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Original article

Heat shock proteins and cell death in situ localisation in hypopharyngeal glands of honeybee (*Apis mellifera carnica*) workers after imidacloprid or coumaphos treatment*

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Abstract – Worker honeybees (*Apis mellifera carnica* Polm.) were treated with imidacloprid or coumaphos. Significant effects of treatment and treatment duration were found on hypopharyngeal glands (HPG) acinus diameter ($P < 0.05$). Differences in the size of acini were evident in all long term (48 h and 72 h) treatments. Short term (24 h) imidacloprid treatment induced heat shock protein 70 (Hsp 70) localisation in nuclei and cytoplasm and Hsp 90 activity was found in most cell cytoplasm. Coumaphos triggered an increased level of programmed cell death, and imidacloprid induced extended necrosis in comparison to coumaphos. In 7–12 day old workers, the level of cell death after 48 hours of imidacloprid treatment was approximately 50% and increased to all cells after 72 hours. Programmed cell death remained at the normal level of approximately 10%. Our results suggest that both pesticide treatments have an influence on the reduced size of HPG and also on the extended expression of cell death.

***Apis mellifera* / immunohistochemistry / pesticide / heat-shock / cell death**

1. INTRODUCTION

Honeybee (*Apis mellifera* L.) workers undergo physiological changes in their behaviour with age. According to their age group they perform a succession of different tasks from nurse bees, through activities inside the brood nest, and finally foraging activities (Winston, 1987). Age dependent changes in exocrine glands also occur (Michener, 1974). Workers become nurse bees within 3–5 days whilst processing ingested pollen, 5–12 day old bees receive nectar and after day 16 they become foragers for the colony (Crailsheim, 1998). The morphological structure of the hypopharyngeal glands (HPG) similarly varies depending on the age of the worker bee (Snodgrass, 1956; Dade, 1962). Programmed cell death need not always undergo classical apoptotosis (Clarke, 1990; Schwartz, 1991; Bowen et al., 1996). This type of cell death can be induced by genetic means (White, 1996). Distinctive extracisterneal acid phosphatase is found accompanying programmed cell death in developmental honeybees (Gregorc and Bowen, 1997). Well developed HPG in young nurse bees (Silva de Moraes et al., 1996) are reduced in size in foragers when they undergo fine structural degeneration (Cruz-Landim and Silva de Moraes, 1977). Programmed cell death in the honeybee midgut has been found during normal honeybee development using scanning electron microscopy (Gregorc and Bowen, 1996) and histochemical and immunohistochemical methods (Gregorc and Bowen, 1997) and has also been evaluated histochemically in larval salivary glands (Silva-Zacarin et al., 2006), in hypopharyngeal glands of

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newly emerged nurse and forager worker *A. mellifera* bees (Silva de Moraes and Bowen, 2000) and also in *A. andreniformis* and *A. florea* workers (Suwannapong et al., 2007). DNA breakdown proceeding nuclear collapse of apoptotic nuclei can be tested using the terminal deoxynucleotidyl transferase mediated dUDP nick end labelling, termed the "TUNEL assay" (Levy et al., 1998; Sgonc and Gruber, 1998). The "ApopTag in situ apoptosis kit" can be used to detect digoxigenin-labelled genomic DNA (Maulik et al., 1998) and the Roche "In situ cell death detection kit" can be used to detect both necrosis and apoptosis. Cell death is a general term describing apoptosis and necrosis and two kits used in our previous experiments on honeybees (Gregorc and Bowen, 2000) had different levels of sensitivity in detecting these two forms of cell deletion. The highly sensitive ISCDDK assay detects both in different tissues (Matylevitch et al., 1998), whereas the DeadEnd kit is more specific to apoptotic cell death.

There are several potential ways that honeybees can be exposed to environmental pollutants, for example through water collection, by contact with foliage, or through contaminated pollen and nectar (Rosiak, 2002; Papaefthimiou et al., 2002). In collecting nectar and pollen from flowers, honeybees may contact pesticides deposited upon the plants. The application and proper dosage of pesticides (acaricides) inside the hive to control parasitic mites could also affect brood and adult bees (Atkins, 1998; Gregorc and Bowen, 2000; Gregorc et al., 2004; Gregorc and Smodiš Škerl, 2007) and queens (Pettis et al., 2004). Imidacloprid is a systemic insecticide used for field and horticultural crops and its metabolites have a potential toxicological relevance to honeybees (Schmuck et al., 2003). Characteristics of imidacloprid toxicity in two subspecies of honeybee *A. m. mellifera* and *A. m. caucasica* have been investigated using contact and oral applications (Suchail et al., 2000; Decourtye et al., 2004).

Heat shock proteins (Hsp) function under normal cellular conditions although they are referred to as the so-called heat-shock or stress proteins. They are considered to be essential components in a number of diverse biological processes. They are highly conserved and contribute to stress tolerance (Feder et al., 1995) and are involved in intracellular protein maturation (Georgopoulos and Welch, 1993). The diverse functions of Hsp 70 are illustrated by their activity in the nucleus, cell organelles and cytosol (Chiang et al., 1989), their participation in protein folding and together with specific soluble or membrane bound partner proteins, they are involved in protein traffic, translocation and gene regulation (Rassow et al., 1995). They are upregulated in response to many stressors (Hendrick and Hartl, 1993). Hsp 70 is important in the maintenance of cellular functions under stress situations (Beckmann et al., 1992).

