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

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Abstract

Several anthropogenic contaminants, including pesticides and heavy metals, can affect honey bee health. The effects of mixtures of heavy metals and pesticides are rarely studied in bees, even though bees are likely to be exposed to these contaminants in both agricultural and urban environments. In this study, the lethal toxicity of Cr alone and in combination with the neonicotinoid insecticide clothianidin and the ergosterol-biosynthesis-inhibiting fungicide propiconazole was assessed in *Apis mellifera* adults. The LD_{50} and lowest benchmark dose of Cr as $Cr(NO_3)_3$, revealed a low acute oral toxicity on honey bee foragers (2049 and 379 mg L^{-1}, respectively) and the Cr retention (i.e. bee ability to retain the heavy metal in the body) was generally low compared to other metals. A modified method based on the binomial proportion test was developed to analyze synergistic and antagonistic interactions between the three tested contaminants. The combination of an ecologically-relevant field concentration of chromium with clothianidin and propiconazole did not increase bee mortality. On the contrary, the presence of Cr in mixture with propiconazole elicited a slight antagonistic effect.

Highlights

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

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

1. Introduction

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

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

In the present study, we addressed the lethal effects of chromium as Cr(III), alone and in combination with the neonicotinoid clothianidin and the ergosterol-biosynthesis-inhibitor (EBI) fungicide propiconazole on honey bees (*Apis mellifera ligustica* L.) following acute oral exposure under laboratory conditions. Chromium is a heavy metal ubiquitous in the environment often found as Cr (III) or (VI). The environmental diffusion of Cr has been increasing in the last years due to mining and industrial activities (Zayed and Terry, 2003). Although Cr(III) is commonly present in animals, it becomes toxic at high concentrations (Di Bona et al., 2011). Since this metal may be accumulated in plant tissues (Oliveira, 2012), honey bees can be exposed to this contaminant by contact and ingestion. As a consequence, chromium can be found in honey (Conti and Botrè, 2001; Porrini et al., 2002; Satta et al., 2012). Honey bees are considered bioindicator of environmental Cr pollution since environmental levels detected in honey bee matrices (i.e. honey, bee body, beeswax) range from 0.005 to 46.52 mg kg^{-1} depending on the matrix considered or on environmental colony location (i.e. rural, urban or industrial area) (Porrini et al., 2002; Satta et al., 2012).

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

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

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

In this study, the LD_{50} of Cr (expressed both in mg L^{-1} sugar syrup and in µg bee^{-1}) and its BMD (expressed in mg L^{-1}) at 48 hours after ingestion were determined for the first time. In addition, possible lethal effects of environmental Cr concentrations in combinations with clothianidin and propiconazole (i.e., binary or ternary mixtures) were investigated and a new statistical method to define synergistic/antagonistic interaction among them was developed *ad hoc*. Finally, Cr bioconcentration ratio in the bee body (i.e., bee Cr concentration/feeding solution Cr concentration), as a measure of honey bee capacity to retain the heavy metal, was estimated.

2. Materials and methods

2.1 Bees and test conditions

Forager honey bees (*Apis mellifera ligustica*) were obtained from three healthy colonies placed in an experimental apiary of CREA-AA (Bologna, Italy). During summer 2015, forager bees were collected using the “Funnel trap” (Medrzycki, 2013). The trap placed at the entrance of the hive allows collecting only forager bees, thus reducing the variability among bee categories (i.e., guard and other in-hive bees). After 30 min of anesthetization with 60% CO_2 in synthetic air, bees were placed in cardboard cages (9.5 cm x 6.5 cm x 5 cm) in groups of 10 (LD_{50} and BMD estimations) or 20 individuals (single pollutants, binary/ternary mixtures exposure experiment) per cage. Three cages per treatment were used. Bees from each colony were randomly distributed in group of 10 (or 20) among treatments to account for genetic diversity (i.e. different colony origin). In addition, to exclude any potential colony effect, a rank-transformed repeated-measures ANOVA analyses (Zimmerman and Zumbo, 1993) was performed for each treatment, with colony as the between-subjects factor and time (4, 24, 48, 72 and 96 h) as the within-subjects factor. In all treatments, no differences among colonies were found (Tables S1 and S2 in the Supplementary Information). During the experiment, the cages were maintained at 25±2 °C and 50-70% of relative humidity in an incubator under complete darkness. The cages were daily rotated to reduce potential differences in the incubator microclimate.

