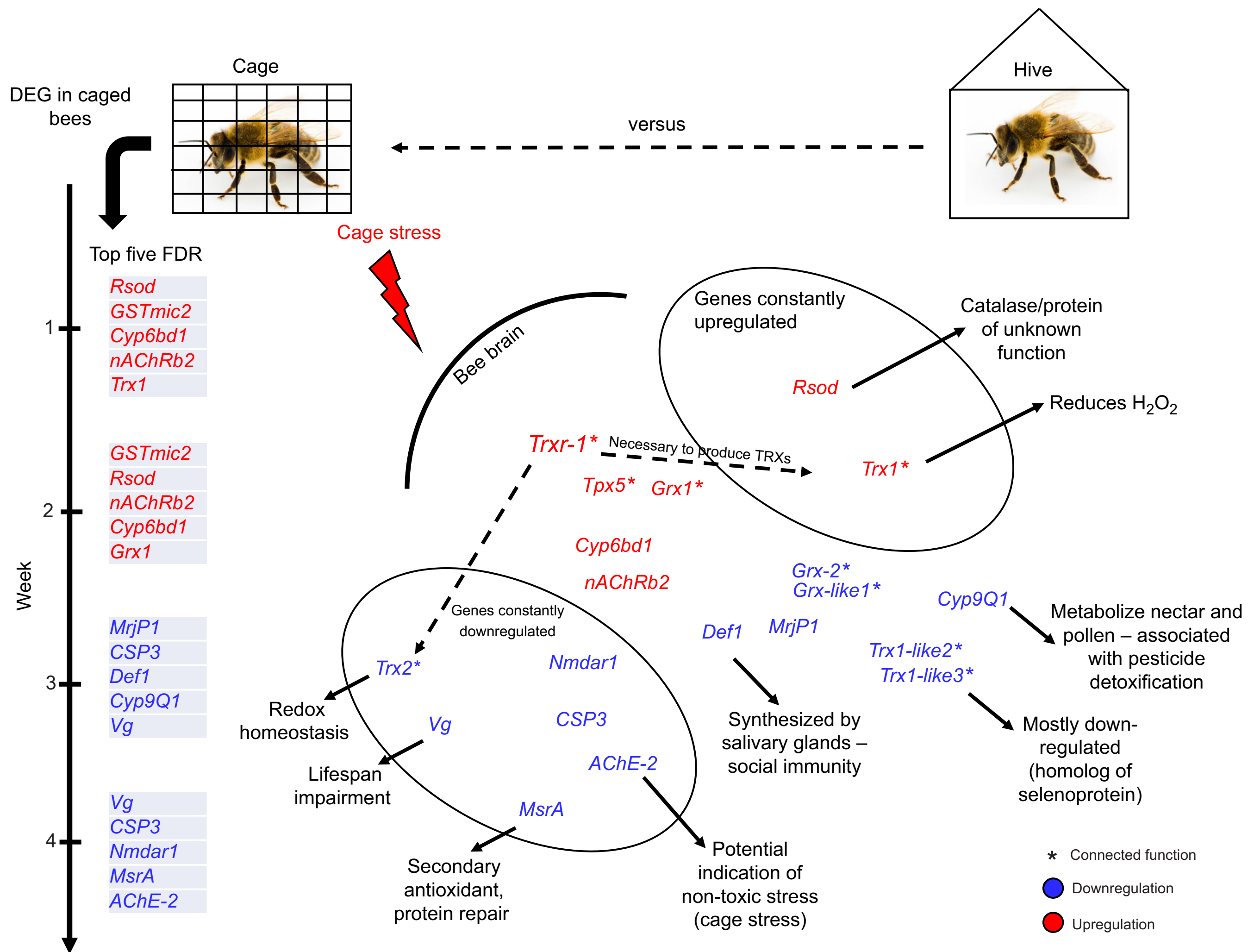

Fig. 6. Illustration of the DEG between caged and hive bees, emphasizing the cage stress factor. The gene regulation for caged bees and the top five significantly regulated genes (FDR<0.05) are displayed per week. Major potential functions for genes are described.

uncharacterized gene, and thioredoxin (*Trx1*) (Fig. 6; Table S2). *Rsod* was described in *Drosophila* as an atypical member of the Cu/ZNSOD family with a highly unusual number of introns (18) and a duplicated SOD domain. Homologous genes were described in *Apis*, protozoa and fish but not found in mammals (Corona and Robinson, 2006). Nonetheless, the function of this gene (*Rsod*) remains unknown in insects. The thioredoxin genes (*Trx*) encode small and highly conserved oxidoreducatse proteins and are required to maintain redox homeostasis of the cell (Holmgren et al., 2005). Thus, we assume that the caging stress challenge may have triggered the bee biological system to mitigate this stress and prevent further cellular damage. This assumption is supported by the significant upregulation of the antioxidant genes and higher protein carbonyl content in caged bees compared with hive bees.

