

Biopesticide spinosad induces transcriptional alterations in genes associated with energy production in honey bees (*Apis mellifera*) at sublethal concentrations

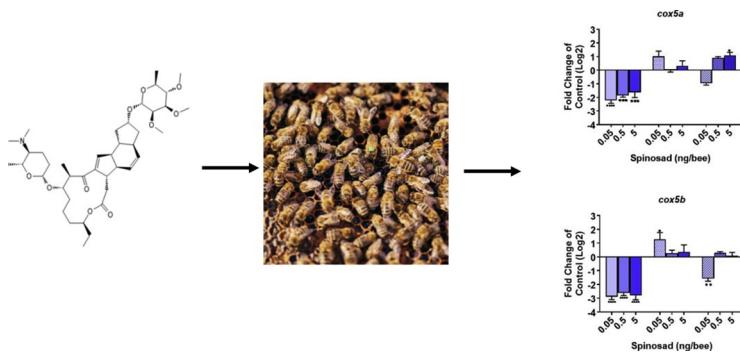
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GRAPHICAL ABSTRACT



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ABSTRACT

Bees experience substantial colony losses, which are often associated with pesticides. Besides synthetic insecticides biological compounds such as spinosad are used in agriculture and organic farming against insect pests. However, potential adverse effect at sublethal concentrations to pollinators are poorly known. Here we aim to determine potential adverse outcome pathways of spinosad and to identify molecular effects by investigating transcriptional alterations in the brain of honey bees. We experimentally exposed bees to three sublethal concentrations of 0.05, 0.5 and 5 ng spinosad/bee, and assessed transcriptional alterations of target genes. Additionally, we evaluated whether spinosad-induced transcriptional alterations were influenced by the time of the year. In April, alterations were most pronounced after 24 h exposure, while in June alterations occurred mostly after 48 h. In July, expressional alterations were often lower but the pattern was more similar to that in June than that in April. Down-regulation of genes encoding acetylcholine receptors, enzymes involved in oxidative phosphorylation (*cox5a*, *ndufb7* and *cox17*), cytochrome P450 dependent monooxygenases (*cyp9q1*, *cyp9q2* and *cyp9q3*) and *insulin-like peptide-1* were among the most significant transcriptional alterations. This suggests adverse effects of spinosad to energy production and metabolism and thus negative consequences on foraging. Together, our study indicates that spinosad causes adverse effects at environmentally realistic concentrations, which may pose a risk to bee populations.

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1. Introduction

In the last decade, important crop pollinators including honey and wild bees have experienced substantial colony losses, which were often associated with exposure to insecticides used in crop protection [1–3]. Not only are bees directly exposed while visiting flowers, they also bring contaminated nectar and pollen to the hive, where larvae and the queen may become exposed to pesticides. Some of the problems have been recognized and three most toxic neonicotinoids have been banned for outdoor applications in Europe and Switzerland (<https://www.efsa.europa.eu/en/press/news/180228>). However, other insecticides may also be harmful to bees, if not at acute than at chronic exposure concentrations. Therefore, less toxic and more sustainable alternatives are needed. One of them is spinosad that has become a pesticide that is even used in organic farming due to its non-synthetic, biological origin.

Spinosad is applied as insecticide to a variety of crops to control for a wide range of insect pests. It is also used as a bioinsecticide in organic farming. Spinosad consists of a mixture of the most biologically active spinosyn A (major component) and spinosyn D. These are naturally produced by the actinomycete *Saccharopolyspora spinosa* and obtained by fermentation [4]. Technical formulations of spinosad show high acute toxicity to honey bees with an oral LC₅₀ (48 h) of 53 ng/bee [5]. Other studies with spinosad report LC₅₀ value of 7.34 mg/L and an inhibition of acetylcholine esterase activity in different organs [6]. In addition, spinosad exhibits high oral and contact toxicities in stingless bees [7]. The acute toxicity to the stingless bee *M. quadrifasciata* was even higher than that of imidacloprid. It also induced sublethal effects on flight activity [7]. Survival of stingless bee larvae was compromised above 114 ng/bee, and in adult workers, walking activity was affected at 2.3 ng/bee [8]. Bumble bees exposed to spinosad in pollen showed brood and adult mortality at 8 mg/kg, which is about twice the level that bees are expected to be exposed to in a worst-case situation. Exposure during larval development to 0.8 mg/kg produced slower foragers [9]. A field-recommended concentration of spinosad was also highly toxic to honey bees. A concentration equivalent to 5% of the lethal spinosad concentration altered the behavioural activity of honey bee workers [10].

Despite its high acute toxicity, spinosad is assumed to be of no high risks to pollinators. This is based on the observation that as dried residue, spinosad was not found to be harmful, and no toxicity was observed under field conditions. Thus, spinosad is regarded as a reduced-risk pesticide by the U.S. EPA [11]. It is assumed to be less harmful than other insecticides to non-target insects. Hence, it is allowed in organic farm production, in which it has become a prominent insecticide. However, only regarding the biological origin does not imply that such an insecticidal compound should be neglected for potential toxicity to non-target insects and cannot be regarded a priori as safe.

In contrast to the acute toxicity, chronic or sublethal toxicity and molecular effects of spinosad to bees are currently unknown. The mode of action of spinosad is based on its neurotoxic activity. Spinosad activates nicotinic acetylcholine receptors and it has an additional activity on gamma-aminobutyric acid (GABA) receptors and alters the function of GABA-gated chloride channels [5,12]. Spinosyn A binds to this receptor and nicotinic acetylcholine receptors at different sites compared to other insecticides, such as nicotine and neonicotinoids [4]. It is suggested that spinosyn A interacts with an unidentified nicotinic acetylcholine receptor subtype and affects nicotinic and GABA receptors through an unknown mechanism. Once inside the insect, spinosyn A is not readily metabolized as shown in tobacco budworm larvae [12].

The lack of knowledge about potential sublethal effects of spinosad and the lack of information on molecular effects prompted us to investigate this biopesticide. Our aim was to evaluate transcriptional responses in the brain of experimentally exposed honey bees, to test the reproducibility of experimental data between independent exposures and to investigate the link between transcriptional alterations and

status of the honey bee hive and food resources between April (spring) and July (summer). To this end, we focused on target genes that play an important role in the physiology of bees and which may be related to the mode of action of spinosad, including acetylcholine receptors and interference with energy metabolism. Our study sheds new lights on adverse implications of this frequently used biopesticide on honey bees and indicates that its risk to pollinators needs further consideration.

2. Material and methods

2.1. Chemicals

Spinosad was purchased from Sigma-Aldrich (Buchs, Switzerland). Stock solutions were prepared in DMSO and diluted into 20% sucrose solution to a final concentration containing 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 situated 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 July 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 20% sucrose solution containing the different spinosad concentrations or 0.1% DMSO as solvent control were done as previously [13,14]. Each exposure experiment consisted of four PET bottles (replicates) with 10 bees per concentration and per three exposure times (24 h, 48 h, 72 h). For RNA extraction three bees were pooled to obtain one pooled RNA sample. Hence, we analysed three bees (pooled to one RNA sample) of each bottle yielding four biological replicates per spinosad concentration.

Exposure concentrations were 0.05, 0.5 and 5 ng spinosad/bee (Table 1). Spinosad stock solutions and spinosad exposure solutions were carefully prepared, nevertheless absorption of spinosad to the wall of the plastic tubes may have occurred. Therefore, the actual exposure concentrations might be slightly below the nominal exposure concentrations. The concentrations were selected on the 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 50% lower than published LD₅₀ concentrations. Indeed, no compound related mortality occurred during exposure.

