DOI: 10.2478/JAS-2014-0008 J. APIC. SCI. VOL. 58 NO. 1 2014

DE GRUYTER OPEN

Original Article

THE PROTEOLYTIC ACTIVITY OF DIAPAUSING AND NEWLY HATCHED RED MASON BEES (*OSMIA RUFA*: MEGACHILIDAE)

Ewa A. Zaobidna*
Kamila Dmochowska
Regina Frączek
Ewa Dymczyk
Krystyna Żółtowska

Department of Biochemistry, Faculty of Biology, University of Warmia and Mazury, 1A Oczapowskiego Street, 10-719 Olsztyn, Poland

*corresponding author: ewa.zaobidna@gmail.com
Received 01 July 2013; accepted 24 April 2014

Abstract

***Osmia rufa* is a solitary bee that is used commercially for pollinating crops. The bee enters obligatory diapause as an imago. The activity of proteolytic enzymes during diapause has not been investigated. We studied the proteinase activity on four substrates – casein, haemoglobin, bovine serum albumin (BSA), and gelatine – during diapause (from October to March) and in newly hatched males and females in April. During diapause, greater fluctuations in enzyme activity levels were noted in males than in females, and a significant decrease in male enzyme activity was observed in January and March. Male enzymes were most effective in decomposing gelatine; whereas, female enzymes were equally effective in hydrolysing gelatine and BSA. The differences in substrate preferences between male and female enzymes were particularly pronounced in October and in the newly hatched individuals. The levels of gelatinolytic activity likely indicate that a high proportion of proteinases in *O. rufa* are elastase-like enzymes. They are involved in the digestion and remodelling of proteins with numerous peptide bonds formed by amino acids with short side-chains.**

Keywords: diapause, *Osmia rufa*, proteolytic enzymes, solitary bees.

INTRODUCTION

Losses in the populations of pollinating insects, mainly honeybees, reduce crops by approximately 8% per annum. Between 1961 and 2007, the number of honeybee colonies decreased by 26.5% in Europe and 49.5% in North America (van Engelsdorp and Meixner, 2010). Solitary bees, including the red mason bee, *O. rufa,* seem to pose a good alternative to *Apis mellifera*. *O. rufa* is a widespread species in North-Central Europe (Conrad et al., 2010) and Northern Africa (Stöcklin et al., 2010) that effectively pollinates 130 plant species (Ruszkowski and Biliński, 1986; Giejdasz and Wilkaniec, 2002). *O. rufa* is much easier and cheaper to breed than the honeybee; thus, it is often used by farmers for crop pollination (Biliński and Teper, 2004).

The majority of research into *O. rufa* focuses on its pollination efficiency, plant preferences, breeding methods, and biology (Giejdasz and Wilkaniec, 2002; Biliński and Teper, 2004). *O. rufa* enters an obligatory diapause and overwinters as an imago inside a cocoon (Giejdasz and Wilkaniec, 2002). The diapause duration is determined mainly by temperature; therefore, the length can be controlled artificially to obtain active pollinators during selected seasons. The first studies on the diapause duration revealed that artificial diapause elongation had a negative effect on the survival and life span of hatched individuals (Giejdasz and Wilkaniec, 2002). This can be attributed to the depletion of energy reserves and lower effectiveness of the antioxidant system (Dmochowska et al., 2012). Wasilewski et al. (2011) demonstrated that vitellogenesis occurs in female *O. rufa* during diapause, and it is accompanied by an increase in protein levels in the ovaries

and a decrease in the protein content of fat bodies. These findings indicate that proteins are rebuilt in diapausing females, which requires proteolytic activity. Previous studies demonstrated that artificially elongated diapauses reduce protein levels in mason bees (Dmochowska et al., 2013); although, the activity and properties of the proteinases involved in those processes have never been studied in red mason bees. Thus, we examined the proteolytic activity levels during natural diapause. In addition, we compared the activity and substrate preferences of proteinases from male and female bees.

