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Mixtures of an insecticide, a fungicide and a herbicide induce
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1 ABSTRACT

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

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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

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

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

74 Imidacloprid (insecticide), difenoconazole (fungicide) and glyphosate (herbicide) are among
75 the pesticides that are frequently detected in beehive matrices (Berg et al., 2018; Chauzat et
76 al., 2011; Mullin et al., 2010). Imidacloprid, together with its metabolite 6-chloronicotinic
77 acid, was the most abundant pesticide in beehive matrices in French apiaries, with a mean
78 concentration of 0.7 µg/kg in honey and 0.9 µg/kg in pollen (Chauzat et al., 2011). However,
79 concentrations of 0.14-0.275 µg/kg in honey, 1.35 µg/kg in pollen and 3-5.09 µg/kg in wax
80 comb were found in other studies (Lambert et al., 2013; Lopez et al., 2016; Nguyen et al.,
81 2009). Imidacloprid belongs to the neonicotinoid family and acts as an agonist of the nicotinic
82 acetylcholine receptors, leading to the disruption of the nervous system through impaired
83 cholinergic neurotransmission (Casida and Durkin, 2013). Glyphosate is the most dominant
84 herbicide worldwide. Its use has increased 15-fold since the introduction of genetically
85 engineered glyphosate-tolerant crops in 1996 (Benbrook, 2016), and it was detected in
86 beehive matrices at concentrations ranging between 17 to 342 µg/kg in honey and 52.4 to 58.4
87 µg/kg in beebread (Berg et al., 2018; El Agrebi et al., 2020; Rubio et al., 2015). It acts by
88 inhibiting the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS), an
89 enzyme necessary for the biosynthesis of aromatic amino acids in plants and some
90 microorganisms, which leads to cell death (Amrhein et al., 1980). Difenoconazole, a curative
91 and preventive fungicide of the triazole family, is authorized for use during full bloom. It has
92 been found at mean concentrations of 0.6 µg/kg in honey, 43 µg/kg in pollen, 270 µg/kg in
93 beebread and 1 µg/kg in wax comb (Kubik et al., 2000; Lopez et al., 2016). It belongs to the
94 ergosterol biosynthesis inhibitor (EBI) fungicides and acts by inhibiting the demethylation of
95 lanosterol (Zarn et al., 2003).

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

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

104

105 2. Materials and Methods

106 2.1. Reagents

107 Triton X-100, monosodium phosphate (NaH_2PO_4), sodium chloride (NaCl), pepstatin A,
108 leupeptin, aprotinin, trypsin, antipain, 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one
109 dibromide (BW284C51), 4-nitrophenyl acetate (*p*-NPA), ethanol, disodium phosphate
110 (Na_2HPO_4), monopotassium phosphate (KH_2PO_4), disodium ethylenediaminetetraacetate
111 dihydrate (EDTA), reduced L-glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB),
112 acetonitrile (CH_3CN), acetylthiocholine iodide (AcSCh), 5,5'-dithiobis(2-nitrobenzoic acid)
113 (DTNB), sodium bicarbonate (NaHCO_3), tris base, D-glucose-6-phosphate disodium salt
114 hydrate (G6P), magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$), β -nicotinamide adenine
115 dinucleotide phosphate hydrate (β -NADP⁺), 4-nitrophenyl phosphate bis(tris) salt (*p*-NPP),
116 sodium hydroxide (NaOH), dimethyl sulfoxide (DMSO) and hydrochloric acid (HCl) were
117 obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Imidacloprid (CAS No
118 138261-41-3), difenoconazole (CAS No 119446-68-3) and glyphosate (CAS No. 1071-83-6)
119 were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Protein solution (Bee
120 Food) was purchased from Remuau Ltd (Barbentane, France).

121

122 2.2. Honey bees

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

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

132

133 2.3. Chronic exposure to pesticides

134 The bees were exposed to the insecticide imidacloprid (I), the fungicide difenoconazole (F)
135 and the herbicide glyphosate (H) individually or in combination. Imidacloprid,
136 difenoconazole and glyphosate were prepared either alone or in binary mixtures (imidacloprid
137 + glyphosate (IH), imidacloprid + difenoconazole (IF), and glyphosate + difenoconazole
138 (HF)) or in a ternary mixture (imidacloprid + glyphosate + difenoconazole (IHF)) at
139 concentrations of 0.1, 1 and 10 µg/L for each substance (equivalent to 0.083, 0.813 and 8.130
140 µg/kg, calculated with a sucrose solution density of 1.23 ± 0.02 (n=10)) in a 60% (w/v)
141 sucrose solution containing a 0.1% (v/v) final concentration of DMSO. The treatments were
142 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:
143 I1, F1, H1, IH1, IF1, HF1 and IHF1; and 10 µg/L: I10, F10, H10, IH10, IF10, HF10 and
144 IHF10. **The primary mother solutions of the individual pesticides were prepared in 100%**
145 **DMSO. These primary solutions were used to generate the mother solutions of the individual**
146 **pesticides or were mixed to obtain the mother solutions of the pesticide mixtures. The**
147 **mother solutions of the pesticides were prepared by serial dilution of the primary mother**
148 **solutions to obtain 1% (v/v) DMSO and stored at -20°C. The sucrose solutions used for**
149 **exposure to pesticides were prepared daily by 10-fold dilution of the mother pesticide**
150 **solutions in sucrose solution to obtain final concentrations of 60% (m/v) sucrose, 1% (m/v)**
151 **proteins and 0.1% (v/v) DMSO. The pesticide concentrations were checked by GC-MS/MS**
152 **according to two analytical methods with RSD < 10% (Paradis et al., 2014; Wiest et al.,**
153 **2011). The control bees were fed a sucrose solution devoid of pesticides. For each modality of**
154 **exposure (including the controls), 14 cages of 30 bees were used.** Each day, the bee mortality
155 and food consumption were recorded, the dead bees were discarded, and the filter paper
156 placed at the bottom of the cage was replaced. For the analysis of the physiological markers,
157 the bees were sampled 10 and 20 days after the beginning of chronic exposure.

