

Fungicides chlorothanolin, azoxystrobin and folpet induce transcriptional alterations in genes encoding enzymes involved in oxidative phosphorylation and metabolism in honey bees (*Apis mellifera*) at sublethal concentrations

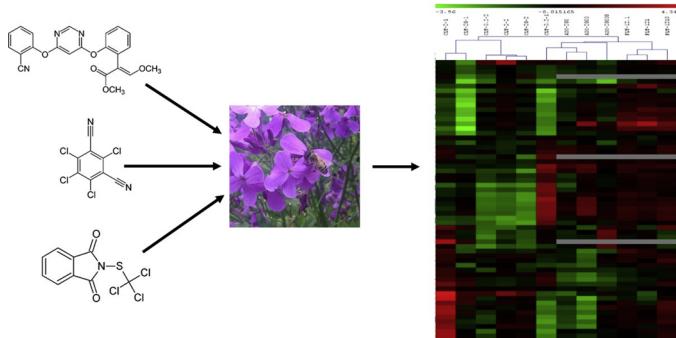
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GRAPHICAL ABSTRACT



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ABSTRACT

Fungicides are highly used for plant protection but their molecular and chronic effects are poorly known. Here, we analyse transcriptional effects in the brain of honey bees of three frequently applied fungicides, azoxystrobin, chlorothanolin and folpet, after oral exposure for 24, 48 and 72 h. Among transcripts assessed were genes encoding proteins for immune and hormone system regulation, oxidative phosphorylation, metabolism, and *acetylcholine receptor alpha 1*. Azoxystrobin and folpet induced minor alterations, including down-regulation of *hbg-3* by azoxystrobin and induction of *ndub-7* by folpet. Chlorothanolin induced strong transcriptional down-regulation of genes encoding enzymes related to oxidative phosphorylation and metabolism, including *cyp9q1*, *cyp9q2* and *cyp9q3*, *acetylcholine receptor alpha 1* and *hbg-3* and *ilp-1*, which are linked to hormonal regulation and behavioural transition of honey bees. Exposures to chlorothanolin in different seasonal times showed different responsiveness; responses were faster and often stronger in April than in June. Chlorothanolin caused the strongest effects and affected transcriptional abundance of genes related to energy production, metabolism and the endocrine system. Disturbed energy production may reduce foraging activity and hormonal dysregulation, such as the transition of nurse bees to foragers. Further analyses are needed to further substantiate potential adverse effects of chlorothanolin in bees on the physiological level.

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1. Introduction

The widespread use of plant protection products (PPPs) in agriculture and private gardens may result in exposure of non-target organisms. Honey bees (*Apis mellifera*) are important pollinators in these areas and can encounter different PPPs. Exposure to PPPs is one among the reasons for population declines of honey and wild bees [1,2] and colony losses [3], often in combination with other factors such as *Varroa destructor* parasites. Bees are exposed while visiting flowers and they bring nectar and pollen to the hive. Thus, foragers may be affected by direct exposure but also the hive colony and larvae can be exposed by contaminated pollen and nectar. Pollen analyses in Europe and North America showed a high incidence of PPPs, and among them were often fungicides [4,5]. Experimental feeding of such common pollen-bound PPP-mixtures delayed foraging with perturbations of the energy metabolism of bees [6].

Thus, bees are often exposed to fungicides, which find widespread use in agriculture. For instance, in France, where about 67'000 tons of PPPs were sold annually between 2011–2015, over 40% were fungicides, of which copper and sulphur were most frequent, but synthetic compounds also find frequent application [7]. The French PPP reduction policy was not effective as PPPs application continues at considerable rate, particularly in grapevines, sugar beet, potatoes and apples, where average treatments per year may be over twenty times [7]. In some countries, including Switzerland, fungicides can also be sprayed when fruit trees are at blossom. This is because of the relatively low acute toxicity of fungicides, which are assumed to be without risks to pollinators. However, whether or not chronic or sublethal effects of fungicides in bees occur is very poorly known.

Fungicides may interact with other PPPs to produce synergistic effects [8]. This was described for combinations between neonicotinoid insecticides (clothianidin) and fungicides (i.e. propiconazole) [9,10], and effects included slow ovary maturation, decreased feeding and survival, and thus, a shortened nesting period in wild bees *Osmia bicornis* [11]. Fungicides that mainly interfere with the metabolism in fungi may also affect metabolism in insects. Triazole fungicides that are cytochrome P450-dependent monooxygenase (cyp) inhibitors, interfere with metabolism in bees and cause down-regulation of mitochondrial-related nuclear genes [12].

In our present study we focus on the transcriptional effects of three largely used fungicides to evaluate potential effects, which are currently unknown. Chlorothanolin is a broad-spectrum fungicide and has been found for instance in bee's wax at concentrations of 47.38 ng/g [13]. Its mode of action in fungi is unclear, but it reduces the fungal intracellular glutathione level [14]. It is often applied to blooming crops when honey bees are present. It affected larval survival upon exposure via contaminated feed with 3.4 mg/L [15]. Other adverse effects on bees are unknown.

Azoxystrobin is a member of the strobilurin fungicides. It is frequently used and was the world's biggest-selling fungicide in 1999 [16]. It inhibits mitochondrial respiration by binding at the Q_o site of cytochrome b, which is part of the cytochrome bc₁ complex in the inner mitochondrial membrane of fungi, but also of other eukaryotes [16]. Thus, it disrupts the energy cycle by halting ATP production. Azoxystrobin was found in poisoned bees in Germany [17], and in 17% of foraging bees collected in grassland in Colorado, USA, where up to 25 ng/g tissue were detected [18]. Contaminated pollen was reported in

Maine, USA, with 0.9 ppb per hive, and also royal jelly was found to contain azoxystrobin in Germany in concentrations of up to 0.91 ng/g [19]. The acute toxicity of azoxystrobin is higher than 200 µg/bee (https://www3.epa.gov/pesticides/chem_search/reg_actions/registration/fs_PC-128810_07-Feb-97.pdf), but very little is known about chronic effects of this important fungicide.

Folpet is a chloralkylthio-fungicide that acts by reacting with thiols, and thus alters proteins and enzymes in fungi [20]. By nonspecific reaction with thiols, folpet reacts with cysteine amino acids in proteins and glutathione, thus affecting the function of many proteins and enzymes. Folpet residues of 3.74 µg/g were detected in a propolis (resinous material produced by bees from plant exudates and buds mixed with bee saliva and wax) sample in Spain [21]. Currently, potential effects of this fungicide to pollinators are unknown.

Due to knowledge gaps in potential adverse effects of these frequently used fungicides and the lack of information on molecular effects, the aim of our study is to evaluate transcriptional responses in the brain of experimentally exposed honey bees. We focus on target genes that play an important role in the physiology of bees and which may be related to the mode of action of the fungicides, including interference with energy metabolism. We focus on the brain as it represents an important target organ for different PPPs. Our analysis at sublethal concentrations sheds new lights to potential adverse implications of bee's exposures to fungicides.

2. Material and methods

2.1. Chemicals

Azoxystrobin, chlorothanolin and folpet (purities of all > 99%) were purchased from Sigma-Aldrich (Buchs, Switzerland). Stock solutions for each compound were prepared in DMSO and diluted into 20% sucrose-solution to a final concentration of 0.1% DMSO.

