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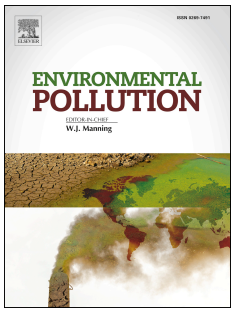

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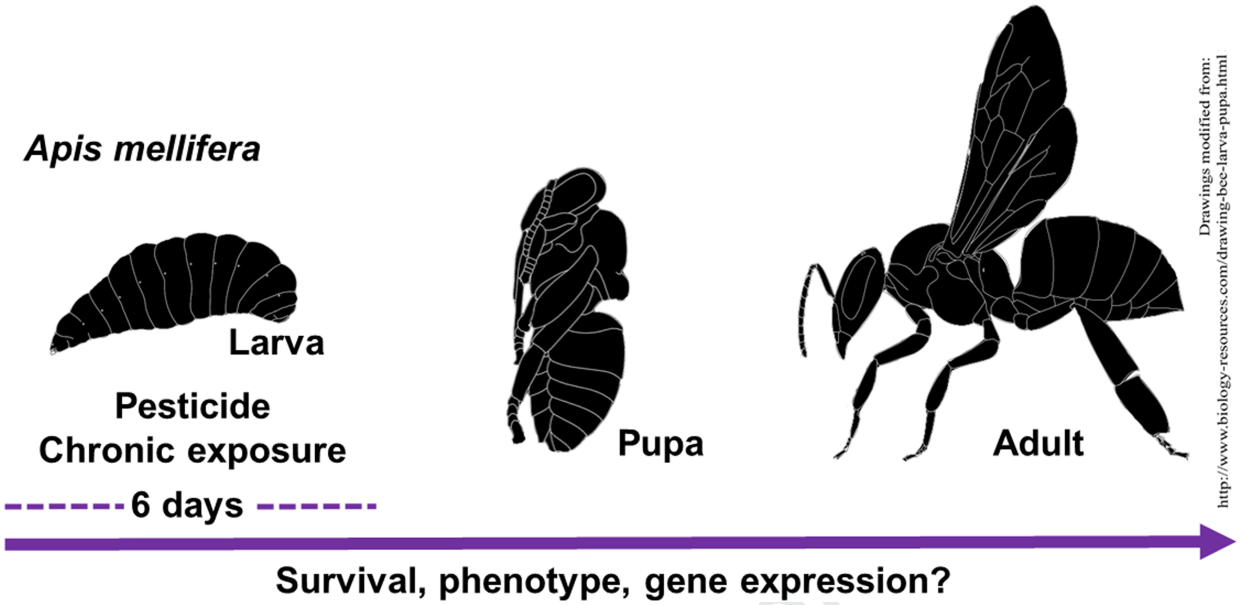

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Frequently encountered pesticides can cause multiple disorders in developing worker honey bee

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Abstract

Pesticide exposure is regarded as a contributing factor to the high gross loss rates of managed colonies of *Apis mellifera*. Pesticides enter the hive through contaminated nectar and pollen carried by returning forager honey bees or placed in the hive by beekeepers when managing hive pests. We used an *in vitro* rearing method to characterize the effects of seven pesticides on developing brood subjected dietary exposure at worse-case environmental concentrations detected in wax and pollen. The pesticides tested include insecticides (chlorpyrifos, imidacloprid), miticides (amitraz, coumaphos, fluvalinate), an herbicide (glyphosate), and a fungicide (chlorothalonil). The larvae were exposed chronically for six days of mimicking exposure during the entire larval feeding period, which is the worst possible scenario of larval exposure. Survival, duration of immature development, the weight of newly emerged adult, morphologies of the antenna and the hypopharyngeal gland, and gene expression were recorded. Survival of bees exposed to amitraz, coumaphos, fluvalinate, chlorpyrifos, and chlorothalonil was the most sensitive endpoint despite observed changes in many developmental and physiological parameters across the seven pesticides. Our findings suggest that pesticide exposure during larvae development may affect the survival and health of immature honey bees, thus contributing to overall colony stress or loss. Additionally, pesticide exposure altered gene expression of detoxification enzymes. However, the tested exposure scenario is unlikely representative of real-world conditions but emphasizes the importance of proper hive management to minimize pesticide contamination of the hive environment or simulates a future scenario of increased contamination.

Keywords: *Apis mellifera*; chronic exposure; honey bee; pesticide exposure

Capsule: The chronic larval exposure to different pesticides affect the survival and health of immature and potentially newly emerged adult honey bees.

Introduction

The honey bee (*Apis mellifera*) is a key pollinator used to provide crop pollination services worldwide (Villalobos 2016). Nonetheless, these services have been threatened by reported high losses of managed honey bees (Aizen et al. 2008, VanEngelsdorp 2010, Steinhauer et al. 2015). A multitude of colony stressors may affect honey bee losses, including malnutrition, parasites, pathogens, poor queens, and pesticides (Aizen and Harder 2009, Ratniek and Carreck 2010, Fairbrother et al. 2014, Staveley et al. 2014, Wilfert et al. 2016, Moritz and Erler 2016). The latter is the most discussed among the possible causes of the high losses reported (Ratnieks and Carreck 2010, Pettis et al. 2012, Moritz and Erler 2016), and is of particular interest since pesticide use is heavily regulated (Rortais et al. 2017). Admittedly, the data so far gathered on the impacts of pesticides on honey bee colony health is conflicting (Eisenstein 2015), with some suggesting detrimental effects on bees (Cresswell et al. 2012, Decourtye et al. 2013, Arena and Sgolastra 2014), and others not (Cresswell, 2011, Cutler et al. 2014, Rolke et al. 2016, Dai et al. 2017, 2018a, 2018b, 2019, Heimbach et al. 2017, 2018). The possible risk of a given pesticide on colony health is based on the pesticide exposure and toxicity to bees (USEPA et al. 2014, EFSA 2013, Rortais et al. 2017).

Pesticide use is a common requirement for crop protection against insect pests and pathogens when producing food, feed, and fiber. However, such scenario of high pesticide inputs led to the observation of at least 161 different compounds and their metabolites in hive products, including in honey, pollen, and wax (Mullin et al. 2010, Krupke et al. 2012, Sanchez-Bayo and Goka 2014). Compounds of diverse physical properties and use patterns have been detected in hives and they include insecticides (Blacquiere 2012, Cresswell et al. 2012), fungicides (Bogdanov 2006, Rosenkranz et al. 2010), herbicides (Johnson 2015), and beekeeper-applied miticides (Bogdanov 2006, Mullin et al. 2010, Rosenkranz et al. 2010, Johnson 2015). The latter is a common tool used to manage populations of *Varroa destructor*, a devastating mite pest of the honey bee (Bogdanov 2006, Mullin et al. 2010, Rosenkranz et al. 2010, Johnson 2015).

Bee exposure to pesticides may take multiple routes (Sgolastra et al. 2019). They may be exposed orally when encountering contaminated resources (nectar, pollen, propolis, and water) collected during foraging flights (Oldroyd 2007), or when consuming contaminated honey, royal jelly or pollen in the hive as immature or adult bees (Martínez-Domínguez et al. 2014, Sanchez-Bayo and Goka 2014). Furthermore, contact exposure may occur when bees are sprayed while foraging, landing on treated crops, encountering compounds used in hives to control colony pests, or as immatures when developing in contaminated wax cells (Johnson et al. 2009, Traynor et al. 2016). Thus, honey bee exposure to pesticides is a real concern, but most studies focus on adult bees (Desneux et al. 2007, Decourtye et al. 2013, Pisa et al 2015, Lopes et al. 2018), while immature bee exposure is equally or perhaps more important, although frequently neglected (Desneux et al. 2007, Tomé et al. 2012, Decourtye et al. 2013, Bernardes et al. 2018). A number of such studies on immature bees have appeared in the literature usually using *in vitro* rearing protocols (Gregorc and Ellis 2011, Wu et al. 2011, Gregorc et al. 2012, Charpentier et al. 2014, Zhu et al. 2014, Dai et al. 2017, 2018a, 2018b, 2019).

The development of assays for assessing pesticide effects on developing honey bees is an answer to regulatory requirements for more data aimed at pollinator risk assessment determining pesticide safety to honey bees (Aupinel et al. 2007, Crailsheim et al. 2013, Schmehl et al. 2016). Historically, pesticide risk assessments predominantly focus on adult bee toxicity data, but the development of laboratory methods for assessing chronic pesticide toxicity to immature bees have improved the current risk assessment process (Croft 1990, Zhu et al. 2014). Guidelines based on single acute exposure and guidance for repeated chronic exposure to a compound were developed for determining pesticide impact on immature honey bees (e.g., (OECD 2013, 2016). Collectively, these developments on the *in vitro* honey bee rearing allowed improved characterization of the chronic pesticide toxicity on developing honey bees.

