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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

glyphosate, and 400 ppm simazine. The respective pesticide concentrations are at or below LC_{50} values known for honey bee larvae (unpublished data). Originally, we wanted to standardize the dose delivered across all pesticides at 400 ppm to bracket the upper residue limit that any of these pesticides have been found in honey bee colonies [1]. However, chlorpyrifos has a low LC_{50} value and fluvalinate/coumaphos LC_{50} values do not fit standard toxicity curves (unpublished data). As such, these three pesticides were administered at different concentrations than were the other pesticides. All applied pesticides were obtained from Chem Service, West Chester, PA, USA.

Prior to administration to the larval diet, each pesticide was diluted individually in an acetone solvent. The diet/pesticide combinations were prepared and stored in 1.5 mL snap-top plastic vials (Fisher Scientific, Pittsburgh, PA, USA). We included two control groups in the study: larvae feeding on diet containing acetone and larvae feeding on an untreated diet. All larvae were sampled on day 6 ($h = 228$), 24 h after the application of the last pesticide treatment. Sampled larvae were fixed in 10% formalin for 24 h, dehydrated in a series of alcohols and xylene, and finally embedded in paraffin wax as described by Gregorc and Bowen [9]. Sections of 5 μm were cut on a 2030 Rechert/Young Microtome (Cambridge Instrument GmbH, Germany), floated on distilled water at 40 °C, collected on cleaned slides, and kept in an drying oven at 60 °C for ~4 h. Slides then were stored at room temperature until later analyses.

2.2. Immunohistology

The paraffin wax was removed from the tissue sections in three washes of xylene and three washes of absolute alcohol. Sections then were rinsed in phosphate buffer solution (PBS, 0.01 M, pH 7.1) and prepared for staining.

2.3. DeadEnd colorimetric TUNEL system

The DeadEnd system (Promega, Madison, WI, USA) labels fragmented DNA of apoptotic cells *in situ* using the TUNEL assay. After applying proteinase K, the larval sections were incubated with the TdT reaction mixture and then with a horseradish peroxidase-labeled streptavidin solution. Diaminobenzidine (DAB) substrate was applied onto the tissue sections to develop a brown reaction product. The sections were counterstained with Mayer's hematoxylin. Negative control labeling was achieved by substituting the deoxynucleotidyl transferase (TdT) enzyme with PBS.

2.4. In situ cell death detection kit, AP (ISCDDK)

Dewaxed and rehydrated tissue sections were incubated with proteinase K (20 μg/mL in 10 mM Tris/HCl, pH 7.4). Labeling was conducted by covering the tissue section with a TUNEL reaction mixture composed of terminal deoxynucleotidyl transferase (TdT) from calf thymus. TdT enzymes with fluorescein were detected using "converter-AP" consisting of anti-fluorescein antibodies from sheep, conjugated with alkaline phosphatase. The substrate solution was obtained using a Vector® Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA, USA). Sections were incubated with the substrate (AP) and washed in tap water for 5 min. Counterstaining was accomplished by transferring the sections into Mayer's hematoxylin and then rinsing the sections under running tap water. As a negative control, we labeled a subgroup with terminal transferase, rather than TUNEL reaction mixture.

2.5. Quantification of cell type and apoptosis

TUNEL labeled tissue slides were used for quantification of cell type and apoptosis as determined using DeadEnd and ISDDK kits. For each treated group of larvae, approximately 300 total cells from at least three larvae on different slides were counted in random fields within the tissue. The results were expressed as the proportion of cells counted that gave positive staining. To confirm reproducibility, 25% of the slides were chosen randomly and scored twice. The proportion of cells that gave positive staining was analyzed by treatment (9 pesticides and 2 controls) with a one way ANOVA for both staining techniques (DeadEnd and ISDDK). Furthermore, we used a two way ANOVA to test the effects of technique, overall treatment and the interaction of treatment × technique on the proportion of cells with positive staining. Prior to all analyses, the proportion data were transformed with an $a \sin \sqrt{x}$ transformation. The untransformed means are reported in the manuscript. Where necessary, we used Student's *T*-tests to compare means, accepting differences at $P \leqslant 0.05$.