In conclusion, our study provides new insights into honey bee gene regulation when bees are exposed to cage stress compared with a typical hive environment. We showed that major honey bee antioxidants were constantly upregulated when bees are caged, including genes with uncharacterized function such as *Rsod* and *Trx1*. Furthermore, to the best of our knowledge, this is the first time that eight genes have been reported to potentially characterize the honey bee caging stress while conducting cage experiments. Our results add a significant contribution to the body of knowledge related to the effect of stressors on honey bee gene regulation.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.A.; Methodology: M.A., S.K.; Software: M.A., K.L.; Validation: M.A., S.K., S.D.S.; Formal analysis: K.L.; Investigation: J.A.; Resources: S.K., K.L., J.A.; Data curation: S.K., K.L., J.A., S.D.S.; Writing - original draft: M.A.; Writing - review & editing: M.A., S.K., K.L., J.A., S.D.S.; Supervision: S.K., J.A., S.D.S.; Project administration: S.D.S.; Funding acquisition: S.K., K.L., J.A., S.D.S.

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Figure S1: Heatmaps for overall data, including caged bees, Time 0 bees and bees in-hive for all target genes. Both heatmaps are generated in order to visualize the highest similarity per target genes and samples, respectively.

Fig. S1

Heatmaps of Overall data / Sorted per sample and gene similarity

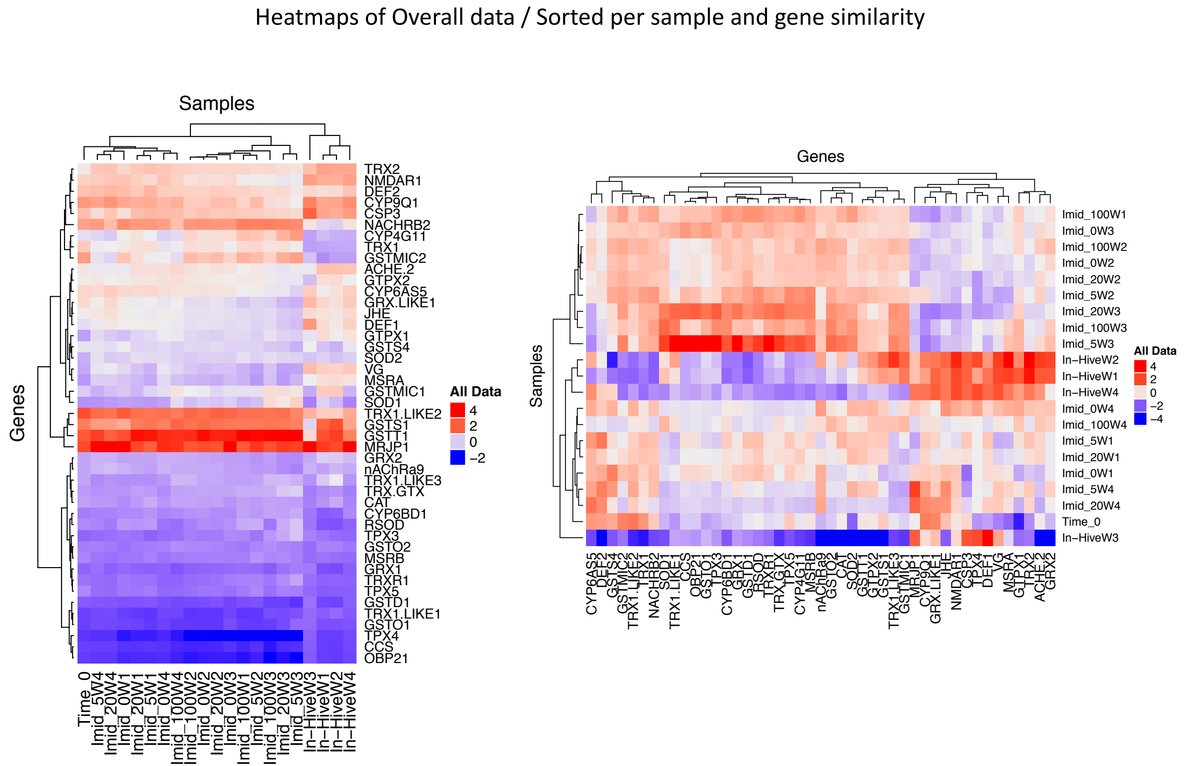

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Figure S2 Weekly analysis of gene expression for caged bees exposed to imidacloprid compared with the control (0 PPB). MDS plots are given per week as well as hierarchical clustering plots. Right set of plots shows no DEGs at any time point.

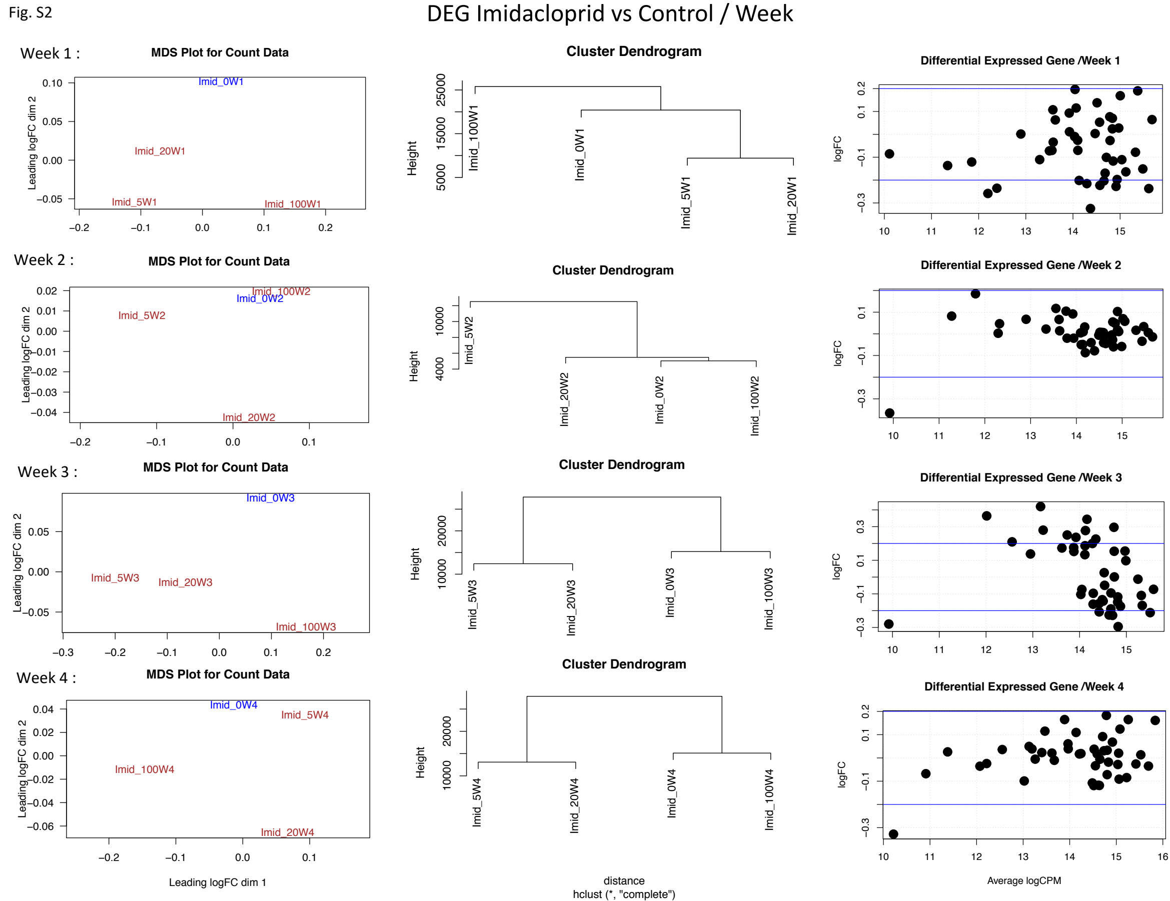

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Figure S3 Shows a multi-dimensional scaling MDS of the DEG between Cage and Hive bees based on log-fold-changes between each pair of RNA samples as well as an hierarchical clustering plot of the same dataset.