All treatments were performed on bees after 1 h starvation period. Test solutions (*vide infra*) were provided using a bulk feeder. For each treatment, the volume provided per cage was defined according to the assumption that, through trophallaxis, all individuals would ingest similar doses of 10 µL (OECD, 1998; Medrzycki et al., 2013). At the end of the exposure phase (maximum 2 h), the complete consumption of the solution was verified by visual inspection of the feeder. After that, bees were fed *ad libitum* with a sugarbeet (Eridania Italia SpA, Italy) syrup solution (sugarbeet:distilled water = 50:50 w/v) until the end of the experiment (96 h). Dead bees were preserved at -20 °C until elemental analysis.

2.2 Chemicals

$Cr(NO_3)_3 \cdot 9H_2O$ (MW 400.15 g mol^{-1}) and $Cr_2(SO_4)_3$ (MW 392.18 g mol^{-1}) were purchased from Carlo Erba (Italy).

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

2.3 Estimation of Cr(III) LD_{50} and BMD

Bees were exposed to different doses of $Cr(NO_3)_3 \cdot 9H_2O$ in a geometric series in order to calculate the dose-response curve and estimate the LD_{50} and BMD of Cr. As defined by a range-finding test, the following Cr concentrations in the sugar syrup solution (50% w/v) were chosen: 514, 1632, 2167, 2667 and 4605 mg Cr L^{-1}. Among treatments, the highest concentration (4605 mg Cr L^{-1}) was excluded in the calculation of the dose-response curve because the solution was not completely consumed by bees at the end of the exposure phase, likely due to its repellent effect. The toxicity of Cr as $Cr_2(SO_4)_3$ was also tested at the Cr concentrations of 302, 932, 1336, 1865, and 2685 mg L^{-1} to evaluate possible effect of the Cr counterion.

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

Control cages were supplied with sugar syrup solution.

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

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

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

A sugar syrup solution (sugar:distilled water = 50:50 w/v), containing 1.5% of acetone and 3.9 mg Cr L^{-1} as $Cr(NO_3)_3 \cdot 9H_2O$, was prepared for the evaluation of the effect of the environmental Cr concentration on bees. Solution aliquots of 10 µL containing 0.039 µg of Cr were provided per-capita to the bees. This concentration was chosen because it falls within the Cr concentrations found in honey bee matrices (Porrini et al., 2002; Satta et al., 2012) and thus it can be considered ecologically relevant.

Binary solutions were prepared by dissolving into 15 ml of acetone: i) 700 mg of propicnazole and 0.074 mg of clothianidin; ii) 700 mg of propiconazole and 3.9 mg of Cr as $Cr(NO_3)_3 \cdot 9H_2O$; iii) 0.074 mg of clothianidin and 3.9 mg of Cr as $Cr(NO_3)_3 \cdot 9H_2O$. All the organic solutions were then diluted with sugar syrup solution up to 1 L of final volume. Aliquots of 10 µL of each binary solution were provided per-capita to the bees.

A ternary solution was prepared by adding to 1 L of the binary solution of propiconazole and clothianidin, 3.9 mg of Cr(III) as $Cr(NO_3)_3 \cdot 9H_2O$. Even in this case, aliquots of 10 µL of the ternary solution were provided per-capita to the bees.

