Resistance of developing honeybee larvae during chronic exposure to dietary nicotine

H. Human[a], C.R. Archer[a], E.E. du Rand[b], C.W.W. Pirk[a] and S.W. Nicolson[a]*

[a]*Department of Zoology and Entomology, University of Pretoria, Pretoria 0002, South Africa*

[b]*Department of Biochemistry, University of Pretoria, Pretoria 0002, South Africa*

*Corresponding author.

E-mail address: swnicolson@zoology.up.ac.za

Abstract

The effects of pesticides on honeybee larvae are less understood than for adult bees, even though larvae are chronically exposed to pesticide residues that accumulate in comb and food stores in the hive. We investigated how exposure to a plant alkaloid, nicotine, affects survival, growth and body composition of honeybee larvae. Larvae of *Apis mellifera scutellata* were reared *in vitro* and fed throughout development on standard diets with nicotine included at concentrations from 0 to 1000 µg/100 g diet. Overall mortality across all nicotine treatments was low, averaging 9.8 % at the prepupal stage and 18.1 % at the white-eyed pupal stage, but survival was significantly reduced by nicotine. The mass of prepupae and white-eyed pupae was not affected by nicotine. In terms of body composition, nicotine affected water content but did not influence either protein or lipid stores of white-eyed pupae. We attribute the absence of consistent negative effects of dietary nicotine to detoxification mechanisms in developing honeybees, which enable them to resist both natural and synthetic xenobiotics.

Keywords: *Apis mellifera scutellata*; body composition; *in vitro* rearing; larval development; lipids; pesticides; protein

1. Introduction

No single causative factor has been identified as responsible for global declines in honeybee (*Apis mellifera* L.) populations (Potts et al., 2010; vanEngelsdorp and Meixner, 2010; Vanbergen et al., 2013). However, there is general agreement that pesticides, whether acting alone or in combination with other stressors, are major threats to pollinators. These include pesticides of environmental origin, encountered by honeybees foraging for nectar, pollen and water, and also miticides used by beekeepers in the control of the *Varroa destructor* mite (Mullin et al., 2010) and small hive beetle *Aethina tumida* (Kanga and Somorin, 2011). They may result in direct poisoning (e.g. Suchail et al., 2000) or subtle sublethal effects. For example, sublethal neonicotinoid exposure can impair learning and memory in honeybee foragers (Williamson and Wright, 2013), and combined pesticide exposure reduces the foraging performance and colony growth of bumblebees (Gill et al., 2012). Pesticide exposure can increase vulnerability to a range of other stressors: for example, laboratory studies have shown increased worker bee mortality and energetic stress due to interactions between *Nosema ceranae* infection and sublethal doses of pesticides (Alaux et al., 2010a; Vidau et al., 2011). At the colony level, previous exposure to sublethal doses of neonicotinoid led to higher infection levels after challenge with *N. ceranae* (Pettis *et al*. 2012).

Compared to adult bees, relatively little work has examined the effects of pesticides on bee larvae; see review for neonicotinoids by Blacquière et al. (2012). However, high levels of agrochemical and miticide residues, including neonicotinoids, have been measured in pollen loads, bee bread, honey and propolis, as well as in the wax cells in which larvae develop and pollen is stored (Chauzat and Faucon, 2007; Chauzat et al., 2006; Mullin et al., 2010; Pettis et al., 2013; Wu et al., 2011). Wax may be a sink for hydrophobic chemicals (Chauzat and Faucon, 2007), which facilitates the transfer of pesticides from contaminated to uncontaminated comb (Wu et al., 2011). Larvae, far from being protected from pesticides in the colony, may thus be chronically exposed to an accumulation of chemical residues with potential additive or synergistic effects. There is evidence for delayed development of larvae reared in comb containing pesticide residues (Wu et al., 2011), and dose-dependent effects on rates of capping, pupation and eclosion were recorded by Yang et al. (2012) when they added imidacloprid solutions directly to cells in the hive containing newly emerged larvae. Interestingly, sublethal doses of imidacloprid used in this study did not affect larvae but did impair learning and memory in the resulting adult bees. In more controlled studies using *in*

vitro rearing of larval honeybees, adverse effects of pesticides often manifest as reduced survival and body mass (Aupinel et al., 2007; Davis et al., 1988; Hendriksma et al., 2011; Zhu et al., 2014). The latter may be due to reduced nutrient assimilation (Bentz and Barbosa, 1990); alternatively, detoxification may be energetically costly, using resources that could otherwise be diverted to growth and development. Tolerance of dietary toxins is most likely due to the presence of metabolic detoxification mechanisms mediated in part by P450 enzymes (Johnson et al., 2012; Mao et al., 2011).