Hsp 90 is reported to be highly specialised in binding protein and has intracellular chaperone properties. It is an abundant type of eukaryotic stress protein (Jakob and Buchner, 1994). Nuclear Hsp 90 has been found in *Xenopus* oocytes that bind tightly to purified histones (Hendrick and Hartl, 1993). Monoclonal antibodies have been used to determine the presence of Hsps in different tissues (Chiang et al., 1989). Anti-Hsp antibodies have been used as markers of the effects caused by toxic metals on terrestrial isopods and terrestrial and marine molluscs (Köhler et al., 1992). Hsps have been described in honeybee larvae after different treatments (Gregorc and Bowen, 1999; Silva-Zacarin et al., 2006).

In this study, we focused on immunocytochemical methods to assay DNA fragmentation and to define the pattern of cell death. We used immunohistochemical methods in order to localise Hsp 70 and Hsp 90 in the HPG of *A. m. carnica* worker bees exposed to pesticides. Another aim of our work was to perform histochemical characterisation of cell death of the HPGs after treating workers with the insecticide imidacloprid or the acaricide coumaphos. Using a combination of Hsp 70 and Hsp 90 and cell death localisation was also hoped to demonstrate whether the applied pesticides were linked in any way to cell damage or death, so we attempted to study the distribution of cell death in treated HPGs. The study of distribution of HSp-s and cell death in HPG of treated bees was considered. Alkaline

phosphatase was employed as a reporter enzyme, fast red for visualization, peroxidase as a reporter enzyme and DAB as a substrate were used in our experiments.

2. MATERIALS AND METHODS

2.1. Treatment of worker honeybees

Worker honeybees (*Apis mellifera carnica* Polm.) emerging from brood comb in an incubator (34.5 °C) were obtained as a mixture from three colonies, and marked on the thorax with a spot of paint. Fifty newly emerged workers per day were marked with the same colour to define their age. Marked bees were put into a queenright host colony with brood of all stages and a population of approximately 10000 workers of diverse age. During the experiment, bee forage conditions were normal for the season. Marked bees were sampled from the hive 30 days after the introduction of the first group of bees. Foragers were collected when returning from flights during peak foraging hours, whilst winter bees were collected in January of the following year. Eight workers from each of four different age groups of bees marked with specific colours were introduced into a wooden cage (7.5 cm × 4 cm × 4 cm) with one side made of clear glass and another of wire mesh. Four separate cages with mixed age groups of bees were established. Age groups were divided as following: first group 1 to 6 days; second group 7 to 12 days; third group 13 to 18; fourth group 19 to 32 days. The cages with bees were kept in a darkened room at 28 (±1) °C. Two treatment solutions were prepared: (1) a solution of imidacloprid 500 ng/kg in 35% sugar/water solution (0.183 mL of manufactured solution: 3.5 mLGaucho/L); (2) a solution of coumaphos (Perizin® 1 mL in 15 g sugar in 50 mL water; Bayer; 32 mg coumaphos/1 mL Perizin®). A 35% (w/v) sugar solution was also prepared for feeding to control cages. Each of the cages was supplied with either 10 mL of the treatment or control sugar solution in the first feeder and water in a second feeder. Worker bees were exposed to treatment solutions ad libitum for 24, 48 or 72 hours, respectively. After treatment only 35% of sugar/water solution was given. Bees were removed after treatments, cooled for a few minutes and then decapitated under a binocular stereomicroscope. The HPGs were dissected in an insect ringer solution (Hyes ringer), fixed in 10% formalin for 24 h, dehydrated in a series of alcohols and xylene, and then finally embedded in wax as described by Gregorc and Bowen (1999). Sections of 5 μm were cut on a Leica microtome, floated on distilled water (42 °C) and collected on cleaned slides. Slides were then stored until the next procedure.

2.2. Sample preparations for immunohistology

Paraffin wax was removed in three changes of xylene and in three changes of absolute alcohol and sections were then rinsed in PBS and were thus prepared for different staining procedures.

2.3. Morphological measurements of HPG

HPG from all immunohistological procedures, from all age groups and from winter bees, were used for morphological measurements. One to three HPG sections of each age group were measured under a Zeiss microscope at 100 × magnification. The diameter of 10 acini per section was measured in μm.

Data from different age groups were statistically analysed with SPSS version 13 (SPSS Inc.; Chicago, IL, USA). Significant differences in acinal diameter were calculated using factorial analysis of a univariate general linear model. The factors analysed were: age of worker bees at different treatments; and time of treatments. Mean acinus diameter of HPGs was compared among the four age groups using one-way ANOVA with treatments as a factor and further compared with time of treatment as a factor. For data testing significant differences, a Scheffe test was applied.

2.4. Immunohistochemical localisation of heat shock proteins

Dewaxed sections were washed under running tap water and placed in PBS (0.01 M, pH 7.1) and incubated with a primary antibody solution. Monoclonal antibodies against heat shock proteins 70 and 90 (Hsp 70 and Hsp 90) were obtained from Sigma. Antibodies were diluted at 1:400 in Tris buffer (pH 8.2) with 1% bovine serum albumin. After incubating the primary antibodies overnight at 4 °C, the sections were covered with a secondary antibody conjugated with alkaline phosphatase and incubated for 1 hour in a humidified

chamber at room temperature. The EnVision System alkaline phosphatase kit (Dako) was used in accordance with the instructions to obtain a red coloured precipitate. Counterstaining was accomplished by directly transferring the sections into Mayer's haematoxylin, and mounted in Faramount aqueous mounting medium. For the control sections the respective primary antibody was omitted. All slides were examined with a Zeiss light microscope.

