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Analysis and evaluation of (neuro)peptides in honey bees exposed to pesticides in field conditions☆

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ABSTRACT

During the last years, declines in honey bee colonies are being registered worldwide. Cholinergic pesticides and their extensive use have been correlated to the decline of pollinators and there is evidence that pesticides act as neuroendocrine disruptors affecting the metabolism of neuropeptides. However, there is a big absence of studies with quantitative results correlating the effect of pesticide exposure with changes on neuropeptides insects, and most of them are conducted under laboratory conditions, typically with individual active ingredients. In this study, we present an analytical workflow to evaluate pesticide effects on honey bees through the analysis of (neuro)peptides. The workflow consists of a rapid extraction method and liquid chromatography with triple quadrupole for preselected neuropeptides. For non-target analysis, high resolution mass spectrometry, multivariate analysis and automatic identification of discriminated peptides using a specific software and protein sequence databases. The analytical method was applied to the analysis of target and non-target (neuro)peptides in honey bees with low and high content of a wide range of pesticides to which have been exposed in field conditions. Our findings show that the identification frequency of target neuropeptides decreases significantly in honey bees with high concentration of pesticides (pesticide concentrations $\geq 500\ \mu g\ kg^{-1}$) in comparison with the honey bees with low content of pesticides (pesticide concentrations $\leq 20\ \mu g\ kg^{-1}$). Moreover, the principal component analysis in non-target search shows a clear distinction between peptide concentration in honey bees with high level of pesticides and honey bees with low level. The use of high resolution mass spectrometry has allowed the identification of 25 non-redundant peptides responsible for discrimination between the two groups, derived from 18 precursor proteins.

1. Introduction

Conservation of pollinator abundance and its role as ecosystem services, contribute decisively in moderating any negative impacts their deficit may provoke in agriculture, food production and environmental sustainability. The European honey bee (*Apis mellifera* L.) is the most commonly managed bee in the world. During recent years, declines in bee colonies are being registered as much in Europe as in other parts of the world (vanEngelsdorp and Meixner, 2010). Various factors have been identified causing the reduction in bee colonies, including parasites, pathogens and pesticide stressor along with other factors such as loss or fragmentation of habitat, invasive species and/or climate change (Sánchez-Bayo et al., 2016; vanEngelsdorp and Meixner, 2010). However, there is a big concern about the possible role that pesticides, particularly neonicotinoids insecticides and organophosphate miticides, may play in honey bee health (Cicero et al., 2017; Fairbrother et al., 2014; Palmer et al., 2013). Honey bees living and foraging near agricultural fields are exposed to pesticides as neonicotinoids (Cicero et al., 2017; Hakme et al., 2017; Palmer et al., 2013) and the extensive use of some of these pesticides has been correlated to the decline of bees and other pollinators (Samson-Robert et al., 2014). In addition, honey bees are also exposed to acaricides, used against *Varroa* in the hives, that can act whether alone or in combination with fungicides showing synergic effects (García et al., 2017; Gómez-Ramos et al., 2016; Sánchez-Bayo et al., 2016).

Neurotoxic insecticides have special importance at sublethal

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levels in honey bees, producing behavioral changes that interfere with foraging behavior, homing success, navigation performance and social communication (Stanley et al., 2016; Tison et al., 2016). Some studies demonstrated that pesticides and other environmental contaminants act as neuroendocrine disruptors capable or acting as agonist/antagonist or modulators of the metabolism of neuropeptides (Waye and Trudeau, 2011). Neuropeptides are 3–100 amino acid residues long, that are produced from precursor proteins by a series of enzymatic processing steps (Lee, 2016). Neuropeptides are key regulators in the majority of physiological and behavioral processes of any animal species, including insects (Boerjan et al., 2010). Some of these substances are involved in food intake of solitary insects such as *Drosophila melanogaster* (Melcher and Pankratz, 2005) and the German cockroach *Blattella germanica* (Pascual et al., 2008) and modulate odor perception and locomotor activity in *Drosophila melanogaster* (Kahsai et al., 2010; Winther et al., 2006). Regarding honey bees, several neuropeptides showed differences in brain abundance in association with nectar or pollen foraging (Brockmann et al., 2009). More recent studies have revealed that the suppression of ovary activation in worker honey bee is probably mediated through steroid and neuropeptide hormone signaling (Cardoen et al., 2012) and neuropeptides appear to have some functions in the honey bee brain that are specifically related to the age-related division of labor (Han et al., 2015; Pratavieira et al., 2014).

Because of the importance of neuropeptides in regulating neural communication and physiological modulation in organisms acting as neurotransmitters, neuromodulators and neurohormones, efforts have been undertaken in recent decades to identify them in a variety of insects, included in *Apis mellifera*, which is the best documented specie among the social insects (Audsley and Weaver, 2006; Boerjan et al., 2010; Han et al., 2015; Nässel and Winther, 2010).

