



# Toxicological status changes the susceptibility of the honey bee *Apis mellifera* to a single fungicidal spray application

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## Abstract

During all their life stages, bees are exposed to residual concentrations of pesticides, such as insecticides, herbicides, and fungicides, stored in beehive matrices. Fungicides are authorized for use during crop blooms because of their low acute toxicity to honey bees. Thus, a bee that might have been previously exposed to pesticides through contaminated food may be subjected to fungicide spraying when it initiates its first flight outside the hive. In this study, we assessed the effects of acute exposure to the fungicide in bees with different toxicological statuses. Three days after emergence, bees were subjected to chronic exposure to the insecticide imidacloprid and the herbicide glyphosate, either individually or in a binary mixture, at environmental concentrations of 0.01 and 0.1 µg/L in food (0.0083 and 0.083 µg/kg) for 30 days. Seven days after the beginning of chronic exposure to the pesticides (10 days after emergence), the bees were subjected to spraying with the fungicide difenoconazole at the registered field dosage. The results showed a delayed significant decrease in survival when honey bees were treated with the fungicide. Fungicide toxicity increased when honey bees were chronically exposed to glyphosate at the lowest concentration, decreased when they were exposed to imidacloprid, and did not significantly change when they were exposed to the binary mixture regardless of the concentration. Bees exposed to all of these pesticide combinations showed physiological disruptions, revealed by the modulation of several life history traits related mainly to metabolism, even when no effect of the other pesticides on fungicide toxicity was observed. These results show that the toxicity of active substances may be misestimated in the pesticide registration procedure, especially for fungicides.

**Keywords** Difenoconazole fungicide · Glyphosate herbicide · Imidacloprid insecticide · Honey bee survival · Toxicological status

## Introduction

During all of their life stages, honey bees are exposed to pesticides used to protect agricultural crops against deleterious species, including insects, mites, plant pathogens, and weeds (Pimentel 2009). At the larval stage and after emergence, honey bees feed on stored honey and bee bread. These products are frequently contaminated by residues of pesticides such as

insecticides, fungicides, and herbicides, leading to chronic exposure to these pesticides (Ostiguy et al. 2019; Piechowicz et al. 2018; Wintermantel et al. 2019). Countless studies have shown sublethal effects of insecticides on honey bees (Belzunces et al. 2012; Meikle et al. 2020). The effects of herbicides have been less well investigated, but an increasing number of studies have reported that these pesticides are toxic to honey bees because they impair key functions, such as metabolism and foraging activity, and negatively impact the gut microbiota (Dornelles and Oliveira 2014; Gonalons and Farina 2018; Motta et al. 2020). Honey bees rarely encounter only a single pesticide in beehive matrices (Kanga et al. 2019; Mullin et al. 2010), and combinations of pesticides, such as insecticides along with herbicides or fungicides, may induce synergistic and additive toxicities (Gonalons and Farina 2018; Johnson et al. 2013; Wang et al. 2019; Wang et al. 2020).

In addition to chronic exposure to residual concentrations of pesticides, honey bees may be acutely exposed to high

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concentrations of pesticides (mainly fungicides) during their foraging flights. Fungicides are authorized for use during full bloom, and many foraging crops require at least one fungicide application during the blooming period to provide feasible management of fungal diseases (Xavier et al. 2020). Based on the standard test methods for the determination of acute oral and contact toxicity to pesticides, fungicides are considered safe to honey bees, with median lethal doses ( $LD_{50}$ ) higher than 100  $\mu\text{g}/\text{bee}$  (Stanley et al. 2015). In these tests, toxicity is generally based on mortality observed 48 h after a single exposure to a pesticide, and the assessment may be prolonged to 96 h after exposure if mortality continues to rise (OCDE 1998; Rortais et al. 2017). However, delayed long-term acute exposures to fungicides are underexamined. In addition, the toxic effects of fungicides are not restricted to their impacts on survival. Fungicides affect larval development and mortality (Mussen et al. 2004), reduce the number of workers in the hive, perturb hive thermoregulation (Meikle et al. 2017), reduce pollen consumption and ATP levels, and increase virus titers in honey bees (Degrandi-Hoffman et al. 2015; Simon-Delso et al. 2014).

Among the pesticides used for plant protection, three pesticides could be considered of high interest because they are widely used and they can induce toxicity in binary and ternary mixtures (Almasri et al. 2020). Imidacloprid is a neonicotinoid insecticide that disrupts the insect nervous system by acting as an agonist of nicotinic acetylcholine receptors (Taillebois et al. 2018). It is among the most frequently detected residues in honey samples. It is detected at concentrations ranging from 0.14 to 0.7  $\mu\text{g}/\text{kg}$  in honey and at a mean concentration of 0.9  $\mu\text{g}/\text{kg}$  in pollen (Chauzat et al. 2011; Lambert et al. 2013; Nguyen et al. 2009). Glyphosate is an herbicide that inhibits the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase, which is essential for the synthesis of some aromatic amino acids in plants (Duke and Powles 2008). It is detected in 27% of honey specimens, at concentrations ranging from 64 to 118  $\mu\text{g}/\text{kg}$ , and in bee bread, at concentrations ranging from 52.4 to 58.4  $\mu\text{g}/\text{kg}$  (Berg et al. 2018; El Agrebi et al. 2020; Rubio et al. 2015). Score® 250 EC is a phytopharmaceutical fungicide preparation containing difenoconazole. This fungicide belongs to the triazole fungicides, which are among the most widely used and ubiquitous fungicides worldwide. Triazoles are active substances that block the conversion of lanosterol into ergosterol in fungi by inhibiting 14 $\alpha$ -demethylase (Zhang 2018). The Score fungicide is applied as a broad-spectrum preventive and curative treatment in fruit trees such as apricot and peach trees and in potato, sugar beet, lettuce, asparagus, and tomato; its use is authorized during the flowering period at label doses of 0.3 to 0.5 L per hectare (equivalent to 75 to 125 g of difenoconazole per hectare) (Anses 2014). Due to the extensive use of difenoconazole during all plant developmental stages, it is frequently detected in beehive matrices at concentrations ranging from 2.8 to 6.7  $\mu\text{g}/\text{kg}$  in honey and at a

mean concentration of 270  $\mu\text{g}/\text{kg}$  in beebread (Abdallah et al. 2017; Kubik et al. 2000).

