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Original Article

CURCUMIN STIMULATES BIOCHEMICAL MECHANISMS OF *APIS MELLIFERA* RESISTANCE AND EXTENDS THE APIAN LIFE-SPAN

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Abstract

We examined the influence of curcumin-supplemented feeding on worker lifespan, *Nosema* resistance, key enzyme activities, metabolic compound concentrations and percentage of the global DNA methylation. Two worker groups (*Apis mellifera*) were set up: 1) control group; workers were fed ad libitum with sucrose syrup; 2) workers were fed with the syrup with the addition of curcumin. Dead workers were removed every two days and the *Nosema* spp. infection levels were assessed. Hemolymph was taken from living workers for biochemical analyses. The global DNA methylation level was analysed using DNA from worker heads and thoraces. The bees that consumed curcumin lived longer and were less infested with *Nosema* spp. The curcumin-treated workers had higher concentrations of proteins, non-enzymatic biomarkers (triglycerides, glucose, cholesterol, Mg^{2+} and Ca^{2+}), uric acid and creatinine, as well as elevated activities of antioxidant enzymes (SOD, GPx, CAT, GST), neutral proteases, protease inhibitors, enzymatic biomarkers (AST, ALT, ALP). The concentrations of albumin and urea, and the activities of acidic and alkaline proteases were higher in the control group. Curcumin decreased global DNA methylation levels especially in older bees in which the natural, age-related level increase was observed. Most of the parameters increased over the apian youth and adulthood, and decreased in older bees. The decrease was markedly delayed in the bees fed with curcumin. Curcumin appeared to be an unexpectedly effective natural bio-stimulator, improving apian health and vitality. This multifactorial effect is caused by the activation of many biochemical processes involved in the formation of apian resistance.

Keywords: antioxidant, *Apis mellifera*, DNA methylation, *Nosema* spp., proteolysis, vitality.

INTRODUCTION

Curcuma longa L. has been used for hundreds of years as a flavor, dye and preservative (Wilken et al., 2011). Curcumin, 1,7-bis[4-hydroxy 3-methoxy phenyl]-1,6-heptadiene-3,5-dione produced by *C. longa* L., is a natural phenol, a compound reported to possess therapeutic properties, including anti-inflammatory, anticarcinogenic and antioxidant activities (Ak and Gülçin, 2008; Wilken et al., 2011). It also may slow down the negative changes in DNA methylation pattern connected with mammalian ageing (Burzyński, 2006). Curcumin displays antimicrobial properties against many bacteria, viruses, malaria, molds, yeast and other fungi (Marathe et al., 2012; Moghadamtousi et al., 2014), but contrary to antibiotics, it has also been described as an immunostimulant in mammals (Zhao et al., 2011).

Global problems of apiculture include colony depopulation and losses, mostly caused by such diseases as varroatosis or nosemosis (Russell et al., 2013), and in a more general scope, by the suppression of apian resistance caused by anthropogenic factors, such as intensive agriculture and commercial apiculture

development (Rosenkranz et al., 2010; Russell et al. 2013). Therefore, natural bioactive preparations/ nutrients that will boost apian resistance are in demand. We suppose that curcumin might be one of these nutrients. *Nosema* spp. spores are common in honeybee colonies and harmfully affect them, particularly under conditions of protein (pollen) shortage, colony hypothermia, etc. - generally always when the apian resistance is suppressed. That is why, in addition to apian life-span, we decided to use the *Nosema* spp. infection level as one of the biological indices of apian resistance and vitality.

The activities of the hemolymphatic proteolytic and antioxidative systems are crucial for honey bee resistance (Weirich et al., 2002; Frączek et al., 2013). These systems complement each other. The antioxidative system ensures protection against reactive oxygen species (ROS). Proteins may undergo scission reactions with certain radicals/oxidants, leading to the direct formation of potentially toxic peptide fragments (Davies, 1986; Ranadive et al., 2011). Various intercellular proteolytic enzymes can recognize, and preferentially degrade, oxidatively damaged proteins to small-sized proteins and amino acids. Moreover, proteases and protease inhibitors activate zymogenes, release proteins from their precursors and receptors, as well as degrading pathogen proteins (Bode et al., 1999; Grzywnowicz et al., 2009).

Aspartate aminotransferase, alanine aminotransferase and alkaline phosphatase are used as indicators of proper liver functioning in mammalians, including humans. Their increased activities may indicate hepatotoxicity, pathological changes and chronic diseases, i.e. deteriorated heath state. Strachecka et al. (2014a, b) and Bajda et al. (2014) found that these enzymes may be also considered as important biomarkers of the health state of honeybees. Decreased activities of the biomarkers have been observed in the hemolymph of bees treated with amphotericin B (antibiotic; Bajda et al., 2014), bromfenvinphos and formic acid (our results, unpubl. data), whereas increased activities were noticed after supplementation of bee feed with such bio-stimulators as caffeine and coenzyme Q10 (Strachecka et al., 2014a, b).

Taking into account all the biological properties of curcumin, it may be hypothesized that curcumin may increase such resistance-related biochemical parameters as activities/concentrations of antioxidants, proteases, protease inhibitors and also some crucial enzymatic and non-enzymatic biomarkers in apian hemolymph (hypothesis 1). Consequently, it may extend apian life-span and reduce the level of infestation by *Nosema* spp. (hypothesis 2).

Global DNA methylation changes are related to honeybee ontogenetic development, ageing, cognitive processes and are connected with apian response to environmental pressure (Burzyński, 2006; Lyko and Maleszka, 2011). DNA methylation levels are elevated in organisms in response to stress caused by environmental pollution (Flores et al., 2013). They decrease, in turn, after supplementation (e.g. caffeine; Strachecka et al., 2014a). Therefore, we suppose that curcumin may influence not only biochemical but also epigenetic characteristics in honeybees, particularly global DNA methylation changes related with ageing (hypothesis 3).

