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

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HIGHLIGHTS

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

GRAPHICAL ABSTRACT

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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 analyse 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.

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

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worldwide (Biesmeijer et al., 2006; Potts et al., 2010) 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. 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 (Fauser-Misslin et al., 2014; Goulson et al., 2015; Abbo et al., 2017; Dance et al., 2017). 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 it by contact and ingestion. Indeed, 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) (Conti and Botrè, 2001; 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 and its BMD at 48 h 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 × 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.

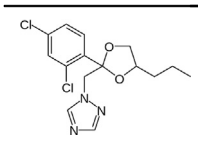
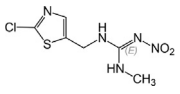
All treatments were performed on bees after 1 h starvation period. Test solutions (*vide infra*) were provided using a bulk feeder. For each treatment, the volume provided per cage was defined according to the assumption that, through trophallaxis, all individuals would ingest similar doses of 10 μL (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 (after 96 h from the exposure phase). 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

Table 1
Main chemical characteristics of agrochemicals under investigation.

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

[a] p*Ka* of the conjugate acid (Tomlin, 2003).