Pesticide Biochemistry and Physiology 170 (2020) 104703

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Effects of selected insecticidal substances on mRNA transcriptome in larvae of *Apis mellifera*

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ARTICLE INFO

Keywords:
Apis mellifera
Larvae
Sublethal dose
Insecticides
Transcriptome

ABSTRACT

For the last decade, scientists have reported a loss of honeybee colonies. Multiple factors like parasites, pathogens and pesticides are dealt as possible drivers of honeybee losses. In particular, insecticides are considered as a major factor of pollinator poisoning. We applied sublethal concentrations of four insecticidal substances to honeybee larval food and analyzed the effects on transcriptome. The aim was to identify candidate genes indicating early negative impacts after application of insecticidal substances. Honeybee larvae were kept in-vitro under hive conditions (34–35 °C) and fed with dimethoate, fenoxycarb, chlorantraniliprole and flupyradifurone in sublethal concentrations between day 3–6 after grafting. Larvae at day 4, 6 and 8 were sampled and their transcriptome analyzed. By use of a RT-qPCR array differences in gene expression of selected gene families (immune system, development detoxification) were measured. Targets mainly involved in development, energy metabolism and the immune system were significantly affected by the insecticidal substances tested, selectively inducing genes of the detoxification system, immune response and nutritional stress.

1. Introduction

Pollination plays an important role in global economics and is estimated to account for world-wide 153 billion Euros per year (Gallai et al. 2009). Most of the important crops are pollinated by insects, mainly by bees (Machado 2004). This makes the honeybee one of the most important farm animals in global agriculture (Hanley et al. 2015).

Since the mid-2000s, honeybee colony losses were reported from all over the world (Van der Zee et al. 2012). The reasons for losses are often complex and interacting, with major drivers including *Varroa destructor* and related viruses and diverse agrochemicals, respectively (van Dooremalen et al. 2018).

Furthermore, the application of insecticides is hypothesized to play another important role in the well-being of individual bees, colony losses and overall decline in beneficial insects (Calatayud-Vernich et al. 2019). Currently different classes of insecticides are used in agriculture that differ in their chemical structure and/or physiological targets, e.g. neonicotinoids. In 2008, the German government restricted the use of distinct neonicotinoids after an inadvertent release of clothianidin dust drift during sowing in South-Germany affecting more than 10,000 honeybee colonies (Pistorius et al. 2008). In 2013, the European Food Safety Authority (EFSA) published a report summarizing toxic effects of neonicotinoids on honeybees triggering a ban of three insecticidal substances (clothianidin, thiamethoxam, imidacloprid) in the field (EFSA 2013).

Flupyradifurone has a similar mode of action as the neonicotinoids and is allowed to use until 2025 in the EU.

Another quite new group of insecticides are anthranilic diamides like chlorantraniliprole. Pesticides that belong to this group stimulate calcium release in muscle cells via ryanodine receptors leading to a detracted muscle regulation and subsequently to death (Qi and Casida 2013).

Another class of insecticides are juvenile hormone analogues like fenoxycarb that interfere with hormonal action (Staal 1975). Hence, insects remain in a juvenile stage inhibiting adult emergence. In the EU, fenoxycarb is permitted to use until 2021. Furthermore, organophosphates like dimethoate are widely used insecticides. They inhibit acetylcholinesterase activity in the target, which leads to an accumulation of acetylcholine and prevents the constant transfer of nerve functions inducing convulsions and death (Larc 1983).

Nevertheless, the application of insecticides is a double-edged sword: First of all, insecticides are used to kill pests to prevent damage from crops. On the other hand, it has to be ensured that beneficial insects (natural predators, pollinators etc.) or non-target animals are not affected. Beneficial arthropods have received the most attention in this regard, as they are important for integrated pest management and

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https://doi.org/10.1016/j.pestbp.2020.104703
Received 4 June 2020; Received in revised form 3 September 2020; Accepted 6 September 2020
Available online 10 September 2020
0048-3575/

pollination (Van Driesche and Bellows 1996, Brittain et al. 2013). In lacewings, Rumpf et al. (1997) showed that inhibition of AChE and glutathione *S*-transferase and mortality are both toxin as well as species specific. In wolf spiders, it was also shown that the inhibition of AChE only disappeared after eight days after exposure (Van Erp et al. 2002). Studies on parasitoids often report negative effects on adult growing up in the pupal stage (Krespi et al. 1991; Saber et al. 2005). Especially the side effects concerning adult honeybees are investigated intensively (for review see Cloyd 2019). However, the health of a colony also depends on honeybee brood. Pollen and nectar contaminated with pesticides may damage the brood and overall colony health (Yang et al. 2012). Therefore, Aupinel et al. (2005) developed an in vitro larval feeding test for evaluating the toxicity of insecticides. On the basis of this methodology, OECD Guideline 237 (single exposure, OECD 2013) and 239 (repeated exposure, OECD 2016) were developed. The mentioned protocols address mortality and abnormal morphological development on honeybee larvae. However, sublethal effects of pesticides are not necessarily detected in such systems, but should be considered in addition in semi-field and field studies (Thompson and Maus 2007). As reported, monitoring the transcriptome can add essential information and serve as an appropriate indicator of sublethal impairment (Li et al. 2019).

