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Insecticides cause transcriptional alterations of endocrine related genes in the brain of honey bee foragers

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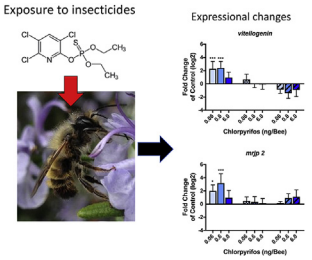
HIGHLIGHTS

- Endocrine activities of pesticides can be assessed in honey bee foragers.
- Pesticides caused transcriptional alterations in brain and HPGs indicative of endocrine activity.
- Chlorpyrifos showed strongest transcriptional alterations.
- Affected genes encode proteins involved in transition of nurse and forager bees.
- *Buffy* and *mrjp* down-regulation and *hbg3 and ilp1* up-regulation are potential endocrine indicators.

GRAPHICAL ABSTRACT



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ABSTRACT

Bees are exposed to endocrine active insecticides. Here we assessed expressional alteration of marker genes indicative of endocrine effects in the brain of honey bees. We exposed foragers to chlorpyrifos, cypermethrin and thiacloprid and assessed the expression of genes after exposure for 24 h, 48 h and 72 h. Chlorpyrifos caused the strongest expressional changes at 24 h characterized by induction of *vitellogenin*, *major royal jelly protein* (*mrjp*) *2* and *3*, *insulin-like peptide* (*ilp1*), *alpha-glucosidase* (*hbg3*) and *sima*, and down-regulation of *buffy*. Cypermethrin caused minor induction of *mrjp1, mrjp2, mmp1* and *ilp1*. The *sima* transcript showed down-regulation at 48 h and up-regulation at 72 h. Exposure to thiacloprid caused down-regulation of *vitellogenin*, *mrjp1* and *sima* at 24 h, and *hbg3* at 72 h, as well as induction of *ilp1* at 48 h. The *buffy* transcript was down-regulated at 24 h and up-regulated at 48 h. Despite compound-specific expression patterns, each insecticide altered the expression of some of the suggested endocrine system related genes. Our study suggests that expressional changes of genes prominently expressed in nurse or forager bees, including down-regulation of *buffy* and *mrjps* and up-regulation of *hbg3 and ilp1* may serve as indicators for endocrine activity of insecticides in foragers.

1. Introduction

The significant decline of insects (Hallmann et al., 2017) and arthropods in biomass and diversity (Seibold et al., 2019) in many countries is of concern, particularly for bees (Lee et al., 2015;

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Ollerton et al., 2014). Although caused by multiple factors (Cameron et al., 2011; Goulson, D.; Nicholls, E.; Botías, C.; Rotheray, 2015; Grab et al., 2019), an important reason is the exposure to insecticides, which in turn has consequences for bird population impacts (Eng et al., 2019). Bees are exposed to many different pesticides applied in agriculture (Gill, R. J.; Ramos-Rodrigeuz, O.; Raine, 2012), including insecticides of the neonicotinoid, pyrethroid and organophosphate class (Mullin et al., 2010; Sanchez-Bayo and Goka, 2014). Besides acute toxicity, these insecticides cause chronic toxicity to the nervous (Decourtye et al., 2004) and immune system of bees (Di Prisco et al., 2013) or on energy allocation (Christen et al., 2019). These plant protection products may affect bee populations by neurotoxicity and associated losses of memory and orientation as documented for neonicotinoids (Henry et al., 2012; Rundlöf et al., 2015; Tsvetkov et al., 2017). Reduction of homing success of honey bees was demonstrated for the neonicotinoid thiacloprid (Tison et al., 2016).

Insecticides are frequently detected as residues in pollen, wax and bees but also in honey. Chlorpyrifos is an organophosphate insecticide that is among the mostly applied insecticide worldwide in agriculture, particularly in orchards, citrus fruits, vegetables and in vineyards. Due to potential human health effects, application of chlorpyrifos has been banned in Switzerland, European countries and in California in 2019. Developmental neurotoxicity has been observed in children (Rauh et al., 2012) and *in vitro* in PC-12 cells (Christen et al., 2017). The mode of action of organophosphates is the inhibition of synaptic acetylcholine esterase. The pyrethroid cypermethrin is also frequently used in many crops and acts by prolonging the open phase of sodium channels in nerve cells. The neonicotinoid thiacloprid activates nicotinic acetyl choline receptors and is now banned as other highly toxic neonicotinoids for outdoor applications in Europe. As other neonicotinoids, thiacloprid may harm bee populations (Ellis et al., 2017). Due to frequent application of insecticides and widespread contamination of pollen (Ostiguy et al., 2019; Sanchez-Bayo and Goka, 2014), it is important to assess potential sublethal adverse effects of these insecticides.

Insecticides can affect the endocrine system, which may compromise the reproduction capacity by decreasing the fertility of queens and drones and ultimately compromise populations. Reproductive effects of these compounds are of growing concern (Christen et al., 2018a). Among others, endocrine disrupting effects were reported in queens by neonicotinoids (Williams et al., 2015). Furthermore, in drones, fertility impairment were reported by fipronil (Kairo et al., 2017) and by the neonicotinoids thiamethoxam and clothianidin (Straub et al., 2016). Among sublethal effects, deltamethrin reduced the fecundity in bees and increased the immature period (Dai et al., 2010). At present, it is unknown whether other insecticides including chlorpyrifos, cypermethrin and thiacloprid cause adverse endocrine effects in bees.

Endocrine effects cannot only be assessed in drones and queens but also in worker bees (Christen et al., 2018a). This poses significant technical and practical advantages. Nurse and forager bees do not only show behavioral and functional disparities but significant differences in the activity and gene expression profile of the hypopharyngeal glands (HPGs) (Ueno et al., 2015) and in the head (Liu et al., 2019). In nurse bees, hypopharyngeal glands secrete mainly major royal jelly proteins (mrps), while forager hypopharyngeal glands secrete mainly α-glycosidase III, an enzyme that converts sucrose into glucose and fructose. Additionally, different genes are expressed in the different castes. As a result, for a given caste, indicator genes in the hypopharyngeal glands were proposed. Nurse bee specific HPG genes are *mrjp2* and *buffy*, and forager hypopharyngeal gland-selective genes are *hbg3* encoding α-glucosidase III, and *mmp1* encoding a matrix metalloproteinase 1 homolog.

Our hypothesis is therefore that expressional alteration of selected genes can be indicative of endocrine disruption in forager bees. Confirmation comes from one study, demonstrating that the insecticide methoprene led to induction of forager-selective genes and repression of nurse bee-selective transcripts (Ueno et al., 2015). Another basis of our hypothesis is the fact that a high number of transcripts differ between nurses and foragers. Thus, expression of specific transcripts may play an important role in behavioral transition in honey bees, and therefore, transcriptional alterations of selected target genes may serve as indicators of endocrine activity of insecticides.

We hypothesize that alteration of a specific set of genes may be indicative for endocrine disruptive effects in worker bees, as foragers can regress to a more nurse-like behavior and physiology. Among the targeted genes are those that show high expression in either nurse or forager bees and its alteration may transmit to physiological and behavioral changes of these castes. The hypothesis is outlined in Fig. S1 (supplementary material) with the proposed set of genes that may be indicative for endocrine effects. Some of these genes have previously been analyzed, which allows a comparison to other insecticides.