158

159 2.4. **Survival rate and food consumption**

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

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

165

166 2.5. Choice of physiological markers

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

177

178 2.6. Tissue preparation and marker extraction

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

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

197

198 2.7. Enzyme assays

199 CaE-3 was assayed in a medium containing the tissue extract, 10 μ M BW284C51
200 (acetylcholinesterase inhibitor), 0.1 mM *p*-NPA as the substrate and 100 mM sodium
201 phosphate pH 7.0. The reaction was monitored at 410 nm (Badiou-Beneteau et al., 2012;
202 Gomori, 1953; Renzi et al., 2016). GST was assayed at 340 nm by measuring the conjugation
203 of GSH to CDNB. The extract was incubated in a medium containing 1 mM EDTA, 2.5 mM
204 GSH as the cosubstrate, 1 mM CDNB as the substrate and 100 mM disodium phosphate pH
205 7.4 (Carvalho et al., 2013). AChE was assayed at 412 nm in a medium containing the tissue
206 extract, 1.5 mM DTNB, 0.3 mM AcSch as the substrate and 100 mM sodium phosphate pH
207 7.0 (Belzunces et al., 1988). G6PDH was measured by following the formation of NADPH at
208 340 nm in a medium containing the tissue extracts, 1 mM G6P as the substrate, 0.5 mM
209 NADP⁺ as the coenzyme, 10 mM MgCl₂ and 100 mM Tris-HCl pH 7.4 (Renzi et al., 2016).
210 ALP was assayed at 410 nm in a medium containing the tissue extract, 20 μ M MgCl₂, 2 mM
211 *p*-NPP as the substrate and 100 mM Tris-HCl pH 8.5 (Bounias et al., 1996). All reactions
212 started after adding the substrate, and the activity was assessed by determining the initial
213 velocity of the enzymatic kinetics, which corresponded to the slope of the tangent at the
214 origin. All enzymatic reactions were followed using a TECAN F500 spectrophotometer.

