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Original Article

THE BODY COMPOSITION AND ENZYMES OF CARBOHYDRATE METABOLISM OF *VARROA DESTRUCTOR*

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Abstract

The aim of the present research was to determine the basic composition of the organic compounds present in extracts from *Varroa destructor*, a parasitic mite of the honeybee. The total protein content was 9.16 ± 0.82 mg/100 mg of body weight, lipid content was 9.81 ± 1.99 mg/100 mg, and carbohydrate content was 26.67 ± 4.52 mg/100 mg. The triacylglycerol content was 2.40 ± 0.86 mg/100 mg and the cholesterol content was 0.14 ± 0.02 mg/100 mg. Thin layer chromatography indicated that phospholipids comprised the major part of the lipid component; cephalins (78%), cerebrosides (16%), and lecithins (6%) were identified in the phospholipid pool. Glucose (23.6 ± 4.52 mg/100 mg) was the main carbohydrate, followed by glycogen (5.43 ± 1.23 mg/100 mg) and trehalose (0.35 ± 0.07 mg/100 mg). Enzyme-linked immunosorbent assays detected two major glycogen metabolism enzymes, glycogen phosphorylase and glycogen synthase. Among the enzymes metabolising disaccharides, maltase (24.7 ± 2.38 µmol/mg protein) and trehalase (14.81 ± 5.21 µmol/mg protein) presented the highest activity. Saccharose and lactose were hydrolysed to a minor extent. These are the first measurements of the basic composition of the mite body. Although these data are not exhaustive, they may serve as the basis for further research on the metabolism of *V. destructor*, particularly concerning lipid metabolism.

Keywords: body composition, carbohydrates, enzymes of carbohydrates metabolism, lipids, proteins, *Varroa destructor*.

INTRODUCTION

Varroa destructor mites are one of the most dangerous ectoparasites of the honeybee *Apis mellifera* (Le Conte et al., 2010; Rosenkranz et al., 2010). The mites feed on the haemolymph of capped brood and mature individuals of *A. mellifera*. They are the vector of many viruses, bacteria, protozoans, and fungi that are dangerous for bees (Ball, 1994; Francis et al., 2013). It is assumed that the pathogens transmitted by the mite may decide the life of both the individual insect as well as the entire honeybee colony if the host's immune system is dysfunctional. The loss of haemolymph is very important in the pathogenesis of varrosis, particularly the loss of structural and energetic compounds critical for brood development. Developmental stages of parasite and the mother mite rob to 25% of the honey brood nutrient reserves (Garedew et al., 2004). How does such extensive feeding by the mite translate into the mite's body composition? Although the literature on varrosis and the *Varroa* mite is extensive (see reviews by Rosenkranz et al. (2010) and by Borsuk et al. (2012)), data on the basic chemical body composition of *V. destructor* are lacking in the available literature.

A recently published series of articles addressed *V. destructor* enzymes, particularly those related to the increasing resistance of mites to pesticides, such as the esterases, glutathione S-transferases, and the p450 oxygenases (Sammataro et al., 2005; Campell et al., 2010; Johnson et al., 2010). The proteolytic enzymes of the parasite were also examined previously (Frączek et al., 2012; 2013; Strachecka et al., 2013). However, the enzymes associated with carbohydrate metabolism, including the metabolism of glycogen and the disaccharides, have not been not rigorously studied (Colin et al., 2001; Frączek et al., 2009). These enzymes seem to be important because the haemolymph of bees, the main source of nutrition of *V. destructor*, is rich in carbohydrates (Božič and Woodring, 1997; Blatt and Roces, 2001; Hrassing and Crailsheim, 2005).
We sought to determine the content of the basic chemical compounds constituting the body of the female *V. destructor* parasitizing a capped brood. Our measurements may be important for understanding the biology and metabolism of this dangerous parasite.

MATERIAL AND METHODS

Biological material. Material was collected at the beginning of July 2011 from apiaries of *A. mellifera* naturally infected with *V. destructor*; two apiaries (six colonies) were located within 20 km of Gdańsk, Poland, and three apiaries (nine colonies) were located near Nidzica, Poland. Immediately following transfer (~1.5 h) to the laboratory, mature female mites (n = 6,500) were isolated from the capped cells of honeybee combs.

