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Exposure of larvae to thiamethoxam affects the survival and physiology of the honey bee at post-embryonic stages☆

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ABSTRACT

Under laboratory conditions, the effects of thiamethoxam were investigated in larvae, pupae and emerging honey bees after exposure at larval stages with different concentrations in the food (0.00001 ng/μL, 0.001 ng/μL and 1.44 ng/μL). Thiamethoxam reduced the survival of larvae and pupae and consequently decreased the percentage of emerging honey bees. Thiamethoxam induced important physiological disturbances. It increased acetylcholinesterase (AChE) activity at all developmental stages and increased glutathione-*S*-transferase (GST) and carboxylesterase *para* (CaE*p*) activities at the pupal stages. For midgut alkaline phosphatase (ALP), no activity was detected in pupae stages, and no effect was observed in larvae and emerging bees. We assume that the effects of thiamethoxam on the survival, emergence and physiology of honey bees may affect the development of the colony. These results showed that attention should be paid to the exposure to pesticides during the developmental stages of the honey bee. This study represents the first investigation of the effects of thiamethoxam on the development of *A. mellifera* following larval exposure.

1. Introduction

The honey bee *Apis mellifera* plays an important role at the economic and environmental levels. It contributes to more than 80% of the total pollination in agriculture and plays an important role in the pollination in ecosystems (Klein et al., 2007; Breeze et al., 2011). Gallai et al. (2009) estimated that the economic value of pollination in global scales is approximately €153 billion per year. However, there is an increasing number of reports on the decline of the bee population worldwide. This decline is characterized by the mass disappearance of bees in the United States (including colony collapse disorder (CCD)) and, in numerous cases, by losses of managed and wild pollinators in Europe and Asia (Stokstad, 2007; Potts et al., 2010). This raises the discussion among beekeepers, researchers, the chemical industry and governmental agencies about the factors involved in this syndrome. Several causes have been identified to explain the decline in the bee population, highlighting beekeeping management, agricultural practices, loss of floral diversity, habitat fragmentation and rarefication, pathogens, parasites and pesticides (Ratnieks and Carreck, 2010; Goulson et al., 2015).

An epidemiological study was performed in 2013 to explain the loss of honey bee colonies in Europe (Chauzat et al., 2013). If we exclude the problems of diseases (e.g., varroosis or nosemosis), the intoxication with pesticides could also, at least partially, explain the colony losses. Among pesticides, neonicotinoid insecticides represent the main family of insecticides used worldwide (Blacquiere

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et al., 2012; Sparks and Nauen, 2014). They act as agonists of the nicotinic acetylcholine receptors of insects (nAChR) (Tomizawa and Casida, 2003). They are characterized by the systemic properties and xylem and phloem transports enable them to be distributed in all plant tissues and to contaminate pollen and nectar, the two main food resources of bees (Rortais et al., 2005). They thus have adverse effects on bees, such as behaviour impairment or morphological and physiological disturbances (Henry et al., 2012; Goulson et al., 2015). Therefore, the effects and the properties of neonicotinoids suggest that they pose the greatest risk to honey bees (Sanchez-Bayo and Goka, 2014).

Within this class of insecticides, thiamethoxam, a second-generation neonicotinoid, can be regarded as an active substance of high concern (Maienfisch et al., 2001). Honey bees can be exposed to thiamethoxam and other neonicotinoids because they usually explore areas in a radius up to 12 km around the hive to collect floral resources, water and resins, increasing the risk of exposure at lethal and sublethal levels (Beekman and Ratnieks, 2000).

In addition to honey bee foragers, larvae could also be exposed to neonicotinoids from residues contained in pollen, nectar, water and wax stored in the hive (Rortais et al., 2005; Desneux et al., 2007; Couvillon et al., 2014; Sanchez-Bayo and Goka, 2014; Johnson, 2015). Thiamethoxam has been found at concentrations ranging from 1 to 100 μg/kg in nectar, pollen and plant secretions (and some other environmental matrices) (Blacquiere et al., 2012; Bonmatin et al., 2014; Kessler et al., 2015) and at a concentration of 0.6 μg/kg in beebread (Giroud et al., 2013).

The post-embryonic period of honey bees may be considered crucial because the exposure to xenobiotics can cause irreversible damage at the cellular, physiological and morphological levels, which can jeopardize the development of the honey bee (Becher et al., 2013; Tavares et al., 2015). At present, the side effects induced by an exposure to neonicotinoids during the post-embryonic period are poorly investigated (Desneux et al., 2007; Blacquiere et al., 2012), although some studies have demonstrated toxicity to bumblebees, which includes an increased mortality, a reduced efficiency of pollen collection and a reduced growth rate (Mommaerts et al., 2010; Whitehorn et al., 2012; Gill et al., 2012; Elston et al., 2013; Laycock et al., 2014).