The systemic neonicotinoids, which have been detected as residues in nectar and pollen, have been blamed for colony losses and shown to affect bees negatively at both individual and colony levels (Blacquière et al., 2012; Decourtye and Devilliers, 2009). Until the development of this class of synthetic insecticides, the chemically related alkaloid nicotine was widely used as an insecticide, and still plays a role in organic farming (Casanova et al., 2002). Both nicotinoids and the related neonicotinoids target nicotinic acetylcholine receptors, but neonicotinoids have the advantage of selective toxicity for insects over vertebrates (Matsuda et al., 2001). Previously, we have found that survival of caged *Apis mellifera scutellata* workers was unaffected by 30 µM (5 ppm) nicotine in sucrose diets, and actually improved in the case of weak colonies (Köhler et al., 2012a). At the colony level, Singaravelan et al. (2006) fed honeybees nicotine in sucrose solutions for 26 days: hatching success and larval survival were not affected by nicotine levels up to 30 µM, which can occur naturally in floral nectar of *Nicotiana* species (Adler et al., 2012).

Here we investigate the effect of dietary nicotine on the survival and development of *A. m. scutellata* larvae. Because nicotine may affect larval development, either directly through nutrient assimilation or indirectly through the costs of detoxification, we predicted that (1) nicotine will reduce survival, slow down development time and reduce prepupal mass; and (2) that nicotine will affect nutritional state, indicated by lipid and protein content at the white-eyed pupal stage.

2. Materials and Methods

2.1. Larval rearing

Frames of *A. m. scutellata* were collected from colonies housed at the experimental farm of the University of Pretoria, South Africa (25º 45’ 11’’S, 28º 15’ 29’’E). Two day old worker honeybee larvae were grafted onto larval food in 48-well microtiter plates (Thermo Fisher Scientific, Rochester, New York), using a protocol slightly modified from Aupinel et al. (2005); see also Crailsheim et al. (2013). The grafted larvae were kept in an incubator (Memmert HCP 108, GmbH + Co. KG, Schwabach, Germany) under constant darkness at 34°C and 95% relative humidity (days 1 to 6). At the prepupal and pupal stages relative humidity was decreased to 80% and 70% respectively. Excluding eight larvae that died after initial grafting, an experiment consisted of five treatments: one control (n=56 larvae per plate) and four nicotine concentrations (n = 48). For each replicate a different colony (n = 5) was used, giving a total of 1240 larvae.

2.2. Larval diets

Three standard diets (A, B and C) were used for the *in vitro* rearing of larvae. All diets contained 50% (w/w) fresh royal jelly (Stakich Inc., USA), glucose and fructose (6 %, 7.5 % and 9 % of each in diets A, B and C), and yeast extract (1 %, 1.5 % and 2 % in diets A, B and C). The four treatment diets were prepared by adding nicotine (1.01 mg/µl, N3876, Sigma Aldrich, Germany) to standard diets (A, B and C) at concentrations of 10, 50, 500 and 1000 µg/100 g diet. All larvae received the standard larval diet (A) without nicotine for the first 24 h after grafting and were thereafter fed on a daily basis with fixed amounts of pre-warmed control or nicotine-containing diets; diet A on day 1 and 2 (10 µl per larva), diet B on day 3 (20 µl) and diet C on days 4-6 (30, 40 and 50 µl respectively).

2.3. Survival, development and body mass

Larvae were removed from the incubator on a daily basis, examined under a stereo microscope and mortality assessed. Dead larvae were removed from the wells and surviving larvae received fresh food. After defaecation, observed as yellow excretions, larvae were transferred into clean pupation plates. Development of larvae was recorded until they reached

the white-eyed pupal stage. Randomly selected prepupal stage larvae (n = 10 per plate) were carefully weighed to 0.1 mg **(**Mettler Toledo MS204S, Switzerland**)** before the transfer to pupal plates and weighed again at the white-eyed pupal stage.

2.4. Body composition: water, lipid and protein

White-eyed pupae were sampled and killed by freezing at -80°C for determination of body water and lipid content, and for protein analyses (n = 6 each).