2.2. Experimental design of laboratory exposures

Adult forager honey bees (*Apis mellifera carnica*) of mixed age were obtained from frames from an outdoor colony placed at a location with no agricultural activity and pesticide use in the Black Forest (Germany, GPS: N 47.7667, E 7.8333) from end of April to June 2018. All used bees were from the same hive. The colony had signs of *Varroa destructor* affection and was previously handled with formic acid (August 2017) and oxalic acid (December 2017). Collection of individual honey bees, transportation to the laboratory, distribution into plastic bottles and exposure to sugar solution with or without fungicide were performed as previously [22,23]. Each exposure experiment consisted of four PET bottles with 10 bees per concentration and per three exposure times (24 h, 48 h and 72 h). After exposure we analysed three bees (pooled to one RNA sample) of each bottle yielding four biological replicates per fungicide concentration. Exposure experiments with chlorothanolin were performed twice (end of April and beginning of June 2018). Exposure experiments with azoxystrobin and folpet were performed in May 2018. The concentrations of fungicides used were selected on basis of existing LD₅₀ values and of estimated environmental concentrations. As concentrations need to be well below acute toxicity, the exposure concentrations were at least 10-times lower than published LD₅₀ concentrations. A summary of used concentrations expressed as ng/bee as

Table 1
Concentrations of fungicides used in the present study.

Compound	Concentration (ng/bee)	Concentration (ng/mL sugar syrup)	LD ₅₀ (oral) in mg/bee
Azoxystrobin	200, 2000 and 20,000	2000, 20,000 and 200,000	> 200 µg/bee (EPA factsheet: azoxystrobin)
Chlorothanolin	0.2, 2 and 20	2, 20 and 200	> 0.2 µg/bee [24]
Folpet	12.1, 121 and 1210	121, 1210 and 12,100	12.1 µg/bee [25]

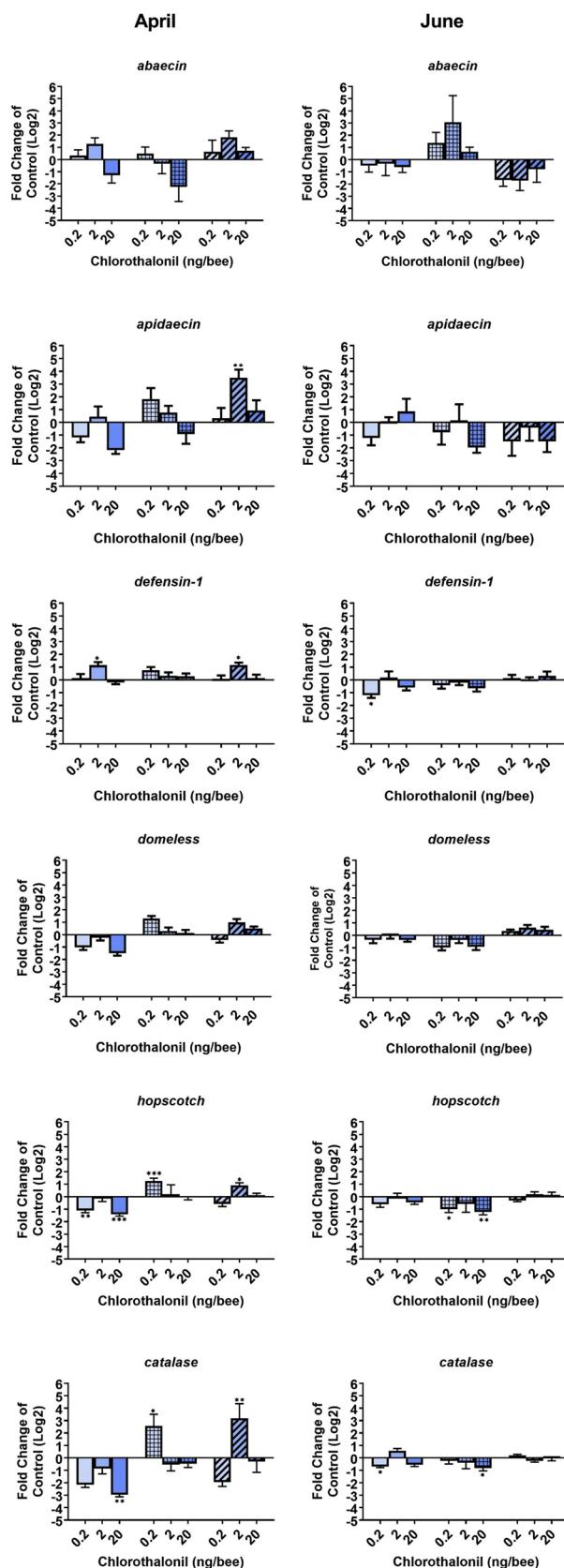


Fig. 1. Abundance of the immune system related transcripts *abaecin*, *apidaecin*, *defensin-1*, *domeless* and *hopscotch* and the stress related transcript *catalase* in the brain of honey bees following exposure in two different experiments to three different concentrations of chlorothalonil for 24 h (plain bars), 48 h (squares) and 72 h (diagonal stripes). Left column: experiment one (end of April 2018), right column: experiment two (beginning of June 2018). Shown are the results of four biological replicates per concentration and exposure time. Significant differences with p -value of ≤ 0.05 are marked with asterisks.

well as ng/mL sugar syrup and of published LD₅₀ values is shown in Table 1. Calculation of ng/mL syrup concentrations are based on a pilot studies, where we determined the daily consumption of sugar syrup per honey bee. In our hands, the honey bees consumed on average 100 μ L per day. As an example, to get a final exposure concentration of 20 ng/bee chlorothalonil, we have to feed 10 bees with 1 mL of a 200 ng/mL chlorothalonil sugar syrup.

During the experiments no mortality that was associated with a fungicide concentration was noted. All the exposed honey bees behaved normally and showed no signs of toxicity. No honey bees were observed that stopped moving or consuming syrup.

2.3. RNA isolation, reverse transcription, and quantitative PCR

The brain of frozen bees was removed in total by opening the cranium using scalpel and forceps. Total RNA of three bee brains were pooled and isolated using TRI Reagent® (Sigma-Aldrich, Buchs, Switzerland) according to the manufacturer's instructions. 1000 ng RNA was reverse transcribed as described before [22,23]. Furthermore, qPCR based on SYBR green fluorescence (SYBR green PCR master mix; Roche) was performed as previously. Primer sequences were taken from literature or self-designed using the NCBI primer-blast tool. Sequences of used primers and primer efficiencies are given in Table S1. For all performed analysis ribosomal protein S5 (*rpS5*) was used as house-keeping gene for normalisation. This selection is based on the stable transcription of *rpS5* after exposure of honey bees to different pesticides as shown in a previous study [23]. Alterations of mRNA abundance in fungicides exposed samples were always compared with the solvent control (0.1% DMSO) samples to determine the effects of fungicides.

2.4. Data processing and statistical analysis

Heat maps of expressional changes were designed by importing analysed qPCR data into MEV 4.9 (Multi Experiment Viewer) software. Differences between treatments were assessed by one-way ANOVA followed by a Bonferroni's multiple comparison test to compare treatment means with respective controls. Results of transcripts are given as means \pm standard error of means. 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 $0.001 > p > 0.0001$.

3. Results

We exposed honey bees to the fungicides azoxystrobin, chlorothalonil and folpet at three different concentrations and at three different exposure times (24, 48 and 72 h) to explore and compare concentration-related and time-related molecular effects in the brain. To analyse for molecular effects, we assessed transcriptional alterations of selected genes including immune system regulating genes, genes involved in oxidative phosphorylation, genes encoding metabolism enzymes and genes linked to hormonal regulation such as *vitellogenin*. Exposures to chlorothalonil were performed in two independent experiments at different times of the year to analyse for season-related activities. The first experiment was conducted end of April, assuming that there are still winter bees in the hive, and the second beginning of June without winter bees. Thus, in the different times of the year, there

were different flowers as natural resources of pollen and nectar. Thereby, we aimed to evaluate the reproducibility of experimental data between independent exposures, and second, whether observed transcriptional alterations are influenced by the status of the honey bee hive and food resources. Further, we aimed to derive gene expression patterns of the individual fungicides, to compare their transcriptional patterns.

