Ecotoxicology and Environmental Safety 132 (2016) 379–389

Contents lists available at ScienceDirect

Ecotoxicology and Environmental Safety

journal homepage: www.elsevier.com/locate/ecoenv

Secondary biomarkers of insecticide-induced stress of honey bee colonies and their relevance for overwintering strength

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ARTICLE INFO

Article history:
Received 29 February 2016
Received in revised form
21 June 2016
Accepted 21 June 2016
Available online 1 July 2016

Keywords:
Apis mellifera
Fenoxycarb
Imidacloprid
Overwintering strength
Field test
Sublethal effects

ABSTRACT

The evaluation of pesticide side-effects on honeybees is hampered by a lack of colony-level bioassays that not only are sensitive to physiological changes, but also allow predictions about the consequences of exposure for longer-term colony productivity and survival. Here we measured 28 biometrical, biochemical and behavioural indicators in a field study with 63 colonies and 3 apiaries. Colonies were stressed in early summer by feeding them for five days with either the carbamate growth regulator fenoxycarb or the neurotoxic neonicotinoid imidacloprid, or left untreated. Candidate stress indicators were measured 8–64 days later. We determined which of the indicators were influenced by the treatments, and which could be used as predictors in regression analyses of overwintering strength. Among the indicators influenced by fenoxycarb were the amount of brood in colonies as well as the learning performance and 24 h-memory of bees, and the concentration of the brood food component 10HDA in head extracts. Imidacloprid significantly affected honey production, total number of bees and activity of the immune-related enzyme phenoloxidase in forager bee extracts. Indicators predictive of overwintering strength but unrelated to insecticide feeding included vitellogenin titer and glucose oxidase-activity in haemolymph/whole body-extracts of hive bees. Apart from variables that were themselves components of colony strength (numbers of bees/brood cells), the only indicator that was both influenced by an insecticide and predictive of overwintering strength was the concentration of 10HDA in worker bee heads. Our results show that physiological and biochemical bioassays can be used to study effects of insecticides at the colony level and assess the vitality of bee colonies. At the same time, most bioassays evaluated here appear of limited use for predicting pesticide effects on colony overwintering strength, because those that were sensitive to the insecticides were not identical with those that were predictive of colony overwintering. Our study therefore illustrates the difficulties involved in evaluating the economic/ecological significance of pesticide-induced stress in honey bee field studies.

1. Introduction

In most countries, pesticides whose intended use implies a possible exposure of honeybees (*Apis mellifera*) have to be tested for negative effects on this economically and ecologically important species in order to achieve homologation (see for example European Union regulation 1107/2009; US-OCSPP-guidelines 850-3020 to 850-340). In recent years, the adequacy of existing testing schemes for plant protection products on honeybees has been questioned, both because of risen public awareness and of new scientific results highlighting the extent and importance of sublethal and/or delayed effects (Abramson et al., 2004; Decourtye et al., 2004; Di Prisco et al., 2013; Dively et al., 2015; Rondeau et al., 2014). Efforts are therefore being made to improve them (EPA, 2012; EFSA, 2013 (revised 2014)).

Studies on entire honey bee colonies are of special importance for pesticide testing. They reflect the most realistic scenario of exposure of the different life stages, and integrate the social stress buffering mechanisms of the species. They therefore are the ultimate way to judge whether effects observed in individual larvae or adults are economically and ecologically relevant (reviewed for the

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http://dx.doi.org/10.1016/j.ecoenv.2016.06.038
0147-6513/

case of neonicotinoids in Fairbrother et al., 2014). Classical endpoints of colony-level studies are direct elements of colony vitality, and usually include the amounts of bees and brood, brood survival, forager mortality, and overwintering success ("primary endpoints", EFSA, 2013 (revised 2014)).

Studies at the colony level face special inherent problems – they are expensive, time-consuming, often lack precision due to difficulties with the standardisation of subjects (colonies), and are generally of low repeatability because of environmental factors that can only partly be controlled (EASAC, 2015; Fairbrother et al., 2014). Some of these problems could be alleviated if additional/alternative colony-level endpoints (biomarkers) could be found that are faster, easier, and/or more precisely/reliably measured than the primary endpoints. While short-term effects on colony strength can be measured relatively soon after the end of exposure, assessment of overwintering success is inherently time consuming. Effects on overwintering success are also particularly sensitive to interactions with uncontrollable environmental factors, because the timespan during which these factors can act is very long (usually >6 months). Secondary endpoints/biomarkers that are predictive of colony overwintering strength (either by themselves, or in combination with classical indicators measured shortly after exposure) would therefore be of particular interest. Although they would likely be subject to interactions with environmental effects as well, the nature of these interactions may differ for different indicators, so that they can be expected to add information to models predicting overwintering strength. The problem with secondary endpoints however is that it is hard to judge of their economic and ecological meaning, i.e. their relationship with primary endpoints/protection goals (EPA, 2012).

