Mixtures of an insecticide, a fungicide and a herbicide induce high toxicities and systemic physiological disturbances in winter Apis mellifera honey bees

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ABSTRACT

Multiple pesticides originating from plant protection treatments and the treatment of pests infecting honey bees are frequently detected in beehive matrices. Therefore, winter honey bees, which have a long life span, could be exposed to these pesticides for longer periods than summer honey bees. In this study, winter honey bees were exposed through food to the insecticide imidacloprid, the fungicide difenoconazole and the herbicide glyphosate, alone or in binary and ternary mixtures, at environmental concentrations (0 (controls), 0.1, 1 and 10 µg/L) for 20 days. The survival of the honey bees was significantly reduced after exposure to these 3 pesticides individually and in combination. Overall, the combinations had a higher impact than the pesticides alone with a maximum mortality of 52.9% after 20 days of exposure to the insecticide-fungicide binary mixture at 1 µg/L. The analyses of the surviving bees showed that these different pesticide combinations had a systemic global impact on the physiological state of the honey bees, as revealed by the modulation of head, midgut and abdomen glutathione-*S*-transferase, head acetylcholinesterase, abdomen glucose-*6*-phosphate dehydrogenase and midgut alkaline phosphatase, which are involved in the detoxification of xenobiotics, the nervous system, defenses against oxidative stress, metabolism and immunity, respectively. These results demonstrate the importance of studying the effects of chemical cocktails based on low realistic exposure levels and developing long-term tests to reveal possible lethal and adverse sublethal interactions in honey bees and other insect pollinators.

Keywords: winter honey bee; pesticide mixtures; synergy; cocktail effects; physiological state

1. Introduction

Despite the 45% global increase in managed honey bee colonies since 1961 (Aizen and Harder, 2009; Faostat, 2008), regional colony losses have been reported in different areas, such as the United States of America (USA) and Europe. In the USA, 31.3% of colonies were lost between 2007 and 2008, while in central Europe, a significant decrease of 25% took place between 1985 and 2005 (Potts et al., 2010; Vanengelsdorp et al., 2008). The reduction in managed beehives is accompanied by a global decrease in the number and diversity of other animal pollinators (Ollerton, 2017). It has been attributed to multiple factors, including the decline in diversity and abundance of flowers, the lack of natural habitat, the presence of parasites and pathogens and exposure to pesticides (Goulson et al., 2015; vanEngelsdorp and Meixner, 2010).

Field surveys have confirmed a transfer from crops to beehive matrices of applied pesticides belonging to the three main classes of insecticides, fungicides and herbicides (Piechowicz et al., 2018; Pohorecka et al., 2012; Skerl et al., 2009). Scientists were interested in knowing the effects of insecticides on honey bees, as these products are considered the most potentially dangerous pesticides to beneficial insects (Brandt et al., 2016; Decourtye et al., 2004; Glavan and Bozic, 2013; Gregorc and Ellis, 2011; Guez et al., 2001; Kessler et al., 2015; Yang et al., 2008). Fungicides and herbicides are considered harmless to honey bees due to their low acute toxicity. Nevertheless, an increasing number of studies are addressing their actual effects (Christen et al., 2019; Cousin et al., 2013; Jaffe et al., 2019; Ladurner et al., 2005; Moffett et al., 1972). In beehive matrices, the phytopharmaceutical products of three main classes can coexist with acaricides used to control infestation by *Varroa destructor* (Chauzat et al., 2009; Chauzat et al., 2006; Mullin et al., 2010). Therefore, honey bees could be continuously exposed to mixtures of pesticides that may exhibit similar or completely different modes of action.

Despite the high probability of honey bee exposure to mixtures of pesticides, only a few studies have focused on their effects on honey bees, and most of them were restricted to the interactions between insecticides (pyrethroids and neonicotinoids) and fungicides (ergosterol biosynthesis inhibitor (EBI) family) (Bjergager et al., 2017; Colin and Belzunces, 1992; Iwasa et al., 2004; Meled et al., 1998; Schmuck et al., 2003; Thompson et al., 2014; Zhu et al., 2017a; Zhu et al., 2017b). Effects varied from no effects to synergism, depending on the pesticides used, the method and duration of exposure, and the concentrations in food. Therefore, there is a large gap in the assessment of pesticide risk in the registration procedure

because the mixtures were never investigated, and further studies are urgently needed in this field.

The losses of honey bee colonies are mostly seen at the end of the winter season (Genersch et al., 2010; Guzmán-Novoa et al., 2010), with approximately 20 to 30% losses in Canada, Europe and the USA (van der Zee et al., 2012). During this period, beehive tasks are performed by a specific category of workers known as winter honey bees. These honey bees can survive up to 6 months (Free and Spencer-booth, 1959), and they rely on the consumption of stored honey and bee bread for survival, exposing them to pesticides for a relatively long period.

Imidacloprid (insecticide), difenoconazole (fungicide) and glyphosate (herbicide) are among the pesticides that are frequently detected in beehive matrices (Berg et al., 2018; Chauzat et al., 2011; Mullin et al., 2010). Imidacloprid, together with its metabolite 6-chloronicotinic acid, was the most abundant pesticide in beehive matrices in French apiaries, with a mean concentration of 0.7 µg/kg in honey and 0.9 µg/kg in pollen (Chauzat et al., 2011). However, concentrations of 0.14-0.275 µg/kg in honey, 1.35 µg/kg in pollen and 3-5.09 µg/kg in wax comb were found in other studies (Lambert et al., 2013; Lopez et al., 2016; Nguyen et al., 2009). Imidacloprid belongs to the neonicotinoid family and acts as an agonist of the nicotinic acetylcholine receptors, leading to the disruption of the nervous system through impaired cholinergic neurotransmission (Casida and Durkin, 2013). Glyphosate is the most dominant herbicide worldwide. Its use has increased 15-fold since the introduction of genetically engineered glyphosate-tolerant crops in 1996 (Benbrook, 2016), and it was detected in beehive matrices at concentrations ranging between 17 to 342 µg/kg in honey and 52.4 to 58.4 µg/kg in beebread (Berg et al., 2018; El Agrebi et al., 2020; Rubio et al., 2015). It acts by inhibiting the enzyme 5- enolpyruvyl- shikimate- 3- phosphate synthase (EPSPS), an enzyme necessary for the biosynthesis of aromatic amino acids in plants and some microorganisms, which leads to cell death (Amrhein et al., 1980). Difenoconazole, a curative and preventive fungicide of the triazole family, is authorized for use during full bloom. It has been found at mean concentrations of 0.6 µg/kg in honey, 43 µg/kg in pollen, 270 µg/kg in beebread and 1 µg/kg in wax comb (Kubik et al., 2000; Lopez et al., 2016). It belongs to the ergosterol biosynthesis inhibitor (EBI) fungicides and acts by inhibiting the demethylation of lanosterol (Zarn et al., 2003).

To understand the effects of pesticide mixtures on winter honey bees, we conducted a study investigating the effects of the insecticide imidacloprid, the fungicide difenoconazole and the

herbicide glyphosate alone or in combinations in winter bees orally exposed at concentrations found in honey and pollen (Berg et al., 2018; Chauzat et al., 2011; Kubik et al., 2000; Nguyen et al., 2009; Thompson et al., 2019). Attention was focused on survival and physiology. The effects on physiological functions were assessed by analyzing the modulation of five physiological markers involved in the nervous system, detoxification, oxidative stress, metabolism and immunity.