The objective of this study was to perform cage tests in honeybee workers to refute or demonstrate the truth of hypotheses 1, 2 and 3. The results of the tests may also be of interest to biomedical scientists, those, who are interested in diet supplementation and beekeepers as the target group to benefit from the applied part of this research.

MATERIAL AND METHODS

Two worker-bee groups (*Apis mellifera*) were set up, 100 wooden cages (12 x 12 x 4 cm), 40 one-day-old workers per cage, in each of the groups. For details see: Strachecka et al. (2014a, b). In the first, the control group, the workers were fed *ad libitum* with sucrose syrup (1:1) using inner cage feeders. In the second group, the workers were fed with the syrup supplemented with curcumin (Linegal Chemicals, Poland) at the concentration of 3 μg/mL (human equivalent dose). All the cages were kept in an air-conditioned chamber (26°C and 65% RH). In each of the two groups, workers from 30 of the 100 cages (in total, 1200 workers) were designated for longevity and *Nosema* spp. infection tests, whereas those from the remaining 70 cages (in total, 2800 workers) were used for biochemical analyses. The procedures described below were started four hours after the start of worker feeding (1-day-old bees).

Procedure 1: In each the groups, dead workers were removed every two days from the 30 longevity-test cages, counted separately for each cage, and next frozen at -25°C for further assessment of the *Nosema* spp. infection level, according to methods described by Pohorecka et al. (2011). The numbers of workers that still remained alive in each the cages were recorded on the 1st, 7th, 14th, 21th and 28th day - in both groups, and on the 34th, 41st and 48th day

- in the curcumin-treated group, where bees lived longer. The apian life-span was expressed as percentages of workers that were still alive.

Procedure 2: In each of the groups and each of the remaining 70 cages, fresh hemolymph was taken 3 to 7 times from 12 to 15 living workers to obtain 3 to 7 pooled samples that contained at least 100 µL of fresh hemolymph. The samplings were performed on the 1st, 7th, 14th, 21th and 28th day of apian life (3 - 7 pooled samples x 5 samplings). Since the bees fed with curcumin lived longer, the samples were additionally collected on the 34th, 41st and 48th day in the curcumin-fed group. All the pooled hemolymph samples were separately placed into sterile Eppendorf tubes containing 200 µL of ice-cooled 0.6% NaCl. The tubes were immediately refrigerated at -25°C for further biochemical analyses. For more details, refer to Strachecka et al.'s (2014a, b) method. Additionally, 10 living workers were randomly captured from all the remaining 70 cages at the same time intervals as for the biochemical tests and refrigerated at -25°C to further analyze global DNA methylation levels.

The following biochemical analyses were performed in the hemolymph from the pooled samples:

- Protein concentration was determined using the Lowry method, as modified by Schacterle and Pollack (1973);
- Proteolytic system activity was determined as follows:
  - activities of acidic, neutral and alkaline proteases according to the Anson method (1938) modified by Strachecka et al. (2010, 2011);
  - activities of natural inhibitors of acidic, neutral and alkaline proteases according to the Lee and Lin method (1995);
  - proteolytic activities after the addition of pepstatin A, PMSF, iodoacetamide, o-phenantroline (diagnostic inhibitors) according to the Lee and Lin method (1995).
- Antioxidant system activity was determined as follows:
  - catalase (CAT) activity according to the Aebi method (1983) modified by Strachecka et al. (2014b);
  - glutathione peroxidase (GPx) activity according to Chance and Maehly (1955) modified by Strachecka et al. (2014b);
  - glutathione S-transferase (GST) activity according to Warholm et al. (1985) modified by Strachecka et al. (2014b);
  - superoxide dismutase (SOD) activity according to Podczasy and Wei (1988) modified by Strachecka et al. (2014b);

All the activities were calculated per 1 mg of protein.

- total antioxidant potential (FRAP) according to Benzie and Strain (1996);
- non-enzymatic antioxidant (albumin, uric acid, urea, creatinine) concentrations according to the colorimetric method using monotests from Cormay (Lublin, Poland).

- Biomarker activity/concentration was determined as follows:
  - activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) using monotests from Cormay (Lublin, Poland) according to the manufacturer's procedure;
  - concentrations of triglycerides, cholesterol, glucose, magnesium (Mg^{2+}) and calcium (Ca^{2+}) using Cormay monotests (Lublin, Poland) according to the manufacturer's procedure.

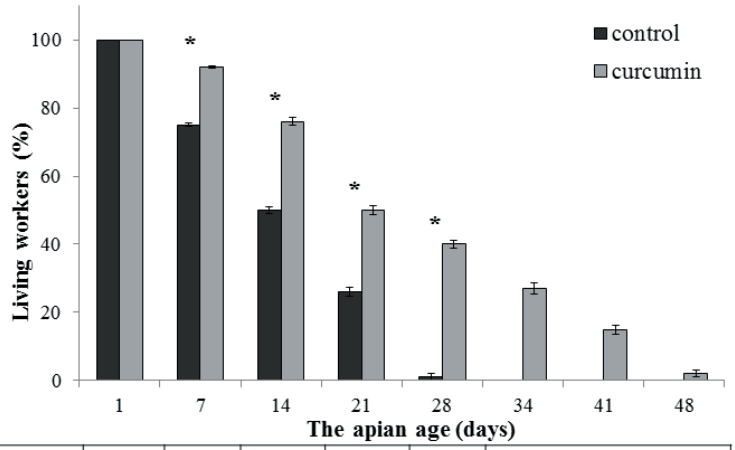
Global DNA methylation levels. After refreezing the workers, DNA was extracted individually from their heads and thoraces using the DNeasy Blood & Tissue Kit (Qiagen. Germany) following the procedure's instructions. Extracted DNA samples were stored at -25°C. The global DNA methylation analyses were performed using an Imprint Methylated DNA Quantification Kit MDQ1 (Sigma, USA) based on the ELISA principle and following the procedure's instructions. Details are provided on the manufacturer's website [electronic resource: http://www.sigmaaldrich.com/life-science/epigenetics/imprint-methylated.html (2015-05-18)], excluding patent-restricted information.