MATERIAL AND METHODS

Bees. The experiments were conducted during the hibernation of *O. rufa* between October 2011 and April 2012. Bees were reared outdoors in the Olsztyn area in accordance with the method proposed by Wójtowski and Wilkaniec (1978). Male and female red mason bees were harvested four times during the study: on 4 October 2011 – in the early diapause period, on 4 January 2012 – in the mid-diapause period, on 15 March 2012 – in the late diapause period, and on 5 April 2012 – when active bees emerged from cocoons in an incubator at 25°C. *O. rufa* (30 females and 20 males) were randomly selected and analysed in each month of the study. The bees were weighed, placed in microcentrifuge tubes (two individuals per tube) and immediately frozen (anaesthetized) in liquid nitrogen. The material was stored at -71°C until analyses.

Preparation of *O. rufa* extracts. The extracts were prepared according to the method described by Dmochowska et al. (2012).

Determination of total proteolytic activity. The proteolytic activities of the bee extracts were determined according to Mendiola et al. (1996) using 1% (w/v) solutions of natural substrates: bovine serum albumin (BSA), gelatine, haemoglobin, and casein in Theorell and Steinhagen buffer, pH 7.5. Incubation mixtures contained 25 µL of the *O. rufa* extract and 125 µL of one of the above substrate solutions. After incubation for 30 min at 37°C, the reaction was stopped by adding 100 µL of 10% trichloroacetic acid (TCA). After 10 minutes, the mixtures were centrifuged for 5 min at 12,000 g, and the absorbance of the supernatant was measured at 280 nm in the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Proteolytic activity was expressed in units (U), where 1U corresponded to 1 mg of peptides released from the substrate per mg of protein in the bees' extract. A total of 120 female bees and 80 male bees were used in the experiment. Enzyme activity levels were determined monthly for each substrate in triplicate.

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

Statistical analysis. Statistical analyses were conducted using the Statistica (Statsoft, V. 9.0, Tulsa, USA) software package. Data was analysed by multi-factor ANOVA and a post-hoc test. The results were considered statistically significant at $p<0.05$.

RESULTS

The protein content ranged from 15.26 mg/mL to 35.69 mg/mL in female bee extracts and from 16.95 mg/mL to 31.55 mg/mL in male bee extracts. The lowest protein levels were noted in hatched bees (April), and the highest in diapausing individuals (January). Protein levels remained relatively stable in males during diapause. In females, a decrease in protein concentrations was observed between January and March, but it was not statistically significant due to high standard deviations. Significantly lower protein concentrations ($p<0.00003$) were reported in hatched males and females than in all diapausing individuals (Fig. 1).

Male and female proteolytic enzymes remained active throughout the experimental period. Female proteinases hydrolysed BSA and gelatine more effectively than casein and haemoglobin throughout the entire period of the study (Fig. 2). For BSA, the observed differences were significant (excluding values for March). Male proteinases hydrolysed gelatine more effectively than the other substrates, and the noted differences were significant (excluding gelatine values for October). There were almost no significant differences in the activity of female enzymes with the same substrates between experimental months, with the exception of the BSA activity in October and March (Fig. 2). The reverse was observed in males (Fig. 3). The activity levels of male enzymes were significantly higher in October than in the late diapause period (excluding gelatine values for March) (Fig. 3).

Greater fluctuations in proteinase activity were observed in males than females. For example, proteolytic activity on gelatine ranged from 0.123 U/mg to 0.34 U/mg in males (Fig. 3) and from 0.138 U/mg to 0.216 U/mg in females (Fig. 2). Male proteinases showed a preference for the gelatine substrate.

