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Piperine as a new natural supplement with beneficial effects on the life-span and defence system of honeybees

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Abstract

Many factors, including pathogens, environmental change and breeding techniques, affect honeybee immunity/resistance, so substances and natural supplements that enhance it are desired. To eliminate the impact of unknown external factors, in 2016 a cage experiment was conducted under constant laboratory conditions (35 °C, 65% relative humidity). Bees in the control group were fed with sugar dissolved in water at ratio 1:1 *ad libitum* with no additives, while the other group was fed with sugar syrup (1:1) supplemented with piperine (3 µg/ml) *ad libitum*. The piperine-treated workers lived 9 days longer compared to the control group. In the piperine-consuming group, protein concentration and the activities of antioxidative enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and glutathione S-transferase (GST), were higher than in the control group. The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were also higher in the piperine-treated group. Neutral and acidic proteases inhibitors, as well as neutral protease activities, were higher in the haemolymph of the piperine-treated workers than in untreated bees. Acidic protease activities in the haemolymph were higher in untreated workers only on days 18 and 32. Alkaline protease activities in the control bees were higher from day 10. From 10 days old, the total antioxidant capacity level was significantly higher in the haemolymph of piperine-treated workers. Piperine decreased DNA methylation levels significantly in the older bees. The compound could have the potential to be a natural diet supplement increasing apian resistance to stress factors.

Introduction

Honeybees (*Apis mellifera* L.) are a key element in maintaining biodiversity and, moreover, provide ecosystem services (Fünfhaus *et al.*, 2018). Through pollination, insects are responsible for a global service worth $215 billion to food production (Goulson *et al.*, 2015). This insect brings huge economic benefits for beekeepers by providing bee products which are not only used as edible products but also as components of medicines in clinical treatments (Al-Lawati *et al.*, 2018). For these reasons, bees are extremely important for humans and the environment and should be preserved.

Constant decline in the number of pollinators, especially honey bees, is being observed due to climatic and environmental changes, Colony Collapse Disorder, the use of pesticides in agriculture, pathogen infestations, parasite infections, spreading of invasive species, over-intensive industrial harvesting of bee products and other threats (Kevan and Viana, 2003; Johnson *et al.*, 2010; vanEngelsdorp *et al.*, 2017; O'Neal *et al.*, 2018; Ptaszyńska *et al.*, 2018*a*, 2018*b*). Other risks include the increasing resistance of various parasites and diseases to drugs, e.g. the *Varroa destructor* mite, the *Nosema* spp. microsporidium and *Paenibacillus larvae* (Pawlowski *et al.*, 2018; Suwannapong *et al.*, 2018). Moreover, bees metabolize antibiotics poorly, which makes their treatment difficult (Raymann *et al.*, 2018). Additionally, some medicines are prohibited by the European Union due to the potential for harmful substances to be incorporated into bee products (Rada *et al.*, 1997). For these reasons, other substances that support the bee immune system without causing side effects, weakening bee colonies and accumulating harmful deposits in honey bees and their products – for instance, dietary supplements – are desired. Moreover, in many regions, a shortage of natural bee forage (pollen and nectar) triggers the need for adequate supplemental diets that may reduce colony losses by alleviating stress (Vannette *et al.*, 2015; Glavinic *et al.*, 2017). Pollen nutrients and extracts from different plant fragments affect gene longevity and the production of some antimicrobial peptides in bees (Alaux *et al.*, 2011; Li *et al.*, 2014; Glavinic *et al.*, 2017). It has also been