Sample preparation (n = 6). Extracts for protein, carbohydrate, and enzymatic analysis were obtained from the isolated mites and prepared according to Frączek et al. (2012). Briefly, 400 mites were washed four times by submerging in 30 mL distilled water with mild shaking for 10 min. The mites were dried, weighed, and ground in a porcelain mortar with 2 mL of 0.9% NaCl in an ice bath. Samples were then placed at 4°C for 1 h, followed by centrifugation at 5,000 x g for 10 min at 4°C. The pellet was discarded, and the supernatant was used for the determination of protein content, carbohydrate content, and disaccharidase activity.

Protein analysis. Protein concentration was determined according to the method of Lowry et al. (1951).

Carbohydrate analysis. Total carbohydrate content was measured using the colorimetric anthrone method (Kłyszejko-Stefanowicz, 1999). Extracts were diluted five-fold. Results were converted to mg of hexose/100 mg of body weight.

Analysis of soluble carbohydrates. Two hundred-microlitre samples of mite extract were evaluated via high-performance liquid chromatography, which was performed according to Dmitryjuk et al. (2009). Carbohydrate chromatography was carried out in a SCL-10A chromatograph equipped with a RID-10A refractometric detector (Shimadzu), on a 250 x 4.6 mm High-Performance Carbohydrate cartridge column (Waters, The Netherlands) at 55°C. The mobile phase was a 75:25 mixture of acetonitrile-deionised water (1.0 mL/min flow rate). Data were processed with Chromax 2005 software (POL-LAB, Warszawa, Poland), and results were converted to μmoles of carbohydrate/100 mg of body weight.

Glycogen content determination. Glycogen was isolated from the extract via the micromethod described by Sölling and Esmann (1975). Ten microlitres of extract were pipetted onto square Whatman No. 3 filter paper (10 mm each side). Glycogen was precipitated on the filter paper by treatment with 5 mL of 10% trichloroacetic acid in 70% ethanol, and then rinsed three times with 5 mL of ethanol for 20 min. The squares were rinsed in cold acetone for 10 min, dried, cut into small pieces, and placed in a tube. Buffer (0.5 mL of 0.2 M acetate buffer, pH 4.8) and 30 μL amyloglucosidase (25.8 mU; A-7255, Sigma) were added to each tube, which were incubated for 15 min at 55°C with careful shaking. At the same time, a standard solution

of glycogen (5 mg/mL) was treated in the same way. Glucose released from glycogen by amyloglucosidase was determined by the enzymatic method using the Cormay (Liquick Cor-Glucose, Cat. No. 2-2010) kit. Results were expressed as µg of glucose/100 mg of tissue.

Determination of disaccharidase activity. The activities of maltase (EC 3.2.1.20), trehalase (EC 3.2.1.28), saccharase (EC 3.2.1.26), and lactase (EC 3.2.1.23) were determined according to Dahlqvist (1968); the method is based on the measurement of glucose released by the enzymes from their specific substrates (maltose, trehalose, saccharose, and lactose, respectively). Samples contained 25 µL of mite extract, 0.375 mL of 0.2 M phosphate buffer (pH 6.6), and 100 µL of 0.5 mM solution of the appropriate substrate. The samples were incubated at 37°C for 2 h. Glucose was measured as above using the Cormay's kit. Activity was expressed as µmoles of glucose released by the enzyme from its substrate per 1 mg of proteins in the mite extract.