Almost 47% of the wax and pollen collected across the United States and Canada in 2007-08 tested positively for both acaricides fluvalinate and coumaphos, and the fungicide chlorothalonil. This sampled pollen also had the insecticides chlorpyrifos and imidacloprid. Fluvalinate, coumaphos, amitraz degradates, and chlorothalonil were also found in 98% of

the tested wax (Mullin et al. 2010). These different compounds were tested in the current work because they represent different categories of pesticides commonly used for various purposes in agriculture and different ways. We examined the impacts of six pesticides (amitraz, coumaphos, chlorothalonil, chlorpyrifos, fluvalinate, and imidacloprid) and one herbicide (glyphosate) on development, physiology, and morphology of honey bees. Glyphosate has not been detected in beehives at the time of our assays, but this herbicide was chosen as a potential contaminator, once it comprises the most used herbicide. Honey bee larvae were exposed chronically for six days during the entire larval feeding period with an artificial diet containing single pesticides at concentrations previously recorded in pollen or wax obtained from managed honey bee colonies (Mullin et al. 2010, Pareja et al. 2011, Table 1). We measured larval and pupal mortality, developmental time, adult eclosion, body mass, detoxification gene expression, hypopharyngeal gland and antenna morphology of the tested individuals to generate a comprehensive data set on possible lethal and sublethal effects of pesticides to developing honey bees. A variety of measurements we performed to illustrate the adverse effects of several pesticides on individual health, homeostasis, behavior, labor and foraging activities, and orientation. These effects are detrimental for the colony survival and development (reviewed in Johnson et al. 2010, Johnson 2015 and Pisa et al. 2015).

Material and methods

In vitro rearing

We used the *in vitro* rearing method developed by Schmehl et al. (2016) as the basis for determining pesticide impact on developing honey bees. Briefly, a honey bee queen was confined on a comb within her hive to acquire known-age eggs for standardization of larval age, as grafted into 48-well tissue culture plates, where the diet was provided, again following (Schmehl et al. 2016). At the end of the larva feeding period, they were allowed to pupate and eventually emerge as adults, starting at about 18 days after grafting. The collection of samples was done during 2013 and 2014.

148 **Pesticide exposure**

149 The pesticides used included three acaricides (amitraz CAS# 33089-61-1, coumaphos
150 CAS# 56-72-4, fluvalinate CAS# 102851-06-9), two insecticides (chlorpyrifos CAS#,
151 imidacloprid CAS# 138261-41-3), one fungicide (chlorothalonil CAS# 1897-45-6) and one
152 herbicide (glyphosate CAS# 1897-45-6). All them were of technical grade (> 99% pure)
153 and purchased from ChemService, Inc. (West Chester, PA, USA). None of the compounds
154 were expired at the time of use.

155 Concentrations of pesticides were selected based upon the levels detected in field
156 hive pollen and wax samples. The pollen and wax concentrations for each pesticide were,
157 respectively: amitraz (181/4,700 ppb), chlorothalonil (10,380/1,545 ppb), chlorpyrifos
158 (127/33 ppb), coumaphos (730/11,555 ppb), fluvalinate (294/28,703 ppb), glyphosate
159 (0.8/54 ppb), and imidacloprid (3.1/377 ppb) (Table 1). These different concentrations
160 corresponded to the concentrations found in the field (Mullin et al. 2010, Pareja et al.
161 2011). Pesticide concentrations varied because pesticides are used in different ways for
162 different purposes in the field. Therefore, it is expected variations on concentrations,
163 depending on the specific concentration used, degradation time, and source of
164 contamination (i.e., pollen or wax) (Böhme et al. 2018).

165 All concentrations are 95th percentile residues detected in pollen and wax, except
166 glyphosate and imidacloprid. A lower percentage of amitraz, chlorothalonil, chlorpyrifos,
167 coumaphos, and fluvalinate was used in comparison to the in hive detected values because
168 there may be pesticide degradation over the time of larval development. Therefore, they
169 would not be exposed to the total concentration found in wax and pollen. Glyphosate
170 residues were not reported in the literature for wax and pollen at the time of the study, and,
171 therefore, the glyphosate concentrations selected were based on simazine, another herbicide
172 previously detected in pollen and wax. The high concentration of 377 ppb imidacloprid was
173 the mean detection of positive wax samples (Mullin et al. 2010).

174 The artificial diet containing the pesticides were daily prepared and individually
175 provided to the larva at the rate of 20, 30, 40, 50 and 60 µL respectively at the 1st day of the
176 feeding period, and then at the 3rd, 4th, 5th and 6th day of the said feeding period (Table 1,

Aupinel et al. 2007, Schmehl et al. 2016). Two control treatments were conducted in parallel and included an untreated (water) negative control and a solvent (acetone) control. The diet consumption was monitored daily during the six days of the larval stage. All larvae that reached the pupal stage and adult emergence have consumed the entire diet allotted (160 µL of diet). Glyphosate and imidacloprid were dissolved in water, while the other pesticides were dissolved in acetone before integrating into the diets. Each pesticide was added to each daily diet (at the appropriate amount by day/larval age) at a volume of 1 µL per larva. The daily dose of active ingredients the larvae consumed is shown in Table 1. For the two controls (water and acetone), 1 µL of each diluent was added into the diet per larva per day. Thirty honey bee larvae from each of three colonies (total of 90 larvae) were used for each concentration per pesticide and control groups. A total of 540 larvae were used for the survival test.

Survival, developmental time and adult body mass

The survival of individual honey bee larvae was monitored daily in each rearing plate, from test initiation (t = 0, grafting) until adult emergence. Dead individuals were recorded daily by the absence of movement and body darkening and were removed from the test. The developmental time (days) from test initiation until the time of pupation and adult emergence were recorded for each bee and used to calculate survival and developmental time endpoints.

Percent survival = (# individuals that reached adulthood / # individuals grafted) * 100

Larval developmental time (in days) = date of pupation initiation – grafting date

Total developmental time (in days) = emergence date – grafting date

Bees that survived until adult emergence were weighed on an analytical scale (Mettler Toledo AL 204) within the first 24 hours after emergence to determine body mass at emergence.

Antennal microanatomy

The left antenna from 15 randomly collected and newly emerged adult workers (five per each of three colonies) was obtained for each treatment and controls and subjected to

scanning electron microscopy (SEM). Only bees with complete extended wings and walking behavior were used. The antennae were fixed in 10 % formalin buffer (0.1 M, pH 7.2), washed in phosphate-buffered saline (PBS, 0.1 M), and dehydrated in acetone solution series (50, 60, 70, 80, 90 and 100 %). They were subsequently dried with HMDS (Hexamethyldisilazane), mounted on aluminum stubs, and metalized with gold using a sputter coating device (Ravaiano et al. 2014). The antennae were visualized using a LEO VP1430 SEM was used to visualize the antennas using fixed magnification (x240) and resolution (72 pixels/inch). The lengths of the scape and flagellum were measured using the software Image-Pro Plus 4.5 (MediaCybernetics, Bethesda, MD, USA).

Hypopharyngeal gland morphometry

Hypopharyngeal glands were dissected from 102 bees (nine pollen level pesticide treatments, eight wax level treatments (17 groups) × six bees per group). Twenty gland cells and 20 nuclei were measured from six bees per treatment. The glands were dissected in phosphate buffered saline pH 7.6 (0.1 M NaCl, 20 mM KH_2PO_4, and 20 mM Na_2HP_4) and fixed in formalin 10% (pH 7.4) overnight at 4°C. The samples were subsequently washed with distilled water, dehydrated in an ethanol series (70, 80, 90, 95 and 100%), and embedded in 2-hydroxyethyl methacrylate historesin (JB-4, Electron Microscopy Sciences). Serial sections (2 µm thick) were obtained with an automatic microtome Leica RM2255 equipped with a glass knife. The sections were stained with toluidine blue (1% toluidine blue and 2% borax in distilled water), dried and slide-mounted using the Eukitt mounting medium (Fluka, St. Louis, MO, USA). The samples were analyzed and photographed under an Olympus BX53 microscope equipped with an Olympus DP 73 digital camera. The acini (i.e., cell clusters) of the hypopharyngeal glands were characterized and analyzed morphometrically, and the cell area and nuclei dimensions were determined from light micrographs using Image Pro-Plus 4.5.

Detoxification gene expression

Detoxification gene expression of six-day-old honey bee worker larvae and newly-emerged adult bees was measured for each treatment group. Eighteen bees at both sampling time

points (six bees per each of three colonies) for each treatment group and controls were sampled and frozen in liquid nitrogen to measure relative expression levels of eight genes (five cytochrome p450s, one glutathione s-transferase, and two cAMP-dependent protein kinases). These genes are associated with known detoxification functions in honey bees (Boncristiani *et al.*, 2012, Schmehl *et al.*, 2014) (Table 2). Samples were stored at -80 °C until processing. Gene expression analyses were conducted for larval and adult samples for six wax treatment groups (fluvalinate-treated individuals were excluded because no treated individuals survived), seven pollen treatment groups, and the two control (water and acetone) groups.