Water and lipid contents were analysed using gravimetric methods. White-eyed pupae were weighed to measure fresh mass, pricked four times with a sterile pin and then dried in glass vials at 60°C. When pupae had reached a constant mass, 1 ml of Folch's reagent (chloroform: methanol 2:1 v/v) was added to each vial using a glass pipette. After 24 h this solvent was removed using a 15 gauge needle and then replaced. After three washes with solvent, the remaining Folch's reagent was allowed to evaporate in a fume hood for 24 h before samples were returned to the drying oven. After 72 h, these white-eyed-pupae were re-weighed to give lipid-free dry mass. The water in each sample was calculated by subtracting the dry mass from the fresh mass of each sample, while the lipid content is the difference between the initial and final dry mass.

For soluble protein determinations, frozen white-eyed pupae were washed with ice cold, double distilled water to remove diet contamination before freeze drying for three days. Pupae were subsequently manually homogenised with a glass rod in 200 µl of lysis buffer, consisting of 40 mM Tris, 7 M urea, 2% (v/v) Triton X-100 and Complete Protease Inhibitor Cocktail tablets (Roche Diagnostics, Mannheim, Germany). These samples were then sonified for 30 min and centrifuged for 10 min at 13 000 x g. The supernatant was collected and diluted 100 fold in a Tris-based buffer, and total protein content was determined by the Bradford assay (Bradford, 1976) using serial dilutions of bovine serum albumin standard (Sigma-Aldrich, Germany) to establish a standard curve. Absorbance was measured at 595 nm using a BioTek Eon microplate reader (Analytical and Diagnostic Products, Johannesburg, South Africa).

2.5. Statistics

Mortality of prepupae and the proportion of larvae reaching the white-eyed stage by day 9 were compared using Chi^2 tests. Body mass was compared using Kruskal-Wallis ANOVA and a multiple comparison of mean ranks for all groups, with nicotine concentration as the grouping variable. Non-parametric statistics were performed since the dependent variable violated the assumptions, normal distribution and homogeneity of variance for parametric test statistics (Pirk et al., 2013). For water, protein and lipid contents of white-eyed pupae, Kruskal-Wallis ANOVAs with multiple comparison of mean ranks for all groups were conducted with nicotine concentration as the grouping, the percentages of water, protein and lipid as dependent variables. Since the variable mass did not violate any assumptions for parametric statistics, mass was compared using parametric ANOVA with nicotine concentration as the independent variable. Significance level was $P \leq 0.05$; data are presented as means ± SD.

3. Results

3.1. Survival and body mass

Development time was not significantly affected by nicotine ($\chi^2 = 1.65$, df = 4), although the number of larvae reaching the white-eyed pupal stage (n=1016) at day 9 ranged between 36%-47%, with the highest nicotine concentration giving the lowest percentage. The overall observed mortality (out of 1240) was low: 9.8 % at the prepupal stage and 18.1 % at the white-eyed pupal stage.

For white-eyed pupae the Chi^2 test to evaluate differences in mortality was significant ($\chi^2 = 9.97$, df = 4, $P < 0.041$; n = 5 x 248 larvae; Fig. 1). The highest mortality of white-eyed pupae was found for the two highest nicotine concentrations. There were no significant effects of nicotine on the mass of prepupae ($F_{4,245} = 0.71$, $P < 0.59$) or white-eyed pupae ($F_{4,180} = 0.45$, $P < 0.77$) (Fig. 2).

3.2. Body composition: water, lipid and protein

Water, lipid and protein contents of the white-eyed pupae are shown in Fig. 3. Nicotine had no effect on either lipid as a percentage of dry mass ($H_{4,149} = 2.99$, $P < 0.56$) or protein as a