2.6. Immunohistochemical localization of PS

Dewaxed and rehydrated tissue sections were placed in PBS (0.01 M, pH 7.1) and incubated with a primary antibody solution. Rabbit antibodies polyclonal to Annexin V were obtained from Abcam (Abcam Inc., Cambridge, MA, USA). Antibodies were used at a concentration of 2 μg/mL in PBS with 1% bovine serum albumin. After incubating the primary antibodies overnight at 4 °C, the sections were covered with biotinylated universal secondary antibodies for 30 min. Alkaline phosphatase reagent also was applied for 30 min. Both reagents were obtained in the Vecastain Universal ABC-AP kit (Vector Laboratories, Burlingame, CA, USA). The substrate solution was obtained using the Vector® Red Alkaline Phosphatase Substrate Kit (Vector Laboratories, Burlingame, CA, USA). Sections were incubated with the substrate (AP) and counterstaining was accomplished by transferring sections into Mayer's hematoxylin. As a control, no primary antibody was applied to the tissue sections. Sections were mounted in Faramount aqueous mounting medium (Dako, Carpinteria, CA, USA). All slides were examined with a Leica light microscope (Leica Microsystems, Germany) at 400× magnification.

3. Results

3.1. DeadEnd colorimetric TUNEL system

The brown reaction product obtained from the Promega DeadEnd kit indicated DAB-positive, and impending apoptotic cell death in all test larvae. Pesticide specific levels of apoptosis detected in the midgut tissue are shown in Table 1. The DAB reaction product was detected in the midguts of all pesticide-treated larvae in larger percentages than in control larvae fed either a diet containing acetone or pure diet (Table 1). In all DAB-positive cells, the brown reaction product was localized to the nuclei. The largest percentages of DAB-positive cells in the midgut epithelium were observed in larvae exposed to simazine, glyphosate, myclobutanil and amitraz (>60%, Table 1). There were some incongruities between the two TUNEL techniques used to estimate cell mortality, but these usually were orders of magnitude differences in the data because the trends detected by both TUNEL techniques were similar (Table 1). In general, pesticides that resulted in high levels of apoptosis as detected by the ISDDK technique resulted in the same as detected by the DeadEnd technique (Table 1). Notably, fluvalinate, on average, resulted in the lowest level of apoptosis of any tested pesticide (Table 1).

The DAB reaction product was observed in columnar midgut epithelial cells in simazine-treated larvae (Fig. 1A) and in nearly all of

Table 1
Mortality in the midgut columnar cells determined using two TUNEL kits, DeadEnd and ISCDDK. Data are mean ± SE proportion cells positively stained, *N* out of at least 300 cells counted from a minimum of 3 larvae. When both treatments were analyzed together, neither technique (DeadEnd or ISCDDK – $F = 1.1$; df = 1123; $P = 0.29$) nor the interaction between technique and treatment ($F = 1.6$; df = 10,123; $P = 0.13$) affected the proportion of cells positively stained. Data in columns followed by the same letter are not different at $P < 0.05$. Student's *T*-tests were used to compare means.

