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Lethal effects of Cr(III) alone and in combination with propiconazole and clothianidin in honey bees

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1 **Lethal effects of Cr(III) alone and in combination with propiconazole and**  
2 **clothianidin in honey bees**

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15

16 **Abstract**

17 Several anthropogenic contaminants, including pesticides and heavy metals, can affect honey bee  
18 health. The effects of mixtures of heavy metals and pesticides are rarely studied in bees, even  
19 though bees are likely to be exposed to these contaminants in both agricultural and urban  
20 environments. In this study, the lethal toxicity of Cr alone and in combination with the  
21 neonicotinoid insecticide clothianidin and the ergosterol-biosynthesis-inhibiting fungicide  
22 propiconazole was assessed in *Apis mellifera* adults. The LD<sub>50</sub> and lowest benchmark dose of Cr as  
23 Cr(NO<sub>3</sub>)<sub>3</sub>, revealed a low acute oral toxicity on honey bee foragers (2049 and 379 mg L<sup>-1</sup>,  
24 respectively) and the Cr retention (i.e. bee ability to retain the heavy metal in the body) was

25 generally low compared to other metals. A modified method based on the binomial proportion test  
26 was developed to analyze synergistic and antagonistic interactions between the three tested  
27 contaminants. The combination of an ecologically-relevant field concentration of chromium with  
28 clothianidin and propiconazole did not increase bee mortality. On the contrary, the presence of Cr in  
29 mixture with propiconazole elicited a slight antagonistic effect.

30

### 31 **Highlights**

- 32 • Low acute oral toxicity of chromium on adults of honey bee foragers
- 33 • Chromium retention in bee body was 20-30% of the quantity ingested
- 34 • No synergistic effect between chromium and propiconazole or clothianidin
- 35 • Slight antagonism between chromium and propiconazole

36

37 **Key words:** heavy metals, pesticides, *Apis mellifera*, ecotoxicology, pollution,  
38 synergism/antagonism

39

## 1. Introduction

40  
41 Bees are extremely important as crop pollinators and to maintain plant biodiversity (Klein et al.,  
42 2007; Ollerton et al., 2011). In the last decades, wild and managed bees have been declining  
43 worldwide thus posing a potential risk to food production and human health (Lautenbach et al.,  
44 2012; Chaplin-Kramer et al., 2014). Abnormal honey bee mortality rates have been observed in US  
45 and in European Countries, with percentages of overwintering colony losses much higher than 10%  
46 rate that is usually considered an acceptable loss threshold value by beekeepers (Lee et al., 2015;  
47 Chauzat et al., 2016). Many factors have been taken into account to explain this phenomenon  
48 (Biesmeijer et al., 2006; Potts et al., 2010; Abbo et al., 2017; Fauser-Misslin et al., 2014; Dance et  
49 al., 2017). Pesticides, malnutrition, pathogens (including *Varroa* mite infestation), climate change,  
50 habitat fragmentation and some beekeeping management practices (e.g. migration activities for  
51 almond pollination in US) are the main factors that affect honey bee survival (Goulson et al., 2015).  
52 However, up to now, these stressors have often been studied individually and the potential synergic  
53 effects of other anthropogenic activities, like heavy metal pollution, have rarely been considered.

54 In fact, although the use of honey bees as environmental bioindicator of heavy metals have been  
55 studying since 1935 (Svoboda, 1961), the effects of these pollutants on bee health have often been  
56 overlooked and only recently they are considered in the framework of bee decline (Moroń et al.,  
57 2012; Exley et al., 2015).

58 In the present study, we addressed the lethal effects of chromium as Cr(III), alone and in  
59 combination with the neonicotinoid clothianidin and the ergosterol-biosynthesis-inhibitor (EBI)  
60 fungicide propiconazole on honey bees (*Apis mellifera ligustica* L.) following acute oral exposure  
61 under laboratory conditions. Chromium is a heavy metal ubiquitous in the environment often found  
62 as Cr (III) or (VI). The environmental diffusion of Cr has been increasing in the last years due to  
63 mining and industrial activities (Zayed and Terry, 2003). Although Cr(III) is commonly present in

64 animals, it becomes toxic at high concentrations (Di Bona et al., 2011). Since this metal may be  
65 accumulated in plant tissues (Oliveira, 2012), honey bees can be exposed to this contaminant by  
66 contact and ingestion. As a consequence, chromium can be found in honey (Conti and Botrè, 2001;  
67 Porrini et al., 2002; Satta et al., 2012). Honey bees are considered bioindicator of environmental Cr  
68 pollution since environmental levels detected in honey bee matrices (i.e. honey, bee body, beeswax)  
69 range from 0.005 to 46.52 mg kg<sup>-1</sup> depending on the matrix considered or on environmental colony  
70 location (i.e. rural, urban or industrial area) (Porrini et al., 2002; Satta et al., 2012).

71 LD<sub>50</sub> of heavy metals are rarely assessed in bee ecotoxicology (Hladun et al., 2013; Di et al. 2016;  
72 Heard et al., 2017; Robinson et al., 2017) and no value is available in literature for Cr as well as its  
73 benchmark dose (BMD) (*i.e.* the estimated lowest dose that produces an adverse response compared  
74 to the negative control).

75 Clothianidin and propiconazole pesticides are commonly applied to various crops such as oilseed  
76 rape, sunflower, fruit trees, maize and cereals (EFSA, 2013a; 2013b; Simon-Delso et al., 2015) and  
77 their residues are often found in honey bee matrices (Lambert et al., 2013; Mullin et al., 2010;  
78 Pistorius et al., 2015; Porrini et al., 2016). Therefore, the co-exposure of bees to these compounds  
79 under field conditions should be investigated.

80 Previous studies have already reported that clothianidin and propiconazole may interact in a  
81 synergistic way in honey bees following acute oral or contact exposure (Biddinger et al., 2013;  
82 Thompson et al., 2014; Sgolastra et al., 2017). However, no information on possible interactions  
83 among Cr and these two pesticides is available.

84 In this study, the LD<sub>50</sub> of Cr (expressed both in mg L<sup>-1</sup> sugar syrup and in µg bee<sup>-1</sup>) and its BMD  
85 (expressed in mg L<sup>-1</sup>) at 48 hours after ingestion were determined for the first time. In addition,  
86 possible lethal effects of environmental Cr concentrations in combinations with clothianidin and  
87 propiconazole (i.e., binary or ternary mixtures) were investigated and a new statistical method to

88 define synergistic/antagonistic interaction among them was developed *ad hoc*. Finally, Cr  
89 bioconcentration ratio in the bee body (i.e., bee Cr concentration/feeding solution Cr concentration),  
90 as a measure of honey bee capacity to retain the heavy metal, was estimated.

91

## 92 **2. Materials and methods**

### 93 *2.1 Bees and test conditions*

94 Forager honey bees (*Apis mellifera ligustica*) were obtained from three healthy colonies placed in  
95 an experimental apiary of CREA-AA (Bologna, Italy). During summer 2015, forager bees were  
96 collected using the “Funnel trap” (Medrzycki, 2013). The trap placed at the entrance of the hive  
97 allows collecting only forager bees, thus reducing the variability among bee categories (i.e., guard  
98 and other in-hive bees). After 30 min of anesthetization with 60% CO<sub>2</sub> in synthetic air, bees were  
99 placed in cardboard cages (9.5 cm x 6.5 cm x 5 cm) in groups of 10 (LD<sub>50</sub> and BMD estimations) or  
100 20 individuals (single pollutants, binary/ternary mixtures exposure experiment) per cage. Three  
101 cages per treatment were used. Bees from each colony were randomly distributed in group of 10 (or  
102 20) among treatments to account for genetic diversity (i.e. different colony origin). In addition, to  
103 exclude any potential colony effect, a rank-transformed repeated-measures ANOVA analyses  
104 (Zimmerman and Zumbo, 1993) was performed for each treatment, with colony as the between-  
105 subjects factor and time (4, 24, 48, 72 and 96 h) as the within-subjects factor. In all treatments, no  
106 differences among colonies were found (Tables S1 and S2 in the Supplementary Information).  
107 During the experiment, the cages were maintained at 25±2 °C and 50-70% of relative humidity in  
108 an incubator under complete darkness. The cages were daily rotated to reduce potential differences  
109 in the incubator microclimate.

