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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 LD₅₀ and lowest benchmark dose of Cr as
23 Cr(NO₃)₃, revealed a low acute oral toxicity on honey bee foragers (2049 and 379 mg L⁻¹,
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⁻¹ 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 LD₅₀ 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 LD₅₀ of Cr (expressed both in mg L⁻¹ sugar syrup and in µg bee⁻¹) and its BMD
85 (expressed in mg L⁻¹) 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% CO₂ in synthetic air, bees were
99 placed in cardboard cages (9.5 cm x 6.5 cm x 5 cm) in groups of 10 (LD₅₀ 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 μL (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 g mol^{-1}) and $\text{Cr}_2(\text{SO}_4)_3$ (MW 392.18 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) 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 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 mg Cr L^{-1} . Among treatments, the highest concentration (4605 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 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⁻¹ 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 LD₅₀ at 24 h
145 for *Apis mellifera* (Ladurner et al., 2005).

146 A clothianidin solution at the concentration of 0.074 mg L⁻¹ 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_{10±95%} 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⁻¹ as Cr(NO₃)₃·9H₂O, 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 propicnazole and
160 0.074 mg of clothianidin; ii) 700 mg of propiconazole and 3.9 mg of Cr as Cr(NO₃)₃·9H₂O; iii)
161 0.074 mg of clothianidin and 3.9 mg of Cr as Cr(NO₃)₃·9H₂O. All the organic solutions were then

162 diluted with sugar syrup solution up to 1 L of final volume. Aliquots of 10 μL 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 μL 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 HNO_3 ($\geq 69\%$ v/v, for trace analysis, Sigma-Aldrich, USA).

177 Single honey bees (mean \pm SE dry weight: 22.75 ± 0.47 mg each) were analyzed for Cr content after
178 dissolution in a mixture of HNO_3 ($\geq 69\%$ v/v, for trace analysis, Fluka, Sigma-Aldrich, USA) and
179 H_2O_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°C for 24 h, five bees were singly spiked with 10 μL 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 LD_{50} values of Cr were estimated at 48 h after exposure phase.

196 The LD_{50} s were estimated with a Probit analysis (Finney, 1952) at 95% CL. The values expressed
197 in mg Cr L^{-1} in the sugar syrup were then transformed in $\mu\text{g bee}^{-1}$ assuming that each bee ingested
198 10 μL 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 LD₅₀ and BMD*

247 The Cr LD₅₀ 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 LD₅₀ are expressed both as mg Cr L⁻¹ sugar syrup and µg Cr bee⁻¹. For the LD₅₀, the
250 CL ranges obtained for Cr as Cr(NO₃)₃ is well overlapped with the range values obtained for Cr as
251 Cr(SO₄)₃, thus excluding possible lethal effects of Cr counterion. The calculated Cr LD₅₀ in *A.*
252 *mellifera* adults equals to 2049 mg L⁻¹ (or 20.5 µg bee⁻¹) which indicates slight toxicity based on the
253 WSDA pesticide's classification (WSDA, 2010), especially when compared to other pollutants

254 (e.g.: Se LD₅₀: 60 mg L⁻¹ (Hladun et al., 2013); Cu LD₅₀: 72 mg L⁻¹ and Pb LD₅₀: 345 mg L⁻¹ (Di et
255 al., 2016); Cd LD₅₀: 18.36 mg L⁻¹ and As LD₅₀: 25.68 mg L⁻¹ (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(NO₃)₃ (BMDL:
259 379 mg Cr L⁻¹, Table 2) is one order of magnitude higher than the highest environmental
260 concentrations found in honey bee matrices (46.52 mg Cr kg⁻¹, 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 α_{AI} coefficient and $p = 0.0880$ for β_{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⁻¹).