Therefore, potential transcript-changes may be suitable to further characterize sublethal effects of selected insecticidal substances in a standardized honeybee larvae feeding assay based on the Guidance Document 239 of the OECD (2016).

Our aim is to develop a honeybee-specific array containing 79 candidate genes (RT-qPCR array) that is able to detect negative effects of sublethal concentrations of a given test substance at an early stage by using a standardized honeybee larvae system. In particular, targets involved in development and detoxification are expected to be regulated.

2. Material and methods

2.1. Source of bees

The experiments were performed in seasons 2018 and 2019 during the egg laying period of queen bees in spring and early summer with colonies of *Apis mellifera* buckfast. We performed our tests with buckfast bees because the Institute for Bee Protection conducts test with these bees since many years. So far, no differences in sensitivity between buckfast and carnica were noted. A comparable sensitivity of the test organisms can be justified by preliminary laboratory tests following standardized OECD protocols, e.g. 237 (2013). These studies met the required validity criteria for the reference substances, indicating a sufficient sensitivity of the test organisms. All colonies were maintained at the Institute for Bee Protection at the Julius Kühn Institute in Braunschweig, Germany. Queens of selected colonies were sister queens. The beehives used to collect freshly hatched honeybee larvae were not checked for virus infections. However, beehives showed no clinical symptoms of virus infections like behavioral and phenotypically abnormalities and were free of notifiable diseases like American foulbrood, Tropilaelaps mites, small hive beetles and varroosis. Furthermore, beehives were only used as donor for freshly hatched honeybee larvae for the in-vitro test system. The colonies were adequately fed and queen-right. No injurious substances (such as antibiotics, varroacides, agrochemicals, etc.) have been used in the hive within four months before our experiments.

2.2. Honeybee larvae test

Honeybee larvae were tested according to the Guidance Document 239 by the OECD (2016): The queens were caged in queen excluders for a maximum duration of 30 h to get a high number of synchronized eggs. After three days of incubation in hive first instar larvae of three replicate colonies were grafted from the combs and individually placed into polystyrene grafting cells in a 48-well plate. For each treatment group, 48 larvae from the three different hives were used, which equates to 16 larvae per replicate. The culture plates were placed into a desiccator (SICCO Star-Desiccator) in an incubator (Memmert BE 600) containing a dish filled with a saturated potassium sulphate (K_2SO_4) solution to maintain the relative humidity at approximately 95% ± 5% and temperature at 34–35 °C. Larvae were fed with artificial diets (A, B, C) containing the test chemicals at sublethal concentrations as follows (OECD 2016):

Diet A (day 1; D1): 50% *w*/w fresh royal jelly, 50% w/w of an aqueous solution containing 2% w/w yeast extract, 12% w/w glucose and 12% w/w fructose.

Diet B (day 3; D3): 50% w/w fresh royal jelly, 50% w/w of an aqueous solution containing 3% w/w yeast extract, 15% w/w glucose and 15% w/w fructose.

Diet C (from days 4–6; D4 to D6): 50% w/w fresh royal jelly, 50% w/w of an aqueous solution containing 4% w/w yeast extract, 18% w/w glucose and 18% w/w fructose.

Dimethoate (BASF, purity 99.9%) and fenoxycarb (HPC Standards, purity 98.3%) were used in 2018 because those two substances are well characterized and no extensive pre-experiments were necessary to identify sublethal concentrations. After first results in 2018 we decided to expand our protocol in 2019 by chlorantraniliprole (HPC Standards, purity 98.1%) and flupyradifurone (HPC Standards, purity 99.9%) because both are potential replacements for banned neonicotinoids. Chemicals were used solely and not in combination. Impurities of the active substances were considered for the calculation of the test concentrations. Organic royal jelly (Cum Natura) was assayed for contaminants and did not contain any residues of plant protection products. The sublethal test concentrations for dimethoate and fenoxycarb, which are commonly used reference substances in honeybee laboratory, semi-field and field studies were derived from the test concentrations given in OECD Guidance Document 239 and own preliminary larval tests. The sublethal test concentration for flupyradifurone mimicked the NOEC of a chronic larval GLP study conducted for the registration of the active substance flupyradifurone in the European Union (EFSA 2015).