110 All treatments were performed on bees after 1 h starvation period. Test solutions (*vide infra*) were  
111 provided using a bulk feeder. For each treatment, the volume provided per cage was defined

112 according to the assumption that, through trophallaxis, all individuals would ingest similar doses of  
113 10  $\mu\text{L}$  (OECD, 1998; Medrzycki et al., 2013). At the end of the exposure phase (maximum 2 h), the  
114 complete consumption of the solution was verified by visual inspection of the feeder. After that,  
115 bees were fed *ad libitum* with a sugarbeet (Eridania Italia SpA, Italy) syrup solution  
116 (sugarbeet:distilled water = 50:50 w/v) until the end of the experiment (96 h). Dead bees were  
117 preserved at  $-20\text{ }^{\circ}\text{C}$  until elemental analysis.

118

## 119 2.2 Chemicals

120  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  (MW  $400.15\text{ g mol}^{-1}$ ) and  $\text{Cr}_2(\text{SO}_4)_3$  (MW  $392.18\text{ g mol}^{-1}$ ) were purchased from  
121 Carlo Erba (Italy).

122 Propiconazole with 98.4% purity and clothianidin with 99% purity were purchased from Sigma-  
123 Aldrich (USA) and from Dr Ehrenstorfer GmbH (Germany), respectively. The main chemical  
124 characteristics of the two pesticides are reported in Table 1.

125

## 126 2.3 Estimation of Cr(III) $\text{LD}_{50}$ and BMD

127 Bees were exposed to different doses of  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  in a geometric series in order to calculate  
128 the dose-response curve and estimate the  $\text{LD}_{50}$  and BMD of Cr. As defined by a range-finding test,  
129 the following Cr concentrations in the sugar syrup solution (50% w/v) were chosen: 514, 1632,  
130 2167, 2667 and  $4605\text{ mg Cr L}^{-1}$ . Among treatments, the highest concentration ( $4605\text{ mg Cr L}^{-1}$ ) was  
131 excluded in the calculation of the dose-response curve because the solution was not completely  
132 consumed by bees at the end of the exposure phase, likely due to its repellent effect. The toxicity of  
133 Cr as  $\text{Cr}_2(\text{SO}_4)_3$  was also tested at the Cr concentrations of 302, 932, 1336, 1865, and  $2685\text{ mg L}^{-1}$   
134 to evaluate possible effect of the Cr counterion.

135 The Cr concentrations in the test solutions were determined by elemental analysis with an  
136 inductively coupled plasma optical emission spectrometer (*vide infra*).

137 Control cages were supplied with sugar syrup solution.

138

#### 139 2.4 Bee treatments with single component solutions, binary and ternary mixtures

140 A propiconazole solution at the concentration of 700 mg L<sup>-1</sup> was prepared by dissolving 700 mg of  
141 the fungicide in 15 mL of acetone (purity >99.0%, Sigma-Aldrich, USA) and then adding sugar  
142 syrup solution (50:50 w/v) up to 1 L of final volume. Aliquots of 10 µL of the solution containing 7  
143 µg of propiconazole were provided per-capita to the bees: the dose was chosen as a non-lethal dose  
144 as previously defined (Sgolastra et al., 2017). This dose corresponds at ~1/9 the oral LD<sub>50</sub> at 24 h  
145 for *Apis mellifera* (Ladurner et al., 2005).

146 A clothianidin solution at the concentration of 0.074 mg L<sup>-1</sup> was prepared by dissolving 0.074 mg  
147 of the insecticide in 15 mL of acetone and then adding the sugar syrup solution up to 1 L of final  
148 volume. Solution aliquots of 10 µL containing 0.74 ng of clothianidin were provided per-capita to  
149 the bees: the dose falls within the range of the LD<sub>10±95%</sub> confidence limit (CL) for clothianidin in  
150 *A. mellifera* as previously estimated (Sgolastra et al., 2017). This dose can be also considered  
151 ecologically relevant since it is within the range of the estimated amount of clothianidin ingested by  
152 a honey bee during a foraging bout (0.11-1.36 ng) (Sgolastra et al., 2017).

153 A sugar syrup solution (sugar:distilled water = 50:50 w/v), containing 1.5% of acetone and 3.9 mg  
154 Cr L<sup>-1</sup> as Cr(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O, was prepared for the evaluation of the effect of the environmental Cr  
155 concentration on bees. Solution aliquots of 10 µL containing 0.039 µg of Cr were provided per-  
156 capita to the bees. This concentration was chosen because it falls within the Cr concentrations found  
157 in honey bee matrices (Porrini et al., 2002; Satta et al., 2012) and thus it can be considered  
158 ecologically relevant.

159 Binary solutions were prepared by dissolving into 15 ml of acetone: i) 700 mg of propiconazole and  
160 0.074 mg of clothianidin; ii) 700 mg of propiconazole and 3.9 mg of Cr as Cr(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O; iii)  
161 0.074 mg of clothianidin and 3.9 mg of Cr as Cr(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O. All the organic solutions were then

162 diluted with sugar syrup solution up to 1 L of final volume. Aliquots of 10  $\mu\text{L}$  of each binary  
163 solution were provided per-capita to the bees.

164 A ternary solution was prepared by adding to 1 L of the binary solution of propiconazole and  
165 clothianidin, 3.9 mg of Cr(III) as  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ . Even in this case, aliquots of 10  $\mu\text{L}$  of the ternary  
166 solution were provided per-capita to the bees.

167 Acetone (15 mL) was diluted to 1 L with the sugar syrup solution as a control (solvent control). In  
168 addition, the syrup solution was also tested on bees as a negative control.

169

### 170 *2.5 Metal content analysis*

171 Metal concentrations in contaminated syrup solution and in honey bee body were measured after 48  
172 h from exposure phase by using an inductively coupled plasma optical emission spectrometer (ICP-  
173 OES) furnished by SPECTRO Analytical Instruments GmbH & Co. (Kleve, Germany) equipped  
174 with a plasma source and an optical detector with a charge-coupled device (CCD) able to quantify  
175 emission wavelengths of elements ranging between 125 and 780 nm. Test solutions were analyzed  
176 for Cr after addition of  $\text{HNO}_3$  ( $\geq 69\%$  v/v, for trace analysis, Sigma-Aldrich, USA).

177 Single honey bees (mean $\pm$ SE dry weight:  $22.75 \pm 0.47$  mg each) were analyzed for Cr content after  
178 dissolution in a mixture of  $\text{HNO}_3$  ( $\geq 69\%$  v/v, for trace analysis, Fluka, Sigma-Aldrich, USA) and  
179  $\text{H}_2\text{O}_2$  (30% v/v, for trace analysis, VWR Prolabo Chemicals, USA) in the ratio of 4:1 (v:v) by  
180 microwave-assisted digestion (*Start D*, Microwave Digestion System, Milestone, USA) before  
181 elemental analysis. The limit of detection (LOD) for Cr was  $0.38 \mu\text{g kg}^{-1}$  bee. For the statistical  
182 analysis, zero value was assigned to concentrations below the limit of detection (*vide infra*).