The aim of our study was to test this hypothesis by experimental exposure of honey bee foragers of mixed age to different concentrations of the insecticides chlorpyrifos, cypermethrin and thiacloprid that are suggested to potentially show endocrine-disrupting activities. These pesticides were also selected on the basis of its high use, occurrence in pollen and known alterations in gene expression in the brain (Christen et al., 2018, 2016; Christen and Fent, 2017). Chlorpyrifos is a highly used organophosphate insecticide in agriculture and household application worldwide. This also holds for the pyrethroid insecticide cypermethrin and the neonicotinoid thiacloprid. Due to adverse effects in the environment and concerns about human health impacts, chlorpyrifos and thiacloprid were banned for agricultural application in Switzerland and other European countries in 2020.

2. Materials and methods

2.1. Chemicals

Cypermethrin (CAS No. 52315-07-8), chlorpyrifos (CAS No. 2921-88-2) and thiacloprid (CAS No. 111988-49-9) were purchased by Sigma−Aldrich (Buchs, Switzerland). DMSO was used to prepare stock solutions of all compounds. Stock solutions were diluted into 20% sucrose solution. The final DMSO concentrations in sucrose solution was 0.1%.

2.2. Experimental design of laboratory exposures

For comparison with data from previous studies and due to easier handling, including practical implications, we focused on forager bees. Adult forager honey bees (*Apis mellifera carnica*) of mixed age were exposed to different concentrations of the insecticides chlorpyrifos, cypermethrin and thiacloprid that were selected due to their high use, transcriptional effects in the brain and potential endocrine effects. Basis for selecting exposure concentrations was their sublethal levels as previously determined (Christen et al., 2016; Christen and Fent, 2017). The concept is depicted in Fig. S1 in the supplementary material. Reported oral LD50 values of chlorpyrifos and cypermethrin were 70 ng/bee and 300 ng/bee, respectively (Sanchez-Bayo and Goka, 2014). Our exposure concentrations were 0.06, 0.6 and 6 ng/bee and 0.3, 3 and 30 ng/bee, respectively. The LD50 value of thiacloprid was 14600 ng/bee (Iwasa et al., 2004) and our nominal exposure

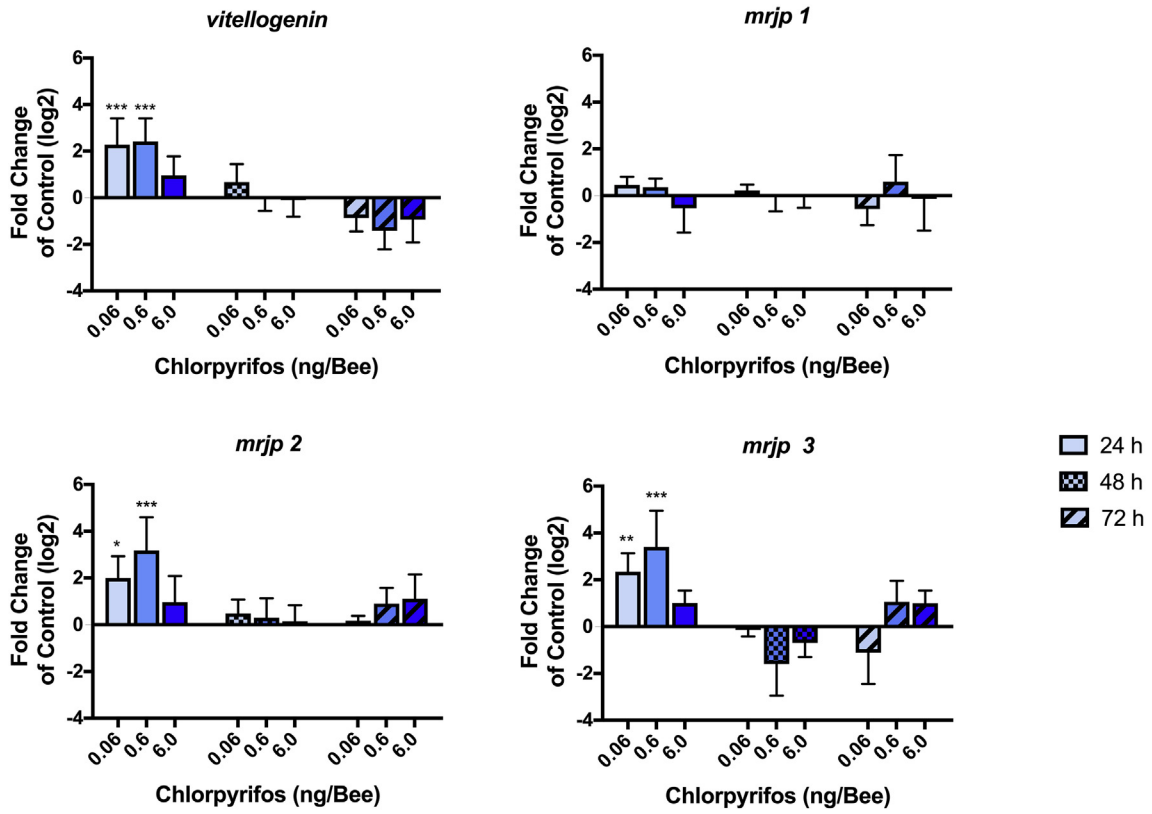

Fig. 1. Abundance of transcripts *vitellogenin*, *mrjp 1*, *mrjp 2* and *mrjp 3* in the brain of honey bees following exposure to three different concentrations of chlorpyrifos for 24 h (plain bars), 48 h (squares) and 72 h (diagonal strips). Shown are means with standard deviation of five biological replicates per concentration and exposure time. Significant differences with *p*-value ≤ 0.05 are marked with one asterisk, $p \leq 0.01$ with two asterisks, $p \leq 0.001$ with three asterisks.

concentrations were 25, 250 and 2500 ng/bee.

We performed experiments with foragers with different ages due to limitation of manpower and resources. This may have resulted in a certain variability in the responses. Honey bee foragers of mixed age (22–35 days old) were taken in June 2019 for insecticide exposures from one outdoor colony from a location with no agricultural activity and pesticide use. Collection of individual honey bees, transportation to the laboratory and exposure to pesticides were done as previously (Christen et al., 2016) with some minor modifications. As in previous exposures, insecticides were dissolved in sucrose solution containing 0.1% DMSO (Christen et al., 2016) with a slight modification in that 8 instead of 10 bees per bottle were used and the consumption of 160 μL thiacloprid solution was controlled followed by uncontaminated sucrose feeding.

In detail, after distribution of 8 bees each to PET bottles, they were fed overnight with 2 mL 20% sucrose solution. The next day, bees in each PET bottle (8 bees) were fed with 160 μL of a 20% sucrose solution containing the appropriate insecticide concentrations or 0.1% DMSO (solvent control) (Table S1). After bees consumed the 160 μL sucrose solution (pesticide exposure or solvent control exposure), they were fed with uncontaminated 20% sucrose solution until the next day. This exposure was repeated each day for 72 h until termination of the experiment.