2.5. Cell death detection

2.5.1. 'In situ cell death detection kit, AP'

After removal of the Paraffin wax from the tissue section further procedures were conducted in accordance with the test kit protocols. Using the 'In situ cell death detection kit, AP' (ISCDDK; Roche), dewaxed and rehydrated tissue sections were incubated with proteinase K (20 μg/mL in 10 mM Tris/HCl, pH 7.4). Labelling was conducted by covering the tissue section with 'TUNEL reaction mixture' composed of terminal deoxynucleotidyl transferase (TdT) from calf thymus. TdT enzyme incorporated fluorescein was detected with "converter-AP" consisting of anti-fluorescein antibody from sheep, conjugated with alkaline phosphatase (included in the kit). The EnVision System alkaline phosphatase kit (Dako) was employed without using the primary antibody added to the kit. Sections were incubated with the substrate (AP) and washed in tap water for 5 min. Counterstaining was accomplished by transferring sections into Mayer's hematoxylin and than rinsed in running tap water. As a negative control, labelling with terminal transferase instead of 'TUNEL reaction mixture' was conducted.

2.5.2. Apop Tag in situ Apoptosis detection kit (Chemiocon)

Dewaxed sections were treated with Proteinase K (20 μg/mL) and endogenous peroxidase was quenched in 3% hydrogen peroxidase in PBS. A working strength TdT enzyme was applied to each section and incubated in a humidified chamber. After incubation, the specimens were put in a Coplin jar containing working strength stop/wash buffer. The sections were washed in PBS and two drops of Anti-Digoxigenin-Peroxidase conjugate were applied to the sections and incubated for 30 min. After washing in PBS, sections were covered with peroxidase substrate. Counterstaining was performed using Mayer's hematoxylin. Slides were mounted in Faramount aqueous mounting medium and examined using a Zeiss light microscope.

The percentage of cells affected by the various treatments, after using different immunohistological methods was established by counting at least 100 cells per sample using a light microscope.

3. RESULTS

3.1. Morphological measurements of HPG

Measurements showed that the average HPG acini diameters of the four age groups were 79.01, 88.37, 110.53 and 77.70 μm respectively. The treatments of coumaphos or imidacloprid and the time of bee exposure to pesticides had significant effect on the HPG acini diameter ($P < 0.05$). HPG mean acinus diameter after 24 h treatment with coumaphos was smaller in all age groups tested compared to the controls ($P < 0.05$). The exception was that the oldest imidacloprid treated bees had larger acini than those treated with coumaphos or the control bees. Changes in the acini diameter after treatments are shown in Figure 1. HPG acini diameters were smaller in the second, third and fourth age group after 48 h of treatment (imidacloprid and coumaphos) compared to the control untreated group ($P < 0.05$). The similar differences in HPG acini diameter were measured in bees after 72 h exposure to treatments ($P < 0.05$). Control, untreated HPG of different bee age groups used for comparison to treated glands were not different among themselves ($P > 0.05$). The influence of the time of exposure to imidacloprid or coumaphos to HPG of four age groups is shown in Figure 2.

3.2. Immunohistological study

3.2.1. Heat shock proteins

3.2.1.1. Imidacloprid treatment

In young (1–6 days) workers exposed to imidacloprid for 24 hours, Hsp 70 activity