2. Materials and Methods

2.1. Reagents

Triton X-100, monosodium phosphate (NaH_2PO_4), sodium chloride (NaCl), pepstatin A, leupeptin, aprotinin, trypsin, antipain, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW284C51), 4-nitrophenyl acetate (*p*-NPA), ethanol, disodium phosphate (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), disodium ethylenediaminetetraacetate dihydrate (EDTA), reduced L-glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), acetonitrile (CH_3CN), acetylthiocholine iodide (AcSCh), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), sodium bicarbonate ($NaHCO_3$), tris base, D-glucose-6-phosphate disodium salt hydrate (G6P), magnesium chloride hexahydrate ($MgCl_2.6H_2O$), β-nicotinamide adenine dinucleotide phosphate hydrate (β-$NADP^+$), 4-nitrophenyl phosphate bis(tris) salt (*p*-NPP), sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO) and hydrochloric acid (HCl) were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Imidacloprid (CAS No 138261-41-3), difenoconazole (CAS No 119446-68-3) and glyphosate (CAS No. 1071-83-6) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Protein solution (Bee Food) was purchased from Remuaux Ltd (Barbentane, France).

2.2. Honey bees

Honey bees were gathered in February 2018 from three colonies of the experimental apiary of the Abeilles & Environnement (Bees & Environment) research unit of INRAE (Avignon, France). The colonies were continuously checked for their health status. The honey bees were mixed together, slightly anesthetized with carbon dioxide and then placed, in groups of 30 honey bees, in plastic cages (6 x 8.5 x 10 cm) with a sheet of filter paper placed on the bottom and replaced daily to maintain hygiene. The honey bees were placed in the dark in incubators at 30°C ± 2°C and 60% ± 10% relative humidity. During the first day, the bees were fed water

and candy (Apifonda®) *ad libitum*. The following day, the few dead bees were removed and replaced, and the chronic exposure to pesticides for 20 days was begun.

2.3. Chronic exposure to pesticides

The bees were exposed to the insecticide imidacloprid (I), the fungicide difenoconazole (F) and the herbicide glyphosate (H) individually or in combination. Imidacloprid, difenoconazole and glyphosate were prepared either alone or in binary mixtures (imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), and glyphosate + difenoconazole (HF)) or in a ternary mixture (imidacloprid + glyphosate + difenoconazole (IHF)) at concentrations of 0.1, 1 and 10 µg/L for each substance (equivalent to 0.083, 0.813 and 8.130 µg/kg, calculated with a sucrose solution density of 1.23 ± 0.02 (n=10)) in a 60% (w/v) sucrose solution containing a 0.1% (v/v) final concentration of DMSO. The treatments were abbreviated as follows: 0.1 µg/L: I0.1, F0.1, H0.1, IH0.1, IF0.1, HF0.1 and IHF0.1; 1 µg/L: I1, F1, H1, IH1, IF1, HF1 and IHF1; and 10 µg/L: I10, F10, H10, IH10, IF10, HF10 and IHF10. The primary mother solutions of the individual pesticides were prepared in 100% DMSO. These primary solutions were used to generate the mother solutions of the individual pesticides or were mixed to obtained the mother solutions of the pesticide mixtures. The mother solutions of the pesticides were prepared by serial dilution of the primary mother solutions to obtain 1% (v/v) DMSO and stored at -20°C. The sucrose solutions used for exposure to pesticides were prepared daily by 10-fold dilution of the mother pesticide solutions in sucrose solution to obtain final concentrations of 60% (m/v) sucrose, 1% (m/v) proteins and 0.1% (v/v) DMSO. The pesticide concentrations were checked by GC-MS/MS according to two analytical methods with RSD < 10% (Paradis et al., 2014; Wiest et al., 2011). The control bees were fed a sucrose solution devoid of pesticides. For each modality of exposure (including the controls), 14 cages of 30 bees were used. Each day, the bee mortality and food consumption were recorded, the dead bees were discarded, and the filter paper placed at the bottom of the cage was replaced. For the analysis of the physiological markers, the bees were sampled 10 and 20 days after the beginning of chronic exposure.

2.4. Survival rate and food consumption

In each cage, the survival rate was recorded daily and expressed as a ratio of the initial population. Every morning, the dead bees were removed for sanitary considerations.

Food consumption was recorded for 20 days by measuring the food consumed daily by the bees in each cage. Individual daily food consumption was calculated by dividing the food consumed per cage by the number of bees that remained alive each day in each cage.

2.5. Choice of physiological markers

The effects of the pesticide combinations on honey bee physiology were assessed by analyzing the modulation of five physiological markers. The markers were chosen to distinguish the systemic and tissue-specific actions of the pesticides alone and in combination. The following two markers common to the three biological compartments (head, midgut and abdomen) were analyzed: CaE-3 and GST. In contrast, one specific physiological marker was chosen in each compartment as follows: AChE in the head, G6PDH in the abdomen and ALP in the midgut. These five markers have been found to be relevant in assessing the effects of pesticides on honey bees in different biological compartments (Badiou-Beneteau et al., 2013; Badiou-Beneteau et al., 2012; Boily et al., 2013; Carvalho et al., 2013; Kairo et al., 2017; Zhu et al., 2017a; Zhu et al., 2017b).

2.6. Tissue preparation and marker extraction

At days 10 and 20, the surviving bees were sampled. To avoid animal suffering, the bees were anesthetized with carbon dioxide, the heads were separated from the rest of the body using a scalpel, and the midguts were obtained by pulling the stinger. The heads, midguts and abdomens (with the intestinal tract removed) were placed in 2 mL microfuge tubes, weighed and stored at -80°C until analysis. For each treatment modality and each type of tissue, 3 tissues were used and pooled to prepare the sample. From this sample, the tissues were homogenized to prepare a single tissue extract. Seven tissue extracts (7 × 3 tissues) were prepared (n=7) for each treatment modality. Each sample was assayed in triplicate. The tissues were homogenized in the extraction medium [10 mM sodium chloride, 1% (w/v) Triton X-100, 40 mM sodium phosphate pH 7.4 and protease inhibitors (2 µg/ml of pepstatin A, leupeptin and aprotinin, 0.1 mg/ml soybean trypsin inhibitor and 25 units/ml antipain)] to make 10% (w/v) extracts. Homogenization was achieved by grinding tissues with a high-speed Qiagen TissueLyser II at 30 Hz for 5 periods of 30 seconds at 30 second intervals. The extracts were centrifuged at 4°C for 20 min at 15000 × $g_{av.}$ and the supernatants were kept on ice for further enzyme assays. Carboxylesterase para (CaE-3) and glutathione-*S*-transferase

(GST) were extracted from the head, midgut and abdomen; acetylcholinesterase (AChE) from the head; glucose-6-phosphate dehydrogenase (G6PDH) from the abdomen; and alkaline phosphatase (ALP) from the midgut.

2.7. Enzyme assays

CaE-3 was assayed in a medium containing the tissue extract, 10 µM BW284C51 (acetylcholinesterase inhibitor), 0.1 mM *p*-NPA as the substrate and 100 mM sodium phosphate pH 7.0. The reaction was monitored at 410 nm (Badiou-Beneteau et al., 2012; Gomori, 1953; Renzi et al., 2016). GST was assayed at 340 nm by measuring the conjugation of GSH to CDNB. The extract was incubated in a medium containing 1 mM EDTA, 2.5 mM GSH as the cosubstrate, 1 mM CDNB as the substrate and 100 mM disodium phosphate pH 7.4 (Carvalho et al., 2013). AChE was assayed at 412 nm in a medium containing the tissue extract, 1.5 mM DTNB, 0.3 mM AcSCh as the substrate and 100 mM sodium phosphate pH 7.0 (Belzunces et al., 1988). G6PDH was measured by following the formation of NADPH at 340 nm in a medium containing the tissue extracts, 1 mM G6P as the substrate, 0.5 mM $NADP^+$ as the coenzyme, 10 mM $MgCl_2$ and 100 mM Tris-HCl pH 7.4 (Renzi et al., 2016). ALP was assayed at 410 nm in a medium containing the tissue extract, 20 µM $MgCl_2$, 2 mM *p*-NPP as the substrate and 100 mM Tris-HCl pH 8.5 (Bounias et al., 1996). All reactions started after adding the substrate, and the activity was assessed by determining the initial velocity of the enzymatic kinetics, which corresponded to the slope of the tangent at the origin. All enzymatic reactions were followed using a TECAN F500 spectrophotometer.