3.1. Transcriptional alteration of immune system related genes and the stress gene catalase

Chlorothanolin showed no relevant alteration in the expression of *abaecin*, *apidaecin*, *defensin-1* and *domeless* in both experiments (Fig. 1). The expression of *hopscotch* was significantly inhibited at 0.2 and 20 ng/bee after 24 h exposure in experiment one, and after 48 h exposure in experiment two (Fig. 1). The *catalase* transcript was down-regulated at 20 ng/bee after 24 h exposure and strongly up-regulated at 0.2 ng/bee after 48 h, and at 2 ng/bee after 72 h of exposure in experiment one. No strong alterations occurred in experiment two (Fig. 1). The exposure of honey bees to azoxystrobin and folpet had no effects on the expression of immune system related transcripts and on the stress gene *catalase* (supplementary material, Figs. S1, S2).

3.2. Transcriptional alteration of cytochrome P450 genes

In comparison to azoxystrobin and folpet that did not induce significant transcriptional alteration of *cyp9q1*, *cyp9q2* and *cyp9q3* (Fig. S3), chlorothanolin led to significant transcriptional down-regulation of *cyp9q1* at 0.2 and 20 ng/bee after 24 h exposure, and induction at 0.2 ng/bee (48 h) and 2 ng/bee (72 h) in experiment one. In experiment two, the *cyp9q1* transcript was significantly down-regulated at 0.2, 2 and 20 ng/bee upon 48 h exposure (Fig. 2). The *cyp9q2* transcript was significantly down-regulated at 20 ng/bee upon 24 h and at 0.2 ng/bee upon 72 h of exposure and significantly up-regulated at 2 ng/bee upon 72 h exposure in experiment one (Fig. 2). In experiment two, *cyp9q2* was significantly down-regulated at 0.2, 2 and 20 ng/bee after 48 h and at 0.2 ng/bee upon the 72 h exposure (Fig. 2). No significant changes of the *cyp9q3* transcript occurred in experiment one, while in experiment two, a significant down-regulation occurred at 2 and 20 ng/bee upon 48 h exposure (Fig. 2).

3.3. Transcriptional alteration of genes related to oxidative phosphorylation

Chlorothanolin altered the expression of all the five analysed transcripts. *Cox5a* and *cox5b* were mainly down regulated after 24 h exposure in April and after 48 h in June (Fig. 3). *Cox6c* did not show a clear expression pattern in April, but was strongly down-regulated after 48 h in June (Fig. 3). *Cox17* was mainly down-regulated after 24 h exposure in April and strongly down-regulated after 48 h in June (Fig. 3). The *ndufb7* transcript was down-regulated after 48 h and up-regulated after 72 h in June (Fig. 3). Exposure to azoxystrobin had no relevant effects on the expression of *cox5a*, *cox5b*, *cox6c*, *cox17* and *ndufb7* (Fig. S4A). Folpet induced the expression of *ndufb7* at 12.1, 121 and 1210 ng/bee upon 24 h exposure but did not alter the abundance of the other transcripts (Fig. S4B).

3.4. Transcriptional alteration of genes linked to hormone system

Chlorothanolin had no effects on the expression of *vitellogenin* in both experiments (Fig. 4). The transcript of *hbg-3* was down-regulated at 20 ng/bee (24 h) and induced at 2 ng/bee (72 h) in experiment one, and down-regulated at 0.2 ng/bee upon 24 h exposure, and at all three concentrations upon 48 h of exposure in experiment two (Fig. 4). The transcript of *ilp-1* was down-regulated at 0.2 and 20 ng/bee (24 h) in experiment one but did not show significant changes in experiment two (Fig. 4).

Exposure to azoxystrobin resulted in a down-regulation of the *hbg-3* transcript at 20 000 ng/bee (24 h) and 2000 ng/bee (72 h). No effects were seen on the expression of *vitellogenin* and *ilp-1* (Fig. S5A). Folpet had no effects on the expression of these transcripts (Fig. S5B).

3.5. Transcriptional alteration of genes encoding major royal jelly proteins

Expression of *mrjp1* and *mrjp2* was significantly down-regulated at 0.2 ng/bee after 24 h in experiment two, while no changes occurred in experiment one (Fig. 5). *Mrjp3* was induced at 2 ng/bee after 72 h in experiment one, and down-regulated at 0.2 ng/bee after 24 h in experiment two (Fig. 5). Exposure to azoxystrobin and folpet had no significant effects on the expression of *mrjp1*, *mrjp2* and *mrjp3* (Fig. S6).

3.6. Transcriptional alteration of genes encoding acetylcholine receptor alpha 1 and alpha 2

The transcript of *acetylcholine receptor alpha 1* was strong down-regulated at 0.2 and 20 ng/bee after 24 h in experiment one and 0.2 ng/bee after 48 h, while it was induced at 2 and 20 ng/bee after 72 h in June (Fig. 6). The transcript of *acetylcholine receptor alpha 2* was strongly down-regulated at 0.2 and 20 ng/bee after 24 h in experiment one (April) and at 0.2 ng/bee after 48 h in experiment two (June), while it was induced at 2 and 20 ng/bee upon the 72 h exposure in June (Fig. 6). Exposure to azoxystrobin and folpet had no significant effects on the expression of *acetylcholine receptor alpha 1* and *alpha 2* (Fig. S7).

3.7. Overall pattern of transcriptional alterations

The heatmap in Fig. 7 depicts all transcriptional alterations of chlorothanolin, azoxystrobin and folpet at all exposures and concentrations. The observed changes were dominated by transcriptional up-regulation but down-regulations also occurred, mostly in some chlorothanolin and azoxystrobin exposures. The transcriptional changes can be grouped in two separate clusters, which share similarities in their expression patterns. One cluster consists of two chlorothanolin concentrations of experiment one and all concentrations of experiment two. The second chlorothanolin exposures (June) build one separate sub-cluster with strong inhibitory effects on different transcripts upon 48 h of exposure. The middle and the high concentration of the first chlorothanolin experiment builds one cluster with strong inhibitory effects on different transcripts upon 24 h, and a strongly increased expression of different transcripts upon 72 h exposure (Fig. 7). Together, this indicates that chlorothanolin induced similar transcriptional alterations in both experiments, but they were not identical. An exception is the 0.2 ng/bee concentration of experiment one, which showed a distinct pattern and grouped into cluster two.

The second cluster consists of the low chlorothanolin concentration in experiment one, and all azoxystrobin and folpet concentrations and exposure times. The azoxystrobin effects group in the same sub-cluster together with the lowest chlorothanolin concentration of the first experiment. This cluster showed down-regulations for different transcripts upon the 72 h exposure (Fig. 7). Transcriptional alterations induced by folpet are distinct from both azoxystrobin and chlorothanolin and build a separate cluster. Folpet showed almost exclusively transcriptional up-regulations but they were weaker than those of the other fungicides (Fig. 7).

To relate the observed effects to concentrations of the fungicides encountered in the environment, we compared the lowest effect concentrations of our study that induced significant transcriptional alterations with published pollen concentrations of the fungicides. The observed lowest effect concentrations of chlorothanolin were within the range of environmentally relevant concentrations, while those of azoxystrobin and folpet were above measured environmental pollen concentrations (Fig. 8).