Type of pesticide	Treatment ↓	DeadEnd technique	ISDDK technique	Both techniques analyzed together
Insecticide	Chlorpyrifos	0.56 ± 0.08, 6abc	0.74 ± 0.04, 6a	0.65 ± 0.05, 12abc
	Imidacloprid	0.51 ± 0.12, 6bcd	0.76 ± 0.02, 4a	0.61 ± 0.08, 10abc
	Amitraz[a]	0.64 ± 0.07, 5abc	0.37 ± 0.03, 4c	0.52 ± 0.06, 9c
	Fluvalinate[a]	0.29 ± 0.04, 7d	0.32 ± 0.02, 3c	0.30 ± 0.03, 10d
	Coumaphos[a]	0.58 ± 0.12, 8abc	0.48 ± 0.03, 5bc	0.54 ± 0.07, 13c
Fungicide	Myclobutanil	0.62 ± 0.11, 5abc	0.78 ± 0.06, 4a	0.69 ± 0.07, 9ab
	Chlorothalonil	0.49 ± 0.08, 7cd	0.66 ± 0.06, 4ab	0.55 ± 0.06, 11bc
Herbicide	Glyphosate	0.74 ± 0.05, 5ab	0.65 ± 0.15, 5a	0.69 ± 0.08, 10ab
	Simazine	0.76 ± 0.06, 7a	0.77 ± 0.06, 7a	0.77 ± 0.04, 14a
Control	Diet with acetone	0.10 ± 0.01, 8e	0.11 ± 0.03, 5d	0.11 ± 0.01, 13e
	Diet only	0.10 ± 0.02, 8e	0.09 ± 0.02, 5d	0.10 ± 0.02, 13e
	ANOVA→	$F = 10.6$; df = 10,71; $P < 0.01$	$F = 16.3$; df = 10,51; $P < 0.01$	Treatment effect: $F = 22.2$; df = 10,123; $P < 0.01$

[a] Used in honey bee colonies to control varroa mites.