183 The Cr recovery from bee matrix exposed to digestion and then analysed by ICP-OES was  
184 determined as follows. After drying at  $100^\circ\text{C}$  for 24 h, five bees were singly spiked with 10  $\mu\text{L}$  of a  
185 Cr standard solution ( $1000 \text{ mg Cr L}^{-1}$ ) for ICP-OES calibration and additional five control bees  
186 were added with the same volume of distilled water. Once the added solutions were reduced by  
187 evaporation (within ca. 2 h), bees were singly mineralized and processed for Cr determination as

188 already described. Cr recovery on spiked bees resulted  $102\pm 1.6\%$  and Cr content of control bees  
189 was always below the LOD.

190

## 191 *2.6 Statistical analysis*

192 The number of dead bees was measured 4, 24, 48, 72 and 96 h after exposure to pollutants (see  
193 Figures S1 and S2 of Supplementary Information for mortality data vs time, corrected with Abbott's  
194 formula for Cr as  $\text{Cr}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$  or  $\text{Cr}_2(\text{SO}_4)_3$ ). Both the BMD intervals (BMDL-BMDU) and  
195  $\text{LD}_{50}$  values of Cr were estimated at 48 h after exposure phase.

196 The  $\text{LD}_{50}$ s were estimated with a Probit analysis (Finney, 1952) at 95% CL. The values expressed  
197 in  $\text{mg Cr L}^{-1}$  in the sugar syrup were then transformed in  $\mu\text{g bee}^{-1}$  assuming that each bee ingested  
198 10  $\mu\text{L}$  of test solution.

199 The Cr BMD intervals were estimated using PROAST version 62.5 (<http://www.proast.nl>). The  
200 BMD approach is considered as an alternative of the no-observed-adverse-effect level (NOAEL)  
201 approach, since it makes a more extended use of available dose-response data and provides a  
202 quantification of their uncertainties (EFSA, 2009). The approach considers the dose-response  
203 information by fitting several mathematical models to the data. Our dose-response data were  
204 analysed according to EFSA (EFSA, 2009, 2017). Briefly, the Bench Mark Response (BMR), also  
205 known as Critical Effect Size, was set at 10% as recommended for quantal data analysis. The BMD  
206 is the dose, derived from the estimated dose-response curve, associated with the BMR. The lower  
207 and upper bounds of the BMD, denoted BMDL and BMDU, correspond to the projection of the  
208 lower and upper 95% one sided confidence bound of BMR, respectively, to the dose axis. The  
209 BMD intervals for each fitted model were reported following the EFSA recommendations (EFSA,  
210 2017) so that the lowest BMDL and highest BMDU from these selected models were then used to  
211 define the final BMD confidence interval.

212 The quantity of Cr retained by single bees (expressed in  $\mu\text{g mg}^{-1}$  of dry body weight) and the metal  
213 bioconcentration ratio (MBR), i.e. the ratio between Cr ingested and Cr found in bee body, were  
214 evaluated with a regression analysis (see Section S3 and Figures S3 and S4 in Supplementary  
215 Information).

216 In the experiment where bees were exposed to pollutants as single compound or binary/ternary  
217 mixtures, Log-rank Kaplan-Meier (K-M) survival analyses with pairwise multi comparison  
218 procedures (Hom-Sidak method) were carried out to compare survival among treatments. Survival  
219 analyses were conducted with SigmaPlot 12.3.

220 For each assessment time (i.e. 4, 24, 48, 72 and 96 h after exposure to pollutants), the binomial  
221 proportion test described in Sgolastra et al. (2017) was used to estimate potential synergism on bee  
222 mortality between the different combinations of chromium and the two pesticides. In addition, the  
223 test was modified in order to assess antagonistic interactions. Since antagonism and synergism were  
224 tested on the same dataset and at five different times, we used a multiple comparison correction  
225 (Holm, 1979) to estimate significance levels for 10 p-values jointly. The null hypotheses that we  
226 were trying to test were:

$$H_0 \equiv p_{AB}^{obs} - p_{AB}^{exp} = p_{AB}^{obs} - (p_A + p_B - p_A \cdot p_B) > 0$$

227 when synergy was expected, and:

$$H_0 \equiv p_{AB}^{obs} - p_{AB}^{exp} = p_{AB}^{obs} - (p_A + p_B - p_A \cdot p_B) < 0$$

228 when antagonism was expected. According to Bliss independence criterion, the expected combined  
229 effect of two substances in an organism is expressed as follows:

$$p_{AB}^{exp} = p_A + p_B - p_A \cdot p_B$$

230 where  $p_A$  and  $p_B$  represent the mortality probability associated with the use of substances A and B,  
231 respectively, and  $p_{AB}^{exp}$  is the expected mortality of their combined effect (see the R script at section  
232 S4 in Supplementary Information).

233

### 234 **3. Results and discussion**

235 Although the co-exposure to heavy metals and pesticides can likely occur in agricultural and urban  
236 environment, their effects in combination have been rarely evaluated in bees (Jumarie et al., 2017).  
237 This study was aimed at assessing the lethal toxicity of Cr alone and in combination with two  
238 common pesticides: the neonicotinoid insecticide clothianidin and the EBI fungicide propiconazole  
239 under laboratory conditions. In general, results from laboratory studies are usually considered  
240 conservative in risk assessment (worst case scenario) since chemicals are better protected by  
241 environmental degradation (Cluzeau, 2002). In addition, data obtained in laboratory conditions are  
242 more reliable and comparable because of the adopted standard methods. However, several  
243 ecologically important effects (i.e. sublethal effects that can affect the whole colony) are difficult to  
244 detect under the same conditions.

245

#### 246 *3.1 Chromium LD<sub>50</sub> and BMD*

247 The Cr LD<sub>50</sub> and BMD intervals (BMDL and BMDU) estimated at 48 hours in the acute oral  
248 toxicity tests are reported in Table 2.

249 The values of LD<sub>50</sub> are expressed both as mg Cr L<sup>-1</sup> sugar syrup and µg Cr bee<sup>-1</sup>. For the LD<sub>50</sub>, the  
250 CL ranges obtained for Cr as Cr(NO<sub>3</sub>)<sub>3</sub> is well overlapped with the range values obtained for Cr as  
251 Cr(SO<sub>4</sub>)<sub>3</sub>, thus excluding possible lethal effects of Cr counterion. The calculated Cr LD<sub>50</sub> in *A.*  
252 *mellifera* adults equals to 2049 mg L<sup>-1</sup> (or 20.5 µg bee<sup>-1</sup>) which indicates slight toxicity based on the  
253 WSDA pesticide's classification (WSDA, 2010), especially when compared to other pollutants

254 (e.g.: Se LD<sub>50</sub>: 60 mg L<sup>-1</sup> (Hladun et al., 2013); Cu LD<sub>50</sub>: 72 mg L<sup>-1</sup> and Pb LD<sub>50</sub>: 345 mg L<sup>-1</sup> (Di et  
255 al., 2016); Cd LD<sub>50</sub>: 18.36 mg L<sup>-1</sup> and As LD<sub>50</sub>: 25.68 mg L<sup>-1</sup> (Heard et al., 2017)).

