THE INFLUENCE OF SUBLETHAL DOSES OF IMIDACLOPRID ON PROTEIN CONTENT AND PROTEOLYTIC ACTIVITY IN HONEYBEES (APIS MELLIFERA L.)

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INTRODUCTION

The use of sublethal doses of neonicotinoid insecticides to which honeybees are exposed can have an effect on the insect's physiology and behavior (Desneux et al. 2007; Teeters et al. 2012). For example, it has been shown that sublethal doses of IMD have an adverse effect on the bee's sense of smell and memory (Kirchner 1999; Decourtye et al. 2004a, b; Williamson et al. 2013). as well as on the insects' intensity of feeding (Yang et al. 2008; Schneider et al. 2012), territorial disorientation (Henry et al., 2012; Hatjina et al. 2012), neurophysiologic changes (Guez et al. 2001a, b; Lambin et al. 2001; Goulson 2013). In recent *in vivo* studies it has also been shown that the sublethal doses of imidacloprid caused both a decrease in the size of HPGs and the respiratory rhythm of *A. mellifera* (Hatjina et al. 2013). Furthermore all above mentioned sublethal effects can also lead to massive losses of honey bee populations (Doucet-Personeni et al. 2003; Lu et al. 2012).

Besides imidacloprid itself, its degradation products released as a result of its metabolism have a further effect on bees (Suchail et al. 2004). Proteins showing enzymatic qualities constitute an interesting group of compounds involved in different biochemical processes in insects. Some of the enzymes fulfill detoxication functions, which is due to the fact, that they take part in the metabolism of pesticides (Shi et al. 2009). Another group is represented by a proteolytic system that catalyzes the hydrolytic degradation of peptide bonds and takes part in many biological processes (Walter and Clélia 1994; Lima et al. 2000; Costa and Cruz-Landim 2005; Strachecka et al. 2008, 2010; Frączek et al. 2013). Many of such proteins are involved in the immunological response and not in the functioning of digestive enzymes (Ji et al. 2004; Jiang et al. 2005).

Phenoloxidase (PO) plays an important role in the immunological response in invertebrates against different pathogens, causing their encapsulation through melanization. In this metabolic pathway, serine proteases are involved, generating active PO that induces the production of melanin (Cerenius and Söderhäll 2004). PO has been analyzed as a parameter of the individual immunity of the honeybee against sublethal doses of imidacloprid (Alaux et al. 2010). The same team of researchers also determined glucose oxidase activity (GOX). The activity of GOX activity was analyzed as a parameter of social immunity. The enzyme takes part in the oxidation of β-D-glucose to D-gluconic acid and peroxide 1 (White et al. 1963). Hydrogen peroxide has antiseptic qualities and is secreted to the larval food and then to honey (Sano et al. 2004; Ohashi et al. 1999). Moreover, the level of hydrogen peroxide in honey is positively correlated with the inhibition of the development of pathogens (Brudzynski 2006). It is worth attention that GOX is mainly expressed in hypopharyngeal glands (HPGs) (Takenaka et al. 1990, Deseyn and Billen 2005). It is also interesting that HPGs take part in the continuation of the synthesis of a large part of proteins out of the whole pool of proteins in bees (Knecht and Kaatz 1990).

THE AIM OF STUDY

The aim of this study was to determine the protein profile and the activity of proteases in the honeybee under the chronic influence of IMD due to their important role in the different biological processes in *A. mellifera*. Our findings can contribute to a better knowledge of the physiological changes induced on honeybees by neonicotinoid insecticides.

RESULTS

Table I. Protein concentration in bee body extracts (in mg/ml). Different capital letters denote the significance of differences for $p<0.01$.

Group	Proteolytic activity of bee body extracts			
	n	August	n	October
BE	80	0.27A	80	0.26A
BE-5	80	0.19BC	80	0.21B
BE-200	80	0.18C	80	0.18C

Table II. Proteolytic activity of bee body extracts (in U/ml). Different capital letters denote the significance of differences for $p<0.01$, and lower-case letters for $p<0.05$.