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confirmed that the use of safe, natural bioactive substances such as caffeine, curcumin and coenzyme Q_{10} (also known as ubiquinone, ubidecarenone, coenzyme Q and often abbreviated to CoQ10) may contribute to increased health of bees and protection against parasites (Strachecka *et al*., 2014*a*, 2015); piperine may be one substance with similar benefits. Moreover, substances such as porphyrins (Ptaszyńska *et al*., 2018*b*) and extracts of polypore mushrooms (Stamets *et al*., 2018), help to destroy pathogens, preventing their development and diminishing the mortality of infected honeybees. Workers treated with bioactive substances (defined as foodstuffs meant to supplement the normal diet and which are concentrated sources of nutrients or other substances with a nutritional value; e.g. caffeine, curcumin and CoQ10) have higher protein concentrations and increased activities of antioxidant enzymes, biochemical markers, neutral proteases and protease inhibitors. The activities of acidic and alkaline proteases are lower in bees that are administrated these substances (Strachecka *et al*., 2014*a*, 2015). The antioxidative system and proteolytic pathway compounds (proteases and their inhibitors) are key substances responsible for individual bee resistance and longevity, particularly in conditions of high stress and senescence (Williams *et al*., 2008; Aurori *et al*., 2014). These compounds interact by mutually activating specific metabolic pathways (Münch *et al*., 2008; Tolfsen *et al*., 2011). The presence of free radicals (which appear in pathological conditions) stimulates the activation of catalase (CAT) and superoxide dismutase (SOD) in the haemolymph (Münch *et al*., 2008). Proteolytic enzymes detect and degrade damaged proteins, thus preventing the formation and accumulation of protein aggregates and mitigating the results of oxidative stress (Cabiscol *et al*., 2000; Zou *et al*., 2006; Münch *et al*., 2008; Bull *et al*., 2012; Parker *et al*., 2012). Moreover, these bioactive substances decrease DNA methylation levels in apian brains. This is the principal gene-silencing mechanism depending on environmental changes (Foret *et al*., 2009).

Piperine [(E,E)-5-(3,4-Methylenedioxyphenyl)-2,4-pentadienoylpiperidide, 1-Piperoylpiperidine] is an alkaloid in the nonvanilloid family, which occurs in berries and roots of the *Piperaceae* family (Badmaev *et al*., 2000; Vasavirama and Upender, 2014). Depending on the source, piperine content in berries varies from about 60 to 100 g/kg. The highest concentrations of piperine are observed in the grain. Pepper grains owe their spicy taste to piperine as well as other compounds, such as chavicine. Previous studies have found that there is no piperine in pollen or nectar, therefore it can be concluded that bees are not exposed to this substance naturally (Srinivasan, 2009; Deng *et al*., 2016).

Analyses on vertebrates have confirmed the positive effect of piperine (Ouyang *et al*., 2013; Lee *et al*., 2018). Studies on mice and rats have shown that piperine possesses analgesic, anticonvulsant and anti-inflammatory properties, which classifies it as one of the agents that counteract epilepsy. The anti-inflammatory character of piperine also protects against oxidative damage by inhibiting or quenching free radicals, reactive oxygen species and hydroxyl radicals, which increases the life-span of cells and organisms (Mittal and Gupta, 2000; Kumar *et al*., 2008; Sharma *et al*., 2010; Bukhari *et al*., 2013; Tasleem *et al*., 2014). Piperine has been found to exert anti-cancer effects and general physiological benefits on organisms (Do *et al*., 2013; Ouyang *et al*., 2013; Reddy *et al*., 2014). However, there appears to be no information in the literature about the application of piperine in insects. However, the existence of beneficial effects on the vertebrates led to the hypothesis that piperine may be a valuable and beneficial honey bee supplement. It was hypothesized that piperine may increase such resistance-related biochemical parameters as activities of antioxidants, proteases, protease inhibitors and also some crucial enzymatic biomarkers in apian haemolymph, extending the apian life-span.

The aim of the current work was to investigate the influence of adding piperine to honey bees' diets on their longevity, selected biochemical parameters and the level of global DNA methylation, to discover whether it should be recommended for apiary testing and in the long-term to be used as a method of disease prevention.