256 As far as the BMD is concerned, a detailed description of the BMD analysis according EFSA  
257 guideline (EFSA 2009; 2011) is reported in section S4 of Supplementary Information (Tables S6  
258 and S7). According to this analysis, the lowest BMD limit determined for Cr as Cr(NO<sub>3</sub>)<sub>3</sub> (BMDL:  
259 379 mg Cr L<sup>-1</sup>, Table 2) is one order of magnitude higher than the highest environmental  
260 concentrations found in honey bee matrices (46.52 mg Cr kg<sup>-1</sup>, Satta et al., 2012). According to our  
261 data, Cr at environmental concentrations poses a relatively low risk to honey bee adults by acute  
262 oral exposure.

263 The effects of Cr have also been addressed in other insect species however it is very difficult to  
264 compare their results to our findings due to the relevant differences in the methodologies adopted.  
265 For example, several studies focused on Cr exposure during larval stage (*Drosophila melanogaster*:  
266 Hepburn et al., 2003; *Bombyx mori*: Tucker et al. 2003; *Galleria mellonella*: Wu and Yi, 2015;  
267 *Hermetia illucens*: Gao et al. 2017), others tested Cr(VI) (*Culex quinquefasciatus*: Sorensen et al.  
268 2006; *Oxya chinensis*: Li et al. 2005) or assessed different endpoints (e.g. genotoxicity and  
269 reproduction in *D. melanogaster*: Hepburn et al., 2003). Finally, other studies dealt with aquatic  
270 insects with exposure via water environment (Warnik and Bell 1969; Rehwoldt et al. 1973).

271

### 272 3.2. Bioaccumulation of chromium in bee body

273 Figure 1 shows the Cr retained in bee body (a) and the MBR (b) as a function of Cr dissolved in the  
274 syrup ingested by the bees. No Cr residues were detected in control bees. Observational data in  
275 Figure 1a,b were fitted with statistical models (see section S3 in Supplementary Information) in  
276 order to model the dependence of Cr retained and MBR datasets on Cr dissolved in syrup.

277 The Cr-retained dataset showed a positive and very significant linear relationship with Cr in the  
278 feeding solution ( $p < 0.001$  for  $\alpha_{AI}$  coefficient and  $p = 0.0880$  for  $\beta_{AI}$ : Table S3 in Supplementary  
279 Information).

280 On the other hand, the MBR data showed a weak increasing trend with Cr in syrup. A non-linear  
281 curve constrained to pass through the origin of coordinates (see section S3.2 in Supplementary  
282 Information) showed a good agreement with the observed MBR points, although its coefficients  
283 were not statistically significant. Similar nonlinear MBR trends with the metal concentrations in the  
284 syrup have been reported for Al, Pb and Cd in honey bee body following chronic exposure  
285 (Gauthier et al., 2016). Remarkably, our data show that Cr accumulated in the bee body was 20-  
286 30% of Cr ingested (0.2–0.3 MBR values) within the tested concentration range (514-2667 mg Cr  
287 L<sup>-1</sup>).

288 In our study, the Cr retention in bee body after acute exposure was generally lower than the values  
289 observed after Al, Pb, Cd and Fe chronic exposure, thus suggesting bee higher ability to eliminate  
290 Cr compared to other heavy metals (Gauthier et al., 2016; Jumarie et al., 2017). Seemingly, the low  
291 toxicity of Cr in bee compared to other heavy metals (Hladun et al., 2013; Di et al., 2016; Heard et  
292 al., 2017; Robinson et al., 2017) might be related to bee ability to eliminate the heavy metal from  
293 the body.

294

### 295 *3.3. Experiment with the mixtures of chromium, clothianidin and propiconazole*

296 Cumulative proportion of surviving bees to Cr, propiconazole and clothianidin as single compounds  
297 and as binary and ternary mixtures are presented in Figure 2. Significant differences among  
298 cumulative survival curves of honey bees exposed to different treatments were found (Log-rank  
299 analysis  $\chi^2 = 87.6$ ,  $df = 8$ ,  $p < 0.001$ ). In order to better highlight differences among treatment effects on

300 bee mortality, pairwise analysis was performed on survival curves of Figure 2 and the p values are  
301 reported in Table 3.

302 In details, the clothianidin and propiconazole combination in the absence (CLO+PRO) or in the  
303 presence of Cr (CLO+PRO+Cr), as well as clothianidin and chromium mixture (CLO+Cr), gave the  
304 lowest bee survival after 96 hours from ingestion (Figure 2). As far as the bee survival within 4  
305 days observation is concerned, no significant differences were observed among the combined  
306 treatments (i.e., CLO+PRO, CLO+PRO+Cr, CLO+Cr); however, the survival rates were  
307 significantly lower than controls (Table 3). On the contrary, after 96 hours from ingestion, bee  
308 exposure to single pollutants (i.e., PRO, CLO and Cr) or to propiconazole and Cr combination  
309 (PRO+Cr) resulted in a more limited mortality if compared to the other treatments (Figure 2). As  
310 reported in Table 3, no significant differences ( $p>0.05$ ) were observed among survival curves of  
311 these treatments and the two controls (negative and solvent controls), thus confirming that our test  
312 doses were sublethal when administered alone.

313 In this study, the binomial proportion test developed for synergism (Sgolastra et al., 2017) was  
314 implemented to evaluate the antagonistic effect of the three pollutants in binary or ternary mixtures  
315 on bee mortality (Table 4). The script of this new procedure is provided as a Supporting data.  
316 Briefly, the implemented test is able to highlight both the synergistic or antagonistic effect size  
317 expressed as a positive or negative difference, respectively, between the observed and expected  
318 mortality probabilities for each pollutants combination at each assessment time. In Table 4, A or B  
319 terms refer to the effect size of single pollutants in binary or in ternary mixture. The lethal effect on  
320 bees of clothianidin and propiconazole combination (A and B terms, respectively, Table 4) was  
321 synergistic for the first 48 hours after ingestion as shown by the significantly ( $p<0.05$ ) positive  
322 values of effect size, in full agreement with previous results (Sgolastra et., 2017). The mechanism  
323 responsible for the synergism between the two pesticides is well known and it is related to the  
324 ability of propiconazole to inhibit the metabolization of clothianidin by cytochrome P450

325 monooxygenases (Berenbaum and Johnson, 2015). According to our data, a similar significant  
326 synergistic effect was also observed in the ternary mixture by considering PRO+Cr (A term) and  
327 CLO (B term) as well as CLO+Cr (A term) and PRO (B term), although within a shorter time  
328 period (4-24 h). Cr contribution to the synergistic effect observed in the ternary mixture with  
329 clothianidin and propiconazole was ruled out by considering the effect size of CLO+PRO (A term)  
330 and Cr (B term).

331 A significant ( $p < 0.05$ ) antagonistic effect in the chromium and propiconazole mixture was revealed  
332 at 72 and 96 hours after ingestion, according to the negative effect size values observed. In the  
333 literature, no information to explain the observed antagonistic effect is available.

334 To exclude any possible complexation of propiconazole by Cr(III) able to decrease the lethal effect  
335 of these stressors in honey bees, a UV study on syrup solution containing propiconazole and Cr as  
336 single compounds and their combination were performed both at the concentration adopted in the  
337 mixture as well as at one order of magnitude higher. The UV spectra (data not shown) did not  
338 reveal visible absorption differences, thus excluding any propiconazole-Cr complex formation.  
339 Likely, the antagonism between propiconazole and Cr may affect their main physiological  
340 detoxification processes in honey bees as bioavailability, uptake, internal transportation,  
341 metabolization, binding at the target site and excretion.