Forager bees were exposed via sucrose solution to the organophosphate chlorpyrifos, the pyrethroid cypermethrin and the neonicotinoid thiacloprid at three different concentrations for three different times of exposure (24, 48 and 72 h) to explore and compare concentration- and time-related effects on the expression of genes indicative of an endocrine disruptive effect in brain and HPG (Christen et al., 2018a).

Each exposure experiment consisted of five replicate PET bottles with 8 bees per concentration and exposure time. For sampling, five bees per bottle were pooled to obtain one RNA sample per replicate. Thus, one pooled RNA sample of each bottle yielded five biological replicates per pesticide concentration. Bees were frozen after sampling at −20 °C until RNA extraction. A summary of used concentrations expressed as ng/bee is given in Table S1. No compound related mortality occurred during exposure.

2.3. RNA isolation, reverse transcription, and quantitative RT-qPCR

The brain of still frozen bees was removed in total by opening the cranium using scalpel and forceps Brains of five bees were pooled for RNA isolation. Total RNA was isolated from the brains using TRI Reagent® (Sigma-Aldrich, Buchs, Switzerland) according to manufacturer's instructions. 1000 ng RNA were reverse transcribed and RT-qPCR based on SYBR green fluorescence (SYBR green PCR master mix; Roche) as described previously (Christen et al., 2016). Primer sequences of used primers are given in Table S2. For normalisation of expressional changes, *ribosomal protein S5* (*rpS5*) was used as house-keeping gene, as this gene shows constant expression and was successfully used in our previous studies. Alterations of mRNA abundance in pesticide exposed samples were compared against the solvent control (0.1% DMSO) samples to determine the effects of compound exposures.

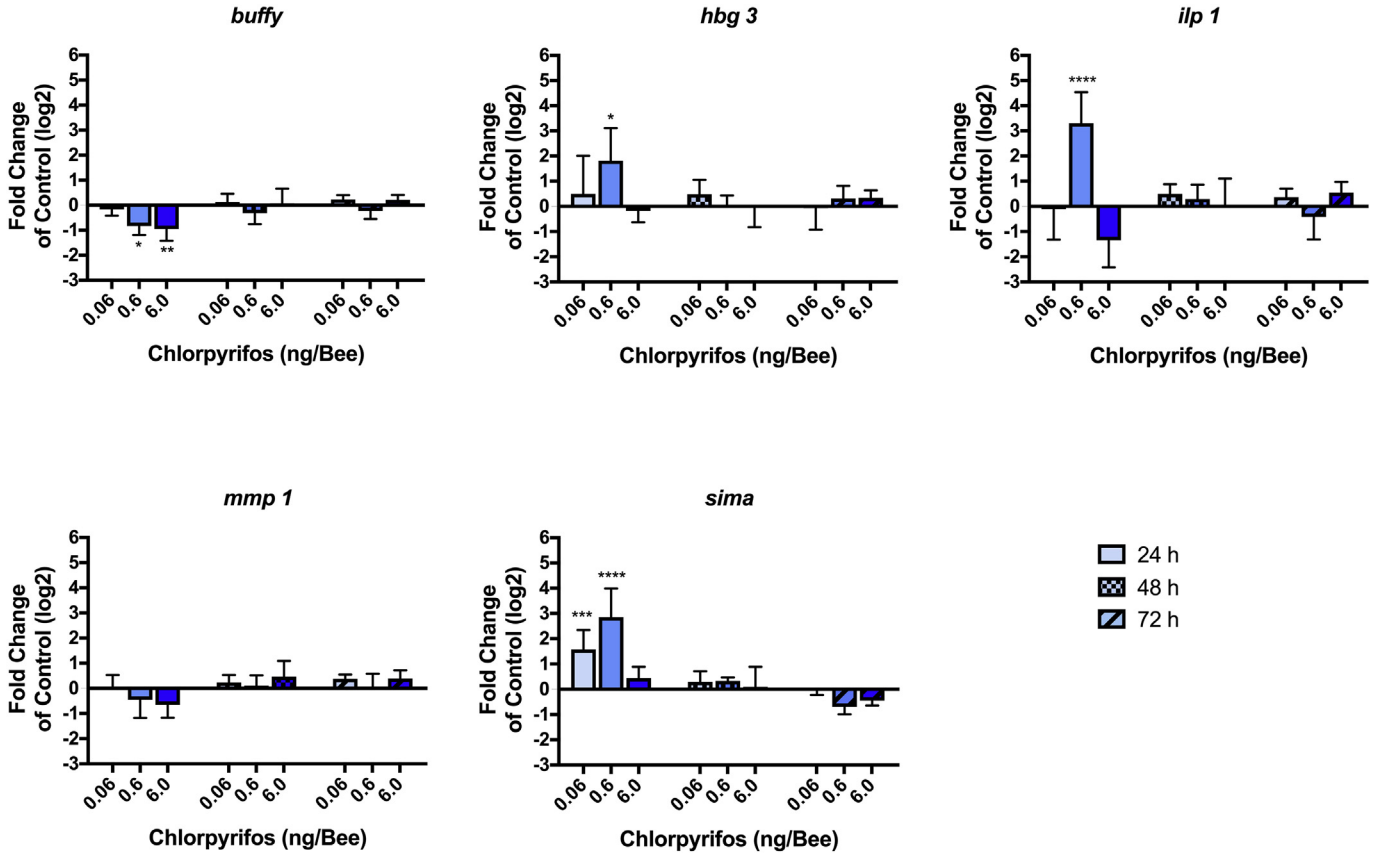

Fig. 2. Abundance of the transcripts *buffy*, *hbg3*, *ilp1*, *mmp1* and *sima* in the brain of honey bees following exposure to three different concentrations of chlorpyrifos for 24 h (plain bars), 48 h (squares) and 72 h (diagonal strips). Shown are the means with standard deviation of five biological replicates per concentration and exposure time. Significant differences with *p*-value $\leq$ 0.05 are marked with one asterisk, p $\leq$ 0.01 with two asterisks, p $\leq$ 0.001 with three asterisks, p < 0.0001 with four asterisks.

2.4. Chemical analysis of chlorpyrifos

To verify exposure concentrations, we analyzed the concentration of one of the used insecticides, chlorpyrifos, in our DMSO stock solutions prior to dilution into sucrose by chemical analysis using an Agilent 1260 Prime Infinity II HPLC system coupled to an Agilent Ultivo triple quadrupole mass spectrometer (Agilent Technologies, Basel, Switzerland). A reversed-phase column Poroshell EC-C_8 (2.1 $\times$ 50 mm, 2.7 μm particle size) (Agilent Technologies) was used for the separation. Details of the analytical method are given in the supplementary material.

2.5. Data processing and statistical analysis

The abundance or transcripts were plotted as Log2 values as commonly done. Differences of mRNA levels between treatments were assessed by one-way ANOVA followed by a Bonferroni's multiple comparison test to compare treatment means with respective controls. Results are given as means ± standard deviation. Differences were considered statistically significant with one asterisk at 0.05 > p > 0.01, two asterisks at 0.01 > p > 0.001 and three asterisks at 0.001 > p > 0.0001. Heat maps of expressional changes were designed by importing analyzed qPCR data into MEV 4.9 (Multi Experiment Viewer) software.