215

216 2.8. Mode of interaction between pesticides

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

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

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

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

237

238 2.9. Statistical analyses

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

249

250 3. Results

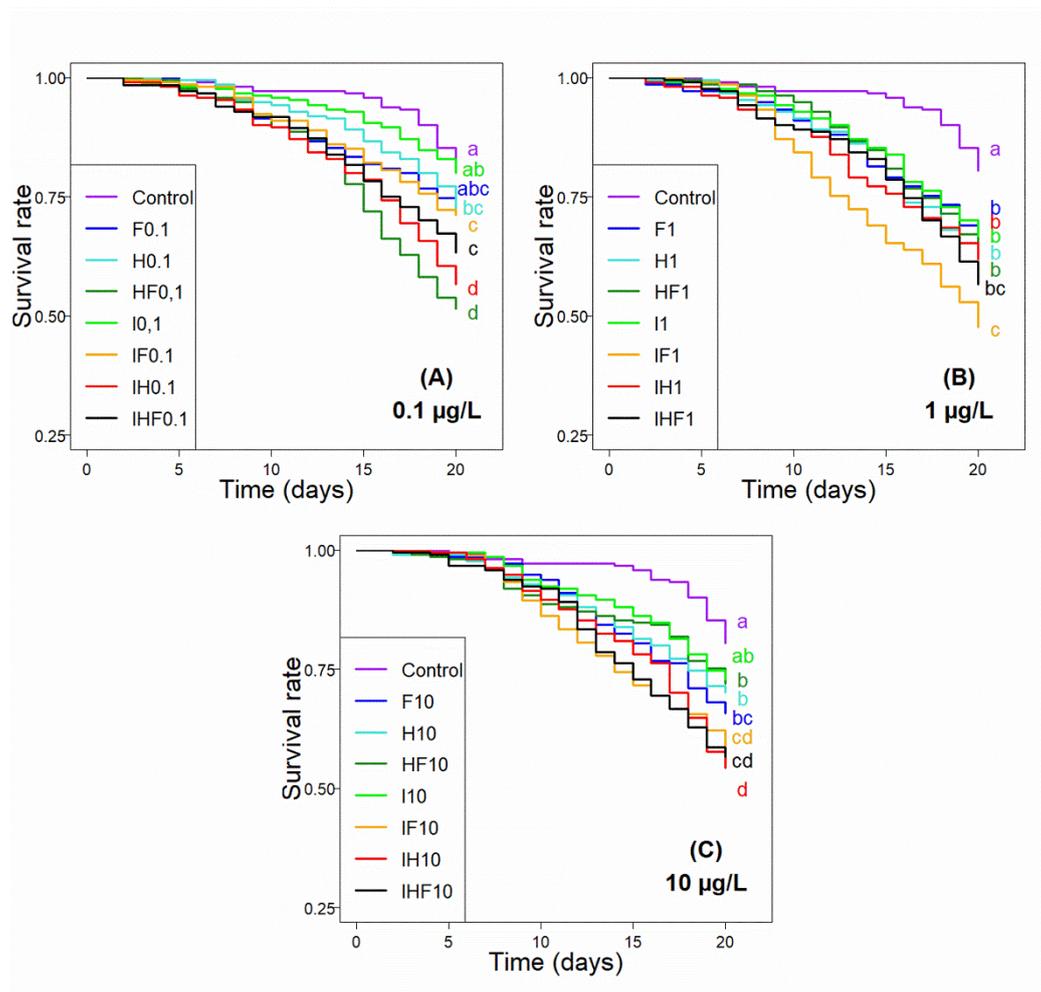
251 3.1. Honey bee survival

252 Exposure to pesticides significantly decreased the survival rate of honey bees at 20 days,
253 except for I0.1, I10 and F0.1, for which no significant difference from the control
254 ($20.0 \pm 2.7\%$) was observed ($p > 0.05$) (Fig. 1A, 1B, 1C and Table S1). Based on mortality
255 rates, the toxicities of pesticides could be ranked as follows: at $0.1 \mu\text{g/L}$, H = IF (28.1%) <

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

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

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270

271

[2-column fitting color image]

272 Fig. 1. Effects of pesticides alone or in combination on honey bee longevity

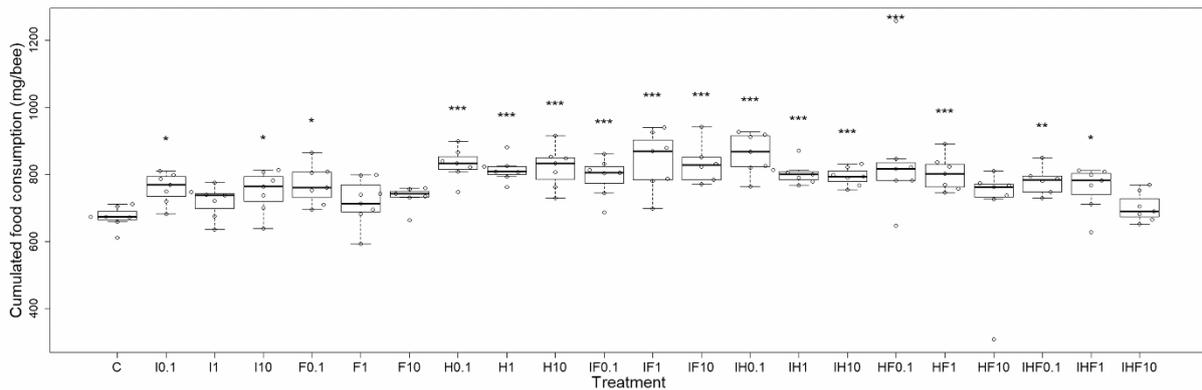
273 For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (Control), difenoconazole
274 (F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid (I), imidacloprid + difenoconazole (IF),
275 imidacloprid + glyphosate (IH) or imidacloprid + glyphosate + difenoconazole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