2.8. Mode of interaction between pesticides

The interaction ratio (IR) was used to define the mode of interaction between pesticides (additive, antagonistic and synergistic) (Colin and Belzunces, 1992; Piggott et al., 2015):

$$\mathrm{IR} = \frac{(Mix - C)}{\sum_{n=0}^{2-3}(P_n - C)}$$

where *Mix* represents the crude mortality of the mixture (binary or ternary), *C* the mortality of the control, and (*Mix* - *C*) the mortality of the pesticide mixture corrected by the control mortality. $\sum_{n=0}^{2-3}(P_n - C)$ represents the sum of the mortalities induced by each pesticide (*n*) in the mixture corrected by the control mortality, which corresponds to the theoretical expected mortality of the mixture. A value of IR = 1 reflects a pure additive effect. However,

considering the variation in the effects, an IR is considered equal to 1 when $0.95 \leq IR \leq 1.05$. When $IR > 1$, the interaction is synergistic. For $IR < 1$, three cases were distinguished: (i) when the mortality of the mixture was lower than the mortality of the lowest toxic substance alone, the interaction was considered purely antagonistic. (ii) When the toxicity of the mixture was higher than the mortality of the most toxic substance but below the expected mortality, the interaction was considered subadditive. In this case, it was not possible to speak in terms of antagonism because the effect of the mixture was higher than the effect of each substance. (iii) When the effect of the mixture was between the effect of the least toxic substance and the effect of the most toxic substance, the interaction was also considered subadditive. In this case, it was also not possible to speak in terms of antagonism because, compared to the most toxic substance, antagonism could be considered, but compared to the least toxic substance, synergy could also be considered. (iv) The effect of the mixture was judged independent when the mixture induced a mortality similar to that of each pesticide.

2.9.Statistical analyses

The statistical analyses were performed using R software (Rstudio Version 1.1.463). The bee survival was analyzed by the Kaplan-Meier method (log-rank test), followed by a post hoc test to compare survival and treatments. The effects of the treatments on food consumption were investigated by comparing the individual cumulative sucrose consumption during the exposure period using the Kruskal-Wallis test, followed by pairwise comparisons using the Wilcoxon rank sum test with a Benjamini-Hochberg correction. The effects of the treatments on the physiological markers were determined by ANOVA, followed by Tukey's HSD test, when the data followed a normal distribution or a Kruskal-Wallis test, followed by a post hoc Dunn test (with Benjamini-Hochberg correction), when the data followed a non-normal distribution.

3. Results

3.1. Honey bee survival

Exposure to pesticides significantly decreased the survival rate of honey bees at 20 days, except for I0.1, I10 and F0.1, for which no significant difference from the control ($20.0 \pm 2.7\%$) was observed ($p > 0.05$) (Fig. 1A, 1B, 1C and Table S1). Based on mortality rates, the toxicities of pesticides could be ranked as follows: at 0.1 µg/L, H = IF (28.1%) <

IHF (35.4%) < IH (43.3%) < HF (49.1%). At 1 µg/L, I (33.3%) < F (34.3%) < H (35.2%) < HF (36.2%) < IH (38.1%) < IHF (43.3%) < IF (52.9%). At 10 µg/L, HF (28.1%) < H (30.0%) < F (34.3%) < IF (41.0%) < IHF (43.3%) < IH (45.7%).

Based on the interaction ratio (IR), which corresponds to the ratio between the obtained mortality of the mixture and the expected mortality (sum of the obtained mortalities of the substances in the mixture), the interaction effects between the pesticides could be grouped into 5 different categories (Table S1): additive, synergistic, subadditive, antagonistic and independent effects. (i) A synergistic effect was observed for all the binary mixtures and the ternary mixture at 0.1 µg/L and for IF1 and IH10. (ii) An additive effect was observed for IF10. (iii) A subadditive effect was observed for IH1, IHF1 and IHF10. (iv) An independent effect was observed for HF1. (v) An antagonistic effect was observed for HF10. The five most toxic pesticide mixtures were ranked as follows based on mortality rates: IF10 (41.0%) < IHF1 (43.3%) = IHF10 = IH0.1 (43.3%) < IH10 (45.7%) < HF0.1 (49.1%) < IF1 (52.9%).

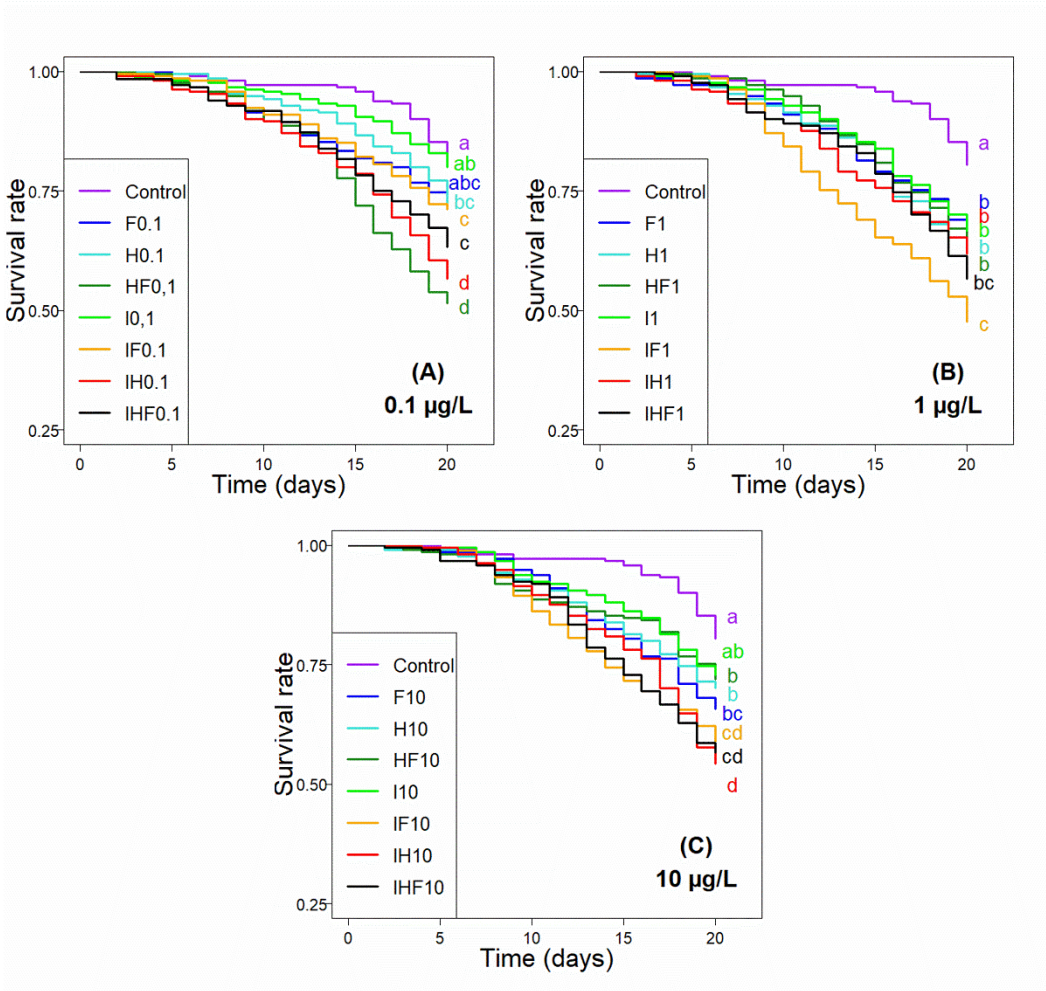

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Fig. 1. Effects of pesticides alone or in combination on honey bee longevity
For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (Control), difenoconazole (F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid (I), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH) or imidacloprid + glyphosate + difenoconazole (IHF), at 0.1 µg/L (A), 1 µg/L (B) and 10 µg/L (C). The data represent the proportion of surviving honeybees exposed to these pesticides. Numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Treatments with different letters are significantly different ($p < 0.05$).