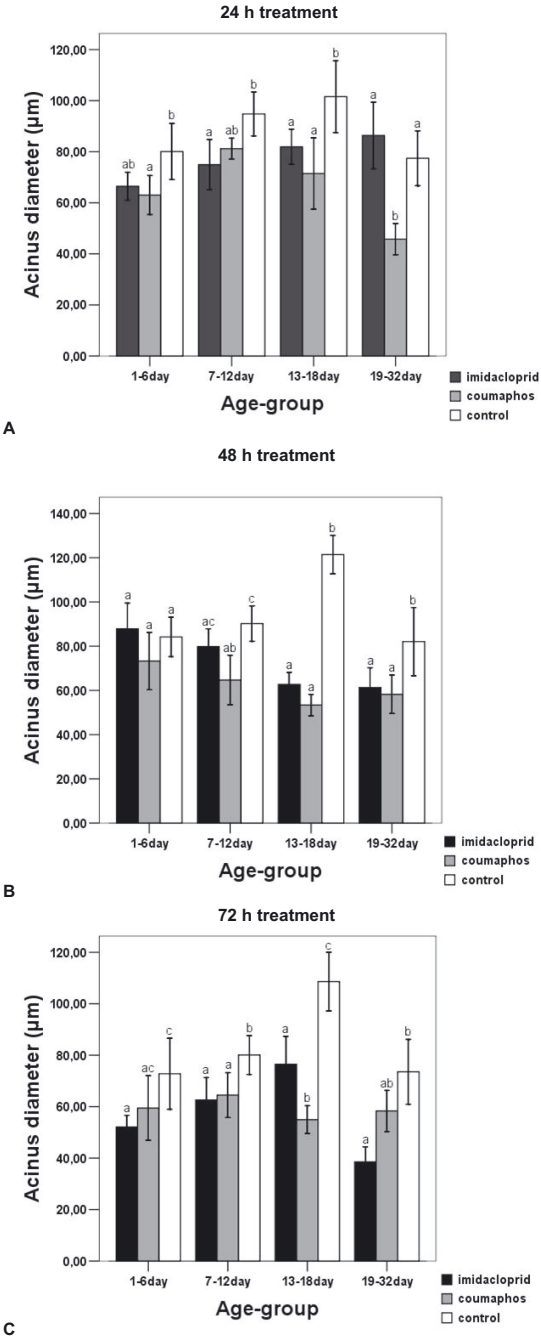

Figure 1. Average HPG acinus diameter (µm) in: (A) bees after 24 h of treatment; (B) bees after 48 h of treatment; and (C) bees after 72 h of treatment with coumaphos or imidacloprid. Bees were divided into four age groups: first (1–6 days); second (7–12 days); third (13–18 days) and fourth age group (18–32 days). Sample sizes are n = 10−30; Same letters indicate that Scheffe tests ($P < 0.05$) established that HPG diameters after imidacloprid or coumaphos treatments of 24, 48 and 72 hours duration were not significantly different.

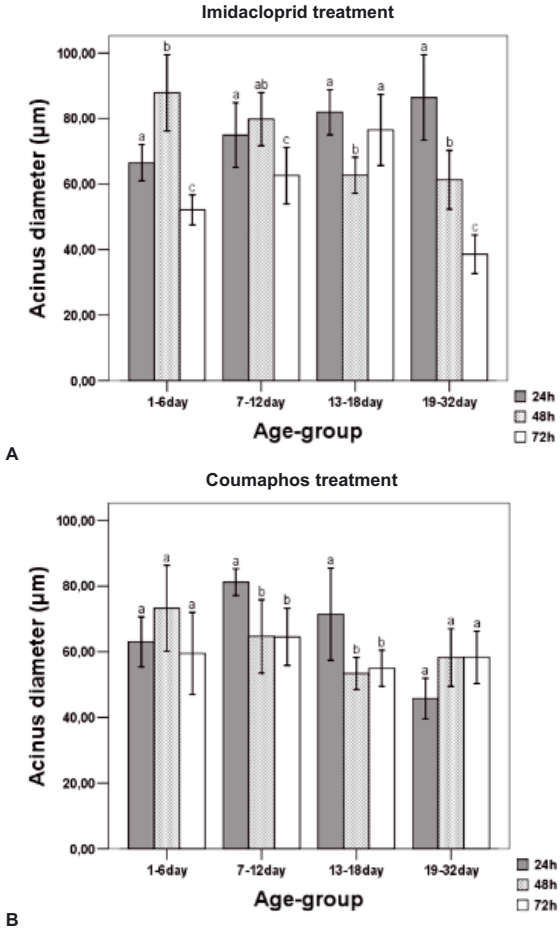

Figure 2. Average (±SE) HPG acinus diameter after 24 h, 48 h or 72 h of: (A) imidacloprid; or (B) coumaphos treatment. Sample sizes are n = 10–30; Same letters indicate that LSD tests ($P < 0.05$) established that HPG diameters after 24, 48 or 72 h exposed to imidacloprid or coumaphos were not significantly different.

was localised in the HPG secretory cells. Red azo-dye reaction product was found dispersed throughout the vacuolated cytoplasm, ducts, and also in the nuclei (Fig. 3A), while Hsp 90 activity was localised in the cytoplasm (Fig. 3B). In older (7–12 days) bees, imidacloprid treatment induced Hsp 70 activity in vacuolated cytoplasm and in sporadic cell nuclei. In HPGs of 13–18 day old bees 24 h after imidacloprid exposure, non intensive red azo-dye reaction product indicative of Hsp 70 was diffused throughout the preserved vacuolated cell cytoplasm. HPGs of the oldest (19–32 days) imidacloprid treated bees did not express intensive Hsp 70 activity after 24 h of treatment and Hsp 90 was localized in sporadic cells.

3.2.1.2. Coumaphos treatment

In HPG of 1–6 day old 48 h coumaphos treated bees, Hsp 70 activity was distributed in cytoplasm but not in the cell nuclei. In older (7–12 days) bees after 24 h of coumaphos treatment, Hsp 70 activity was localised in the vacuolated cytoplasm, whilst after 48 h of treatment, most of the nuclei exhibited Hsp 70 and Hsp 90 activity (Fig. 3C). Coumaphos in 13–18 day old bees induced Hsp 70 activity