3. Results

3.1. Alteration of gene expression by insecticides in the brain

We assessed expressional changes of genes in the brain of honey bee foragers proposed to be indicative of endocrine activity. According to our concept (Fig. S1), they are prominently expressed in nurse bees or forager bees and included *vitellogenin*, *mrjp1*, *mrjp2*, *mrjp3*, *sima*, *buffy*, *hbg3*, *ilp1*, *mmp1*. We analyzed the expression of transcripts mainly expressed in nurse bees, such as *vitellogenin*, *mrjps* and *sima*, and transcripts mainly expressed in foragers, inculding *buffy*, *hbg3*, *ilp1* and *mmp1*.

3.1.1. Chlorpyrifos

Chemical analysis verified that the concentrations of the chlorpyrifos stock solutions were close to nominal (Table S3). This indicates that exposure levels were close to nominal values in our experiments. Therefore, the other insecticides are also assumed to be at nominal concentrations. Exposure to chlorpyrifos led to

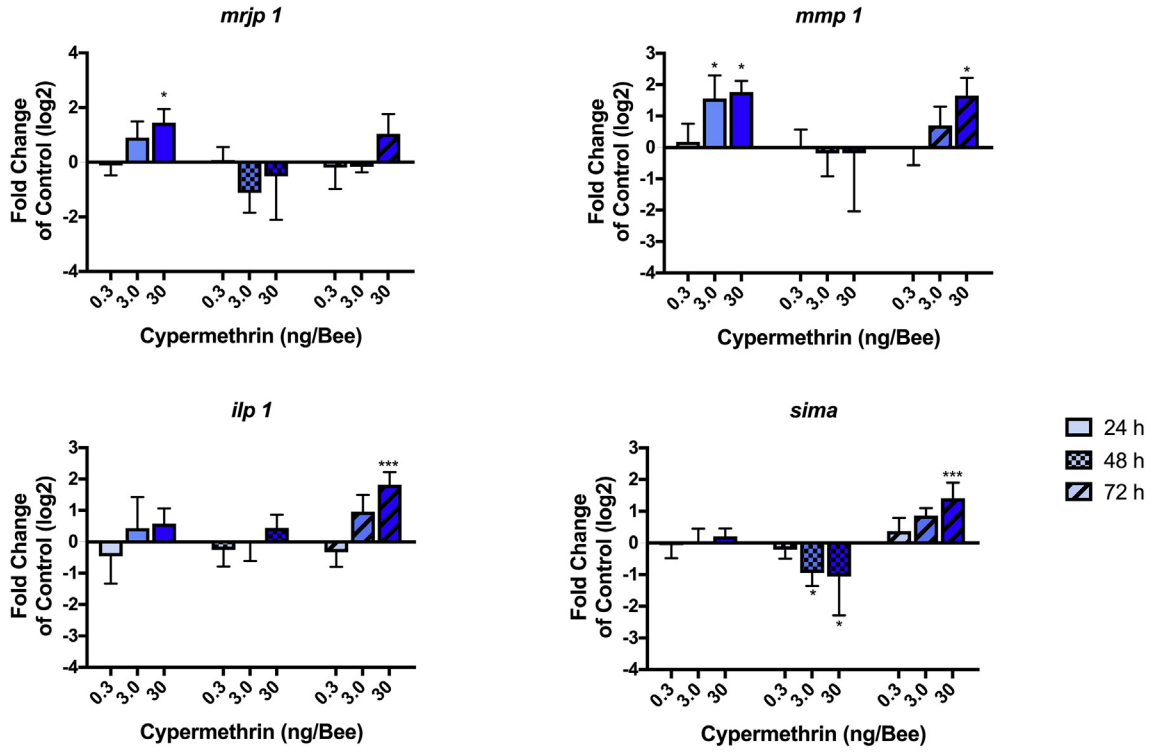

Fig. 3. Abundance of the transcripts *mrjp1*, *mmp1*, *ilp1* and *sima* in the brain of honey bees following exposure to three different concentrations of cypermethrin for 24 h (plain bars), 48 h (squares) and 72 h (diagonal strips). Shown are the means with standard deviation of five biological replicates per concentration and exposure time. Significant differences with *p*-value $\leq$ 0.05 are marked with one asterisk, p $\leq$ 0.001 with three asterisks.

significant up-regulation of genes after 24 h exposure but not at later exposure times of 48 h and 72 h. Transcripts of *vitellogenin*, *mrjp 2*, *mrjp 3* and *sima* were up-regulated at 0.06 and 0.6 ng/bee chlorpyrifos, and *hbg3* and *ilp1* at 0.6 ng/bee (Figs. 1 and 2). In contrast, *buffy* was down-regulated at 0.6 and 6 ng/bee (Fig. 2). All significant effects occurred at 24 h. Almost no significant effects occurred at the highest chlorpyrifos concentration, suggesting that there was no classical concentration-response relationship.

3.1.2. Cypermethrin

Exposure to cypermethrin induced only minor transcriptional changes with significant up-regulation of a few transcripts. Significant up-regulation occurred for the *mrjp1* transcript after exposure to 30 ng/bee at 24 h and for the *mrjp2*, *ilp1* and *sima* transcript at 72 h (Fig. 3 and S2). The *mmp1* transcript showed a significant up-regulation after exposure to 3 and 30 ng/bee after 24 h and to 30 ng/bee after 72 h (Fig. 3).

3.1.3. Thiacloprid

Thiacloprid led to transcriptional alterations of a number of genes. Significant down-regulation occurred for *vitellogenin* at 250 ng/bee after the 24 h exposure, *mrjp1*, *buffy* and *sima* at 250 and 2500 ng/bee after 24 h, and *hbg3* at 2500 ng/bee after 72 h (Fig. 4). Significant up-regulation occurred for the transcripts *buffy* at 25 ng/bee after 48 h, *ilp1* at 25 ng/bee after 24 h and at 25 and 2500 ng/bee after 48 h, and *mmp1* at 25 and 2500 ng/bee after 48 h (Fig. 4). Additional transcripts, *vitellogenin* and *hbg3*, showed alterations at one concentration and at one time-point, while *mrjp2* and *mrjp3* did not show significant alterations (Fig. S3).

3.2. Overall pattern of transcriptional alterations of genes indicative for endocrine system regulation

The heatmap in Fig. 5 summarizes transcriptional alterations induced by chlorpyrifos, cypermethrin and thiacloprid at all concentrations and exposure times. The observed changes were characterized by strong up-regulations at 24 h by chlorpyrifos and down-regulations for the other insecticides and exposure times. Generally, the expression pattern of chlorpyrifos differed from that of cypermethrin and thiacloprid, which were more similar. Moreover, transcriptional alterations changed with exposure times. Chlorpyrifos led to up-regulation at 24 h but was transient and did not occur after longer exposures. Each pesticide showed a rather specific expression pattern with similarities to the other compounds, particularly between cypermethrin and thiacloprid. Thus, transcriptional changes can be grouped into three separate clusters, which share similarities in their expression patterns (Fig. 5). One cluster consists of all three chlorpyrifos concentrations. All three thiacloprid concentrations and the lowest cypermethrin concentration build a second cluster and the middle and high cypermethrin concentration a third cluster. The low and middle chlorpyrifos concentrations in cluster one show a very similar expression pattern, which is also found in cluster two for the middle and the high concentration of thiacloprid.