3.2. Effects of exposure to pesticides on food consumption behavior

Food consumption was monitored daily. In general, at the end of the exposure period, it appeared that the food consumption was higher in the exposed bees (Fig. 2 and Table S2). This higher consumption was significant for all exposure conditions except F1, I1, F10 and I10 for pesticides alone, and HF10 and IHF10 for the mixtures. The five highest individual cumulative consumption levels were ranked as follows: H0.1 (831.4 mg/bee) < IF10 (834.3 mg/bee) < IF1 (840.3 mg/bee) < HF0.1 (851 mg/bee) < IH0.1 (862.7 mg/bee) (control = 672.4 ± 33.0 mg/bee). At 0.1 µg/L, the bees exposed to imidacloprid alone or in IF, IH or IHF exhibited a cumulative food consumption of 759.7, 792.6, 862.7 and 781.9 mg/bee, respectively. Therefore, on the basis of a food density of 1.23 ± 0.02 (n = 10) and pesticide concentrations, each honey bee ingested 62, 64, 70 and 63 pg of imidacloprid, which corresponded to ca. 1/60, 1/58, 1/53 and 1/58 of the imidacloprid LD_{50} (LD_{50} = 3.7 ng/bee (Schmuck et al., 2001)). At 1 µg/L, the bees exposed to imidacloprid alone or in IF, IH or IHF exhibited a cumulative food consumption of 719.3, 840.3, 804.2 and 758.4 mg/bee, respectively. Therefore, each honey bee ingested 584, 682, 653 and 615 pg of imidacloprid, which corresponded to ca. 1/6, 1/5, 1/6 and 1/6 of the imidacloprid LD_{50}. At 10 µg/L, the bees exposed to imidacloprid alone or in IF, IH and IHF exhibited a cumulative food consumption of 749.3, 834.3, 794.1 and 702.5 mg/bee, respectively. Therefore, each honey bee ingested 6081, 6770, 6445 and 5701 pg of imidacloprid, respectively, which corresponded to ca. 1/0.6, 1/0.6, 1/0.6 and 1/0.7 of the imidacloprid LD_{50}. The $LD_{50\ values}$ of difenoconazole and glyphosate are equal to or higher than 100 µg/bee (National Center for Biotechnology Information). Therefore, for difenoconazole and glyphosate at 0.1, 1 and 10 µg/L, each honey bee ingested $1/1.6x10^6$, $1/1.7x10^5$ and $1/1.8x10^4$ of the LD_{50}, respectively (Table S2).

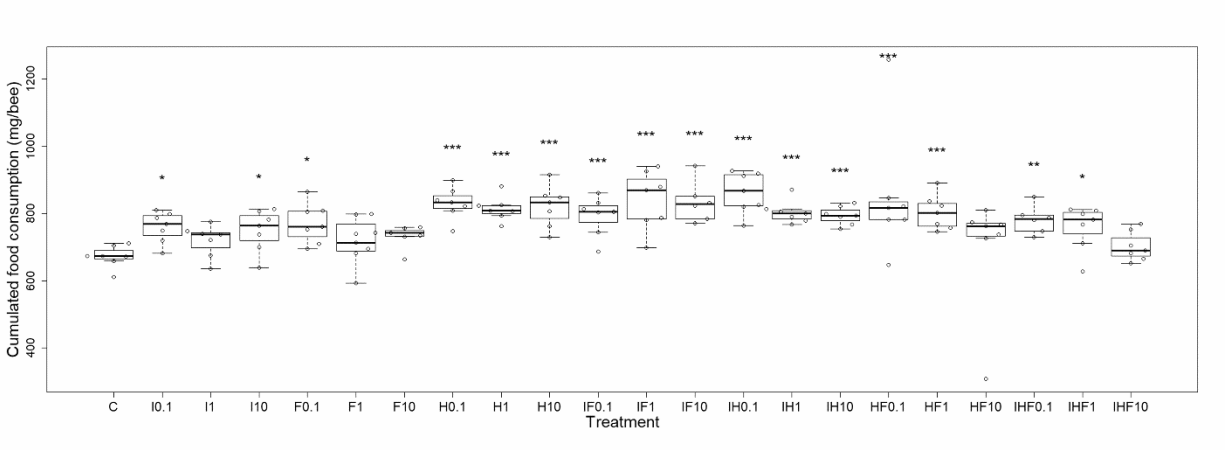

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Fig. 2. Effects of pesticides alone or in combination on food consumption

For 20 days, winter honey bees were fed sucrose solutions containing no pesticide (C, control), difenoconazole (F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid (I), imidacloprid + difenoconazole (IF), imidacloprid + glyphosate (IH) or imidacloprid + glyphosate + difenoconazole (IHF), at 0.1 µg/L, 1 µg/L, and 10 µg/L. Food consumption was followed during the 20 days of exposure by measuring the food consumed daily by the bees alive in each cage. Box plots represent the cumulated individual consumption (mg/bee) for 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 the Benjamini-Hochberg correction. The numbers after the abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

3.3. Effect of exposure to pesticides on the physiological status of honey bees

The physiological status of the honey bees was examined by studying the modulation of physiological markers in different compartments to distinguish the local from the systemic effects of the pesticides (Table 1). The responses of the honey bee markers to the exposure to the pesticides alone or in combination were analyzed after 10 and 20 days of chronic exposure to concentrations of 0.1 µg/L and 1 µg/L (Fig. 3, Fig. 4, Table S3 and Table S4). The lowest concentrations were chosen because they are particularly environmentally relevant. To render the data comparable, the enzymatic activities are expressed as percentages of the control values (Zhu et al., 2017a).

Table 1. Distribution of common and specific physiological markers across honey bee tissues

	Head	Abdomen	Midgut
Common markers	CaE-3	CaE-3	CaE-3
	GST	GST	GST
Specific markers	AChE	G6PDH	ALP

Repartitioning of physiological markers across honey bee compartments. The following three tissues were investigated: head, abdomen and midgut. In each tissue, 1 specific marker (AChE in the head, G6PDH in the abdomen and ALP in the midgut) and 2 common markers (CaE-3 and GST) were considered.