Materials and methods

The experiment was carried out on 8000 1-day-old worker bees (*Apis mellifera carnica*, Pollmann; collected from an apiary belonging to the University of Life Sciences in Lublin – 51° 13′31″N, 22°38′07″E), prepared according to the method of Strachecka *et al*. (2016) in June and July 2017. Bees were settled into 200 wooden cages (40 bees/cage) with a volume of 576 cm^3 each ($12 \times 12 \times 4$ cm^3, length/height/width), with a sliding front window, air vents located on the side and a feeder (a syringe with a modified adaptor) on the top. Optimal conditions were guaranteed in an air-conditioned chamber – constant temperature at 35 °C and 65% relative humidity. Prior to the experiment, bee colonies were inspected for the absence of *Nosema* spp., *Varroa destructor* signs and evaluated for general colony condition in order to ensure that the bees were in excellent condition. The bees were divided into two equal groups: the control group and the piperine-supplemented experimental group, 100 cages in each group. Thirty cages in each group were designated for longevity tests (Procedure 1) and 70 for laboratory analyses (Procedure 2 and 3). The bees in the control group were fed with sugar (Diamant, Toruń, Poland) dissolved in water at ratio 1:1 *ad libitum* with no additives, while the other group was fed sugar syrup (1 : 1) supplemented with piperine (Sigma Aldrich, Saint Louis, Missouri, USA) at a concentration of 3 µg/ml *ad libitum*. The most effective dose of piperine was selected on the basis of previous pilot studies. Piperine was added directly into sugar syrup with no buffers.

Procedure 1 – determining bee life-span

Longevity tests were performed according to the method of Fries *et al*. (2013). The front window of each cage was taken out every second day: dead bees were removed using tweezers and counted to determine their life-span.

Procedure 2 – collecting haemolymph and biochemical analyses

Haemolymph was collected from randomly chosen bees from 70 cages destined for biochemistry from each group on the 1st, 4th, 10th, 18th, 25th and 32nd day of the experiment. Since the bees fed with piperine lived longer, haemolymph samples were additionally collected on the 37th and 41st day in the piperine-fed group. Haemolymph was drawn from the dorsal sinus of each bee using the capillary puncturing method between the 3rd and 4th tergite (Strachecka *et al*., 2014*a*, 2014*b*). Glass capillaries (20 µl; the 'end to end' type; without anticoagulant; Medlab Products, Raszyn, Poland) filled with the haemolymph were placed immediately in sterile Eppendorf tubes (2 ml) containing

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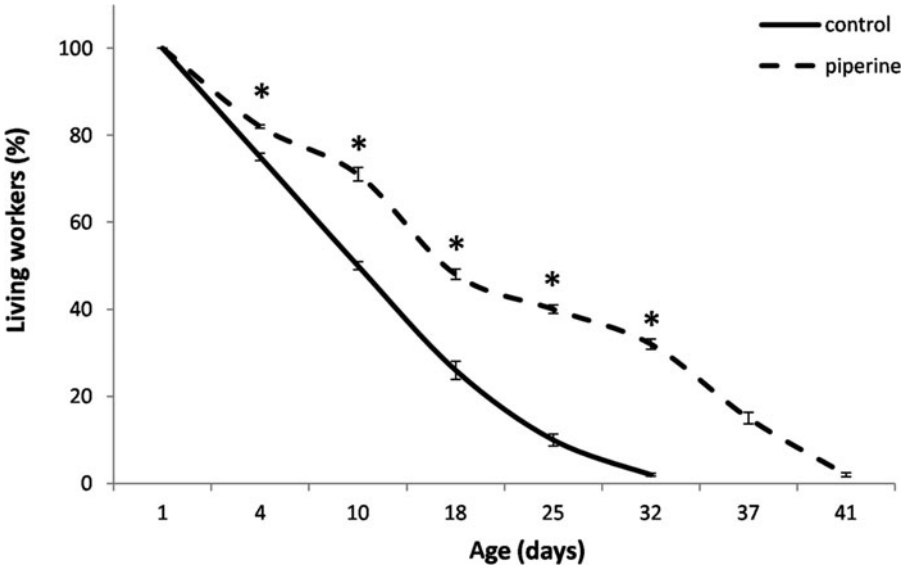

Fig. 1. Longevity of the workers in the control and piperine-treated group (means ± S.E.; $N = 1200$ workers in each group). The asterisks indicate significant differences ($P \leqslant 0.01$;) between the group averages for longevity within a given apian age (except for 1-day-old workers).

150 µl of ice-cooled 0.6% sodium chloride (NaCl; Braun, Melsungen, Germany). Five to ten pooled samples of approximately 100 µl haemolymph (five capillaries × 20 µl haemolymph; one capillary contain haemolymph collected from one to two bees) were obtained for each age/sampling. The samples were refrigerated immediately at −40 °C for subsequent biochemical analysis.