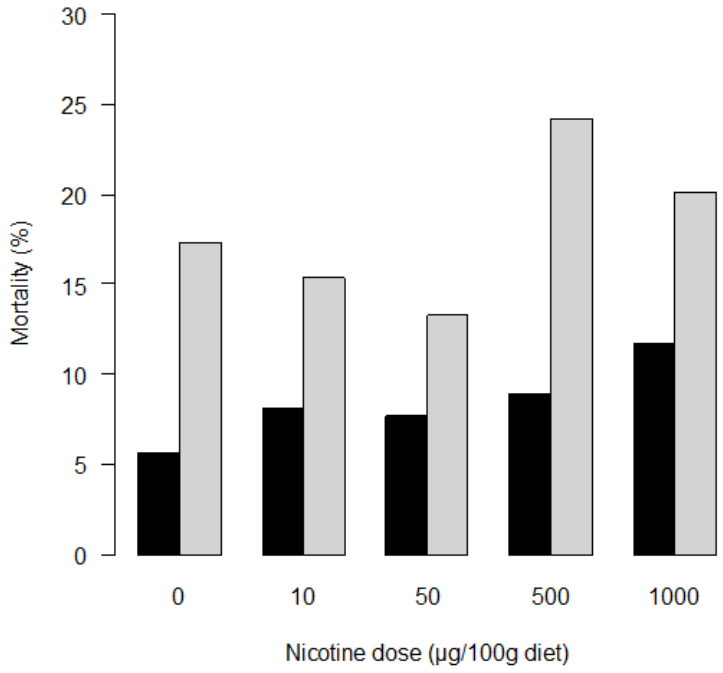

Fig. 1. Effect of nicotine concentration on the mortality (% of larvae that survived initial grafting) of prepupae (black bars) and white-eyed pupae (grey bars For white eyed pupae, mortality was significantly affected by nicotine concentration.

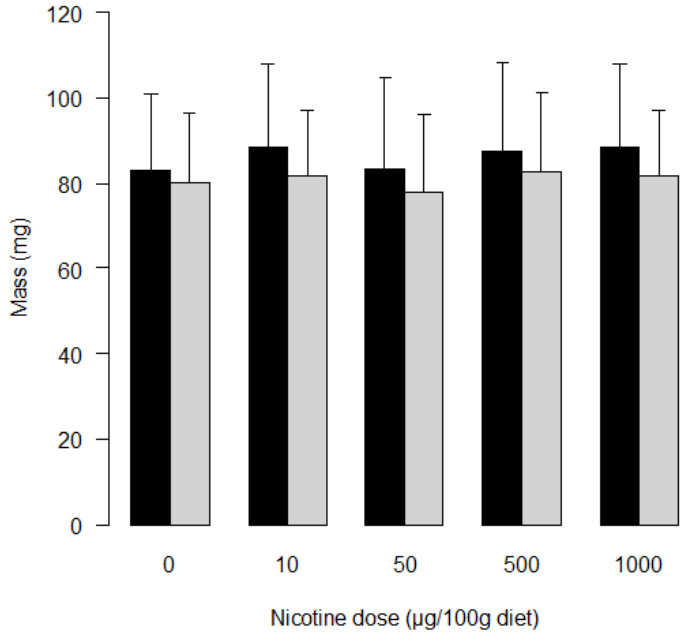

Fig. 2. Effect of nicotine concentration on body mass (mg) of prepupae (black bars) and white-eyed pupae (grey bars). Data are presented as means ± SD. Body mass of both prepupae and white-eyed pupae was unaffected by nicotine.

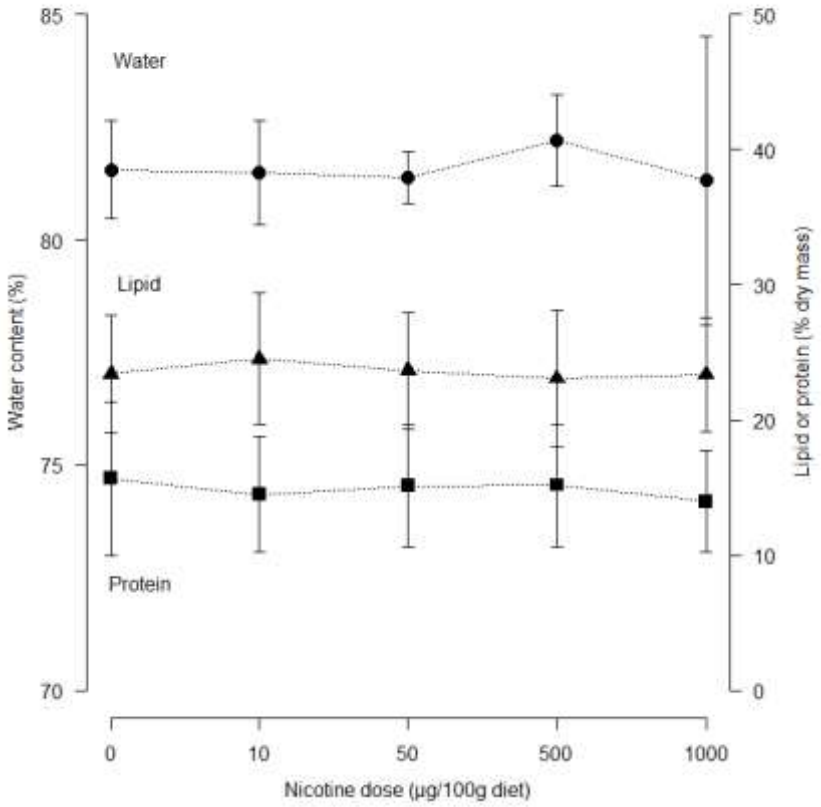

Fig. 3. Effect of nicotine concentration on body composition of white-eyed pupae: water content (% wet mass, ●), protein content (% dry mass, ▲) and lipid content (% dry mass, ■). Data are presented as means ± SD. Lipid and protein contents were unaffected by nicotine. However, water content was significantly lower on 50 µg/100 g nicotine than on a concentration of 500 µg/100 g.