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

2.5 Metal content analysis

Metal concentrations in contaminated syrup solution and in honey bee body were measured after 48 h from exposure phase by using an inductively coupled plasma optical emission spectrometer (ICP-OES) furnished by SPECTRO Analytical Instruments GmbH & Co. (Kleve, Germany) equipped with a plasma source and an optical detector with a charge-coupled device (CCD) able to quantify emission wavelengths of elements ranging between 125 and 780 nm. Test solutions were analyzed for Cr after addition of HNO_3 (≥ 69% v/v, for trace analysis, Sigma-Aldrich, USA).

Single honey bees (mean±SE dry weight: 22.75±0.47 mg each) were analyzed for Cr content after dissolution in a mixture of HNO_3 (≥ 69% v/v, for trace analysis, Fluka, Sigma-Aldrich, USA) and H_2O_2 (30% v/v, for trace analysis, VWR Prolabo Chemicals, USA) in the ratio of 4:1 (v:v) by microwave-assisted digestion (*Start D*, Microwave Digestion System, Milestone, USA) before elemental analysis. The limit of detection (LOD) for Cr was 0.38 µg kg^{-1} bee. For the statistical analysis, zero value was assigned to concentrations below the limit of detection (*vide infra*).

The Cr recovery from bee matrix exposed to digestion and then analysed by ICP-OES was determined as follows. After drying at 100°C for 24 h, five bees were singly spiked with 10 µL of a Cr standard solution (1000 mg Cr L^{-1}) for ICP-OES calibration and additional five control bees were added with the same volume of distilled water. Once the added solutions were reduced by evaporation (within ca. 2 h), bees were singly mineralized and processed for Cr determination as already described. Cr recovery on spiked bees resulted 102±1.6% and Cr content of control bees was always below the LOD.

2.6 Statistical analysis

The number of dead bees was measured 4, 24, 48, 72 and 96 h after exposure to pollutants (see Figures S1 and S2 of Supplementary Information for mortality data *vs* time, corrected with Abbott's formula for Cr as $Cr(NO_3)_3{\cdot}9H_2O$ or $Cr_2(SO_4)_3$). Both the BMD intervals (BMDL-BMDU) and LD_{50} values of Cr were estimated at 48 h after exposure phase.

The LD_{50}s were estimated with a Probit analysis (Finney, 1952) at 95% CL. The values expressed in mg Cr L^{-1} in the sugar syrup were then transformed in µg bee^{-1} assuming that each bee ingested 10 µL of test solution.

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

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

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

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

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

when synergy was expected, and:

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

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

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

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

3. Results and discussion

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

3.1 Chromium LD_{50} and BMD

The Cr LD_{50} and BMD intervals (BMDL and BMDU) estimated at 48 hours in the acute oral toxicity tests are reported in Table 2.

The values of LD_{50} are expressed both as mg Cr L^{-1} sugar syrup and µg Cr bee^{-1}. For the LD_{50}, the CL ranges obtained for Cr as $Cr(NO_3)_3$ is well overlapped with the range values obtained for Cr as $Cr(SO_4)_3$, thus excluding possible lethal effects of Cr counterion. The calculated Cr LD_{50} in *A. mellifera* adults equals to 2049 mg L^{-1} (or 20.5 µg bee^{-1}) which indicates slight toxicity based on the WSDA pesticide’s classification (WSDA, 2010), especially when compared to other pollutants (e.g.: Se LD_{50}: 60 mg L^{-1} (Hladun et al., 2013); Cu LD_{50}: 72 mg L^{-1} and Pb LD_{50}: 345 mg L^{-1} (Di et al., 2016); Cd LD_{50}: 18.36 mg L^{-1} and As LD_{50}: 25.68 mg L^{-1} (Heard et al., 2017)).