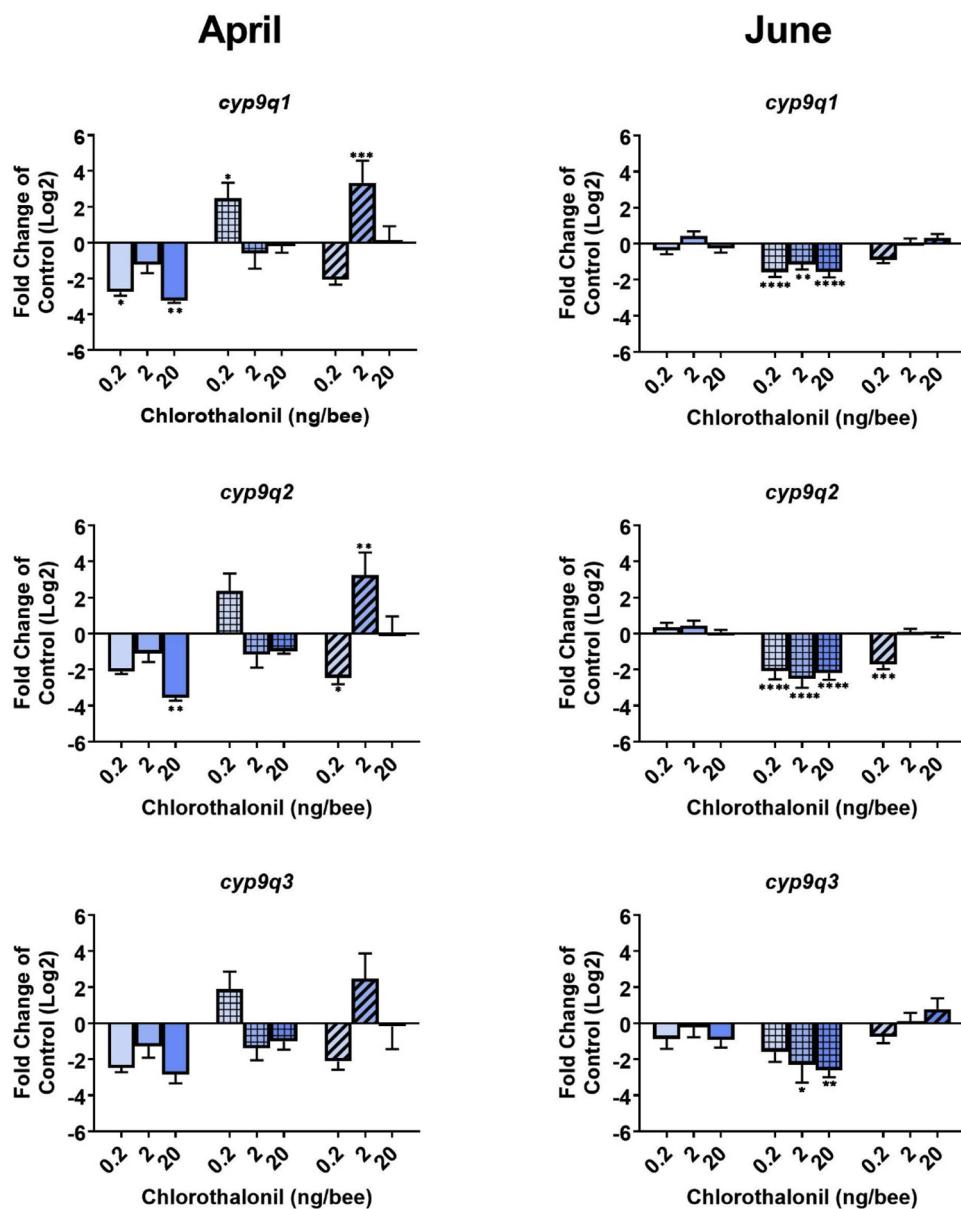


Fig. 2. Abundance of cytochrome P450 dependent monooxygenase gene transcripts *cyp9q1*, *cyp9q2* and *cyp9q3* in the brain of honey bees following exposure in two different experiments to three different concentrations of chlorothalonil for 24 h (plain bars), 48 h (squares) and 72 h (diagonal strips). Left column: experiment one (end of April 2018), right column: experiment two (beginning of June 2018). Shown are the results of four biological replicates per concentration and exposure time. Significant differences with p -value of ≤ 0.05 are marked with asterisks.

4. Discussion

Here we show for the first time significant molecular effects of fungicides in the brain of honey bee workers. Transcripts of different important physiological pathways such as immune system, oxidative phosphorylation, detoxification and endocrine regulation were analysed. Of the three investigated fungicides, chlorothalonil showed strongest effects characterized by differential transcriptional expression of genes encoding enzymes and proteins related to oxidative phosphorylation, detoxification enzymes, *acetylcholine receptor alpha 1*, and endocrine regulation. Azoxystrobin induced weaker effects on differential expression of genes encoding enzymes/proteins involved in oxidative phosphorylation and the endocrine system, while folpet basically only affected the *ndufb-7* transcript encoding a part of an enzyme of the complex I of the oxidative phosphorylation cascade of enzymes. Although in general being similar, effects of chlorothalonil were stronger and faster in April than in June, which indicates that there is a

different sensitivity in different times of the year.

4.1. Effects on oxidative phosphorylation

All fungicides exhibited differential expressional effects on nuclear genes encoding enzymes of the mitochondrial oxidative phosphorylation. The gene product of *ndufb-7* builds, together with other proteins, the NADH-dehydrogenase of complex I and enzymes encoded by *cox5a*, *cox5b*, *cox6c* and *cox17* that belong to the cytochrome c oxidase of complex IV [32]. Strong and mainly inhibitory effects were induced by chlorothalonil at all exposure concentrations. Azoxystrobin altered the expression of *ndufb-7*, *cox5b* and *cox17* (Fig. 3), while folpet only affected the expression of *ndufb-7* (Fig. S4). This indicates a different potency of these fungicides. Exposure led to altered expression of essential enzymes involved in the function of oxidative phosphorylation, the key process in energy metabolism.