In this study, we focused on determining the acute toxicity and long-term effects of a single spray application of a fungicide preparation on honey bees. We aimed to study the influence of a past exposure of individuals to pesticides on the toxicity induced by fungicides to determine whether the toxicological status of honey bees may significantly modulate the toxicity of fungicides. Attention was focused on the effect of these exposure combinations on survival, food consumption, and metabolism by analyzing the variations in six life history traits. In this protocol, we intentionally omitted the chronic treatments applied alone because, in the toxicity tests used in the pesticide registration procedure, the history of the bees is not known. We aimed only to determine the extent to which the toxicological status of the bees could modulate the apparent toxicity of fungicides to elucidate toxicological impacts for the pesticide registration.

## Materials and methods

### Materials

Imidacloprid (CAS No. 138261-41-3) and glyphosate (CAS No. 1071-83-6) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Score® 250 EC (difenoconazole) was purchased from Syngenta France S.A.S. Bee food® Protein solution was purchased from Remuau Ltd. (Barbentane, France). The Bee Boost® (PseudoQueen) pheromone preparation was purchased from Intko Supply Ltd. (Vancouver, Canada).

### Honey bees

The study was conducted on emergent *Apis mellifera* honey bees (less than 24 h old) collected from brood frames from three beehives that were continuously checked for their health status in the experimental apiary of the *Abeilles & Environnement* Research Unit (Bee & Environment Research Unit) of INRAE (Avignon, France). Emergent honey bees were collected, in mid-April 2018, directly from brood frames devoid of adult bees and placed in an incubator at  $33 \pm 2$  °C with  $60 \pm 5\%$  relative humidity. The emerging bees from the three beehives were mixed together and randomly distributed in groups of 30 honey bees housed in plastic cages ( $6 \times 8.5 \times 10$  cm). A small piece of Bee Boost® (PseudoQueen), releasing queen mandibular pheromone, and a small wax foundation sheet were deposited on the top of the cage to mimic the hive environment. For hygienic purposes, a sheet of filter paper was placed on the bottom of each cage and changed daily. During the first 2 days post-emergence, the honey bees were fed water, pollen, and candy ad libitum, and the few dead bees were

removed and replaced with bees of the same age set aside for this purpose. It should be noted that the genetic differences between the three colonies are levelled out by the mixing of the bees from these colonies during sampling.

### Chronic exposure to pesticides

At the beginning of the third day after emergence, water, pollen, and candy were removed from the cages and replaced with a 60% (w/v) sucrose solution containing 0.1% (v/v) dimethyl sulfoxide (DMSO) and 1% (v/v) Bee Food® protein solution, to which imidacloprid and glyphosate were added, either alone or in binary mixtures, at concentrations of 0 (control), 0.01, and 0.1 µg/L. These two concentrations were equivalent to 0.0083 and 0.083 µg/kg, respectively, calculated according to a sucrose solution density of  $1.23 \pm 0.02$  ( $n = 10$ ). They were chosen because imidacloprid and glyphosate are frequently found in honey and pollen at these contamination levels (Bridi et al. 2018; Karise et al. 2017; Pareja et al. 2019). Chronic exposure was maintained until day 30 after emergence. Each treatment consisted of 14 cages ( $n = 14$ ) with 30 honey bees per cage. The 10-fold (10×) concentrated working pesticide solutions were prepared in 1% (v/v) DMSO via the serial dilution of primary mother solutions and were stored at  $-20^\circ\text{C}$ . The 10× working solutions were diluted 10-fold in a 66.67% (w/v) sucrose solution to obtain a feeding syrup containing 60% sucrose, 0.1% DMSO, and 1% (v/v) Bee Food® protein solution, plus or minus the pesticides at the desired concentrations. The working and feeding solutions were checked by GC-MS/MS and LC-MS/MS according to two analytical methods, with RSD < 10% (Oulkar et al. 2017; Paradis et al. 2014).

### Exposure to fungicide spraying

We mimicked an environmental situation in which honey bees were firstly chronically exposed to pesticides (imidacloprid and glyphosate) through food from the time of their emergence. The bees were then exposed to the fungicide when they were 10 days old, an age at which they can initiate their first flight outside the hive (Requier et al. 2020).

Honey bees were exposed to Score® 250 EC by spraying in a Potter-type tower at an application rate of 0.3 L/ha (75 g/ha), which is the application rate recommended for use on peach, apricot, and cherry trees to fight fungal diseases such as *Monilia spp.* during flowering. The Potter-type tower mimics agricultural spray application during which foragers are exposed by contact to pesticides at the field application rate. The formulation was freshly prepared before spraying using tap water to mimic the method performed by the farmers when treating their crops. Spray application was performed 10 days after emergence on honey bees that were chronically exposed to imidacloprid or glyphosate, either alone or in a binary

mixture, for 1 week. The honey bees in each cage were slightly anesthetized with CO<sub>2</sub> and placed together on a 200 cm<sup>2</sup> Plexiglas disc. The disc was immediately subjected to rotation at 23 rpm to achieve homogenous spraying (Colin and Belzunces 1992; Poquet et al. 2014). Deposition was previously calibrated to achieve a rate of  $2.14 \pm 0.14$  µL/cm<sup>2</sup> (214 L/ha). The calibration accuracy was checked after every three spraying events. The bees that were exposed to the fungicide by spray application without being chronically exposed to the other pesticides were designated group F. The bees that were chronically exposed to the insecticide (imidacloprid) at 0.01 or 0.1 µg/L and then to the fungicide were designated groups I0.01F and I0.1F, respectively. The bees that were chronically exposed to the herbicide (glyphosate) at 0.01 or 0.1 µg/L and then to the fungicide were designated groups H0.01F and H0.1F, respectively. The bees that were chronically exposed to the insecticide-herbicide mixture at 0.01 or 0.1 µg/L and then to the fungicide were designated groups IH0.01F and IH0.1F, respectively. Control honey bees (C) were fed a 60% sucrose solution containing 0.1% (v/v) DMSO and 1% (v/v) Bee Food® protein solution from the third day after emergence onward. Then, 10 days after emergence, they were slightly anesthetized with CO<sub>2</sub> and sprayed with tap water using the Potter-type tower.

### Survival and food consumption

The number of dead bees was recorded daily at 8:30 a.m. until the end of the chronic exposure period. Dead bees were removed, and the sheet of filter paper was replaced to maintain hygienic conditions. The sucrose solutions were replaced with freshly prepared solutions, and individual food consumption was calculated by dividing the total daily food consumed per cage by the number of honey bees remaining alive each day. To obtain an accurate measurement of food consumption, an evaporation control was included.