As far as the BMD is concerned, a detailed description of the BMD analysis according EFSA guideline (EFSA 2009; 2011) is reported in section S4 of Supplementary Information (Tables S6 and S7). According to this analysis, the lowest BMD limit determined for Cr as $Cr(NO_3)_3$ (BMDL: 379 mg Cr L^{-1}, Table 2) is one order of magnitude higher than the highest environmental concentrations found in honey bee matrices (46.52 mg Cr kg^{-1}, Satta et al., 2012). According to our data, Cr at environmental concentrations poses a relatively low risk to honey bee adults by acute oral exposure.

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

3.2. Bioaccumulation of chromium in bee body

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

The Cr-retained dataset showed a positive and very significant linear relationship with Cr in the feeding solution ($p<0.001$ for α_{A1} coefficient and $p=0.0880$ for β_{A1}: Table S3 in Supplementary Information).

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

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

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

Cumulative proportion of surviving bees to Cr, propiconazole and clothianidin as single compounds and as binary and ternary mixtures are presented in Figure 2. Significant differences among cumulative survival curves of honey bees exposed to different treatments were found (Log-rank analysis $\chi^2=87.6$, df=8, $p<0.001$). In order to better highlight differences among treatment effects on bee mortality, pairwise analysis was performed on survival curves of Figure 2 and the p values are reported in Table 3.

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

In this study, the binomial proportion test developed for synergism (Sgolastra et al., 2017) was implemented to evaluate the antagonistic effect of the three pollutants in binary or ternary mixtures on bee mortality (Table 4). The script of this new procedure is provided as a Supporting data. Briefly, the implemented test is able to highlighten both the synergistic or antagonistic effect size expressed as a positive or negative difference, respectively, between the observed and expected mortality probabilities for each pollutants combination at each assessment time. In Table 4, A or B terms refer to the effect size of single pollutants in binary or in ternary mixture. The lethal effect on bees of clothanidin and propiconazole combination (A and B terms, respectively, Table 4) was synergistic for the first 48 hours after ingestion as shown by the significantly ($p<0.05$) positive values of effect size, in full agreement with previous results (Sgolastra et., 2017). The mechanism responsible for the synergism between the two pesticides is well known and it is related to the ability of propiconazole to inhibit the metabolization of clothianidin by cytochrome P450 monooxygenases (Berenbaum and Johnson, 2015). According to our data, a similar significant sinergistic effect was also observed in the ternary mixture by considering PRO+Cr (A term) and CLO (B term) as well as CLO+Cr (A term) and PRO (B term), although within a shorter time period (4-24 h). Cr contribution to the synergistic effect observed in the ternary mixture with clothianidin and propiconazole was ruled out by considering the effect size of CLO+PRO (A term) and Cr (B term).

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

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

5. Conclusions

The calculated LD_{50} of chromium as $Cr(NO_3)_3$ in *A. mellifera* adults (2049 mg L^{-1} syrup solution or 20.5 µg bee^{-1}) indicates low toxicity. Acute exposure to Cr at concentration higher than 379 mg L^{-1} (BMDL) may cause lethal effects to honey bee forgers. However, these concentrations are 10-100 times higher than the level usually found in honey bee matrices, thus confirming moderate Cr risks for honey bee foragers. In addition, honey bees showed higher ability to eliminate Cr (low Cr MBR) compared to other heavy metals (Al, Pb, Cd and Fe). However, Cr effect on mortality of bee larvae or behavioural perturbation that might chronically affect colony could not be ruled out.

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

Competing interests

We have no competing interests.

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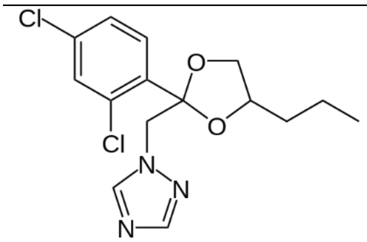
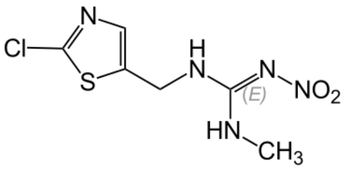
Table 1. Main chemical characteristics of agrochemicals under investigation.