Honey bee energy metabolism can be affected by many PPPs

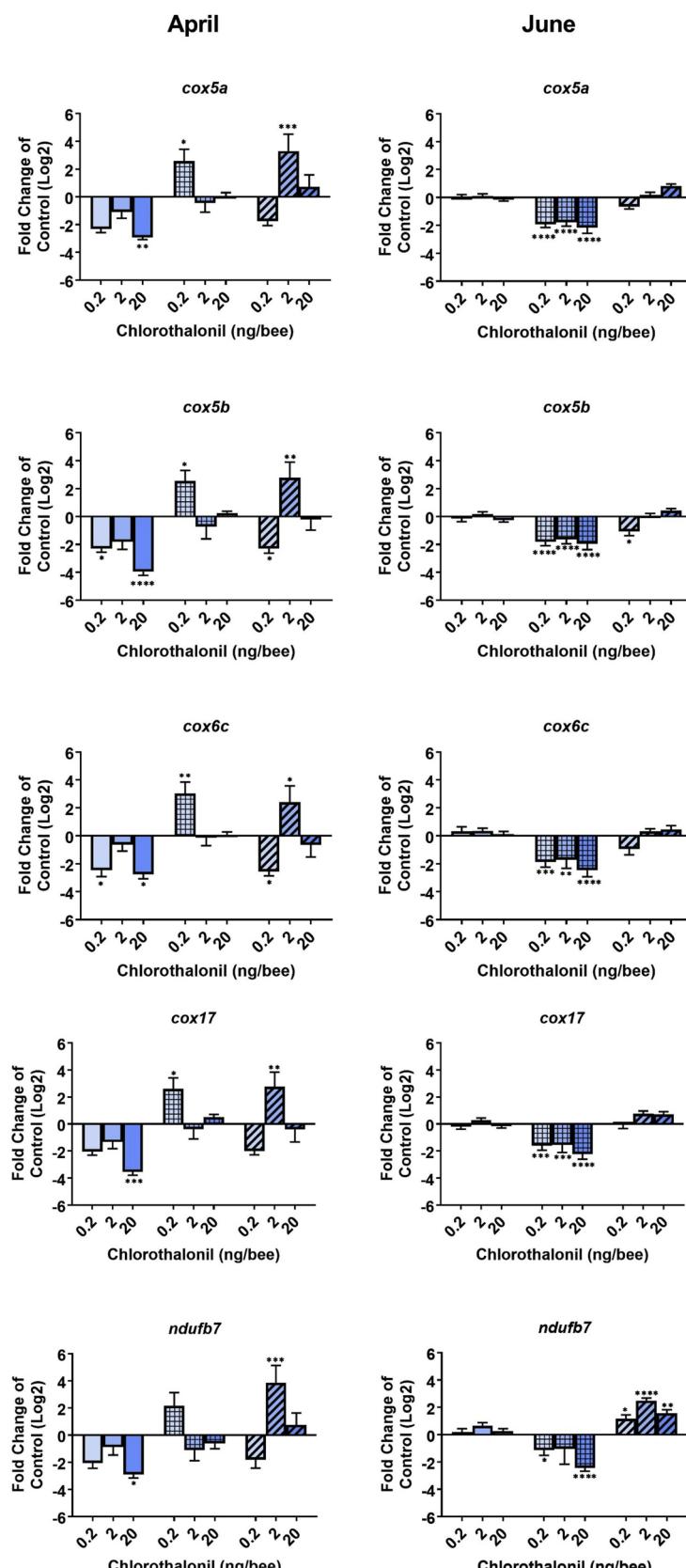


Fig. 3. Abundance of the transcripts *cox5a*, *cox5b*, *cox6c*, *cox17* and *ndufb7* encoding nuclear genes of the mitochondrial oxidative phosphorylation in the brain of honey bees following exposure in two different experiments to three different concentrations of chlorothalonil for 24 h (plain bars), 48 h (squares) and 72 h (diagonal strips). Left column: experiment one (end of April 2018), right column: experiment two (beginning of June 2018). Shown are the results of four biological replicates per concentration and exposure time. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

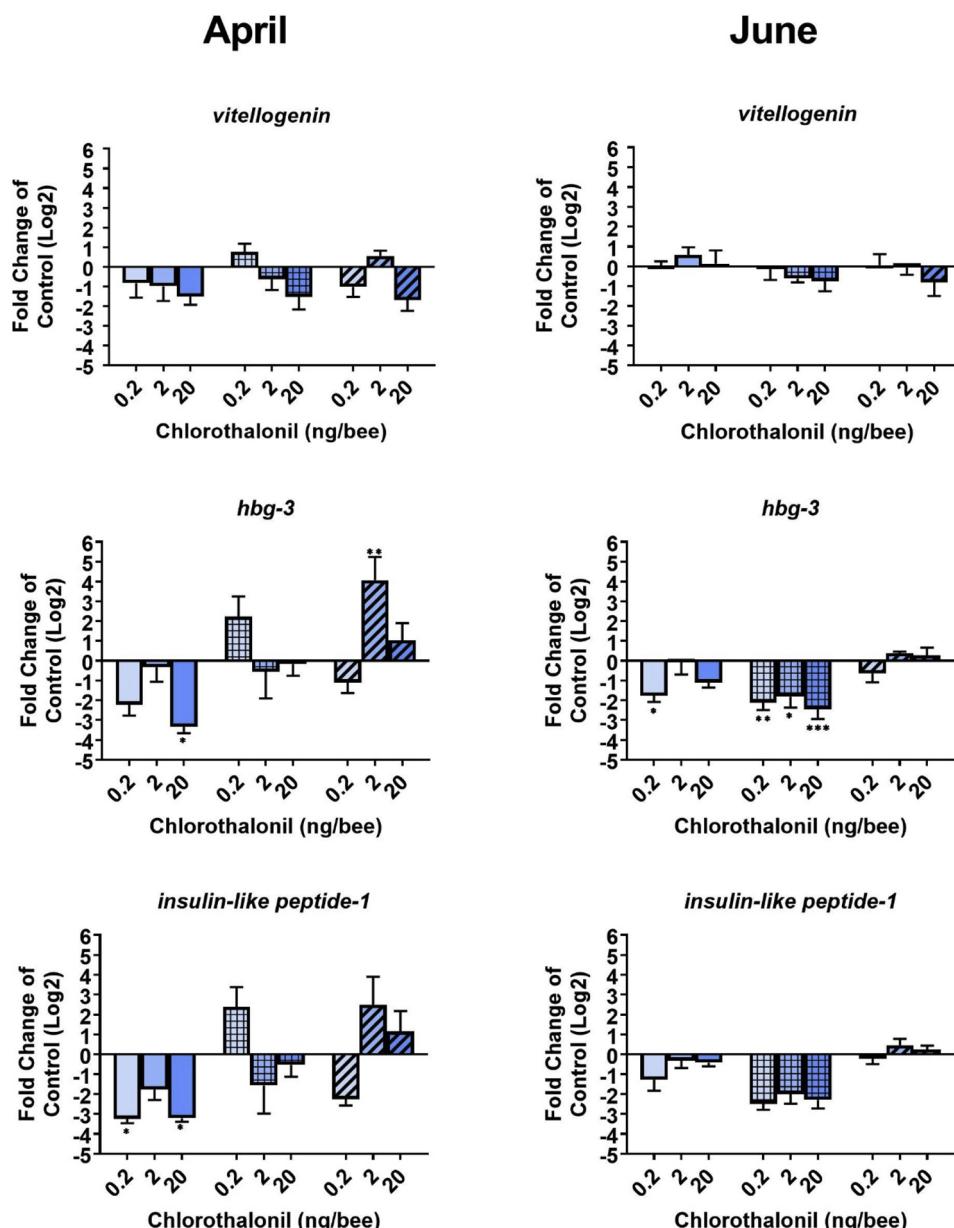


Fig. 4. Abundance of the transcripts *vitellogenin*, *hbg-3* and *ilp-1* in the brain of honey bees following exposure in two different experiments to three different concentrations of chlorothalonil for 24 h (plain bars), 48 h (squares) and 72 h (diagonal strips). Left column: experiment one (end of April 2018), right column: experiment two (beginning of June 2018). Shown are the results of four biological replicates per concentration and exposure time. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

including the neonicotinoid thiamethoxam [33]. Imidacloprid and fipronil reduced mitochondrial activity and ATP production in the brain and thorax of bees [34]. Also the fungicides diniconazole, fludioxonil, dithianon and difenoconazole displayed negative effects on mitochondrial energy metabolism in flight muscles of bumble bees [35]. Nicotine affected oxidative phosphorylation, characterized by up-regulation of 15 and down-regulation of four proteins [36]. Phytochemicals in nectar and pollen can also interfere with honey bee energy metabolism. Quercetin, one of the main flavonols of pollen, led to expressional alteration of transcripts encoding enzymes of oxidative phosphorylation, characterized by down-regulation of *cox5a*, *cox5b*, *cox6c*, *cox17* and *ndufb-7* [32].