### Choice of physiological markers

The effects of the three pesticides on bee physiology were assessed by investigating the activity of six life history traits, mainly related to metabolism. These physiological markers are relevant for assessing the physiological perturbations induced by pesticides in different honey bee compartments (Almasri et al. 2020; Badiou-Beneteau et al. 2012; Carvalho et al. 2013; Degrandi-Hoffman et al. 2015; Kairo et al. 2017; Nicodemo et al. 2014). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head, abdomen, and thorax. Carboxylesterases (CaE-2, CaE-3) were analyzed in the abdomen and midgut. Glucose-6-phosphate dehydrogenase (G6PDH) was analyzed in the head. Lactate dehydrogenase (LDH) was analyzed in the head and midgut, and

**Table 1** Distribution of common and specific physiological markers in honey bee tissues

Head	Abdomen	Midgut	Thorax
GA3PDH	GA3PDH	LDH	GA3PDH
G6PDH	CaE-3	CaE-3	ATP
LDH	CaE-2	CaE-2	

adenosine triphosphate (ATP) was analyzed in the thorax (Table 1).

GA3PDH plays an important role in energetic metabolism. It catalyzes the sixth step of glycolysis through the reversible conversion of glyceraldehyde-3-phosphate into 1,3-bisphosphoglyceric acid with nicotinamide adenine dinucleotide ( $\text{NAD}^+$ ) as a cosubstrate (Nicholls et al. 2012). CaEs are multifunctional enzymes with different isoforms involved in the detoxification of xenobiotics, such as pesticides, and in lipid metabolism (Badiou-Beneteau et al. 2012; Ross et al. 2010). G6PDH is involved in metabolism and antioxidant defense. It catalyzes the first step of the pentose phosphate pathway, thus generating precursors for nucleotide synthesis and reduced nicotinamide adenine dinucleotide phosphate (NADPH); NADPH is in turn used to generate reduced glutathione (GSH), which plays an important role in protection against oxidative damages (Efferth et al. 2000). LDH is a metabolic enzyme involved in the regeneration of  $\text{NAD}^+$  by catalyzing the reduction of pyruvate into lactate under anaerobic conditions.  $\text{NAD}^+$  is used by GA3PDH during glycolysis (Tornheim 2018). ATP is the molecular unit of the intracellular energy currency, and it plays a role in signal transduction involving kinases and adenylate cyclase (Dunn and Grider 2020).

### Tissue preparation and enzyme extraction

On days 20 and 30 after the start of chronic exposure (23 and 33 days after emergence), the surviving bees were sampled. Their heads, abdomens, and midguts were extracted according to Almasri et al. (2020). Briefly, to avoid animal suffering, the bees were first anesthetized with carbon dioxide, and their heads were separated from the rest of their bodies using a scalpel. Then, their midguts were obtained by pulling on the stinger, and the abdomen was recovered. The heads, midguts, and abdomens (devoid of the intestinal tract) were separately placed in 2 mL microfuge tubes, weighed, and stored at  $-80^\circ\text{C}$  until analysis. For each treatment and each type of tissue, 3 tissue specimens were pooled to prepare the sample. Seven samples ( $7 \times 3$  tissues) were prepared ( $n = 7$ ) for each treatment. During enzymatic analyses, each sample was assayed in triplicate. For each sample, the pooled tissues were homogenized in the extraction medium to prepare a 10% (w/v) tissue extract using a high-speed Qiagen TissueLyser II operated at

30 Hz in 5 periods of 30 s with 30 s intervals. The extraction medium consisted of 10 mM NaCl, 1% (w/v) Triton X-100, 40 mM sodium phosphate, pH 7.4, and protease inhibitors (2  $\mu\text{g/mL}$  pepstatin A, leupeptin, and aprotinin, 0.1 mg/mL soybean trypsin inhibitor, and 25 units/mL antipain). The extracts were then centrifuged at  $4^\circ\text{C}$  for 20 min at  $15,000 \times g_{\text{av}}$ , and the supernatants were kept on ice for further enzyme assays.

### Analysis of physiological life history traits

Physiological traits were spectrophotometrically assayed at  $25^\circ\text{C}$  in the head, thorax, midgut, and abdomen tissues of the same bees. GA3PDH was assayed on the basis of the conversion of 1,3-bisphosphoglyceric acid (1,3-BPG) into glyceraldehyde-3-phosphate (GA3P). In this reaction, 3-phosphoglyceric acid (3-PGA) is converted into 1,3-BPG by phosphoglycerate kinase (PGK), and 1,3-BPG is converted into glyceraldehyde-3-phosphate (GA3P) by GA3PDH in the presence of reduced nicotinamide adenine dinucleotide (NADH), whose transformation into its oxidized form ( $\text{NAD}^+$ ) is followed at 340 nm. The reaction medium contained 7 mM 3-PGA, 120  $\mu\text{M}$  NADH, 2 mM magnesium sulfate ( $\text{MgSO}_4$ ), 1.2 mM ATP, 4 mM L-cysteine-HCl neutralized with sodium bicarbonate ( $\text{NaHCO}_3$ ), 1 mM ethylenediaminetetraacetic acid (EDTA), 5 units  $\cdot\text{mL}^{-1}$  3-phosphoglycerate kinase (3-PGK), and 80 mM triethanolamine buffer, pH 7.6 (Kairo et al. 2017; Renzi et al. 2016). CaE-2 and CaE-3 were monitored according to their specific respective substrates  $\beta$ -naphthyl acetate ( $\beta$ -NA) and p-nitrophenyl acetate (p-NPA) at 515 and 410 nm, respectively (Badiou-Beneteau et al. 2012). G6PDH activity was determined by following the formation of the reduced form of  $\text{NADP}^+$  (NADPH) at 340 nm. The reaction medium contained 10 mM magnesium chloride ( $\text{MgCl}_2$ ), 0.5 mM nicotinamide adenine dinucleotide phosphate ( $\text{NADP}^+$ ), 1 mM glucose-6-phosphate (G6P), and 100 mM Tris-HCl pH 7.4 (Renzi et al. 2016). LDH activity was determined by measuring the regeneration of  $\text{NAD}^+$  at 340 nm. The reaction medium contained 0.2 mM NADH, 5 mM disodium ethylenediaminetetraacetate dihydrate (EDTA), 2 mM sodium pyruvate, and 50 mM triethanolamine, pH 7.6 (Al-Lawati et al. 2009; Bergmeyer and Gawehn 1978). ATP concentrations were quantified using an ATPlite<sup>TM</sup> assay kit (PerkinElmer®, MA, USA) by comparing luminescence values to a seven-point standard curve (0.01–2  $\mu\text{M}$ ).

