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Mixtures of herbicides and metals affect the redox system of honey bees

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HIGHLIGHTS

- Co-exposure to atrazine, glyphosate and Cd lowered bee carotenoid contents.
- Co-exposure to high levels of Fe decreased the accumulation of Cd in bees.
- Orally ingested Fe was bioconcentrated in bees.
- Fe induced high level of lipid peroxidation.
- Bees exposed to Fe had lower levels of 9-*cis*-RA and 13-*cis*-RA.

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ABSTRACT

The increasing loss of bee colonies in many countries has prompted a surge of studies on the factors affecting bee health. In North America, main crops such as maize and soybean are cultivated with extensive use of pesticides that may affect non-target organisms such as bees. Also, biosolids, used as a soil amendment, represent additional sources of metals in agroecosystems; however, there is no information about how these metals could affect the bees. In previous studies we investigated the effects of environmentally relevant doses of herbicides and metals, each individually, on caged honey bees. The present study aimed at investigating the effects of mixtures of herbicides (glyphosate and atrazine) and metals (cadmium and iron), as these mixtures represent more realistic exposure conditions. Levels of metal, vitamin E, carotenoids, retinaldehyde, *at*-retinol, retinoic acid isomers (9-*cis* RA, 13-*cis* RA, *at*-RA) and the metabolites 13-*cis*-4-oxo-RA and *at*-4-oxo-RA were measured in bees fed for 10 days with contaminated syrup. Mixtures of herbicides and cadmium that did not affect bee viability, lowered bee α- and β-carotenoid contents and increased 9-*cis*-RA as well as 13-*cis*-4-oxo-RA without modifying the levels of *at*-retinol. Bee treatment with either glyphosate, a combination of atrazine and cadmium, or mixtures of herbicides promoted lipid peroxidation. Iron was bioconcentrated in bees and led to high levels of lipid peroxidation. Metals also decreased zeaxanthin bee contents. These results show that mixtures of atrazine, glyphosate, cadmium and iron may affect different reactions occurring in the metabolic pathway of vitamin A in the honey bee.

1. Introduction

In many countries, the overwintering losses of honey bee colonies have risen beyond the 20% level during the last decades (Aizen and Herder, 2009). This concern prompted numerous studies to identify the causes affecting the health of bees, among them agricultural practices that may modify the quality of the bee environment and its nutritional status and hence its development, immune system and neuronal system (Wu et al., 2011; Boily et al., 2013; Pettis et al., 2013). In the province of Quebec, wide-row corn and soybean are the main crops, together occupying 665 kha in 2012 and consuming 46% of the pesticide use in 2008 (ISQ and MAPAQ, 2013; Gorse and Rivard, 2011). Glyphosate and atrazine are still widely used in these crops in the United States and Canada. They are available for the foraging bees from pollen, nectar, water, and dust (Krupke et al., 2012). Also, in addition to natural and chemical fertilizers, biosolids (municipal wastewater treatment

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sludge) are used as soil amendment to stimulate rooting. Although this practice follows restriction criteria based on the sludge's metal content, these biosolids represent a significant source of metals as they can contain up to 3 mg/kg cadmium (Cd) and 150 mg/kg lead (MDDEP, 2012). Metals in soil are available to plants, and therefore to foraging bees. For example, Cd accumulates in all parts of the maize (*Zea mays*) plant (Xu et al., 2013; Wang et al., 2016) and may exhibit a five-fold concentration in the pollen of the partridge pea (*Anisacanthus linearis*) compared to the flower part of the plant (Henson et al., 2013). Very few is known about how these metal contents may affect pollinators. Nonetheless, pesticides and metals found in pollen are brought back to the hive (Mullin et al., 2010; Lambert et al., 2012), and metals were shown to affect the overall hive health status, as revealed by lower honey production, increased dead pupae in the capped cells, and lowered relative pupae growth index (Di et al., 2016; Hladun et al., 2016). Atrazine, glyphosate and metals may induce oxidative stress, in other words, an imbalance between the level of pro-oxidant chemical species and the antioxidative defense, which may result in lipid peroxidation (Thornton et al., 2010; Jasper et al., 2012; Keshk et al., 2014; Nwani et al., 2010).

Alpha-tocopherol (vitamin E) and carotenoids, provided by diet, protect against oxidative damage. In vertebrates, carotenoids can also be oxidized into retinaldehyde (RALD), which in turn may be reversibly reduced to form all-*trans* retinol (ROH) or be oxidized into retinoic acid (RA) by a unidirectional pathway. In insects as well as in vertebrates, retinoids are essential for a number of biological functions including vision, reproduction and immune system response. In *Drosophilia*, imbalanced RA levels may lead to blindness or developmental malformations (von Lintig et al., 2001; Nakamura et al., 2007). For bees, pollen represents an important source of α- and β-carotene (Mărgăoan et al., 2014), which are converted into retinoids by the carotenoid isomeroxygenase NinaB, responsible for both the oxidative cleavage and the isomerization of carotenoids (Oberhauser et al., 2008). In vertebrates, there are three isomers for RA: all-*trans* retinoic acid (*at*-RA), 9-*cis* retinoic (9-*cis*-RA) and 13-*cis* retinoic acid (13-*cis*-RA). The transcriptional activity of RA isomers involves *at*-RA, 9-*cis*-RA and 13-*cis*-RA binding to the nuclear receptor RAR, whereas the RXR receptor reacts only with 9-*cis*-RA (Bastien and Rochette-Egly, 2004). The affinity of the 13-*cis*-RA isomer for the RAR receptor is much lower compared to that of *at*-RA; however, the possibility of an intracellular isomerization of 13-*cis*-RA to *at*-RA has been suggested (Tsukada et al., 2000; Veal et al., 2002). The cellular level of 13-*cis*-RA may, indirectly, play a role in RA activity. In vertebrates, RA isomers are converted into various polar metabolites, including *at*-4-oxo-RA and 13-*cis*-4-oxo-RA. The CYP26A1/B1/C1 isoforms of the CYP450 superfamily play a major role in the first step of the oxidative catabolism of *at*-RA leading to the formation of *at*-4-OH-RA (Armstrong et al., 2005; Petkovitch, 2001). Thus, controlling synthesis, isomerization and degradation finely regulates RA signaling.