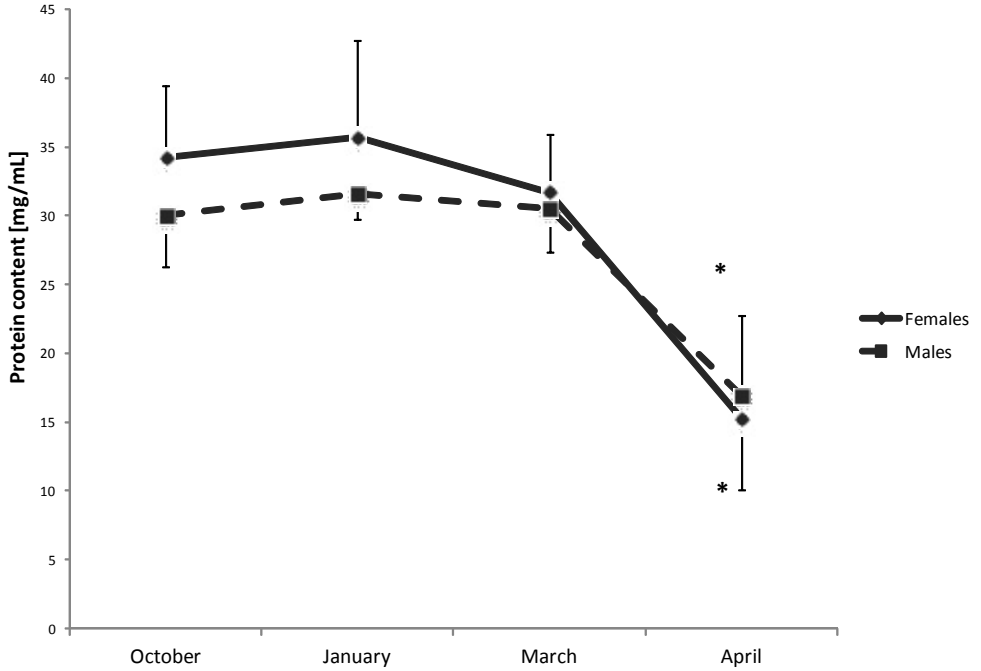

Fig. 1. Protein content in female and male *O. rufa.* Asterisks represent statistically significant differences ($p<0.05$) in the protein content of females and males in the studied period.

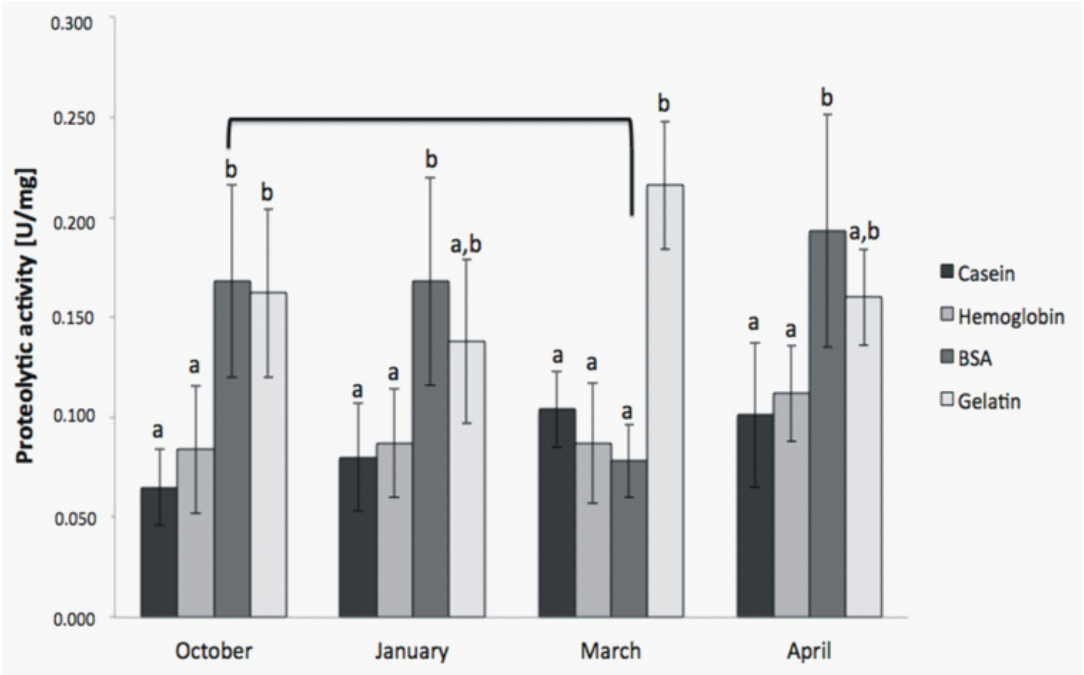

Fig. 2. The mean activity of proteinases from female *O. rufa.* Different letters (a, b) at the top of the bars represent significant differences among the enzyme activities for different substrates in the appropriate month. Brackets represent significant differences between enzyme activities for a specific substrate.