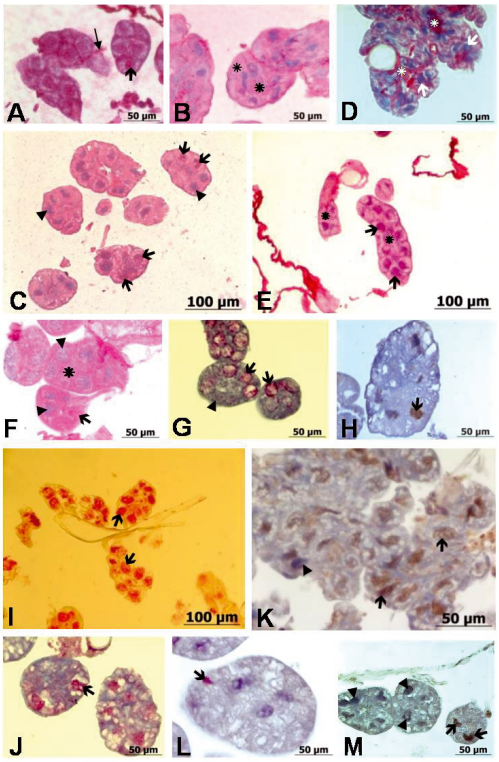

Figure 3. A-M. A. 2 day old worker bee after 24 h of imidacloprid treatment. Staining with Hsp 70 monoclonal antibody. Red azo-dye reaction product dispersed throughout the cytoplasm, cell nuclei (➔) and ducts (→) in glandular cells. B. 6 day old worker bee after 24 h of imidacloprid treatment. Staining with Hsp 90 monoclonal antibody. Activity found in cytoplasm (❋). C. HPG of 9 day old bee after 48 h of coumaphos treatment with sporadic Hsp 70 positive nuclei (➔). Note negative nuclei (▼). D. 20 day old bee after 48 h of coumaphos treatment. Staining with Hsp 70 monoclonal antibody. Fast red reaction product is diffused throughout the cell cytoplasm (❋) and nuclei remain Hsp 70 negative (➔). E. 4 day old untreated worker bee. Staining with Hsp 90 monoclonal antibody. Fast red reaction product is diffused throughout the cytoplasm (❋), and in the nuclei (➔). F. 12 day old untreated bee. Staining with Hsp 70 monoclonal antibody. Fast red reaction product is diffused throughout the cell cytoplasm (❋), and in sporadic nuclei (➔) of HPG cells. Note negative cell nuclei (▼). G. 4 day old bee after 48 h of imidacloprid treatment. Cell death was detected by the TUNEL technique using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP for DNA labelling, and anti-fluorescein alkaline phosphatase conjugated antibody (ISCDDK), fast red was used for visualization, and counterstaining with haematoxylin. The figure shows dense red azo-dye staining localised to the nuclei (➔) of the glandular cells. Note some negative cell nuclei (▼). H. 6 day old bee after 48 h of imidacloprid treatment. Staining of formalin-fixed, paraffin-embedded HPG and the terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labelling (TUNEL) technique using "ApopTag" kit. Peroxidase conjugated anti-digoxigenin secondary antibody and DAB as a substrate were used to obtain specific brown reaction product. DAB reaction product is localised in sporadic cell nuclei (➔). I. 7 day old bee after 72 h of imidacloprid treatment, with all dense red azo-dye staining localised to the nuclei (➔). J. 11 day old bee after 48 h of coumaphos treatment. Cell death was detected by the TUNEL technique (ISCDDK). Dense red azo-dye staining localised to the nuclei (➔). K. 21 day old bee after 72h of coumaphos treatment. ApopTag kit indicates DAB positive cell nuclei (➔) characteristic for cell death in the HPG. Note sporadic non apoptotic cell (▼). L. 15 day old untreated bee. Cell death was detected by the TUNEL technique (ISCDDK). Dense red azo-dye staining localised to the sporadic nuclei (➔). M. 2 day old untreated bee. DAB reaction product is localised in sporadic cell nuclei (➔). Note negative nuclei (▼).

diffused throughout the vacuolated cytoplasm of HPG cells after 24 h treatment and Hsp 90 activity was found in sporadic cell nuclei, and remained throughout the cytoplasm. HPG cells from the oldest (19–32 days) age group, showed Hsp 70 activity in the cytoplasm and ducts after 48 h of coumaphos treatment (Fig. 3D).

3.2.1.3. Untreated bees

In the untreated workers (1–6 days), fast red reaction product indicative of Hsp 90 activity was dispersed in the cytoplasm, and intense activity was localised in the glandular cell nuclei and cell ducts (Fig. 3E). In control untreated HPG, however, Hsp 70 were localized in sporadic cell nuclei and in the cytoplasm (Fig. 3F).

3.2.2. Cell death

3.2.2.1. Imidacloprid treatment

The 'In situ cell death detection kit, AP' (ISCDDK) identified red azo-dye reaction product in the nuclei of dying HPG cells and the ApopTag kit DAB substrate gave brown staining. When using ISCDDK, the level of positive nuclei in 1 to 6 day old bees, characteristic for cell death HPG cells after imidacloprid treatment was determined in the majority of glandular cell nuclei (Fig. 3G), but compared to the ApopTag kit, sporadic DAB positive cell nuclei were found after 48 h of imidacloprid treatment (Fig. 3H).

ISCDDK in older (7–12 days) bees indicated red azo-dye reaction product in approximately 100% of HPG cell nuclei 72 h after treatment (Fig. 3I). In imidacloprid treated 13–18 day old bees an increased level of cell death after using ISCDDK was at a comparable level to that obtained using ApopTag estimated at the level of 10%.

3.2.2.2. Coumaphos treatment

Coumaphos treatment induced an increased level of red azo-dye reaction product in nuclei in the majority of HPG cells when using the ISCDDK in 1 to 6 day old bees after 48 h of coumaphos treatment induced sporadic DAB positive HPG cells detected using ApopTag.

The level of positive nuclei characteristic for cell death in HPG secretory cells 7–12 days old bees using ISCDDK was found in most of the cells after 48 h of coumaphos treatment (Fig. 3J). The ApopTag kit indicated high level of DAB positive glandular cell nuclei in HPG cells of 19–32 old bees (Fig. 3K).