RNA isolation. Abdomens of both larvae and adults were prepared on dry ice and homogenized in lysis buffer with zirconium beads using a FastPrep-24 instrument with CoolPrep adapter (MP Biomedicals; Solon, Ohio). RNA was isolated following the manufacturer's protocol using the Qiagen Miniprep kit (Qiagen 74106), QIAshredder columns (Qiagen 79656), and DNAse treated with Turbo DNA-free reagents (Life Technologies AM1907).

cDNA synthesis. RNA was quantified with Nanodrop 2000 and samples of 2 µg were synthesized into cDNA using a High Capacity cDNA Reverse Transcription Kit (Invitrogen 4374966), again following the manufacturer's protocol.

qPCR experiments. cDNA was pooled into four separate groups per treatment to run RealTime quantitative PCR with SYBR Green master mix (BioRad 172-5265) using a CFX-96 RT-PCR instrument (BioRad; Hercules, California). The RNA of four individual bee samples was pooled into one sample group (2 µg of each sample was pooled); 12-16 bees were quantified per treatment (bees 1- 4 are group one, bees 5-8 are group two, and so on with three or four groups representing 12 larvae or 16 individual bee samples). Gene expression activity was measured for two target genes and the housekeeping reference genes (Actin/EIF-s8) on each qRT-PCR plate. Samples representing the control groups were analyzed on every plate in parallel with the samples from each treatment group. All groups were assayed using three technical replicates. Each qPCR plate included water and no-enzyme control to check for false amplification. Primer specificity was tested by analyzing the melting curves of the real-time PCR product for a single peak.

Data Analyses. Analyses were based on a statistical comparison of the cycle threshold (Ct) values of treatment groups compared to the controls. The expression for each candidate detoxification gene was normalized using the geometric mean of two housekeeping genes, *eIF-S8*, and *actin*, before any statistical comparisons (Vandesompele *et al.*, 2002, Grozinger 2003, Huising and Flik, 2005).

Statistical Analysis

Survival analyses were conducted using the procedure LIFETEST, which uses Kaplan-Meyer estimators (SAS, 2008). The bees surviving through emergence were treated as censored data. Developmental time and body mass were subjected to Kruskal-Wallis' test with Dunn's test to recognize significant differences between treatment groups. The total area and nuclei area of the hypopharyngeal cells were subjected to analysis of variance (ANOVA GLM) and Tukey's HSD test when applicable. The assumptions of normality and homoscedasticity were checked before data analyses (PROC UNIVARIATE) (SAS, 2008). Numbers of normal (i.e., straight, clean, and with all segments, Fig. S3 A) and deformed antenna (i.e., bent or with unshed pupal cuticle, Figs. S3 B-D) were analyzed using Kruskal-Wallis test. Gene expression data were determined using RT-qPCR experiments. Normalized expression of two conditions was compared to calculate $\Delta\Delta$Ct, as the relative fold change in gene expression, which is based on the quantity of mRNA. A positive fold change is up-regulated, while a negative fold change is down-regulated. Statistical analyses are presented as heat-maps.

Results

Survival, developmental time and adult body mass

The average survival was 100% and 99% for the negative and solvent control treatments when pesticide at wax contaminated levels was tested, while survival was 99% and 98% when pollen contamination levels were tested. The high survival in the controls validates the design of both experiments according to OECD guideline No. 239 (OECD, 2016), which requires minimum 70% emergence, e.g., up to 30% mortality in the control groups. Honey bee survival to adulthood was significantly reduced with exposure to any of the test pesticides at the residue levels found in wax (Log-rank test: χ^2=848.01; df= 8; P<0.001). This reduction in survival was most pronounced for larvae exposed to the three miticides used against *Varroa* mites (amitraz, coumaphos, and fluvalinate, Fig.1A). Fluvalinate wax residue killed all developing larvae before reaching the pupal stage. The fungicide chlorothalonil and the two insecticides imidacloprid and chlorpyrifos also significantly reduced bee survival (Fig. 1A). Although the reduction in survival of glyphosate-exposed larvae was statistically different from the control, 16% mortality usually is considered incidental in larval studies because OECD guidance (No. 239) accepts up to 30% mortality in the control groups (OECD, 2016). Survival to adulthood was also reduced for pesticide contamination at pollen levels, except for imidacloprid and glyphosate (Log-rank test: χ^2=400.36; df= 8; P<0.001). The three miticides amitraz, coumaphos, and fluvalinate along with the fungicide chlorothalonil, also reduced bee survival. The most significant reduction in larval survival occurred with the insecticide chlorpyrifos (20 ng/bee), where only 15% of adult emergence was observed (Fig. 1B).

Developmental time to pupation was significantly delayed by amitraz (75 ng/bee), coumaphos (1850 ng/bee), chlorothalonil (250 ng/bee), and chlorpyrifos (5.3 ng/bee) ($H_{1,7}$=198.40; P<0.001, Fig. S1A), as was to adult emergence by chlorothalonil (250 ng/bee) ($F_{1,7}$=86.46; P<0.001, Fig. S1C) at the concentrations found in wax. Developmental time to pupation was significantly delayed (0.5-1 day in average) with pollen contamination levels by amitraz (2.9 ng/bee), chlorothalonil (1660 ng/bee), chlorpyrifos (20 ng/bee), coumaphos (120 ng/bee), and fluvalinate (0.29 ng/bee) ($F_{1,8}$=250.88; P<0.001, Fig. S1B), as was to adult emergence by amitraz (2.9 ng/bee) and

chlorothalonil (1660 ng/bee) ($F_{1,8}$=73.17; P<0.001, Fig. S1D). These initial delays in starting pupation are presented in Figure S2, where a plate containing chlorothalonil (pollen) concentration-treated diet shows larvae of variable sizes and at different stages of development.

The body mass of newly emerged adult bees was about 15% significantly reduced by amitraz (75 ng/bee), chlorothalonil (250 ng/bee) and chlorpyrifos (5.3 ng/bee) at the concentrations found in wax ($H_{1,7}$=80.45; P<0.001), and by coumaphos (120 ng/bee), fluvalinate (0.29 ng/bee), amitraz (2.9 ng/bee), chlorothalonil (1660 ng/bee), and chlorpyrifos (20 ng/bee) at the concentrations found in pollen ($H_{1,8}$=114.29;P<0.001, Fig. 2).

Antennae and hypopharyngeal gland morphologies

Malformed antennae were observed among a small percentage (up to 20%) of the newly emerged workers after larval exposure to all pesticides, except amitraz ($H_{1,1}$=5.41; P=0.02; Table 3). Shortened flagella and scapes were the most common deformities observed in antennae (Fig. S3).

Hypopharyngeal gland cells of newly-emerged bees were significantly smaller for bees in several of the pesticide treatment groups relative to those in the control groups (Fig. 3). Bees exposed to coumaphos, chlorothalonil, and chlorpyrifos at the concentrations found in wax ($F_{1,7}$=6.59; P<0.001; Fig. 3A) and to all seven pesticides at the levels found in pollen exhibited smaller cells than those of the control ($F_{1,8}$=16.07; P<0.001; Fig. 3B). Workers exposed to glyphosate, imidacloprid, coumaphos, chlorothalonil, and chlorpyrifos at the concentrations found in wax ($F_{1,7}$=8.89; P<0.001; Fig. 3C) and all pesticides except glyphosate at the levels found in pollen ($F_{1,8}$=13.12; P<0.001; Fig. 3D) also exhibited reduced nucleus size. The cells in most of the pesticide-treated bees were altered in structure, while cells in the control groups were round and displayed normal nuclei and cytoplasm morphologies (Fig. S4A, B). For example, acini from imidacloprid- and chlorothalonil-exposed bees were pyramidal (S4C), while those exposed to chlorpyrifos and chlorothalonil at concentrations found in wax were smaller, with dark nuclei and a white halo around the nucleus (Fig. S4D). Acini in bees exposed to coumaphos and

fluvalinate concentrations found in pollen had enlarged cell ductules (Fig. S4E). In general, acini from bees exposed to coumaphos and fluvalinate showed weak or non-stained regions in the cytoplasm, suggestive of cell damage (Fig. S4E).

Expression of detoxification genes

The expression levels of the target genes were modified in six-day-old larvae and newly emerged adults exposed to pesticides as larvae (Figs. 4, 5). There was a general pattern of downregulation of the target genes in pesticide-exposed larvae and an upregulation in adults ($P<0.05$, Figs 4, 5). The p450 genes were of notable interest in the larval gene expression (Fig. 4), as significant gene downregulation was observed for CYP305D1 (chlorpyrifos), CYP9Q1 (chlorothalonil, chlorpyrifos), CYP9Q2 (amitraz, chlorpyrifos, coumaphos, glyphosate, and imidacloprid), CYP9Q3 (amitraz, chlorothalonil, chlorpyrifos, coumaphos, and imidacloprid), and CYP9S1 (amitraz, coumaphos, and imidacloprid). Gene downregulation was also observed for GSTD1 (chlorothalonil, chlorpyrifos) and PKAC1 (imidacloprid). While not significant, an approximately 3-fold increase in gene expression was observed for CYP305D1 (fluvalinate) and GSTD1 (chlorpyrifos). In newly emerged adults, none of the target genes were downregulated (Fig. 5). The majority of the upregulated genes were in response to chlorothalonil (five of eight genes studied) and chlorpyrifos (six of eight genes studied) exposure. Significant upregulation was observed for CYP9Q1 (chlorpyrifos), CYP9Q2 (amitraz, chlorothalonil, chlorpyrifos, and coumaphos) CYP9Q3 (chlorothalonil, coumaphos), CYP9S1 (chlorothalonil, chlorpyrifos), GSTD1 (chlorothalonil, chlorpyrifos), PKAc1 (chlorothalonil, chlorpyrifos, glyphosate, and imidacloprid) and PKAr1 (chlorpyrifos, glyphosate, and imidacloprid). Significant mortality in the coumaphos and fluvalinate wax-concentrations prevented any quantification of gene expression in the adults. A summary of all the observed effects across the different studies is presented in Table 4.