### Statistical analysis

Statistics were performed using RStudio version 1.1.463 statistical software. Survival analyses were performed using the packages *survival* and *survminer* (Kassambara and Kosinski 2018; Therneau 2015), and the Kaplan-Meier method followed by a post hoc test was used for the comparison of survival



between treatments. The Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank test (with Benjamini-Hochberg correction) was employed to compare the cumulative individual food consumption between treatments. The effects of the treatments on enzymatic activities were determined by ANOVA followed by Tukey's HSD test when the data followed a normal distribution or by the Kruskal-Wallis test followed by the post hoc Dunn's test (with Benjamini-Hochberg correction using the *agricolae* package (de Mendiburu 2013)) when the data followed a nonnormal distribution. Principal component analyses (PCAs) were performed using the FactoMineR package to compare the different treatments according to their effects on physiological markers.

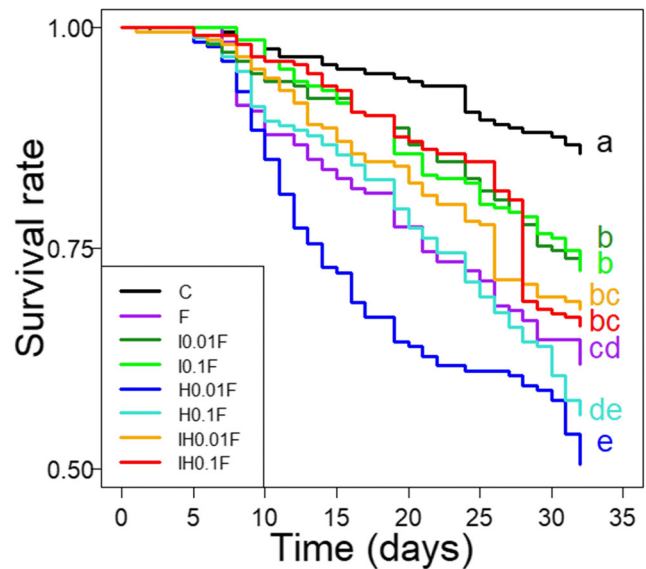
## Results

### Effects of exposure to pesticides on honey bee survival

The results regarding the toxicity of difenoconazole, either alone or preceded by chronic exposure to imidacloprid and glyphosate, are summarized in Table S1. The mortality recorded under all treatments was higher than that in the control group, which was below 15% at 30 days after emergence. The spray application of difenoconazole (F) induced a cumulative mortality of 37.8%. The I0.01F and I0.1F treatments induced toxicities lower than that induced by F. The IH0.01F and IH0.1F treatments induced a toxicity identical to that induced by F. Chronic exposure to glyphosate followed by the spray application of difenoconazole (H0.01F and H0.1F) induced toxicities higher than that induced by F, but the difference was only significant under H0.01F, which induced mortality exceeding 49% after 30 days (Fig. 1 and Table S1).

### Effects of exposure to pesticides on food consumption

The effects of the pesticide treatments on the feeding behavior of the honey bees were determined by comparing individual cumulative food consumption between the different treatments (Fig. 2 and Table S2). The honey bees exposed to the different pesticide treatments consumed an amount of food equal to that of the control group. When we compared the cumulative food consumption between honey bees that were chronically exposed to the pesticides at 0.01  $\mu\text{g/L}$  followed by the spray application of difenoconazole, we noted significantly higher food consumption in the honey bees that were chronically exposed to glyphosate at 0.01  $\mu\text{g/L}$  than in those subjected to the other treatments (Fig. 2 and Table S2).

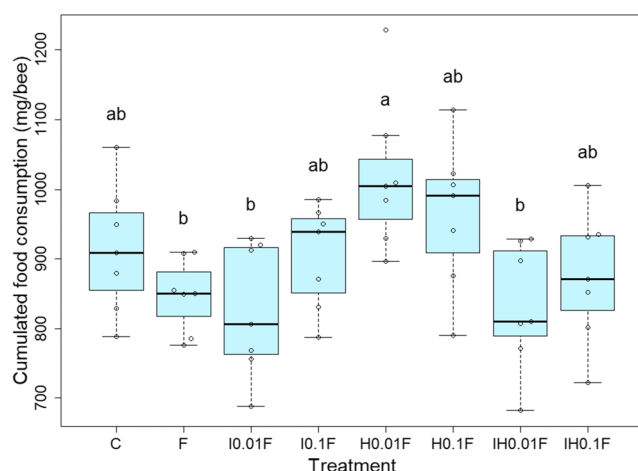


**Fig. 1** Effect of chronic exposure of honey bees to pesticides on the toxicity of difenoconazole. Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H), or a binary mixture of imidacloprid + glyphosate (IH) at 0.01 or 0.1  $\mu\text{g/L}$ . On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). The survival rate was followed for 33 days after emergence (30 days after the start of chronic exposure). The data represent the mean proportion of surviving honey bees. The mortalities were analyzed from 7 replicates of 30 bees per treatment using the Kaplan-Meier method followed by a post hoc test for the comparison of survival between treatments. The numbers after the abbreviations for each treatment refer to the concentrations of the pesticides in the feeding solution. Treatments with different letters are significantly different ( $p < 0.05$ )

### Physiological effects

To determine the physiological effects of chronic exposure to pesticides followed by the spray application of difenoconazole, we examined the modulation of six biomarkers mainly involved in metabolism. The responses of the physiological markers were determined at 20 and 30 days after the start of chronic exposure, which corresponded to 10 and 20 days, respectively, after the spray application of difenoconazole. The concentration of 0.01  $\mu\text{g/L}$  was chosen because a higher mortality was recorded when this concentration was used than when the concentration applied was 0.1  $\mu\text{g/L}$ . The enzymatic activities recorded on days 20 and 30 were expressed as percentages of their respective control values to render the data comparable (Fig. 3, Fig. 4, Table S3, and Table S4).