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 pooled bee brains was isolated using TRI Reagent® (Sigma-Aldrich, Buchs, Switzerland) according to the manufacturer's instructions. 1000 ng RNA was reverse transcribed as described before [13,14]. 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 NCBI primer-blast tool. Sequences of used primers are given in Table 2. For all performed analyses ribosomal protein S5 (rpS5) was used as house-keeping gene for normalization. Alterations

Table 1

Concentrations of spinosad used in the present study.

Compound	Concentration (ng/bee)	Concentration (ng/mL sugar syrup)	LD ₅₀ (oral) in ng/bee
Spinosad	0.05, 0.5 and 5	0.5, 5 and 50	53 [5]

of mRNA abundance in spinosad-exposed samples were always compared with the solvent control (0.1% DMSO) samples to determine the effects of spinosad.

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 (analysis of variance) with Brown Forsythe Test and Bartlett's test to check the equality of variances, followed by a Bonferroni's multiple comparison test to compare treatment means with respective controls. We used the GraphPad Prism software. Results of transcripts are given as means \pm 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 $0.001 > p > 0.0001$.

3. Results

To explore concentration-related and time-related molecular effects we exposed honey bees to concentrations of 0.05, 0.5 and 5 ng spinosad/bee for three different exposure times (24, 48 and 72 h). We focused on the brain tissue, because it is easily accessed, well suitable for gene expression analyses and a major target of this and other neurotoxic insecticides [13,14]. To analyse for season-related effects, we performed three independent experiments. The first experiment was conducted at the end of April, assuming that there are still winter bees in the hive and the spectrum of natural sources of pollen and nectar is still limited. The second experiment was performed in June and the third experiment in July, when no winter bees and a different spectrum of natural resources of pollen and nectar occurred. Honey bees exposed to 20% sucrose solution containing 0.1% DMSO were used as controls, no additional controls containing only 20% sucrose were run. The effects of 0.1% DMSO versus controls were already analysed before [22]. DMSO at this concentration changed the expression of eight transcripts. Gene ontology (GO)-term analysis showed that these transcripts belong to immune system pathways (innate immune response and defence against bacteria) and response to external stimuli. However, there was no effect of DMSO on transcripts of genes encoding enzymes involved in energy metabolism or other target genes of spinosad. Therefore, the effects of DMSO can be neglected in the present study.

3.1. Transcriptional alterations of acetylcholine receptor alpha 1 and alpha 2

All three analysed spinosad concentrations caused strong expressional down-regulation of acetylcholine receptor alpha 1 and alpha 2 after 24 h in April, and after 48 h in June (Fig. 1). In July, these transcripts showed little alteration in response to spinosad exposure (Fig. 1).

3.2. Transcriptional alteration of genes related to oxidative phosphorylation

Transcripts of all five nuclear genes encoding enzymes involved in oxidative phosphorylation in mitochondria were strongly down-regulated after 24 h in all spinosad concentrations in April (Fig. 2). In June, strong down-regulation occurred for cox5a, ndufb7 and cox17 after 48 h exposure in all concentrations. In July, the two highest spinosad concentrations caused down-regulation of ndufb7 expression after 48 h, and of cox17 after 24 h, whereas the expression of cox5b was induced after 48 h exposure to 0.05 and 5 ng/bee spinosad (Fig. 2).

3.3. Transcriptional alterations of genes related to immune system regulation

Exposure of honey bees to spinosad had only minor effects on the expression of immune system related genes. Transcripts of domeless and

hopscotch were significantly down-regulated upon 24 h exposure to 0.05 and 0.5 ng/bee spinosad in April. Expression of both transcripts were also significantly down-regulation after 48 h exposure to 0.5 ng/bee in June. In July, only the transcript of domeless was significantly down-regulated after 72 h exposure to 0.5 and 5 ng/bee spinosad (Fig. 3).

3.4. Transcriptional alteration of genes encoding cytochrome P450-dependent monooxygenases

Spinosad caused strong expressional down-regulation of all three cytochrome P450 transcripts upon 24 h exposure in April (Fig. 4). In June, the expression of cyp9q1, cyp9q2 and cyp9q3 was again down-regulated upon 48 h of exposure (Fig. 4). In July, the cyp9q2 transcript showed a significant alteration in abundance. This transcript was down-regulated upon 48 h exposure to 0.5 ng/bee and up-regulated after 72 h exposure to 5 ng/bee spinosad (Fig. 4).

3.5. Transcriptional alteration of genes encoding major royal jelly proteins

Strong transcriptional alterations of genes encoding major royal jelly proteins were caused by spinosad in June and July but not in April. Transcripts of mrjp1, mrjp2 and mrjp3 were down-regulated after 24 h of

Table 2
Primer sequences used for quantitative qPCR analysis.

Primer	Direction	5' Sequence 3'	Source
<i>Housekeeping gene: ribosomal protein s5</i>	Forward Reverse	AATTATTTGGCTGCTGGAATTG TAACGTCCAGCAGAATGTGGTA	[15]
<i>abaecin</i>	Forward Reverse	CAGCATTTCGATACGTACCA GACCAGGAAACGTTGGAAAC	[15]
<i>acetylcholine receptor α1</i>	Forward Reverse	GAAATACGTGGCGATGTTGC GTGGTATCGTACAGGCTCGG	[13]
<i>acetylcholine receptor α2</i>	Forward Reverse	CCGAACTCTACGTACCGAGC TCGAACGCTATCTCGCAGC	[13]
<i>apidaecin</i>	Forward Reverse	TTTGCCCTAGCAATTCTTGTG GAAGGTCGAGTAGGCAGGATCT	[16]
<i>cox5a</i>	Forward Reverse	TCCGATGATGGACCAAGA AGGTACAAGATCCAGCCGC	Self-designed
<i>cox5b</i>	Forward Reverse	TGGATGTTGTTACATGATGGC AAAGTGTGCAACTTGAGTAAG	Self-designed
<i>cox6c</i>	Forward Reverse	TCGCTTACAGAACACATCTACA ACGAAGCTGAGGCTTGGTAA	Self-designed
<i>cox17</i>	Forward Reverse	AACCTTGTGTTGCTGT ATGTCCTCTATAAATCCC	Self-designed
<i>cyp9q1</i>	Forward Reverse	TCGAGAAGTTTTCCACCG CTCTTCCCTCCGATG	[17]
<i>cyp9q2</i>	Forward Reverse	GATTATCGCCATTATTACTG GTTCTCCTCCCTCTGAT	[17]
<i>cyp9q3</i>	Forward Reverse	GTCGGGGAAAATGAATC GGTCAAATGGTGGTGCAC	[17]
<i>defensin-1</i>	Forward Reverse	CTGCACCTGTTGAGGATGAA GGCCAAGCACTGTCATTAAC	[16]
<i>domeless</i>	Forward Reverse	TTGTGCTCTGAAAATGCTG AACCTCCAAATCGCTCTGTG	[15]
<i>hb-g</i>	Forward Reverse	TACCTGGCTTCGTCAC ATCITCGGTTTCCAGAGAATG	[18]
<i>hopscotch</i>	Forward Reverse	ATTATGGCATCGTGAACAA CTGTTGGGGATTGTTGGTG	[15]
<i>Ilp-1</i>	Forward Reverse	GCTCAGGCCCTGTCGAAAAGT CGTGTATCCACGACCCTTG	[19]
<i>mrjp1</i>	Forward Reverse	CACAGCCCAAGATGGAATT AAGAACGCGCACTTGTGA	[20]
<i>mrjp2</i>	Forward Reverse	GGAAAGGGAGGGCTAGTCTC TCGATCGTCATTTGGCATA	[20]
<i>mrjp3</i>	Forward Reverse	ATTGCCGTAACGCCACTAC CAATCGATGGAAGGAATCGT	[20]
<i>ndufb7</i>	Forward Reverse	TAGAGAACGCAATAGGC TGCTCTTCACAACTAAATC	Self-designed
<i>vitellogenin</i>	Forward Reverse	GCAGAATACATGGACGGTGT GAACAGTCTCGGAAGCTTG	[21]