Transcriptional down-regulation of enzymes of the oxidative phosphorylation may have negative consequences on the energy production of the bees. Besides delivery of ATP as energy source for numerous biochemical processes, brain metabolism regulates the aggressive

behaviour of honey bees. Transcripts of different complexes of the oxidative phosphorylation are differently expressed in aggressive honey bees compared to non-aggressive honey bees (comparison between African honey bee (aggressive) and European honey bee (non-aggressive), and between older foragers (more aggressive) and younger foragers (less aggressive)). Complex I and complex IV showed less activity in the brain of aggressive compared to non-aggressive honey bees [37]. In addition, bees treated with complex I and complex V inhibitors showed a more aggressive behaviour compared to untreated bees [38]. The transcriptional down-regulation of enzymes involved in oxidative phosphorylation by chlorothalonil and azoxystrobin may have consequences on energy production and behaviour in honey bees as the oxidative phosphorylation capacity is higher in nurse bees compared to foragers [39].

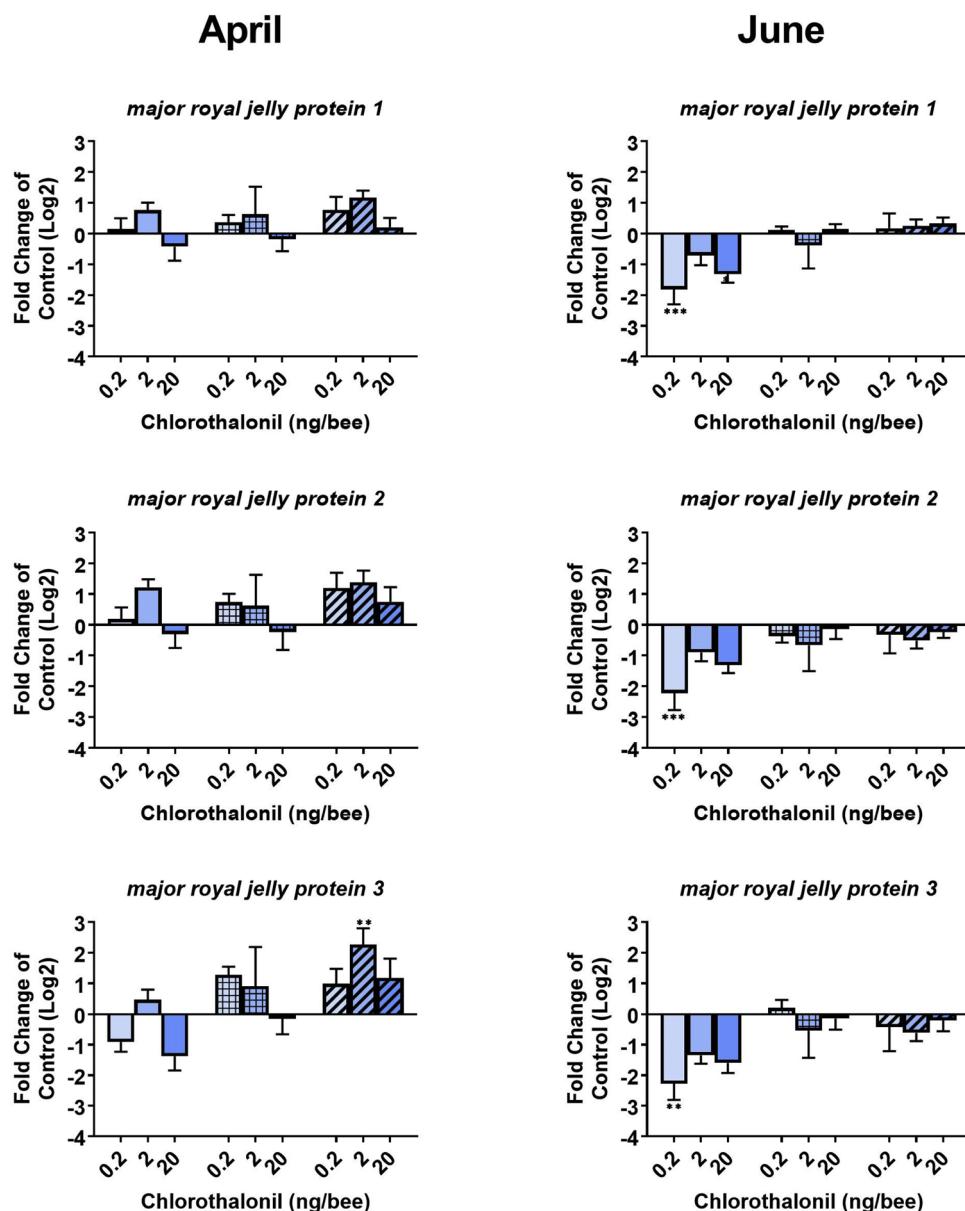


Fig. 5. Abundance of the major royal jelly protein transcripts *mrjp1*, *mrjp2* and *mrjp3* in the brain of honey bees following exposure in two different experiments to three different concentrations of chlorothalonil for 24 h (plain bars), 48 h (squares) and 72 h (diagonal stripes). Left column: experiment one (end of April 2018), right column: experiment two (beginning of June 2018). Shown are the results of four biological replicates per concentration and exposure time. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

4.2. Effects on hormonal regulation

Chlorothalonil led to expressional down-regulation of *hbg-3* and *ilp-1* (Fig. 4), while azoxystrobin led to a down-regulation of *hbg-3* (Fig. S5). The gene product of *hbg-3* is involved in the transition of nurse bees to foragers. In foragers, the hypopharyngeal glands are shrinking, and at the same time, the expression of *hbg-3* is increasing. *Hbg-3* is involved in the procession of nectar to honey [40]. Both processes are controlled by the juvenile hormone [41]. As azoxystrobin changed the expression of *hbg-3*, it may have adverse effects on the hormonal regulation of honey bees and may disturb the development of foragers. Similarly, the gene product of *ilp-1* is also involved in the transition of nurse bees to foragers. This gene shows higher expression in foragers than in nurse bees and exposure to the juvenile hormone analogue methoprene led to induction of *ilp-1* [27]. In our study, chlorothalonil induced a “nurse-like” expression profile of *hbg-3* and *ilp-1* in foragers. Expressional alteration of *hbg-3* and *ilp-1* by exogenous compounds may indicate an

endocrine disruptive activity [42]. Therefore, such effects may result in altered development of honey bee in colonies.

Major royal jelly proteins 1, 2 and 3 are the most abundant proteins in food jelly [43]. Mrjp1 and mrjp2 display antibacterial activity, whereas mrjp3 has nutritional functions [44]. Mrjp proteins are produced in the hypopharyngeal gland. In nurse bees, *mrjp2* is up-regulated in the hypopharyngeal gland [26]. Exposure of honey bees to 20-hydroxyecdysone, a bee hormone regulating, among others, the transition of nurse bees to foragers [45,46], led to down-regulation of *mrjp1*, *mrjp2* and *mrjp3* [47]. In our study, we found that chlorothalonil led to expressional down-regulation of *mrjp1*, *mrjp2* and *mrjp3*, and azoxystrobin to down-regulation of *mrjp1* and *mrjp2* (Fig. 5, Fig. S6). Consequently, both fungicides may display adverse effects on the hormonal regulation of honey bees and disturb the development of foragers.