GA3PDH showed changes on days 20 and 30 in the heads, abdomens, and thoraxes. Head GA3PDH activity increased to 127% of the control activity under I0.01F at day 20 and decreased to 81.2% under H0.01F at day 30. At day 20, abdomen GA3PDH activity decreased to 18.3% and 42% of the control activity under I0.01F and IH0.01F, respectively. At



**Fig. 2** Effect of exposure to pesticides on food consumption. Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H), or a binary mixture of imidacloprid + glyphosate (IH) at 0.01 or 0.1  $\mu\text{g/L}$ . On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). Box plots represent the cumulative individual consumption (mg/bee) at day 30 determined from 7 cages of 30 bees per treatment. Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise comparisons using the Wilcoxon rank sum test with Benjamini-Hochberg correction. The numbers after the abbreviations for each treatment refer to the concentrations of the pesticides in the feeding solution. Treatments with different letters are significantly different ( $p < 0.05$ )

day 30, abdomen GA3PDH activity increased to 748.6% of the control activity under H0.01F. At day 20, thorax GA3PDH activity increased to 130.8% of the control activity under the I0.01F and decreased to 57.6% of the control activity under IH0.01F. At day 30, the activity of this enzyme increased to 110.6% and 104% of the control activity under H0.01F and IH0.01F, respectively. LDH activity showed no change in the head at day 20 but it increased under all exposure modalities at day 30, with 191.6% of the control activity observed under F, 207.7% under I0.01F, 266.8% under H0.01F, and 221.8% under IH0.01F.

LDH activity showed no change in the midgut at days 20 and 30. CaE-2 activity showed no change in the abdomen at days 20 and 30 or in the midgut at day 20 but it increased in the midgut to 124.3% of the control activity under F at day 30. CaE-3 activity showed no change in the abdomen at day 30. However, it increased to 160% of the control activity under H0.01F at day 20. In the midgut, CaE-3 activity showed no change at day 20, but it decreased to 65.5% of the control activity under H0.01F at day 30.

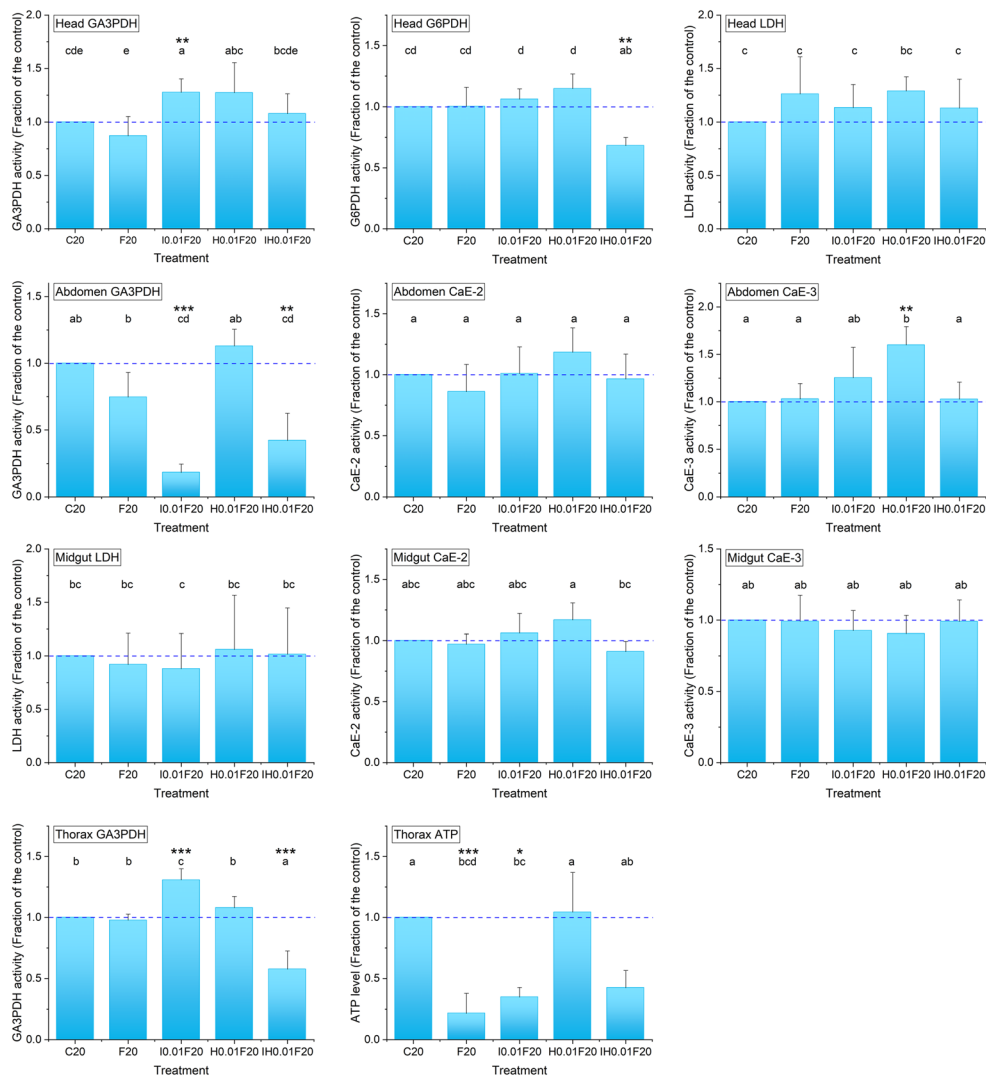
G6PDH activity showed no change at day 30, but it decreased to 68.1% of the control activity at day 20 under IH0.01F. At day 20, ATP level decreased to 21.6% and 34.9% of the control level under F and I0.01F, respectively. However, at day 30, it increased to 227% of the control level under IH0.01F.

PCA was conducted to differentiate the treatments based on their effects on the physiological markers (Fig. 5a and c). The correlation circles obtained at days 20 and 30 were not sufficient to distinguish the physiological activity levels, as the sum of the two axes on the two sampling dates did not exceed 42% (Fig. 5b and d). The results of PCA at day 20 (Fig. 5a) indicated similarity of the physiological status of the honey bees under the different treatments 20 days after the beginning of chronic exposure, while the results of PCA at day 30 (Fig. 5c) indicated the separation of I0.01F and IH0.01F from the other treatments.

## Discussion

Honey bees are exposed to a wide variety of fungicides during their foraging activity, especially when farmers treat their crops in the blooming period to fight fungal diseases (Fisher et al. 2017; Xavier et al. 2020). Despite reports on the frequent acute exposure of foragers to fungicides and their persistence in hive environments (Blaga et al. 2020; Piechowicz et al. 2018), the majority of studies on fungicides have focused on their potential to increase the toxicity of other pesticides, such as neonicotinoids and pyrethroids (Biddinger et al. 2013; Colin and Belzunces 1992; Manning et al. 2017; Wade et al. 2019; Zhu et al. 2017a), instead of studying their individual effects. Our study of the effect of a single spray application of difenoconazole at the recommended field application rate revealed delayed toxicity. Mortality started to occur 3 days after spray application and continued to rise, reaching 23.4% (corrected mortality) 20 days after spray application, which is a far-from-negligible effect. To the best of our knowledge, no previous studies have evaluated the possible delayed toxicity of difenoconazole, particularly in its Score® 250 EC form, to honey bees. Studies on other triazole fungicides, such as propiconazole, have failed to demonstrate significant toxicity to honey bees (Ladurner et al. 2005; Stanley et al. 2015). However, these studies were based on an observation period not exceeding 72 h following topical propiconazole application. Thus, considering the relatively high and delayed toxicity of difenoconazole, it is legitimate to hypothesize that the toxicity of all fungicides is likely underestimated. This emphasizes the importance of further studies aimed at investigating the long-term effects of acute exposure to pesticides, especially to reveal the actual toxicity of fungicides.