279

280 3.2. Effects of exposure to pesticides on food consumption behavior

281 Food consumption was monitored daily. In general, at the end of the exposure period, it
282 appeared that the food consumption was higher in the exposed bees (Fig. 2 and Table S2).
283 This higher consumption was significant for all exposure conditions except F1, I1, F10 and
284 I10 for pesticides alone, and HF10 and IHF10 for the mixtures. The five highest individual
285 cumulative consumption levels were ranked as follows: H0.1 (831.4 mg/bee) < IF10 (834.3
286 mg/bee) < IF1 (840.3 mg/bee) < HF0.1 (851 mg/bee) < IH0.1 (862.7 mg/bee) (control = 672.4
287 ± 33.0 mg/bee). At 0.1 µg/L, the bees exposed to imidacloprid alone or in IF, IH or IHF
288 exhibited a cumulative food consumption of 759.7, 792.6, 862.7 and 781.9 mg/bee,
289 respectively. Therefore, on the basis of a food density of 1.23 ± 0.02 (n = 10) and pesticide
290 concentrations, each honey bee ingested 62, 64, 70 and 63 pg of imidacloprid, which
291 corresponded to ca. 1/60, 1/58, 1/53 and 1/58 of the imidacloprid LD₅₀ (LD₅₀ = 3.7 ng/bee
292 (Schmuck et al., 2001)). At 1 µg/L, the bees exposed to imidacloprid alone or in IF, IH or IHF
293 exhibited a cumulative food consumption of 719.3, 840.3, 804.2 and 758.4 mg/bee,
294 respectively. Therefore, each honey bee ingested 584, 682, 653 and 615 pg of imidacloprid,
295 which corresponded to ca. 1/6, 1/5, 1/6 and 1/6 of the imidacloprid LD₅₀. At 10 µg/L, the bees
296 exposed to imidacloprid alone or in IF, IH and IHF exhibited a cumulative food consumption
297 of 749.3, 834.3, 794.1 and 702.5 mg/bee, respectively. Therefore, each honey bee ingested
298 6081, 6770, 6445 and 5701 pg of imidacloprid, respectively, which corresponded to ca. 1/0.6,
299 1/0.6, 1/0.6 and 1/0.7 of the imidacloprid LD₅₀. The LD₅₀ values of difenoconazole and
300 glyphosate are equal to or higher than 100 µg/bee (National Center for Biotechnology
301 Information). Therefore, for difenoconazole and glyphosate at 0.1, 1 and 10 µg/L, each honey
302 bee ingested $1/1.6 \times 10^6$, $1/1.7 \times 10^5$ and $1/1.8 \times 10^4$ of the LD₅₀, respectively (Table S2).

303



304

305

[2-column fitting image]

306

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

307

For 20 days, winter honey bees were fed sucrose solutions containing no pesticide (C, control), difenoconazole

308

(F), glyphosate (H), glyphosate + difenoconazole (HF), imidacloprid (I), imidacloprid + difenoconazole (IF),

309

imidacloprid + glyphosate (IH) or imidacloprid + glyphosate + difenoconazole (IHF), at 0.1 µg/L, 1 µg/L, and

310

10 µg/L. Food consumption was followed during the 20 days of exposure by measuring the food consumed daily

311

by the bees alive in each cage. Box plots represent the cumulated individual consumption (mg/bee) for 7 cages of

312

30 bees per treatment. Statistical analyses were performed using the Kruskal-Wallis test followed by pairwise

313

comparisons using the Wilcoxon rank sum test with the Benjamini-Hochberg correction. The numbers after the

314

abbreviations of each treatment refer to the concentrations of the pesticides in the sucrose solution. Asterisks

315

indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$).

316

317

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

318

The physiological status of the honey bees was examined by studying the modulation of

319

physiological markers **in different compartments to distinguish the local from the systemic**

320

effects of the pesticides (Table 1). The responses of the honey bee markers to the exposure to

321

the pesticides alone or in combination were analyzed after 10 and 20 days of chronic exposure

322

to concentrations of 0.1 µg/L and 1 µg/L (Fig. 3, Fig. 4, Table S3 and Table S4). **The lowest**

323

concentrations were chosen because they are particularly environmentally relevant. To render

324

the data comparable, the enzymatic activities are expressed as percentages of the control

325

values (Zhu et al., 2017a).