Determination of glycogen synthase and glycogen phosphorylase. Enzyme-linked immunosorbent assays (ELISAs) were performed to measure the amounts of these two enzymes. Briefly, mite extract was prepared by grinding 40 mg of *V. destructor* with 4 mL of phosphate-buffered saline (PBS, pH 7.4) followed by centrifugation at 5000 x g for 10 min at 4°C. The supernatant was diluted with PBS (pH 7.4) in the range of 1:2 to 1:32 v/v. Microtitre plates (Corning, Sigma) were coated with 100 µL of diluted extracts, incubated for 24 h at 4°C, and washed three times with 200 mL of PBS containing 0.05% Tween 20. Sites that remained uncoated by mite antigen were blocked with 1% gelatine in PBS for 2 h, and the plates were washed again with PBS. Primary polyclonal rabbit antibodies against glycogen synthase (SAB 4300648, Sigma) or against glycogen phosphorylase (SAB 2900042, Sigma) were diluted from 1:1000 to 1:4000 in 1% gelatine in PBS. Then, 100 µL of secondary antibody (anti-rabbit IgG-peroxidase antibody; A6667, Sigma) diluted 1:5000 with 1% gelatine in PBS were applied and the plates were rinsed. Next, 1 mL of peroxidase substrate was added (0.9% hydrogen peroxide in 50 mM citrate-phosphate buffer, pH 5.0, and 0.4 mg of o-phenyldiamine) and incubated for 30 min at 37°C. The reaction was stopped with 5 M HCl, and absorbance was measured with a microplate reader (ELISA, ASYS UVM 340 Biogenet with Micro Win 2000 software) at 492 nm. ELISA steps were performed at 37°C with shaking. Negative controls consisted of samples without antibodies against glycogen synthase or glycogen phosphorylase. Results were expressed as nmol enzyme/100 mg of body weight. The analyses for each extract were conducted three times.

Lipid extraction (n = 3). Lipids were extracted from 200 mg of *V. destructor* by grinding the mites with 4 mL chloroform/methanol (2:1 v/v) according to the method of Fochl et al. (1957). The lipid precipitate was dissolved with 1.0 mL of absolute ethanol, and the lipid solution was used for subsequent analyses.

Determination of total lipid content. Lipid content was assayed by the sulfo-phospho-vanillin reaction (Frings et al., 1972). Twenty microlitres of the ethanol solution of extracted lipids were added to 0.2 mL of concentrated sulphuric acid. Samples were placed in boiling water for 10 min, cooled, and 10 mL of sulfo-phospho-vanillin reagent (prepared as in the orginal method by Frings et al. (1972) were added. After a 15-min incubation at 37°C, the absorbance was measured at 540 nm. Lipid content was expressed as mg/100 mg of body weight.

Thin layer chromatography (TLC). Ten microlitres of lipid-extract solution were spotted on commercial TLC plates (Silica gel G 60, Merck, ref. 5727). The standard lipid mixture included cholesterol, glyceryl tripalmitate, ceramide, phosphatidylethanolamine, and phosphatidylcholine (all from Sigma). Chloroform-methanol-water (60:30:5 by volume) was used as the mobile phase. After drying, the plates were exposed to iodine vapours; lipids appeared as yellow-brown spots. These spots were immediately scratched

off into Eppendorf tubes, extracted with 0.2 mL of absolute ethanol, and centrifuged for 5 min at 500 x g. Individual classes of phospholipids in the supernatant were measured with the method of Chalvardjian and Rudnicki (1970).

Measurement of triacylglycerols. Triacylglycerol levels were determined in the solution of extracted lipids using the Randox kit for triglycerides (TRIGLYCERIDES GPO-PAP, TR210) according to the manufacturer's instructions.

Cholesterol measurement. Cholesterol levels were measured in the solution of extracted lipids using the Pointe Scientific (Poland) kit (Cholesterol no. C7509-400) according to the manufacturer's instructions.
Measurements were repeated 3 - 6 times. Results are shown as means with SD.

RESULTS

Proteins and lipids were present in extracts from the body of *V. destructor* in nearly equal amounts (~9 mg/100 mg of mite body weight), and the carbohydrate level was approximately three times higher (Tab. 1). TLC revealed three major phospholipid fractions in the lipid extracts (Fig. 1): 78% were lipids with an Rf corresponding to the cephalins, 16% to the cerebrosides, and 6% to the lecithins. Triacylglycerols and cholesterol were also present (Tab. 1, Fig. 1).
Among the soluble carbohydrates, glucose was the major component (up to 97.21%), followed by trehalose (up to 1.46%); maltose, saccharose, and fructose were present in trace quantities (Tab. 1). Substantial levels of glycogen were present (5.43 ± 1.23 mg/100 mg). ELISA indicated that glycogen phosphorylase and glycogen synthase were present in the mite extracts; positive reactions were observed with a 32-fold dilution of mite extract and 1:1,000 dilutions of each antibody. Assuming that the antibodies react with the enzymes at a 1:1 ratio, glycogen phosphorylase was present at ~0.825 nmol/100 mg body weight and glycogen synthase was present at 0.967 nmol/100 mg of body weight. These estimates may not be strictly accurate due to the lack of specific antibodies against *V. destructor* proteins (we used commercial rabbit antibodies against human enzymes).
Among the enzymes metabolising the disaccharides, maltase (α-1,4-glycosidase) exhibited the highest activity. Trehalase's activity was nearly half that of maltase. Saccharose and lactose were hydrolysed much less intensely (Tab. 1).