The sublethal test concentration for chlorantraniliprole was selected based on a chronic larval GLP study available to the Institute for Bee Protection due to its activity in the zonal risk assessment of plant protection products in accordance with § 41 (3) Point 2 of the German Plant Protection Act (PflSchG). However, this study and its results are confidential and not publicly accessible. From day three (D3) until day six (D6) after grafting the test chemicals were fed chronically at a constant concentration to the larvae resulting in a cumulative dose per larva on D6 according to Table 1. The stock solution of dimethoate was prepared by dissolving dimethoate in deionized water. The stock solutions of fenoxycarb, flupyradifurone and chlorantraniliprole were prepared by dissolving the chemicals in pure acetone. Subsequent dilutions were performed using the same solvents. The content of test solution in the final diet was 10% *w*/w for dimethoate, 0.5% w/w for fenoxycarb, flupyradifurone and chlorantraniliprole. According to the latter, we added an additional acetone control containing 0.5% of pure acetone to preclude effects of the solvent itself. All larvae were fed once a day, except on D2, with a multi stepper pipette (Eppendorf Multipette M4). Larvae were not touched or drowned during feeding. On D4, D6 and D8 alive larvae were sampled, washed with PBS and quick-frozen for further transcriptome analysis (Fig. 1).

2.3. Transcriptome analysis

2.3.1. RNA extraction and cDNA synthesis

RNA extraction was performed following the instructions of the Zymo Research Quick RNA Microprep Kit (Thermo Scientific). One larva was homogenized in 600 µl of lysis buffer and incubated at room temperature for 30 min. 300 µl of lysate was transferred into a RNase-free tube and an equal volume of ethanol was added, the mixture was

Table 1
List of all sample groups and corresponding ingredients (a.s. = active substance).

Group	Treatment	Concentration (mg a.s./kg diet)	Cumulative doses (µg a.s./larva)	Active ingredient	LD50 (µg/bee)
C	Control	–	–	Water	
CS	Solvent control	–	–	Water + acetone	
DM1	Treatment 1	0.33	0.05	Dimethoate	1.9 (Aupinel et al. 2007)
DM2	Treatment 2	0.65	0.1	Dimethoate	see DM1
DM3	Treatment 3	1.29	0.2	Dimethoate	see DM1
Cl	Treatment 4	0.72	0.11	Chlorantraniliprole	> 0.027 (Dinter et al. 2009)
Fl	Treatment 5	10	1.54	Flupyradifurone	1.2 (Carleton 2014)
Fe	Treatment 6	0.32	0.05	Fenoxycarb	No effect on larvae (Aupinel et al. 2007)

transferred onto a column tube. After several washing steps and DNA digestion RNA was eluted using 15 µl DNase/RNase free water. The concentration of total RNA was determined with a DS-11+ spectrophotometer (DeNovix). RNA samples were stored at −20 °C. For cDNA synthesis, 1 µg of total RNA was reverse-transcribed using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) following the protocol of Scholven et al. (2009), resulting cDNA stored at −20 °C.

2.3.2. RT-qPCR array

Reverse transcribed quantitative polymerase chain reaction (RT-qPCR) was used to quantify the expression levels of selected gene families: A qPCR array containing 79 honeybee-specific primers-pairs was designed in regard to different gene families to quantify distinct physiological pathways (primer list shown in supplementary material S1). Expression analysis was performed by means of SYBR Green detection chemistry using the Biozym Blue S'Green 2× Mix and the PikoReal96 Cycler (Thermo Scientific). All reactions were carried out using clear MicroAmp® Fast 96-Well Reaction Plates (Thermo Scientific) that were sealed with adhesive films. The following RT-qPCR protocol was applied: denaturation at 95 °C for 2 min, followed by 40 amplification cycles including 95 °C for 5 s and 60 °C for 20 s, the fluorescence signal was acquired at 60 °C. A subsequent melting curve (60–90 °C) was performed as quality control with continuous fluorescence measurement and final cooling to room temperature (Scholven et al. 2009).

PCR reactions were performed using a master mix containing 2 × Mix SYBR Hi-ROX with a 1:5 dilution of respective RT-reaction. After dispensing 9 µl of master mix in respective sample wells of a 96 well plate, 1 µl of each forward and reverse primer (each 4 µM) was added (Pawar et al. 2016) and amplified as mentioned above. Samples containing water instead of RT-reaction served as negative controls. Relative quantification was analyzed by means of the comparative C_T (ΔΔCT) method. The presented data reflect the means of three biological replicates. In order to normalize our expression data, we utilized different housekeeping genes (samples DM1–3 and Fe: Enolase, GAPDH and RPL13a; samples C and F: GAPDH, COX6c and ATP Synthase beta). Reference genes were evaluated via geNorm 3.5 (Vandesompele et al. 2002) and Normfinder (Andersen et al. 2004).

2.4. Statistical analysis

Statistical analysis was performed using SPSS version 25.0 (SPSS, Inc., Chicago, IL, USA). Fold changes were compared using one-way ANOVA following a pairwise contrast comparison. All data are expressed as $\log_2$ ratios.