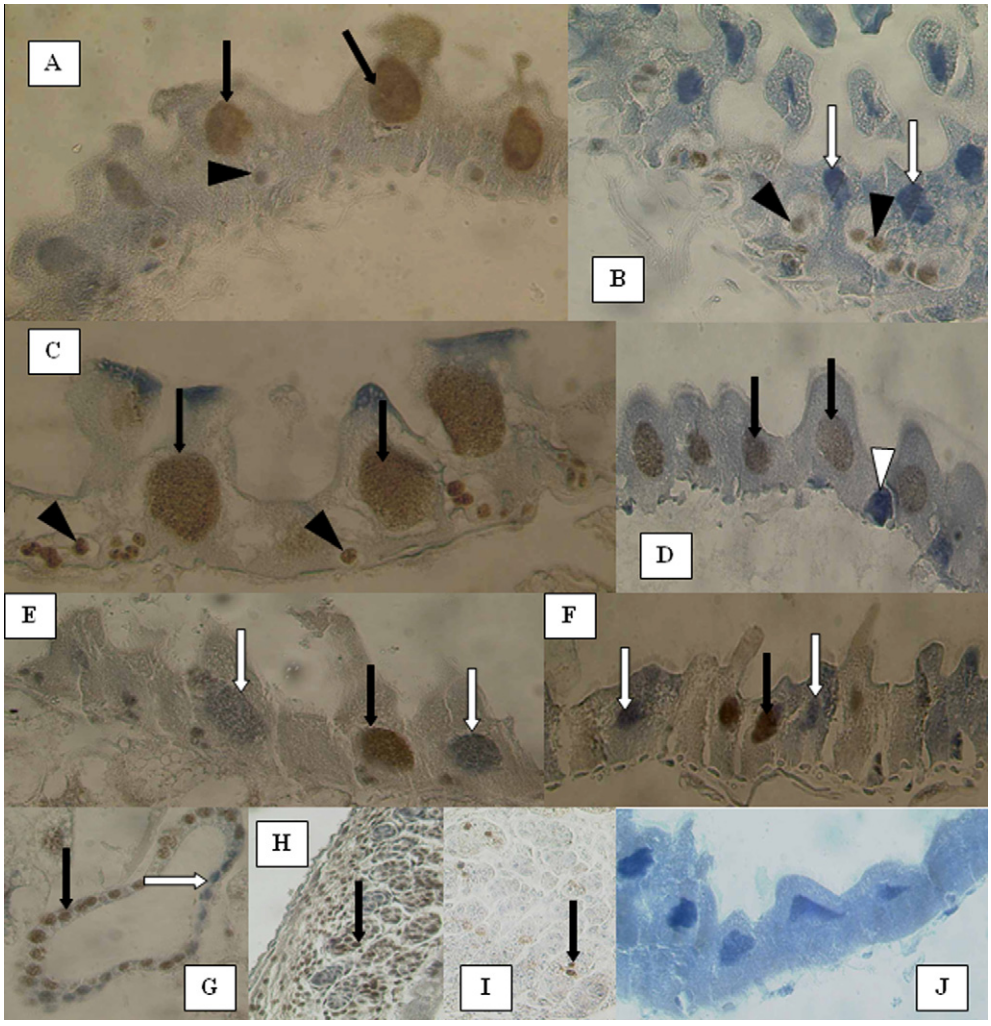

Fig. 1. Staining of formalin-fixed, paraffin-embedded larvae on which the DeadEnd colorimetric apoptosis detection system (Promega, Madison, WI, USA) was used. Larvae were 6-day-old, 24 h after the last of four consecutive daily pesticide treatments and prepared for immunohistology. Peroxidase conjugated anti-digoxigenin secondary antibody and DAB as a substrate were used to obtain a specific brown reaction product. The DAB reaction product localized to the nuclei of the columnar midgut epithelial cells is indicated by black arrows and the DAB reaction product localized to the regenerative cells is indicated by a black arrow head. When the DAB reaction product is absent, either the arrow (columnar midgut epithelial cells) or arrow head (regenerative cells) is white. Panel 1A shows the midgut, of a simazine-treated larva. The DAB reaction product is localized to the nuclei of the most of the epithelial columnar and regenerative cells. Panel B shows chlorpyrifos-treated larva with the DAB reaction product localized to the regenerative epithelial cells but not the columnar ones. Panel C shows an amitraz treated larva where the DAB reaction product was localized in columnar and regenerative epithelial cells. Panel D shows sections of the midgut of an imidacloprid treated larva with the DAB reaction product localized in the nuclei of columnar cells but not in regenerative cells. Panel E shows a chlorothalonil treated larva. The DAB reaction product was found sporadically in the migut columnar epithelial cells. Panel F shows an untreated larva where only ~10% DAB positive midgut epithelial cells were found. Panel G shows an amitraz treated larva where DAB positive and negative salivary glands cells were seen. Panel H shows an imidacloprid treated larva with indicative DAB staining in ovariole nurse cells. Panel I shows a control larva where the DAB reaction was distributed sporadically in ovariole nurse cells. Panel J shows a control section of an imidacloprid treated larva where endogenous peroxidase was quenched successfully and enzyme incubation was omitted. No DAB reaction product was found. Magnification of all panels: 400×.

the regenerative cells in chlorpyrifos-treated larvae (Fig. 1B). Furthermore, there were midgut regions in amitraz treated larvae with both DAB-positive columnar and regenerative epithelial cells (Fig. 1C), and regions in imidacloprid-treated larvae with columnar DAB-positive and regenerative negative cells (Fig. 1D). In larvae with high proportions of DAB-positive cells, the positive cells were localized in compartmental areas of the midgut, but tissues also were observed containing only solitary DAB-positive cells (chlorothalonil treated larvae, Fig. 1E). In untreated larvae, ~10% of the midgut epithelial cells were DAB-positive (Fig. 1F).

The DAB reaction product also was localized in the salivary glands and the ovaries of treated larvae. Salivary gland tissue expressed high levels of DAB-positive cells in larvae exposed to amitraz (Fig. 1G). Similar levels also were found in salivary glands in simazine, imidacloprid, glyphosate, myclobutanil or fluvalinate treated larvae. In the ovarian tissue, high levels of DAB-positive nurse cells were found in imidacloprid-treated larvae (Fig. 1H). In ovaries of larvae treated with the remaining pesticides, the DAB reaction product was found in similar amounts as in ovaries of untreated larvae. At normal tissue turnover, up to 20% of nurse cells were DAB-positive (Fig. 1I). Negative control sections showed no presence of the DAB reaction product, and endogenous peroxidase also was quenched successfully (Fig. 1J).