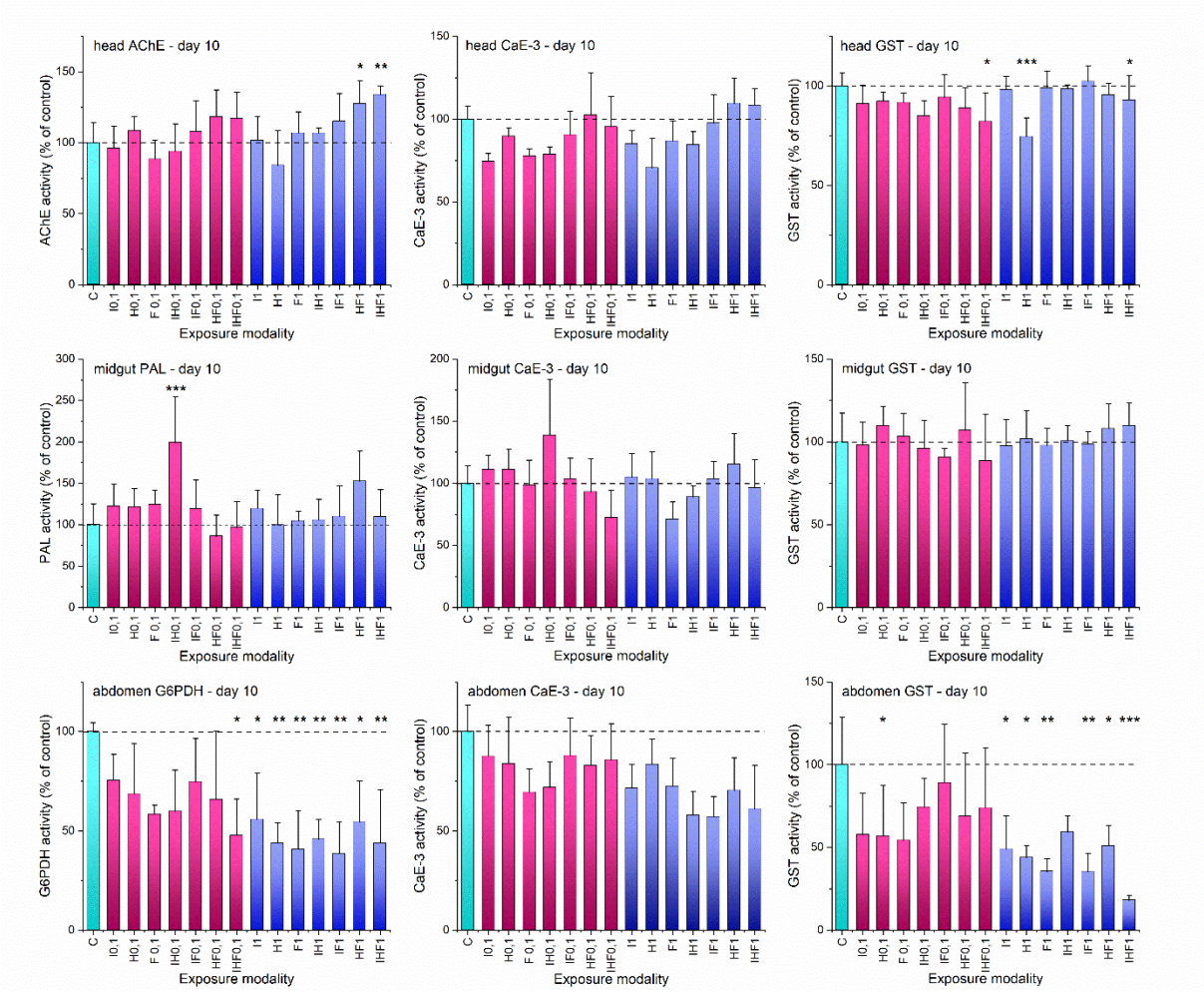

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Fig. 3. Physiological impacts of pesticides alone or in combination in winter bees after 10 days of exposure
For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (C, control), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF). The impact of the exposure to pesticides on the physiology of the surviving honey bees at day 10 was investigated through an analysis of 2 common markers in the head, abdomen and midgut (GST and CaE-3) and 3 specific markers

(AChE in the head, G6PDH in the abdomen and ALP in the midgut). To make the data comparable, the enzymatic activities were expressed as percentages of the control values. Numbers after the abbreviation of each treatment refer to the concentration of the pesticide in the sucrose solution. The exposure modalities above and below the dashed horizontal line indicate increases and decreases in enzymatic activity, respectively, compared to the control (C). Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

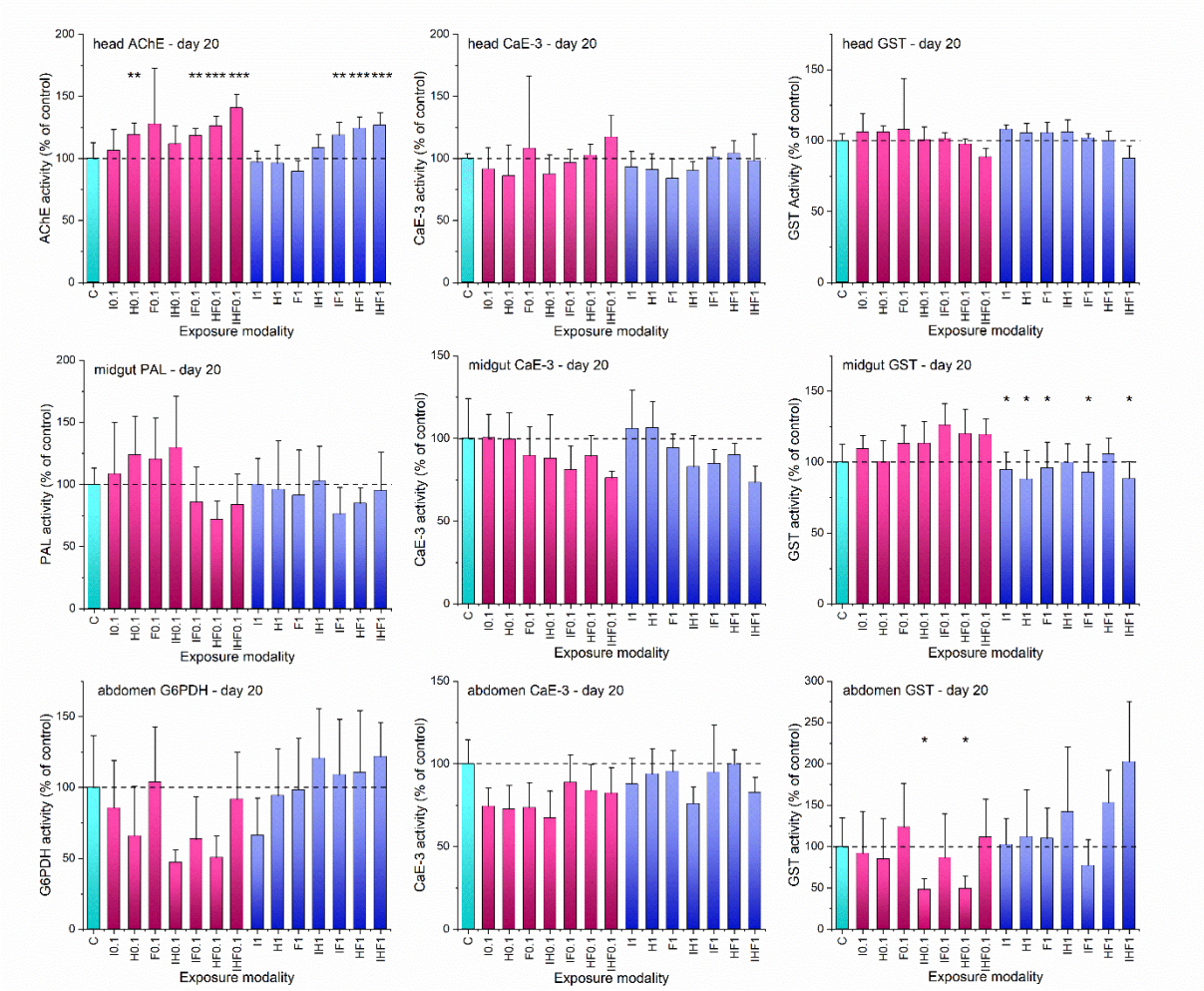

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Fig. 4. Physiological impacts of pesticides alone or in combination in winter bees after 20 days of exposure
For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (C, control), imidacloprid (I), glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF), glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF). The impact of the exposure to pesticides on the physiology of the surviving honey bees at day 20 was investigated through an analysis of 2 common markers in the head, abdomen and midgut (GST and CaE-3) and 3 specific markers (AChE in the head, G6PDH in the abdomen and ALP in the midgut). To make the data comparable, the enzymatic activities were expressed as percentages of the control values. Numbers after the abbreviation of each treatment refer to the concentration of the pesticide in the sucrose solution. The exposure modalities above and below the dashed horizontal line indicate increases and decreases in the enzymatic activity, respectively, compared to the control (C). Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