Discussion

Honey bees are frequently exposed to toxic compounds in the environment, including both naturally occurring plant toxins and synthetic pesticides (Mullin et al. 2010, Pareja et al. 2011, Sanchez-Bayo and Goka 2014). Colony exposure to pesticides is not restricted to agricultural landscapes where pesticides are used for crop protection, but in urban and suburban landscapes and from in-hive uses to manage honey bee pests (Bogdanov 2006, Mullin et al. 2010, Spivak et al. 2010, Goulson 2015, Johnson 2015). While adult bees are initially exposed to a given pesticide, developing bees may, in turn, be exposed orally through contaminated pollen and nectar provisioning or by translocation of contaminants in the wax to the diet or directly to the larvae (Martínez-Domínguez et al. 2014, Sanchez-Bayo and Goka 2014, Böhme et al. 2018). In our assays, larvae of honey bees were exposed to different classes of frequently encountered pesticides (i.e., insecticide, acaricide, fungicide, and herbicide). We performed oral/chronical exposure, covering both dietary exposures through the royal jelly and brood food, and contact exposure from the surrounding wax cell, resembling natural conditions within the hives.

Royal jelly is consumed during the first three days of larval worker development, but pesticide residues in royal jelly are minimal and not expected to be a primary route of exposure (Böhme et al. 2018). The larvae consume nectar and pollen for the remainder of the larval development as a source of carbohydrates and proteins (Brodschneider and Crailsheim 2010), with pollen comprising approximately 3% of this diet (USEPA et al. 2014). The test levels we selected in our study were based upon pesticide residues previously identified in wax and pollen matrices to simulate a worst-case exposure scenario given that wax is not consumed directly and that pollen volume in the larval diet is minimal. These test levels provide the basis for our mechanistic understanding of how frequently-encountered pesticides in the environment may impact the development and functionality of honey bees. It remains unclear what percentage of the residues in wax are bioavailable and transferred to the larval diet or via a contact in normal hive conditions, especially if the pesticides are lipophilic (Shimshoni et al. 2019).

To evaluate the risk of honey bee larval exposure to pesticides, the OECD Guidance Document No. 239 recommends an *in vitro* chronic exposure during four days, e.g., dosing initiating approximately 2-3 days after the larval eclosion (emergence from their egg, OECD 2016). In our study, we initiated the pesticide exposure on the same day of larval eclosion, giving larvae two extra days of exposure over that suggested by OECD. We added two additional days of exposure because there is no available data indicating the potential impact on honey bees as the larvae are exposed to pesticides in the entire larval stage, e.g., six days. Therefore, our results represent chronic exposure during the whole larval development phase, which covers both dietary exposures through the royal jelly and brood food, and contact exposure from the surrounding wax cell (see doses on Table 1). Although it is almost a consensus that exposure via royal jelly is considered minimal, pesticide residues are commonly encountered in royal jelly (Martínez-Domínguez et al. 2014). Therefore, exposure to low pesticide concentrations during the first two-three days of development may be considered to identify potential pesticide risks and improve the risk assessments of plant protection products (Sanchez-Bayo & Goka 2014, Traynor et al. 2016, Böhme et al. 2018).

Pesticide exposure during the larval stage of development at concentrations previously identified in the environment caused multiple adverse effects to honey bees. Survival was impacted by at least the maximum tested concentrations in the honey bee larval diet for all seven pesticides, regardless of whether the compound was an insecticide, fungicide, or herbicide. Additionally, a diverse set of sublethal effects was also observed, supporting the work of others (Fairbrother et al. 2014, Gregorc and Ellis 2011, Wu et al. 2011, Gregorc et al. 2012, Charpentier et al. 2014, Pisa et al. 2015, Dai et al. 2017, 2018a, 20018b, 2019). The traditional toxicological measurements are limited to survival, growth, and reproduction (USEPA et al. 2014), and a key goal of our study was to determine if these traditional toxicity measurements, particularly survival for our study design, are satisfactory for assessing the pesticide risk to developing bees. In our study, we investigated sublethal impacts on individual bees that may occur when chronically exposed to pesticides during larval development (Table 4). Sublethal effects are not required in the OECD Guidance Document No. 239. However, other regulatory agencies, as USEPA, have

437 required data weights from emerged bees exposed in the larval stage as part of the checklist
438 of risk assessment for registration of new molecules (confidential information).

439 The data indicate that the tested pesticide concentrations found in wax and pollen,
440 except glyphosate and imidacloprid, significantly delayed total developmental time. To our
441 knowledge, this is one of the first studies to present evidence that larvae exposed to
442 pesticides may experience delayed maturation and lower body mass at emergence (Nicholls
443 et al. 2017). Dai et al. (2018b) demonstrated that exposure to concentrations below 46000
444 ppb amitraz, below 25000 ppb coumaphos, and 6000 ppb fluvalinate did not affect
445 developmental time. In contrast, developmental delays were observed from 181 and 4700
446 ppb amitraz, 730 and 11555 ppb coumaphos, and as low as 294 ppb fluvalinate. Similar
447 differences occurred in other publications reporting non-effects with other compounds such
448 as imidacloprid, chlorothalonil, and chlorpyrifos (Dai et al. 2018a, c). We believe these
449 discrepancies between our results and those of Dai et al. (2018a, 2018b, 2019) may result
450 from the added two-day exposure of larvae to pesticides in our study since the above-cited
451 studies were performed with similar *in vitro* rearing method. However, other factors such as
452 seasonality, queen lineages, and nutrition can be a source of variability to pesticide
453 susceptibility (Klein et al. 2017). Honey bee larvae consume only royal jelly during their
454 first three days of development, and royal jelly is known to have only trace amounts of
455 pesticides (Böhme et al. 2018), suggesting that a four-day exposure per OECD guidelines is
456 protective of the immature life stage of the honey bee. Developmental delays like those we
457 found, regardless of the duration of exposure, may lead to diverse negative impacts,
458 including disruption of social colony structure and even increase in the reproductive
459 potential of *Varroa* (Al Ghamdi and Hoopingarner 2004).

460 Body mass of newly emerged adult worker bees exposed to several of the pesticides
461 in the study was reduced; however, the data suggest that survival is a more sensitive
462 endpoint than body mass. For emerged adult bees, significant differences in body mass
463 were only observed when there was a minimum of 41% mortality detected between the
464 control and a given treatment (Figure 2B). Previous studies have demonstrated that amitraz,
465 coumaphos, and fluvalinate did not predictably impact larval body mass after chronic

466 exposure (Dai et al., 2018a, b, c). Body mass does not appear to add value in assessing the
467 risk of a particular chemical to developing honey bees based upon our data.

468 Sublethal effects of pesticide exposure on hypopharyngeal glands have been
469 reported in the literature, but the pesticide concentrations tested were not within realistic
470 field exposure levels (Heylen et al. 2011, Hatjina et al. 2013). Hypopharyngeal glands are
471 essential for larval nutrition and development because these glands produce components of
472 brood food (Crailsheim et al. 1992, Hrassnigg and Crailsheim 1998). The size of
473 hypopharyngeal glands was observed to be one of the most sensitive endpoints from our
474 study. There was a reduction in the cell size for individual pesticides, even when survival
475 was not significantly different from the controls (Fig. 3). Additional studies are required to
476 validate whether these observations occur at the colony-level when exposed to pesticides at
477 a sublethal level and whether a difference in hypopharyngeal gland size from ~250-300
478 μm^2 to ~150-200 μm^2 has implications for colony health. It is a labor-intensive process to
479 collect size data on the hypopharyngeal glands, and future studies may find that these
480 measurements do not add value for assessing the risk to bees.

481 Antennal morphology was deformed in at least one bee, and never more than three
482 bees (20% of the sampled bees) for all tested pesticides except amitraz. Others noted an
483 impact of fluvalinate and coumaphos on the development of queen antennae, but not
484 emerged adult workers (Haarmann et al. 2002). It is unclear from our data why deformities
485 were not more uniform with pesticide exposure. These observed deformities do not seem
486 due to the experimental design, impact of grafting or *in vitro* rearing method, since no
487 changes were qualitatively observed in either of the control groups. Further empirical data
488 are needed to characterize better if and how pesticide exposure could result in antennal
489 deformities.