3.2. In situ cell death detection kit, AP (ISCDDK)

Twenty-four hours after honey bee larvae were exposed to the last of four pesticide treatments, the red azo-dye reaction product was found in increased levels of the midgut columnar-cell nuclei and also in the midgut regenerative-epithelial cells. In chlorpyrifos-treated larvae, the level of positive-reaction product in the columnar midgut cells (Fig. 2A) had risen to ~74% (Table 1). In simazine, myclobutanil, imidacloprid, chlorpyrifos, chlorothalonil and glyphosate-treated larvae, the level of positive columnar epithelial cells with red azo-dye reaction product was ⩾65% (Table 1). Simazine induced localization of red azo-dye reaction product to the columnar and regenerative cells (Fig. 2B). In coumaphos-treated larvae, the reaction product was found in ~48% of all columnar and regenerative epithelial cells (Fig. 2C). The reaction product in the salivary glands was found in myclobutanil-treated larvae, where a majority of cells were positive (Fig. 2D). In untreated larvae, low amounts of reaction products were observed, though sporadic cells were positive (Fig. 2E). The red azo-dye product in the ovarian tissue of all treated and untreated control larvae ranged from 5% to 10% (Fig. 2F).

3.3. Immunohistochemical localization of PS

In the pesticide-treated larvae, the red azo-dye reaction product detected by Annexin V, which characteristically localizes PS, was found to be present abundantly in the midgut epithelium, salivary glands and ovaries. Thus, it was possible to delineate the PS boundary at the apical columnar cell membrane in the brush border and at the basal cell cytoplasm bound to basal membrane by immunostaining of Annexin V. The red azo-dye reaction product was localized and bound to the apical brush border in chlorpyrifos-treated larvae (Fig. 3A). Annexin V staining spread throughout the midgut epithelium cells noticeably, where immunostaining was diffuse and the entire cell cytoplasm of glyphosate-treated tissue was stained (Fig. 3B). In the glyphosate-treated larvae, Annexin V was abundant and PS was localized in the basal and apical cell cytoplasm (Fig. 3C). Staining of the cytoplasm in a group of columnar cells at the basal area was uneven and spotty and bound to the basal membrane in simazine-treated larvae (Fig. 3D). Red azo-dye was present abundantly in salivary glands of mycobutanil (Fig. 3E) and ovaries of glyphosate-treated larvae (Fig. 3F). Staining was less intensive in salivary gland cells in untreated larvae (Fig. 3G). In untreated larvae, Annexin V was present in some sections of the midgut epithelium and immunostaining was bound to the apical and basal cell membrane (Fig. 3H) while the cytoplasm of the midgut cells was not stained. Results indicate that Annexin V binds to cells of the midgut, salivary glands and ovaries of all pesticide-treated larvae abundantly while in untreated larvae Annexin V binding was not as evident. In both groups of control larvae, the general morphology of the epithelium was unchanged.

4. Discussion

Honey bee larvae reared in an incubator and treated with one of nine pesticides undergo subclinical, cellular changes that can be detected using immunohistochemical methods. ISCDDK showed