Statistical analysis. Curcumin treatment effects (Ai) and the significance of differences between the group averages were determined using two-way ANOVA and the least significant analysis procedures (LSD) with SAS statistical software (SAS Institute Version 9.13., 2002-2003 license 86636). The model was Yijk = µ + Ai + Bj + eijk (Bj is the age, i.e. the collective effect). Bj was taken into consideration as a source of variation but it was not considered in detail in this paper (Figures), which is focused on the curcumin effects only.

The numbers of *Nosema* spp. spores were compared between the control and curcumin-treated workers using the G-test with William's correction, and the significance of differences in the levels of infection between these groups was tested using the nonparametric Mann-Whitney U test (compare: Kuszewska and Woyciechowski, 2014; Strachecka et al., 2014a, b).

RESULTS

The caged workers that consumed curcumin lived longer and were less infected with *Nosema* spp. than those from the control group (Fig. 1). The percentage of the long-lived bees was also greater in the curcumin-treated group. The workers consuming curcumin had higher concentrations of hemolymph proteins (Fig. 2). Generally, curcumin consumption increased the activities of antioxidant enzymes (SOD, CAT, GPx and GST; Fig. 3), the total antioxidant potential (FRAP; Fig. 4), the activities of neutral proteases and their inhibitors (acidic, neutral and alkaline; Fig. 5), as well as the enzymatic biomarker activities (AST, ALT and ALP; Fig. 6). The workers fed with curcumin had increased concentrations of non-enzymatic biomarkers (triglycerides, glucose, cholesterol, Mg^{2+} and Ca^{2+}; Fig. 7), uric acid and creatinine (Fig. 8). It should be emphasized that the values of most of the parameters increased in



Nosema spores no.								
control	0	0	9 *	20 *	28 *	There were no living workers		
curcumin	0	0	0 *	0 *	0 *	2	2	3

Fig. 1. Longevity of workers and intensity of *Nosema* spp. infections (table) in the control and curcumin-treated group. The asterisks indicate significant differences (P≤0.01) between the group averages both for longevity and *Nosema* spp. infections within a given apian age (except for 1-day-old workers).

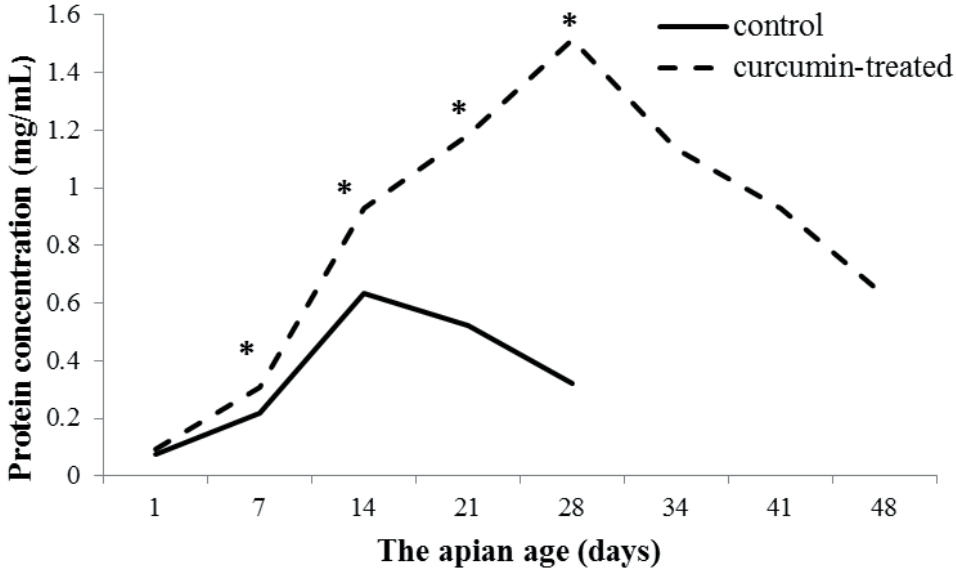


Fig. 2. Protein concentrations in worker hemolymph in the control and curcumin-treated group. The asterisks indicate significant differences (P≤0.01) between the group averages within a given apian age (except for 1-day-old workers).