3. Results

Based on RT-qPCR the RNA expression of candidate genes from whole worker larvae was analyzed at D4, D6 and D8 after grafting (Fig. 2). In pre-experiments, we could validate the expression of 79 out of initial 95 honeybee-specific target genes (honeybee qPCR Array) in

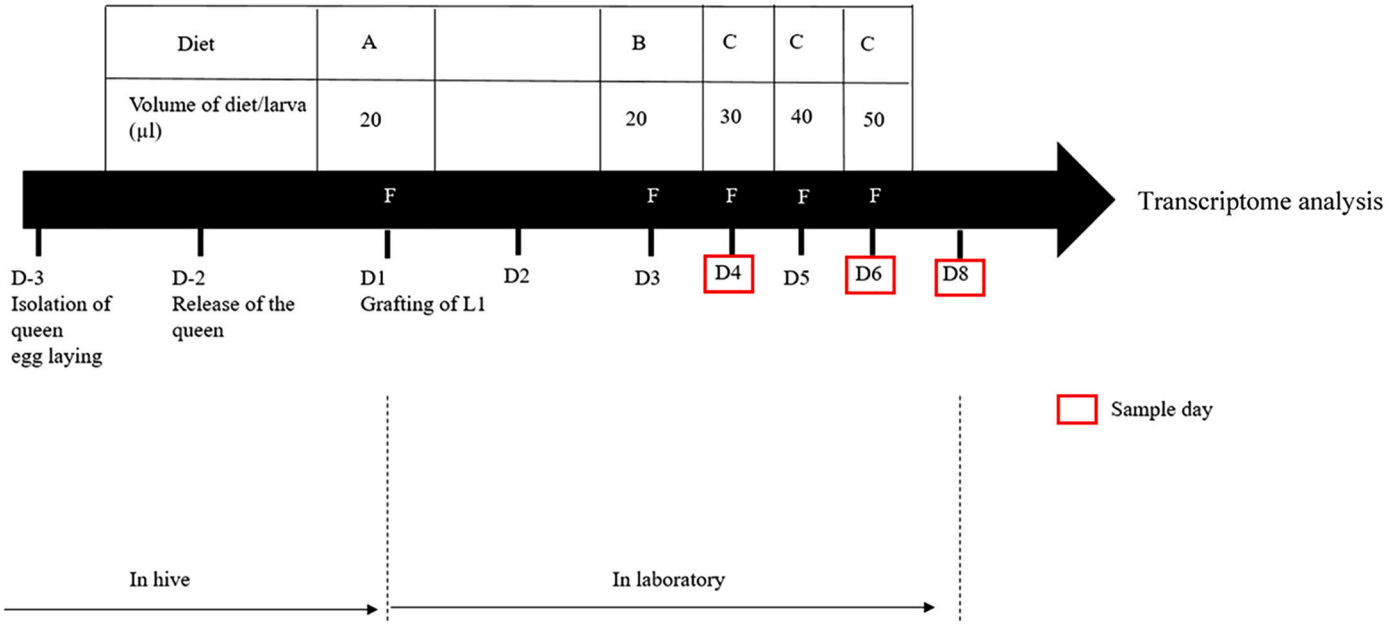

Fig. 1. Experimental design of larval test introduced by Aupinel et al. (2005, changed after OECD Guidance Document 239, 2016). D-3 and D-2, D1-D8 correspond to days before or after grafting of L1 larvae; F indicates feeding days; A, B and C represent the different diets; red squares indicate sample days. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