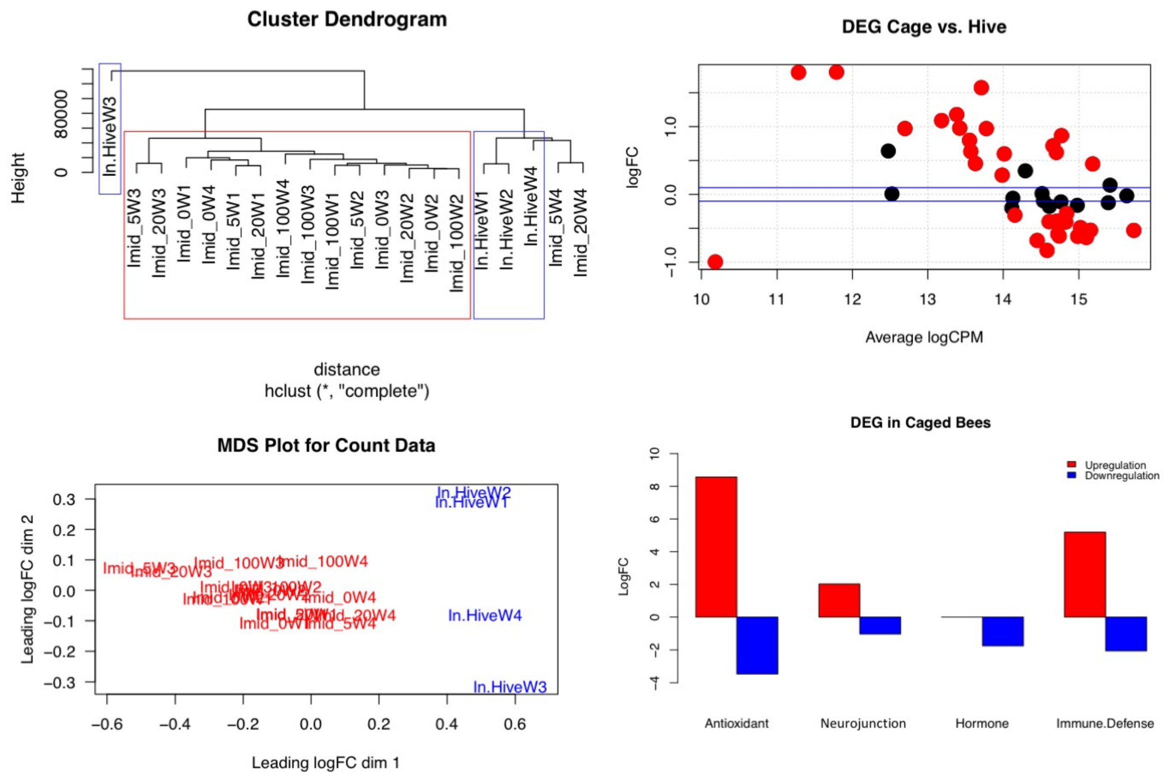

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Figure S4: General view of the cages used in this experiment. Protein patties were provided to caged bees inside the red robber plugs, water and syrup treatment through both upper syringes. View of bees feeding on syrup and patty inside the cages.

Fig. S4

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Table S1. Descriptive list of the studied genes targeted by RNA-seq

