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Cell death localization *in situ* in laboratory reared honey bee (*Apis mellifera* L.) larvae treated with pesticides

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ABSTRACT

In this study, cell death detected by DNA fragmentation labeling and phosphatidylserine (PS) localization was investigated in the honey bee (*Apis mellifera* L.) midgut, salivary glands and ovaries after treating larvae with different pesticides offered via an artificial diet. To do this, honey bee larvae reared in an incubator were exposed to one of nine pesticides: chlorpyrifos, imidacloprid, amitraz, fluvalinate, coumaphos, myclobutanil, chlorothalonil, glyphosate and simazine. Following this, larvae were fixed and prepared for immunohistologically detected cellular death using two TUNEL techniques for DNA fragmentation labeling and Annexin V to detect the localization of exposed PS specific *in situ* binding to apoptotic cells. Untreated larvae experienced ~10% midgut apoptotic cell death under controlled conditions. All applied pesticides triggered an increase in apoptosis in treated compared to untreated larvae. The level of cell death in the midgut of simazine-treated larvae was highest at 77% mortality and statistically similar to the level of cell death for chlorpyrifos (65%), imidacloprid (61%), myclobutanil (69%), and glyphosate (69%) treated larvae. Larvae exposed to fluvalinate had the lowest midgut columnar apoptotic cell death (30%) of any pesticide-treated larvae. Indications of elevated apoptotic cell death in salivary glands and ovaries after pesticide application were detected. Annexin V localization, indicative of apoptotic cell deletion, had an extensive distribution in the midgut, salivary glands and ovaries of pesticide-treated larvae. The data suggest that the tested pesticides induced apoptosis in tissues of honey bee larvae at the tested concentrations. Cell death localization as a tool for a monitoring the subclinical and sub-lethal effects of external influences on honey bee larval tissues is discussed.

1. Introduction

Globally, the environment around honey bee (*Apis mellifera*) colonies can be contaminated with toxic chemicals from industrial, agricultural and domestic activities. In many cases, these chemicals are pesticides which encompass an array of compounds designed to repel or kill insects (insecticides), plants (herbicides), fungi (fungicides) and other organisms considered pests. Though honey bees are non-target organisms for most pesticide applications, they nevertheless can be exposed to pesticides while collecting pollen and nectar from flowers, collecting resins from various plants, drinking water from rivers/lakes/ponds/etc., breathing, and during flight (if the pesticides are airborne). These pesticides may be brought back inadvertently to the colony where their levels are concentrated further in the waxy nest infrastructure. In surveys of North American honey bee colonies conducted in 2007 and 2008, investigators found 121 different pesticides and metabolites in wax, pollen, bees, and corresponding hive samples [1], thus illustrating the need to understand how pesticides may affect individual honey bees and the social colonies in which they reside.

Many of the pesticides to which honey bees are exposed have insecticidal properties and may be harmful to bees. For example, pesticides are known to lower the developmental rate of queen honey bees, increase the occurrence of queen rejection, and lower queen weight [2–4], affect honey bee cardiotoxicity [5], and affect forager bee mobility and communicative capacity [6], among other effects documented in the literature. In our effort, we broaden the study of pesticide effects on honey bees by investigating pesticide effects on cell death and localization in pesticide-treated, honey bee larvae.

There are many reasons to look at pesticide effects in bee larvae tissues. First, toxic effects of pesticides have been shown to manifest in mammalian tissue and alter enzymatic levels, blood biochemistry and tissue histology [7], thus providing evidence that toxins can affect tissues in pesticide-exposed organisms. Second, histological changes in treated individuals provide a rapid detection method for the effects of toxicants, especially chronic irritants, in various tissues and organs [8]. Third, many of the studies where

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the effects of pesticides on honey bees are discussed focus on toxin effects on adult bees rather than immature ones, resulting in a lack of information concerning the latter. Fourth, previous immunocytochemical studies of cell death and the localization of heat-shock proteins in larval honey bee tissues after acaricide application have fostered a better-understanding the adverse effects acaricides may have on bees [9–11]. Finally, there is an overall lack of histopathological studies on pesticide treated animal tissues. For all of these reasons, we studied the effects of pesticides on larval honey bees at the cellular level.

To determine pesticide effects on the cellular tissues of larval honey bees, we looked specifically at unintentional cell death (necrosis) and programmed cell death (apoptosis) [12]. Necrotic cell death is induced by external influences with evident morphological changes: i.e. the chromatin condenses and clumps are formed at the nuclear periphery [12]. Necrosis refers to the *post mortem* changes that occur following the death of the cell [13]. Apoptosis on the other hand presents a range of morphological symptoms including cell shrinkage and chromatin margination, the latter of which is followed by DNA fragmentation and the formation of apoptotic bodies [14]. Apoptosis originally was defined as the physiological death of cells and tissues associated with developmental remodeling [15] and can be induced by genetic [16] and non-genetic [17] means.