342

## 343 **5. Conclusions**

344 The calculated LD<sub>50</sub> of chromium as Cr(NO<sub>3</sub>)<sub>3</sub> in *A. mellifera* adults (2049 mg L<sup>-1</sup> syrup solution or  
345 20.5 µg bee<sup>-1</sup>) indicates low toxicity. Acute exposure to Cr at concentration higher than 379 mg L<sup>-1</sup>  
346 (BMDL) may cause lethal effects to honey bee foragers. However, these concentrations are 10-100  
347 times higher than the level usually found in honey bee matrices, thus confirming moderate Cr risks  
348 for honey bee foragers. In addition, honey bees showed higher ability to eliminate Cr (low Cr MBR)

349 compared to other heavy metals (Al, Pb, Cd and Fe). However, Cr effect on mortality of bee larvae  
350 or behavioural perturbation that might chronically affect colony could not be ruled out.

351 Chromium at environmental concentration ( $3.9 \text{ mg L}^{-1}$ ) ingested alone or in combination with  
352 sublethal doses of clothianidin and propiconazole did not significantly decrease the survival rate in  
353 bees. A modified binomial proportion test-based method was developed to analyse pairwise  
354 synergistic and antagonistic interactions between the three stressors for each assessment time.  
355 Significant synergistic effects were observed in bees in the first 48 hours after ingestion in the  
356 mixture clothianidin and propiconazole either in the presence or in the absence of chromium,  
357 whereas antagonistic effects were observed in the binary mixture of propiconazole and Cr at 72 and  
358 96 hours after ingestion.

359

## 360 **Competing interests**

361 We have no competing interests.

362

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368

## 369 **Reference**

370 Abbo, P.M., Kawasaki, J.K., Hamilton, M., Cook, S.C., DeGrandi-Hoffman, G., Li W.F., Liu, J.,  
371 Chen Y.P., 2017. Effects of Imidacloprid and *Varroa destructor* on survival and health of  
372 European honey bees, *Apis mellifera*. *Insect. Sci.* 24 (3), 467-477. doi: 10.1111/1744-  
373 7917.12335.

374 Berenbaum, M.R., Johnson R.M., 2015. Xenobiotic detoxification pathways in honey bees. *Curr.*  
375 *Opin. Insect. Sci.* 10, 51–58.

376 Biddinger, D.J., Robertson, J.L., Mullin, C., Frazier, J., Ashcraft, S., Rajotte, E.G., et al., 2013.  
377 Comparative toxicities and synergism of apple orchard pesticides to *Apis mellifera* (L.) and  
378 *Osmia cornifrons* (Radoszkowski). *PloS One* 8(9), e72587.  
379 <http://doi.org/10.1371/journal.pone.0072587>.

380 Biesmeijer, J.C., Roberts, S.P.M., Reemer, M., Ohlemüller, R., Edwards, M., Peeters, T., et al.,  
381 2006. Parallel Declines in Pollinators and Insect-Pollinated Plants in Britain and the  
382 Netherlands. *Science*, 313 (5785), 351–354. <http://doi.org/10.1126/science.1127863>.

383 Chaplin-Kramer, R., Dombek, E., Gerber, J., Knuth, K.A., Mueller, N.D., Mueller, M., et al.,  
384 2014. Global malnutrition overlaps with pollinator-dependent micronutrient production. *Proc.*  
385 *R. Soc. B* 281: 20141799. <http://dx.doi.org/10.1098/rspb.2014.1799>.

386 Chauzat, M.-P., Jacques, A., Laurent, M., Bougeard, S., Hendriks, P., & Ribière-Chabert, M., 2016.  
387 Risk indicators affecting honeybee colony survival in Europe: one year of surveillance.  
388 *Apidologie* 47, 348-378. <http://doi.org/10.1007/s13592-016-0440-z>.

389 Cluzeau, S., 2002. Risk assessment of plant protection products on honey bees. In: *Honey Bees:*  
390 *Estimating the Environmental Impact of Chemicals*. Edited by James Devillers and Minh-Hà  
391 Pham-Delègue. London and New York. Taylor and Francis.

392 Conti, M.E., Botrè, F., 2001. Honeybees and their products as potential bioindicators of heavy  
393 metals contamination. *Environ. Monit. Assess.* 69(3), 267–82.

394 Dance, C., Botías, C., Goulson, D., 2017. The combined effects of a monotonous diet and exposure  
395 to thiamethoxam on the performance of bumblebee micro-colonies. *Ecotoxicol. Environ.*  
396 *Safety* 139, 194-201. <https://doi.org/10.1016/j.ecoenv.2017.01.041>.

397 Di, N., Hladun, K.R., Zhang, K., Liu, T.X., Trumble, J.T., 2016. Laboratory bioassays on the  
398 impact of cadmium, copper and lead on the development and survival of honeybee (*Apis*  
399 *mellifera* L.) larvae and foragers. *Chemosphere* 152, 530-538.

400 Di Bona, K.R., Love, S., Rhodes, N.R., McAdory, D., Sinha, S.H., Kern, N., et al., 2011.  
401 Chromium is not an essential trace element for mammals: effects of a “low-chromium” diet. *J*  
402 *Biol. Inorg. Chem.* 16, 381–390. <http://doi.org/10.1007/s00775-010-0734-y>.

403 EFSA (European Food Safety Authority), 2009. Guidance of the Scientific Committee on a request  
404 from EFSA on the use of the benchmark dose approach in risk assessment. *EFSA J.* 7(6),  
405 1150. <http://doi.org/10.2903/j.efsa.2009.1150>.

406 EFSA (European Food Safety Authority), 2013a. Conclusion on the peer review of the pesticide  
407 risk assessment for bees for Clothianidin. *EFSA J.* 11(1), 3066.  
408 <http://doi.org/10.2903/j.efsa.2013.3066>.

409 EFSA (European Food Safety Authority), 2013b. Reasoned opinion on the review of the existing  
410 maximum residue levels (MRLs) for ethoxyquin according to Article 12 of Regulation ( EC ).  
411 *EFSA J.* 11(396), 3231. <http://doi.org/10.2903/j.efsa.2013.3231>.

412 EFSA (European Food Safety Authority), 2017. Update: Guidance on the use of the benchmark  
413 dose approach in risk assessment. *EFSA J.* 15(1), 4658. doi:10.2903/j.efsa.2017.4658.

414 Exley, C., Rotheray, E., Goulson, D., 2015. Bumblebee pupae contain high levels of aluminium.  
415 *PloS One* 10(6), e0127665. <http://doi.org/10.1371/journal.pone.0127665>.

416 Fauser-Misslin, A., Sadd, B. M., Neumann, P., Sandrock, C., 2014. Influence of combined pesticide

417 and parasite exposure on bumble bee colony traits in the laboratory. *J. Appl. Ecol.* 51, 450–  
418 459. <http://doi.org/10.1111/1365-2664.12188>.

419 Finney, D.J., 1952. *Probit Analysis*. 2<sup>nd</sup> edition. Cambridge University Press. 318 pp.

420 Gao, Q., Wang, X.Y., Wang, W.Q., Lei, C.L., Zhu, F., 2017. Influences of chromium and cadmium  
421 on the development of black soldier fly larvae. *Environ. Sci. Pollut. Res.* 24, 8637-8644.

422 Gauthier, M., Aras, P., Jumarie, C., Boily, M., 2016. Low dietary levels of Al, Pb and Cd may  
423 affect the non-enzymatic antioxidant capacity in caged honey bees (*Apis mellifera*).  
424 *Chemosphere* 144, 848–854. <http://doi.org/10.1016/j.chemosphere.2015.09.057>.