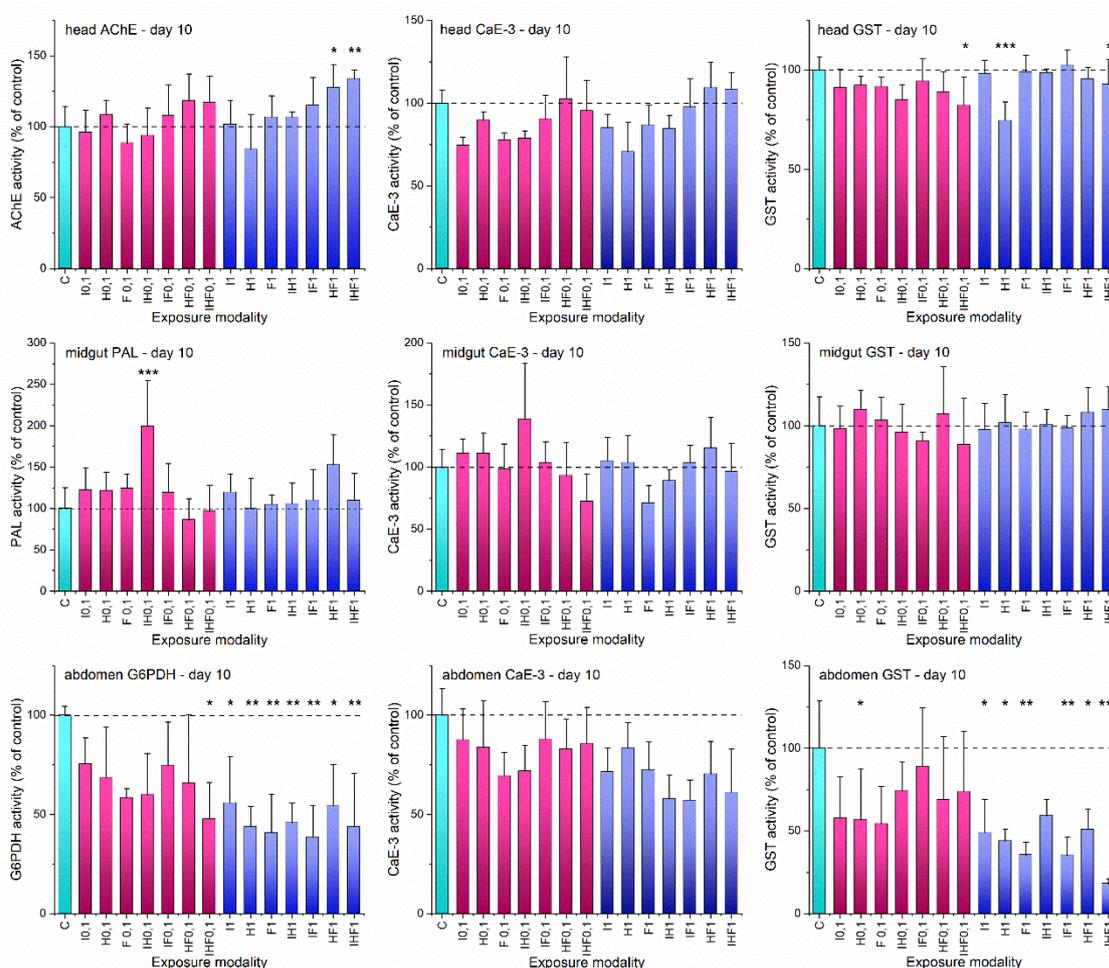
326

327 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

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

331



332

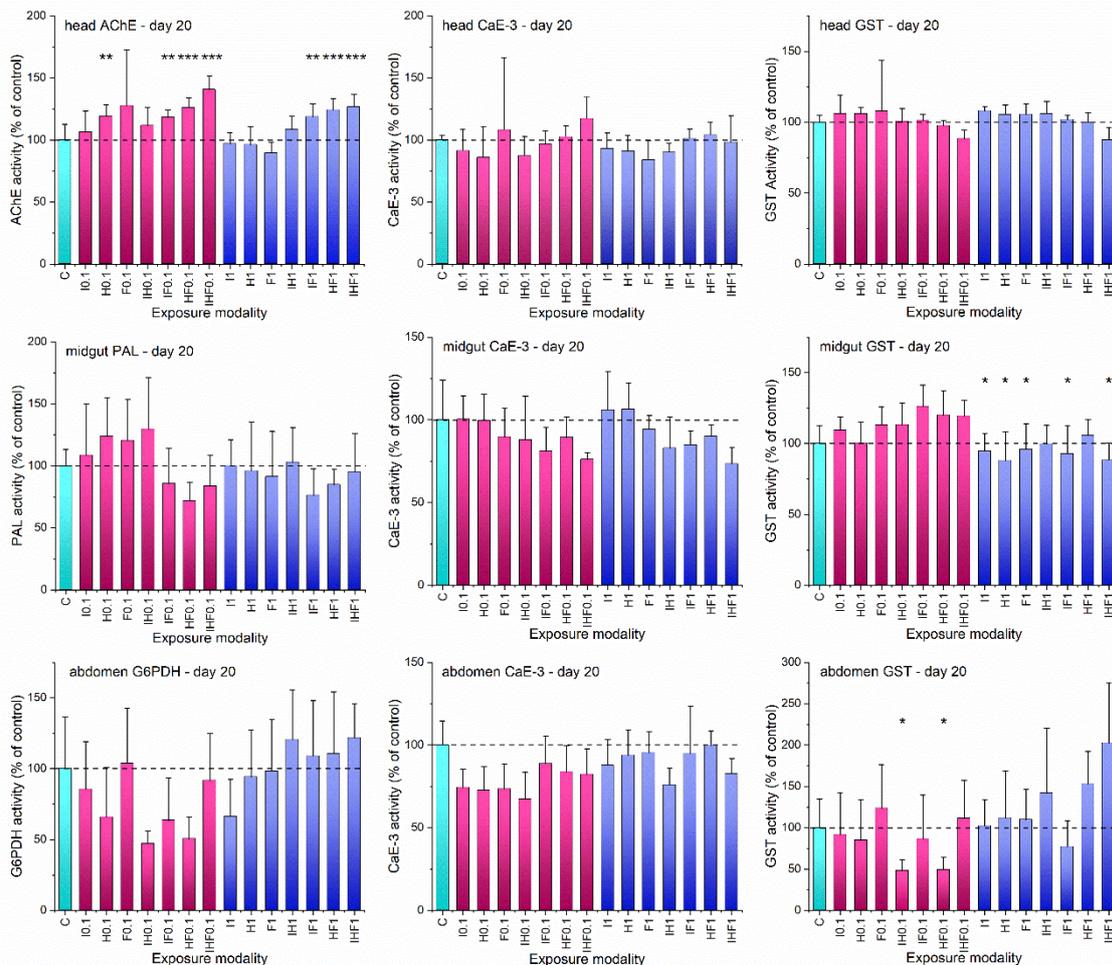
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[2-column fitting image]

