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Proteomic and metabolomic analysis reveals rapid and extensive nicotine detoxification ability in honey bee larvae

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ABSTRACT

Despite potential links between pesticides and bee declines, toxicology information on honey bee larvae (*Apis mellifera*) is scarce and detoxification mechanisms in this development stage are virtually unknown. Larvae are exposed to natural and synthetic toxins present in pollen and nectar through consumption of brood food. Due to the characteristic intensive brood care displayed by honey bees, which includes progressive feeding throughout larval development, it is generally assumed that larvae rely on adults to detoxify for them and exhibit a diminished detoxification ability. We found the opposite. We examined the proteomic and metabolomic responses of *in vitro* reared larvae fed nicotine (an alkaloid found in nectar and pollen) to understand how larvae cope on a metabolic level with dietary toxins. Larvae were able to effectively detoxify nicotine through an inducible detoxification mechanism. A coordinated stress response complemented the detoxification processes, and we detected significant enrichment of proteins functioning in energy and carbohydrate metabolism, as well as in development pathways, suggesting that nicotine may promote larval growth. Further exploration of the metabolic fate of nicotine using targeted mass spectrometry analysis demonstrated that, as in adult bees, formation of 4-hydroxy-4-(3-pyridyl) butanoic acid, the result of 2′C-oxidation of nicotine, is quantitatively the most significant pathway of nicotine metabolism. We provide conclusive evidence that larvae are capable of effectively catabolising a dietary toxin, suggesting that increased larval sensitivity to specific toxins is not due to diminished detoxification abilities. These findings broaden the current understanding of detoxification biochemistry at different organizational levels in the colony, bringing us closer to understanding the capacity of the colony as a superorganism to tolerate and resist toxic compounds, including pesticides, in the environment.

1. Introduction

Honey bees (*Apis mellifera* L.) develop through complete metamorphosis. What differentiates honey bees from solitary holometabolous insects is that they feed their larvae progressively throughout larval development and have a nutrition-based mechanism of caste determination (Buttstedt et al., 2016; Winston, 1987). Larvae are primarily fed jelly produced by the hypopharyngeal and mandibular glands of nurse bees (Haydak, 1970). From day three, larvae destined to become workers are fed some pollen and honey in addition (Haydak, 1970) and even receive a little nectar (DeGrandi-Hoffman and Hagler, 2000; Nixon and Ribbands, 1952). Consequently, larvae are directly exposed to secondary metabolites and pesticides present in floral nectar and pollen (Adler et al., 2012; Blacquière et al., 2012; Chauzat et al., 2006, 2011). Secondary metabolites and pesticides have also been detected in bee bread (stored pollen mixed with glandular secretions), honey and royal jelly (Chauzat et al., 2006, 2011; Isidorov et al., 2009; Kretschmar and Baumann, 1999; London-Shafir et al., 2003; Mullin et al., 2010; Smodiš Škerl et al., 2010). In addition, high levels of pesticide residues were also measured in wax samples

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from the brood nest where larvae develop (Chauzat and Faucon, 2007; Mullin et al., 2010). Subsequently, larvae are exposed orally and topically to naturally occurring toxins as well as synthetic toxicants.

Despite the potential links between pesticides and bee declines, toxicology information on honey bee larvae is scarce and detoxification mechanisms in this development stage are virtually unknown. Due to the characteristic intensive brood care displayed by honey bees (Heimken et al., 2009), it is generally assumed that the larvae rely on adult bees to detoxify for them and that this development stage exhibits a diminished ability to detoxify dietary toxins. Most published work in this area tends to focus exclusively on adult bees and on the acute toxicity of dietary toxins. Compared to the extensive research on adults, only a few studies have examined the sublethal effects or larval responses to pesticide or plant secondary metabolite exposure (Arnold et al., 2014; Aupinel et al., 2007; Davis, 1989; Gregorc and Ellis, 2011; Mao et al., 2015; Singaravelan et al., 2006; Zhu et al., 2014). Where sublethal effects of secondary plant metabolites are concerned, ecologically relevant concentrations of nicotine (found in the pollen and nectar of *Nicotiana* species) did not impact survival of larval honey bees negatively (Human et al., 2014; Singaravelan et al., 2006). Microcolonies of bumblebees provided with lupanine treated pollen (an alkaloid found in pollen and nectar of *Lupinus* species), which was fed to larvae by workers, produced fewer and smaller males (Arnold et al., 2014). *p*-Coumaric acid, a phenolic found in pollen, differentially regulates genes involved in caste determination and influences the development fate of female larvae in *A. mellifera* (Mao et al., 2015).