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_{50} of chromium as $Cr(NO_3)_3$ in *A. mellifera* adults (2049 mg L^{-1} syrup solution or
345 $20.5 \text{ } \mu\text{g bee}^{-1}$) indicates low toxicity. Acute exposure to Cr at concentration higher than 379 mg L^{-1}
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 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

363 **Acknowledgements**

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368

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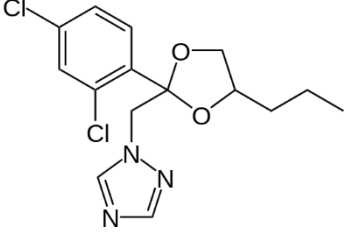
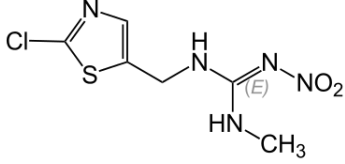
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549 **Table 1.** Main chemical characteristics of agrochemicals under investigation.

Chemical structure	Abbreviation	Molecular weight (g mol ⁻¹)	p <i>K</i> _a
	PRO	342.22	1.09*
	CLO	249.67	11

550 * p*K*_a of the conjugate acid (Tomlin, 2003)

551

552 **Table 2.** Lowest and highest benchmark doses* (BMDL and BMDU, respectively) and lethal
 553 dose** (LD₅₀) of Cr following acute oral exposure to Cr(NO₃)₃ or Cr₂(SO₄)₃ in *Apis mellifera* at 48
 554 h after ingestion. In brackets, the 95% CLs for LD₅₀ values.

Compound	BMDL-BMDU mg Cr L ⁻¹	LD ₅₀ (±95% CLs)			
		χ^2	p	mg Cr L ⁻¹	µg Cr bee ⁻¹
<i>Cr(NO₃)₃·9H₂O</i>	379-1670	0.341	>0.05	2049 (1674-2508)	20.5 (16.7-25.1)
<i>Cr₂(SO₄)₃</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).