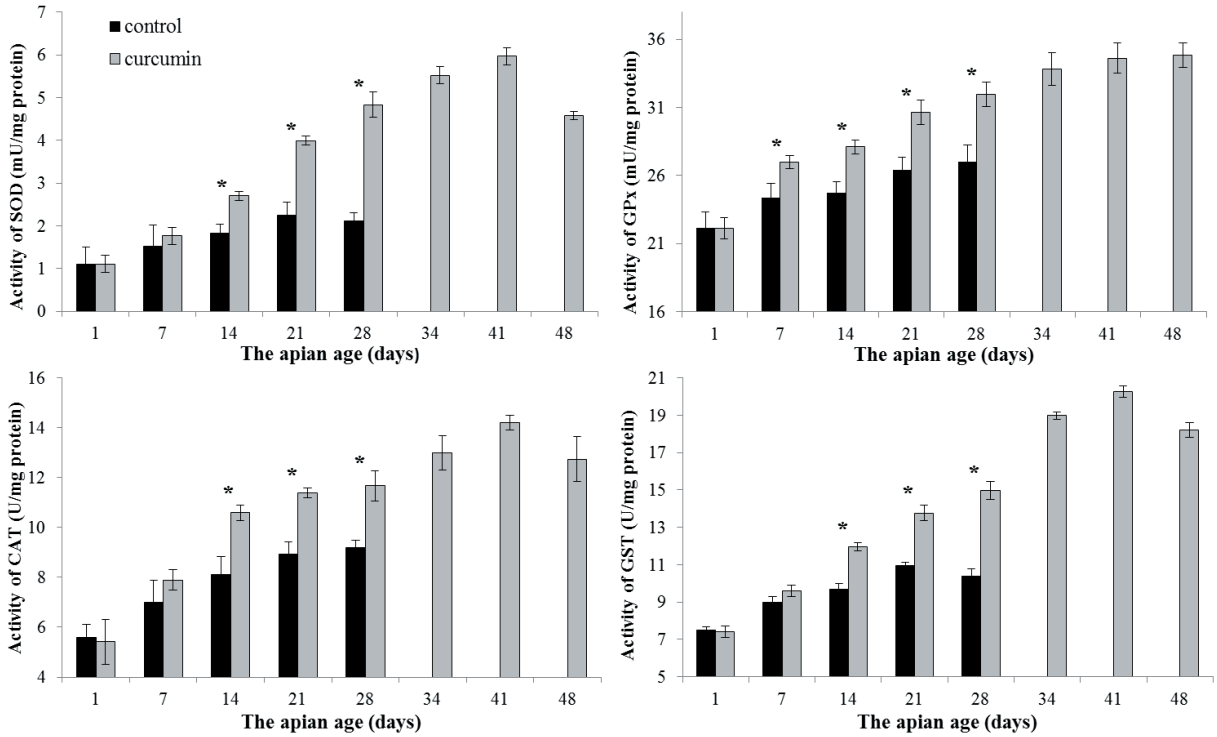


Fig. 3. Activities of enzymatic antioxidants in worker hemolymph in the control and curcumin-treated group. The asterisks indicate significant differences (P≤0.01) between the group averages within a given apian age and each of the individual enzymes. SOD - superoxide dismutase; GPx - peroxidase; CAT - catalase; GST - glutathione; S-transferase.

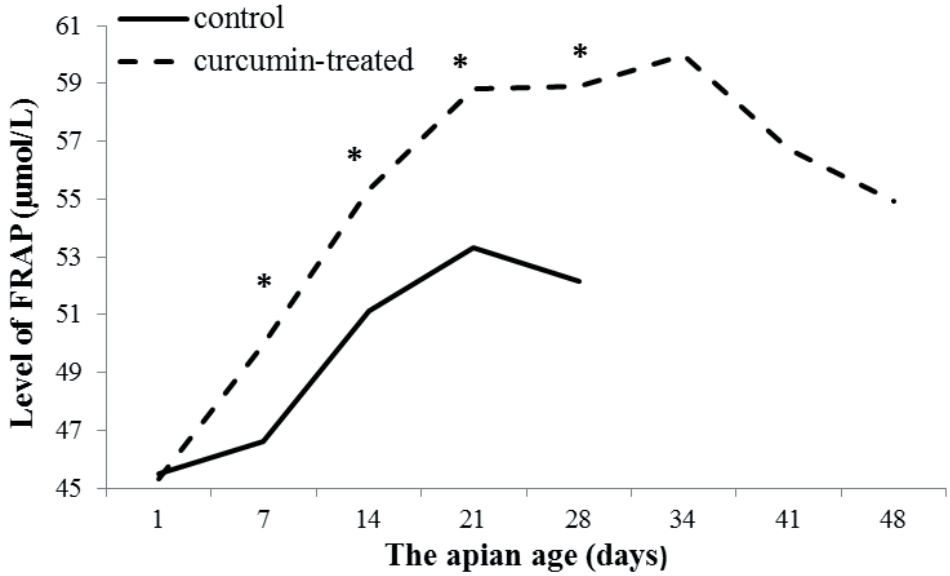


Fig. 4. Levels of the total antioxidant potential (FRAP) in worker hemolymph in the control and curcumin-treated groups. The asterisks indicate significant differences (P≤0.01) for comparisons between the group averages within a given apian age (except for 1-day-old workers).

worker youth and adulthood, and then decreased in older bees and that curcumin significantly delayed this decrease (Fig. 3 - 8). The concentrations of albumin and urea (Fig. 8) and the activities of acidic and alkaline proteases (Fig. 5) were higher in the control group. Only the concentration of glucose in the control group decreased with age (Fig. 8).

The global DNA methylation level increased with age in both groups (Fig. 9). The increase, however, was markedly slower and observed later in the workers that consumed curcumin. Generally, curcumin consumption decreased DNA methylation levels, particularly in adult and older workers.