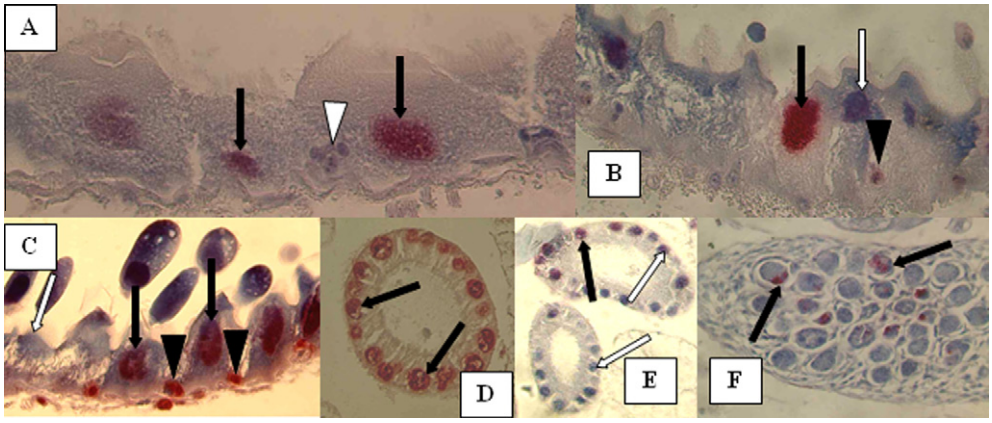

Fig. 2. Sections of formalin-fixed, paraffin-embedded, 6-day-old larvae, 24 h after the last of four consecutive daily pesticide treatments. Cell death was detected using the TUNEL technique ISCDDK (Roche). TdT-mediated dUTP for DNA labeling was employed, followed by the application of anti-fluorescein alkaline phosphatase conjugated antibody, using fast red for visualization, and counterstaining with haematoxylin. Dense red azo-dye staining localized to the nuclei of the midgut epithelial cells, in the salivary gland cells, or in ovary nurse cells indicative of impending cell death is indicated by a black arrow. Reaction product localization to the regenerative cells is indicated by a black arrow head. Where the reaction product is absent, either the arrow (midgut epithelial cells) or arrow head (regenerative cells) is white. Panel A shows a chlorpyrifos-treated larva with red azo-dye staining localized to the midgut columnar epithelial-cell nuclei but not the regenerative epithelial cells. Panel B shows midgut epithelium in a simazine-treated larva. The red azo-dye reaction product localized to the columnar and regenerative cells. Panel C shows a midgut epithelium section of a coumaphos-treated larva. The red azo-dye reaction product localized to the columnar and regenerative epithelial cells. Panel D shows salivary gland tissue of a myclobutanil-treated larva where the majority of cells were alkaline phosphatase positive. Panel E shows untreated, control larvae with red azo-dye reaction product to sporadic salivary gland cells. Panel F shows the red azo-dye product sporadically in nurse cells in ovaries of a simazine-treated larva. Magnification of all panels: 400×.