Global protein concentrations in the pooled samples were determined using the Lowry method modified by Schacterle and Pollack (1973). Total antioxidant capacities were determined according to the Benzie and Strain (1996) method.

The following antioxidant enzymes were analysed in haemolymph from the pooled samples:

- superoxide dismutase (SOD) according to the Podczasy and Wei (1988) method;
- catalase (CAT) according to the Aebi (1984) method;
- glutathione peroxidase (GPx) according to the Chance and Maehly (1955) method;
- glutathione S-transferase (GST) according to the method of Warholm *et al.* (1985) method.

Descriptions of these methods have been presented by Strachecka *et al.* (2014*a*, 2014*b*, 2016). All the activities were calculated per 1 mg of protein.

Alkaline, neutral and acidic proteases were determined with the Anson (1938) method modified by Strachecka *et al.* (2010). The activities of natural inhibitors of acidic, neutral and alkaline proteases were determined according to the method of Lee and Lin (1995).

The activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) were measured with the kinetic method using monotests from Cormay (Lublin, Poland) according to the manufacturer's procedure.

Procedure 3 – DNA methylation investigation

To investigate global DNA methylation, ten randomly chosen live bees were caught from different cages from each group dedicated for laboratory analysis on the 1st, 4th, 10th, 18th, 25th, 32nd, 37th and 41st day and refrigerated in sterile 2 ml Eppendorf tubes at −25 °C. Next, the brain of each bee was isolated and brain DNA was extracted with a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. An MDQ1-96RXN Imprint Methylated DNA Quantification Kit (Sigma Aldrich, Saint Louis, Missouri, USA) was used to determine global DNA methylation.

Statistical analysis

The effects of piperine treatment and significance of differences between the group averages were determined using one-way ANOVA and the least significant analysis procedures (LSD) with $P \leqslant 0.01$ taken as indicating significance, using SAS statistical software (SAS Institute Version 9.13). Cage was a random effect and group (the control and piperine-treated group) was a fixed effect. The Bliss transformation ($y = \text{arc sin } [x/100]0.5$) was used to process the percentages of DNA 5-methylocytosine.

Results

The addition of piperine to sugar syrup consumed by the bees increased the honeybee life-span by 9 days (approximately 22%) compared to the control group (Fig. 1; $P \leqslant 0.01$).

Protein concentration was significantly higher in the piperine-supplemented group than in the control group (Fig. 2; $F = 19.601$, $P < 0.001$). Protein concentration rapidly dropped in the haemolymph after the 32nd day of age in the piperine-treated workers. This result cannot be compared with the haemolymph of the control group workers due to the fact that almost none of them reached this age.

Total antioxidant capacity levels were significantly higher ($F = 5.509$, $P < 0.001$) in the haemolymph of the piperine-treated workers from the 10th day of age (Fig. 3). High levels of the total antioxidant potential were maintained in the haemolymph of the piperine-supplemented workers until the death of all the bees.

Supplementation with piperine caused a significant increase in the activities of antioxidant enzymes, such as superoxide

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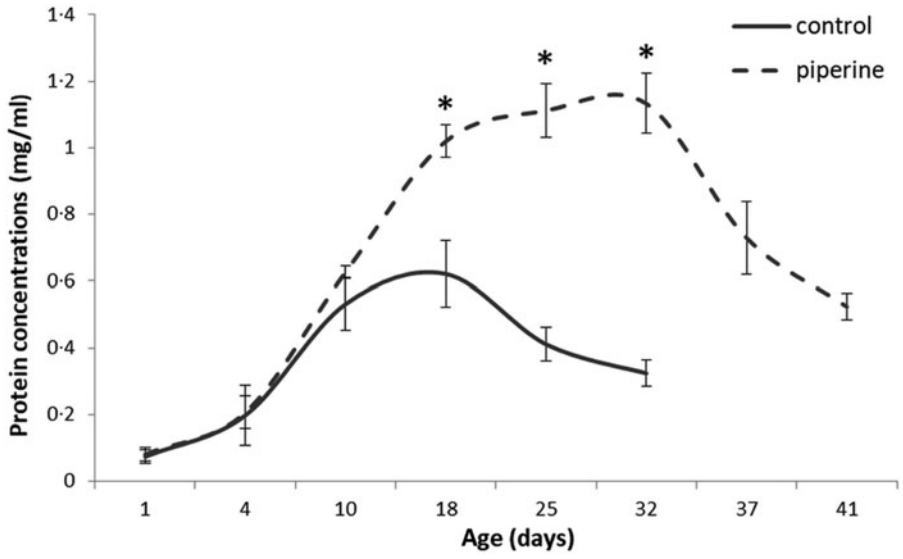