N	Gene code	Description/known gene function	Gene ID: NCBI/Beebase	References
Target genes				
Antioxidant				
1	Cat	Catalase	443552	Alburaki et al., 2017a
2	GstO1	Pyrimidodiazepine synthase	552118	Corona and Robinson, 2006
3	GstO2	Glutathione S-transferase omega-1	726823	Corona and Robinson, 2006
4	GstS1	Glutathione S-transferase S1	552304	Corona and Robinson, 2006; Liu et al., 2016
5	GstS4	Glutathione S-transferase S4	411045	Alburaki et al., 2017a; Corona and Robinson, 2006
6	GSTmic1	Microsomal glutathione S-transferase 1	410837	Corona and Robinson, 2006
7	GSTmic2	Uncharacterized LOC725853/Protein coding	725853	Corona and Robinson, 2006
8	GstT1	Glutathione S-transferase theta-1	552314	Corona and Robinson, 2006
9	Gtpx1	Glutathione peroxidase-like 1	494523	Corona and Robinson, 2006
10	Gtpx2	Probable phospholipid hydroperoxide glutathione peroxidase	726269	Corona and Robinson, 2006
11	GstD1	Glutathione S-transferase D1	409490	Alburaki et al., 2017a; Corona and Robinson, 2006
12	Sod1	Superoxide dismutase 1	409398	Corona and Robinson, 2006
13	Sod2	Superoxide dismutase 2	410082	Corona and Robinson, 2006
14	MsrA	Methionine sulphoxide reductase A	409097	Corona and Robinson, 2006
15	MsrB	Methionine sulphoxide reductase B	724494	Corona and Robinson, 2006
16	Trx-1	Thioredoxin, mitochondrial	410120	Corona and Robinson, 2006
17	Trx-2	Thioredoxin-2	409451	Corona and Robinson, 2006
18	Trx/Gtx	Glutaredoxin 3	409355	Corona and Robinson, 2006
19	Trx1-like1	Thioredoxin-like protein 1	550734	Corona and Robinson, 2006
20	Trx1-like2	Uncharacterized LOC725664/Protein coding	725664	Corona and Robinson, 2006
21	Trx1-like3	Endoplasmic reticulum resident protein 44	552191	Corona and Robinson, 2006

N	Gene code	Description/known gene function	Gene ID: NCBI/Beebase	References
22	Trxr-1	Thioredoxin reductase 1	410032	Corona and Robinson, 2006
23	Tpx3	Thioredoxin peroxidoase 3	408540	Corona and Robinson, 2006
24	Tpx4	Thioredoxin peroxidoase 4	551975	Corona and Robinson, 2006
25	Tpx5-6	Peroxiredoxin-6	411852	Corona and Robinson, 2006
26	CCS	Copper chaperone for superoxide dismutase	552629	Corona and Robinson, 2006
27	Grx-like1	Uncharacterized LOC411159/Protein coding	411159	Corona and Robinson, 2006
28	Grx1	Glutaredoxin-C4	727309	Corona and Robinson, 2006
29	Grx2	Glutaredoxin-related protein 5, mitochondrial	552835	Corona and Robinson, 2006
30	Rsod	Uncharacterized LOC413369/Protein coding	413369	Corona and Robinson, 2006
Immune defense/sensory				
31	Def1	Defensin-1	406143	Aronstein and Saldivar, 2005; Richard et al., 2012
32	Def2	Defensin-2	413397	Aronstein and Saldivar, 2005; Richard et al., 2012
33	CSP3/ASP	Chemosensory protein 3	406094	Briand et al., 2002
34	Cyp4g11	Cytochrome P450 4G11	409469	Gong and Diao, 2017
35	Cyp6as5	Cytochrome P450 6AS5	409677	Alptekin et al., 2016
36	Cyp6bd1	Cytochrome P450 6k1	551560	Li et al., 2014
37	Cyp9q1	Cytochrome P450 9e2	410492	Mao et al., 2011
38	Obp21	Odorant binding protein 21	551935	Iovinella et al., 2011
Development/hormone				
39	Vg	Vitellogenin	406088	Amdam et al., 2006; Bordier et al., 2017; Nelson et al., 2007
40	Jhe/Est	Juvenile hormone esterase	406066	Bordier et al., 2017
41	Mrjp1	Major royal jelly protein1		Buttstedt et al., 2014
Neuro-junction				

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N	Gene code	Description/known gene function	Gene ID: NCBI/Beebase	References
42	AChE-2	Acetylcholinesterase 2	406104	Alburaki et al., 2017a
43	nAChRa9	Nicotinic acetylcholine receptor alpha9 subunit	411303	Alptekin et al., 2016
44	nAChRb2	Nicotinic acetylcholine receptor beta2 subunit	726079	Alptekin et al., 2016
45	Nmdar1	NMDA receptor 1	406079	Mussig et al., 2010
Housekeeping genes				
1	Ancr1	AncR-1 non-coding nuclear RNA	100049571	Alburaki et al., 2017a
2	Camkii	Calcium/calmodulin-dependent protein kinase II	551691	Alburaki et al., 2017a
3	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase 2	XM_393605.6	Alburaki et al., 2017a
4	E2F	Transcription factor E2F4	XM_006566781.2	Alburaki et al., 2017a

Genes included are involved in various processes: antioxidant, immune defense, nervous system regulation and hormone production. Target genes were standardized against four housekeeping genes.