In this study, we clearly show that the toxicological status of bees influences the apparent toxicity of pesticides. Prior chronic exposure situations have induced three clear cases of interaction between the fungicide and the other pesticides: (i) toxicity lower than that of the fungicide alone, for an insecticide; (ii) toxicity higher than that of the fungicide alone, for an herbicide, and (iii) no change in fungicide toxicity, for a pesticide mixture. However, the past exposure and toxicological



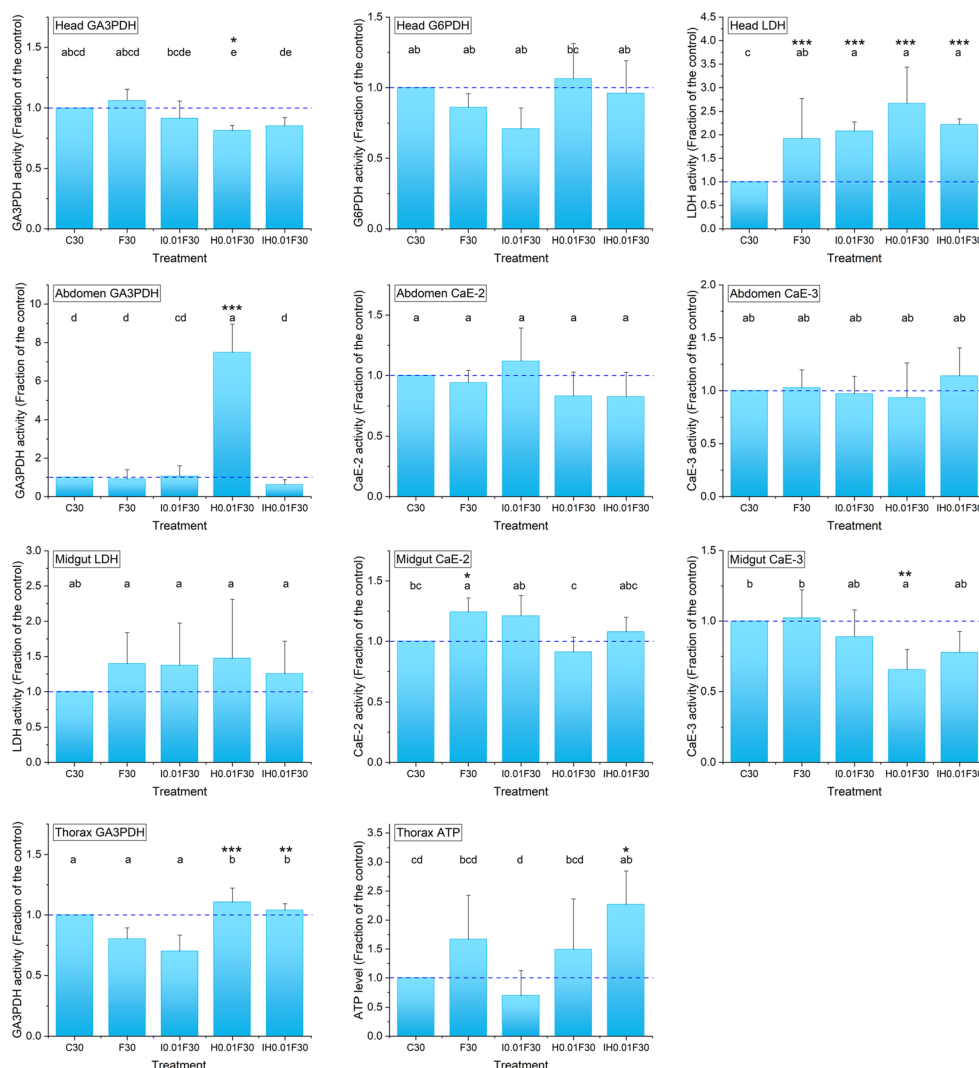
**Fig. 3** Physiological effects of exposure to pesticides in honey bees at day 20. Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H), or a binary mixture of imidacloprid + glyphosate (IH) at 0.01 or 0.1  $\mu\text{g/L}$ . On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head (h), abdomen (a), and thorax (t). Carboxylesterases (CaE-2, CaE-3) were analyzed in the

abdomen and midgut (m). Glucose-6-phosphate dehydrogenase (G6PDH) was analyzed in the head. Lactate dehydrogenase (LDH) was analyzed in the head and midgut, and adenosine triphosphate (ATP) was analyzed in the thorax. The data represent the mean tissue activities from 7 repetitions ( $n = 7$ ) performed in triplicate at day 20 and are expressed as percentages of the mean control value. Data with different letters are significantly different ( $p < 0.05$ ). Asterisks indicate significant differences from the control group: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . The dotted lines indicate the levels in controls

status of bees are not known when performing toxicity tests for pesticide registration procedures. Thus, these three cases would have resulted in the underestimation of fungicide toxicity, overestimation of fungicide toxicity, or no effect on the toxicity estimation, respectively, regardless of the effects that the prior chronic exposures would have been able to induce. This highlights the importance of not using only one or a few toxicological studies in the registration dossier to assess the acute toxicity of pesticides to bees before assigning a first tier value. This is particularly true if we consider that very large variations in the estimates of acute toxicity can be observed, independent of the quality of the experimenter. These

variations could be attributed in part to genetic differences in the bees used in toxicological studies. However, considering the huge variation in the 48-h contact  $\text{LD}_{50}$  values of substances such as the pyrethroid insecticide deltamethrin (1.5 to 67 ng/bee) or the nicotinoid insecticide imidacloprid (6.7 to 102 ng/bee) (Atkins et al. 1981; European commission 2002; Nauen et al. 2001; Suchail et al. 2000), the toxicological past of an individual could also play a role in the observed heterogeneity of pesticide toxicity.