Although there are several methods for the analysis of neuropeptides, also known as neuropeptidomics, mass spectrometry (MS), with its qualitative and quantitative capabilities, is ideally suited to the task (Lee, 2016; Yin et al., 2011). MS enables fast, sensitive, accurate and high-throughput analyses of neuropeptides without *a priori* knowledge of the peptide's identity, resulting in the identification of previously unknown neuropeptides (Lee, 2016). Two types of ionization are commonly used in the analysis of neuropeptides, electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI), each of them having its own advantages. Direct tissue analysis by MALDI-based MS is usually performed by a simple sequence of steps, whereas ESI-MS can be coupled more easily with separation methods (Lee, 2016). Liquid chromatography (HPLC)- MS has proved to be particularly useful for the identification and quantification of neuropeptides, primarily due to its capability to unambiguously characterize peptides in complex biological samples (Yin et al., 2011). LC and nano-scale LC coupled to high resolution MS, using quadrupole time-of-flight (Q-TOF) or Orbitrap, have been used in some recent studies for the analysis of neuropeptides in animal-brain tissue (Yin et al., 2011), including honey bees (Han et al., 2015). Brain extract is a very complex matrix and in this context, high resolution is decisive in the discrimination of very similar compounds. Neuropeptides are typically identified with both MS and MS/MS fragmentation data, normally using neuropeptide prohormone databases to facilitate neuropeptide identification (Lee, 2016). MS has been used to characterize hundreds of putative signaling peptides in a range of animals (Yin et al., 2011). In honey bees, 158 neuropeptides derived from 22 precursor proteins have been identified in the brain using MS/MS techniques (Han et al., 2015). In addition, several MS-based measurement approaches have been developed and enable relative quantitation of peptide levels in biological samples, including correlating peptide levels to specific conditions or behaviors (Han et al., 2015; Lee et al., 2016; Yin et al., 2011). LC-MS/MS techniques, using relative quantitation, have been used to investigate connections between social behavior and bioactivities of neuropeptides (Han et al., 2015) including the regulation of foraging activity in honey bees (Brockmann et al., 2009; Schoofs et al., 2017) and labor division (Han et al., 2015). However, the physiological and behavioral functions of most neuropeptides in honey bees remain largely unknown (Han et al., 2015; Schoofs et al., 2017). Study of neuropeptide function is a challenging task, as it is known that more than one neuropeptide can be involved in the regulation of a physiological activity and multifunctionality is common for brain peptides (Nässel, 2002).

In this work, a new analytical method using a rapid and simple extraction method and LC with triple quadrupole (LC-QqQ-MS/MS) and high resolution MS (LC-QTOF-MS/MS) has been successfully applied for the target and not-target analysis of (neuro)peptides in honey bees with low and high content of pesticides to which bees have been exposed in field conditions. Neuropeptide differences, in concentration and detection frequency, were compared between the group of honey bees with low level content of pesticides and the group of honey bees with a high level of content. To our knowledge, this is the first work that studies the effects of pesticides in honey bees in relation with the presence and concentration of neuropeptides. This is of great importance for better understanding the neuronal basis of pesticide exposure of honey bees in the field.

2. Material and methods

2.1. Chemical reagents

A set of 12 neuropeptides were selected for the validation study. The neuropeptides were chosen as representatives of different neuropeptides families identified in *Apis* sp. (Brockmann et al., 2009; Han et al., 2015). The neuropeptides included in this study were supplied by Phoenix Pharmaceuticals Inc. (Burlingame, CA) at analytical grade (purity >95%). Individual standard stock solutions of the compounds were prepared in methanol 1% formic acid, at concentration of 200 mg L^{-1} and stored at −20 °C. Working standard solutions, at different concentrations, were prepared by appropriate dilution of the stock solutions with the mobile phases in a proportion of methanol/water (2:8 v/v). HPLC-grade methanol and formic acid (purity 98%) were supplied by Sigma-Aldrich (Steinheim, Germany). LC-MS grade water was obtained from Fisher Scientific (Geel, Belgium). Sodium chloride was purchased from J.T.Baker (Deventer, The Netherlands). Anhydrous magnesium sulfate was supplied by Panreac (Barcelona, Spain).

2.2. Sample collection and classification

Bee samples were collected from July to September of 2016 by beekeeper collaborators of 60 different apiaries distributed at diverse locations in Spain. Samples containing high level of pesticides were collected in apiaries close to areas of high intensive agricultural production with conventional practices of pesticide applications. Samples with low level of pesticides were collected in apiaries near fields with low agricultural production or intermediate areas near agricultural fields with conventional use of pesticides. Each collected sample was composed of approximately 500 adult forager honey bees (*Apis mellifera iberica*) from at least six