490 Pesticide impacts on several physiological pathways have been reported for adult
491 honey bee workers. These impacts include changes in the activity of genes associated with
492 detoxification enzymes, immunity, and development (Boncristiani et al. 2012, Mao et al.
493 2011, 2013, Derecka et al. 2013, Schmehl et al. 2014). However, little is known about the
494 function of detoxification genes when larvae are exposed to a toxin (Gregorc et al. 2012,
495 Derecka et al. 2013). Even though honey bees are reported to exhibit low detoxification

activity (Claudianos et al. 2006), we characterized gene expression across three families of detoxification enzymes (i.e., cytochrome P450, glutathione s-transferase, and cAMP catalytic protein kinase) in larvae and the resulting emerged adults. As a general trend, the detoxification gene expression was up-regulated in the emerged adults, but down-regulated during the larval stage for pesticide-treated individuals.

Past studies demonstrated that adults exposed to pesticides upregulate detoxification genes (Boncristiani et al. 2012, Mao et al. 2011, Schmehl et al. 2014). On the other hand, the exposure of honey bees to sublethal concentrations of imidacloprid changed the expression pattern of 578 genes (out of 15,314) more than two-fold after the emergence when each honey bee larva consumed two ng of active ingredient (Wu et al. 2017). In our study, multiple genes were upregulated in the newly-emerged adults exposed to amitraz, coumaphos chlorothalonil, imidacloprid, chlorpyrifos, and glyphosate, even though pesticide exposure occurred almost two weeks earlier during the larval stage of development. It can be suggested that the pesticides elicit the expression of detoxification genes in honey bee throughout its development to adult. Nevertheless, the persistence of this signalizing is not yet understood. The downregulation of detoxification genes during larval exposure suggests that larvae utilize different mechanisms to counter xenobiotics in their environment or that they may be more susceptible to some pesticides. The energetic cost of development combined with social protection through colony-level buffering mechanisms may explain the differences in gene expression patterns between exposed larvae and the resulting adults. The downregulation of detoxification genes during the larval stage might be connected with other disorders like low survival, mal-regulation of the development, and morphological disorders. However, these conclusions need to be made with caution because these mechanisms have to be better investigated to explain how low pesticide concentrations can disturb honey bee larvae.

The herbicide and fungicide tested impacted survival and other physiological parameters. Our data suggest that herbicides and fungicides should not be excluded from honey bee safety assessments despite their different primary targets in fungi and plants. The EPA recognizes the need for and requires acute and chronic toxicity tests for honey bee adult and larval exposures to provide laboratory data as part of the registration and

registration review process for chemicals, including most insecticides, fungicides, and herbicides (USEPA 2016a). Physiological measurements are not generally collected as part of the regulatory framework; however, our data identified physiological changes (e.g., changes in the gene expression) even when survival was not affected. Glyphosate at low concentrations (0.8 ppb) that amounted to a total cumulative dose of 0.13 ng was found to change gene expression patterns and acini size. Indirect effects on honey bee health may also be occurring through the disruption of the gut microbiota when glyphosate is ingested (Motta et al. 2018). Our results are not replicated in other studies that identified glyphosate to be safe to honey bee colonies at concentrations up to 300 ppm (Thompson et al. 2014). One limitation of our study is that only two concentrations were selected, thus limiting our ability to derive a dose-response for the particular compounds. The selection of multiple test levels would have given us more confidence in the interpretation of our data for implications at the colony level.

The fungicide chlorothalonil impacted all the endpoints tested (Table 4) at concentrations as low as 1.45 ppm amounting to a total cumulative dose of 0.25 µg/bee. The honey bee oral acute study that was conducted to support chlorothalonil US registration (MRID 00036935) demonstrated only 14% mortality at 181 µg/adult bee, which is a dose about 720 fold higher than what we observed in immature bees. Here the death of bees exposed to chlorothalonil was about 45% for cumulative doses of 0.25 and 1.66 µg/larva. Chlorothalonil is a general-use fungicide commonly found in honey bee hives (Mullin et al. 2010) and classified as non-toxic to honey bees by the U.S. EPA (USEPA 1999). However, there are concerns in the literature that chlorothalonil may synergize other compounds (Zhu et al. 2014) or honey bee pathogens such as *Nosema* spp. (Pettis et al. 2013), thus affecting honey bees. Dai et al. (2018b) tested multiple concentrations of chlorothalonil on individual developing honey bees and observed toxicity when concentrations were greater than 10 ppm. They concluded that chlorothalonil did not present risk of developing larvae based on BeeREX (https://www.epa.gov/sites/production/files/2015-11/beerexv1.0.xlsx), a pollinator risk assessment tool developed by the USEPA. Colony-level effects data would be considered valuable to assess the real-world risk of chlorothalonil to honey bee colonies based upon

the conflicting results between our study and earlier conclusions made in the literature (Dai et al. 2018b). One of the primary purposes of laboratory studies is to generate conservative toxicity data on individual bees that can be used in combination with exposure data to assess risk (USEPA et al. 2014). If the laboratory assessment cannot exclude a threat to a particular chemical, more realistic studies at the colony-level can be conducted to refine the risk assessment and what residue concentration is considered safe for use when a specific product is used according to the label.

Larvae were more tolerant to imidacloprid than adults when compared to the adult toxicity data in the literature (Abbo et al. 2017, Raymann et al. 2018), which supports earlier findings (Yang et al. 2012, Dai et al. 2019). A concentration as high as 377 ppb caused 30% mortality, but Mullin et al. (2010) only found imidacloprid in 2.1% of bee matrices (wax and pollen), with a mean detection of 3.1 ppb in pollen. Colony-level effects from imidacloprid exposure were observed at concentrations higher than 25 ppb (USEPA 2016b). The risk from imidacloprid does not appear to be relevant based on the frequency and magnitude of residues in the environment and the toxicity data observed in both the laboratory and at the colony level (USEPA 2016b).

Chlorpyrifos, the other insecticide investigated in our study, impacted survival and every physiological parameter measured at concentrations as low as 33 ppb. Chlorpyrifos is frequently detected in bee matrices (Mullin et al. 2010, Sanchez-Bayo and Goka 2014) and has previously been shown to impact larval honey bee survival (Zhu et al. 2014, Dai et al. 2019). Further studies at the colony-level are needed to validate what concentrations of chlorpyrifos disrupt the function and long-term survival of honey bees for better risk characterization.

Effective *Varroa* management is necessary for maintaining the bee colony health (Bogdanov 2006, Rosenkranz et al. 2010, Johnson et al. 2015) and reducing the spread of viruses (Martin et al. 2012, Dainat and Neumann 2013). Nonetheless, the results obtained indicate that amitraz, coumaphos, and fluvalinate in larval diet can negatively impact developing honey bees chronically exposed to these pesticides. The high concentrations in the wax can lead to significant decreases in survival if fed to the larvae, and lower concentrations were still observed to impact several physiological parameters in bees.

There has been a clear reduction of fluvalinate and coumaphos use in the United States due to *Varroa* resistance, but this has increased beekeeper dependence on other chemicals for adequate control. It is unclear how much chemical is translocated from the wax to the larval diet, but our data support the recommendation to dispose of frames that are three-five years old as a sound management practice to prevent in-hive residues from reaching critical thresholds (Keystone Policy Center 2019).

The present study demonstrated that laboratory-reared honey bee workers exposed to field-relevant concentrations of six pesticides commonly encountered in pollen and wax matrices, and the herbicide glyphosate could compromise the survival of honey bee workers when exposed during the larval development. Survival was the most sensitive endpoint for the seven tested pesticides. The majority of the physiological parameters altered in our study such as gene expression, development, weights, and morphology, only manifest when survival was significantly affected (Dai et al. 2018a, b), likely making them unnecessary endpoints in toxicological tests conducted for regulatory purposes. However, sublethal effects on weights and development of honey bees would impact the whole colony survival and function, and this is the reason which agencies as USEPA has requested weight data in chronic larval studies with honey bees; therefore, colony-level data are valuable to determine whether effects observed in the laboratory are replicated in a more realistic scenario. The absence of a dose-response study design prevents us from generating a robust toxicity endpoint for use in risk assessment; however, our study is one of the first to consider a multi-faceted approach to how developmental and physiological parameters can be adversely affected by pesticides in exposed larvae.

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Table 1. Amount of active ingredient consumed daily (D) and in total by each honey bee larva. Each pesticide level selected was based on published concentrations detected in wax and pollen as denoted by superscript letters and the footnotes. The diet was provisioned on D1, D3-D6 (160 µl total).