This study on the influence of the toxicological status of honey bees on the toxicity of difenoconazole revealed several unexpected and interesting findings. First, chronic exposure to



**Fig. 4** Physiological effects of exposure to pesticides in honey bees at day 30. Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H), or a binary mixture of imidacloprid + glyphosate (IH) at 0.01 or 0.1  $\mu\text{g/L}$ . On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head (h), abdomen (a), and thorax (t). Carboxylesterases (CaE-2, CaE-3) were analyzed in the

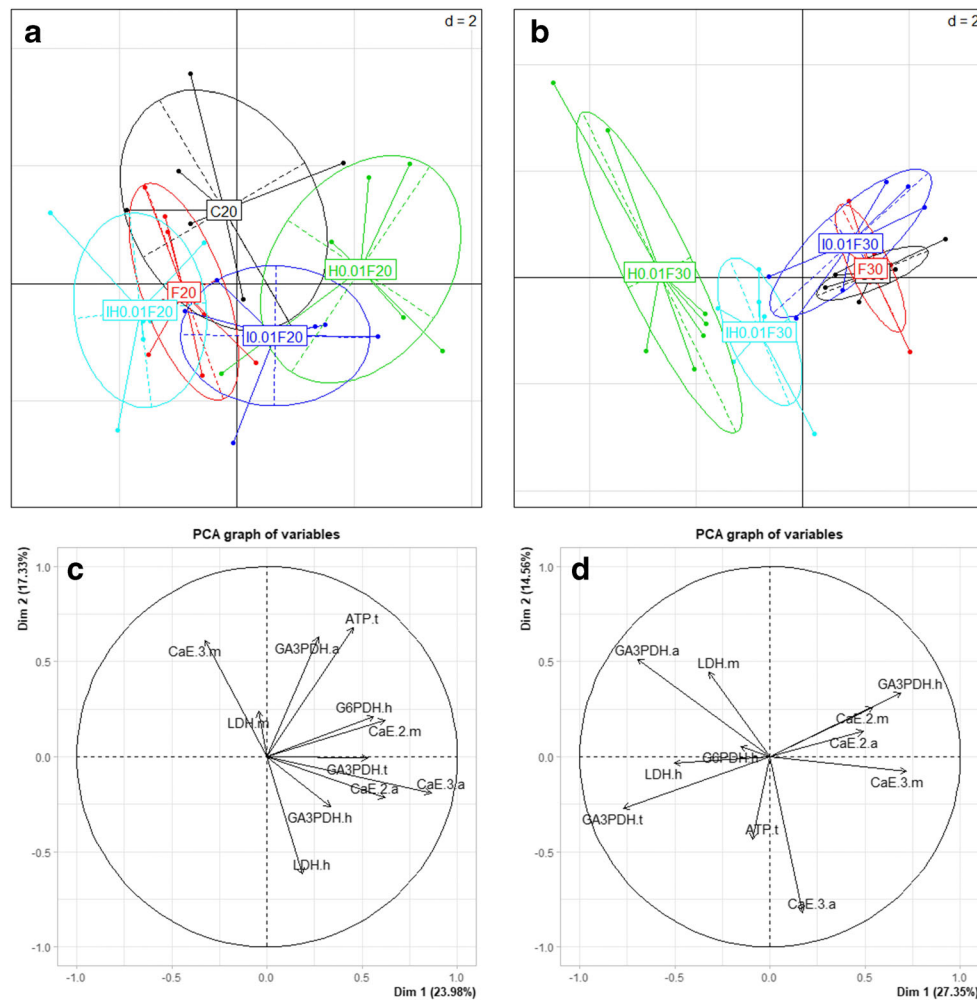
abdomen and midgut (m). Glucose-6-phosphate dehydrogenase (G6PDH) was analyzed in the head. Lactate dehydrogenase (LDH) was analyzed in the head and midgut, and adenosine triphosphate (ATP) was analyzed in the thorax. The data represent the means of tissue activities from 7 repetitions ( $n = 7$ ) performed in triplicate at day 30 and are expressed as percentages of the mean control value. Data with different letters are significantly different ( $p < 0.05$ ). Asterisks indicate significant differences from the control group: \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ . The dotted lines indicate the levels in controls

the insecticide imidacloprid at both tested doses in association with difenoconazole resulted in toxicity lower than that induced by the fungicide alone. This result is very surprising considering that neonicotinoids, including imidacloprid, and azole fungicides have been shown to induce synergistic interactions (Schmuck et al. 2003; Thompson et al. 2014; Zhu et al. 2017b). Second, for the herbicide glyphosate, the association of chronic exposure with difenoconazole induced the greatest effect at the lowest dose. Third, exposure to a greater number of substances did not consistently cause higher toxicity because no modulation of difenoconazole toxicity was observed when the binary mixture was used, regardless of the

concentration. These results demonstrate that the effects induced by mixtures of toxic substances are difficult to predict; they do not vary in a predictable manner according to the dose or number of substances involved, and they result from complex interactions that may increase or decrease the toxicity of the substances.

The mechanism by which difenoconazole toxicity is differentially influenced by imidacloprid and glyphosate might involve the metabolism of xenobiotics. In this study, chronic exposure to glyphosate made honey bees more susceptible to difenoconazole, whereas imidacloprid made honey bees less susceptible, and the binary imidacloprid-





**Fig. 5** Physiological effects of pesticides in honey bees exposed to a concentration of 0.01  $\mu\text{g/L}$ . Three days after emergence, honey bees were chronically exposed to food containing no pesticides (C), imidacloprid (I), glyphosate (H), or a binary mixture of imidacloprid + glyphosate (IH) at 0.01. On the 10th day after emergence, honey bees were exposed to the fungicide Score® 250 EC (F) via spray application at a field application rate of 0.3 L/ha (75 g/ha). Glyceraldehyde-3-phosphate dehydrogenase (GA3PDH) was analyzed in the head (h), abdomen (a), and thorax (t). Carboxylesterases (CaE-2, CaE-3) in the abdomen and

midgut (m). Glucose-6-phosphate dehydrogenase (G6PDH) in the head. Lactate dehydrogenase (LDH) in the head and midgut and adenosine triphosphate (ATP) in the thorax. Principal component analyses (PCAs) (a and c) provide visual representations of the physiological states of honey bees exposed to pesticides at day 20 (a) and day 30 (c). The correlation circles (b and d) indicate the significance of the enzymes in the PCA representations in honey bees exposed to the pesticides at day 20 (b) and day 30 (d)