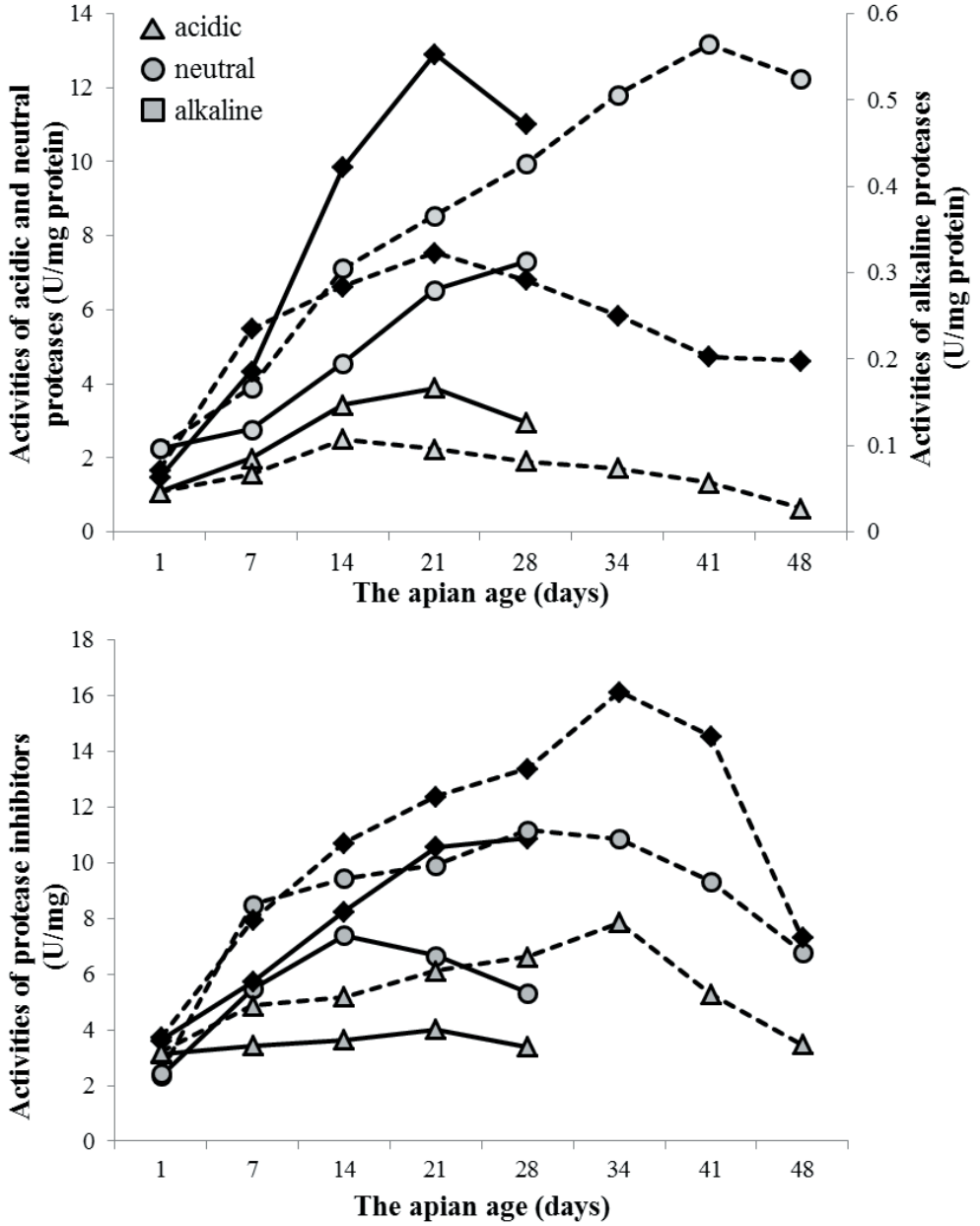


Fig. 5. Mean activities of proteases and protease inhibitors (U/mg) in the hemolymph of workers in the control (solid line) and curcumin-treated groups (dashed line). The group averages within a given apian age and each of the individual enzyme types were always significant ($P \leq 0.01$) beginning from the 7th day of age.

DISCUSSION

We confirmed that curcumin substantially extended worker life-span and suppressed *Nosema* spp. infection (Fig. 1). A similar mechanism was observed during the reduction in fungal cell ergosterol. The reduction in ergosterol production results in accumulations of the biosynthetic ergosterol precursor, which leads to cell death via ROS generation (Moghadamtousi et al., 2014). The bee organism is protected from the effects of ROS by antioxidants. First, curcumin safeguards against ROS directly, because curcumin is a lipophilic polyphenol which is also an antioxidant. The antioxidant capacities come by virtue of its chemical structure. Curcumin consists of two methoxylated phenols connected with two α, β unsaturated carbonyl groups that exist in a stable enol form. Curcumin inhibits lipid peroxidation using linoleate, a polyunsaturated fatty acid that is able to be oxidized and form a fatty acid radical. Curcumin acts as a chain-breaking antioxidant at the 3' position, resulting in an intermolecular Diels-Alder reaction and neutralization of the lipid radicals (Masuda et al., 2001). Secondly, curcumin increased mRNA expressions for such antioxidants as catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD)

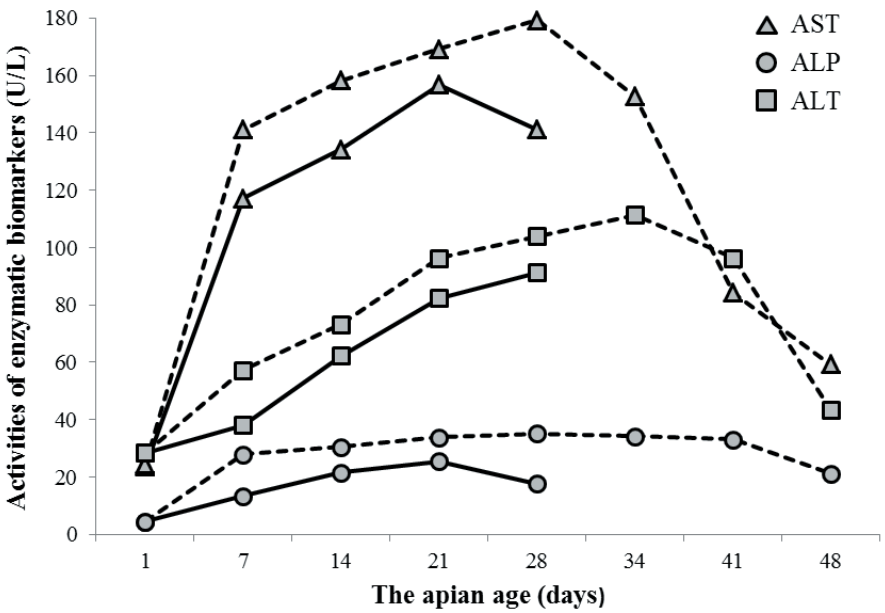


Fig. 6. Mean activities of aspartare aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in the hemolymph of workers in the control (solid line) and curcumin-treated groups (dashed line). The group averages within a given apian age and each of the individual enzyme types were always significant (P≤0.01) beginning from the 7th day of age.