At 0.1 µg/L, head, midgut and abdomen CaE-3 and midgut GST were not modulated by all types of exposure at day 10 and day 20. Head AChE was not modulated at day 10. However, at day 20, its activity was 119% of the control activity (127.5 ± 16.0 mUA.min^{-1}.mg of tissue^{-1}) for H, 126% for HF and 141% for IHF. Head GST, abdomen G6PDH, and midgut ALP underwent modulation at day 10. For IHF, these modulations corresponded to a decrease in head GST (82% of control activity (115.3 ± 7.5 mUA.min^{-1}.mg of tissue^{-1})) and a decrease in abdomen G6PDH (48% of control activity (2.07 ± 0.53 mUA.min^{-1}.mg of tissue^{-1})). For IH, midgut ALP increased to 199% of the control activity (10.86 ± 2.75 mUA.min^{-1}.mg of tissue^{-1}). Conversely, no modulation was observed at day 20 for any of these latter enzymes. A decrease in abdomen GST was observed at 10 and 20 days. At 10 days, GST decreased to 57% of the control activity (116.1 ± 33.3 mUA.min^{-1}.mg of tissue^{-1}) for H. At day 20, GST decreased to 48% of the control activity (83.0 ± 28.7 mUA.min^{-1}.mg of tissue^{-1}) for IH and 49% for HF.

At 1 µg/L, head, midgut and abdomen CaE-3 and midgut ALP were not modulated for all types of exposure at day 10 and day 20. Head and abdomen GST underwent modulation at day 10. Head GST decreased to 75% of the control activity (115.3 ± 7.5 mUA.min^{-1}.mg of tissue^{-1}) for H and 93% for IHF. Abdomen GST decreased for all types of exposure except IH: 49% of the control activity for I; 44% for H; 36% for F; 35% for IF; 51% for HF and 18% for IHF (116.1 ± 33.3 mUA.min^{-1}.mg of tissue^{-1} for the control). Conversely, head and abdomen GST were not modulated at day 20. Abdomen G6PDH decreased at day 10 for all types of exposure: 56% of the control activity for I; 44% for H; 41% for F; 46% for IH; 38% for IF; 55% for HF and 44% for IHF (12.1 ± 0.5 mUA.min^{-1}.mg of tissue^{-1} for the control). However, no modulation was observed at day 20. Midgut GST was not modulated at day 10 but was modulated at day 20. Its activity decreased with all exposure types except IH and HF: 95% of the control activity for I; 88% for H; 96% for F; 93% for IF and 88% for IHF (147.9 ± 18.8 mUA.min^{-1}.mg of tissue^{-1} for the control). At day 10, head AChE increased to 128% of the control activity (127.7 ± 18.5 mUA.min^{-1}.mg of tissue^{-1}) for HF and 134% of the control activity for IHF. At day 20, the activity of AChE increased to 124% of the control (127.5 ± 16.0 mUA.min^{-1}.mg of tissue^{-1}) for HF, 127% of the control for IHF and 119% of the control for IF.

When comparing the dose effect of each type of exposure on physiological markers (comparison of the effects at 0.1 and 1 µg/L), no dose effect could be observed for I alone. The effects of H on all markers were similar at both concentrations except for AChE at day 20

and head GST at day 10 (H0.1 > H1). F had the same effect on all markers at both concentrations except for AChE at day 20 (F0.1 > F1). The effect of IH on CaE-3, ALP, and abdomen GST was not similar at both concentrations. The effect of IH on head CaE-3 at day 10 and on abdomen CaE-3 and GST at day 20 was lower at 0.1 µg/L than at 1 µg/L. Conversely, the effect of IH on midgut CaE-3 at days 10 and 20 and on abdomen CaE-3 and midgut ALP at day 10 was higher at 0.1 µg/L than at 1 µg/L. The effect of IF on midgut GST at day 20 was higher at 0.1 µg/L than at 1 µg/L. Depending on the concentration, the IF mixture modulated abdomen GST at day 10 (IF0.1 > IF1) and abdomen G6PDH at day 10 (IF0.1 > IF1). The effect of HF was dose-dependent only on the activity of GST in the abdomen at day 20 (HF0.1 < HF1). The effect of the ternary mixture IHF on abdomen GST at day 10 and on midgut GST at day 20 was higher at 0.1 µg/L than at 1 µg/L (IHF0.1 > IHF1) (Table S5).

4. Discussion

Honey bees that emerge at the end of the summer are considered winter bees. These bees can live up to 6 months (Free and Spencer-booth, 1959) and, therefore, are chronically exposed to pesticide residues throughout the winter. In this study, the mixtures induced relatively high toxicity even though the winter honey bees were exposed for only 20 days to these three pesticides, alone or in binary and ternary mixtures, at concentrations equal to or even less than the environmental concentrations detected in beehive matrices. Thus, determining the effect of these pesticides on colony winter survival is highly important.

4.1. Pesticide combinations are more toxic to honeybees than individual pesticides

In this study, these three pesticides alone or in combination affected the survival of winter honey bees at all tested exposure concentrations, except for I0.1, I10 and F0.1. Concerning imidacloprid, the toxicity was less pronounced than that previously observed at the same concentrations on summer bees, where 50% mortality was reached after 8 days of chronic exposure at all concentrations (Suchail et al., 2001). In contrast, imidacloprid toxicity was much more pronounced than that observed in young summer bees after 14 days of exposure at 1 µg/L (Gonalons and Farina, 2018). The differences in imidacloprid toxicity could be attributed to seasonal variations (Decourtye et al., 2003; Meled et al., 1998; Piechowicz et al.,

2016), genetic differences (Smirle and Winston, 1987), the age of the bees or the exposure duration.

Herbicides and fungicides were considered nontoxic to honey bees for a long time. Concentrations of imidazole fungicides and glyphosate up to 0.084 and 35 mg/L, respectively (Zhu et al., 2017a), were shown to be nonlethal. However, in this study, chronic exposure to glyphosate and difenoconazole (except for F0.1) was lethal. All pesticide combinations alter honey bee survival and are more toxic than pesticides alone, except HF10, which exhibits an antagonistic effect. Thus, the tier approach implemented in the pesticide registration procedure, which is first based on acute toxicity, shows great limits in detecting pesticides toxic to bees.