glyphosate mixture did not affect difenoconazole toxicity. This complex type of interaction could be linked to the inhibition of cytochrome P450 (CYP450) enzymes involved in the metabolism of glyphosate and imidacloprid by difenoconazole (Berenbaum and Johnson 2015). The inhibition of CYP450 can lead to the accumulation of glyphosate instead of its less toxic metabolite aminomethylphosphonic acid (AMPA) (Blot et al. 2019) and, hence, to a high impact of the glyphosate-difenoconazole interaction on mortality. Conversely, the accumulation of imidacloprid instead of its toxic metabolites (5-OH-imidacloprid and an olefin derivative) moderates imidacloprid-difenoconazole toxicity (Suchail et al. 2001). The absence of an effect of the binary mixture on

difenoconazole toxicity could be due to a compensation mechanism mediating a trade-off between an increase in toxicity associated with glyphosate and a decrease in toxicity associated with imidacloprid. Thus, the physiological parameters of the bees (including vitality) might exhibit very different responses, depending both on the type of parameter and the toxicant. Therefore, the presence of pesticides at residual concentrations in the hive environment during early developmental stages could result in two situations dramatic for honey bees. The first situation would involve adult honey bees, which would be more susceptible to fungicidal spray application during foraging. The second situation would result in the use of honey bees less susceptible to the fungicide, during the toxicity tests

applied in the registration procedure for pesticides, which would lead to an underestimation of fungicide toxicity.

The modulation of physiological life history traits may indicate the involvement of metabolic disruption in the modulation of fungicide toxicity according to the toxicological status of honey bees. Overall, the results of the present study revealed some modulation of all studied physiological markers in honey bees exposed to difenoconazole either alone or associated with prior chronic exposure to glyphosate and/or imidacloprid (individually or in a binary mixture). The spray application of difenoconazole alone induced delayed metabolic changes in honey bees, reflected by a decrease in ATP levels in the thorax at day 20 and increases in LDH in the head and CaE-2 in the midgut at day 30. These results are consistent with previous findings showing the alteration of honey bee thermoregulation by difenoconazole via the modulation of energetic metabolism, possibly by inhibiting ATPases (Olivari et al. 1991; Vandame and Belzunces 1998). Difenoconazole and other triazole fungicides, such as propiconazole, also alter the levels of metabolites such as lactate and acetate, which are associated with energy metabolism in fish (Souders et al. 2019; Tabassum et al. 2016; Teng et al. 2018).

Difenoconazole induced physiological alterations that were more pronounced when the bees were chronically exposed to imidacloprid and glyphosate, either individually or in a binary mixture, from the time of emergence. The increase in the negative physiological impacts of difenoconazole resulting from chronic exposure to pesticides was expected because both glyphosate and imidacloprid are known to induce metabolic disruptions and oxidative stress in honey bees and other animals (Avigliano et al. 2014; Burchfield et al. 2019; Nicodemo et al. 2014; Powner et al. 2016).

The differences in the effects of the different treatments on the physiological markers, between day 20 and day 30 after emergence, allow an estimation of the effects linked to honey bee aging. The difference in the effects of treatments associated with honey bee aging was examined via PCAs at days 20 and 30. At day 20, the analysis groups all of the treatments together, whereas at day 30, H0.01F and IH0.01F were clearly separated from the other treatments, reflecting distinct physiological conditions. At day 20, I0.01F had the greatest physiological impact on the honey bees, mainly through the disruption of metabolism revealed by the modulation of GA3PDH and LDH in the head, GA3PDH and ATP in the thorax, and GA3PDH in the abdomen. At day 30, H0.01F and IH0.01F had the greatest impacts on honey bee physiology, resulting in a higher number of affected enzymes than in the control and the other treatments. At day 30, the major changes in physiological markers observed in honey bees exposed to treatments H0.01F and IH0.01F coincided with higher mortalities under these treatments. This may reflect a strong correlation between the observed lethal effects and the metabolic alterations. Thus, the interference between pesticides and

metabolism could induce symptoms similar to nutrient deficiency (Degrandi-Hoffman et al. 2015). This phenomenon could disrupt key physiological functions that rely on carbohydrate oxidation, such as flight (Thompson and Suarez 2009) and honey bee thermoregulation (Heinrich and Esch 1994), which are two essential functions at both the individual and colony levels. Flight, which involves muscle contractions at high frequency, and thermoregulation, which involves a tetanic contraction of flight muscles, are great consumers of carbohydrates (Brandt and Huber 1979; Suarez et al. 2005). Hence, all toxicants that can negatively interfere with carbohydrate metabolism could potentially alter these functions. It has been previously demonstrated that hypothermia can be induced in the honey bee by pesticides acting at neural level (organophosphorus and pyrethroid insecticides) and at metabolic level (azole fungicides) (Schmaranzer et al. 1987; Vandame and Belzunces 1998). However, the possibility of a hypothermia elicited by pesticides that impair the mobilization of energetic resources, such as the pyrethroid insecticide deltamethrin, must be carefully considered (Bounias et al. 1985).

## Conclusion

This study demonstrates that a single spray application of a fungicide at a registered field dosage induces delayed toxicity that compromises the survival of foragers. The toxicological status of individuals may change the susceptibility of bees to the fungicide by increasing, decreasing, or not affecting the toxicity of the fungicide. The effects of prior exposure to different pesticides on the susceptibility of bees to a fungicide are not directly linked to the concentration or the number of substances to which bees are exposed. The fungicide difenoconazole elicits delayed metabolic disruptions that are more pronounced when honey bees are exposed to residual concentrations of other pesticides, such as imidacloprid and glyphosate. These findings emphasize the importance of putting more effort toward adopting new risk assessment approaches that take into consideration the delayed and long-term effects of acute exposures to fungicides at their field application rates. It appears necessary to extend the period during which the effects are observed to more than 96 h and to include several studies in the pesticide registration dossier to consider possible coexposure situations that could lead to an underestimation of the toxicity of fungicides.

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**Availability of data and materials** The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Author contribution** Conceptualization: Luc P. Belzunces and Jean-Luc Brunet; formal analysis: Hanine Almasri and Luc P. Belzunces; funding acquisition: Luc P. Belzunces; investigation: Hanine Almasri, Daiana Antonia Tavares, Deborah Sené, and Sylvie Tchamitchian; methodology: Hanine Almasri and Luc P. Belzunces; project administration: Luc P. Belzunces; resources: Sylvie Tchamitchian, Marianne Cousin, and Michel Pélissier; supervision: Luc P. Belzunces and Jean-Luc Brunet; visualization: Hanine Almasri and Luc P. Belzunces; writing — original draft: Hanine Almasri and Luc P. Belzunces; writing — review & editing: Luc P. Belzunces, Hanine Almasri, and Jean-Luc Brunet.

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## Declarations

**Ethics approval and consent to participate** Not applicable.

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