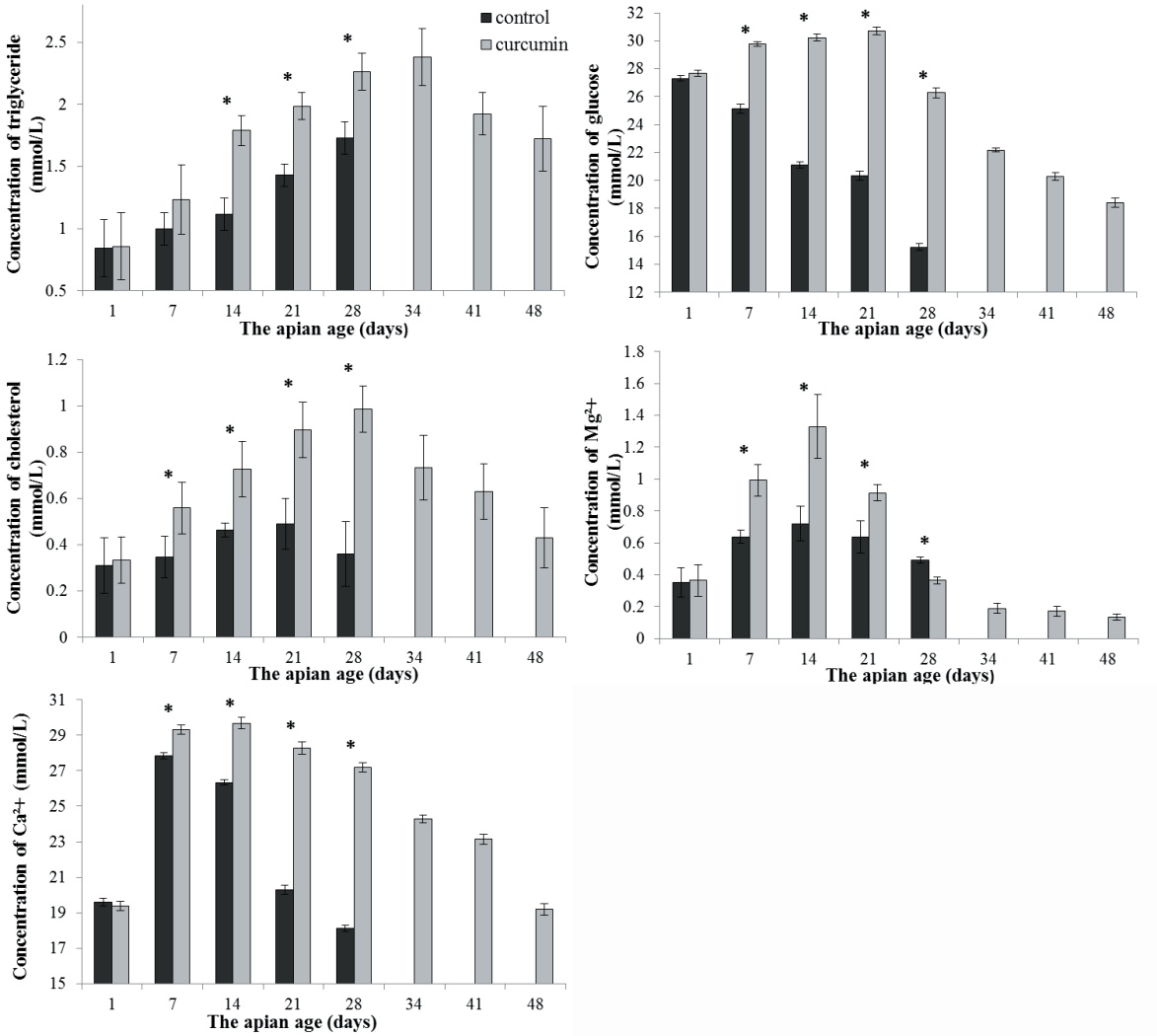


Fig. 7. Concentrations of non-enzymatic biomarkers in the hemolymph of workers in the control and curcumin-treated group. The asterisks indicate significant differences (P≤0.01) for comparisons made within a given apian age and each of the individual biomarkers.