Table S2. Differentially expressed genes (DEGs) between caged bees and in-hive bees across 4 weeks

Gene		FDR	logFC	Regulation
Week 1				
1	Rsod	8.79E-11	2.052154955	Up
2	GSTmic2	1.51E-10	1.283773182	Up
3	cyp6bd1	5.62E-07	1.502223105	Up
4	nAChRb2	1.41E-06	0.760356888	Up
5	Trx-1	1.79E-05	0.815020997	Up
6	Vg	2.85E-05	−0.68666675	Down
7	GSTmic1	0.000105929	−0.625977048	Down
8	Grx1	0.000147178	1.466580514	Up
9	MsrA	0.000147178	−0.640895526	Down
10	Tpx5	0.000326753	1.151666352	Up
11	GstD1	0.000529584	1.626628738	Up
12	Cyp4g11	0.003272353	0.538929922	Up
13	Trx1-like2	0.004426979	0.409447152	Up
14	Trxr-1	0.004426979	0.894300369	Up
15	Gtpx1	0.007143152	−0.45210316	Down
16	Trx1-like3	0.007416819	−0.53308333	Down
17	Trx-2	0.007416819	−0.396484705	Down
18	Nmdar1	0.010552677	−0.390262699	Down
19	AChE-2	0.013164542	−0.384722407	Down
20	CCS	0.013164542	1.789933422	Up
21	Sod1	0.022290396	−0.443546	Down
22	CSP3	0.029977795	−0.347780712	Down
23	Grx-like1	0.038464198	−0.345534644	Down
24	Grx2	0.044258894	−0.395457615	Down
25	GstS1	0.045643338	−0.303379857	Down

Gene		FDR	logFC	Regulation
				Up=12 Down=13
Week 2				
1	GSTmic2	5.63E-10	1.125683012	Up
2	Rsod	1.38E-09	1.920755161	Up
3	nAChRb2	1.34E-07	0.816655652	Up
4	Cyp6bd1	2.17E-06	1.359483887	Up
5	Grx1	6.34E-06	1.676160098	Up
6	MsrA	6.34E-06	–0.750403485	Down
7	Trx1	1.50E-05	0.793851856	Up
8	Vg	1.50E-05	–0.691147669	Down
9	Cyp4g11	3.29E-05	0.736912478	Up
10	GstD1	4.01E-05	1.850627106	Up
11	Tpx5	0.000248075	1.141077705	Up
12	Nmdar1	0.001369615	–0.462428888	Down
13	Trx1-like2	0.001746155	0.435546394	Up
14	Trx1-like3	0.002126055	–0.540989241	Down
15	Trx2	0.003455925	–0.412143627	Down
16	GSTmic1	0.004504549	–0.442902891	Down
17	MsrB	0.005116035	0.773936843	Up
18	Trxr-1	0.008514979	0.807396999	Up
19	CCS	0.012821076	1.79540416	Up
20	GstS1	0.012821076	–0.29896178	Down
21	Tpx3	0.021384737	0.670350958	Up
22	AChE-2	0.022404618	–0.339632338	Down
23	Cyp9q1	0.022960491	–0.314596096	Down
24	CSP3	0.036059913	–0.295797369	Down
				Up=14 Down=10

	Gene	FDR	logFC	Regulation
Week 3				
1	Mrjp1	8.48E-30	−1.334501094	Down
2	CSP3	8.01E-23	−1.378281782	Down
3	Def1	4.70E-21	−1.48284558	Down
4	Cyp9q1	7.19E-17	−1.26844048	Down
5	Vg	1.03E-16	−1.415441134	Down
6	Nmdar1	3.76E-15	−1.219803445	Down
7	Grx-like1	5.39E-15	−1.306168202	Down
8	Cyp6as5	9.79E-08	−0.932079671	Down
9	Sod1	3.25E-07	1.453492666	Up
10	Trx2	3.33E-07	−0.843916112	Down
11	Jhe	3.50E-07	−0.926640082	Down
12	MsrA	3.26E-06	−0.912448636	Down
13	GstS4	6.02E-05	−0.787091217	Down
14	MsrB	0.000167022	1.515519968	Up
15	Trx/Gtx	0.000168527	1.189757184	Up
16	GstO2	0.000209972	1.510206221	Up
17	DefF2	0.000292519	−0.640178689	Down
18	Cyp4g11	0.000370339	0.825928994	Up
19	Trxr-1	0.000606566	1.441878171	Up
20	Rsod	0.000842094	1.255522862	Up
21	Cyp6bd1	0.000895332	1.107718079	Up
22	Tpx5	0.000895332	1.490533886	Up
23	Gtpx1	0.004267108	−0.562430366	Down
24	Trx1-like2	0.004267108	−0.441305624	Down
25	Obp21	0.006305542	3.045853631	Up
26	CCS	0.007839115	2.109938747	Up