4.2. Increased concentrations of pesticides are not always linked to increased toxicity

In terms of dose-effect relationships, in general, it appears that the highest concentration was not the most dangerous, and the highest mortalities were observed at the intermediate concentration of 1 µg/L. This bell-shaped non-monotonic dose response relationship (NMDR) (high response at intermediate doses and lower responses at low and high doses) was previously observed for imidacloprid and glyphosate (Boily et al., 2013; Suchail et al., 2001; Vazquez et al., 2018). Three main hypotheses might explain this profile (Lagarde et al., 2015). The first is the plurality of molecular targets, i.e., each xenobiotic has several molecular targets of different affinities that may induce opposite effects across the range of the tested concentrations. The second hypothesis is the metabolic hypothesis (Suchail et al., 2001), which proposes that detoxification enzymes are induced at high but not at low concentrations. This hypothesis is consistent with the action of glyphosate, whose main metabolite, aminomethylphosphonic acid (AMPA), was shown to be nontoxic to honey bees (Blot et al., 2019). However, the metabolic hypothesis is not consistent with the action of imidacloprid because all metabolites were shown to be toxic to honey bees after chronic exposure (Suchail et al., 2001). The third hypothesis is receptor desensitization, where at high concentrations, numerous receptors are bound to xenobiotics, leading to a downregulation phenomenon (Lagarde et al., 2015).

The mixture of EBI fungicides with imidacloprid or glyphosate was shown in different studies to have no synergistic action (Iwasa et al., 2004; Thompson et al., 2014; Zhu et al., 2017b) or to induce a synergistic effect (Biddinger et al., 2013). However, these studies were based on acute contact exposure. Therefore, it is not possible to directly compare these results with

462 those of our study in which the mixtures induced an increase in mortality after chronic oral
463 exposure. On the other hand, in two studies based on chronic oral exposure, the imidacloprid-
464 fungicide and/or imidacloprid-glyphosate mixture did not show a synergistic or additive effect
465 (Gonalons and Farina, 2018; Zhu et al., 2017a). The differences in the mixture effects
466 between the different studies could be attributed to multiple factors: (i) The age of exposed
467 honey bees, with newly emerged honey bees in the studies of Gonalons and Farina (2018) and
468 Zhu et al. (2017b), and adult honey bees in our study. (ii) The duration of exposure, which did
469 not exceed 14 days in the studies of Gonalons and Farina (2018) and Zhu et al. (2017b) but
470 was 20 days in our study. (iii) The type of exposure, with the active ingredient in our study
471 and in the study of Gonalons and Farina (2018) and with the formulated products in the study
472 of Zhu et al. (2017b). (iv) Seasonal variability, which could be reflected by the use of winter
473 honey bees in our study and summer or spring honey bees in the two previously cited studies.
474 (v) The concentrations of the active ingredients constituting the mixtures, which were lower
475 in our study when compared to the studies of Zhu et al. (2017b) and Gonalons and Farina
476 (2018).

477 In this study, all binary mixtures had a differential effect on mortality in terms of both dose
478 dependence and number of substances present in the mixture. Regarding the differential dose
479 effect, HF induced a synergistic effect at 0.1 µg/L, an independent effect at 1 µg/L and an
480 antagonistic effect at 10 µg/L. IF induced a synergistic effect at 0.1 and 1 and an additive
481 effect at 10 µg/L. IH induced a synergistic effect at 0.1 and 10 µg/L and a subadditive effect
482 at 1 µg/L. The ternary mixture induced a subadditive effect at 1 and 10 µg/L and a synergistic
483 effect at 0.1 µg/L. Interactions between substances can occur not only through the primary
484 biological targets responsible for the expected effect (insecticide, herbicide or fungicide) and
485 common metabolic pathways, if they exist in the honey bee, but also through secondary
486 targets responsible for non-intentional effects. Because primary and secondary targets may
487 have different affinities for these substances, the effects induced could depend on the internal
488 body concentration and, therefore, the exposure level. Hence, substances may interfere by
489 blocking or activating metabolic pathways triggered by the substances in the mixtures, which
490 explains why the nature and importance of the effects vary with the doses (Lagarde et al.,
491 2015). However, at 0.1 µg/L, the mortality induced by IHF was lower than those induced by
492 IH and IF, leading us to conclude that increasing concentration or number of substances does
493 not always increase the toxicity of a mixture. This finding exemplifies that the toxicity of a

mixture is not merely the sum of the toxicity of the substances or the basic sum of the individual modes of actions.

4.3. Pesticides modulate feeding behavior through an increase in food consumption

Bees exposed to imidacloprid, difenoconazole and glyphosate, alone or in mixtures, consume more food than unexposed bees. Different hypotheses could explain this high consumption. (i) A higher food consumption could be triggered by energetic stress due to an increase in intermediary metabolism induced by the pesticides or the spoliation of energetic resources as has been shown for pyrethroids (Bounias et al., 1985). (ii) Honey bees could display a preference for sucrose solutions containing glyphosate and imidacloprid, as previously shown (Kessler et al., 2015; Liao et al., 2017). In contrast, a study has shown a decrease in food consumption after exposure to mixtures of the formulated products of imidacloprid with tetraconazole and of imidacloprid with glyphosate (Zhu et al., 2017a). This finding suggests that the decrease in food consumption could be attributed to adjuvants present in the formulated products that might have a repellent feeding effect. However, the effect on food consumption could also depend on the concentration of the pesticides to which honey bees are exposed. In our study, the presence of pesticides elicited a higher food consumption, whereas in the study conducted by Zhu et al. (2017b), at higher concentrations, the pesticides elicited a lower food consumption. Thus, active substances, adjuvants or both could induce concentration-dependent effects on food consumption depending on their affinities to the biological target.

The honey bees received a cumulative dose of imidacloprid equivalent to 1/60, 1/6 and 1/0.6 of the LD_{50} at 0.1, 1 and 10 µg/L, respectively. However, for glyphosate and difenoconazole, the cumulative quantity ingested was, at least, equivalent to $1/1.52x10^{6}$, $1/1.57x10^{5}$ and $1/1.65x10^{4}$ of the LD_{50} at 0.1, 1 and 10 µg/L. Despite cumulative exposure ratios of difenoconazole and glyphosate at least 10 000 times less than the LD_{50}, these two pesticides caused significant increases in mortality except for F0.1. Therefore, pesticides that are considered harmless to honey bees (high $LD_{50,}$ superior to 100 µg/bee) can become dangerous even at very low concentrations after long-term exposure. This highlights the importance of an in-depth revision of the current risk assessment schemes used in the pesticide registration procedure (Sgolastra et al., 2020).

4.4. Pesticides induce perturbations in the detoxification process, nervous system, defense against oxidative stress, metabolism and immunity

CaE-3, along with the other carboxylesterases, is involved in the metabolism of xenobiotics by catalyzing the hydrolysis of substrates containing amide, ester and thioester bonds. It is also involved in lipid metabolism (Badiou-Beneteau et al., 2012; Ross et al., 2010). In our study, head, midgut and abdomen CaE-3 were not significantly modulated by any type of exposure. However, the activity of this enzyme was reported to decrease after acute exposure to 2.56 ng bee^{-1} thiamethoxam (neonicotinoid) (Badiou-Beneteau et al., 2012) and at $LD_{50}/20$ of fipronil (Carvalho et al., 2013). Several studies have shown differential expression of carboxylesterases (CaEs) after exposure to pesticides (Badiou-Beneteau et al., 2012; Zhu et al., 2019; Zhu et al., 2017a; Zhu et al., 2017b). Thus, measuring only overall CaE activity with nonspecific substrates could mask the differential modulation of several isoforms, including CaE-3.

AChE is a neural enzyme hydrolyzing the neurotransmitter acetylcholine in cholinergic synapses (Badiou et al., 2007). AChE was found to be involved in learning and memory processes (Gauthier et al., 1992; Guez et al., 2010). Its activity was significantly increased for HF1 and IHF1 at day 10 and for IF, HF and IHF at 0.1 and 1 µg/L at day 20. Therefore, the increase in AChE activity is closely related to the duration of exposure and the concentrations of the pesticides forming the mixture. This reflects a delayed effect of the pesticide combinations on the nervous system and reveals the importance of studies on the effects of these pesticide combinations on the behavior and cognitive functions of honey bees.