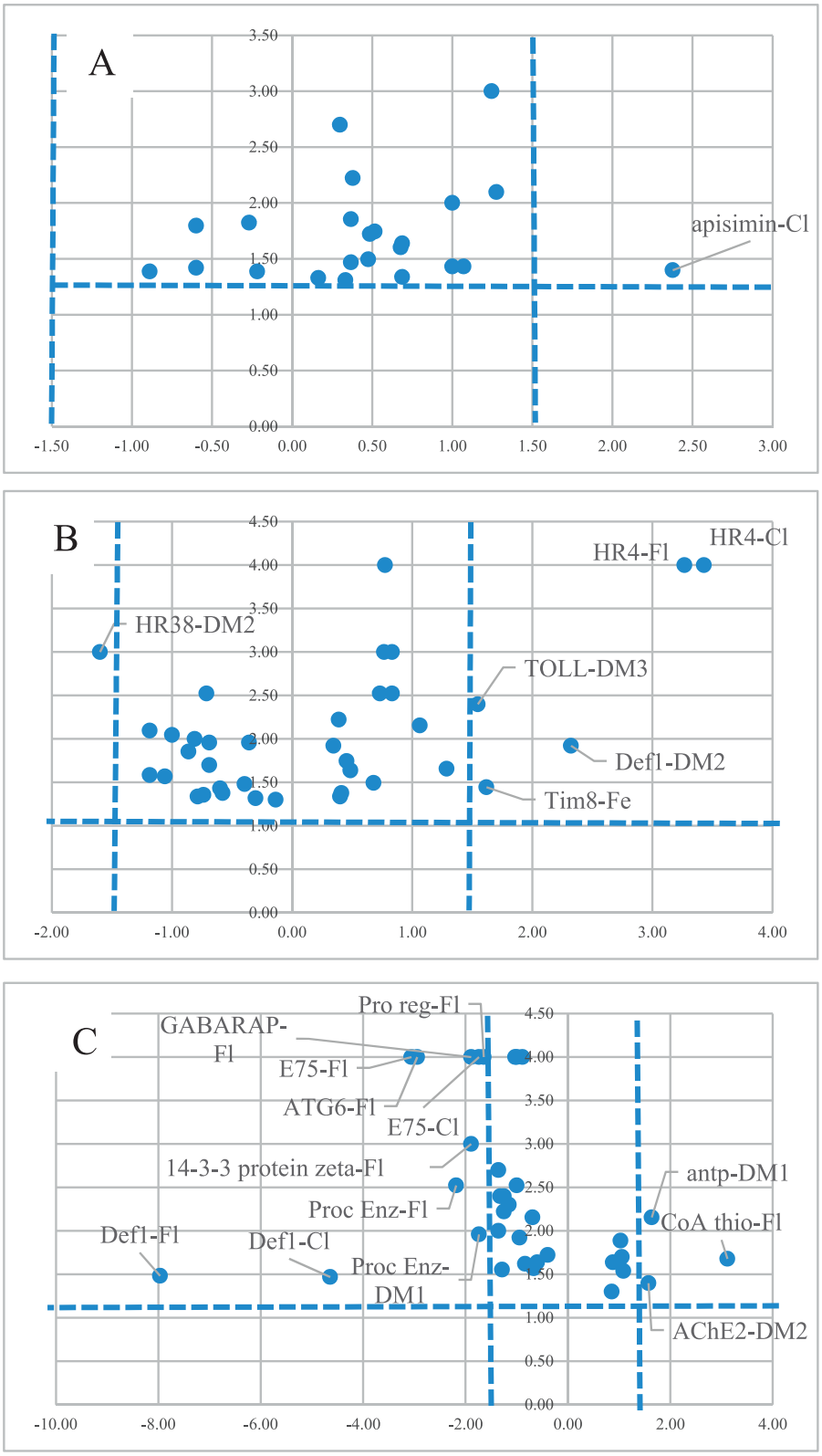

Fig. 2. Volcano plot of significantly dysregulated targets after pesticide exposition at days 4 (A), 6 (B) and 8 (C). Every spot corresponds to one target and reflects the mean of three replicates. For further analysis we used targets that showed a $\log_2$ ratio of 1.5 or more. Dotted lines represent thresholds of significance (ordinate) and fold change (abscissa). Identifiers after the target names correspond to treatment groups: DM1 = 0.05 µg dimethoate, DM2 = 0.1 µg dimethoate, DM3 = 0.2 µg dimethoate, Cl = chlorantraniliprole, Fl = flupyradifurone, Fe = fenoxycarb. We analyzed three replicates. Complete names of targets are mentioned in the text or S1.

Table 2
List of all significantly dysregulated targets that show a $\log_2$ ratio of at least ± 1.5.

Day	Treatment	Target	log2 ratio	*p* value
4	Chlorantraniliprole	apisimin	2.38	< 0.05
6	0.2 µg dimethoate	TOLL	1.55	< 0.01
6	Fenoxycarb	Tim8	1.62	< 0.05
6	0.1 µg dimethoate	Def1	2.32	< 0.01
6	Flupyradifurone	HR4	3.27	< 0.001
6	Chlorantraniliprole	HR4	3.43	< 0.001
6	0.1 µg dimethoate	HR38	−1.60	0.001
8	Flupyradifurone	Def1	−7.97	< 0.05
8	Chlorantraniliprole	Def1	−4.64	< 0.05
8	Flupyradifurone	E75	−3.06	< 0.001
8	Flupyradifurone	ATG6	−2.94	< 0.001
8	Flupyradifurone	Proc Enz	−2.18	< 0.01
8	Flupyradifurone	GABARAP	−1.89	< 0.001
8	Flupyradifurone	p zeta	−1.89	0.001
8	Chlorantraniliprole	E75	−1.74	< 0.001
8	0.05 µg dimethoate	Proc Enz	−1.74	< 0.05
8	Flupyradifurone	Pro reg	−1.64	< 0.001
8	0.1 dimethoate	AChE2	1.58	< 0.05
8	0.05 µg dimethoate	antp	1.64	< 0.01
8	Flupyradifurone	CoA thio	3.12	< 0.05

our larvae treatment system (data not shown). Overall, we monitored no differences in mortality or development between control and treatment groups and consider our applicated doses as sublethal.