Gene		FDR	logFC	Regulation
27	Trx1	0.007928216	0.629539606	Up
28	AChE-2	0.013108948	–0.479519602	Down
29	Tpx3	0.014996185	0.803097403	Up
30	Cat	0.017555048	0.759332849	Up
31	GstS1	0.042206547	0.381846224	Up
32	GstO1	0.048847638	0.957056383	Up
				Up=16 Down=16
Week 4				
1	Vg	0.001242008	–0.606664482	Down
2	CSP3	0.00252203	–0.503672436	Down
3	Nmdar1	0.002523729	–0.489465881	Down
4	MsrA	0.006270594	–0.542183971	Down
5	AChE-2	0.007054297	–0.467422182	Down
6	GSTmic2	0.007054297	0.635050548	Up
7	Trx2	0.007054297	–0.430007244	Down
8	Grx-like1	0.012104495	–0.444598377	Down
9	Def1	0.01305566	–0.44637647	Down
10	Trx1	0.015380605	0.546124297	Up
11	Rsod	0.016919141	0.877252547	Up
12	Gtpx1	0.020410695	–0.422238441	Down
13	Cyp9q1	0.028824892	–0.374761958	Down
14	Tpx5	0.028824892	0.875701234	Down
				Up=3 Down=11
Overall weeks				
1	Trx1	2.31E-13	0.716399134	Up
2	MsrA	5.77E-13	–0.679235231	Down
3	Rsod	5.82E-12	1.574315294	Up

Gene		FDR	logFC	Regulation
4	Vg	5.82E-12	−0.826047318	Down
5	GSTmic2	2.35E-09	0.867077354	Up
6	Tpx5	2.98E-09	1.177112211	Up
7	Trx2	6.22E-09	−0.490134369	Down
8	Nmdar1	1.49E-08	−0.617713476	Down
9	CSP3	3.57E-08	−0.634702226	Down
10	Cyp6bd1	7.29E-08	0.970961205	Up
11	Cyp4g11	3.27E-07	0.618538837	Up
12	AChE-2	1.33E-06	−0.40628725	Down
13	Grx-like1	1.33E-06	−0.584585069	Down
14	Gtpx1	1.41E-06	−0.401699776	Down
15	MsrB	1.41E-06	0.798387461	Up
16	Def1	1.56E-06	−0.613786308	Down
17	Trxr-1	9.85E-06	0.977293279	Up
18	Grx1	1.33E-05	1.089440238	Up
19	Trx/Gtx	1.53E-05	0.598159979	Up
20	Cyp9q1	2.37E-05	−0.529039749	Down
21	CCS	6.18E-05	1.802497238	Up
22	GstD1	8.11E-05	0.971662373	Up
23	nAChRb2	9.14E-05	0.449655327	Up
24	Mrjp	9.30E-05	−0.530617812	Down
25	Jhe	0.000116388	−0.392980093	Down
26	Obp21	0.00012842	1.797821637	Up
27	GstO2	0.000806508	0.455560596	Up
28	Grx2	0.002324083	−0.305279916	Down
29	Tpx3	0.008824973	0.633043926	Up
30	Cat	0.009449226	0.284131552	Up

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	Gene	FDR	logFC	Regulation
31	Cyp6as5	0.012545948	–0.281873672	Down
32	Tpx4	0.030356733	–0.995920357	Down
				Up=17 Down=15

Genes are sorted descending from most (false discovery rate, FDR<0.001) to least (FDR<0.05) significant differential. logFC is the log fold-change and the regulation is for bees in cages. Table generated by the EdgeR package.