334

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

341 (AChE in the head, G6PDH in the abdomen and ALP in the midgut). To make the data comparable, the
 342 enzymatic activities were expressed as percentages of the control values. Numbers after the abbreviation of each
 343 treatment refer to the concentration of the pesticide in the sucrose solution. The exposure modalities above and
 344 below the dashed horizontal line indicate increases and decreases in enzymatic activity, respectively, compared
 345 to the control (C). Asterisks indicate significant differences from the control group (* $p \leq 0.05$; ** $p \leq 0.01$;
 346 *** $p \leq 0.001$).
 347
 348



349

350

351

352 Fig. 4. Physiological impacts of pesticides alone or in combination in winter bees after 20 days of exposure
 353 For 20 days, winter honey bees were fed sucrose solutions containing no pesticides (C, control), imidacloprid (I),
 354 glyphosate (H), difenoconazole (F), imidacloprid + glyphosate (IH), imidacloprid + difenoconazole (IF),
 355 glyphosate + difenoconazole (HF), or imidacloprid + glyphosate + difenoconazole (IHF). The impact of the
 356 exposure to pesticides on the physiology of the surviving honey bees at day 20 was investigated through an
 357 analysis of 2 common markers in the head, abdomen and midgut (GST and CaE-3) and 3 specific markers
 358 (AChE in the head, G6PDH in the abdomen and ALP in the midgut). To make the data comparable, the
 359 enzymatic activities were expressed as percentages of the control values. Numbers after the abbreviation of each
 360 treatment refer to the concentration of the pesticide in the sucrose solution. The exposure modalities above and
 361 below the dashed horizontal line indicate increases and decreases in the enzymatic activity, respectively,
 362 compared to the control (C). Asterisks indicate significant differences from the control group (* $p \leq 0.05$;
 363 ** $p \leq 0.01$; *** $p \leq 0.001$).
 364

364

[2-columns fitting image]

365 At 0.1 µg/L, head, midgut and abdomen CaE-3 and midgut GST were not modulated by all
366 types of exposure at day 10 and day 20. Head AChE was not modulated at day 10. However,
367 at day 20, its activity was 119% of the control activity (127.5 ± 16.0 mUA.min⁻¹.mg of tissue⁻¹)
368 ¹) for H, 126% for HF and 141% for IHF. Head GST, abdomen G6PDH, and midgut ALP
369 underwent modulation at day 10. For IHF, these modulations corresponded to a decrease in
370 head GST (82% of control activity (115.3 ± 7.5 mUA.min⁻¹.mg of tissue⁻¹)) and a decrease in
371 abdomen G6PDH (48% of control activity (2.07 ± 0.53 mUA.min⁻¹.mg of tissue⁻¹)). For IH,
372 midgut ALP increased to 199% of the control activity (10.86 ± 2.75 mUA.min⁻¹.mg of
373 tissue⁻¹). Conversely, no modulation was observed at day 20 for any of these latter enzymes.
374 A decrease in abdomen GST was observed at 10 and 20 days. At 10 days, GST decreased to
375 57% of the control activity (116.1 ± 33.3 mUA.min⁻¹.mg of tissue⁻¹) for H. At day 20, GST
376 decreased to 48% of the control activity (83.0 ± 28.7 mUA.min⁻¹.mg of tissue⁻¹) for IH and
377 49% for HF.

378 At 1 µg/L, head, midgut and abdomen CaE-3 and midgut ALP were not modulated for all
379 types of exposure at day 10 and day 20. Head and abdomen GST underwent modulation at
380 day 10. Head GST decreased to 75% of the control activity (115.3 ± 7.5 mUA.min⁻¹.mg of
381 tissue⁻¹) for H and 93% for IHF. Abdomen GST decreased for all types of exposure except
382 IH: 49% of the control activity for I; 44% for H; 36% for F; 35% for IF; 51% for HF and 18%
383 for IHF (116.1 ± 33.3 mUA.min⁻¹.mg of tissue⁻¹ for the control). Conversely, head and
384 abdomen GST were not modulated at day 20. Abdomen G6PDH decreased at day 10 for all
385 types of exposure: 56% of the control activity for I; 44% for H; 41% for F; 46% for IH; 38%
386 for IF; 55% for HF and 44% for IHF (12.1 ± 0.5 mUA.min⁻¹.mg of tissue⁻¹ for the control).
387 However, no modulation was observed at day 20. Midgut GST was not modulated at day 10
388 but was modulated at day 20. Its activity decreased with all exposure types except IH and HF:
389 95% of the control activity for I; 88% for H; 96% for F; 93% for IF and 88% for IHF ($147.9 \pm$
390 18.8 mUA.min⁻¹.mg of tissue⁻¹ for the control). At day 10, head AChE increased to 128% of
391 the control activity (127.7 ± 18.5 mUA.min⁻¹.mg of tissue⁻¹) for HF and 134% of the control
392 activity for IHF. At day 20, the activity of AChE increased to 124% of the control ($127.5 \pm$
393 16.0 mUA.min⁻¹.mg of tissue⁻¹) for HF, 127% of the control for IHF and 119% of the control
394 for IF.