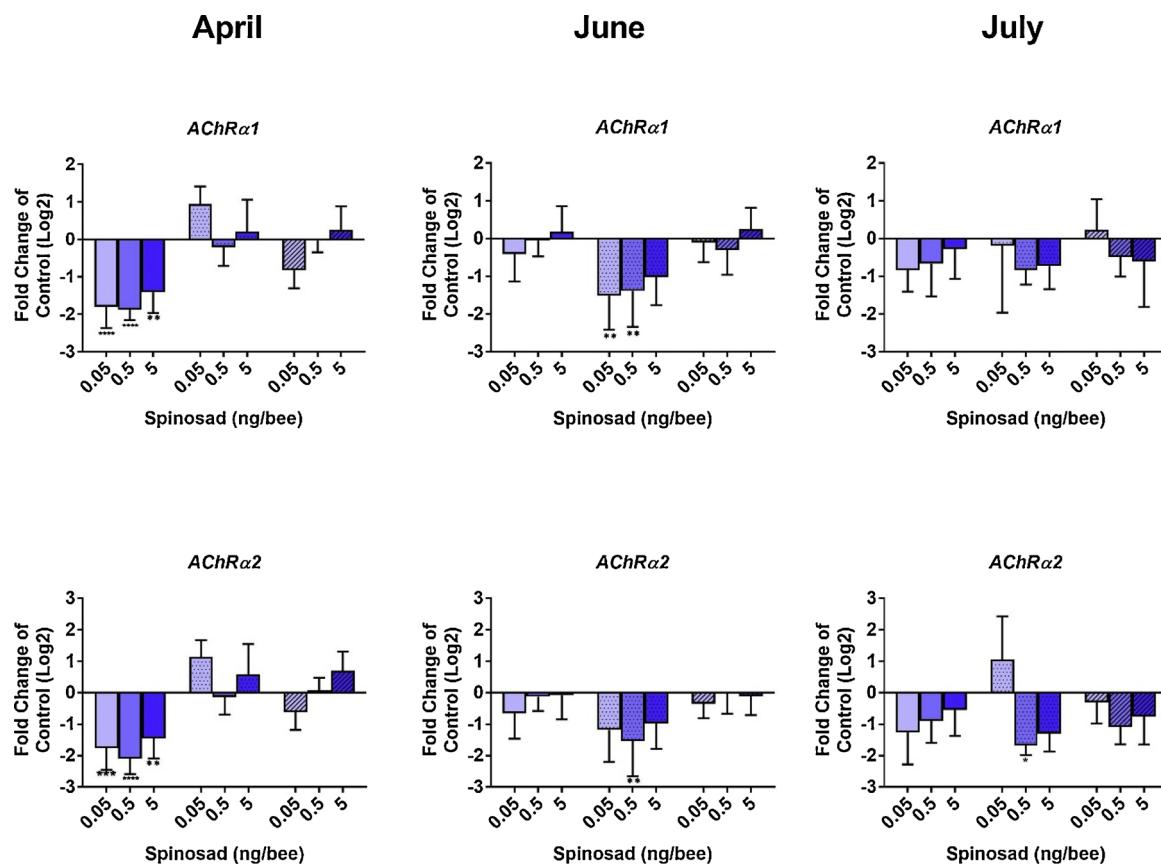


Fig. 1. Abundance of acetylcholine receptor alpha 1 and alpha 2 transcripts in the brain of honey bees following exposure in three different experiments to three different concentrations of spinosad (light blue: 0.05, blue: 0.5 and dark blue: 5 ng/bee) for 24 h (plain bars), 48 h (dots) and 72 h (diagonal stripes). Left column: experiment one (April 2018), middle column: experiment two (June 2018) and right column: experiment three (July 2018). Shown are the results of four biological replicates per concentration and exposure time and standard deviations. Significant differences with p-value of ≤ 0.05 are marked with asterisks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

exposure in June. In July, the transcript of *mrjp1* was upregulated upon 48 h of exposure and the *mrjp3* transcript was downregulated upon 24 h of exposure (Fig. 5).

3.6. Transcriptional alteration of genes linked to hormone system

Only minor expressional alterations were observed in transcripts of genes encoding proteins associated with the hormone system. The transcript of *insulin-like peptide 1* was down-regulated after 24 h at 0.5 ng/bee in April, and after 48 h at 0.05 and 0.5 ng/bee in June. In July, the transcript of *hbg-3* was induced upon 24 h exposure to 0.05 ng/bee (Fig. 6). Spinosad caused no significant expressional alterations of *vitellogenin* (Fig. 6).

3.7. Overall pattern of transcriptional alterations

The heatmap in Fig. 7 depicts all transcriptional alterations of spinosad at all exposure times and concentrations. The observed changes were dominated by transcriptional down-regulations. Alterations form two separate clusters, which share similarities in their patterns. One cluster consists of the responses observed in the exposure experiment in April, and the second cluster consists of responses found in exposure experiments in June and July. Thus, the expression pattern in June and July are similar and distinct from that in April. In April, spinosad caused strong down-regulation at 24 h of genes encoding enzymes involved in oxidative phosphorylation, *acetylcholine receptor alpha 1*, *cyp9q1* and *cyp9q2*. In contrast, in June, strong down-regulation occurred at 48 h, involving transcripts of *cyp9q1*, *cyp9q2*, *cyp9q3*, *domeless*, *hopscotch*, *acetylcholine alpha 1* and *2*, *cox5a* and *cox17*. In July,

weaker transcriptional changes occurred, except for a few genes, including down-regulation of *acetylcholine receptors alpha1* and *alpha2*, *ndufb7*, *cox17*, *cyp9q2* and *mrjp3*.

We compared the lowest effect concentration (LOEC) for transcriptional alteration of our study with environmental concentrations of spinosad. Fig. 8 demonstrates that the LOEC of our study was within the range of environmentally relevant concentrations of spinosad in pollen.

4. Discussion

Here we show for the first time significant molecular effects of the biopesticide spinosad in the brain of honey bee workers at environmentally realistic exposures. Transcripts of genes encoding proteins of important physiological pathways such as neuronal signalling, immune system regulation, oxidative phosphorylation, metabolism and endocrine regulation were analysed. To analyse for seasonal effects, three independent experiments were performed in different times of the year. The first experiment was conducted in April when still winter bees are in the bee hive, the second experiment in June, when the bee hive is fully developed and the third experiment in July, when the bee population starts to decline. Spinosad induced strongest effects in April after 24 h of exposure. Strong and similar effects were caused in June but this occurred at 48 h of exposure. Only minor effects occurred in July.