			Amount of active ingredient consumed per day (µg)					Total consumed per bee (µg)
Pesticide	**Matrix**	**Concentration (ppb[1])**	**D1 + D2 (in 20 µl of diet)**	**D3 (in 20 µl of diet)**	**D4 (in 30 µl of diet)**	**D5 (in 40 µl of diet)**	**D6 (in 50 µl of diet)**	**Total (in 160 µl of diet)**
amitraz	wax	4700[a]	0.09401	0.09401	0.14111	0.18821	0.23502	0.75200
chloropyrifos	wax	33[a]	0.00066	0.00066	0.00099	0.00132	0.00165	0.00528
chlorothalonil	wax	1545[a]	0.03092	0.03092	0.04635	0.06182	0.07725	0.24720
coumaphos	wax	11555[a]	0.23111	0.23111	0.34665	0.46223	0.57775	1.84880
fluvalinate	wax	28703[a]	0.57406	0.57406	0.86109	1.14812	1.43515	4.59248
glyphosate	wax	54[a2]	0.00108	0.00108	0.00162	0.00216	0.00271	0.00864
imidacloprid	wax	377[b]	0.00754	0.00754	0.01131	0.01508	0.01885	0.06032
amitraz	pollen	181[a]	0.00362	0.00362	0.00543	0.00724	0.00905	0.02896
chloropyrifos	pollen	127[a]	0.00254	0.00254	0.00381	0.00508	0.00635	0.02032
chlorothalonil	pollen	10380[a]	0.20762	0.20762	0.31140	0.41521	0.51911	1.66080
coumaphos	pollen	730[a]	0.01461	0.01461	0.02190	0.02921	0.03651	0.11680
fluvalinate	pollen	294[a]	0.000036	0.000036	0.000055	0.000074	0.000092	0.00029
glyphosate	pollen	0.8[a2]	0.000016	0.000016	0.000024	0.000032	0.000040	0.00013
imidacloprid	pollen	3.1[a]	0.000023	0.000023	0.000035	0.000047	0.000058	0.00019

[a] Mullin et al 2010 [b] Pareja et al. 2011.

[1]parts per billion (µg/L)

[2]Glyphosate residues are not reported in the literature for wax and pollen at the time of the study, and therefore the glyphosate concentrations selected were based on simazine, another herbicide previously detected in pollen and wax.

Table 2. Gene descriptions and primer sequences used for *q*RT-PCR to check the relative expression levels of eight candidate genes in larval and adult honey bees exposed to field-relevant concentrations of pesticides as feeding larvae. The genes of interest are predominantly associated with pesticide detoxification (five cytochrome P450 detoxification genes, one gluthatione *S*-transferase and two cAMP-dependent kinase genes). The expression of each candidate gene was normalized to the geometric mean of the two housekeeping genes: actin and eIF-S8 (Vandesompele et al. 2002, Grozinger 2003, Huising and Flik, 2005)

Candidate	**Description**	**Forward primer**	**Reverse primer**
CYP9S1	Cytochrome P450- detoxification	CTAATTTTCGCGTTCCCAAA	CTCCCGTTACGTTTGTCGAT
CYP305D1	Cytochrome P450- detoxification/hormone function	TCGATCTTTTTCTCGCTGGT	TTGCTTTGTCCTCCATGTTG
CYP9Q1	Cytochrome P450- detoxification/insecticide metabolism	TCGAGAAGTTTTTCCACCG	CTCTTTCCTCCTCGATTG
CYP9Q2	Cytochrome P450- detoxification	GATTATCGCCTATTATTACTG	GTTCTCCTTCCCTCTGAT
CYP9Q3	Cytochrome P450- detoxification	GTTCCGGGAAAATGACTAC	GGTCAAAATGGTGGTGAC
GST-D1	Gluthatione s–transferase- detoxification/metabolism	GCCGCTTCAAAAGAAGTACG	GTGGCGAAAACAAGGATGAT
PKA-C1	cAMP – dependent kinase type 1	TCCATTTTTGGTCTCCTTGC	GTAAAAGCGCGAATGTGGTT
PKA-R1	cAMP – dependent kinase type 1 regulatory subunit	GAAGCAATTATTCGGCAAGG	TCACCGAAACTTCCACCTTC
eIF3-S8	Housekeeping	TGAGTGTCTGCTATGGATTGCAA	TCGCGGCTCGTGGTAAA
Actin	Housekeeping	CCTAGCACCATCCACCATGAA	GAAGCAAGAATTGACCCACCAA

Table 3. Number and percentage of emerging adult honey bees with deformed antennae within each treatment group

Treatment	Matrix	Pesticide concentration (ppb[1])	Number of bees sampled	Number sampled bees with deformed antennae	Percent (%) sampled bees with deformed antennae [2] (%)
control water	-	-	30	0	0
control acetone	-	-	30	0	0
amitraz	wax	4700	15	0	0
amitraz	pollen	181	15	0	0
chlorpyrifos	wax	33	15	2	13.3
chlorpyrifos	pollen	127	15	2	13.3
chlorothalonil	wax	1545	15	1	6.7
chlorothalonil	pollen	10380	15	3	20.0
glyphosate	wax	54	15	1	6.7
glyphosate	pollen	0.8	15	0	0
imidacloprid	wax	377	15	2	13.3
imidacloprid	pollen	3.1	15	1	6.7
coumaphos	pollen	730	15	1	6.7
fluvalinate	pollen	294	15	1	6.7

[1]parts per billion (µg/L)

[2]There were significant differences between untreated bees (control water and control acetone) and pesticide-treated bees (all pesticide treatments pooled) per Kruskal-Wallis' test (P = 0.002). Analyses did not include coumaphos and fluvalinate wax treatments due to limited bee survival to adulthood in those groups.

Table 4. Summary of all effects observed on honey bees that were chronically exposed to pesticides while larvae relative to those on honey bees in the control groups.

Pesticide	Matrix	Pesticide concentration (ppb)	Lethal and sublethal effects						
			Survival	Gene expression (larvae)	Gene expression (adults)	Developmental delays	Body mass	Antennal abnormalities	Acini
amitraz	wax	4700	x	x		x	x		
	pollen	181	x	x	x	x	x		x
chlorothalonil	wax	1545	x	x	x	x	x	x	x
	pollen	10380	x	x	x	x	x	x	x
chloropyrifos	wax	33	x	x	x	x	x	x	x
	pollen	127	x	x	x	x	x	x	x
coumaphos	wax	11555	x			x			
	pollen	730	x	x	x	x	x	x	
fluvalinate	wax	28703	x						
	pollen	294	x			x	x	x	x
glyphosate	wax	54	x					x	
	pollen	0.8		x	x				x
imidacloprid	wax	377	x						x
	pollen	3.1		x	x			x	x

A significant effect relative to the control in each measured endpoint is denoted with an ‘x’.