Group	Protein concentration in bee body extracts			
	n	August	n	October
BE	80	9.0A	80	6.7C
BE-5	80	8.4B	80	5.4D
BE-200	80	6.5C	80	4.5E

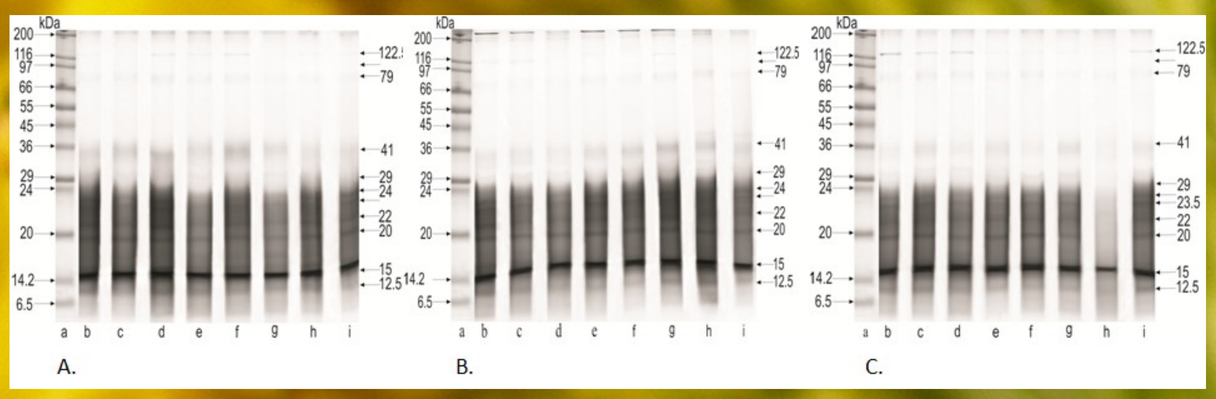

Figure 1. SDS-PAGE of *Apis mellifera* body extract proteins collected in August from groups: A, BE; B, BE-5; C, BE-200; lane a, molecular weight markers (the Wide Range Sigma Marker TM 6.5-200 kDa); lanes b-i, different colonies

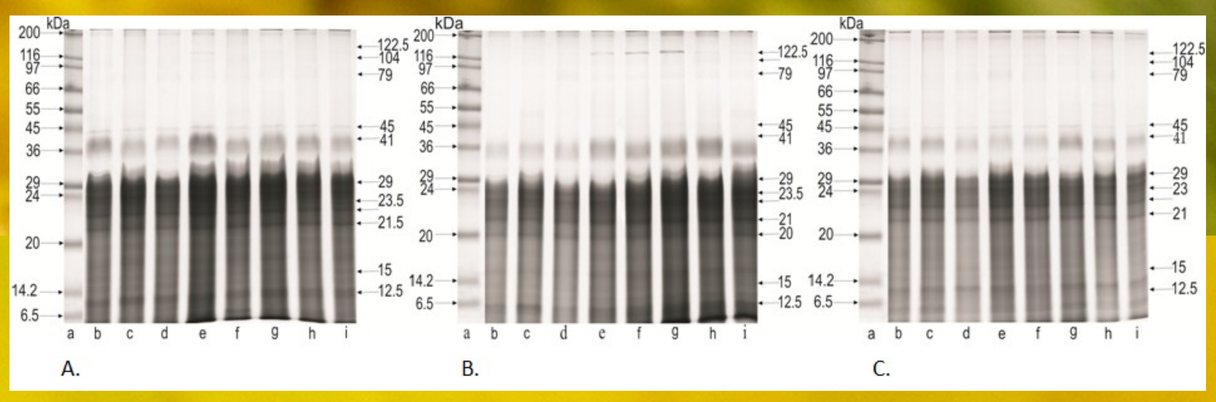

Figure 2. SDS-PAGE of *Apis mellifera* body extract proteins collected in October from groups: A, BE; B, BE-5; C, BE-200; lane a, molecular weight markers (the Wide Range Sigma Marker TM 6.5-200 kDa); lanes b-i, different colonies

MATERIAL AND METHODS

Honey bee colonies and treatments

The study was carried out on *Apis mellifera carnica* colonies in North-East Poland (53°53'49" *N longitude, 20°13'27" E latitude*) during summer 2013. A number of 24 colonies were randomly assigned to 3 experimental groups. Colonies from group BE (control group) were given food free from imidacloprid, while the food administered to colonies from group BE-5 and BE-200 was contaminated with 5 and 200 ppb of imidacloprid, respectively. The bees were fed with both bee syrup (Apifortuna) and pollen pastry made from fresh pollen loads and inverted syrup Apifortuna in the ratio 2.5:1.4. The colonies were given 5.5 kg of the liquid food and 0.3 kg of the pastry in two portions for a total period of 2.5 months. The survival status of the colonies was assessed several times during the experiment and their overwintering ability was finally assessed in March 2014.