425 Goulson, D., Nicholls, E., Botías, C., Rotheray, E.L., 2015. Bee declines driven by combined stress  
426 from parasites, pesticides, and lack of flowers. *Science* 347, 1255957-1-1255957-9.  
427 <http://doi.org/10.1126/science.1255957>.

428 Heard, M. S., Baas, J., Dorne, J.-Lou, Lahive, E., Robinson, A.G., Rortais, A., et al., 2017.  
429 Comparative toxicity of pesticides and environmental contaminants in bees: Are honey bees a  
430 useful proxy for wild bee species? *Sci. Total. Environ.* 578, 357–365.  
431 <http://doi.org/10.1016/j.scitotenv.2016.10.180>.

432 Hepburn, D.D.D, Xiao, J., Bindom, S., Vincent, J.B., O'Donnell, J., 2003. Nutritional supplement  
433 chromium picolinate causes sterility and lethal mutations in *Drosophila melanogaster*. *PNAS*  
434 100, 3766–3771.

435 Hladun, K.R., Kaftanoglu, O., Parker, D.R., Tran, K.D., Trumble, J.T., 2013. Effects of selenium on  
436 development, survival, and accumulation in the honeybee (*Apis mellifera* L.). *Environ.*  
437 *Toxicol. Chem.* 32, 2584–2592. <http://doi.org/10.1002/etc.2357>.

438 Holm, S., 1979. A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* 6, 65–70.

- 439 Jumarie, C., Aras, P., Boily, M., 2017. Chemosphere Mixtures of herbicides and metals affect the  
440 redox system of honey bees. *Chemosphere* 168, 163–170.  
441 <http://doi.org/10.1016/j.chemosphere.2016.10.056>
- 442 Klein, A.-M., Vaissière, B.E., Cane, J.H., Steffan-Dewenter, I., Cunningham, S.A., Kremen, C.,  
443 Tschamntke, T., 2007. Importance of pollinators in changing landscapes for world crops. *Proc.*  
444 *R. Soc. B* 274, 303-313. <http://doi.org/10.1098/rspb.2006.3721>.
- 445 Ladurner, E., Bosch, J., Kemp, W.P., Maini, S., 2005. Assessing delayed and acute toxicity of five  
446 formulated fungicides to *Osmia lignaria* Say and *Apis mellifera*. *Apidologie* 36, 449–460.
- 447 Lambert, O., Piroux, M., Puyo, S., Thorin, C., L’Hostis, M., Wiest, L., et al., 2013. Widespread  
448 Occurrence of Chemical Residues in Beehive Matrices from Apiaries Located in Different  
449 Landscapes of Western France. *PLoS One* 8(6), 1–12.  
450 <http://doi.org/10.1371/journal.pone.0067007>.
- 451 Lautenbach, S., Seppelt, R., Liebscher, J., Dormann, C.F., 2012. Spatial and temporal trends of  
452 global pollination benefit. *PloS One*, 7(4), e35954.  
453 <http://doi.org/10.1371/journal.pone.0035954>.
- 454 Lee, K.V., Steinhauer, N., Rennich, K., Wilson, M.E., Tarpy, D.R., Caron, D.M., et al., 2015. A  
455 national survey of managed honey bee 2013–2014 annual colony losses in the USA.  
456 *Apidologie* 46, 292–305. <http://doi.org/10.1007/s13592-015-0356-z>.
- 457 Li, L.J., Zhang, F., Liu, X.M., Guo, Y.P., Ma, E.B., 2005. Oxidative stress related enzymes in  
458 response to chromium(VI) toxicity in *Oxya chinensis* (Orthoptera: Acridoidae). *J. Environ. Sci.*  
459 17, 823-826.
- 460 Medrzycki, P., 2013. Funnel trap – a tool for selective collection of exiting forager bees for tests. *J.*  
461 *Apic. Res.* 52, 122–123. <http://doi.org/10.3896/IBRA.1.52.3.02>.

- 462 Medrzycki, P., Giffard, H., Aupinel, P., Belzunces, L.P., Chauzat, M.-P., Claßen, C., et al., 2013.  
463 Standard methods for toxicology research in *Apis mellifera*. J. Apic. Res. 52, 1–60.  
464 <http://doi.org/10.3896/IBRA.1.52.4.14>.
- 465 Moroń, D., Grześ, I.M., Skórka, P., Szentgyörgyi, H., Laskowski, R., Potts, S.G., Woyciechowski,  
466 M., 2012. Abundance and diversity of wild bees along gradients of heavy metal pollution. J.  
467 Appl. Ecol. 49(1), 118–125. <http://doi.org/10.1111/j.1365-2664.2011.02079.x>.
- 468 Mullin, C.A, Frazier, M., Frazier, J.L., Ashcraft, S., Simonds, R., Vanengelsdorp, D., Pettis, J.S.,  
469 2010. High levels of miticides and agrochemicals in North American apiaries: implications for  
470 honey bee health. PloS One 5(3), e9754. <http://doi.org/10.1371/journal.pone.0009754>.
- 471 OECD, 1998. Guideline for testing of chemicals. Test No. 213: Honey bees, acute oral toxicity test.  
472 OECD, Paris, France (1998).
- 473 Oliveira, H., 2012. Chromium as an environmental pollutant: Insights on induced plant toxicity.  
474 Journal of Botany, Article ID 375843, 1-8. <http://doi.org/10.1155/2012/375843>.
- 475 Ollerton, J., Winfree, R., Tarrant, S., 2011. How many flowering plants are pollinated by animals?  
476 Oikos 120(3), 321–326. <http://doi.org/10.1111/j.1600-0706.2010.18644.x>.
- 477 Pistorius J., Wehner A., Kriszan M., Bargaen H., Knäbe S., Klein O., Frommberger M., Stähler M.,  
478 Heimbach U., 2015. Application of predefined doses of neonicotinoid containing dusts in field  
479 trials and acute effects on honey bees. B Insectology 68, 161-172.
- 480 Porrini, C., Ghini, S., Girotti, S., Sabatini, A.G., Gattavecchia, E., Celli, G., 2002. Use of honey  
481 bees as bioindicators of environmental pollution in Italy. In: Honey Bees: Estimating the  
482 Environmental Impact of Chemicals. Edited by James Devillers and Minh-Hà Pham-Delègue.  
483 London and New York. Taylor and Francis.
- 484 Porrini, C., Mutinelli, F., Bortolotti, L., Granato, A., Laurenson, L. et al., 2016. The Status of

485 Honey Bee Health in Italy: Results from the Nationwide Bee Monitoring Network. PLoS One,  
486 11(5): e0155411. <http://doi.org/10.1371/journal.pone.0155411>.

487 Potts, S., Roberts, S., Dean, R., Marris, G., Brown, M., Jones, R., et al., 2010. Declines of managed  
488 honey bees and beekeepers in Europe. *J. Apic. Res.* 49, 15-22. DOI: 10.3896/IBRA.1.49.1.02.

489 Rehwoldt, R., Lasko, L., Shaw, C., Wirhowski, E., 1973. The acute toxicity of some heavy metal  
490 ions toward benthic organisms. *Bull. Environ. Contam. Toxicol.* 10, 291-294.

491 Robinson, A., Hesketh, H., Lahive, E., Horton, A.A., Svendsen, C., Rortais, A., et al., 2017.  
492 Comparing bee species responses to chemical mixtures: Common response patterns? PLoS  
493 One 12(6): e0176289. <https://doi.org/10.1371/journal.pone.0176289>.