Fig. 2. Protein concentrations in worker haemolymph in the control and piperine-treated group (means ± S.E.; N = 5–10 pooled samples [9–15 workers in each pooled sample] in each sampling, in each group;). The asterisks indicate significant differences ($P \leqslant 0.01$) between the group averages within a given apian age.

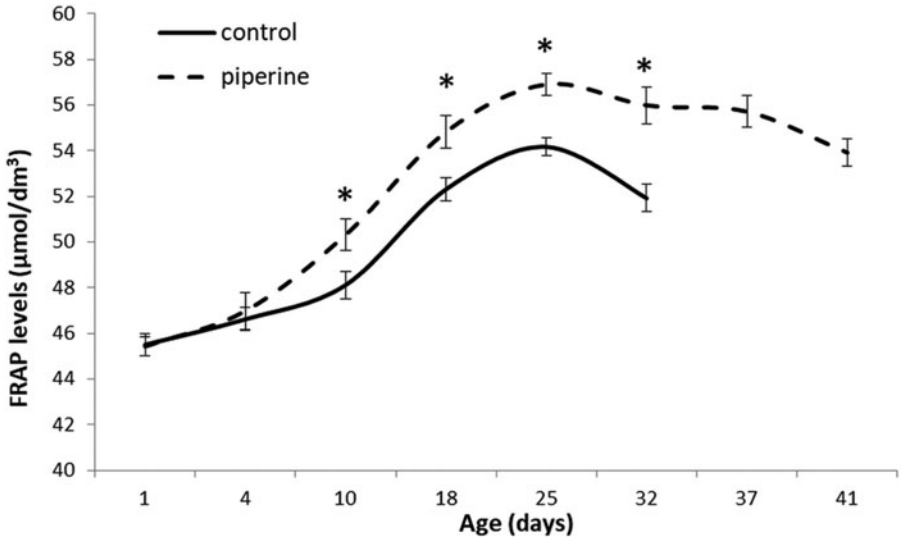

Fig. 3. Levels of the total antioxidant potential in worker haemolymph in the control and piperine-treated groups (means ± S.E.; N = 5–10 pooled samples [9–15 workers in each pooled sample] in each sampling, in each group). The asterisks indicate significant differences ($P \leqslant 0.01$) for comparisons between the group averages within a given apian age.

dismutase (SOD; between the 10th and 32nd day, $F = 7.698$, $P = 0.001$), glutathione peroxidase (GPx; between the 4th and 32nd day, $F = 9.882$, $P < 0.001$), catalase (CAT; between the 10th and 32nd day, $F = 3.949$, $P < 0.001$) and glutathione S-transferase (GST; between the 10th and 32nd day, $F = 5.509$, $P < 0.001$) (Fig. 4), as well as the activities of aspartate aminotransferase (AST; between the 10th and 32nd day, $F = 51.492$, $P < 0.001$), alanine aminotransferase (ALT; in the 4th, 10th and 25th day, $F = 27.218$, $P < 0.001$) and alkaline phosphatase (ALP; in the 18th, 25th and 32nd day, $F = 23.252$; $P < 0.001$) (Fig. 5) in honeybee haemolymph.