In the present study, we screened potential secondary endpoints for the measurement of insecticide-induced stress in bee colonies. Our approach was to induce varying levels of insecticide stress, and measure the reactions of the prospected endpoints, in order to identify those that show colony-level sensitivity for the types of insecticides tested and could therefore be useful for mechanistic studies of insecticide effects at the colony level. Moreover, the relevance of observed effects for colony productivity and vitality was assessed by studying the relationship between the tested secondary endpoints and the primary endpoint overwintering strength. Because our approach required that widely varying levels of stress were applied to the experimental colonies, insecticides were administered at concentrations that were partly higher than those to which bees would be exposed in non-experimental field settings. The two substances used, the neonicotinoid imidacloprid and the carbamate compound fenoxycarb, exhibit two very different modes of action (neurotoxic effect vs. hormonal dysregulation). Their choice was additionally motivated by the fact that imidacloprid, as a neonicotinoid, is part of the group of insecticides that have been at the centre of the controversy regarding the adequacy of current testing procedures (recently reviewed by Blacquière et al., 2012; Fairbrother et al., 2014), while fenoxycarb is frequently used as positive control in toxicological studies in *Apis mellifera* and is known for long-lasting effects on colony development (EASAC, 2015; OECD, 2014; Thompson et al., 2005). The biochemical and morphometrical indicators tested were chosen to reflect foraging efficiency, brood rearing, immune status and age composition of the colony. Many of them were inspired by research on sublethal pesticide effects in *Apis mellifera*. These include the indicators of immune status, development of the hypopharyngeal glands, and learning performance, all three known to be affected by both neonicotinoids (Aliouane et al., 2009; Di Prisco et al., 2013; Hatjina et al., 2013) and insect growth regulators (Abramson et al., 2004; Heylen et al., 2011; Pinto et al., 2000).

2. Materials and methods

2.1. Colonies

Colonies used were artificial swarms set up with 2 kg of bees of mixed age, previously treated against *Varroa destructor* with oxalic acid (40 mL of a 3.5% w/v solution in 50% w/v sucrose), and young, naturally-mated queens of the subspecies *A. m. carnica*. They were installed in hive boxes containing 11 frames of 825 cm^2 (German Standard). In order to increase the representativeness of results, daughters of nine different mother queens were used. Sister groups were of different size 5–11 and had been mated in different locations. These queens were randomly attributed to treatment groups. The colonies were allowed to develop for three weeks before the start of insecticide exposure, to make sure that all brood stages were present at the start of the experiment.

2.2. Insecticide exposure and blinding of study

The aim of the exposure was not to evaluate effects of the insecticides under field-realistic conditions, but to cause measurable stress at variable levels in order to compare the sensitivity of stress indicators, as well as their relationship to overwintering success. The methodology followed for insecticide exposure was modelled on protocols used for semi-field tests for honeybee risk assessment (EPPO, 2010), modified to allow greater control of the dose and concentration administered. In order to allow direct exposure of forager bees, but still make sure that each colony only received the intended treatment, each hive was placed within a tent of 4×5 m, containing nearly no flowering plants. Before placing the hives inside the tents, stores of honey and pollen were checked to make sure that they were still similar, and that no starvation could take place. The insecticides were offered in dissolved form in 50% w/v sucrose. Imidacloprid (98.7% pure; HPC, Cunnersdorf, Germany) was directly dissolved in the sucrose solution, while fenoxycarb (99.3% pure; HPC, Cunnersdorf, Germany) was added from stock solutions in dimethyl sulfoxide (DMSO; final concentration of solvent in the sucrose solution 0.5% v/v). In order to maximise uptake and at the same time allow direct exposure of forager bees, the total feeding volume of 1 L/day was split in two portions fed in separate feeders. Of these, one was placed within the hive box, the other outside of it. The solution in the feeders was renewed daily and the volume left over from the previous day was measured for the calculation of insecticide uptake. Feeding lasted for five consecutive days. Three different concentrations of imidacloprid (1000, 200 and 50 μg/L) and three of fenoxycarb (80, 20 and 5 mg/L) were administered. A further group was fed with pure sucrose solution, containing neither insecticides nor DMSO (control). For comparison, field-relevant concentrations of imidacloprid in nectar are in the area of 0.7 to 10 μg/L (Cresswell, 2011). Nectar concentrations of fenoxycarb can be expected to be low because of its low water solubility, but concentrations in pollen of plants treated at field-realistic doses during blossoming are in the range of 7.5 to 217 mg/kg (Gretenkord and Drescher, 1996, as cited in Tasei, 2002). Each concentration of each of the two insecticides was fed to a group of 9 colonies, which later was spread evenly over the three apiaries. Together with the 9 control-colonies-63 colonies were used in the study. Because of logistical limitations, these 63 colonies had to be established, exposed and observed in two batches of 31 and 32 colonies, with an offset of one week. Since it was not possible to divide the nine colonies of each treatment and the control evenly into two groups, each treatment and the control were represented by either four or five colonies in each of the two batches. After the end of insecticide feeding, the tags on the hive boxes were exchanged by a person otherwise not involved in the experiment (and not employed by any of the participating