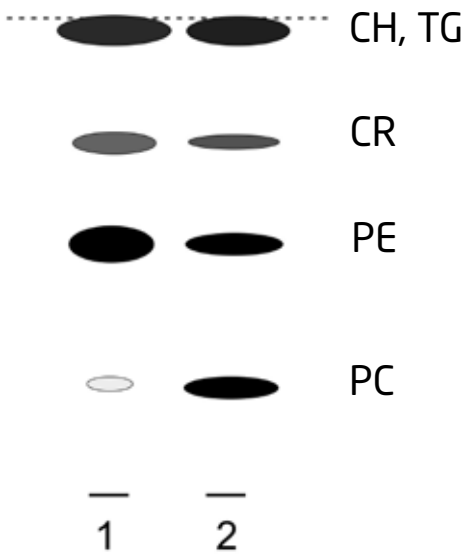

Fig. 1. Schematic of the separation of lipids in the *V. destructor* mite extract by TLC with chloroform-methanol-water (60:30:5 by volume). 1 - mite extract; 2 - standard lipid solution for comparing the Rf values of individual lipids. The size of the spots and the scale of their gray correspond to the contents of the individual lipid fractions in the sample (see Tab.1). CH - cholesterol; CR - cerebroside; TG - triacylglycerol; PE - phosphatidylethanolamine (cephalin); PC - phosphatidylcholine (lecithin).

Table 1.

Proteins, lipids, carbohydrates, glycogen-metabolising enzymes, and disaccharidases in *V. destructor* extracts

Concentration (mg/100 mg of body weight)		
	Range	Mean ± SD
Proteins	8.28 – 10.24	9.16 ± 0.82
Lipids		
Total	9.04 – 12.15	9.81 ± 1.99
Triacylglycerols	1.15 – 2.94	2.40 ± 0.86
Cephalins	3.68 – 6.65	5.78 ± 1.43
Lecithins	0.15 – 0.52	0.35 ± 0.15
Cerebrosides	0.73 – 1.69	1.09 ± 0.37
Cholesterol	0.12 - 0.16	0.14 ± 0.02
Carbohydrates		
Total	14.77 – 35.67	26.67 ± 4.52
Glycogen	4.07 – 6.65	5.43 ± 1.23
Trehalose	0.114 – 0.466	0.351 ± 0.072
Maltose	0.026 – 0.063	0.043 ± 0.018
Saccharose	0.001 – 0.003	0.002 ± 0.001
Glucose	14.55 – 30.25	23.63 ± 3.44
Fructose	0.002 – 0.004	0.003 ± 0.001
Enzymes of glycogen metabolism (nmol/100 mg tissue)*		
Glycogen phosphorylase	0.825	
Glycogen synthase	0.967	
Disaccharidases (μmol/mg protein)		
Maltase	19.67 – 28.54	24.70 ± 2.38
Trehalase	10.26 – 17.38	14.81 ± 5.21
Saccharase	1.89 – 4.02	3.09 ± 1.02
Lactase	0.63 – 1.43	1.03 ± 0.34

* These analyses have been carried out three times. Their results did not differ from each other.

DISCUSSION

Lack of data on the chemical composition of the mite body renders difficult the discussion of our results. We will therefore discuss them in the context of *V. destructor* nutritional status. The honeybees' food represents a high-energy diet (Božič and Woodring, 1997; Blatt and Roces, 2001; Hrassing and Crailsheim, 2005) to which the parasite has almost unlimited access, and thus the parasite stores fewer of its reserves as glycogen and lipids compared to other parasites which risk extended periods of starvation (Von Brand, 1973). According to Garedew et al. (2004), *V. destructor* is strictly dependent on the frequent intake of haemolymph from the host; it utilises only 2.2% of the energy contained in that haemolymph. During the first 6 hours of starvation, the mites lost on average ~50% of their wet weight, mainly water (Garedew et al., 2004) Thus, the share of lipids and non-lipid compounds in the dry mass of mites remained almost unchanged during starvation and was very similar to the share in control, non-starved *V. destructor* (Garedew et al., 2004), indicating that the rates of utilisation of lipids and non-lipid compounds are similar. These results may also suggest that there is no preference for using

lipids as the energetic reserve. Garedew et al. (2004) did not provide measurements of lipid content, only percentages, thus hindering more precise comparisons with the results of the present investigation.