RNA-targets found significantly regulated depending on the insecticide treatments were depicted in Table 2. Only transcripts with a $\log_2$ ratio of 1.5 or − 1.5 were considered for further analysis as listed in Table 2. A complete list of all dysregulated targets is presented in the supplementary material (S2). At least 15 distinct transcripts were found significantly regulated after treatment with insecticidal substances compared to control honeybee larvae, most of them at D8.

In detail, on D4 only apisimin was found upregulated in groups treated with chlorantraniliprole ($\log_2$ ratio to control: 2.38; one-way ANOVA $F_{(3,8)} = 19.91, p < 0.05$).

On D6, larvae treated with 0.1 µg dimethoate showed a downregulation of HR38 (hormone receptor-like in 38, $\log_2$ ratio to control: −1.6; one-way ANOVA $F_{(5,12)} = 0.444, p < 0.01$) and an upregulation of Def1 (defensin 1, $\log_2$ ratio to control: 2.32; one-way ANOVA $F_{(5,12)} = 8.61, p = 0.01$). When treated with 0.2 µg dimethoate, TOLL was upregulated ($\log_2$ ratio to control: 1.55; one-way ANOVA $F_{(5,12)} = 5.95, p < 0.01$). Fenoxycarb application upregulated Tim8 (translocase of the inner membrane 8) significantly ($\log_2$ ratio to control: 1.62; one-way ANOVA $F_{(5,12)} = 6.43, p < 0.05$). Treatments with chlorantraniliprole ($\log_2$ ratio to control: 3.43; one-way ANOVA $F_{(3,8)} = 27.33, p < 0.001$) and flupyradifurone ($\log_2$ ratio to control: 3.27; one-way ANOVA $F_{(3,8)} = 27.33, p < 0.001$) led to a significant upregulated expression of HR4 (hormone receptor 4).

Most dysregulated targets were found at D8. Def1 (defensin 1) showed the highest downregulation under treatment with either flupyradifurone ($\log_2$ ratio to control: −7.97; one-way ANOVA $F_{(3,8)} = 31.96, p < 0.05$) or chlorantraniliprole ($\log_2$ ratio to control: −4.64; one-way ANOVA $F_{(3,8)} = 31.96, p < 0.05$). Furthermore, flupyradifurone ($\log_2$ ratio to control: −3.06; one-way ANOVA $F_{(3,8)} = 32.69, p < 0.001$) and chlorantraniliprole ($\log_2$ ratio to control: −1.74; one-way ANOVA $F_{(3,8)} = 32.69, p < 0.001$) significantly reduced the expression of E75 (Ecdysone-inducible protein). The target proclotting enzyme (Proc. Enz.) is associated to the immune system and was dysregulated in groups fed with flupyradifurone ($\log_2$ ratio to control: −2.18; one-way ANOVA $F_{(3,8)} = 7.83, p < 0.01$) and 0.05 µg dimethoate ($\log_2$ ratio to control: −1.74; one-way ANOVA $F_{(5,12)} = 0.164, p < 0.01$). Flupyradifurone also led to a downregulation of autophagy related targets like ATG6 (autophagy-related gene 6) ($\log_2$ ratio to control: −2.94; one-way ANOVA $F_{(3,8)} = 17.41, p < 0.001$) and GABARAP (Gamma-aminobutyric acid receptor-associated protein)($\log_2$ ratio to control: −1.89; one-way ANOVA $F_{(3,8)} = 18.81, p < 0.001$). Additionally, application of flupyradifurone followed a dysregulation of the enzymes 26S proteasome regulatory subunit 10B (Pro reg) ($\log_2$ ratio to control: −1.64; one-way ANOVA $F_{(3,8)} = 40.38, p < 0.001$) and 3-ketoacyl-CoA thiolase (CoA thio)($\log_2$ ratio to control: 3.12; one-way ANOVA $F_{(3,8)} = 21.6, p < 0.01$). The kinase 14–3-3 protein zeta was also downregulated after exposition to flupyradifurone ($\log_2$ ratio to control: −1.89; one-way ANOVA $F_{(3,8)} = 13.12, p = 0.001$). After exposition to 0.05 µg dimethoate the developmental associated target antp (antennapedia) was upregulated ($\log_2$ ratio to control: 1.64; one-way ANOVA $F_{(5,12)} = 3.54, p < 0.01$). Application of 0.1 µg dimethoate was accompanied by an upregulation of acetylcholinesterase 2 (AChE2)($\log_2$ ratio to control: 1.58; one-way ANOVA $F_{(5,12)} = 0.829, p < 0.05$).