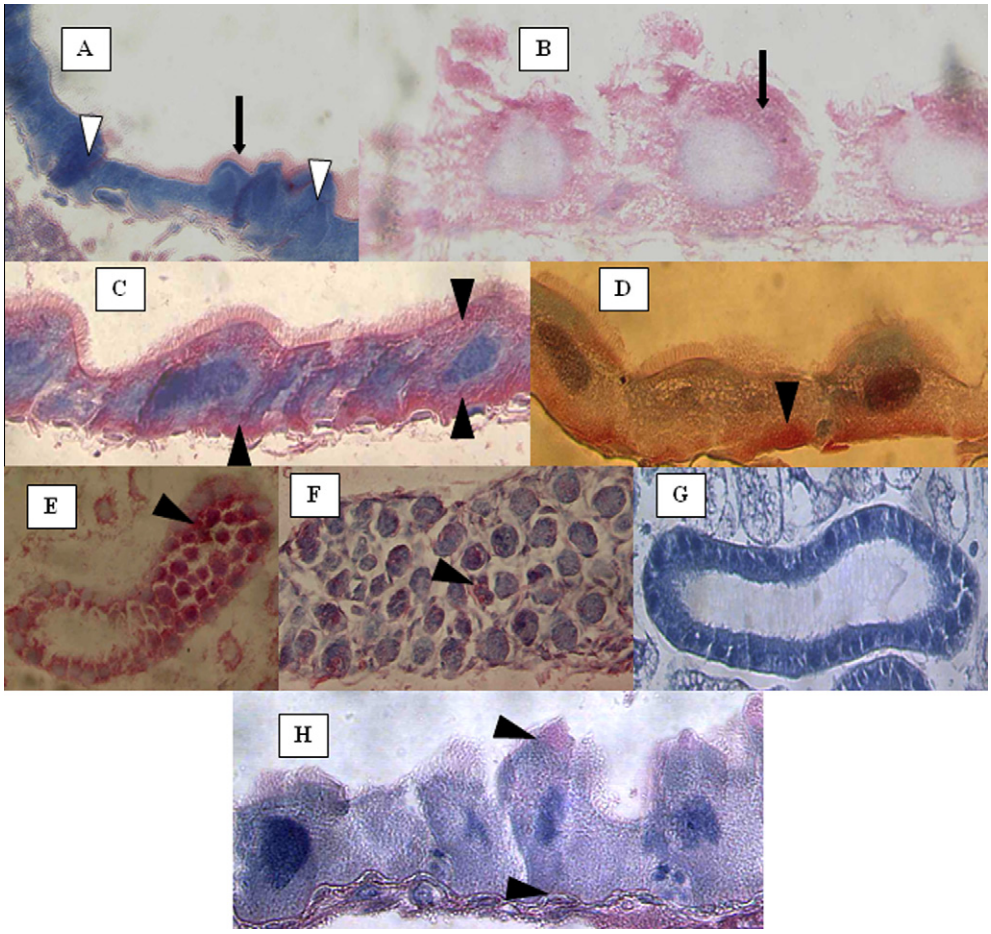

Fig. 3. Immunohistochemical localization of Annexin V cells of formalin-fixed, paraffin-embedded 6-day-old larvae, 24 h after the last of four consecutive daily pesticide treatments. Panel A shows the red azo-dye reaction product bound to the apical brush border (black arrow) in a chlorpyrifos-treated larva. The reaction product was not localized in the midgut cells (white arrow head). Panel B shows cells from a glyphosate-treated larva and indicates Annexin V staining throughout the remaining midgut epithelium cytoplasm (black arrow). Panel C shows a midgut section of a glyphosate-treated larva with red azo-dye staining indicating PS localized to the basal and apical cell cytoplasm (black arrow head). Panel D indicates that an alkaline phosphatase reaction product in a simazine-treated larva was localized to the cytoplasm of the columnar cells at the basal area (black arrow head). Panel E shows red azo-dye localized in the salivary gland tissue of a myclobutanil-treated larva (black arrow head). Panel F shows a glyphosate-treated larva in which red azo-dye was localized to ovarian nurse cells (black arrow head). Panel G shows no intensive staining in the salivary gland tissue of a larva consuming untreated food. Panel H shows cells from an untreated, control larva with red azo-dye reaction product to some sections of the midgut epithelium bound to the apical and basal cell membrane (black arrow head). The cell cytoplasm of the midgut cells was not stained. Magnification of all panels: 400×.

comparable levels of apoptosis with that shown using the DeadEnd kit. Both TUNEL kits indicated induction of DNA strand breaks after pesticide treatments and differences in apoptosis levels in the tissue sections. There were variations in the distribution of apoptosis, which was uneven and inconsistent. ISCDDK was found to demonstrate DNA-fragmentation after both apoptotic and necrotic cell death [22,23].

There were differences in apoptosis appearance in the midgut and apoptotic cells were observed randomly in the epithelium of pesticide-treated larvae. Normal apoptotic cell death level in the epithelium observed in both groups of control larvae (untreated diet and acetone treated diet) was ~10%. Observed elevated death rates in the midgut columnar cells and in ovarian or salivary gland cells of pesticide-treated larvae may be triggered by an apoptotic pathway after pesticide application. All applied pesticides induced significant apoptotic cell death in the larvae midgut as demonstrated through the use of both TUNEL kits. Necrosis, which usually is caused by a lethal accident or disease as opposed to a programmed process, can be detected by TUNEL as found in Orita et al. [23] and in previous experiments where larvae were water-treated [10].

Interestingly, fluvalinate resulted in the lowest levels of observed cell death of any pesticide-treated larvae. Fluvalinate has been used in the US for over two decades to control *Varroa destructor* Anderson and Trueman, the varroa mite. Our data suggest that honey bee larvae may have developed some level of resistance to fluvalinate exposure. Equally interesting is that the herbicides glyphosate and simazine and fungicides myclobutanil and chlorothalonil induced elevated apoptotic cell death in an insect. Though unclear how this may affect honey bees at the individual organism or colony level, the data suggest that herbicides and fungicides cannot be presumed innocuous to bees. Regardless, the level of stress-induced apoptosis related to pesticide treatment in bee larvae in our experiment was comparable to that experienced by two invasive bivalves exposed to a molluscicide [35].