Chemical structure	**Abbreviation**	**Molecular weight** (g mol^{-1})	**p*Ka***
	PRO	342.22	1.09*
	CLO	249.67	11

* pKa of the conjugate acid (Tomlin, 2003)

Table 2. Lowest and highest benchmark doses* (BMDL and BMDU, respectively) and lethal dose** (LD_{50}) of Cr following acute oral exposure to $Cr(NO_3)_3$ or $Cr_2(SO_4)_3$ in *Apis mellifera* at 48 h after ingestion. In brackets, the 95% CLs for LD_{50} values.

Compound	**BMDL-BMDU** mg Cr L^{-1}			**LD_{50}** (±95% CLs)	
		χ^2	p	mg Cr L^{-1}	µg Cr bee^{-1}
$Cr(NO_3)_3 \cdot 9H_2O$	379-1670	0.341	>0.05	2049 (1674-2508)	20.5 (16.7-25.1)
$Cr_2(SO_4)_3$	43-1250	0.270	>0.05	3458 (1917-6237)	34.6 (19.2-62.4)

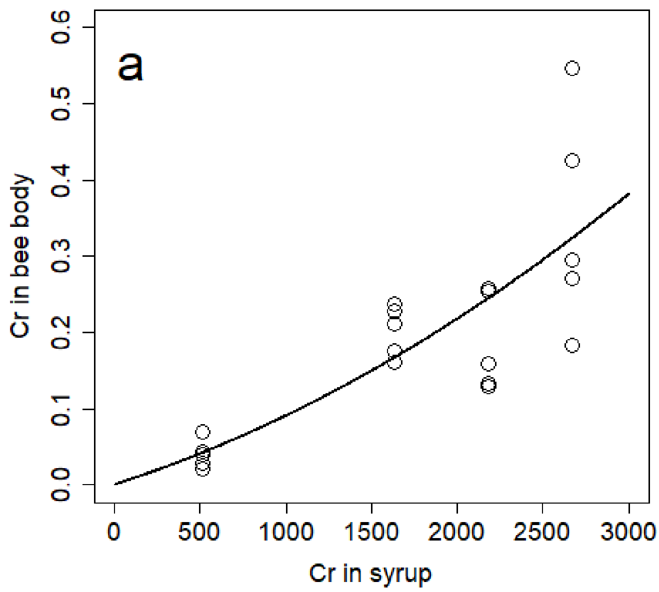
*Obtained with PROAST version 62.5; **Obtained with Probit analysis

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

Table 4. Effect size for binary (PRO+CLO; PRO+Cr, CLO+Cr) and ternary (PRO+CLO+Cr) mixtures at each assessment time (4, 24, 48, 72, and 96 h). A or B terms refer to the effect size of single pollutants in binary or in ternary mixture. A positive or negative difference indicates synergistic or antagonistic effect. Significance levels (Holm-corrected for multiple comparisons) for 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



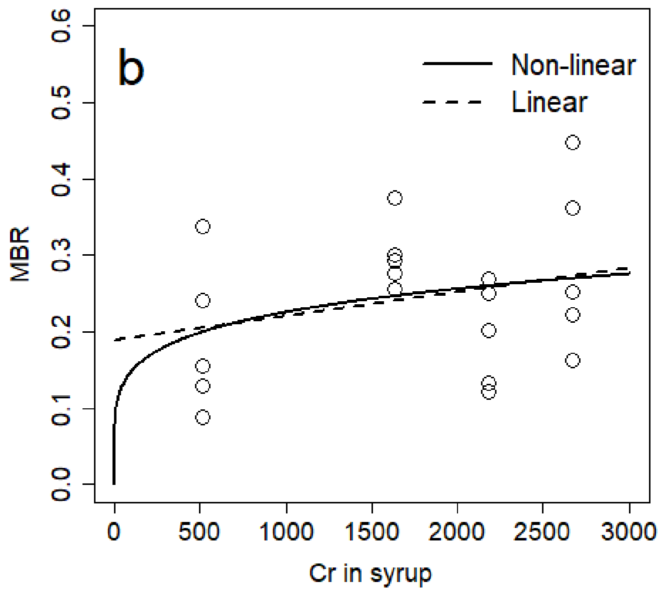


Figure 1. Results of regression analysis to the a) Cr-retained and b) MBR observations. Observational data points are shown as empty dots. Figures also show a) parabola (solid line) and b) nonlinear (solid line) and linear (dashed line) curves fitted to the data. Analytic expressions for each