The activities of neutral and acidic protease inhibitors, as well as the activities of neutral proteases, were higher in the haemolymph of piperine-treated workers than in untreated bees. There were no significant differences between the groups (piperine-treated or control) for alkaline protease inhibitor activities in the haemolymph. The acidic protease activities in the haemolymph were higher in workers from the control group only on the 18th and 32nd day. On the other hand, starting from the 10th day, the alkaline protease activities in haemolymph of the control bees were higher than in haemolymph obtained from the bees in the experimental group (Fig. 6).

The mean level of global DNA methylation in the control group was significantly higher (6.5–13.4%) in the brains of workers from the age of 18 days than the corresponding level in the brains of the bees in the piperine-treated group (Fig. 7; between the 18th and 32nd day, $F = 13.309$, $P < 0.001$).

Discussion

Metabolic effects of piperine, an antioxidant, anti-inflammatory and protective compound, in honeybees, were confirmed in the present study. Of all its properties (Chopra *et al.*, 2016), strengthening immunity may be particularly important for bees. Moreover, piperine and its derivatives enhance the bioavailability of various nutrients including vitamins and minerals (Bhardwaj *et al.*, 2002; Chopra *et al.*, 2016). This property is especially necessary for bees whose diet is, increasingly, stoichiometrically unbalanced (Filipiak *et al.*, 2017). In the present study, piperine changed the longevity of workers and their metabolism through

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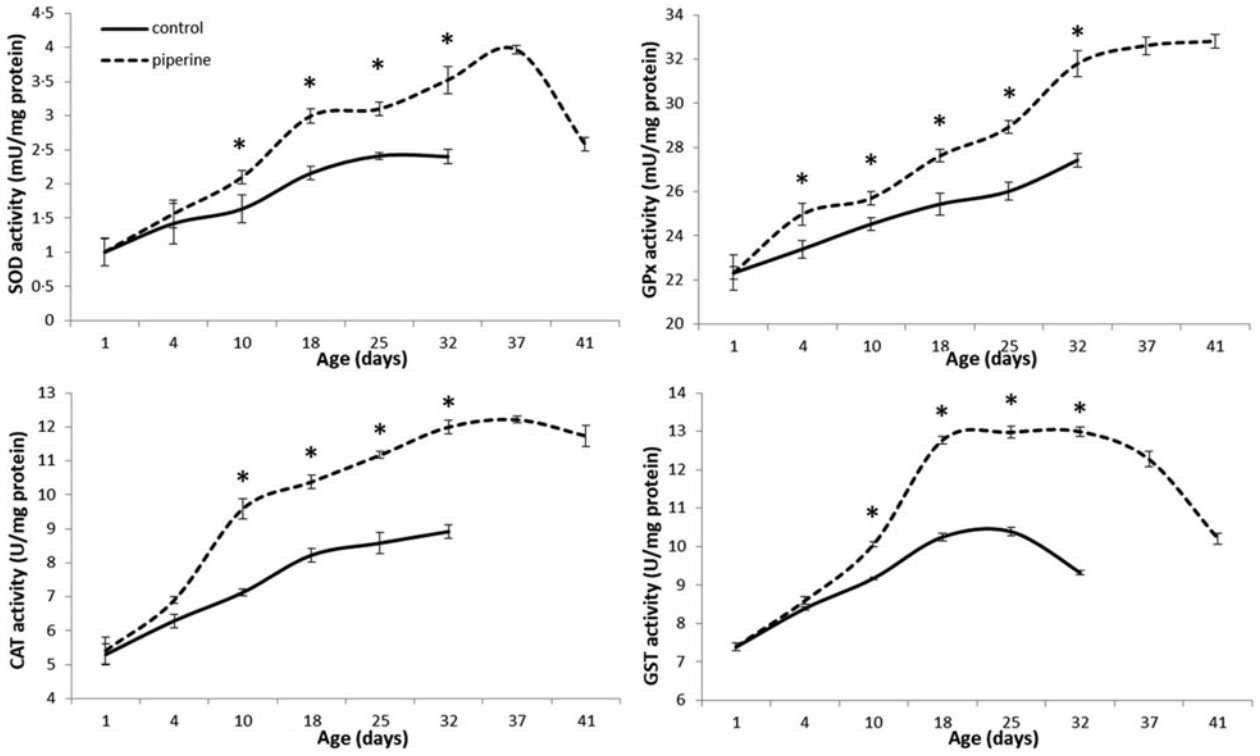