3.2.2.3. Untreated bees

Sporadic positive cell nuclei were found using ISCDDK in the untreated control bees of the all ages groups of bees (Fig. 3L). Sporadic cells were also found to be DAB positive after applying ApopTag to all age groups (Fig. 3M).

The sequence of identified red azo-dye reaction product or brown staining product obtained by DAB substrate observed in the HPG of bees in all treatments and age groups after applying Hsp 70, Hsp 90, ISCDDK or ApopTag kit, respectively, is shown in Table I.

4. DISCUSSION

4.1. Acinal diameter

The HPG acinus diameter in coumaphos and imidacloprid treated workers was found to be reduced compared to the control untreated workers. Difference in the acinus size was evident in all long term (72 h) treatments, where untreated HPG acini were larger in all age groups, and it appears that treatment also influenced their secretory function. To better understand these results, it must be noted that HPG acinus diameter is about 80 μm in newly emerged bees measured in vivo (Crailsheim and Stolberg, 1989; Deseyn and Billen, 2005). It should therefore be noted that in our study acinal diameter was smaller due to the use of histological samples which become smaller during the histological procedures.

Acinal diameter which increased in size until the age of 13–18 days was comparable to that of winter workers (Cruz-Landim and Hadek, 1969; Deseyn and Billen, 2005). Our

Table I. Staining localization in HPG of different age-group honey bee workers, treatments and time of treatments.

Bee age-group (days)	Treatment	Treatment duration (hours)	Staining	Staining localization in HPG
1–6	Imidacloprid	– 24	Hsp 70	Nuclei, cytoplasm
		– 48	Hsp 70	Cytoplasm
		– 48	Hsp 90	Cytoplasm, ducts
		– 24, – 48	ISCDDK	Majority cell nuclei
		– 48	ApopTag	Approx. 50% cell nuclei
	Coumaphos	– 24, – 48	Hsp70	Cytoplasm, sporadic cell nuclei
		– 24, – 48, – 72	Hsp90	No cellular activity; only in ducts
		– 24, – 48, – 72	ISCDDK	Majority cell nuclei
		– 48	ApopTag	Sporadic cell nuclei
	Control (untreated)		Hsp70, Hsp90	Cytoplasm, cell nuclei
			ISCDDK	Sporadic cell nuclei
			ApopTag	Sporadic cell nuclei
7–12	Imidacloprid	– 48	Hsp70	Cytoplasm, sporadic cell nuclei
		– 48	Hsp90	No activity
		– 48	ISCDDK	Approx. 50% cell nuclei
		– 72	ISCDDK	Approx. 100% cell nuclei
		– 48, – 72	ApopTag	Approx. 10% cell nuclei
	Coumaphos	– 24	Hsp70, Hsp90	Cytoplasm
		– 48	Hsp70, Hsp90	Sporadic cell nuclei
		– 48	ISCDDK	Majority cell nuclei
		– 24, – 48	ApopTag	Majority cell nuclei
	Control (untreated)		Hsp70	Cytoplasm, sporadic cell nuclei
			Hsp90	Cytoplasm, all cell nuclei
			ISCDDK, ApopTag	Sporadic cell nuclei
13–18	Imidacloprid	– 24, – 72	Hsp70, Hsp90	Cytoplasm, sporadic cell nuclei
		– 24, – 72	ISCDDK	Approx. 50% cell nuclei
		– 48	ISCDDK	Approx. 100% cell nuclei
		– 24, – 72	ApopTag	Approx. 50% cell nuclei
	Coumaphos	– 24	Hsp70	Cytoplasm
		– 24, – 48	Hsp90	Sporadic cell nuclei, cytoplasm
		– 24, – 48	ISCDDK	Majority cell nuclei
		– 24, – 48	ApopTag	Sporadic cell nuclei
	Control (untreated)		Hsp70, Hsp 90	Cell nuclei
			ISCDDK	Sporadic cell nuclei
			ApopTag	Sporadic cell nuclei
19–32	Imidacloprid	– 24, – 48	Hsp 70	Not intensive activity
		– 24, – 48	Hsp90	Sporadic cell nuclei, ducts
		– 24	ISCDDK	Approx. 30% cell nuclei
		– 24, – 48	ApopTag	Approx. 100% cell nuclei
	Coumaphos	– 48, – 72	Hsp70, Hsp90	Cytoplasm, HPG ducts
		– 72	ISCDDK	Approx. 50% cell nuclei
		– 48, – 72	ApopTag	Approx. 50% cell nuclei
	Control (untreated)		Hsp70, Hsp90	Cytoplasm, cell nuclei
			ISCDDK, ApopTag	Sporadic cell nuclei

findings also correspond with the published literature suggesting that nurse bees have larger acini than older foraging bees (Crailsheim and Stolberg, 1989; Sasagawa et al., 1989; Hrassning and Crailsheim, 1998; Huang et al., 1994). Keeping honeybee workers in small cages without brood for 24, 48 or 72 hours can also have an influence by producing smaller acini. The untreated bees thus had less developed acini than they might have had if kept in colonies (Crailsheim and Stolberg, 1989; Crailsheim et al., 1993; Lass and Crailsheim, 1996).