In April and June strong down-regulation occurred in the expression of genes encoding *acetylcholine receptors alpha 1* and *alpha 2*, enzymes of the oxidative phosphorylation and cytochrome P450 enzymes. In July, down-regulation was weaker but occurred also for some genes. Although generally similar, effects of spinosad were stronger and faster in April than in June but were weaker in July. This indicates that

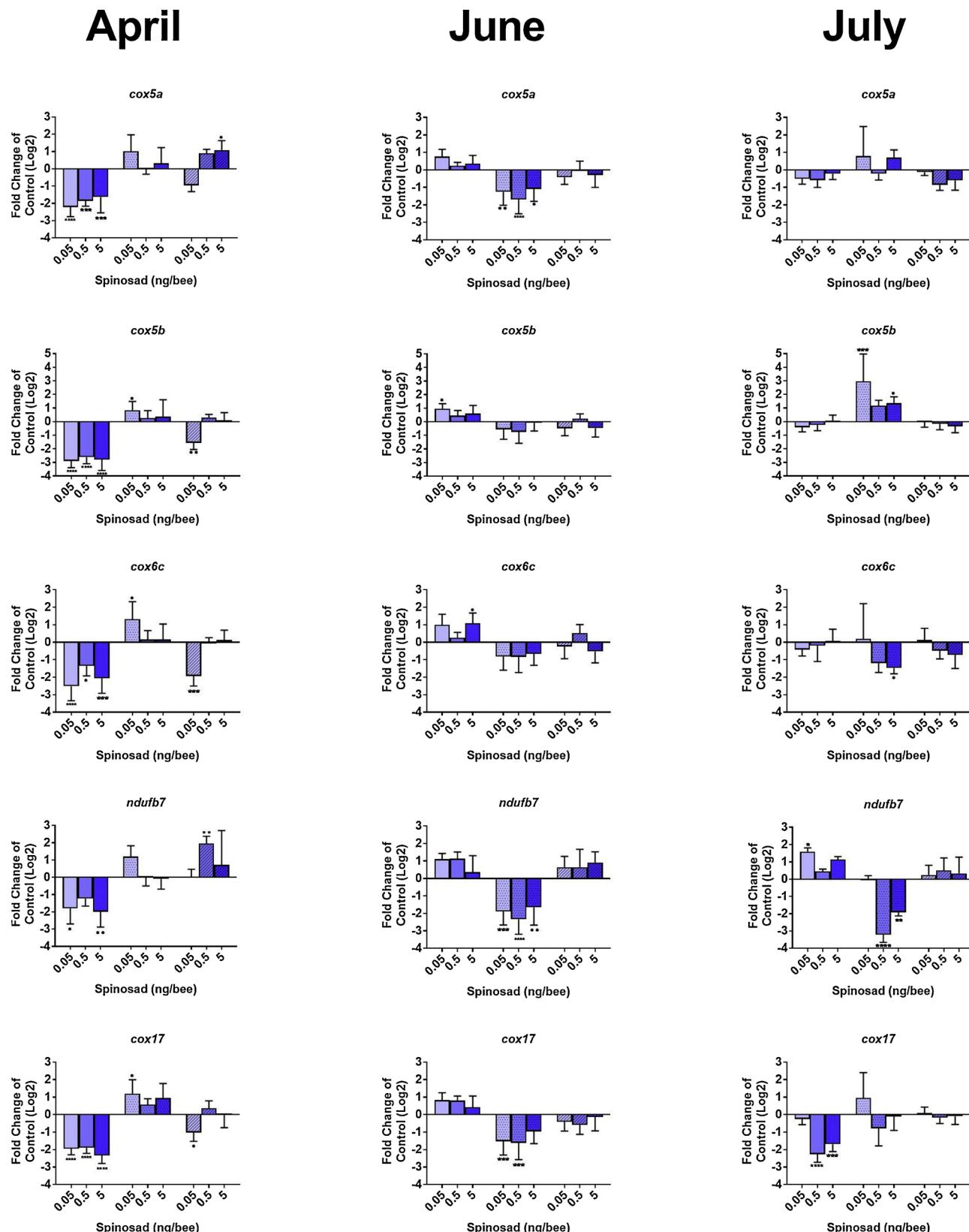


Fig. 2. Abundance of transcripts *cox5a*, *cox5b*, *cox6c*, *cox17* and *ndufb7*, which are nuclear genes encoding enzymes involved in mitochondrial oxidative phosphorylation in the brain of honey bees following exposure in three different experiments to three different concentrations of spinosad (light blue: 0.05, blue: 0.5 and dark blue: 5 ng/bee) for 24 h (plain bars), 48 h (dots) and 72 h (diagonal stripes). Left column: experiment one (April 2018), middle column: experiment two (June 2018) and right column: experiment three (July 2018). Shown are the results of four biological replicates per concentration and exposure time and standard deviations. Significant differences with p-value of ≤ 0.05 are marked with asterisks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