4. Discussion

In our analysis, 15 out of 79 transcripts were found regulated; four of them are related to the immune system (apisimin, TOLL, Def1, Proc Enz). Apisimin and Def1 belong to the group of antimicrobial peptides (AMPs, Wu et al. 2018). According to the *Drosophila* model the expression of AMP genes is induced by the TOLL pathway (Hultmark 2003) which was found in parallel significantly upregulated in our system. In our study on D4 and D6 the mentioned targets were upregulated in accordance with reports published recently for adult workers after larval pesticide exposure (Glavinic et al. 2019). It may be concluded that these pesticides might also stimulate the expression of antimicrobial associated genes. On contrary, Def1 was strongly downregulated ($\log_2$ ratio < −4) on D8 in groups treated with chlorantraniliprole and flupyradifurone. This might be due to the observations that pesticide exposure degenerated the Malpighian tubules (Rossi et al. 2013) or reduced the total amount of hemocytes (Brandt et al. 2016), further compromising AMP production. The proclotting enzyme (Proc. Enz.) is an intracellular protease zymogen. The active form, clotting enzyme, is part of the coagulation-inducing activity in hemolymph (Nakamura et al. 1985). Another part of insect immune response is the prophenoloxidase-cascade which leads to the activation of phenoloxidase (Söderhäll and Cerenius 1998). Furthermore, prophenoloxidase was reported as part of the hemolymph clotting system (Karlsson et al. 2004). The observed downregulation of proclotting enzyme in our study could indicate for further impairments in coagulation and phenoloxidase cascades and therefore negatively impact the honeybee defense response.

Another group of dysregulated genes is associated to development (HR38, HR4, E75, antp). In honeybees, HR38 and candidate genes of the ecdysteroid pathway are regulated by neural activity (Fujita et al. 2013). In insect larvae, the ecdysteroid system is also responsible for molting and embryonic development (for review see Myers 2003). The observed dysregulated targets HR38, HR4 and E75 are part of this developmental hormone cascade (Thummel 2002). In our study, the application of 0.1 µg dimethoate on D6 and chlorantraniliprole on D8 led to a downregulation of HR38 and E75. This observation might be explained by damaging production sites of ectysteroids: honeybees' ovary, brain and fat body (Yamazaki et al. 2011). In opposite, chlorantraniliprole and flupyradifurone upregulated HR4 on D6. Their possible influences on insect hormone systems is quite unclear. Our results show that sublethal concentration of pesticides might disrupt the insect endocrine system and interrupt the hormone balance. Additionally, our findings indicate for a possible linkage between pesticide response and hormonal regulation in insects. It is known that the Nrf2-Keap1 signaling pathway contributes to mammalian and insect xenobiotics response (Sykiotis and Bohmann 2008). Further research is necessary towards the interplay of pesticide exposure and hormonal systems in insects. The last developmental target that was significantly upregulated is the homeobox gene antp. Homeobox genes control body

segmentation by activating downstream genes (Carroll 1995). The Antennapedia gene (antp) is required for the development of the thoracic segments (Schneuwly and Gehring 1985). The induced phenotype leads to a transformation of antennae into second thoracic legs, but may also turn dorsal head into dorsal thorax (Duncan und Lewis 1982). Based on these results the observed upregulation of antp in our study may interfere with developmental processes during larval morphogenesis.

We also found induced expression of a translocase of the inner mitochondrial membrane (Tim8) after fenoxycarb application in larvae of D6. Tim8, together with Tim13, is like other complexes involved in the import of proteins into the mitochondrion (Dudek et al. 2013). The function of the Tim8/Tim13 complex becomes vital under sub-optimal conditions when the membrane potential of the mitochondria decreases (Paschen et al. 2000). In our study fenoxycarb might lead to this stress induced membrane potential reduction but other Tim proteins in our array were not affected. The fact that there are multiple transport systems for similar proteins may reflect the importance of inner membrane proteins (Leuenberger et al. 1999) and therefore redundant transport mechanisms.

Further results showed the dysregulation of three enzymes on D8 after flupyradifurone application: 14–3-3 protein zeta, 26S proteasome regulatory subunit 10B and 3-ketoacyl-CoA thiolase). There are two isotypes of 14–3-3 proteins known in insects: epsilon and zeta (Skoulakis and Davis 1998). In *Drosophila*, null mutants of just one isotype lead to high mortality during larval development (Acevedo et al. 2007). Furthermore, 14–3-3 proteins participate in neuronal differentiation (Chang and Rubin 1997), or organ development (Le et al. 2016). Hence, the observed downregulation of 14–3-3 protein zeta might impair larval development.