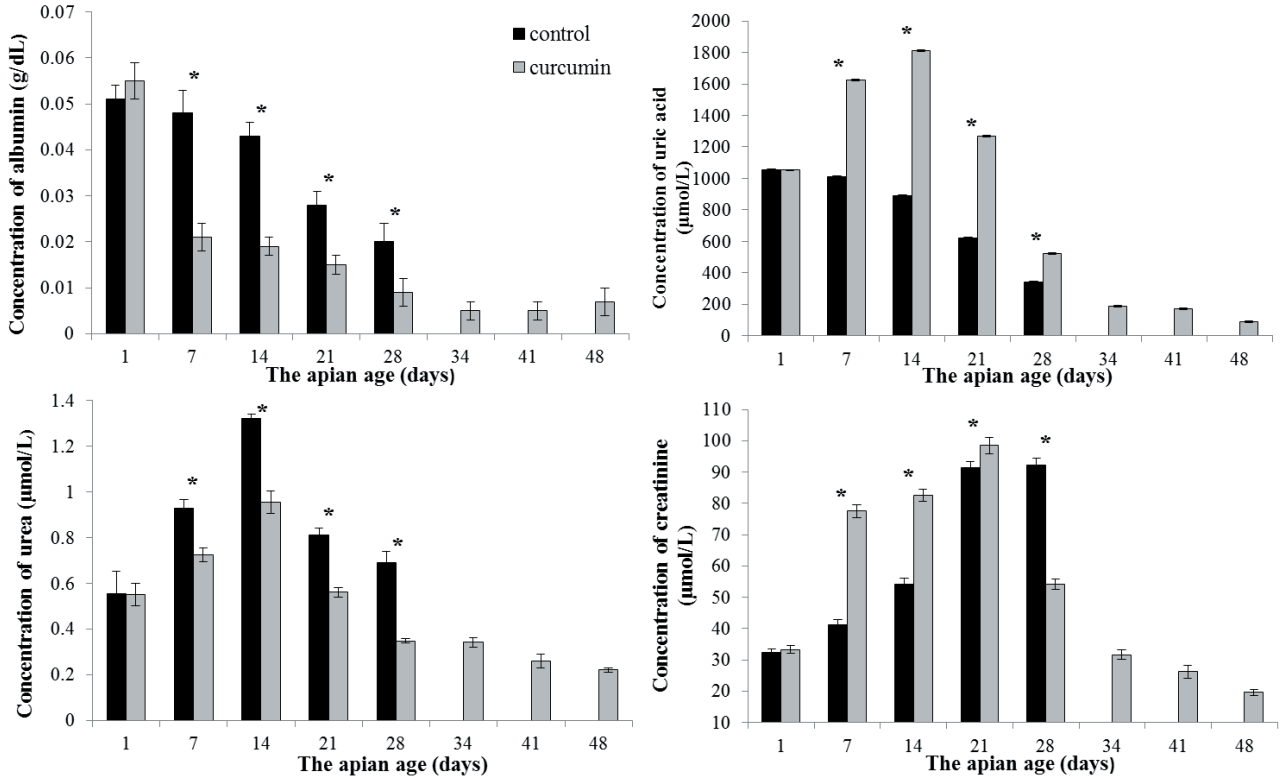


Fig. 8. Concentrations of non-enzymatic antioxidants in the hemolymph of workers in the control and curcumin-treated groups. The asterisks indicate significant differences (P≤0.01) for comparisons between the group averages within a given apian age and each of the individual antioxidants.

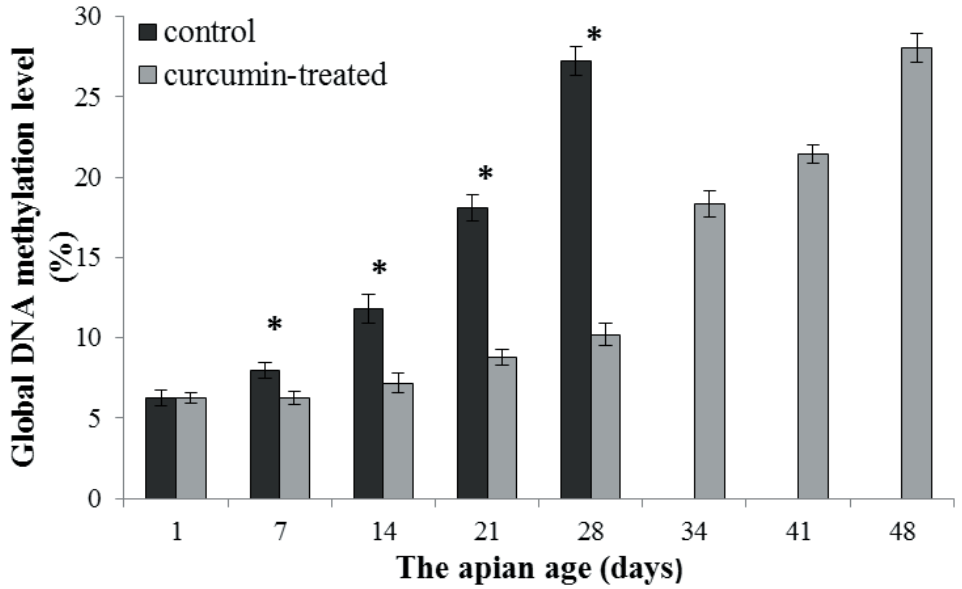


Fig. 9. Mean global DNA methylation levels (%) in workers from the control and curcumin-treated groups. The asterisks indicate significant differences (P≤0.01) for comparisons between the group averages within a given apian age (except for 1-day-old workers).

and glutathione S-transferase (GST) (Soliman et al., 2014). The increased activities of these compounds were observed in the hemolymph of bees that consumed curcumin in our study (Fig. 3). Curcumin is known to augment the antioxidant potential especially through SOD (Joe and Lokesh, 1994; Cheng et al., 2005), which converts superoxide radical to H_2O_2. Further on, H_2O_2 is converted to molecular oxygen and H_2O by either CAT or GPx. Moreover, GPx can reduce lipid peroxides and other organic hydroperoxides that are highly cytotoxic products. Thus, SOD, CAT and GPx constitute the principal

components of the antioxidant system and their deficiencies can cause oxidative stress. In addition, GSH conjugates with xenobiotic substances with the aid of GST (El-Bahr, 2013; Trujillo et al., 2013). Consequently, our results show that curcumin not only improves the antioxidative potential in mammals (Trujillo et al., 2013), but also, and even particularly, in bees. It should be emphasized that curcumin had a greater impact on almost all the biochemical characteristics, and consequently on the life-span and health of caged workers, than we could ever expect. Additionally, the concentrations of albumin, urea and creatinine (non-enzymatic antioxidants) were lower in the curcumin-treated group than those in the control group (Fig. 8). Murugan and Pari (2007) suggested that curcumin and tetrahydrocurcumin safeguard against kidney dysfunction through the inhibition of the synthesis of non-enzymatic antioxidants in diabetic rats. Additionally, curcumin induces apoptotic cell death by cycle arrest in the S and G2/M phases (Sharma et al., 2005; Ye et al., 2012) and may help to remove damaged cells, e.g. in the digestive track after *Nosema* infections.