Chemical analysis indicated that phospholipids (structural lipids) form the majority of the mite's lipid fraction (Tab. 1). Triacylglycerols, the preferred energetic material, comprised 24.5% of the lipid pool, a proportion that corresponds to the composition of the lipid fraction in honeybee haemolymph (Mikulecky and Bounias, 1997). Our preliminary analysis of fatty acids by gas chromatography revealed the presence of acids with chain lengths of C12 to C24 in the lipids of the mite (unpublished data), with compositions similar to those of fatty acids from honeybee larvae (Van der Vorst et al., 1983).

Arthropods, including insects, do not synthesise cholesterol (Law and Wells, 1989); their steroids originate from their diet. Hence, honeybee haemolymph is the only source of steroids for *V. destructor* (Hartfelder and Feldlaufer, 1997). It is known that the steroid content of *A. mellifera* depends on diet, which mainly consists of pollen (Szczęsna, 2006). The haemolymph steroid content of worker honeybees ranges from 0.1 nmol/mg to 0.5 nmol/mg during the lunar cycle (Mikulecky and Bounias, 1997). Based on the cholesterol content measured here, we calculate that *V. destructor* accumulates ~3.6 nmol cholesterol/100 mg of body weight. Comparison of the steroid content of the mite body and of the honeybee haemolymph suggests that very efficient mechanisms exist for the capture and accumulation of these compounds in the parasite. They may be very important to the parasite as the starting material for synthesising steroid hormones, including moulting hormones (ecdysteroids) (Hartfelder and Feldlaufer, 1997), and for the development of consecutive generations (Rosenkranz et al., 2010).

Energy originating from the carbohydrates of bee haemolymph, which are not used immediately, is stored by the mites as glycogen and trehalose. The parasite may metabolise glycogen effectively because it possesses glycogen phosphorylase and glycogen synthase (Tab. 1). Due to the small amount of available test material and the complicated procedure for determining glycogen synthase activity, which requires the use of radioisotopes, we opted to estimate the activities of both enzymes based on their protein content. ELISA indicated similar levels of the two enzymes (Tab. 1), which suggests similar activities that may allow a continual glycogen presence in tissues (although glycogen levels may fluctuate depending on energy demand). This situation is similar to that of other parasites, such as *Ascaris suum* and *Schistosoma mansoni* (Donahue et al., 1981; Tielens et al., 1990). As expected, trehalose comprised the largest part of the disaccharide pool, probably due to its high content in bee haemolymph (Blatt and Roces, 2001; Hrassing and Crailsheim, 2005). Endogenous synthesis of trehalose by mite enzymes may also occur. On the other hand, the high glucose content measured in the present investigation was surprising. For *V. destructor*, we expected to observe a proportion of soluble sugars that was similar to that found in bee haemolymph (higher levels of trehalose than of glucose; Blatt and Roces (2001)). The quantity of glucose in the body of the parasite corresponds to the high activity of enzymes releasing it from trehalose (trehalase) and from oligosaccharides, polysaccharides, glycolipids, and glycoproteins (α-1,4-glycosidases such as maltase). These results are consistent in that regard with our earlier studies (Frączek et al., 2009).

Our earlier studies addressed the composition of *V. destructor* proteins, focusing on the role of the proteolytic system in the active intake and digestion of honeybee haemolymph proteins (Frączek et al., 2012). The results of the present investigation complement our previous observations with data concerning lipids and carbohydrates. Based on the results of Garedew et al. (2004) and our own measurements, it may be assumed that carbohydrates are an important energy source used in a way that is compatible with the use of lipids by the female *V. destructor*. Our results yield insight into the basis of the mite's metabolism and physiology of digestion that are ultimately reflected in the chemical composition of its body.

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