Chlorpyrifos and thiacloprid led to down-regulation of *buffy*, while cypermethrin and thiacloprid induced the *mmp1* transcript. Thus, chlorpyrifos changed the expression of transcripts normally expressed in nurse bees, while thiacloprid changed the expression of two transcripts mainly expressed in nurse bees and two transcripts normally expressed in foragers. Cypermethrin changed the

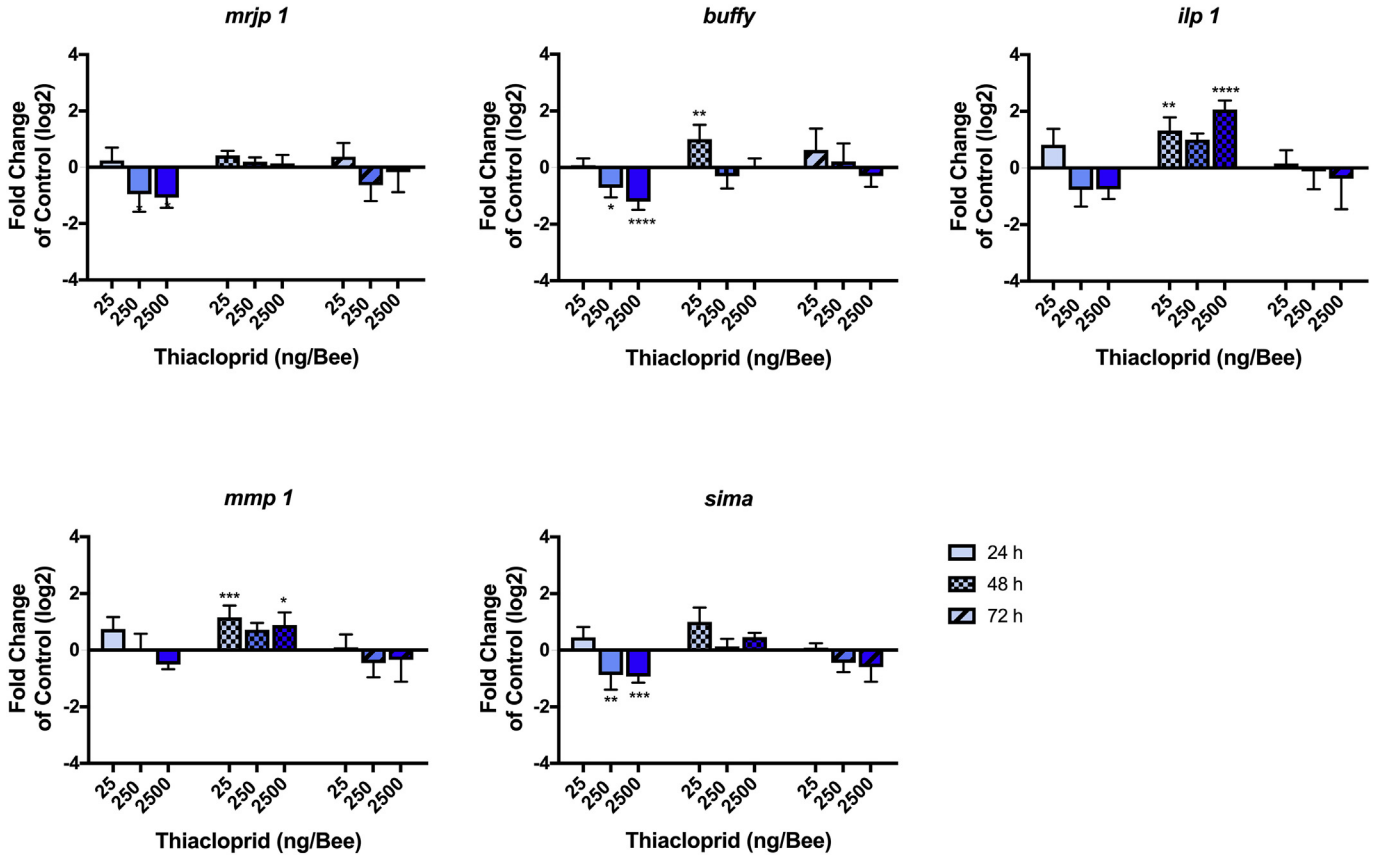

Fig. 4. Abundance of the transcripts *mrjp 1*, *buffy*, *ilp1*, *mmp1* and *sima* in the brain of honey bees following exposure to three different concentrations of thiacloprid for 24 h (plain bars), 48 h (squares) and 72 h (diagonal strips). Shown are the means with standard deviation of five biological replicates per concentration and exposure time. Significant differences with *p*-value ≤ 0.05 are marked with one asterisk, $p \leq 0.01$ with two asterisks, $p \leq 0.001$ with three asterisks, $p < 0.0001$ with four asterisks.

expression of one transcript, which is normally expressed either mainly in nurse bees or in foragers. The transcript *sima* that is mainly expressed in nurse bees was changed by all three insecticides, although not in the same direction.

3.3. Environmental implications

Based on reported concentrations of chlorpyrifos, cypermethrin and thiacloprid in nectar and pollen, daily uptake of these pesticides was estimated and compared to the lowest effect concentrations (LOECs) in our study. Estimates were based on daily uptake of 0.041 mg/day pollen and 43 mg/day or 292 mg/day nectar as derived from the BeeRex model (https://www.epa.gov/pesticide-science-and-assessing-pesticide-risks/models-pesticide-risk-assessment#beerex). The data indicate that the LOECs of chlorpyrifos, cypermethrin and thiacloprid are in the range of residues found in nectar but above levels in pollen (Fig. 6). Therefore, transcriptional effects suggested to be associated with endocrine disruptive activities occur at environmentally relevant nectar concentrations of these insecticides.

4. Discussion

In this study, we tested the hypothesis that insecticides alter the expression of genes in the brain that may be involved in behavioral transition from nurse bees to foragers, and thus, are indicative of an endocrine activity (Christen et al., 2018a). Foragers can be used for assessing endocrine activities, as they can regress to a more nurse-like behavior and physiology. This was the conceptual basis of our experiments. Our findings indicate that all tested insecticides altered the expression of the selected genes but the direction of transcriptional alterations, the concentration-response relationships and time courses varied between the different insecticides. Despite this variability our hypothesis that the endocrine activity of pesticides can be assessed in worker bees on the molecular level is supported by our findings.