In March and April, their gelatinolytic activity was significantly higher than for the other substrates ($p<0.00003$). The gelatinolytic activity of proteinases in hatched males was 2-fold higher (0.340 U/mg) than in October (0.176 U/mg) and nearly 3-fold higher than in January (0.123 U/mg). During the initial diapause period (October), the proteolytic activities for haemoglobin ($p<0.002$) and casein ($p<0.0003$) were significantly higher in males than in females. In January, proteinase activity levels for BSA were higher in females than in males ($p<0.00003$). The activity of extracts from hatched insects differed significantly in relation to BSA (higher in females, $p<0.0003$) and gelatine (extremely high in males, $p<0.0003$) (Fig. 2 and 3).

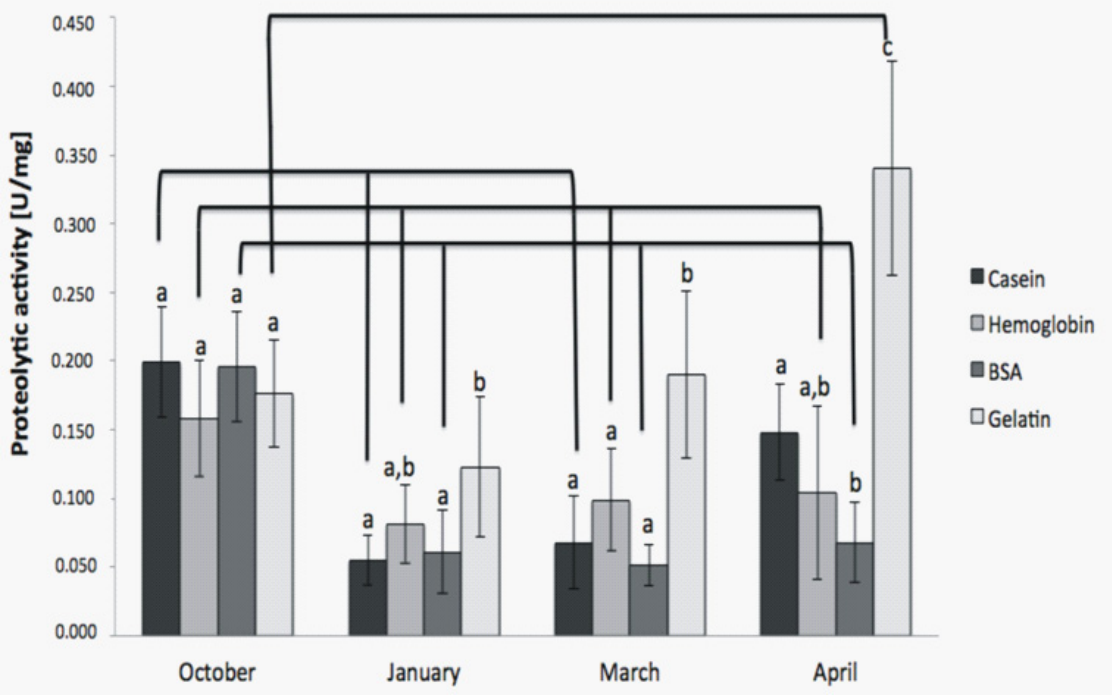

Fig. 3. The mean activity of proteinases from male *O. rufa*. Different letters (a, b) at the top of the bars represent significant differences among the enzyme activities for different substrates in the appropriate month. Brackets represent statistically significant differences between enzyme activities for a specific substrate.