Biological samples

The bee samples were collected for analysis twice during the study period: in late August (3 weeks after feeding started) and again in beginning of October 2013 (10 weeks after feeding started), in order to determine differences due to the exposure time. Each sample group contained 20-30 adult honeybees. The bees were first weighed, then placed in Eppendorf tubes and immediately were frozen (anaesthetized) in liquid nitrogen. The bee material was stored at -70°C until use.

Crude extract preparations from bees

Extract from each one sample of bees was obtained by homogenizing whole bees on ice for 2 minutes with 0.9% NaCl at 1:10 (w/v) ratio in an Omni TH-02 (5,000-35,000RPM, OMNI International, USA) homogenizer. The homogenates were then centrifuged at 2500 *g* for 15 min at 4°C for debris removal and at 20000 *g* for 40 min at the same temperature for supernatant clarification (Felicioli et al. 2004). The insect body extracts from all groups were coded accordingly to the group (as BE, BE-5 and BE-200). All collected materials were stored at -70 until use.

Determination of protein content

Protein content was determined by the spectrophotometric method at 280 nm wavelength (Aitken and Learmonth 1996). The results obtained were expressed in mg of protein contained in mL of supernatant.

Estimation of molecular weight

The molecular weights of active proteins were estimated by using 12.5% polyacrylamide gel (Laemmli 1970) and an electrophoresis process was carried out under semi-denaturing conditions. The samples of protein extract (BE), (BE-5) and (BE-200), containing 15 µg proteins, were mixed with loading buffer (0.125 M-Tris-HCl buffer pH 6.8, 4% SDS, 0,18 M-glycerol, 1x10-5 M-bromophenol blue, without boiling). No β-mercaptoethanol was added. Electrophoresis was carried out in a SE 250 mini vertical electrophoresis unit (Amersham-Biosciences, Sweden) at 80 V for about 4 h at room temperature, using an electrophoresis buffer containing 0.125 M Tris-HCl, 0.192 M-glycine and 0.1% SDS (pH 8.5). After electrophoresis, the gels were stained with 0.1% (w/v) Colloidal Coomassie brilliant blue G-250 solution according to Neuhoff et al. (1985). The mix of proteins (Sigma, 6.5-200 kDa) were used as molecular weight markers. The gels were scanned with image scanner (GE Healthcare Life Science, with Lab Scan software, USA), and finally analyzed by densitometry using KTE Gel Scan software (Kucharczyk, Poland).

Determination of proteolytic activity

The proteolytic activity of the bee extracts was determined according to Mendiola et al. (1996), by using natural substrate as 1% (w/v) gelatin solution in 0.1 M Theorell and Steinhagen buffer (Küster and Thiel 1993) at pH 7.5. The incubation mixtures contained: 25µl of each one extracts (BE), (BE-5) and (BE-200) and 125 µl gelatin 1 solution and were allowed to incubate for 30 minutes at 37°C. The reaction was terminated by the addition of 100 µl of 10% trichloroacetic acid (TCA). After careful mixing and a 10 min incubation at 4°C, the samples were centrifuged at 14 000 g for 15 min at 4°C. The concentration of peptides released from gelatin by proteases was determined in the supernatant (Frączek et al. 2012). Proteolytic activity was expressed in units (U) expressing the mg peptides released per mg protein in bee extract.

Zymography

Zymography was performed with SDS-PAGE, using 12.5% polyacrylamide gels containing 0.1% gelatin in the presence of sodium dodecyl sulphate (SDS), according to Felicioli et al. (2004). Proteins in the clarified extracts from each bee sample containing 15 µg proteins were used in the applied probe. Electrophoresis was carried out at 80 V for about 4 h at 4°C. After electrophoresis, the gels were washed with gentle shaking at room temperature with 2% (v/v) aqueous solution of Triton X-100 for 30 min to remove SDS and restore the full activity of the peptidases and then rinsed with 0.1 M Theorell and Steinhagen buffer pH 7.5. The gels were transferred to petri dishes filled with the above mentioned buffer and incubated for 9 h at 37°C, and then stained in 0.1% (w/v) Colloidal Coomassie brilliant blue G-250. The active fractions appeared as unstained bands on the blue background of gels. Then the gels were scanned.

Statistical analysis

A Two-way ANOVA was carried out using 'dose' (0, 5 and 200ppb) and 'exposure period' (3 and 10 weeks) as fixed effect factors. Significance of differences between the means was determined by the Duncan's multiple range test. Statistical analysis was conducted using the software package *Statistica* (Statsoft, V. 12.0).