To study honey bee health, the approach involving the use of biomarkers appears to be particularly pertinent to assess the physiological responses of honey bees after exposure to xenobiotics and to understand the mechanisms involved in the toxicity and the adaptation to environmental changes (Jovanovic-Galovic et al., 2004; Badiou-Beneteau et al., 2012; Boily et al., 2013; Carvalho et al., 2013; Badawy et al., 2015). Some biomarkers are particularly used to assess the physiological effects of environmental stressors. Acetylcholinesterase (AChE, EC 3.1.1.7) is an enzyme that controls the neuronal activity of cholinergic synapses (Badiou et al., 2008). Carboxylesterases (CaE, EC 3.1.1.1) and glutathione-*S*-transferase (GST 2.5.1.1.8) are phase I and phase II enzymes involved in the detoxification and endocrine systems (Yu et al., 1984; Maxwell, 1992; Diao et al., 2006). Alkaline phosphatase (ALP, EC 3.1.3.1) hydrolyses the phosphate group of different substrates and is involved in the absorption of substances, in intestinal integrity and homeostasis, and in the immunity process (Moss, 1992; Millán, 2006; Lallès, 2010).

In this study, we have investigated the effects of thiamethoxam in larvae, pupae and emerging workers following exposure at the larval stage. The study was focused on the success of post-embryonic development and on the physiological disturbances assessed by the modulation of the biomarkers AChE, GST, CaE*p* and ALP.

2. Materials and methods

2.1. Chemicals

Thiamethoxam (98.5% pure) was purchased from Dr. Ehrenstorfer GmbH. Yeast extract, D-glucose, D-fructose, antipain, aprotinin, leupeptin, pepstatin A, soybean trypsin inhibitor, monosodium phosphate, sodium chloride (NaCl), Triton® X-100, acetylthiocholine iodide (AcSCh.I), 5,5'dithio-bis(2,nitrobenzoic acid) (DTNB), reduced L-glutathione (GSH), ethylenediaminetetraacetic acid (EDTA), 1-chloro-2,4-dinitrobenzene (CDNB), Trizma® base (Tris), hydrochloric acid (HCl), magnesium chloride ($MgCl_2$), *p*-nitrophenyl phosphate (*p*-NPP), 1,5-bis(4-allyldimethylammonium-phenyl)pentan-3-one-dibromide (BW284C51) and *p*-nitrophenyl acetate (*p*-NPA) were obtained from Sigma Aldrich (France). Royal jelly was purchased from Ickowicz Apiculture (Bollène, France).

2.2. Collection of honey bee larvae and maintenance during development

Four colonies of *Apis mellifera* honey bees, previously checked for their health status, were selected from the experimental apiary of the *Abeilles & Environment* Research Unit (INRA, Avignon, France). Each colony had 6 to 7 brood frames and was supervised during the experiments to ensure good condition of the individuals. Larvae rearing was performed according to the method developed by Aupinel et al. (2005, 2007) and adopted by OECD (2013). To obtain larvae of a known age, three days before the experiment, combs containing empty cells were previously equipped with a queen excluder and placed in the hive for egg laying. The fourth day, 1st instar larvae were transferred into plastic queen-starter-cells and placed in an incubator under controlled conditions (34 ± 2 °C and 95 ± 5% relative humidity (RH)). At the 7th day, the RH was changed to 80% for the pupation period. On the 15th day (emergence period), each plate was individually sealed with a thin layer of beeswax so that each cell was individualized. In each cell, orifices were made on the top to enable air exchange. The plates were individually accommodated in pots upright, simulating colony conditions. Temperature and RH were identical to those of the pupation period. To feed the emerging honey bees, candy and distilled water were provided ad libitum.

2.3. Larvae feeding

Larvae were provided with food at the daily intakes recommended for each developmental stage (OECD, 2013). Food was composed of 1 volume of royal jelly and 1 volume of an aqueous solution containing 12% (w/v) glucose, 12% fructose and 2% yeast extract (diet A); 15% glucose, 15% fructose and 3% yeast extract (diet B); or 18% glucose, 18% fructose and 4% yeast extract, plus or minus (control) thiamethoxam (diet C). The daily feeding of larvae (volume per diet and per day) was performed from the 1st day (grafting) to the 6th day, except for the 2nd day, which was considered a period of acclimatization. The diet was: 20 μL of diet A on the 1st day, 20 μL of diet B on the 3rd day, and 30, 40, and 50 μL of diet C on the 4th, 5th and 6th days, respectively.

2.4. Exposure to thiamethoxam

To expose larvae, three concentrations of thiamethoxam were selected: 0.00001, 0.001 and 1.44 ng/μL of the diet. The two lowest concentrations were close to the levels of residues found in nectar, pollen and beebread (Rortais et al., 2005; Desneux et al., 2007; Mullin et al., 2010; Blacquiere et al., 2012; Krupke et al., 2012;