DISCUSSION

Proteinases and their inhibitors are found in all tissues and play various roles in insect biology. Most importantly, they are the digestive enzymes of the alimentary system. Proteolytic activity plays an important role in the degradation of cell proteins, tissue remodelling during metamorphosis, immune responses, and hemolymph clotting (Lima et al., 2000; Otlewski et al., 2001; Deraison, 2004; Malone et al., 2004; Evans et al., 2006; Strachecka and Grzywnowicz, 2008; Strachecka et al., 2010; Grzywnowicz et al., 2009; Andrejko and Mizerska-Dudka, 2011; Frączek et al., 2013). Proteinase activity is important for *O. rufa* that feed on high protein diets composed mainly of pollen (Brodschneider and Crailsheim, 2010). These bees require an effective proteolytic system to digest proteins present in their diet.

The red mason bee enters an obligatory diapause and overwinters as an imago inside a cocoon (Giejdasz and Wilkaniec, 2002). The diapause of bee species of the genus *Osmia* can be divided into two stages. A rapid decrease in respiration intensity was observed at the beginning of the first diapause stage (lasting about 100 days), followed by a plateau during which respiratory intensity remained stable or decreased very slowly (Bosch and Kemp, 2003; Sgolastra et al., 2010). Respiration intensity is an indicator of metabolic rate. The imago stage is achieved before diapause, which could suggest that metabolic processes slowed down to a level supporting the fulfilment of the insect's basic metabolic needs. Wasilewski et al. (2011) demonstrated that this is not always the case. They determined that, during oogenesis in diapausing female red mason bees, fat body proteins are rebuilt and used for vitellogenin synthesis. Proteolytic enzymes participate in those processes. In this study, proteinases were active throughout the entire diapause period. Enzyme activity levels were measured in total body extracts instead of the extracts from fat bodies or the ovaries; therefore, our results cannot be directly compared to the findings of Wasilewski et al. (2011). Despite this, our observations suggest that enzymes that use casein as their main substrate could be involved in vitellogenesis because their activity patterns in females correspond to the changes in the protein content of *O. rufa* fat bodies reported by Wasilewski et al. (2011). Casein is a phosphoprotein, like vitellogenin (Adamson and Reynolds, 1996).

The observed differences in the effectiveness of *O. rufa* in decomposing various substrates suggest the presence of a heterogeneous range of proteolytic enzymes in the red mason bee. The noted levels of gelatinolytic activity suggest the proteolytic enzymes of red mason bees have a catalytic preferences for peptide bonds formed by amino acids with short side-chains. The proteinases of

another solitary bee, *Megachile rotundata*, demonstrated similarly high levels of activity on gelatine (Felicioli et al., 2004). The gelatinolytic activity in *O. rufa* increased significantly during the late phase of diapause in females and in newly hatched males. Similar fluctuations in the activity of proteolytic enzymes – trypsin, chymotrypsin, elastase, and aminopeptidases – were observed during diapause in *Lymantria dispar* (Lee et al., 1998). In *Culex pipiens,* the expression of trypsin and chymotrypsin genes decreased at diapause and increased after it (Robich and Denlinger, 2005). A similar process could take place in *O. rufa* males where nearly all proteinases were characterized by lower activity levels during the winter dormancy. The caseinolytic and gelatinolytic activities of proteinases increased significantly in newly hatched males. This process was less pronounced in females, perhaps due to oogenesis. In the late phase of diapause, the observed increase in proteolytic activity levels in both sexes, in particular in hatching individuals, could be related to the insects' preparations for active life and food intake. A similar increase in proteolytic activity levels was observed after hatching in other insects, including *M. rotundata* and *Bombyx mori* (Indrasith et al., 1988; Ikeda et al., 1990; Felicioli et al., 2004).
The substrate preferences of the analysed enzymes suggest there are sex-related differences. Female and male bees differ in their genomic ploidy levels, which could lead to variations in gene expression, including of protease genes, between the sexes. Szolderits and Crailsheim (1993) described differences in the activities of proteolytic enzymes in *A. mellifera* males and females. They attributed the observed variations between the sexes to differences in their nutrition strategies and various tasks performed by drones and workers. In red mason bees, there are greater differences between the physiological tasks of males and females, since - unlike honey bee workers - *O. rufa* are fertile.