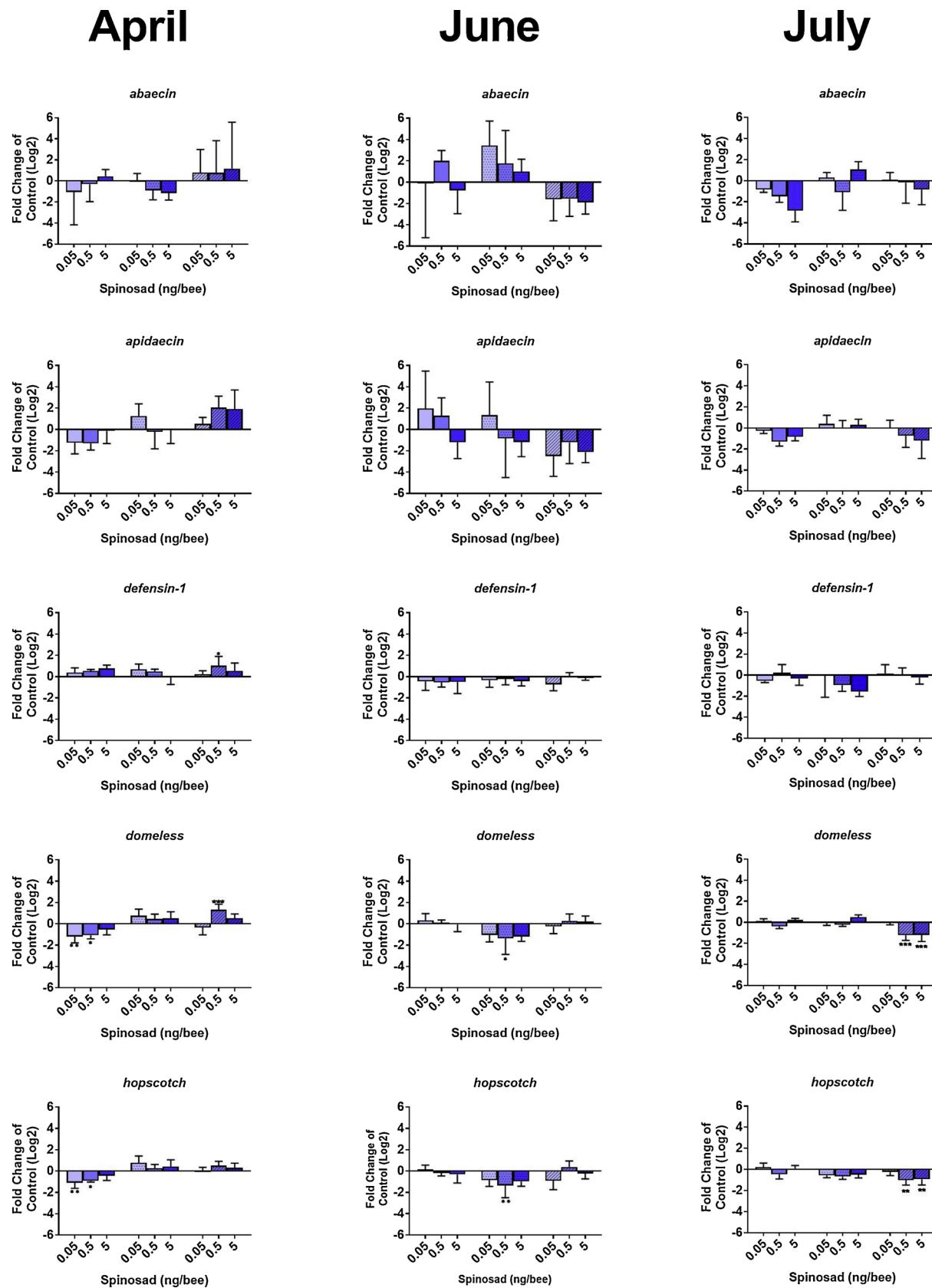


Fig. 3. Abundance of transcripts of genes related to immune system regulation such as *abaecin*, *apidaecin*, *defensin-1*, *domeless* and *hopscotch* in the brain of honey bees following exposure in three different experiments to three different concentrations of spinosad (light blue: 0.05, blue: 0.5 and dark blue: 5 ng/bee) for 24 h (plain bars), 48 h (dots) and 72 h (diagonal stripes). Left column: experiment one (April 2018), middle column: experiment two (June 2018) and right column: experiment three (July 2018). Shown are the results of four biological replicates per concentration and exposure time and standard deviations. Significant differences with p -value of ≤ 0.05 are marked with asterisks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

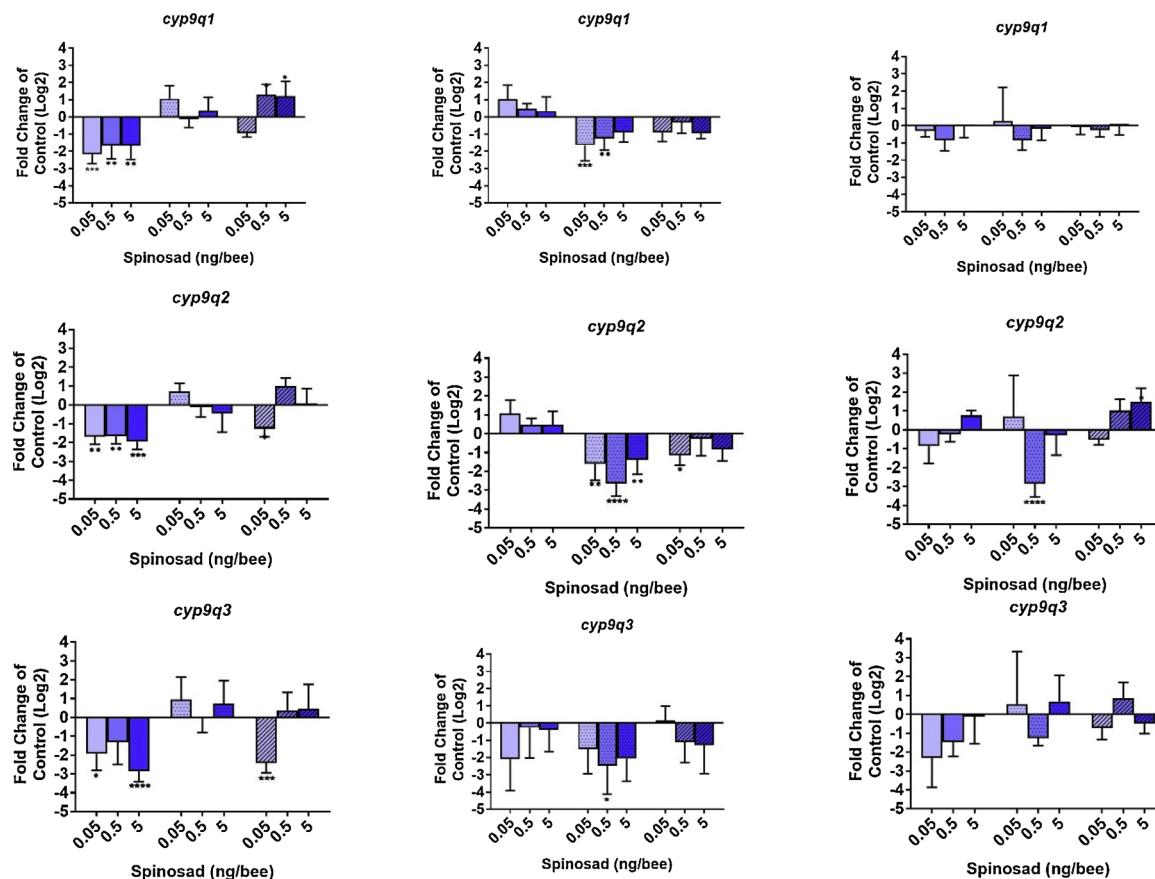


Fig. 4. Abundance of cytochrome P450 dependent monooxygenase gene transcripts *cyp9q1*, *cyp9q2* and *cyp9q3* in the brain of honey bees following exposure in three different experiments to three different concentrations of spinosad (light blue: 0.05, blue: 0.5 and dark blue: 5 ng/bee) for 24 h (plain bars), 48 h (dots) and 72 h (diagonal stripes). Left column: experiment one (April 2018), middle column: experiment two (June 2018) and right column: experiment three (July 2018). Shown are the results of four biological replicates per concentration and exposure time and standard deviations. Significant differences with p -value of ≤ 0.05 are marked with asterisks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spinosad caused transcriptional alterations with different potency and different temporal behaviour in different times of the year.

4.1. Effects on acetylcholine receptor transcripts

In the insect nervous system, acetylcholine receptors (AChR) mediate fast cholinergic transmission and they are important targets of insecticides [23]. Agonists of the AChRs such as neonicotinoids caused changes in behaviour, poor navigation and foraging of honey bees [24,25] and bumble bees [26]. In our previous studies, we demonstrated that different insecticides induced gene transcripts encoding *acetylcholine receptor alpha 1* and *alpha 2*. Insecticides including neonicotinoids, such as acetamiprid, clothianidin, imidacloprid and thiamethoxam, organophosphates, such as chlorpyrifos and malathion, and the pyrethroide cypermethrin caused an up-regulation, while chlorantraniliprole caused a down-regulation [13,14]. In the present study, we observed a strong down-regulation of the *acetylcholine receptor alpha 1* and *alpha 2* transcripts in response to spinosad (Fig. 1). This may be regarded as a compensation reaction of the honey bee to the sustained activation of acetylcholine receptors by spinosad, as it agonistically activates nicotinic acetylcholine receptors [12]. The importance of this target is also shown by the fact that the nicotinic acetylcholine receptor subunit alpha 6 is associated with the development of spinosad resistance in various pests [27–29].