Imidacloprid, and even more so, coumaphos, clearly caused a decrease of HPG acinal sizes. In the literature, regression has also been found to occur in newly emerged bees due to soybean trypsin inhibitor (Babendreier et al., 2005). To put these results in context, it is clear that even the short treatment time (24 h) greatly decreased acinal diameter. By the second or third day of treatment with imidacloprid, bees somehow developed their HPGs, but coumaphos seemed to seriously retard their development. We believe that this kind of observed reduction in HPG size can have several effects on the colony well-being, as was suggested by Maurizio (1954). Affected workers can not produce enough royal jelly, and their task partitioning may change earlier than in unaffected bees. We therefore conclude that one method for determining the sub-lethal effects of pesticides could be the morphological measurement of HPGs.

4.2. Hsp 70 and Hsp 90 activity

Our immunohistochemical data showed that short term imidacloprid treatment (24 h) retained Hsp70 activity in nuclei and cytoplasm. In young workers after 48 hours exposed to imidacloprid, Hsp 70 activity was not present in nuclei, but was localized in the cytoplasm. In the second (7–12 days old) age workers, Hsp 70 activity was localised to cytoplasm and sporadic nuclei after imidacloprid treatment and the activity of both Hsps was more intensive in untreated workers. In workers of the third (13–18 days) and fourth (19–32 days) age groups, Hsp 70 and Hsp 90 activity was not characteristically present in nuclei after imidacloprid or coumaphos treatments, but remained mainly in the cytoplasm. Hsp 90 was found intensively in most cell cytoplasm and in some gland ducts after 48 h and 72 h treatment in all four age groups. Hsp 90 activity in cell nuclei increased with increasing age of the bees.

In young coumaphos treated workers, Hsp 70 activity was present in the cytoplasm and in some of the cell nuclei. Furthermore, enhanced Hsp 90 activity was found in cytoplasm and nuclei of untreated HPG. In 7–12 day old bees Hsps were localized in the cell nuclei, as in the untreated HPG, after 48 h of coumaphos treatment which did exhibit more intensive Hsp 70 localisation in nuclei in comparison to untreated bees. Hsp activity seemed to be more intensive and preserved in coumaphos treated bees compared to those treated with imidacloprid. There were some differences between Hsp 70 and Hsp 90 localisation compared to control tissue where enhanced activity of both Hsps was localized in the cytoplasm and nuclei.

Heat shock proteins are considered to be essential components in a number of diverse biological processes and function under normal cellular conditions. They contribute to stress tolerance by functioning as molecular chaperones (Feder et al., 1995) and are involved in intracellular protein maturation (Georgopoulos and Welch, 1993). Hsp 70 and Hsp 90 activity is regularly found in untreated HPG cells. Because of diverse functions, their activity is in the nucleus, cell organelles and cytosol where they stimulate protein transport into the endoplasmic retriculum, mitochondria and nucleus (Chiang et al., 1989). The Hsp 70 and Hsp 90 families are thus present in a wide variety of unstressed cells (Garrido et al., 2001), and in this study we detected Hsps in the cytoplasm and/or nuclei of the HPG, under both normal and stress conditions.

In unstressed cells, members of the Hsp 70 family function as ATP-dependent molecular chaperones by assisting the folding of newly synthesized polypeptides, the assembly of multi-protein complexes and the transport of proteins across cellular membranes (Beckmann et al., 1990; Shi and Thomas,

1992). Members of the Hsp 70 family recognize nascent proteins and correct any aggregation that may occur during their folding and assembly into oligomeric structures (Pelham, 1986). Both Hsp 70 and Hsp 90 families have been widely used in ecosystem biomonitoring, whilst high levels of their expression in organisms under stress serve to protect cells and to prevent the induction of cell death (Bierkens, 2000). Silva-Zacarin et al. (2006) also suggested that Hsp 70 has an antiapoptotic effect. Hsp activity was found in the coumaphos treated HPG in comparison to those treated with imidacloprid. Together with morphological alterations, coumaphos treatment seemed to cause less stress in the glandular cells with regard to Hsp activity.

4.3. Cell death

In the youngest group of imidacloprid treated workers, a higher level of cell death in the HPGs was found using ISCDDK compared to the results obtained using the ApopTag kit. A high level of ISCDDK is indicative of cell necrosis caused by the imidacloprid, while programmed cell death remained at the low level indicated by the ApopTag kit as was shown in our previous results in treated honeybee midguts (Gregorc and Bowen, 2000). Coumaphos triggered an increased level of necrosis detected by ISCDDK in up to 6 day old workers and also some level of apoptosis. Imidacloprid also induced extended necrosis in HPG. In the second (7–12 days) age group, the level of cell death expressed after 48 hours treatment with imidacloprid detected with ISCDDK was approximately 50%, and increased to 100% after 72 hours treatment. Necrotic and apoptotic cell death thus increased with the prolonged time of imidacloprid treatment. In these workers the difference between programmed cell death and necrosis was high. Programmed cell death detected with the ApopTag kit remained at a low level, specific for normal midgut tissue turnover of approximately 5 to 10% (Gregorc and Bowen, 1997). In the HPG of untreated honeybees we found of about 10% programmed cell death using the ApopTag kit. Increased cell death in the imidacloprid or coumaphos treated bees is due to necrosis, as assayed by the ISCDDK.