In previous studies, we observed changes in vitellogenin mRNA and protein levels caused by exposure of honey bees to plant protection products, such as neonicotinoids and other insecticides [22,23], thus

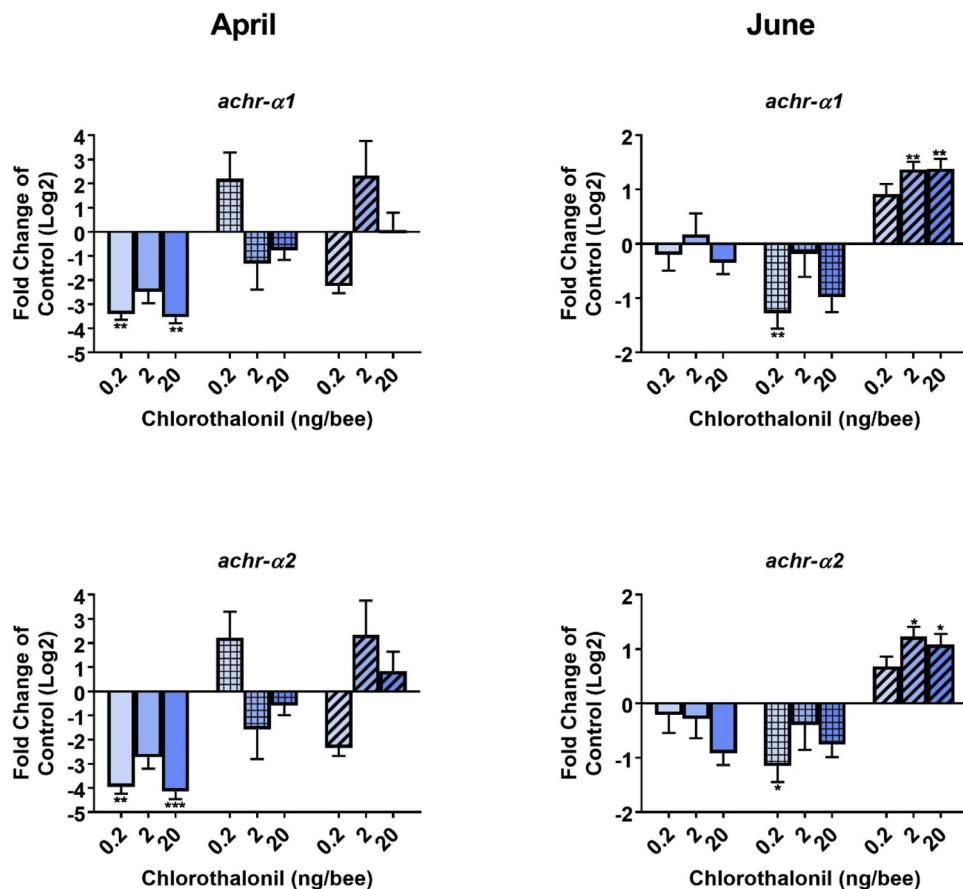


Fig. 6. Abundance of the acetylcholine receptor transcripts *achr- α 1* and *achr- α 2* in the brain of honey bees following exposure in two different experiments to three different concentrations of chlorothalonil for 24 h (plain bars), 48 h (squares) and 72 h (diagonal stripes). Left column: experiment one (end of April 2018), right column: experiment two (beginning of June 2018). Shown are the results of four biological replicates per concentration and exposure time. Significant differences with p-value of ≤ 0.05 are marked with asterisks.

we also analysed this target gene expression for fungicides in the present study. This is important, as vitellogenin is an important regulator of life-span and foraging behavior, and changes in expression may have significant physiological effects. However, none of the three fungicides affected the expression of vitellogenin.

4.3. Effects on metabolism enzymes

Cytochrome P450-dependent monooxygenases are important detoxification enzymes of endogenous and exogenous substrates. For instance, *cyp9q1*, *cyp9q2* and *cyp9q3* contribute to detoxification of plant protection products in honey bees [12]. Pesticides can also change cytochrome P450 levels. Our present study shows that chlorothalonil decreased the expression of *cyp9q1*, *cyp9q2* and *cyp9q3* (Fig. 2), whereas azoxystrobin and folpet had no effect (Fig. S3).

The organophosphate acephate elevated cypP450 activity in exposed bees [48]. The neonicotinoid imidacloprid increased the level of the *cyp9q3* transcript [49]. Expressional down-regulation of the *cyp9q1*, *cyp9q2* and *cyp9q3* transcripts occurred after exposure of honey bees to the organophosphates chlorpyrifos and malathion, the pyrethroide cypermethrin and the ryanidin receptor activator chlorantraniliprole [23]. Different expression levels of cytochrome P450 are found between nurse bees and foragers; foragers express much higher levels of *cyp9q1*, *cyp9q2* and *cyp9q3* as they collect pollen and nectar, which may be potentially contaminated with PPPs [50]. Chlorothalonil induces a “nurse-like” expression pattern of the analysed *cyp* transcripts in foragers. This was also detected upon exposure of foragers to the organophosphates chlorpyrifos and malathion, the pyrethroid cypermethrin and the ryanidin receptor activator chlorantraniliprole [23]. As *cyp9q1*, *cyp9q2* and *cyp9q3* enzymes contribute to detoxification of plant protection products in honey bees [12], their down-regulation may lead to a reduced metabolism and detoxification of chlorothalonil.

4.4. Effect of seasonal exposure time in chlorothalonil exposures

We performed two exposure experiments with chlorothalonil, the first in April and the second in June to investigate the reproducibility of the data between different exposure times and to assess the effects at different seasonal times. Generally, effects were stronger in April than in June and effects occurred upon 24 h exposure, while in June they occurred at 48 h (Fig. 7). One exception was the expression of major royal jelly proteins, which showed almost no change in April, but a strong down-regulation in June (24 h exposure) (Fig. 7). In April, most significant changes occurred in the highest concentration, whereas in June, all concentrations exhibited significant changes (Fig. 7). The reason for this different reaction is not known but it is possibly based on the different composition of the exposed bee sample in the different times of the year. Thus, honey bees in April react faster than in June probably due to differences in the composition of the honey bee populations. In April, there are still some winter bees found in the honey bee population and the colony just started to thrive. The ratio between nurse bees and foragers consists of about 2/3 nurse bees and 1/3 foragers. In June, the honey bee hive is fully developed, the amount of nurse bees and foragers is balanced (<https://www2.hu-berlin.de/bienenkunde/index.php?id=112>). In addition, a reason for the different reaction of the bees between April and June might be the temperature and the composition of flowering plants with different phytochemical residues in pollen and nectar. In June, the metabolic rate is on a higher level [51].

Although the experiments were performed in the laboratory under controlled conditions, it should be noted that bees came from outdoor hives held under field conditions. We therefore, hypothesize that temperature and food source changes may have influenced the performance of the honey bees in our laboratory exposures in addition to the composition of the exposed bee samples. Thus, we assume that the current