The transcriptional effect of spinosad on AChR transcripts differed from that of neonicotinoids that have a similar agonistic action on acetylcholine receptors but bind to other sites of the receptors. While neonicotinoids caused an induction of AChR transcripts, spinosad

caused a down-regulation. Although transcriptional alterations are regarded as compensatory reaction to pesticide exposure, the distinct reactions of neonicotinoids and spinosad are currently not understood but may be based on the different binding sites to the AChR by the different pesticides, and consequently, affecting compensatory transcriptional reactions differently.

4.2. Transcriptional effects on oxidative phosphorylation enzymes

Spinosad displayed strong, mainly inhibitory effects on expression of genes encoding enzymes of oxidative phosphorylation, a key process in energy production from glucose (Fig. 2). Together with other proteins, the protein encoded by *ndufb-7* builds the NADH-dehydrogenase of complex I and proteins encoded by *cox5a*, *cox5b*, *cox6c* and *cox17* belong to the cytochrome c oxidase complex IV [30]. Adverse effects of pesticides on honey bee energy metabolism are well known. For instance, mitochondrial activity and ATP production was reduced in the brain and thorax of bees exposed to imidacloprid and fipronil [31]. Negative effects of several plant protection products on mitochondrial energy metabolism in the flight muscle of bumble bees were also observed [32]. Additionally, phytochemicals in nectar and pollen can also negatively interact with honey bee energy metabolism [30].

Expressionally down-regulation of enzymes essential for oxidative phosphorylation may have negative impacts on honey bee energy production when translated to the enzyme level.

Besides lower or lack of delivery of ATP as energy source for numerous biochemical processes, brain metabolism may also be affected causing behavioural changes. When comparing aggressive honey bees

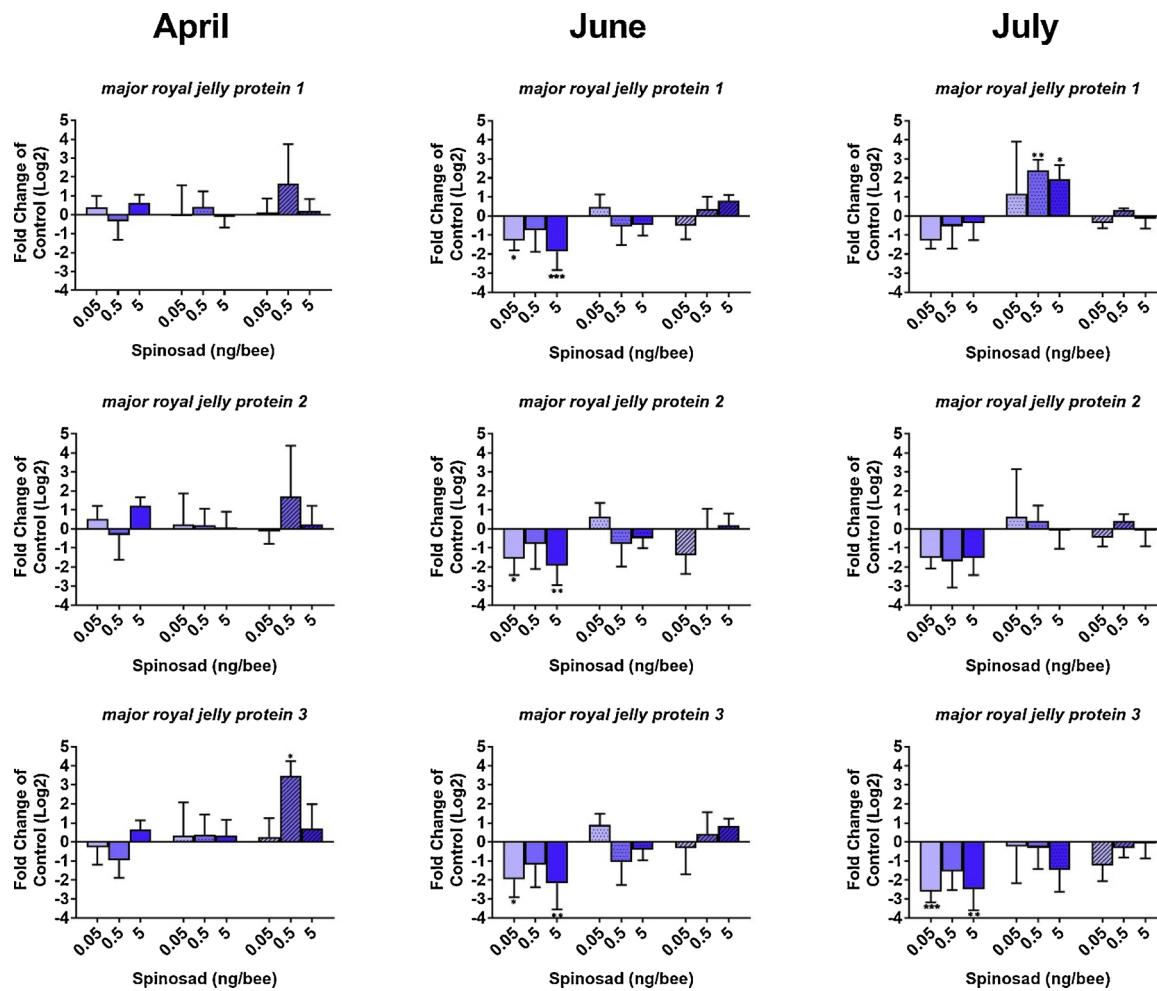


Fig. 5. Transcript abundance of genes encoding major royal jelly proteins *mrjp1*, *mrjp2* and *mrjp3* in the brain of honey bees following exposure in three different experiments to three different concentrations of spinosad (light blue: 0.05, blue: 0.5 and dark blue: 5 ng/bee) for 24 h (plain bars), 48 h (dots) and 72 h (diagonal strips). Left column: experiment one (April 2018), middle column: experiment two (June 2018) and right column: experiment three (July 2018). Shown are the results of four biological replicates per concentration and exposure time and standard deviations. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to non-aggressive honey bees, transcripts of the different complexes of the oxidative phosphorylation were differently expressed. In the brain of aggressive honey bees, complex I and complex IV show lower activity compared to non-aggressive honey bees [33]. Treatment of bees with complex I and complex V inhibitors stimulated aggressive behaviour [34]. As spinosad strongly inhibited the expression of transcripts encoding proteins of complex I and IV, the question arises, whether this may have implications not only on energy production but also on the behaviour of honey bees. In addition, oxidative phosphorylation capacity is different in different honey bee castes. In the head of nurse bees, the oxidative phosphorylation capacity is higher compared to foragers [35]. Taken together, exposure of nurse bees to spinosad through pollen and nectar may cause an accelerated transition to foragers.

4.3. Effects on detoxification system

Cytochrome P450 dependent monooxygenases, carboxylesterases and glutathione transferases are the major enzyme families responsible for the metabolism and detoxification of foreign compounds [36]. Several studies showed that cytochrome P450 enzymes such as *cyp9q1*, *cyp9q2* and *cyp9q3* are responsible for the degradation of various classes of plant protection products [37,38]. Pesticide exposure may have negative effects on the honey bee detoxification system. Tau-fluvalinate caused up-regulation of the *cyp9q3* transcript and bifenthrin up-

regulated *cyp9q1* and *cyp9q2* and decreased expression of *cyp9q3* [17]. Coumaphos and fluvalinate caused transcriptional up-regulation of several P450 genes of the CYP3 family that is involved in detoxification of insecticides [39,40]. Additionally, altered expression of *cyp9q1*, *cyp9q2* and *cyp9q3* was observed after exposure of honey bees to the organophosphates chlorpyrifos and malathion, the pyrethroid cypermethrin and chlorantraniliprole [14]. Imidacloprid resulted in the up-regulation of P450 transcripts belonging to the CYP4, CYP6 and CYP9 families, all known to contribute to insecticide resistance [34] in honey bees larvae [41].