Figures

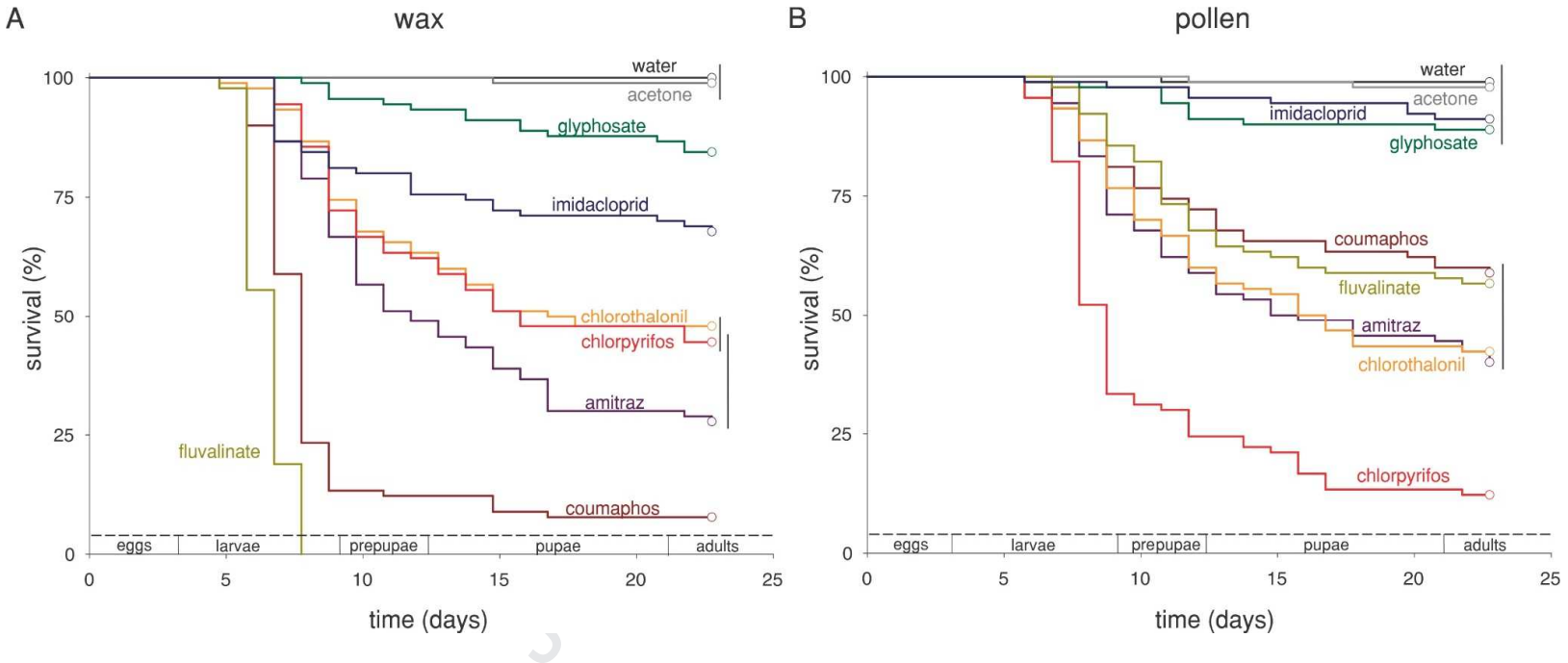

Figure 1. Survival plots of honey bee workers fed with diets containing pesticides at concentrations detected in wax (A) and pollen (B) during their development as larvae. Individual larvae were chronically exposed to pesticides for a period of six days *in vitro***.** Survival curves grouped by the same vertical line are not significantly different per Holm-Sidak's test (P>0.05). **Wax cumulative dose:** glyphosate: 0.0086 µg/bee, imidacloprid: 0.06 µg/bee, chlorothalonil: 0.25 µg/bee, chlorpyrifos: 0.005 µg/bee, amitraz: 0.75 µg/bee, coumaphos: 1.85 µg/bee, fluvalinate: 4.59 µg/bee. **Pollen cumulative dose:** glyphosate: 0.00013 µg/bee, imidacloprid: 0.00019 µg/bee, chlorothalonil: 1.66 µg/bee, chlorpyrifos: 0.020 µg/bee, amitraz: 0.029 µg/bee, coumaphos: 0.12 µg/bee, fluvalinate: 0.00029 µg/bee

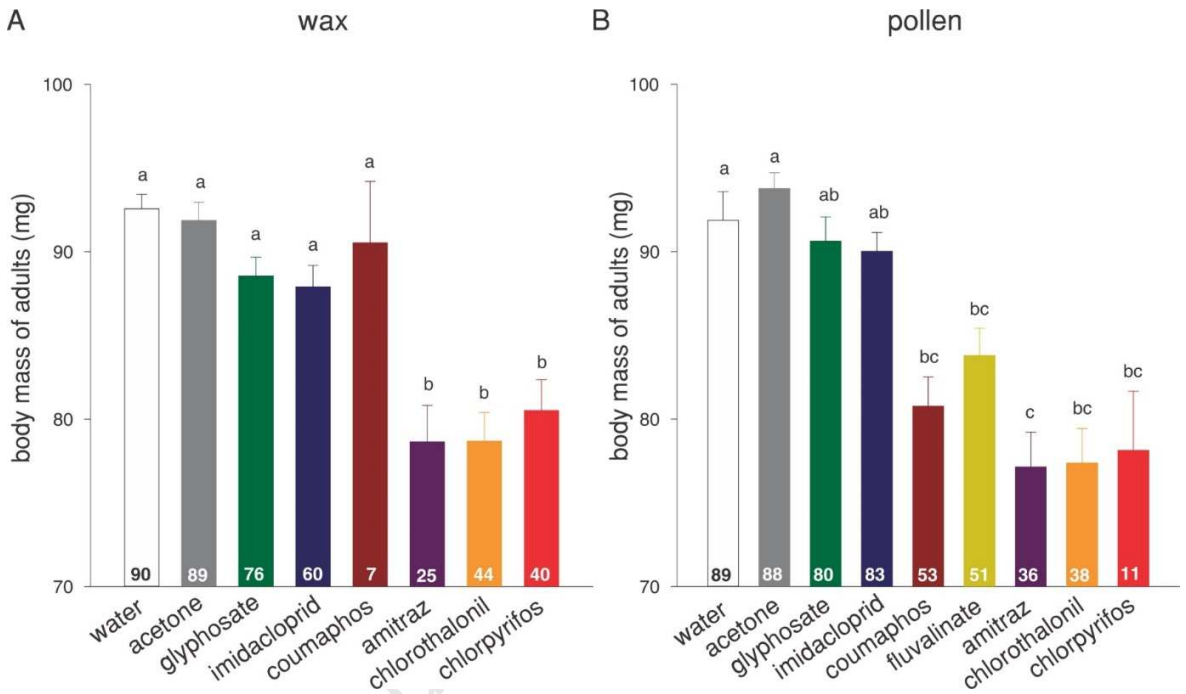

Figure 2. Body mass (mg) of newly emerged adult worker honey bees reared exposed to pesticide residues found in wax (A) or pollen (B) as larvae. The number (N) of bees that emerged from a total of 90 larvae reared for each treatment (wax, and pollen) and were weighed are indicated at the base of each treatment bar. The means ± standard errors are represented for each treatment. Treatments grouped by the same letter are not significantly different per Tukey's HSD test (P>0.05).

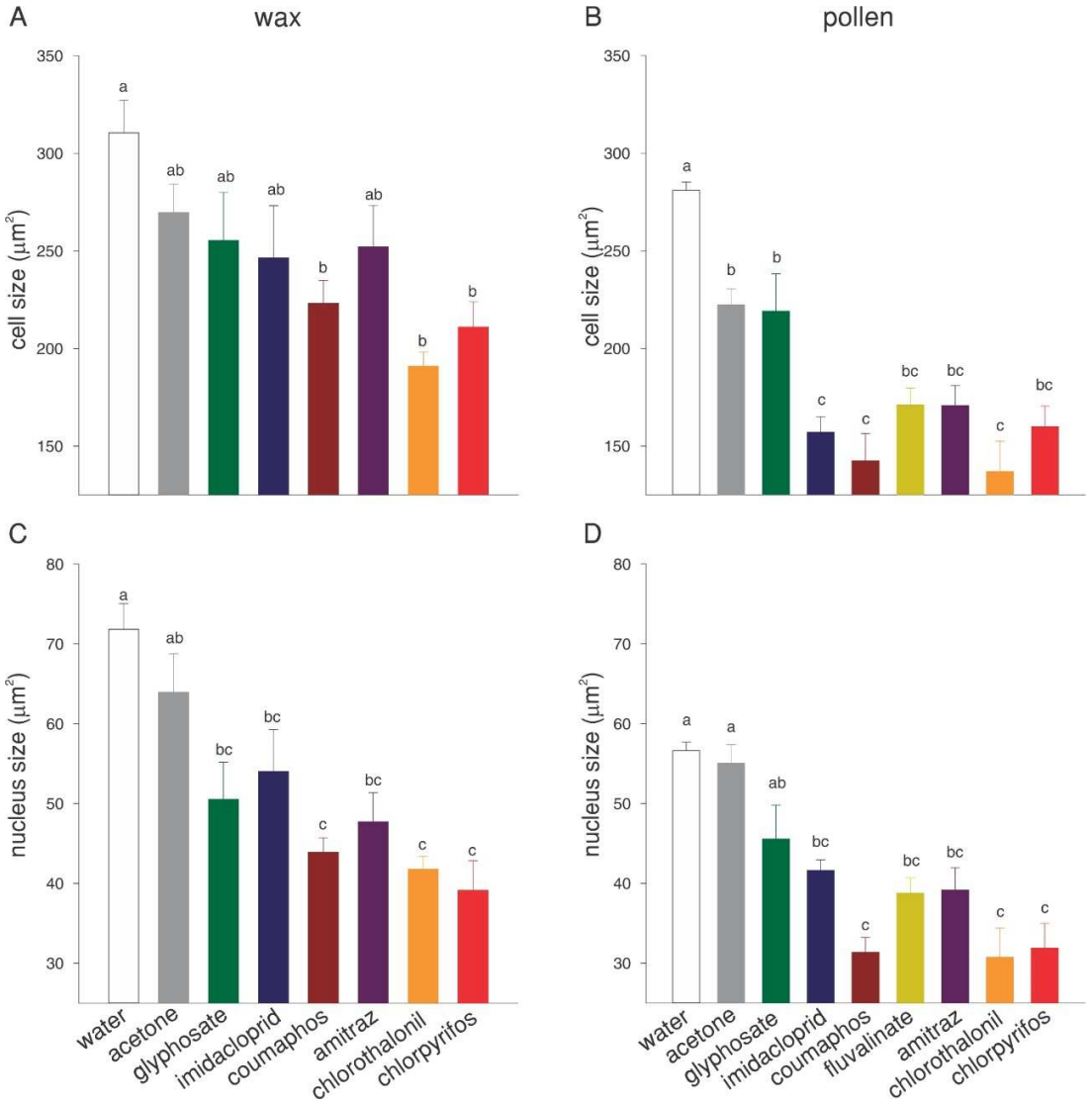
A
wax
B
pollen
C
D
cell size (µm²)
nucleus size (µm²)
water
acetone
glyphosate
imidacloprid
coumaphos
fluvalinate
amitraz
chlorothalonil
chlorpyrifos

Figure 3. Morphometry of cells and nuclei of hypopharyngeal glands of newly emerged adult worker honey bees reared exposed to pesticide residues found in wax (A, B) or pollen (C,D) as larvae. The area of 120 cells and 120 nuclei were measured from six bees (20 of each) per treatment group. The means ± standard errors are represented. Treatments with the same letter are not significantly different per Dunn's test (P>0.05).