Imidacloprid thus does not cause increased programmed cell death as is the case with coumaphos. Increased cell death accompanying programmed cell death and necrosis in coumaphos treated workers were assayed by the ApopTag kit and ISCDDK, respectively. In the third age group, prolonged HPG treated with imidacloprid exhibited extensive cell death and was assayed by the ISCDDK. The difference in sensitivity in comparison to the ApopTag technique was high. ISCDDK appears to show both programmed and necrotic cell deletion (Gregorc and Bowen, 2000) as was clearly seen in untreated HPG tissue assayed by both ISCDDK and Apop Tag, where sporadic cells were positive.

Our results showed that in biological and toxicological, in situ studies of worker HPG treated with imidacloprid or coumaphos, determination of Hsp 70 and Hsp 90, localisation and use of ISCDDK and ApopTag kit can be useful immunohistochemical methods. It is possible to demonstrate and better understand any possible adverse (sub-lethal) effects on tissue in vivo. A combination of the immunohistochemical assays may help to detect the cellular responses of honeybee HPG to two widely used pesticides, imidacloprid in the field and coumaphos in the honeybee colony itself. The methods used are potentially powerful tools for the detection of subclinical changes and the evaluation of threshold effects of chemical substances. Further research should be performed in order to establish honeybee tissues and organs as a model for detection of environmental influences.

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Localisation in situ des protéines du choc thermique et de la mort cellulaire dans les glandes hypopharyngiennes des ouvrières de l'abeille (*Apis mellifera carnica*) après traitement à l'immidaclopride et au coumaphos.

***Apis mellifera carnica* / immunohistochimie / pesticides / mort cellulaire / choc thermique**

Zusammenfassung – In situ Lokalisierung von Hitzeschockproteinen und Zelltod in Hypopharynxdrüsen von Arbeiterinnen der Honigbiene (*Apis mellifera carnica*) nach Imidacloprid oder Coumaphos Behandlung. Arbeiterinnen der Honigbiene (*Apis mellifera carnica* Polm.), die in einem Brutschrank geschlüpft waren, wurden auf dem Thorax markiert und in weiselrichtige Völker gegeben. Danach wurden altersspezifische Gruppen aus jeweils acht markierten Arbeiterinnen gebildet und in Holzkäfigen gehalten. Die erste Gruppe bestand aus 1 bis 6 Tage alten Arbeiterinnen, die zweite aus 7 bis 12 Tage alten, die dritte Gruppe aus 13 bis 18 Tage alten und die vierte Gruppe aus 19 bis 32 Tage alten. Diese erhielten als Behandlung entweder: (1) eine Imidacloprid-Lösung (500 ng/kg in 35 % Zuckerwasser), (2) eine Coumaphos-Lösung oder (3) als Kontrolle 35 % (w/v) Zuckerwasser. Die Arbeiterinnen konnten diese Lösungen über 24, 48 oder 72 Stunden ad libitum aufnehmen. Am Ende der jeweiligen Behandlungsperioden wurden die Hypopharynxdrüsen dieser Bienen entnommen, fixiert und in Wachs eingebettet. Für alle Alters- und Behandlungsgruppen wurden immunhistologische Präparate angefertigt und 'morphometrische Messungen durchgeführt, die statistisch ausgewertet wurden. Entwachste Schnitte wurden mit Primärantikörpern gegen die Hitzeschockproteine 70 und 90 (Hsp 70 und Hsp 90) inkubiert. Zelltodereignisse wurden mittels TUNEL Reagenz des 'In situ cell death detection kit, AP' (Roche) erfasst. Als zweites Detektionsverfahren für Zelltod verwendeten wir das Apop Tag in situ Apoptosis detection kit (Chemiocon). Damit erfassten wir die Prozentsätze an Zellen, die behandlungsbedingten Zelltod oder Hitzeschockproteine aufwiesen. Wir konnten signifikante Unterschiede im Acinus-Durchmesser der Drüsen in Abhängigkeit von der Behandlung und der Behandlungsdauer erkennen, wobei die Unterschiede in der Acinus-Größe vor allem bei den Langzeitbehandlungen (48 und 72 Std.) deutlich waren. Bei der Kurzzeitbehandlung (24 Std.) mit Imidacloprid wurde Hsp 70 induziert und war sowohl im Zytoplasma als auch im Zellkern zu finden, während Hsp 90 im Zytoplasma der meisten Zellen nachweisbar war. Die Hsp-Aktivität war bei Coumaphos behandelten Gruppen deutlicher als bei den Imidacloprid behandelten. Coumaphos führte außerdem zu höheren Zelltodraten als Imidacloprid, während nach Imidaclopridbehandlung mehr Nekroseschäden zu sehen waren. Bei den 7–12 Tage alten Arbeiterinnen lag die Zelltodrate nach 48 stündiger Imidacloprid-Behandlung bei 50 % und erfasste bei längerer Behandlung (72 Std.) nahezu alle Zellen. Die normalen Zelltodraten bei Kontrollen lagen bei 10 %. Wir schliessen daraus, dass beide Pestizide die Größe der Hypopharynxdrüsen negativ beeinflussen können und dass eine längere Exposition zum Zelltod führt. Unser Versuchsansatz erlaubt es auch, subletale Effekte auf bestimmte Organe erkennen zu können. Diese Nachweisverfahren können damit Hilfsmittel darstellen, um die zelluläre Antwort in Hypopharynxdrüsen nach Feldeinsätzen von Imidacloprid, bzw. von Coumaphos in Bienenvölkern zu erfassen.

***Apis mellifera* / Immunhistochemie / Pestizide / Hitzeschockproteine / Zelltod**

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