CypP450 enzymes are known to be involved in the resistance of houseflies to spinosad. Of all cypP450 transcripts, 60–80% were differently expressed in resistant strains compared to wild type strains [42], and resistant *Helicoverpa armigera* populations showed an increase in cypP450 enzymes [43]. Spinosad caused a strong and transient down-regulation of *cyp9q1*, *cyp9q2* and *cyp9q3* in honey bees (Fig. 4). If translated to the enzyme level, this implies that the metabolism and detoxification capacity is negatively affected, which may result in higher toxicity of this and other pesticides.

4.4. Seasonal effect on gene expression

Expressional changes varied with exposure duration from 24 h to 72 h in all experiments. However, transcriptional alterations caused by

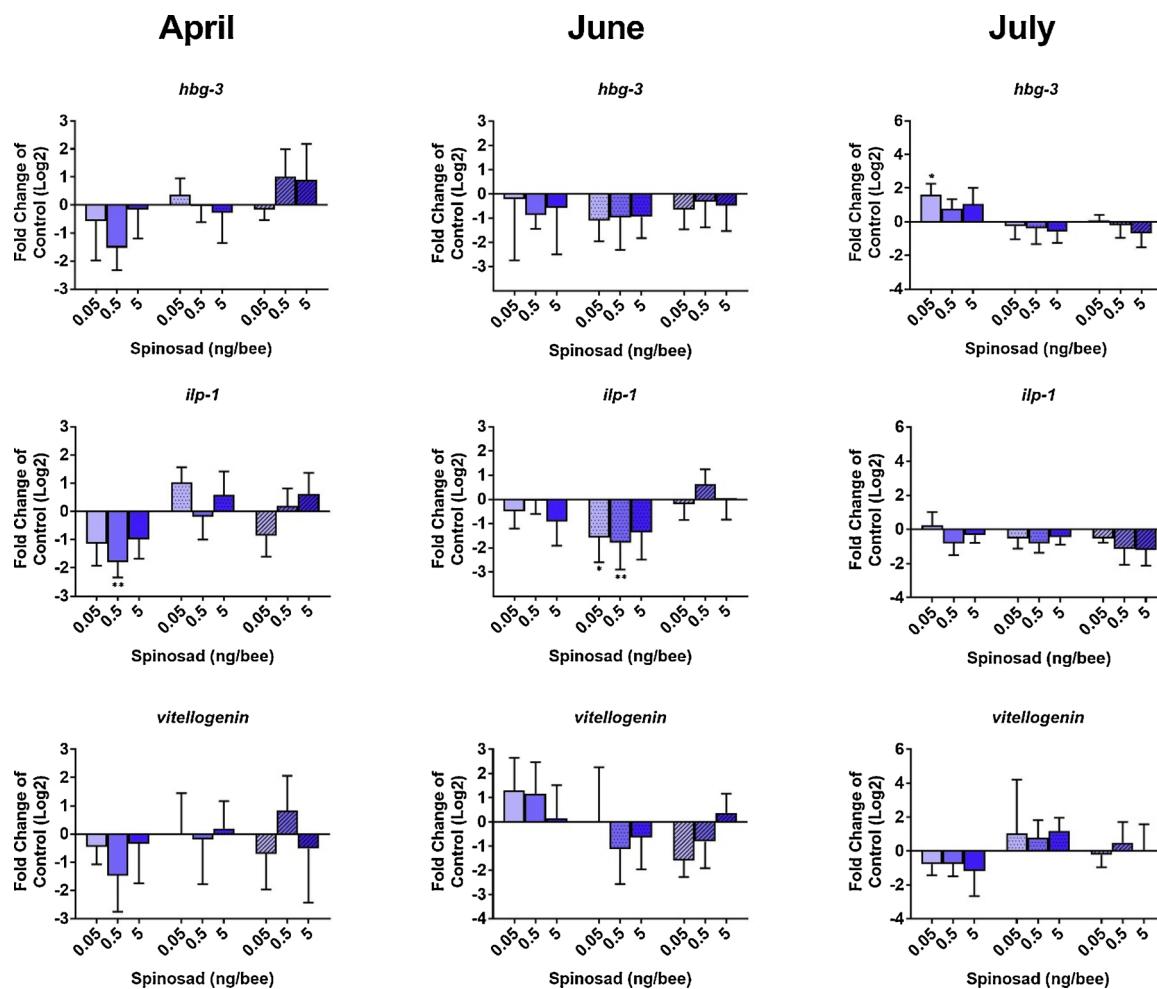


Fig. 6. Abundance of transcripts of genes related to hormonal activity such as *hb-g-3*, *ilp-1* and *vitellogenin* in the brain of honey bees following exposure in three different experiments to three different concentrations of spinosad (light blue: 0.05, blue: 0.5 and dark blue: 5 ng/bee) for 24 h (plain bars), 48 h (dots) and 72 h (diagonal stripes). Left column: experiment one (April 2018), middle column: experiment two (June 2018) and right column: experiment three (July 2018). Shown are the results of four biological replicates per concentration and exposure time and standard deviations. Significant differences with p -value of ≤ 0.05 are marked with asterisks. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

spinosad were strongest in April and weakest in July. Moreover, strongest alterations occurred after 24 h in April, while in June, they occurred after 48 h (Fig. 7). Furthermore, the pattern of expressional changes is similar in June and July and distinct from that in April. These differences are not due to technical reasons, as transcriptional data are reproducible between different years when assessed at the same time of the year [44]. Reasons for the different sensitivity of expressional changes in different times of the year are likely based on other factors, including different composition of the bee population, different temperature and natural plant secondary products in nectar and pollen to which bees are exposed.

In April, the honey bee hive just starts to develop, has still a bigger proportion of winter bees inside, and the ratio between nurse bees and foragers is two thirds to one third. In June, the honey bee hive is fully developed without winter bees and the ratio between nurse bees and foragers is balanced (<https://www2.hu-berlin.de/bienenkunde/index.php?id=112>). In addition to different composition of exposed bees and the state of the bee population, different temperatures may also be relevant. In June and July, the metabolic rate of honey bees is higher due to higher temperature [45] and this may make bees more sensitive in July. This may also be a reason for the similarity of expressional changes in June and July in comparison to April.

Another reason might be the composition of flowers on which honey bees are foraging and feeding. In spring, mainly phacelia, wild lupine,

penstemon, fireweed and various fruit trees are sources of pollen and nectar. In summer, there is a shift to butterfly milkweed, mints, bergamot, coneflowers and sunflowers and in late summer, interesting flowers for bees are aster and goldenrod (<https://door.extension.wisc.edu/files/2010/05/Deutsch-BeeKeeperTalk2.pdf>). Due to differences in secondary plant products in nectar and pollen of different flowers, honey bees are exposed to different secondary plant products throughout the year. This may also influence the reactivity of honey bees to pesticides.