CONCLUSIONS

Further biochemical research should be conducted on the useful crop pollinator, *O. rufa*. The observed differences in the effectiveness of breaking down substrates suggest the presence of a heterogeneous range of proteolytic enzymes in the red mason bee. Proteolytic activity levels determined in diapausing female and male red mason bees suggest there are protein transformations occurring during winter dormancy. Our results suggest that, similar to other insects, the proteolytic activity patterns in *O. rufa* are sex-dependent and could be controlled by a diapause programme specific to them. In late diapause, the observed increase in proteolytic activity levels in both females and males, particularly in hatching individuals, could be related to the insects' preparations for active life and food intake.

REFERENCES

Adamson N. J., Reynolds E. C. (1996) Characterization of casein phosphopeptides prepared using alcalase: determination of enzyme specificity. Enzyme and Microbial Technology 19(3): 202-207.

Aitken A., Learmonth M. (1996) Protein determination by UV absorption. In: Walker J. (Ed.) The protein protocols handbook. Humana Press. Totowa, New Jersey 3-6. DOI: 10.1007/978-1-60327-259-9_1

Andrejko M., Mizerska-Dudka M. (2011) Elastase B of *Pseudomonas aeruginosa* stimulates the humoral immune respons in the great wax moth, *Galleria mellonella.* Journal of Invertebrate Pathology 107(1): 16-26.

Biliński M., Teper D. (2004) Rearing and utilization of the red mason bee – *Osmia rufa* L. (Hymenoptera, Megachilidae) for orchard pollination. Journal of Apicultural Science 48(2): 69-74.

Bosch J., Kemp W. P. (2003) Effect of wintering duration and temperature on survival and emergence time in males of the orchard pollinator *Osmia lingaria* (Hymenoptera: Megachilidae). Environmental Entomology 32: 711-716.

Brodschneider R., Crailsheim K. (2010) Nutrition and health in honey bees. Apidologie 41(3): 278-294.

Conrad T., Paxton R. J., Barth F. G., Francke W., Ayasse M. (2010) Female choice in the red mason bee, *Osmia rufa* (L.) (Megachilidae). Journal of Experimental Biology 213: 4065-4073.

Deraison M. C. (2004) Isolement, caractérisation et cibles de nouveaux inhibiteurs deprotéases pour la création de plantes transgéniques résistantesaux pucerons. Universite Paris XI UFR Scientique d'orsay 1: 1-241.

Dmochowska K., Giejdasz K., Fliszkiewicz M., Żółtowska K. (2012) Changes in the antioxidative system of the red mason bee (*Osmia rufa)* (Hymenoptera: Megachilidae) induced by artificially elongated diapause. Journal of Apicultural Science 56(1): 99-106.

Dmochowska K., Giejdasz K., Fliszkiewicz M., Żółtowska K. (2013) Prolonged postdiapause: Influence on some indicators of carbohydrate and lipid metabolism of the red mason bee, *Osmia rufa*. Journal of Insect Science 13: 77.

Evans J. D., Aronstein K., Chen Y. P., Hetru C., Imler J. L., Jiang H., Kanost M., Thompson G. J., Zou Z., Hultmark D. (2006) Immune pathways and defence mechanisms in honey bee *Apis mellifera*. Insect Molecular Biology 15(5): 645-656.

Felicioli A., Donadio E., Balestreri E., Montagnoli G., Felicioli R., Podestà A. (2004) Expression profile of water-soluble proteinases during ontogenesis of *Megachile rotundata:* an electrophoretic investigation. Apidologie 35(6): 595-604.

Frączek R. J., Żółtowska K., Lipiński Z., Dmitryjuk M. (2013) The mutual influence of proteins from *Varroa destructor* extracts and from honeybee haemolymph on their proteolytic activity – in vitro study. Acta Parasitologica 58(3): 317-323.

Giejdasz K., Wilkaniec Z. (2002) Individual development of the red mason bee (*Osmia rufa* L., Megachilidae) under natural and laboratory conditions. Journal of Apicultural Science 46(1): 51-57.

Grzywnowicz K., Ciołek A. (Strachecka), Tabor A., Jaszek M. (2009) Profiles of body-surface proteolytic system of honey bee queens, workers and drones: Ontogenetic and seasonal changes in proteases and their natural inhibitors. Apidolgie 40(1): 4-19.