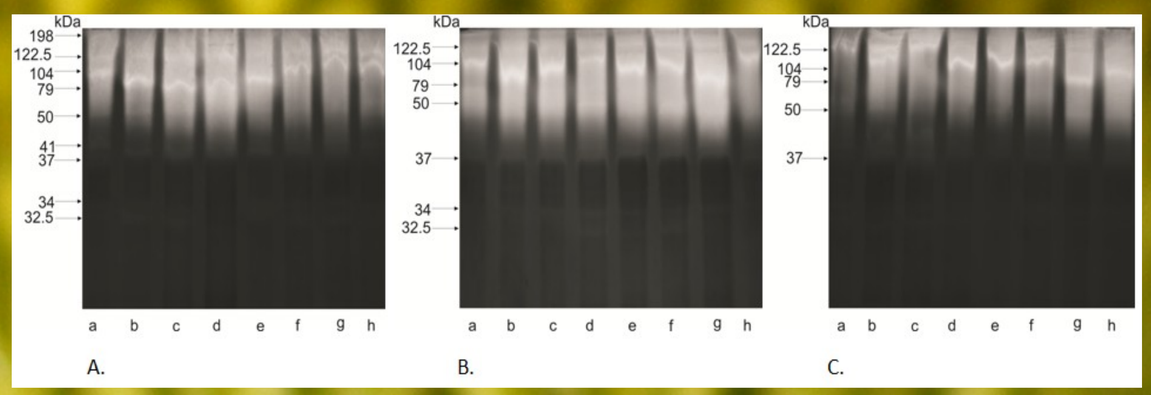

Figure 3. Zymogram of *Apis mellifera* body extract proteases collected in August from groups: A, BE; B, BE-5; C, BE-200; lane a-h, different colonies

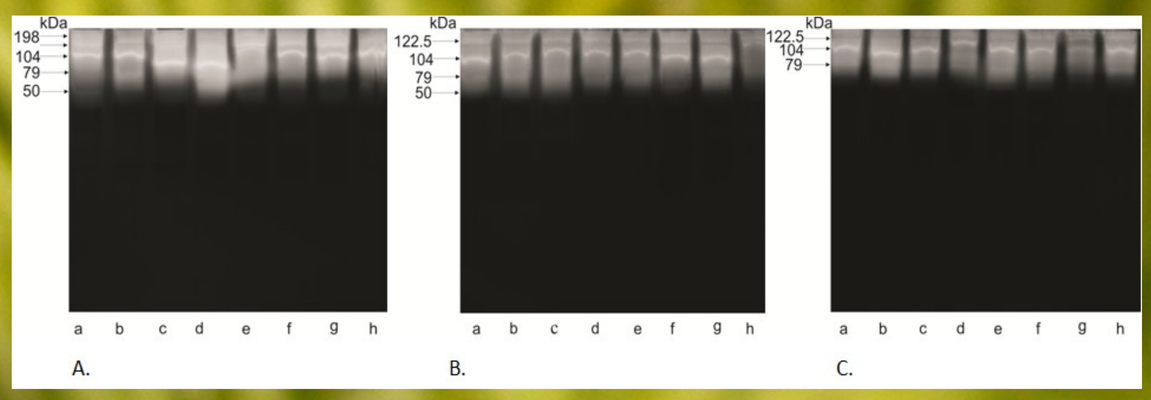

Figure 4. Zymogram of *Apis mellifera* body extract proteases collected in October from groups: A, BE; B, BE-5; C, BE-200; lane a-h, different colonies

CONCLUSIONS

Our studies show that a reduction in protein biosynthesis and a decrease in the level of proteolytic activity in *A. mellifera* could be a result of the bees' exposure to sublethal doses of IMD even for the duration of 3 weeks. Longer duration of exposure increases the impact of IMD in relation to protein levels but not in relation to proteolytic activity. Furtheremore, an intensified adverse effect of IMD was observed for the 200 ppb dose (still sublethal although very high). However, there are still questions to be answered as: what is the impact of this reduced protein synthesis and activity on the 1 honeybee function or colony development. Although this was not the scope of our study, the indirect effect observed on the colony development, meaning the de-population of several colonies used for the experiment, shows that a malfunction on colony level occurs and this is more profound in increased levels of the pesticide. The reduced protein content in our study, probably, signified the reduced quality of royal jelly, therefore, a change in the feeding behaviour towards the queen, thus a stress factor resulting in reduced egg laying and depopulation of the colonies.