The proteolytic system is involved in the mechanism of apoptosis (Dhule et al., 2012). Enzymes involved in the proteolytic system, both in the hemolymph and on the cuticle, are essential components of insect resistance barriers (Grzywnowicz et al., 2009; Frączek et al., 2013; Strachecka et al., 2014a, b). Their activities are also associated with the activity of the antioxidant system (Davies, 1986) because the proteolytic enzymes contribute to the amelioration of the consequences of oxidative damage (Salmon et al., 2010). Additionally, these enzymes affect the normal course of the following processes: phagocytosis, melanization, cellular adhesion, molecule recognition, generation of reactive intermediates of oxygen and nitrogen, activation of proapoptotic molecules, synthesis of cytokines and antimicrobial peptides, enzyme activation, and molecular and hormonal signalling (Bode et al., 1999; Evans et al., 2006). These processes, among others, are involved in the proper functioning of the resistance systems, as well as in the improvement of vitality, lifespan extension and stabilisation of metabolic functions in honeybees (Münch et al., 2008; Amdam, 2011). It has been revealed that curcumin increases protease activities in mammalian cells (Dhule et al., 2012), and our results confirmed that this phenomenon also occurs in bees (Fig. 5). Increased activities of caspases and metalloproteinases (Dhule et al., 2012; Ye et al., 2012) were mainly observed in human cells, whereas, in bees, increases were observed in the activity of aspartic and serine proteases. Similar effects were observed in the caffeine- or coenzyme Q10-treated workers (Strachecka et al., 2014a, b).

Following curcumin administration, an increase was observed in the activities of the enzymatic biomarkers (AST, ALT, ALP; Fig. 6). Fan et al. (2014) and Yu et al. (2011) suggested that curcumin decreased serum AST and ALT levels in rat and mouse livers. Bajda et al. (2014) observed that the activities of these enzymes were lower in the hemolymph of bees treated with amphotericin B. The same was noticed in our earlier studies of workers treated with formic acid and bromfenvinphos (unpublished results). This study confirmed and developed the view that the mechanism of AST, ALP and ALT activation in bees (Strachecka et al., 2014a, b) is opposite to that of mammalians. Increased activities in mammalians may indicate hepatotoxicity, pathological changes and chronic diseases, but in bees they seems to be indicators of improved health state and resistance.

Concentrations of non-enzymatic biomarkers were higher in the workers treated with curcumin than in the control group (Fig. 7). Caffeine, which is a curcumin analogue (Anand et al., 2008), has similar effects (Strachecka et al., 2014a). Curcumin reduces blood glucose concentration in diabetic rats (Zhang et al., 2013). Arshami et al. (2012) and Seo et al. (2008) observed that curcumin decreased concentrations of triglycerides, cholesterol, glucose, total proteins and calcium in the blood of hen and mice. In bees, these biomarkers are energy sources and their increase is necessary to reduce the effects of stress (Bajda et al., 2014; Strachecka et al., 2014a, b). In mammalians, their increased activities may indicate pathological changes and chronic diseases.

Our studies of the resistance-related compounds in bees are highly innovative and therefore there is a lack of publications on their activity/concentration in bees. There are publications available that relate to this subject in birds and mammals. However, a detailed discussion concerning the biochemical response of the compounds in bees *versus* birds/mammals seems to be too speculative. Therefore, only directions for further analyses were emphasized in this paper.

We confirmed the results of Lyko and Maleszka (2011) that global DNA methylation levels increase as workers advance in age, but we also found that only curcumin slowed this process markedly (Fig. 9). It is believed that, in mammalian cells, curcumin regulates the expression of genes that are critically involved in the regulation of cellular signalling

pathways (including NF-κB, Akt, MAPK and other pathways; Reuter et al., 2011). These signalling pathways could be regulated by miRNAs. Molecular docking of interaction between curcumin and DNMT1 suggested that curcumin covalently blocks the catalytic thiolate of DNMT1 to exert its inhibitory effect on DNA methylation (Liu et al., 2009; Kuck et al., 2010). Similar down-regulation of DNMT1 at the mRNA and protein level was observed in cells in leukemia, melanoma and in breast cancer in which cases curcumin induces a global decrease of DNA methylation activity (Fu and Kurzrock, 2010; Teiten et al., 2013). Consequently, curcumin appeared to be an effective epigenetic switch in bees.

Many apian resistance improving biochemical changes, which were triggered by curcumin, suggested that the decrease of the level of the *Nosema* infection was not the only reason for extending the life of workers fed with this compound.

CONCLUSION

This study is the first report on the effect of curcumin on many health-related biochemical parameters in workers. The response seems surprisingly clear and significant, because this compound, by extending the apian life-span and decreasing the levels of the *Nosema* spp. infections, increased protein concentrations and the activities of the antioxidative and proteolytic systems and enzymatic markers, but decreased global DNA-methylation level at the same time. Therefore, curcumin unexpectedly turned out to be an effective natural bio-stimulator, influencing apian health and vitality. This multifactorial effect is caused by the activation of many biochemical processes involved in the formation of apian resistance which also seems to be connected with the epigenetic changes.

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