Exposure to field realistic levels of in-hive pesticides, fungicides and miticides negatively impacts brood survival and development. Larvae reared in comb containing pesticide residues demonstrated delayed development and increased mortality (Berry et al., 2013; Wu et al., 2011), as well as reduced rates of capping, pupation and eclosion (Yang et al., 2007). Chronic exposure of larvae to dietary pesticides at sublethal levels reduced survival rates (Zhu et al., 2014) as well as causing impaired learning and memory in the ensuing adults (Yang et al., 2007). On a molecular level, changes in gene expression with pesticide exposure were variable but generally minor when a targeted gene approach was used: most notable was higher expression of prophenoloxidase-activating enzyme, an enzyme involved in humoral immunity, and lower expression of a hexameric larval storage protein involved in larval development (Gregorc et al., 2012). A study that explored genome-wide changes in gene expression with pesticide exposure in larvae reported differential expression of 300 transcripts (Derecka et al., 2013): notably, a cluster of genes encoding detoxifying enzymes was overexpressed while expression of genes involved in energy metabolism was reduced. Lipid metabolism was also altered, with lipid profiles indicating effects not only on lipids involved in energy metabolism, but structural lipids as well (Derecka et al., 2013).

This finding of up-regulation of detoxification genes in combination with down-regulation of genes involved in energy metabolism in larvae exposed to pesticides is surprising. Since it is widely assumed that metabolic detoxification mechanisms in insects are energetically expensive (Berenbaum and Zangerl, 1994; Cresswell et al., 1992; Guedes et al., 2006; Kliot and Ghanim, 2012): however, the absence of evidence for costs has also been reported (Castañeda et al., 2009; Kliot and Ghanim, 2012). Previously, it has been demonstrated that dietary nicotine had no significant adverse effects on lipid and protein reserves in honey bee larvae (Human et al., 2014), suggesting negligible energetic or metabolic costs associated with the observed nicotine tolerance. However, in adult bees nicotine tolerance is associated with oxidative detoxification coupled with an increase in energy metabolism (Du Rand et al., 2015). It is possible that nicotine exposure imposes an energetic demand on larvae and that the larvae compensate by down-regulating specific functions which are not necessarily reflected by changes in body mass or body composition.

Nicotine is a highly toxic alkaloid found primarily in the plant family Solanaceae, including tomato, potato, green pepper and tobacco. It is a broadly effective defence against herbivores, with a mode of action resembling that of synthetic neonicotinoids; and is used as a nonsynthetic insecticide in the form of tobacco tea in organic farming methods (Isman, 2006). Nicotine mimics acetylcholine at the neuromuscular junction in mammals, causing twitching, convulsions and even death (Steppuhn et al., 2004; Tomizawa and Casida, 2003). In susceptible insects, the same mode of action is observed in the ganglia of the central nervous system (Tomizawa and Casida, 2003). Only a few insect species such as *Myzus persicae* (susceptible strains LC_{50} < 30 ppm; resistant strains LC_{50} > 200 ppm), *Bemisia tabaci* (resistant strains LC_{50} = 2000–10 000 ppm) and *Manduca sexta* are known to tolerate nicotine in their diet (Bass et al., 2013; Kliot et al., 2014; Snyder et al., 1994).

In the present study, we use an integrated mass spectrometry-based proteomic and metabolomic approach to address the question of whether and how honey bee larvae respond to a dietary alkaloid, using nicotine as model compound. The pyridine alkaloid nicotine occurs naturally in the *Solanaceae* such as tobacco, tomato, potato and green pepper. It is a broadly effective plant defence metabolite against herbivores that binds to the nicotinic acetylcholine receptors at neuromuscular junctions (Tomizawa and Casida, 2003; Steppuhn et al., 2004); and is used as a nonsynthetic insecticide in the form of tobacco tea in organic farming methods (Isman, 2006). In *Nicotiana* species, leaves contain up to 2000 ppm nicotine while the levels in pollen and nectar are 23 ppm and 0.1–5 ppm, respectively (Adler et al., 2012; Detzel and Wink, 1993; Tadmor-Melamed et al., 2004). We also explored the metabolic fate of the ingested alkaloid in order to shed more light on the detoxification ability of larvae. While genome-wide measurements of mRNA expression levels have undoubtedly revealed important cell-specific processes, there is often only a weak correlation between RNA levels and the abundance of the corresponding proteins (Vogel and Marcotte, 2012). Protein profiles provide indirect insight into the regulation of metabolic flux, while the metabolite profiles provide direct knowledge of relative metabolite concentrations reflecting the metabolic or physiological state. This study is also noteworthy for identifying detoxification processes in healthy honey bee larvae sampled from colonies historically free from routine in-hive preventative treatment for *Varroa destructor* and other diseases. The described data set is free from the confounding effects of miticide and disease treatment and establishes a reference point for normal detoxification processes in studies investigating the synergistic effects of pesticides, disease and malnutrition on honey bee larvae.

2. Materials and methods

2.1. In vitro larval rearing

Frames with open brood were collected from strong, queen right *Apis mellifera scutellata* colonies maintained at the University of Pretoria apiary (Pretoria, South Africa) during late summer (January–April 2013). Two day old worker honey bee larvae were grafted onto larval food in 48-well microtiter plates (Thermo Fisher Scientific, Rochester, New York), using the protocol from Aupinel et al. (2005) and Crailsheim et al. (2013). The grafted larvae (Fig. 1) were kept in an incubator (Humidity chamber HCP108,