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

398 and head GST at day 10 (H0.1 > H1). F had the same effect on all markers at both
399 concentrations except for AChE at day 20 (F0.1 > F1). The effect of IH on CaE-3, ALP, and
400 abdomen GST was not similar at both concentrations. The effect of IH on head CaE-3 at day
401 10 and on abdomen CaE-3 and GST at day 20 was lower at 0.1 µg/L than at 1 µg/L.
402 Conversely, the effect of IH on midgut CaE-3 at days 10 and 20 and on abdomen CaE-3 and
403 midgut ALP at day 10 was higher at 0.1 µg/L than at 1 µg/L. The effect of IF on midgut GST
404 at day 20 was higher at 0.1 µg/L than at 1 µg/L. Depending on the concentration, the IF
405 mixture modulated abdomen GST at day 10 (IF0.1 > IF1) and abdomen G6PDH at day 10
406 (IF0.1 > IF1). The effect of HF was dose-dependent only on the activity of GST in the
407 abdomen at day 20 (HF0.1 < HF1). The effect of the ternary mixture IHF on abdomen GST at
408 day 10 and on midgut GST at day 20 was higher at 0.1 µg/L than at 1 µg/L (IHF0.1 > IHF1)
409 (Table S5).

410

411 4. Discussion

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

419

420 4.1. Pesticide combinations are more toxic to honeybees than individual pesticides

421 In this study, these three pesticides alone or in combination affected the survival of winter
422 honey bees at all tested exposure concentrations, except for I0.1, I10 and F0.1. Concerning
423 imidacloprid, the toxicity was less pronounced than that previously observed at the same
424 concentrations on summer bees, where 50% mortality was reached after 8 days of chronic
425 exposure at all concentrations (Suchail et al., 2001). In contrast, imidacloprid toxicity was
426 much more pronounced than that observed in young summer bees after 14 days of exposure at
427 1 µg/L (Gonalons and Farina, 2018). The differences in imidacloprid toxicity could be
428 attributed to seasonal variations (Decourtye et al., 2003; Meled et al., 1998; Piechowicz et al.,

429 2016), genetic differences (Smirle and Winston, 1987), the age of the bees or the exposure
430 duration.

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

439

440 4.2. Increased concentrations of pesticides are not always linked to increased toxicity

441 In terms of dose-effect relationships, in general, it appears that the highest concentration was
442 not the most dangerous, and the highest mortalities were observed at the intermediate
443 concentration of 1 µg/L. This bell-shaped non-monotonic dose response relationship (NMDR)
444 (high response at intermediate doses and lower responses at low and high doses) was
445 previously observed for imidacloprid and glyphosate (Boily et al., 2013; Suchail et al., 2001;
446 Vazquez et al., 2018). Three main hypotheses might explain this profile (Lagarde et al.,
447 2015). The first is the plurality of molecular targets, i.e., each xenobiotic has several
448 molecular targets of different affinities that may induce opposite effects across the range of
449 the tested concentrations. The second hypothesis is the metabolic hypothesis (Suchail et al.,
450 2001), which proposes that detoxification enzymes are induced at high but not at low
451 concentrations. This hypothesis is consistent with the action of glyphosate, whose main
452 metabolite, aminomethylphosphonic acid (AMPA), was shown to be nontoxic to honey bees
453 (Blot et al., 2019). However, the metabolic hypothesis is not consistent with the action of
454 imidacloprid because all metabolites were shown to be toxic to honey bees after chronic
455 exposure (Suchail et al., 2001). The third hypothesis is receptor desensitization, where at high
456 concentrations, numerous receptors are bound to xenobiotics, leading to a downregulation
457 phenomenon (Lagarde et al., 2015).