Glyphosate increased AChE activity in the bees exposed to 0.1 µg/L. This finding contradicts the results showing that both newly emerged and adult honey bees exposed for up to 14 days during the summer period to glyphosate or its formulated product Roundup, at concentrations ranging from 2.5 to 10 ng/bee (Boily et al., 2013) and 35 mg/L, exhibit a decrease in AChE activity (Zhu et al., 2017a). The difference in the effect of glyphosate between our study and the previously cited studies could be attributed to seasonal variability. This hypothesis is supported by studies showing that the adverse effects of pesticides may be higher in summer bees than in winter bees. This higher sensitivity of summer bees has been shown in terms of the effects of imidacloprid on learning performance (Decourtye et al., 2003) and the synergistic effect of the pyrethroid insecticide deltamethrin and the azole fungicide prochloraz (Meled et al., 1998). These alterations in AChE activities might explain, at least in part, the impairment of cognitive behaviors, sucrose responsiveness and olfactory learning observed in

559 honey bees after exposure to glyphosate (Balbuena et al., 2015; Gonalons and Farina, 2018;
560 Herbert et al., 2014).

561 GST is a multifunctional enzyme involved in protection against oxidative stress and is a
562 phase II enzyme involved in the detoxification of xenobiotics. It can also contribute to phase I
563 detoxification by sequestering toxicants (Berenbaum and Johnson, 2015; du Rand et al.,
564 2015). GST activity was mainly decreased after exposure to pesticides in the head, abdomen
565 and midgut. This decrease could hypothetically be due either to inhibition of this enzyme or to
566 a downregulation by these pesticides. However, noncovalent inhibition could not be detected
567 because of the dilution of the tissue components during the step of tissue homogenization and
568 the assay procedure (at least 1/200-fold final dilution). In addition, a covalent inhibition of
569 GST by pesticides has never been reported, even with electrophilic pesticides such as
570 organophosphorus insecticides or herbicides that include glyphosate. Thus, the decrease in
571 GST activity, associated with the absence of inhibition, is consistent with GST
572 downregulation, which is also consistent with the 4-fold downregulation of GST S1, which is
573 responsible for fighting against oxidative stress, in the heads of honey bee larvae exposed to
574 imidacloprid (Wu et al., 2017). Furthermore, no phase II metabolites in imidacloprid
575 metabolism, including those that could be conjugated to glutathione, were found in the honey
576 bee (Suchail et al., 2004). This could be explained either by an absence of conjugation with
577 GST, by the production of GST conjugates at undetectable levels, or by drastic
578 downregulation of GST by imidacloprid. Thus, the decrease in GST activity may indicate a
579 decrease in the honey bee capacities to detoxify these pesticides and to fight against oxidative
580 stress that takes place after exposure to imidacloprid and glyphosate (Contardo-Jara et al.,
581 2009; Gauthier et al., 2018; Jasper et al., 2012; Lushchak et al., 2009).

582 G6PDH is the primary enzyme of the pentose phosphate pathway that generates NADPH and
583 is involved, among other things, in the regeneration of reduced glutathione, which contributes
584 to the fight against oxidative stress (Thomas et al., 1991). G6PDH activity decreased after 10
585 days of exposure to all modalities at 1 µg/L. However, it is improbable that this decrease is
586 due to oxidative stress. Indeed, in the presence of oxidative stress, glyceraldehyde-3-
587 phosphate dehydrogenase (GAPD) is inhibited (Chuang et al., 2005), which induces a
588 deviation of glycolysis towards the pentose phosphate pathway and an increase in G6PDH
589 activity (Nicholls et al., 2012; Renzi et al., 2016).

590 ALP is an enzyme of the digestive tract involved in adsorption and transport mechanisms
591 through the gut epithelium (Vlahović et al., 2009) and in immune response (Chen et al.,

2011). The activity of ALP was not modulated after 10 and 20 days of exposure. Thus, imidacloprid, glyphosate and difenoconazole did not affect the activity of ALP. This finding strongly contrasts with the results of other studies that showed a modulation of ALP in bees exposed to other pesticides, such as fipronil and spinosad, and following infection by Nosema (Carvalho et al., 2013; Dussaubat et al., 2012; Kairo et al., 2017). Thus, the apparent absence of ALP modulation in our study could reflect either an absence of effect or the occurrence of a compensatory phenomenon.

4.5. The effect of exposure to pesticides is systemic and tissue-specific

By comparing the dose effect of IH on CaE-3, it is possible to notice that for the same exposure duration, the effect of IH on CaE-3 at 0.1 and 1 µg/L differed among the biological compartments. For the modulations of CaE-3 at day 10, IH0.1 < IH1 in the head and IH0.1 > IH1 in the midgut and abdomen. For the modulations of CaE-3 at day 20, IH0.1 > IH1 in the gut and IH0.1 < IH1 in the abdomen. This complex profile of modulations was also found for both head and midgut GST after exposure to *Bt* spores and to *Nosema*-fipronil combination (Kairo et al., 2017; Renzi et al., 2016), thus confirming a spatially differential response due to the specificity of each tissue and to the occurrence of pesticide metabolism not only in the gut but also in other honey bee compartments (Suchail et al., 2004).

GST activity was modulated in the head, midgut and abdomen. In addition, AChE was modulated in the head, G6PDH in the abdomen and ALP in the midgut. These results indicate that the effects of the exposure to pesticides are not localized in the midgut (and in turn in the abdomen), which is considered the primary site of interaction with the ingested pesticide, but are spread across all biological compartments, leading to a systemic response that could explain the severe impact on honey bee survival.

The effects of the pesticides on physiological markers were determined in surviving bees after 10 and 20 days of daily exposure. The results at day 10 revealed a massive modulation of all physiological markers except CaE-3 and midgut GST. However, a less pronounced effect was detected at day 20 with a higher number of non-modulated enzymes (CaE-3, head GST, ALP and G6PDH were not modulated). This lower effect at day 20 suggests that the honey bee population at day 10 was composed of both sensitive and resistant individuals, while the population that survived until the twentieth day mainly contained honey bees that were more resistant to these pesticides alone or in combination. However, this hypothesis could be ruled out because the progression of mortality during this period was approximately linear,

indicating that the honey bees were sensitive to the pesticides and were unable to compensate for the increase in exposure duration.

5. Conclusion

This study demonstrates that chronic exposure to insecticides, herbicides and fungicides, alone or in combination, may induce high toxicity via systemic action in winter honey bees and constitutes a threat to these workers in two ways. The first is a direct drastic effect on survival, with a mortality that exceeded 50% after only 20 days of exposure, which can endanger the colony. The second involves a systemic action of these pesticides that alters honey bee physiology through metabolism, immunity, the nervous system, detoxification and antioxidant defenses. A severe loss of the winter bee population may compromise colony development during the spring, which might explain the high winter losses encountered in many regions. If such cocktail effects occurred in summer bees, this would have drastic impacts on colonies that could largely explain the bee population decline, especially because summer bees are more susceptible to pesticides and pesticide combinations than winter bees.

This study also reveals that the standard 10-day chronic toxicity test, used during pesticide risk assessment procedures, may not always be reliable in detecting the potential toxicities of pesticides. In addition, this study highlights the difficulty in predicting the cocktail effects of pollutants because the toxicity of the mixture is not always directly linked to the number of substances or the exposure level.

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