percentage of dry mass ($H_{4,149} = 0.86$, $P < 0.94$); however, it had an significant effect on the water content of pupae ($H_{4,149} = 10.95$, $P < 0.03$) with a nicotine concentration of 50 µg/100 g resulting in a significantly lower water content than a concentration of 500 µg/100 g ($Z = 3.09$, $P < 0.02$).

4. Discussion

Although we are beginning to understand how pesticides interact with a range of other environmental stressors in adult honeybee workers to drive population declines, our understanding of larval responses to pesticide exposure is comparatively poor. Given that larval health is vital to colony survival in honeybees, we must improve our understanding of how larvae respond to pesticide exposure. Here, we find that larvae of *A. m. scutellata* were

remarkably unaffected by the inclusion of nicotine in their diet, even at fairly high concentrations. Although survival was reduced in white-eyed pupae fed high doses of nicotine, pupae were able to tolerate lower doses without experiencing elevated mortality. Nevertheless, water content of the larvae was significantly affected by nicotine concentration. In the absence of a clear trend (Fig. 3), we could speculate that the effect is caused by detoxification processes involving the P450 cycle resulting in production of metabolic water. Additionally, despite possible metabolic costs of detoxification, nicotine did not have deleterious effects on development time, growth or body composition. Here we discuss the potential mechanisms that may underpin this resistance to nicotine.

Nicotine can be present in both pollen and nectar of *Nicotiana* species (Detzel and Wink, 1993; Adler et al., 2012) but bees are more likely to be exposed through its continuing use as a botanical insecticide (Casanova et al., 2002). The lack of consistent, negative effects of nicotine on the survival of developing honeybee larvae supports previous studies showing limited effects of nicotine in sucrose solutions on survival of caged workers (Köhler et al., 2012a) and on hatching success and survival of honeybee larvae in 'minihives' maintained in enclosures (Singaravelan et al., 2006). The latter authors, however, found that 50 ppm nicotine reduced larval survival, with the highest mortality in day 3 larvae: this is 5x higher than the highest concentration in our experiments. Increasingly it appears that the association between nicotine and honeybee survival is complex. Recently, we have found (Archer et al., in press) that low temperatures and nicotine interacted to reduce survival in African honeybee workers fed low protein diets, but that nicotine exposure alone did not reduce survival. Under certain conditions nicotine can even improve survival (Köhler et al. 2012a), perhaps due to its antipathogenic effects (Zaidi et al., 2012). Although little is known of the effects of xenobiotics on solitary bees it appears that solitary bees may also show some resistance to toxin exposure. For example, the nectar alkaloid gelsemine had no effect on offspring performance of *Osmia lignaria* when provisions were supplemented with gelsemine-containing nectar (Elliott et al., 2008). Abbott et al. (2008) found little effect of imidacloprid on *O. lignaria* or clothianidin on *Megachile rotundata* when they injected these neonicotinoids into pollen provisions or fed the larvae with contaminated pollen.

To help clarify the effects of toxins on bee larvae it is vital that we adopt standardised methodological approaches. This is because the dose, time of first exposure and duration of exposure may all influence the response of bee larvae to pesticides (Davis et al., 1988; Davis,

1989). Chronic feeding of the toxin, as in our study, ensures accurate measurement of doses of the tested substance, whereas with a single feeding the volume ingested is unknown, because bees are always provided with a surplus of food (Aupinel et al., 2005). The *in vitro* larval rearing technique has been standardised for assessing effects of pesticides on honeybee brood; acute effects (24/48 h) are investigated by including the toxin in the diet on day 4 of larval development (second instar), and chronic effects by feeding the toxin from day 1 to day 6 (Aupinel et al., 2007; Zhu et al. 2014). Interestingly, Zhu et al. (2014) recorded the greatest mortality after four days of chronic feeding of pesticides to honeybee larvae; this toxicity is likely to be undetected in a conventional assay of acute effects.