Ikeda M., Sasaki T., Yamashita O. (1990) Purification and characterization of proteases responsible for vitellin degradation of the silkworm, *Bombyx mori*. Insect Biochemistry 20(7): 725-734.

Indrasith L. S., Sasaki T., Yamashita O. (1988) A unique protease responsible for selective degradation of a yolk protein in *Bombyx mori*. Journal of Biological Chemistry 263(2): 1045-1051.

Lee K. Y., Valaitis A. P., Denlinger D. L. (1998) Activity of gut alkaline phosphatase, proteases and esterase in relation to diapause of pharate first instar larvae of the gypsy moth, *Lymantria dispar*. Archives of Insect Biochemistry and Physiology 37(3): 197-205.

Lima P. R. M., Brochetto-Braga M. R., Chaud-Netto J. (2000) Proteolytic activity of Africanized honey bee (*Apis mellifera*: Hymenoptera, Apidae) venom. Journal of Venomous Animals and Toxins 6(1): 104-113.

Malone L. A., Todd J. H., Burgess E., Christeller J. T. (2004) Development of hypopharyngeal glands in adult honey bees fed with a Bt toxin, a biotin-binding protein and a protease inhibitor. Apidologie 35(6): 655-664.

Mendiola J., Alonso M., Marquetti M. C., Finlay C. (1996) *Boophilus microplus,* multiple proteolytic activities in the midgut. Experimental Parasitology 82(1): 27-33.

Otlewski J., Jaskólski M., Buczek O., Cierpliński T., Czapiński H., Krowarsch D., Smalas A. O., Stachowiak D., Szpineta A., Dadlez M. (2001) Structure - function relationship of serine protease - protein inhibitor interaction. Acta Biochimica Polonica 48(2): 419-428.

Robich R. M., Denlinger D. L. (2005) Diapause in the mosquito *Culex pipiens* evokes a metabolic switch from blood feeding to sugar gluttony. Proceedings of the National Academy of Sciences of the USA (PNAS) 102(44): 15912-15917.

Ruszkowski A., Bilinski M. (1986) Food plants and economical importance of mason-bee (Osmia Pz., *Hoplitis* Klug, *Anthocopa* Lep. - *Hymenoptera, Megachilidae*). Pszczelnicze Zeszyty Naukowe 30: 63-87.

Sgolastra F., Bosch J., Molowny - Horas R., Maini S., Kemp W. P. (2010) Effect of temperature regime on diapause intensity in an adult-wintering Hymenopteran with obligate diapause. Journal of Insect Physiology 56(2): 185-194.

Stöcklin R., Favreau P., Thai R., Pflugfelder J., Bulet P., Mebs D. (2010) Structural identification by mass spectrometry of a novel antimicrobial peptide from the venom of the solitary bee *Osmia rufa* (Hymenoptera: Megachilidae). Toxicon 55(1): 20-27.

Strachecka A., Grzywnowicz K. (2008) Aktywność inhibitorów proteaz na powierzchni ciała pszczoły miodnej. Medycyna Weterynaryjna 64(10): 1256-1259.

Strachecka A., Gryzińska M., Krauze M. (2010) The influence of environmental pollution on the protective proteolytic barrier of the honey bee *Apis mellifera*'s body surface. Polish Journal of Environmental Studies 19(4): 855-859.

Szolderits M. J., Crailsheim K. (1993) A comparison of pollen consumption and digestion in honeybee (*Apis mellifera carnica*) drones and workers. Journal of Insect Physiology 39(10): 877-881.

van Engelsdorp D., Meixner M. D. (2010) A historical review of managed honey bee populations in Europe and the United States and the factors that may affect them. Journal of Invertebrate Pathology 103: 80-95.

Wasielewski O., Giejdasz K., Wojciechowicz T., Skrzypski M. (2011) Ovary growth and protein levels in ovary and fat body during adult-wintering period in the red mason bee, *Osmia rufa*. Apidologie 42: 749-758.

Wójtowski F., Wilkaniec Z. (1978) Rearing and utilization of solitary bees settled in nest traps. Instrukcja Wdrożeniowa AR Poznań 1: 1-10.