We used multiple cell death assays to determine the effects of various pesticides on honey bee larvae. The first method we used to determine the progression of cell death *in situ* was the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling) method which assesses DNA breakdown preceding the nuclear collapse of apoptotic nuclei [18] and consists of the visualization of fragmented DNA in the nucleus [19]. Cell death previously has been characterized using the terminal TUNEL technique method in the honey bee midgut [10,20] and larval salivary glands [11] where the death of salivary gland tissues in honey bee larvae was detected [21]. We decided to use two TUNEL methods in our experiment because others have provided data which show that different TUNEL kits can indicate different levels of cell death in target tissues [10]. For example, the *in situ* cell death detection kit AP was unable to differentiate between apoptosis and necrosis in different human tissues [22]. Furthermore, DNA fragmentation and a TUNEL-positive reaction can occur after different kinds of cell death using various kits. Regardless, immunocytochemical methods assaying DNA fragmentation [24] are useful techniques for detecting impending apoptosis due to larval exposure to pesticides while feeding [25].

The second method we employed to monitor cell death was through our use of Annexin V to detect the localization of exposed phosphatidylserine (PS) specific *in vivo* binding to apoptotic cells. In dying cells, PS is externalized actively to the plasma membrane's outer leaflet parallel to the extracellular environment [26]. Most forms of cell death share the phenomenon of cell surface expression of PS [27]. Externalization of PS is an early event in the sequence of steps leading to cell death which starts well before changes in the cell nuclei and plasma membrane integrity are compromised [28]. PS on the cell surface can be detected using Annexin V, a member of the annexin protein family that binds in a calcium-dependent way to PS-containing membranes [29]. The Annexin V affinity assay discriminates among living cells, cells in the early phase of cell death and (secondary) necrotic cells that have a compromised cell membrane [30].

In our study, induced cell death and PS localization was investigated in honey bee midguts after treating larvae with one of nine different pesticides offered via an artificial diet. The tested pesticides (with insecticide class in parentheses) included 2 fungicides [myclobutanil (azole), chlorothalonil (substituted benzene)], 2 herbicides [simazine (triazine), glyphosate (phosphonoglycine)], and 5 insecticides/miticides [fluvalinate (pyrethroid), imidacloprid (nicotinoid), coumaphos (organophosphate), chlorpyrifos (organophosphate), amitraz (amidine)] and represent a range of modes-of-actions and pesticide families. With the exception of glyphosate, all have been found as residues in honey bee colonies [1]. Immunohistological methods using both TUNEL assays and Annexin 5 were employed in order to reduce the probability of extraneous artifacts [25], in an attempt to define the specific modes of cell death, and for the broad quantification of cell death observed in larval midguts. We hypothesized that increased apoptotic cell death (determined using the TUNEL technique) occurs in pesticide-treated larvae in comparison to untreated larvae and that PS exposure on the plasma membrane of apoptotic cells (determined using Annexin V) would be present in pesticide-treated larvae.

2. Materials and methods

2.1. Larval rearing, treatment and sampling

Experiments were conducted at the University of Florida Honey Bee Research and Extension Laboratory, Department of Entomology and Nematology, Gainesville, FL. Queens in three production honey bee colonies housed in 10-frame Langstroth-style equipment were confined to a section of newly-drawn comb using a metal queen excluder cage (~10 × 10 × 3 cm) at time $t = -12$ h. The caged queen and frame were returned to the center of the brood nest where worker bees could access and tend the queen. After 24 h of queen confinement, $t = 12$ h [31,32], we removed the queen from the cage and replaced the cage on the comb as before but this time for 108 h (from $t = 0$) to allow the eggs to hatch and larvae to reach an appropriate age for grafting. During this time, worker bees were able to access the comb to feed the developing larvae. At 108 h, we removed the test frames (now containing 36 ± 12 h old larvae) from the colonies and took them to the laboratory.

At the laboratory, the larvae were grafted to sterile, 96-well tissue culture plates (well volume = 0.32 mL, Fisher Scientific, Pittsburgh, PA, USA). Prior to grafting the larvae into plates, we pipetted 20 μL of larval diet into the bottom of each cell. The diet had a pH that ranged from 4.0 to 4.5 and consisted of 50% royal jelly (Glory Bee Foods, Eugene, OR, USA), 6% D-glucose (Fischer Chemical, Fair Lawn, NJ, USA), 6% D-fructose (Fischer Chemical, Fair Lawn, NJ), 37% double distilled water, and 1% yeast extract (Bacto™, Sparks, MD, USA) by volume [32]. Prior to adding the diet to each cell, we pre-warmed it to 35 °C in an incubator (Percival Scientific Inc., Perry, IA, USA).

Each subsequent day, we transferred larvae to a clean culture plate provisioned with fresh diet. The amount of artificial diet provided to each larva depended on the larva's age. We fed larvae 20 μL of diet at hours 108 and 132, 30 μL on hour 156, 40 μL on hour 180, and 50 μL on hour 204 [33,34]. At 204 h post oviposition (larvae are 132 ± 12 h old), we transferred the larvae to a 48-well plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA, wells were 13 × 17 mm) because the growing larvae were too large to handle delicately in a 96-well plate. Throughout the study, trays containing larvae were incubated in the dark at 35 °C and ~96% RH [31].

To test the effects of pesticides on developing larvae, specific pesticide concentrations were mixed with the larval diet daily for 4 days beginning the second day larvae were in the laboratory (132 h = 60 h old larvae). Nine treatment groups of larvae were established in all, each group being composed of 12 treated larvae. Each group of test larvae was treated with 1 of the following pesticide concentration: 1.6 ppm chlorpyrifos, 400 ppm imidacloprid, 400 ppm amitraz, 200 ppm fluvalinate, 100 ppm coumaphos, 400 ppm myclobutanil, 400 ppm chlorothalonil, 400 ppm