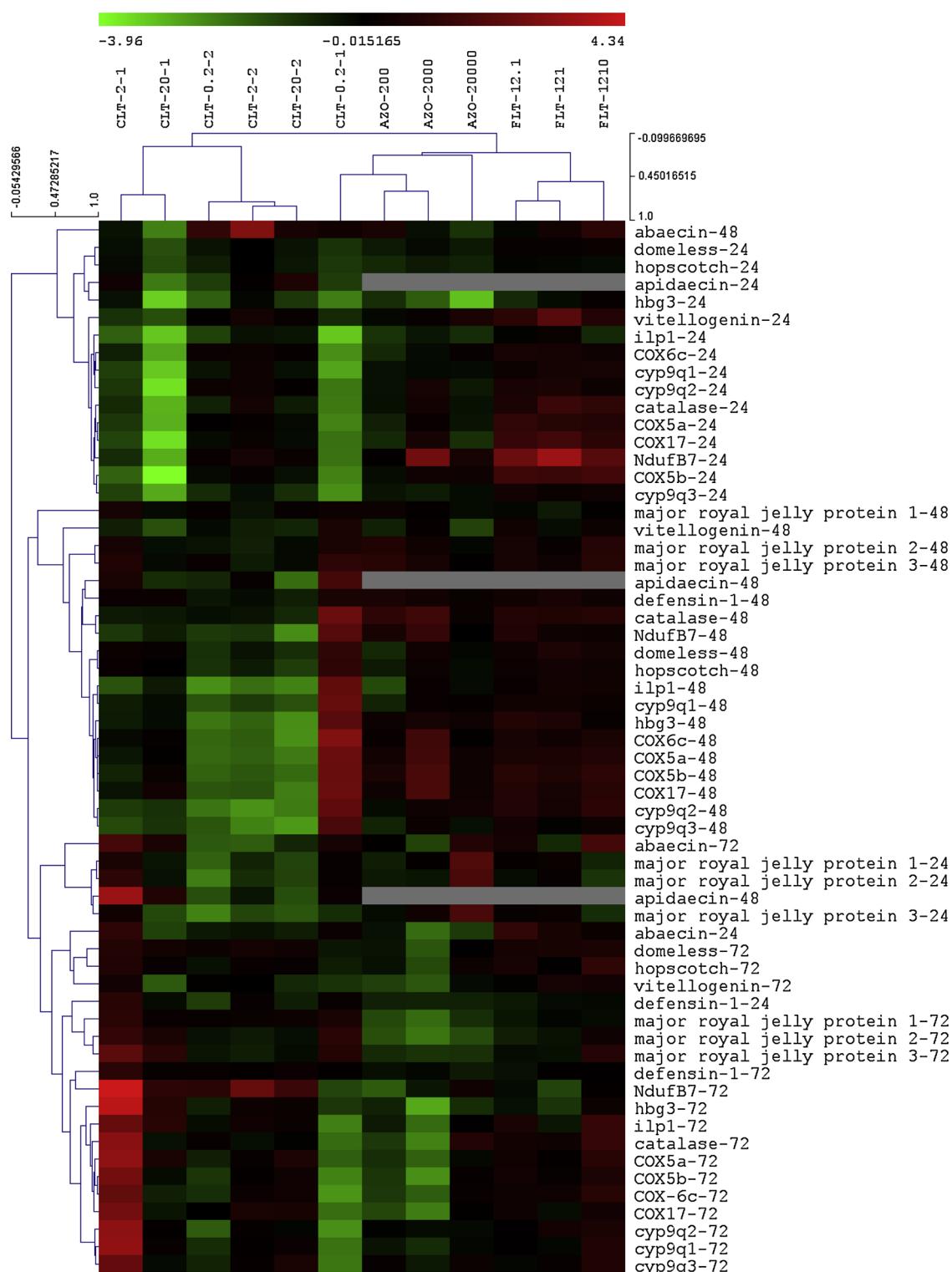


Fig. 7. Heat map showing all obtained transcriptional alterations for both chlorothanolin experiments (experiment one and two are named CLT1 and CLT2), azoxystrobin (AZO) and folpet (FLT) at different concentrations and exposure times of 24 h, 48 h and 72 h (key: 24, 48, 72). Concentration groups and experiment number 1 and 2 are shown above, transcripts and exposure time are listed to the right. The magnitude of transcriptional alterations is given in green (down-regulation) or red (up-regulation), while no changes are given in black. Grey, not analysed.

environmental situation, at least in the beginning of the exposure, affected the bee's responsiveness.

In our present study, we performed experiments with mixed-aged honey bees as previously [22,23]. The sensitivity and responsiveness of honey bees to pesticide exposure can be affected by the age of the individual bee. Our approach was aimed to obtain robust and biologically

relevant data. To this end, we used mixed age honey bees. In case significant effects in such a heterogeneous population occur, they would have higher relevance (as this is the normal situation) than with a homogenous age-controlled population. Another aspect is the better technical feasibility.

In the present study, we did not find a clear correlation between

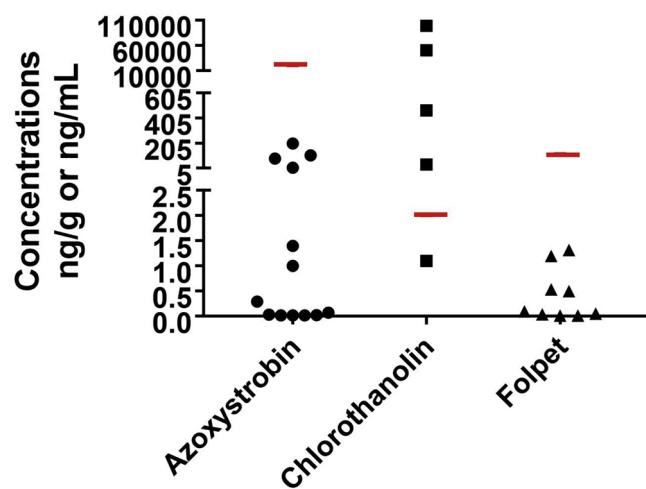


Fig. 8. Comparison of lowest effect concentrations (LOEC) defined as significant transcriptional alterations of gene transcripts of the present study with reported pollen concentrations of azoxystrobin, chlorothanolin and folpet taken from [28–31]. Red lines show the LOEC observed in our study.

exposure concentration and amplitude of transcriptional alteration (no dose-response curves were observed), similar to our previous studies. This is not expected. However, only little is known about the responsiveness in bees, as transcriptional effects are and were very rarely investigated. There was also some variability in the responsiveness. One factor that influences uptake of the fungicides, and consequently, the response, is the trophallaxis of honey bees. This may result in an unequal distribution of the fungicides between the 10 honey bees in the exposure bottle. Therefore, some honey bees may be exposed to lower and some to higher concentrations. This can only be circumvented by feeding single bees. However, in a study with three different time-points and three different concentrations this is practically not feasible.

Exposure of 10 bees per replicate and concentration is an approach according to OECD guidelines. The differences in reaction between 24 h, 48 h and 72 h may partly be due to an adaption of the honey bees to the exposed fungicide due to induction of metabolism enzymes. Therefore, effects were lower after 72 h.

We carefully prepared the different fungicides concentrations. For each treatment, we checked the complete consumption of the sugar solution containing the test substance. This certifies that the exposure concentration is very close to the nominal concentration. However, actual concentrations should be demonstrated by analytical chemical measurements in forthcoming experiments.

4.5. Comparison of lowest observed effect concentrations with environmental levels

Data on environmental concentrations of the here analysed fungicides are sparse. Acoxystrobin concentrations in pollen range from a few ng/g up to 102 ng/g (Fig. 8), but the majority of measured concentrations was below 2 ng/g [31]. Environmental concentrations of folpet are in the low ng/g range (Fig. 8) [31]. Such levels are below the lowest observed effect concentrations of acoxystrobin and folpet of this study (Fig. 8). The range of measured environmental chlorothanolin concentrations is very broad. The lowest detected concentration was 1.1 ng/g and the highest 98,900 ng/g pollen [30] (Fig. 8). The lowest observed effect concentration of chlorothanolin in our study was 2 ng/g and thus below most of the detected pollen concentrations (Fig. 8). Therefore, expressional alteration of target genes involved in oxidative phosphorylation, metabolism, endocrine disruption, and the acetylcholine receptor are environmentally relevant.

5. Conclusion

Chlorothanolin showed strongest effects of the analysed fungicides. Marked down-regulation occurred for transcripts of genes linked to metabolism/detoxification, oxidative phosphorylation and hormone system. The observed effects were season-related; honey bees reacted often faster to chlorothanolin exposure in April than in June, probably due to different composition of the bees sampled (consisting of more nurse bees in April), and differences in temperature and flowering plants on which bees are foraging. Acoxystrobin and folpet had only minor effects on the analysed endpoints and may be regarded as of much lower risk than chlorothanolin to honey bees.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.jhazmat.2019.05.056>.

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