Our analysis was focused on the brain of forager bees. However, in our brain sampling, we did not specifically separate the hypopharyngeal glands from the brain tissue, thus parts of the hypopharyngeal glands may have also been included. Therefore, the observed transcriptional alterations cannot only be assigned to expressional changes in the brain alone but may include the HPGs too. The hypopharyngeal glands undergo physiological changes from nurse to forager bees and each caste is characterized by expression of a specific set of genes (Christen et al., 2018a; Ueno et al., 2015). Additionally, in the brain, genes are also differentially expressed in both castes (Liu et al., 2019). The genes selected in our study are normally regulated by ecdysone and juvenile hormone and suggested here to be differentially regulated by insecticides that exhibit an endocrine activity. Differential expression of the genes is suggested to be a result of the direct or indirect interaction of the pesticides with biological receptors (hormone

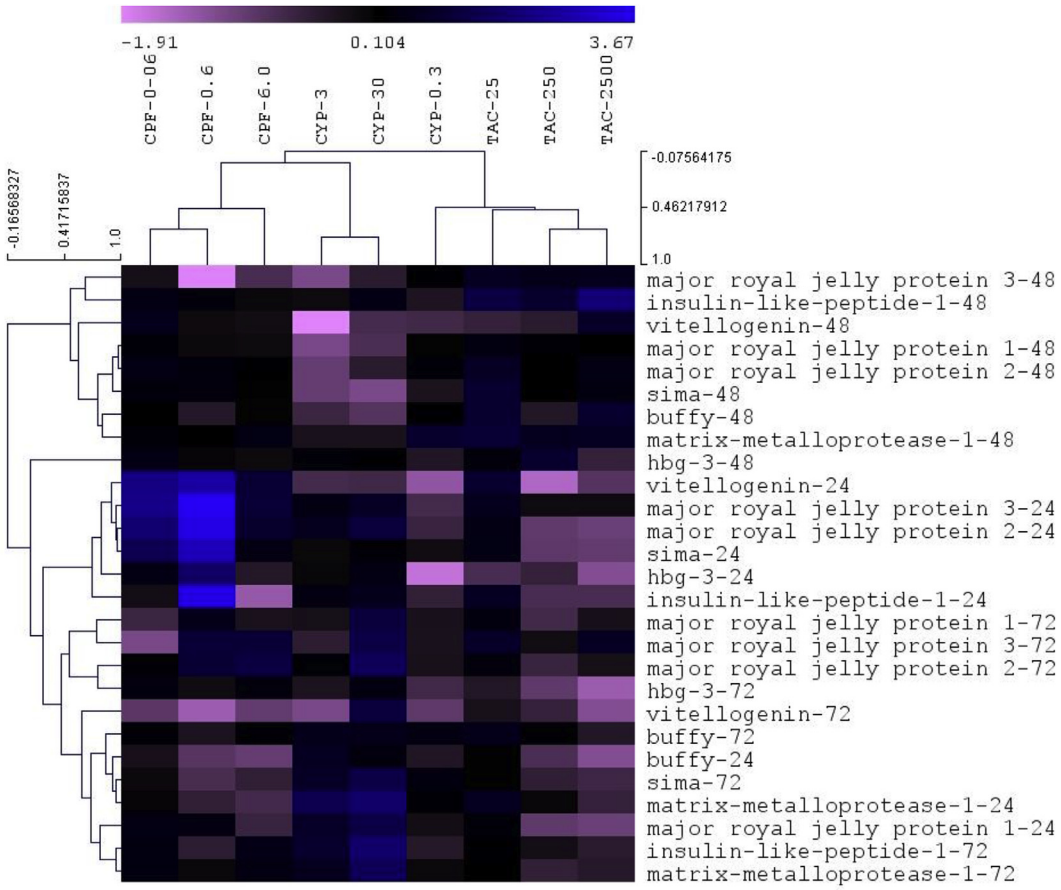

Fig. 5. Heat map showing all obtained transcriptional alterations for all three insecticides at different concentrations and exposure times of 24 h, 48 h and 72 h. Insecticides including concentrations are shown above and transcripts are listed to the right including exposure times. The magnitude of transcriptional alterations is given in pink (down-regulation) or blue (up-regulation), while no changes are given in black. CPF, chlorpyrifos; CYP, cypermethrin; TAC, thiacloprid. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

receptors), regulatory elements and processes, which in turn regulate the transcription of the genes.

4.1. Consequences of transcriptional responses of target genes

In nurse bees, expression of *mrjp2, vitellogenin* and *buffy* is higher than in foragers, while expression of *hbg3*, *mmp1, ilp1, ecdysone receptor*, *methoprene tolerant* (*met*), *E74* and *krüppel homolog 1* (*Kr-h1*) is higher in foragers than in nurse bees (Ueno et al., 2015). In our study, we assessed five transcripts which occur at high abundance in nurse bees (*vitellogenin*, *mrjp1*, *mrjp2*, *mrjp3*, *sima*) and four transcripts with high abundance in foragers (*buffy*, *hbg3*, *ilp1*, *mmp1*) upon exposure of foragers to insecticides.

Exposure of worker bees to the juvenile hormone analogue methoprene via application to their heads for 24 h led to up-regulation of forager-selective genes *hbg3* and *mmp1* and down-regulation of nurse bee-selective genes *mrjp2*. Additionally, a trend for down-regulation of *buffy* in the HPGs was observed (Ueno et al., 2009). Thus, our findings in the brain tissue that probably included parts of the HPGs are in line with the response to methoprene in the HPGs.

Exposure of mixed-age foragers to chlorpyrifos induced the expression of genes that are normally over-expressed in nurse bees. Transcripts of *vitellogenin*, *mrjp2*, *mrjp3*, *ilp1, hbg3* and *sima* were induced, and *buffy* was down-regulated. On the other hand, exposure to thiacloprid caused down-regulation of *vitellogenin*, *mrjp1*, *sima* and *hbg3*, as well as induction of *ilp1*. Thus, expression of indicator genes occurred but differed between the compounds. Alteration of these genes also occurred with cypermethrin. The expression of *sima*, a transcript over-expressed in nurse bees, and *mmp1*, a transcript induced in foragers, were altered.

Although the expression pattern varied between the insecticides, we identified these genes to have thepotential for detection of endocrine activities of pesticides, particularly the transcript of *buffy* that was down-regulated by chlorpyrifos and thiacloprid, *mmp1* that was upregulated by cypermethrin and thiacloprid, *sima* and the *mrjps* that were differentially regulated by all insecticides (Table 1). The direction of expressional alterations varied and was constant for two insecticides. Some transcripts were down-regulated by one insecticide and up-regulated by another (Table 1). However, the importance of these findings lies in the fact that there is an expressional change of these gene transcripts *per se*. The here studied insecticides chlorpyrifos, cypermethrin and thiacloprid induce neurotoxicity but act by different modes of action. This makes it plausible that the insecticides also differ to some extent in their transcriptional responses. Changes in expression of these hormone-associated genes in the worker bee brain and HPGs may translate to proteins, and subsequently, to physiological