494 Satta, A., Verdinelli, M., Ruiu, L., Buffa, F., Salis, S., Sassu, A., Floris, I., 2012. Combination of  
495 beehive matrices analysis and ant biodiversity to study heavy metal pollution impact in a post-  
496 mining area (Sardinia, Italy). *Environ. Sci. Pollut. Res.* 19(9), 3977–3988.  
497 <http://doi.org/10.1007/s11356-012-0921-1>

498 Sgolastra, F., Medrzycki, P., Bortolotti, L., Renzi, T., Tosi, S., Bogo, G., et al. 2017. Synergistic  
499 mortality between a neonicotinoid insecticide and an ergosterol-biosynthesis-inhibiting  
500 fungicide in three bee species. *Pest. Manag. Sci.* 73, 1236–1243.  
501 <http://doi.org/10.1002/ps.4449>

502 Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs, C.,  
503 Wiemers, M., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of  
504 action and metabolites. *Environ. Sci. Pollut. Res.* 22, 5–34. [http://doi.org/10.1007/s11356-](http://doi.org/10.1007/s11356-014-3470-y)  
505 [014-3470-y](http://doi.org/10.1007/s11356-014-3470-y).

506 Sorensen, M.A., Jensen, P.D., Walton, W.E., Trumble, J.T., 2006. Acute and chronic activity of  
507 perchlorate and hexavalent chromium contamination on the survival and development of *Culex*

508 *quinquefasciatus* Say (Diptera: Culicidae). Environ. Pollut. 144, 759-764.

509 Svoboda, J., 1961. Prumyslové otravy vcel arsenem (Industrial poisoning of bees by arsenic). Ved.  
510 Pr. Vyzk. Ustavu Vcelarskeho CSAZV 2, 55–60.

511 Thompson, H.M., Fryday, S.L., Harkin, S., Milner, S., 2014. Potential impacts of synergism in  
512 honeybees (*Apis mellifera*) of exposure to neonicotinoids and sprayed fungicides in crops.  
513 Apidologie 45, 545–553. <http://doi.org/10.1007/s13592-014-0273-6>.

514 Tomlin, C.D.S., 2003. ed. The e-Pesticide Manual: a World Compendium. 13th ed. Surrey, UK:  
515 British Crop Protection Council. Version 3.0.

516 Tucker, F.B., Wang, K., Lu, S., Xu, L., 2003. The influence of form and quantity of chromium on  
517 the development and survival of two silkworm (*Bombyx mori* L.) races. J. Environ. Sci. 15,  
518 744-748.

519 Warnick S.L., Bell H.L., 1969. The Acute Toxicity of Some Heavy Metals to Different Species of  
520 Aquatic Insects. J. Water Pollut. Control. Fed. 41, 280-284.

521 WSDA (Washington State Department of Agriculture), 2010. Pollinator protection requirements for  
522 Section 18 Emergency Exemptions and Section 24(c) special local need registration in  
523 Washington State. (AGR PUB 631–225.) 9 pp. Olympia, WA: Registration Services Program,  
524 Pesticide Management Division, Washington State Department of Agriculture.

525 Wu, G., Yi, Y., 2015. Effects of dietary heavy metals on the immune and antioxidant systems of  
526 *Galleria mellonella* larvae. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 167, 131-139.

527 Zayed, A.M., Terry, N., 2003. Chromium in the environment: Factors affecting biological  
528 remediation. Plant and Soil 249, 139–156. <http://doi.org/10.1023/A:1022504826342>.

529 Zimmerman, D.W., Zumbo, B.D., 1993. Relative power of the Wilcoxon test, the Friedman test,

530 and repeated measures ANOVA on ranks. *J. Exp. Educ.* 62, 75-86.

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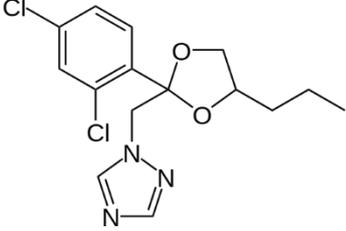
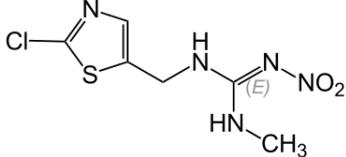
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549 **Table 1.** Main chemical characteristics of agrochemicals under investigation.

Chemical structure	Abbreviation	Molecular weight (g mol <sup>-1</sup> )	p <i>K</i> <sub>a</sub>
	PRO	342.22	1.09*
	CLO	249.67	11

550 \* p*K*<sub>a</sub> of the conjugate acid (Tomlin, 2003)

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552 **Table 2.** Lowest and highest benchmark doses\* (BMDL and BMDU, respectively) and lethal  
 553 dose\*\* (LD<sub>50</sub>) of Cr following acute oral exposure to Cr(NO<sub>3</sub>)<sub>3</sub> or Cr<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub> in *Apis mellifera* at 48  
 554 h after ingestion. In brackets, the 95% CLs for LD<sub>50</sub> values.

Compound	BMDL-BMDU mg Cr L <sup>-1</sup>	LD <sub>50</sub> (±95% CLs)			
		$\chi^2$	p	mg Cr L <sup>-1</sup>	µg Cr bee <sup>-1</sup>
<i>Cr(NO<sub>3</sub>)<sub>3</sub>·9H<sub>2</sub>O</i>	379-1670	0.341	>0.05	2049 (1674-2508)	20.5 (16.7-25.1)
<i>Cr<sub>2</sub>(SO<sub>4</sub>)<sub>3</sub></i>	43-1250	0.270	>0.05	3458 (1917-6237)	34.6 (19.2-62.4)

555 \*Obtained with PROAST version 62.5; \*\*Obtained with Probit analysis

556

557 **Table 3.** Pairwise p comparison results obtained with Holm-Sidak multicomparison test based on  
 558 Log-rank Kaplan-Meier survival analyses. Significantly different comparison with  $p < 0.05$  (PRO:  
 559 propiconazole; CLO: clothianidin; Negative control: sugar syrup solution; Solvent control: sugar  
 560 syrup solution with 1.5% acetone).

<b>Pairwise p comparison</b>	<b>Negative control</b>	<b>Solvent control</b>	<b>Cr</b>	<b>CLO</b>	<b>PRO</b>	<b>CLO+PRO</b>	<b>PRO+Cr</b>	<b>CLO+Cr</b>
<b>Solvent control</b>	0.925	-	-	-	-	-	-	-
<b>Cr</b>	0.439	0.923	-	-	-	-	-	-
<b>CLO</b>	0.161	0.843	0.952	-	-	-	-	-
<b>PRO</b>	0.91	0.857	0.954	0.899	-	-	-	-
<b>CLO+PRO</b>	<0.001	<0.001	<0.001	0.002	<0.001	-	-	-
<b>PRO+Cr</b>	0.927	0.941	0.906	0.67	0.947	<0.001	-	-
<b>CLO+Cr</b>	0.001	0.044	0.425	0.857	0.069	0.183	0.022	-
<b>CLO+PRO+Cr</b>	<0.001	0.002	0.035	0.18	0.004	0.942	0.001	0.923

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563 **Table 4.** Effect size for binary (PRO+CLO; PRO+Cr, CLO+Cr) and ternary (PRO+CLO+Cr)  
 564 mixtures at each assessment time (4, 24, 48, 72, and 96 h). A or B terms refer to the effect size of  
 565 single pollutants in binary or in ternary mixture. A positive or negative difference indicates  
 566 synergistic or antagonistic effect. Significance levels (Holm-corrected for multiple comparisons) for  
 567 differences are shown within parentheses, i.e. (\*):  $p < 0.05$ ; (\*\*):  $p < 0.01$ ; (\*\*\*):  $p < 0.001$ .