458 The mixture of EBI fungicides with imidacloprid or glyphosate was shown in different studies
459 to have no synergistic action (Iwasa et al., 2004; Thompson et al., 2014; Zhu et al., 2017b) or
460 to induce a synergistic effect (Biddinger et al., 2013). However, these studies were based on
461 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 $\mu\text{g/L}$, an independent effect at 1 $\mu\text{g/L}$ and an
480 antagonistic effect at 10 $\mu\text{g/L}$. IF induced a synergistic effect at 0.1 and 1 and an additive
481 effect at 10 $\mu\text{g/L}$. IH induced a synergistic effect at 0.1 and 10 $\mu\text{g/L}$ and a subadditive effect
482 at 1 $\mu\text{g/L}$. The ternary mixture induced a subadditive effect at 1 and 10 $\mu\text{g/L}$ and a synergistic
483 effect at 0.1 $\mu\text{g/L}$. **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 $\mu\text{g/L}$, 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**

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

496

497 4.3. Pesticides modulate feeding behavior through an increase in food consumption

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

515 The honey bees received a cumulative dose of imidacloprid equivalent to 1/60, 1/6 and 1/0.6
516 of the LD₅₀ at 0.1, 1 and 10 µg/L, respectively. However, for glyphosate and difenoconazole,
517 the cumulative quantity ingested was, at least, equivalent to 1/1.52x10⁶, 1/1.57x10⁵ and
518 1/1.65x10⁴ of the LD₅₀ at 0.1, 1 and 10 µg/L. Despite cumulative exposure ratios of
519 difenoconazole and glyphosate at least 10 000 times less than the LD₅₀, these two pesticides
520 caused significant increases in mortality except for F0.1. Therefore, pesticides that are
521 considered harmless to honey bees (high LD₅₀, superior to 100 µg/bee) can become dangerous
522 even at very low concentrations after long-term exposure. This highlights the importance of
523 an in-depth revision of the current risk assessment schemes used in the pesticide registration
524 procedure (Sgolastra et al., 2020).

525

526 4.4. Pesticides induce perturbations in the detoxification process, nervous system, defense
527 against oxidative stress, metabolism and immunity

528 CaE-3, along with the other carboxylesterases, is involved in the metabolism of xenobiotics
529 by catalyzing the hydrolysis of substrates containing amide, ester and thioester bonds. It is
530 also involved in lipid metabolism (Badiou-Beneteau et al., 2012; Ross et al., 2010). **In our**
531 **study**, head, midgut and abdomen CaE-3 were not **significantly** modulated by any type of
532 exposure. However, the activity of this enzyme was reported to decrease after acute exposure
533 to 2.56 ng bee⁻¹ thiamethoxam (neonicotinoid) (Badiou-Beneteau et al., 2012) and at LD₅₀/20
534 of fipronil (Carvalho et al., 2013). Several studies have shown differential expression of
535 carboxylesterases (CaEs) after exposure to pesticides (Badiou-Beneteau et al., 2012; Zhu et
536 al., 2019; Zhu et al., 2017a; Zhu et al., 2017b). Thus, measuring only overall CaE activity
537 with nonspecific substrates could mask the differential modulation of several isoforms,
538 including CaE-3.

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

547 **Glyphosate increased AChE activity in the bees exposed to 0.1 µg/L. This finding contradicts**
548 **the results showing that both** newly emerged and adult honey bees exposed for up to 14 days
549 during the summer period to glyphosate or its formulated product Roundup, at concentrations
550 ranging from 2.5 to 10 ng/bee (Boily et al., 2013) and 35 mg/L, exhibit a decrease in AChE
551 activity (Zhu et al., 2017a). The difference in the effect of glyphosate between our study and
552 the previously cited studies could be attributed to seasonal variability. This hypothesis is
553 supported by studies showing that the adverse effects **of pesticides may be higher in summer**
554 **bees than in winter bees. This higher sensitivity of summer bees has been shown in terms of**
555 **the effects of** imidacloprid on learning performance (Decourtye et al., 2003) **and the**
556 **synergistic effect of the pyrethroid insecticide deltamethrin and the azole fungicide prochloraz**
557 **(Meled et al., 1998)**. These alterations in AChE activities might explain, at least in part, the
558 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.,

592 2011). The activity of ALP was not modulated after 10 and 20 days of exposure. Thus,
593 imidacloprid, glyphosate and difenoconazole did not affect the activity of ALP. This finding
594 strongly contrasts with the results of other studies that showed a modulation of ALP in bees
595 exposed to other pesticides, such as fipronil and spinosad, and following infection by *Nosema*
596 (Carvalho et al., 2013; Dussaubat et al., 2012; Kairo et al., 2017). Thus, the apparent absence
597 of ALP modulation in our study could reflect either an absence of effect or the occurrence of a
598 compensatory phenomenon.

599

600 4.5. The effect of exposure to pesticides is systemic and tissue-specific

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

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

616 The effects of the pesticides on physiological markers were determined in surviving bees after
617 10 and 20 days of daily exposure. The results at day 10 revealed a massive modulation of all
618 physiological markers except CaE-3 and midgut GST. However, a less pronounced effect was
619 detected at day 20 with a higher number of non-modulated enzymes (CaE-3, head GST, ALP
620 and G6PDH were not modulated). This lower effect at day 20 suggests that the honey bee
621 population at day 10 was composed of both sensitive and resistant individuals, while the
622 population that survived until the twentieth day mainly contained honey bees that were more
623 resistant to these pesticides alone or in combination. However, **this** hypothesis could be ruled
624 out because the progression of mortality during this period was approximately linear,

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

627

628 5. Conclusion

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

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

645

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653

654

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