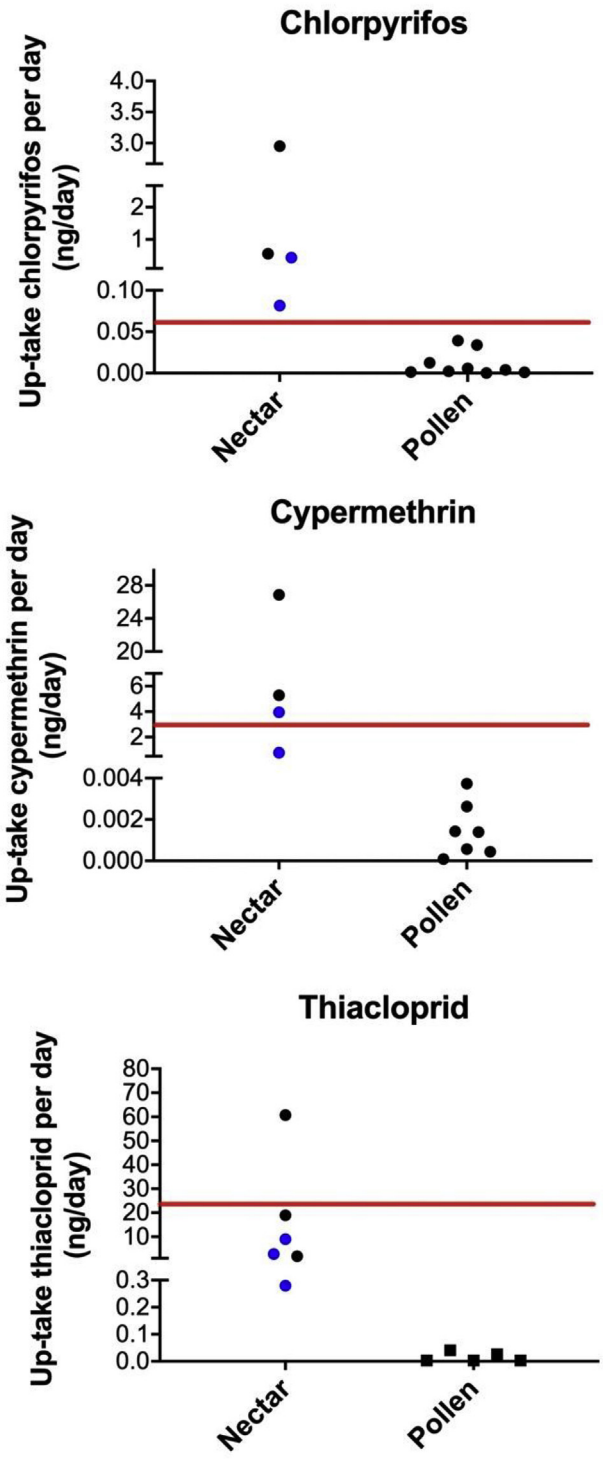

Fig. 6. Comparison between lowest observed effect concentration in the present study (red line) and estimated uptake of chlorpyrifos, cypermethrin and thiacloprid by foragers trough nectar and pollen. As basis, the daily consumption of nectar by foragers is assumed to be 43 or 292 mg/day and of pollen 0.041 mg/day according to the BeeREX model. Concentrations of chlorpyrifos, cypermethrin and thiacloprid are from the literature (Cutler et al., 2014; Sanchez-Bayo and Goka, 2014). Metabolism was not considered. Black circles: daily uptake of pesticides by consumption of 43 mg nectar per day, blue circles: daily uptake of pesticides by consumption of 292 mg nectar per day, black squares: daily uptake of pesticide by consumption of 0.041 mg pollen per day, red line: LOECs in the present study. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

outcomes such as behavioral alterations of foragers.

Despite some variability in the expression pattern of the target genes, all insecticides led to alteration of *buffy*, *mrjp1* and *sima*. Expression of these and additional genes in the HPGs (Ueno et al., 2015) and brain (Ben-Shahar et al., 2002; Whitfield et al., 2003) correlated with worker behaviour and age. Consequently, expressional changes in the brain genes goes along with changes in the behaviour. Age-related transition from nursing to foraging is associated with changes in mRNA abundance in the brain for a considerable number of genes. Among them is the *foraging* gene encoding a cGMP-dependent protein kinase, which is up-regulated in the brain of foragers. The genes *buffy* and *mmp1* are involved in intracellular signal transduction and extracellular matrix degradation in the HPGs, respectively. Due to differential expression of these genes in HPGs, they can serve as indicator genes of the behavioral state of worker bees. In our sampling, we also included parts of the HPGs. Both *buffy* and *mrjp2* are expressed in nurse bee HPGs, whereas *hbg3, ilp1* and *mmp1* are expressed in forager HPGs (Fig. S1). The molecular mechanisms underlying the expressional regulation of these genes related to HPG physiology and behavior remains to be investigated (Ueno et al., 2015).

Our data suggest that these genes are indicator genes for endocrine activity of pesticides, similar to methoprene that induced forager-selective genes *mmp1*, *hbg 3*, ecdysone receptor (*ecr*) and methoprene tolerant (*met*) and repressed nurse bee-selective transcripts of *buffy* and *mrjp2* (Ueno et al., 2015). In particular, chlorpyrifos and thiacloprid led to down-regulation of *buffy* in our study. Further studies are needed to show how these expressional changes translate to behavioral alterations. Ultimately, these changes may compromise the thriving of bee populations.

In addition to transition of nurse to forager bees with major changes in the HPGs, there are changes in cellular mitochondrial activity and redox environment in the head of bees. Caste differences occur in oxidative metabolism and mitochondrial physiology. Nurse bees have a higher oxidative phosphorylation capacity than foragers (Cervoni et al., 2017). The expression of *sima*, a transcript showing higher expression in nurse bees, was altered in our study after exposure to all three pesticides, although not in the same direction. This gene on chromosome 5 is involved in hypoxia transcriptional response (Azevedo et al., 2011). Transcript levels change with caste differences and are higher in worker larvae, thus mRNA levels also change during larval development. This gene represents the honey bee homolog of *hif1α* in *Drosophila*. The higher mitochondrial activity in nurse than foragers bees leads to higher concentrations of H_2O_2, which is known to stabilize hif1α. The transcriptional alteration of *sima* found in our study may thus complement the series of genes that indicate an alteration of caste development by pesticides in bees.

A recent RNA-sequencing study revealed a high number of differentially expressed genes in the head between nurse and forager bees (Liu et al., 2019,). Thus, there are additional genes not determined in our study that are involved in transition of nurse to forager bees, including prominently expressed genes, such as *foraging*, *malvolio*, *dop1*, *Kr-h1* and *HR38* (Liu et al., 2019). *Foraging* and *malvolio* are among many genes that play a causal role in the division of labor of honey bees, *dop1, Kr-h1* and *HR38* are upregulated in foragers compared to nurse bees (Liu et al., 2019), whereby Wnt signaling was suggested to be involved in the modulation of honey bee behavior by regulating the neuronal function of the brain. Potentially, Wnt signaling may be involved in the behavioral transition in addition to its role in a variety of processes (development, cell proliferation, cell motility) and maintaining and protecting neural connection. Dysregulation of Wnt signaling can lead to behavioral disorders (Maguschak and Ressler, 2012). On this basis, further studies should test, whether or not these genes have

the potential to serve as additional indicators of endocrine effects of pesticides but this needs to be tested in forthcoming studies.