A	B	4 h	24 h	48 h	72 h	96 h
CLO	PRO	0.1900(**)	0.3650(***)	0.3181(**)	0.1322	0.0978
Cr	PRO	0.0167	0.0003	-0.1069	-0.2811(*)	-0.3244(*)
CLO	Cr	0.0342	0.0850	-0.0247	-0.1197	-0.0978
CLO+PRO	Cr	0.0973	-0.0553	-0.1467	-0.2193	-0.2307
PRO+Cr	CLO	0.2683(***)	0.3033(***)	0.2181(*)	0.0356	-0.0022
CLO+Cr	PRO	0.2500(**)	0.2200 (*)	0.1569	-0.0500	-0.1133

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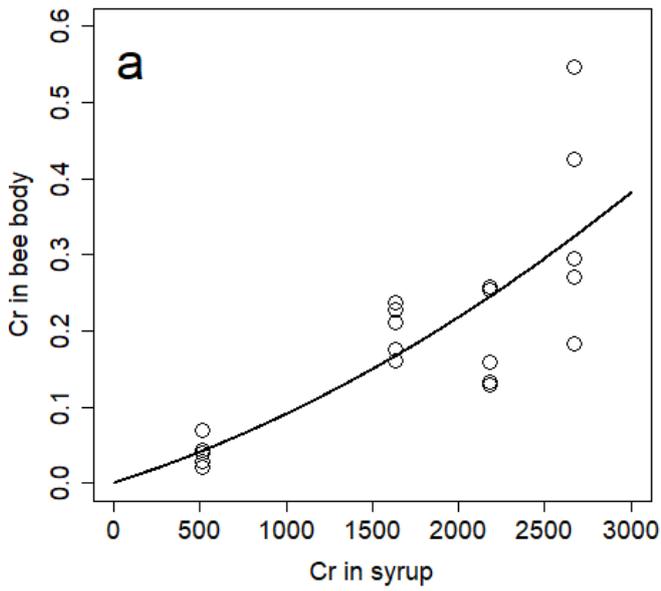
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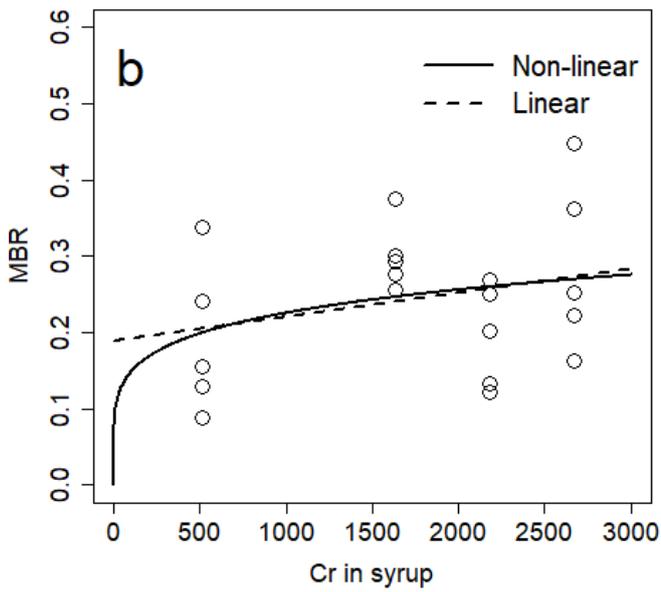
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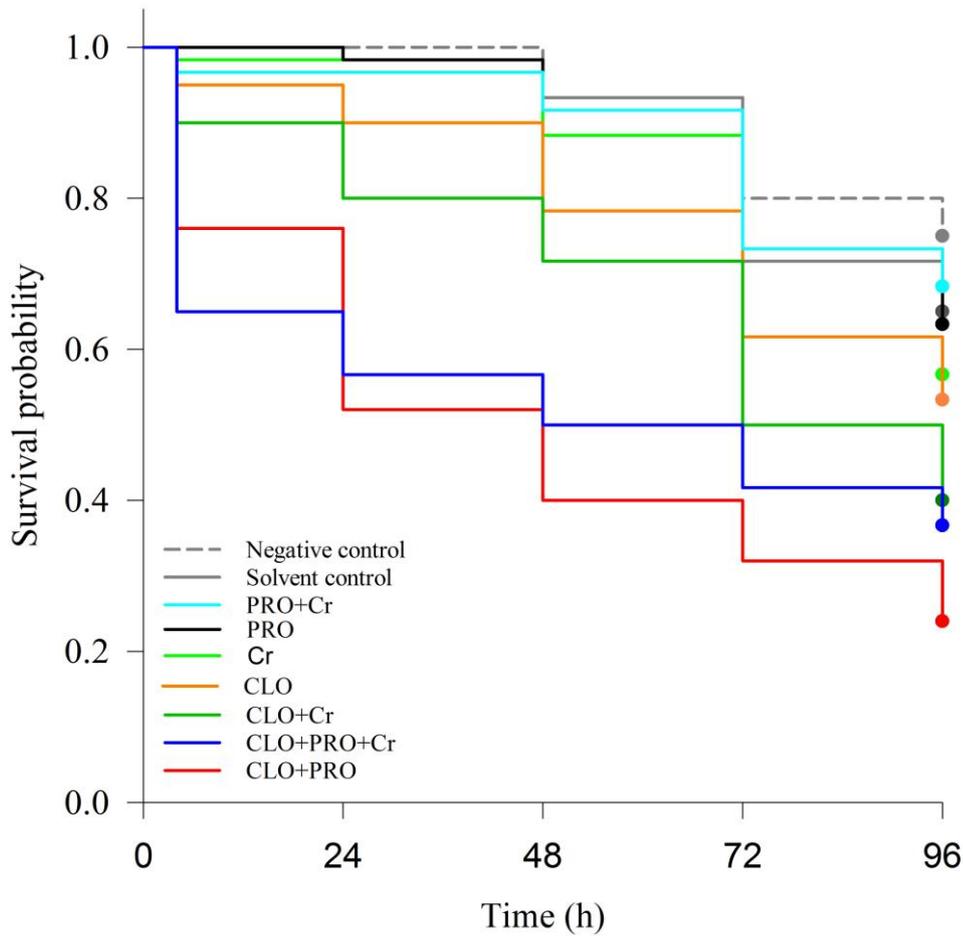
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581 Figure 1. Results of regression analysis to the a) Cr-retained and b) MBR observations.  
 582 Observational data points are shown as empty dots. Figures also show a) parabola (solid line) and b)  
 583 nonlinear (solid line) and linear (dashed line) curves fitted to the data. Analytic expressions for each

584 curve can be found in the Supplementary data. The parabola in a) and the non-linear curve in b) are  
585 forced to pass through the origin of coordinates (0, 0).

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589 Figure 2. Cumulative proportion of surviving *Apis mellifera* foragers orally exposed to  
590 propiconazole (PRO, 700 mg L<sup>-1</sup>), clothianidin (CLO, 0.074 mg L<sup>-1</sup>) and Cr (3.9 mg L<sup>-1</sup>) as single  
591 pollutants or binary and ternary mixtures. Negative control (sugar syrup solution) and solvent  
592 control (sugar syrup solution with 1.5% acetone) are reported for comparison.

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