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

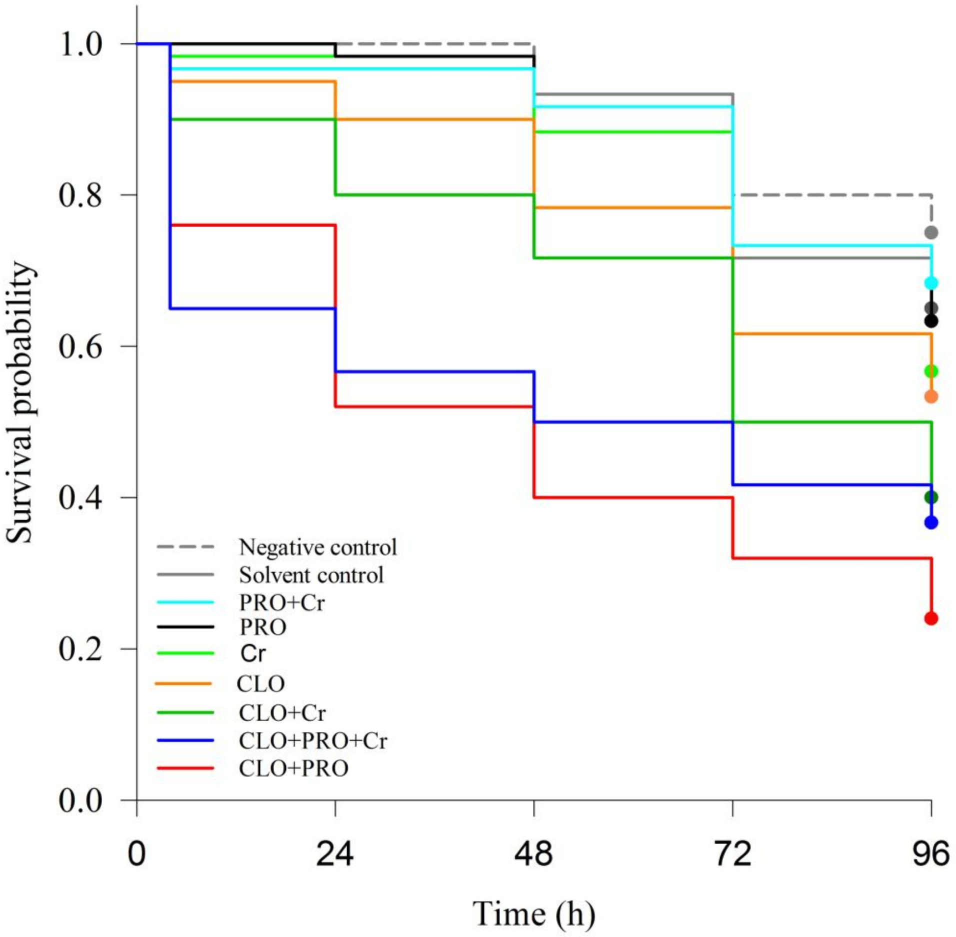


Figure 2. Cumulative proportion of surviving *Apis mellifera* foragers orally exposed to propiconazole (PRO, 700 mg L^{-1}), clothianidin (CLO, 0.074 mg L^{-1}) and Cr (3.9 mg L^{-1}) as single pollutants or binary and ternary mixtures. Negative control (sugar syrup solution) and solvent control (sugar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