We predicted that, even in the absence of effects on survival, nicotine might reduce larval growth and development, either because it reduces nutrient assimilation efficiency or because the energetic demands of detoxification drive trade-offs between investment in detoxification and growth. In keeping with this, dietary nicotine has negative effects on the development and digestive physiology of caterpillars, but researchers have tended to use higher concentrations of nicotine in artificial diets than those in our study. Studies on the tobacco hornworm *Manduca sexta* have used 0.1% or 0.5% wet weight, based on the normal range found in tobacco (Bentz and Barbosa, 1990; Harvey et al., 2007). An even higher concentration of 0.5% dry mass in diets of *Spodoptera eridania* had adverse effects on growth efficiency, and elevated respiration rates suggested a metabolic cost to detoxification of nicotine (Cresswell et al., 1992). However, the moderate doses of nicotine used in our study had no effect on lipid or protein reserves in bee larvae, indicating that they can tolerate toxin exposure without incurring substantial metabolic costs. Similarly, consumption of gelsemine by bumblebees had no effect on oocyte development as an indicator of protein utilization or on haemolymph carbohydrate concentrations, used as a proxy for the energetic cost of ingesting gelsemine (Manson and Thomson, 2009).

There are few data available on the body composition of honeybee larvae. Hrassnigg and Crailsheim (2005) reviewed differences in the physiology of drones and workers, and provided tables summarising the content of water, glycogen, lipids and protein for different life stages. Many of the cited references are very old. We measured protein averaging 23.7% dry mass, similar to the approximately 20% dry mass recorded by Hepburn et al. (1979) for prepupae of *A. m. scutellata*. These authors used the method of Lowry et al. (1951) which, like the Bradford method, is a colorimetric assay that measures the concentration of protein in

solution based on a protein-dependant colour change (due to a dye complexing with proteins). In contrast, use of total nitrogen (determined by the Kjeldahl method) as an indicator of body protein gave a much higher value of 40% dry mass (Melampy et al., 1940): this includes other sources of nitrogen such as chitin and uric acid (although the latter should be minimal after defaecation). This lack of data means it is not clear how energy metabolism and storage are affected by exposure to pesticides. However, Derecka et al. (2013) found changes in lipid metabolism in honeybee larvae when hives were treated with low doses of imidacloprid. The stress resistance of bee larvae may be influenced by diet composition. For example, higher protein may benefit insects that need to upregulate their P450 enzymes and other detoxification pathways. In adult honeybees, Wahl and Ulm (1983) showed that the quality of pollens fed to young bees affected their subsequent response to pesticides, and Alaux et al. (2010b) found effects of pollen diets on immunocompetence. The resistance of laboratory-reared larvae to fungal parasites in the genus *Aspergillus* is enhanced by supplementing the diet with pollen if the royal jelly component of the larval diets is reduced (Foley et al., 2012). It is possible that our larvae may have shown stronger effects of nicotine if they were reared on a less than optimal diet – as we found with adult bees (Archer et al., in press). There is also a need to look at nicotine in combination with other stresses: the study by Köhler et al. (2012b) reported a decrease in worker survival when workers were exposed to the stress of dietary nicotine in combination with lipopolysaccharide (LPS) injection.

At the metabolic level, resistance mechanisms of insects to both plant toxins and synthetic insecticides involve the induction of detoxification enzymes such as cytochrome P450s, which transform xenobiotics into less toxic and more soluble compounds that can be excreted (Després et al., 2007). The lack of effect of nicotine on honeybee larvae may be due to good detoxification abilities (du Rand et al., unpublished data). Compared to other insect genomes, the honeybee genome encompasses far fewer xenobiotic-metabolising P450 genes (Claudianos et al., 2006). However, an expansion is observed in the CYP6AS subfamily (Mao et al. 2009) and a similar expansion in the CYP6 family in lepidopteran genomes is related to the xenobiotic detoxification ability of these insects (Li et al., 2003). The dominance and redundancy of the CYP6AS family in the honeybee genome may in part be responsible for the ability of bees to detoxify both natural (Mao et al., 2009) and synthetic (Mao et al., 2011; Suchail et al., 2003) xenobiotics present in their diet. An alternative - but not mutually exclusive - explanation for the resistance to pesticides suggested by our results could be the higher genetic variability of African honeybee populations. Although all major

stressors are present, this may have resulted in selection for more resistant honeybees (Dietemann et al 2009). This could be tested by experiments comparing the response to xenobiotics of both larval and adult stages of different honeybee subspecies.

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