Pairwise p comparison	Negative control	Solvent control	Cr	CLO	PRO	CLO+PRO	PRO+Cr	CLO+Cr
Solvent control	0.925	-	-	-	-	-	-	-
Cr	0.439	0.923	-	-	-	-	-	-
CLO	0.161	0.843	0.952	-	-	-	-	-
PRO	0.91	0.857	0.954	0.899	-	-	-	-
CLO+PRO	<0.001	<0.001	<0.001	0.002	<0.001	-	-	-
PRO+Cr	0.927	0.941	0.906	0.67	0.947	<0.001	-	-
CLO+Cr	0.001	0.044	0.425	0.857	0.069	0.183	0.022	-
CLO+PRO+Cr	<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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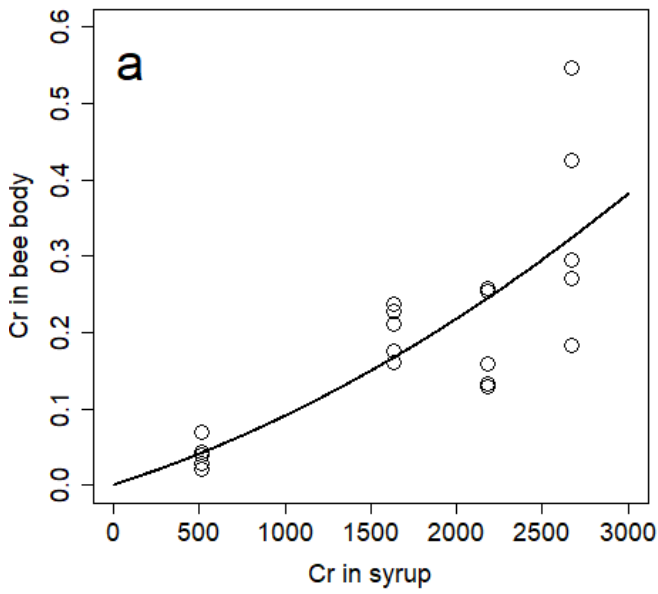
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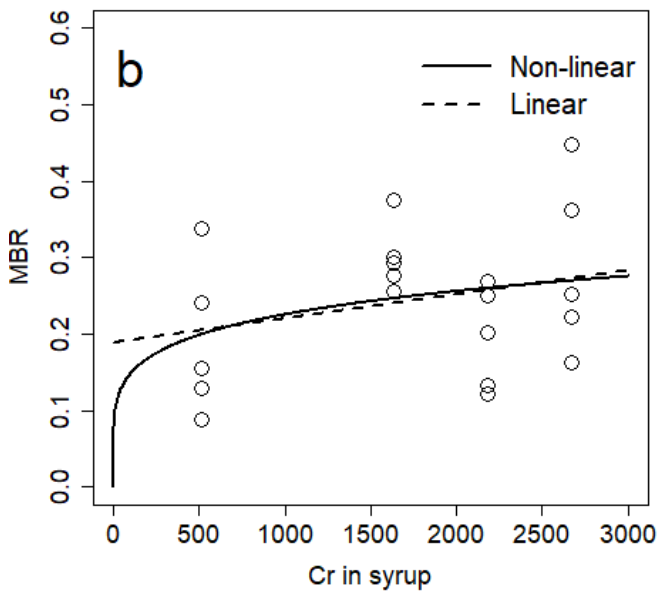
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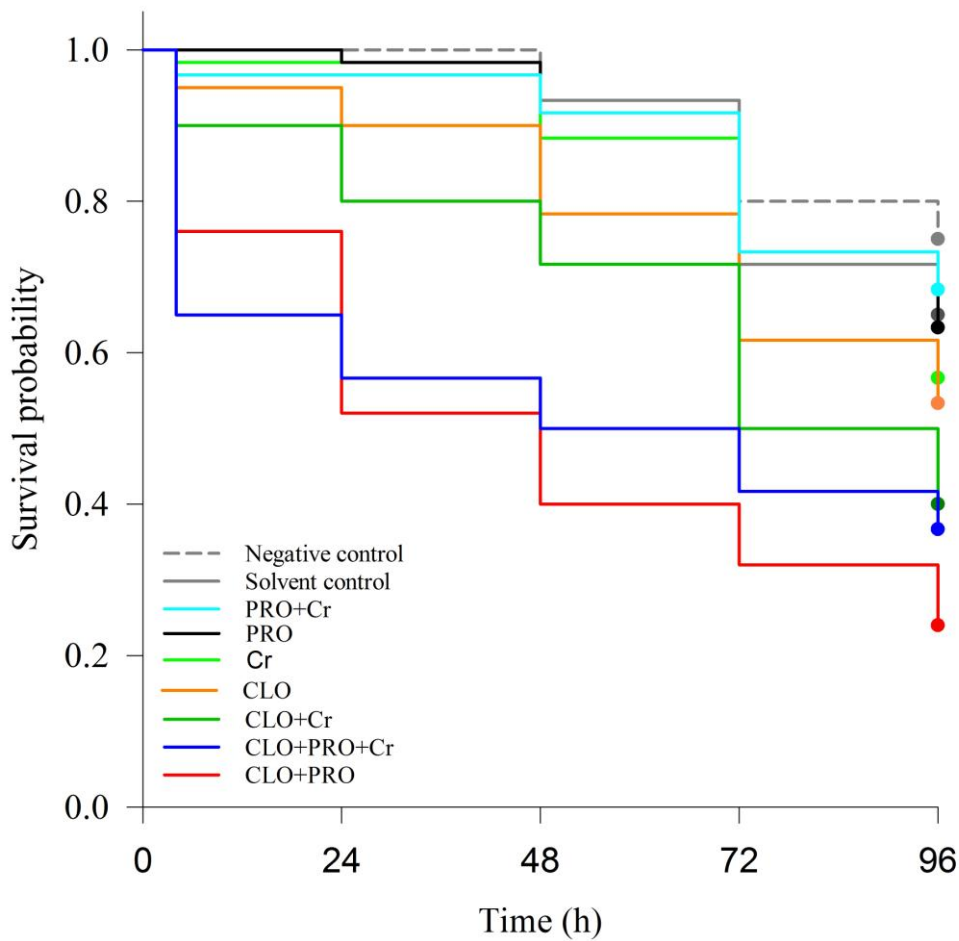
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581 Figure 1. Results of regression analysis to the a) Cr-retained and b) MBR observations.
 582 Observational data points are shown as empty dots. Figures also show a) parabola (solid line) and b)
 583 nonlinear (solid line) and linear (dashed line) 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⁻¹), clothianidin (CLO, 0.074 mg L⁻¹) and Cr (3.9 mg L⁻¹) 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.

593