4.5. Comparison of lowest observed effect concentrations with environmental levels

Despite sparse data, residue concentrations of spinosad in pollen, nectar, honey or bees are poorly known. Treatment of sweet corn with 40 g/ha spinosad caused pollen concentrations of 320 ng/g [46]. In pollen collected by honey bees in areas with different agricultural activities spinosad concentrations were 0.87–1.01 ng/g in non-agriculture areas, 0.57–0.64 ng/g in areas with untreated maize and 0.16–0.44 ng/g in treated maize areas [47]. In honey, spinosad concentrations were up to 20 ng/g [48]. The lowest effect concentration of spinosad in our study was 0.5 ng/g, and thus below most detected pollen concentrations (Fig. 8). This leads to the conclusion that spinosad caused expressional alteration of target genes involved in neuronal signalling, oxidative

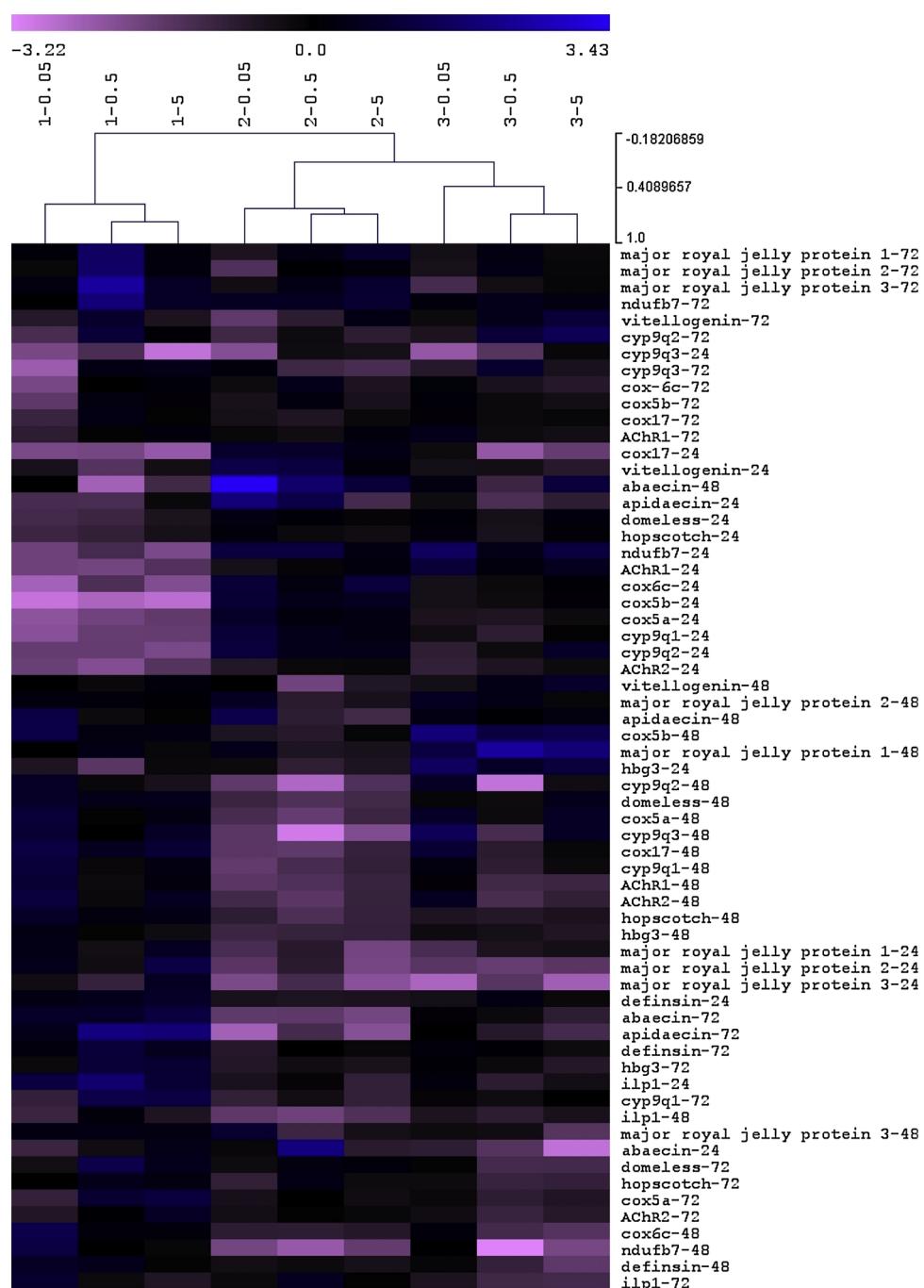


Fig. 7. Heat map showing all obtained transcriptional alterations for all three experiments at different concentrations of spinosad (0.05, 0.5, 5 ng/bee) and exposure times of 24 h, 48 h and 72 h (given after gene name as 24, 48 and 72). Concentrations and experiments are shown above (key: 1, April; 2, June; 3, July) and transcripts are listed to the right. The magnitude of transcriptional alterations is given in pink (down-regulation) or blue (up-regulation), while no changes are given in black. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

phosphorylation and detoxification at environmentally relevant concentrations. Spinosad is applied to treat many fruit trees (cherries, plums, apples, pears), strawberries, raspberries, vines and different vegetables (<https://www.psm.admin.ch/de/produkte/D-6175>) at a time, when the honey bee hives and wild bee colonies are developing and when honey bees react most sensitive to spinosad according to our data. If energy production is negatively affected during this time in bees, foraging activity and colony development may be adversely affected. Furthermore, development of larvae may be negatively affected.

5. Conclusions

Our study indicates that spinosad induces expressional alterations of important genes in the brain of honey bee workers at concentrations that may occur in pollen of treated plants. Spinosad caused mainly transcriptional down-regulation of genes encoding acetylcholine receptors and enzymes involved in oxidative phosphorylation and metabolism. Consequently, spinosad causes lower energy production (ATP) and reduced metabolism/detoxification in the brain of honey bee. We

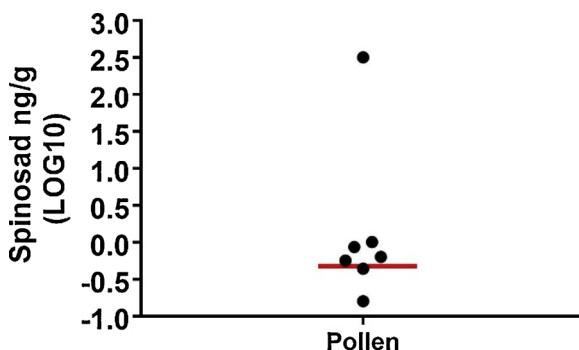


Fig. 8. Comparison of lowest effect concentrations (LOEC, red line) defined as significant transcriptional alterations of gene transcripts of the present study with reported pollen concentrations of spinosad [46–48]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

also observed that transcriptional alterations were more pronounced in April than in July. In addition, the time-course differed; transcriptional changes occurred faster in April than later in the year. This is probably based on different composition of bee population and plant secondary products that affects honey bee sensitivity to pesticides exposure and therefore may influence gene expression. As spinosad is applied before and after flowering of fruit trees, April is a very critical time for honey bees with regard to spinosad exposure, as energy metabolism of foragers may be compromised. Thus, this may negatively influence the striving of bee populations in case the effects are not transient. Together, our data clearly indicate that the biopesticide spinosad induces adverse transcriptional effects in bees at environmentally realistic concentrations. Further studies are needed to investigate whether they translate to physiological effects such as altered behaviour and foraging activities.

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