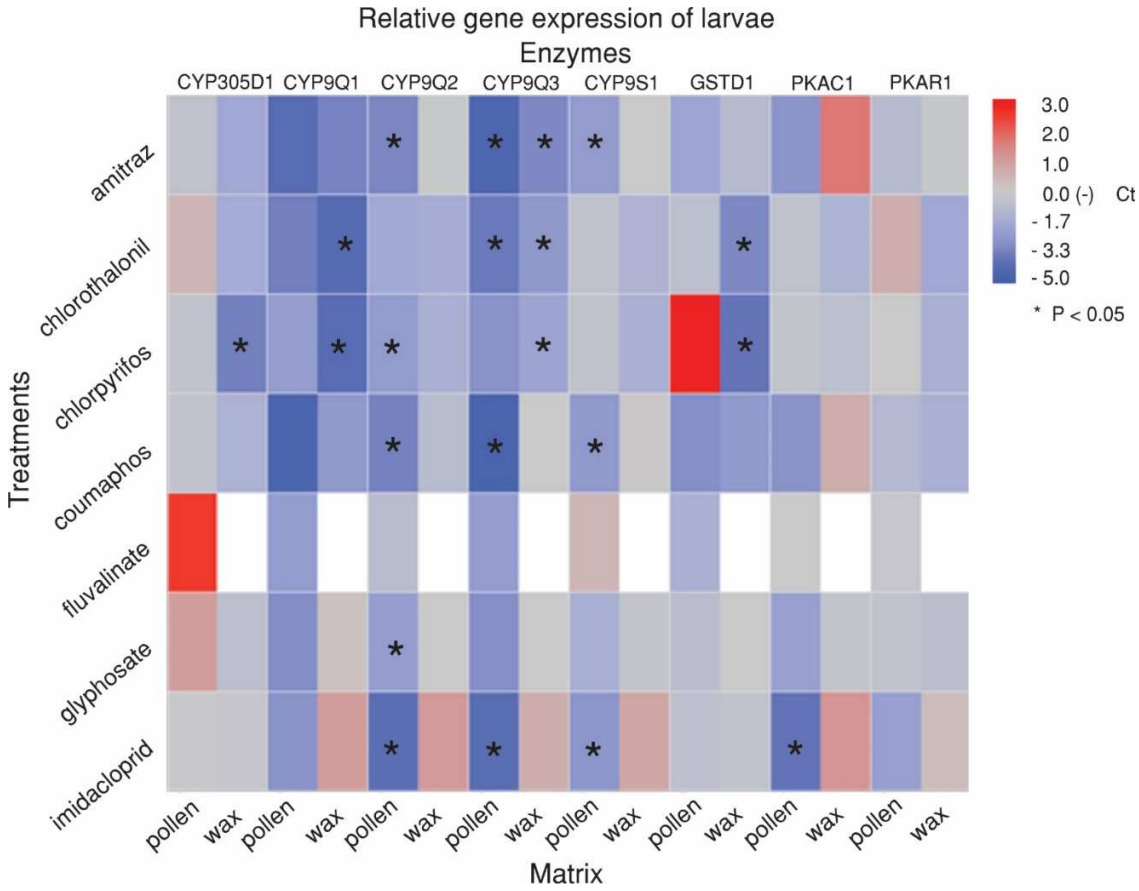

Figure 4. Heatmap displaying the relative gene expression results of honey bee D6 larvae exposed to field-relevant pesticide concentrations detected in wax and pollen, with pesticides on the y-axis and concentration levels (wax or pollen) on the x-axis. Target genes of interest are displayed across the top (CYP305D1, CYP9Q1, CYP9Q2, CYP9Q3, CYP9S1, GSTD1, PKAC1, PKAR1). Up-regulated genes are displayed with varying degrees of red intensity and down-regulated genes are displayed with varying degrees of blue intensity depending on amount of

change in expression relative to that in the control groups. Gene expression data were normalized relative to the geometric mean of the two reference genes Actin and EIF-S8. Significant changes in gene expression ($P<0.05$) from the respective control (negative or solvent) are denoted with asterisks. The white boxes indicate that no analyses were performed due to high mortality of the bees prior to each relevant sampling interval.

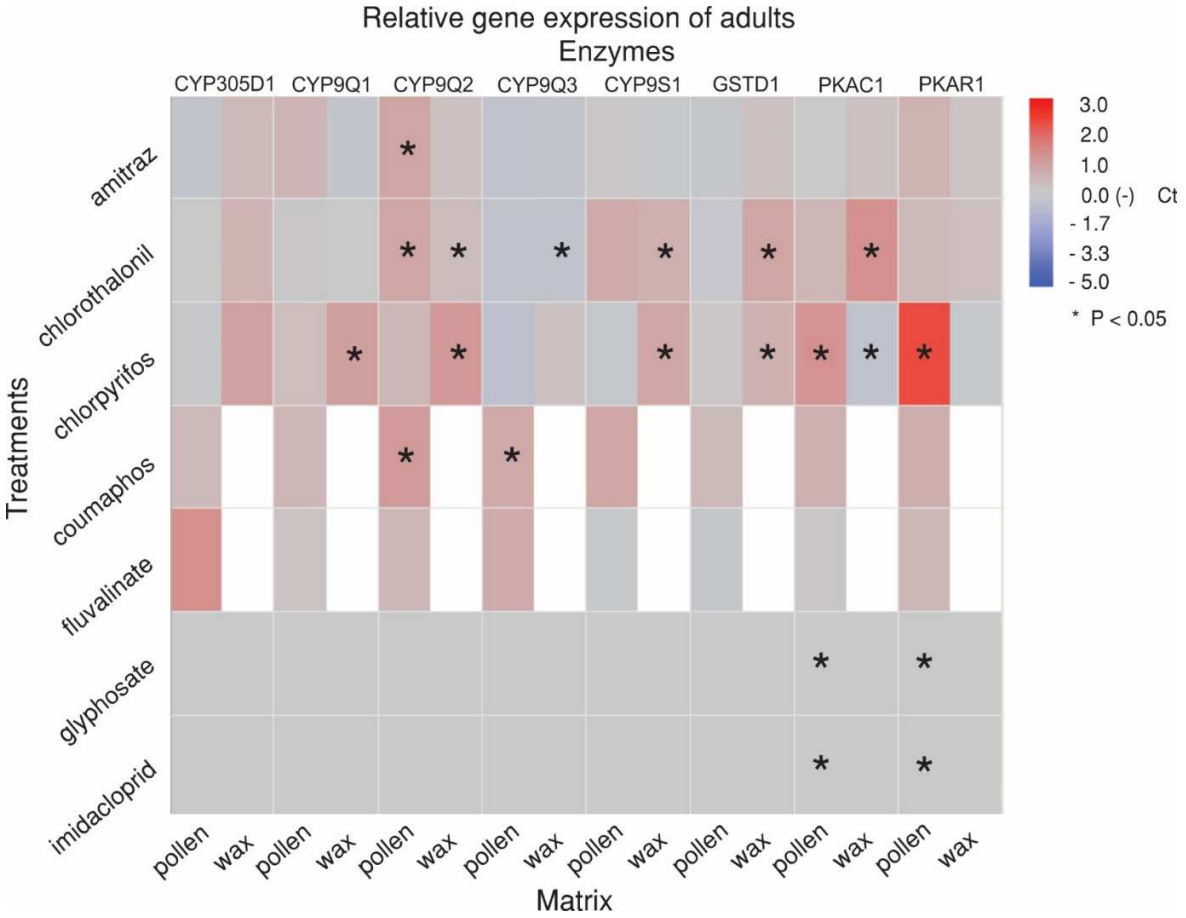

Figure 5. Heat map displaying the relative gene expression results of newly emerged adult workers exposed to pesticide concentrations detected in wax and pollen during the larval stage of development, with pesticides on the y-axis and concentration levels (wax or pollen) on the x-axis. Target genes of interest are displayed across the top (CYP305D1, CYP9Q1, CYP9Q2, CYP9Q3, CYP9S1, GSTD1, PKAC1, PKAR1). Up-regulated genes are displayed with varying degrees of red intensity and down-regulated genes are displayed with varying

degrees of blue intensity depending on amount of change in expression relative to that of the control groups. Gene expression data were normalized relative to the geometric mean of the two reference genes Actin and EIF-S8. Significant changes in gene expression ($P<0.05$) from the respective control (negative or solvent) are denoted with asterisks. The white boxes indicate that no analyses were performed due to high mortality of the bees prior to each relevant sampling interval.

Highlights

- The effects of chronic exposure of seven pesticides were studied in *Apis mellifera*
- Larvae were fed on diet with pesticides at concentrations detected in wax and pollen
- Diet was contaminated with acaricides, insecticides, an herbicide, and a fungicide
- The survival, body mass, phenotype, gene expression, and development were studied
- The exposure to pesticides chronically affected the health of immature honey bees

Conflict of interest

The authors declare none conflict of interest.