In a previous study we have shown that both β-carotene and retinol levels decreased when bees were exposed to atrazine or glyphosate via contaminated syrup (Hedrei Helmer et al., 2015). We have also shown that Cd, at environmentally relevant concentrations in the syrup, is available for bees and increased the level of metallothionein-like proteins (Gauthier et al., 2016). The aim of the present study was to pursue our investigation of the effects of herbicides and metals on bee health by testing more realistic exposure conditions, being herbicide and metal mixtures. Carotenoids were measured for their antioxidant properties as well as for their involvement in RA metabolism. Retinoic acid was studied because of its crucial role in development and neuronal function. Cadmium and iron (Fe) were chosen for the following reasons: i) both metals may promote oxidative stress (Wu et al., 2016); ii) one of our previous studies shows the induction of stress proteins in bees exposed to Cd; iii) Cd may interfere with RA metabolism in some species (Cui and Freedman, 2009; Lee et al., 2006); iv) up to 850 ppm Fe were measured in samples of pollen from maize fields.

2. Material and methods

2.1. Chemicals

Butylated hydroxytoluene (BHT), malondialdehyde (MDA), carotenoids (lutein zeaxanthin, α- and β-cryptoxanthin, α- and β-carotene), retinol (ROH), vitamins A (all-*trans*-retinol) and E (α-tocopherol) standards were purchased from Sigma-Aldrich Ltd. (Oakville, ON, Canada). Antipain dihydrochloride, pepstatin A, tris-(2-carboxyethyl)phosphine hydrochloride (TCEP), 7-fluorobenzo-2-oxa-1,3-diazole-4-sulfonic acid ammonium (SBD-F) and 2-thiobarbituric acid (TBA) were purchased from Sigma-Aldrich Ltd. HPLC-grade solvents were used. Cadmium, as $CdCl_2$, was purchased from Sigma-Aldrich Ltd., whereas Fe, as $FeCl_2 \cdot 4H_2O$, was bought from Alfa Aesar – Thermo Fisher Scientific (Ward Hill, MA, US). Commercial formulations of atrazine (Aatrex®: 480 g active matter/L) and glyphosate (Credit Extreme®: 540 g active matter/L) were purchased from Les Moulins Mondou (Mirabel, QC, Canada).

2.2. Honey bee exposure

Honey bees (from the same healthy beehive) were taken from the frame without brood during the summer of 2014 and exposed to both herbicides and Cd (August 12–22) as well as to combinations of Cd and Fe (August 25 to September 4). Bees were placed in acrylic cages (3 cages, 32–38 bees/cage) and were fed a sucrose solution (50% w/w) ad libitum, as already performed in a prior study (Hedrei Helmer et al., 2015). Bees were exposed to syrup containing 0.03 mg/L Cd, 0.12 mg/L atrazine or glyphosate, alone or as mixtures. The exposure level to metals was based on the metal concentrations measured in maize pollen sampled in 2012, as determined in a previous study (Gauthier et al., 2016): Cd and Fe contents ranged from 0.01 to 0.03 ppm and from 90 to 850 ppm, respectively. The level of atrazine and glyphosate was based on herbicide concentrations measured in crops and vegetation (Krupke et al., 2012) and was estimated considering the quantity of active matter in the respective commercial formulations. Considering the daily consumption of syrup of 41 µl/bee estimated previously (Hedrei Helmer et al., 2015), the level of herbicides in the syrup led to a daily dose of 5.0 ng/bee, which represents less than 1% of the LD_{50} for atrazine and glyphosate (ARLA, 2007). Control cages were supplied with a sucrose solution only. The control and contaminated sugar solutions were prepared about 10 days prior to exposure, stored at −20 °C and thawed at room temperature before use. The sugar solutions of all cages (three replicates per experimental condition) were changed daily for the duration of the 10-day test. Cages were equipped with two feeders for syrup (1.5-ml capacity each). These were weighed before and after daily changes to estimate the consumption of syrup per cage/day adjusted for dead bees. Mortality was recorded every 24 h. After 10 days, the surviving bees were anesthetized/euthanized by placing the cages in an insulated container with dry ice for 5 min and stored at −80 °C until analysis. Pools of bees ($n = 7$ to 10; randomly mixed between replicates for each dose of contaminant) were analyzed for retinoids, carotenoids, α-tocopherol, peroxidation of lipids and protein content determination.

2.3. Metal content analyses

Metal concentrations in contaminated syrup as well as metal