Another downregulated enzyme was a subunit of the 26S proteasome, which might indicate an overall repression of this protease. The 26S proteasome is a large enzymatic complex in which proteins are degraded into peptides and amino acids (Voges et al. 1999). In addition, the 26S proteasome is not only responsible for protein degradation but is also involved in further biological processes, e.g. both induction as well as suppression of apoptosis (Orlowski 1999, Grimm und Osborne 2000). Especially concerning metamorphosis during insect ontogeny programmed cell death (apoptosis) is an important factor. In the moth *Manduca sexta*, Löw et al. (2001) could show that the 26S proteasome is involved in the apoptosis of ventral muscle cells that are no longer needed in the imago.

The observed downregulation in our experiments therefore could lead to an enrichment of abnormal polypeptides in the cytosol activating two processes: (1) apoptosis is initiated, e.g. by activation of the proapoptotic protein p53; (2) heat shock proteins can be activated (Gabai et al. 1998). One may assume that the cell's protective system against aberrant protein accumulation is impaired by insecticide exposure.

The possible impact of flupyradifurone on cellular degradation is also supported by the downregulation of two mainly autophagy associated targets on D8, ATG6 and GABARAP. Connections between autophagy and apoptosis include pathways that regulate both processes (Crighton et al. 2006). In particular, it could be shown that ecdysone plays an important role in apoptosis and autophagy during insect metamorphosis: In holometabolous insects ecdysone concentrations increases at the end of larval development and prior to pupation mediating the activation of apoptosis genes (Tian et al. 2012) and ATG genes (Baehrecke 2000). Therefore, the observed downregulation of ATG6 and GABARAP can be explained as follows: Larvae on D8 are prior to pupation and normally ecdysone concentrations rise to induce metamorphosis and upregulation of apoptosis and autophagy associated genes. In treated larvae flupyradifurone can cause severe cell damage which might inhibit ecdysone release by a possible retinoid dependent signaling cascade and prolonged larval stage to repair damaged tissue (Halme et al. 2010).

In contrast, 3-ketoacyl-CoA thiolase shows an upregulation after flupyradifurone application. This enzyme catalyzes the final step of fatty acid β-oxidation (Uchida et al. 1992). Słowińska et al. (2019) explained an upregulation of this thiolase with a higher synthesis of hexamerins, larval storage proteins and source of amino acids. This assumption does not account for our results because expression of hexamerins were found unregulated. The increased breakdown of lipids may indicate for a higher energy consumption in larval metabolic rate because intermediates of β-oxidation are also used as precursors in other biosynthetic pathways (Akram 2014). Based on these assumptions we could argue that flupyradifurone treated larvae showed increased metabolic rates due to both activated detoxification mechanisms and immune responses (Ganeshan and Chawla 2014).

The last dysregulated target was AChE2 on D8 after dimethoate exposure. Acetylcholinesterase (AChE) is a key enzyme in synaptic transmission. In most insects, there are two loci identified and one AChE is higher expressed as the main catalytic enzyme (Kim and Lee 2013). In our experiments AChE2 was upregulated as reported after acute exposure by Williamson et al. (2013). It is assumed that an induced expression is a physiological adaptation to compensate the AChE inhibition (Bass and Field 2011). The increased expression in our study might therefore counteract the inhibition of AChE2. This observation was not made for larvae treated with 0.2 µg dimethoate, probably due to the higher variability of the data sets (data not shown).

The applied concentrations of the substances in our setup are higher than the concentrations found in the field (Cripe et al. 2003, Kar et al., 2013, Carleton 2014, Sanchez-Bayo and Goka 2014). Nevertheless, it has been shown that sublethal concentrations have an effect on honeybee larvae without increasing mortality or impairing the phenotype. Furthermore, we could validate the RT-qPCR array used for transcriptome analysis.

5. Conclusion

Based on the Guidance Document 239 on Honeybee Larval Toxicity Test following Repeated Exposure (OECD) the potential effects on mRNA transcripts of four different insecticidal substances in sublethal concentrations were investigated. By use of a honeybee-specific 79 RT-qPCR-array significant differences on the expression of selected transcripts identified targets mainly involved in larval development, the endocrine as well immune system were detected. Chronic feeding of sublethal doses of insecticides led to increasing host reactions until D8 (time-dependency). During our treatment chlorantraniliprole and flupyradifurone application showed stronger effects on changed gene expression in honeybee larvae than fenoxycarb and dimethoate. However, our RT-qPCR array was able to detect significant endogenous effects when treating larvae with sublethal concentrations of the tested substances, although no increased mortality or abnormal larval development was detected in parallel. Further research concerning protein expression and the link of protein expression in larval stages with adverse sublethal effects on individual adult bees and bee colonies in field-realistic scenarios are needed to validate our results in prospect of a holistic and more sensitive risk assessment for tested agrochemicals in the honeybee.

Funding

This work at FUB was supported by the Deutscher Berufs- und Erwerbsimkerbund (DBIB).

Declaration of Competing Interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2020.104703.

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