In a previous study, we found that chlorpyrifos and cypermethrin caused transcriptional alterations of additional genes. Chlorpyrifos led to induction of cytochrome P450-dependent monooxygenase transcripts *cyp9q2* and *cyp9q3* at concentrations between 0.06 and 6 ng/bee. Cypermethrin, on the other hand, induced transcripts of *acetylcholine receptor alpha 1* and *2*, and altered transcripts of *cyp9q1* and *cyp9q3* at 0.3−30 ng/bee. Both insecticides led to induction of *vitellogenin*. While induction of *vitellogenin* by chlorpyrifos was confirmed in our current study, this was not the case for cypermethrin. Reasons for the differences may be related to differences in the reactivity of different bee populations used and/or due to trophallaxis.

4.2. Potential mechanisms and consequences

The mechanisms behind the observed expressional changes and their consequences remain poorly known. The insecticides may have acted directly on the expression of the target genes in brain and HPGs, or alternatively, they may have caused changes in hormone titers, such as ecdysone and juvenile hormone, and their signaling. This indirect effect may have resulted from an interference of the insecticides with hormone synthesis and degradation. In this case, expressional changes of the genes assessed in our study would be the result of altered hormone titers. Furthermore, it has to be shown in future studies whether the insecticides act to both the brain and HPGs, or whether to HPGs alone, by clearly separating both organs and by evaluating expressional responses separately. Thus far, it is known that HPGs are involved in the production of mrjps and α-glucosidase III. Nurses and foragers differ in mRNA levels of specific genes including *buffy*, *mrps* and others (*vitellogenin*, *hbg3*, *mmp1, Kr-h1*), and this is associated with differences in behavior.

Together, these transcriptional alterations may ultimately cause changes in age-development and behavior of worker bees. Thus, endocrine active pesticides may alter normal development and behavior of workers. Nurse bees would prematurely develop into foragers or foragers change to a nurse bee like behavior. This results in changes of foraging activity and brood care, which ultimately transfers to shrinking of colony size and may compromise thriving of populations.

4.3. Limitations

4.3.1. Response in relation to concentration and exposure time

The feasibility of our concept is shown by the results of the investigated insecticides. However, it has also some limitations. As we used foragers with different age, this may have resulted in a certain variability in the responses. It would be interesting to evaluate foragers of the same age to confirm our data or on age-controlled nurse bees in addition. An amendment would be that gene expression of pesticide-exposed foragers are compared with age-specific nurse bees.

Despite this limitation, a common feature in our findings was the expressional alteration of the suggested target genes by all insecticides, but the extent and direction of the changes and the time-course varied. While significant effects were visible for chlorpyrifos after 24 h, effects of cypermethrin and thiacloprid occurred at 48 and 72 h. Thus, there is no cumulative effect in exposure time. In case of chlorpyrifos, one of the reasons for the response at 24 h, but not at later exposure times, might be metabolism of this insecticide. The biotransformation of this organophosphate may lead to metabolites that did not affect expression of the assessed genes. Metabolism occurs by acetylcholinesterase (Jackson et al., 2011).

The data showed variability in extent and direction of transcriptional expression with concentration and duration of exposure. There were no classical concentration-response relationships. This has previously been observed with these insecticides on other gene transcripts and with different pesticides, including fungicides (Christen et al., 2019b) or the bio-pesticide spinosad ( ). As generally only little is known about the responsiveness of genes in bees to pesticide exposure on the molecular level and on the concentration-dependence, reasons for the non-classical concentration-response relationships are not known.

We focused on a limited number of indicator genes but there are additional genes involved in the transition of nurses to foragers that need consideration. They belong to the wnt signalling and the transcripts *foraging*, *malvolio*, *Kr-h1*, *dop1* and *HR38* are potential candidate genes (Liu et al., 2019). Besides direct neurotoxic action expressional alterations are the first molecular reaction of the bees to insecticides but the physiological outcomes of the altered gene expression, such as the behavioural change, has to be shown. The connection between expressional changes and behaviour changes has been demonstrated previously. In forthcoming experiments, the transcriptional alterations should be connected to physiological and behavioural outcomes. Furthermore, more endpoints in the endocrine pathway need to be assessed on the protein level or on hormone titers.

5. Conclusions

Our study with chlorpyrifos, cypermethrin and thiacloprid indicates that expressional changes of selected target genes in the brain of exposed honey bees are indicative for endocrine activity of pesticides at environmentally relevant concentrations. Despite some limitations (mixed age foragers, variability in time-response, lack of classical concentration-response relationships), our concept

Table 1
Summary of transcriptional alterations of insecticides. Shown are the selected genes that showed either significant alterations at two concentrations or at two time points. For each gene, direction of alteration and significance level is given.

Transcript	Chlorpyrifos	Cypermethrin	Thiacloprid	Normally mainly expressed in
Vitellogenin	↑ **	~	~	Nurse bees
mrjp1	~	~	↓ *	Nurse bees
mrjp2	↑ **	~	~	Nurse bees
mrjp3	↑ **	~	~	Nurse bees
buffy	↓ *	~	↓ **	Nurse bees
sima	↑ ***	↓ * (↑ 72 h)	↓ **	Nurse bees
hbg3	~	~	~	Foragers
ilp1	~	~	↑ **	Foragers
mmp1	~	↑ *	↑ **	Foragers

↑ upregulated, ↓ downregulated, ~ no significant expressional changes or at only concentration or time point. Significant differences with *p*-value of * ≤0.05, ** ≤0.01 and *** ≤0.001 are marked with asterisks. n.d. not determined.

outlined in Fig. 1 is supported by the data from insecticides acting by different mechanisms of action. Together, the data support and confirm our concept that expressional changes of a set of selected target genes in the brain of honey bees may be indicative for endocrine-disrupting effects. This holds true for different classes of pesticides that occur in nectar at environmentally relevant concentrations. Besides *buffy* and *mrjps*, more genes are involved in the transition of nurse to forager bees, and thus may represent indicator genes, such as *hbg3*, *mmp1*, *foraging*, *malvolio*, *dop1*, *Kr-h1* and *HR38*. The advantage of our concept is to study worker bees, which is significantly easier compared to detection of endocrine effects in queens (reduction of fecundity) or drones (decrease of sperms). Our proposed concept for identification of endocrine activities of pesticides in the worker bee brain has the potential for further investigation and validation. Further studies with additional pesticides are needed to validate the concept with additional genes, considering bees of same age and specific tissues such as HPGs and brain, to evaluate whether separation of brain and hypopharyngeal glands is needed and to what extent our proposed concept needs amendment and refinement. Furthermore, studies should investigate to what extent the observed transcriptional alterations translate to physiology and behaviour.

Credit author statement

Karl Fent, Conceptualization, Validation, Visualization, Writing and editing paper, Supervision, Management and acquisition of financial support. **Tiffany Haltinger**, Performance of experiments, Data curation, Visualization. **Petra Kunz**, Writing - reviewing and editing. **Verena Christen**, Conceptualization, Methodology, Visualization, Supervision, Writing-reviewing.

Declaration of competing interest

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

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Appendix A. Supplementary data

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