In our experiment, the tested insecticides, fungicides and herbicides induced an elevated level of apoptosis in the larval midgut. In previous experiments, lower concentrations of coumaphos applied to adult worker bees did not trigger increased levels of apoptosis in hypopharyngeal glands compared to that in untreated bees [20]. In contrast, honey bee larvae treated with acaricides experienced apoptosis and stress-induced, necrotic cell deletion [10], indicating that these different types of cell death can occur simultaneously after exposure to pesticides [32].

Follicular maturation during oogenesis involves necrosis along with apoptosis [36] and investigators have shown that necrosis

potentially can accompany apoptosis during normal development as shown in experiments with mouse cell embryos [37]. Thus, necrotic and apoptotic cell death often occur simultaneously during many pathological processes, as seen in the present study, and during normal processes such as tissue renewal, embryogenesis, and immune response.

In our study, we confirmed elevated levels of apoptosis in larvae treated with pesticides. The epithelial-cell nuclei remained morphologically unchanged but became TUNEL-positive, indicating that the DNA was fragmented but not different from neighboring cell nuclei otherwise. It is possible that the induced larval cell apoptosis trigged by pesticide treatment in our study may have been a reversible process in the midgut tissue, one from which the affected larvae could recover. On the other hand, the appearance of apoptosis may precede further tissue deletion, the development of necrosis in the midgut cells, or cell death altogether. The TUNEL method thus is a useful diagnostic tool to monitor subclinical changes in honey bee larvae induced by external influences.

The apoptosis of regenerative cells observed in the basal area of the epithelium of pesticide-treated larvae may function to maintain the proper ratio of cells in the midgut, i.e. large numbers of regenerative cells may die to compensate for the inadequate number of epithelial cells. This apoptotic mechanism has been suggested for *Drosophila* cell mechanisms which cause dying germline and follicle cells in *Drosophila* ovaries [38]. Other investigators observed that the percentage of epithelial cells labeled with digoxigenin using the ISCDDK increased to 70% in 3-day-old larvae when to the larvae were treated with formic acid [39]. The high cell death levels detected using ISCDDK likely indicated accidental cell death leading to necrosis, triggered by necrotic injury [39]. Further studies should be performed to establish whether higher pesticide concentrations can decrease apoptosis and increase necrosis in honey bee larvae and how this may affect clinical symptoms or larvae mortality.

Our data indicate that Annexin V has a widespread distribution in pesticide-treated larvae being found in the midgut epithelium, salivary glands and ovaries. Microscopic analyses on cellular localization of Annexin V would help to obtain information on its function. Intracellular and extracellular localizations of Annexin V have been reported in human cardiac muscle and vary based on changes in disease states [40]. It has also been reported that in the ischemic rat heart, Annexin V leaked from cardiac cells into the extracellular space and that the cardiac cell membrane was stained intensely by the anti-Annexin V antibody [41]. In our study, the varied localization of Annexin V suggests that this protein is related closely to apoptotic cell death in tissues of bee larvae exposed to pesticides. These findings may contribute to a better understanding of potential cell injury during and after pesticide exposure, especially due to the possibility of false-negatives produced using TUNEL kits [28]. Moreover, cell death detection and quantification can be more accurate and potential artifacts can be reduced by using more than one assay [25].

Collectively, our data indicate that the nine test pesticides can induce apoptosis in tissues of honey bee larvae reared in an incubator. The data also suggest that the pesticide concentrations we tested were tolerable to larvae because apoptosis likely was initiated as a protective mechanism in the midgut, salivary glands or ovaries, though further expansion into necrosis, tissue deletion and larval death is a potential development of these events. Future studies will be necessary to explore the effects and modes of action of different concentrations of these pesticides on larvae at the cellular and tissue levels. The quantification of cell death could be used to monitor the subclinical and sub-lethal effects of applied pesticides on larval tissue. Honey bee larvae reared *in vitro* could be used in the future as models for studying the effects of chemicals on living tissues at the cellular level.

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