Fig. 4. Activities of enzymatic antioxidants in worker haemolymph in the control and piperine-treated group (means ± s.e.; $N = 5–10$ pooled samples [9–15 workers in each pooled sample] in each sampling, in each group). The asterisks indicate significant differences ($P \leqslant 0.01$) between the group averages within a given apian age and for each of the individual enzymes. SOD, superoxide dismutase; GPx, glutathione peroxidase; CAT, catalase; GST, glutathione S-transferase.

modifications in the concentrations and activities of many biochemical parameters. Piperine has a protective influence not only on vertebrates (Kapoor *et al.*, 2009) but also on bees by increasing the activity of the antioxidant system. The higher activities of antioxidant enzymes, such as superoxide dismutase, peroxidase, catalase and glutathione S-transferase triggered by piperine treatment suggest that this bio-stimulant may promote detoxification (Nahak and Sahu, 2011; Bukhari *et al.*, 2013; Tasleem *et al.*, 2014). Piperine protects against oxidative damage by inhibiting or suppressing free radicals, reactive oxygen species and hydroxyl radicals, which cause destructive and lethal cellular effects by oxidizing membrane lipids, cellular proteins, DNA and enzymes, thus shutting down cellular respiration (Srinivasan, 2007; Umar *et al.*, 2013). Piperine acts as a hydroxyl radical scavenger at low concentrations, but at higher concentrations it activates the Fenton reaction, resulting in increased generation of hydroxyl radicals (Mittal and Gupta, 2000). Amino acids in cells, especially L-amino acids, enhance the properties and effects of piperine not only in mammals but also in honeybees (Matysiak *et al.*, 2014; Paarakh *et al.*, 2015; Donkersley *et al.*, 2017). Piperine treatment may protect from cancer growth, bacterial and fungal infections. In future studies, the influence of piperine on *Nosema* spp. and bacterial infections in honeybees should be tested. Oxidative imbalance and decreased endogenous antioxidants lead to the release of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase enzymes (ALP) (Mehta *et al.*, 2012). However, the mechanism of AST, ALP and ALT activation in bees is opposite to that in mammals (Bajda *et al.*, 2014; Strachecka *et al.*, 2014*a*, 2014*b*, 2016), as increased activities seem to be indicators of improved health and resistance (Łoś and Strachecka, 2018). In the current study, higher activities of these enzymatic biomarkers in the haemolymph of piperine-treated workers compared with untreated bees indicates a reduction of oxidative stress and support of the defence systems, similar to curcumin, caffeine, CoQ10 and vitamin C (Farjan *et al.*, 2012; Strachecka *et al.*, 2014*a*, 2014*b*, 2015). The antioxidant system and biomarkers interact with the proteolytic system. Proteolytic enzymes contribute to the amelioration of the consequences of oxidative damage. Piperine induced increased activity of acidic, neutral and alkaline protease inhibitors as well as that of neutral proteases in the haemolymph of the workers. These enzymes are involved in the mechanism of apoptosis and are essential components of insect resistance barriers (Grzywnowicz *et al.*, 2009; Dhule *et al.*, 2012; Strachecka *et al.*, 2014*a*, 2014*b*). They also participate in other processes such as phagocytosis, melanization, cellular adhesion, synthesis of cytokines and antimicrobial peptides, enzyme activation and hormonal signalling (Griesch and Vilcinskas, 1998). Antioxidant, proteolytic and biomarker systems are involved in the proper functioning of the resistance systems, as well as in the improvement of vitality and stabilization of metabolic functions in honeybees and consequently in life-span extension. Moreover, the current study confirmed the findings of Lyko and Maleszka (2011) that global DNA methylation levels increase as workers advance in age, but also found that piperine slowed this process markedly. The reason for this may be the effect of piperine on DNA methylation, with changes in other components of the epigenome possibly induced due to the trilateral relationship that exists between DNA methylation, histone covalent modifications and non-coding RNAs. Moreover, piperine, just as curcumin, regulates the expression of genes that are critically involved in the regulation of cellular signalling pathways

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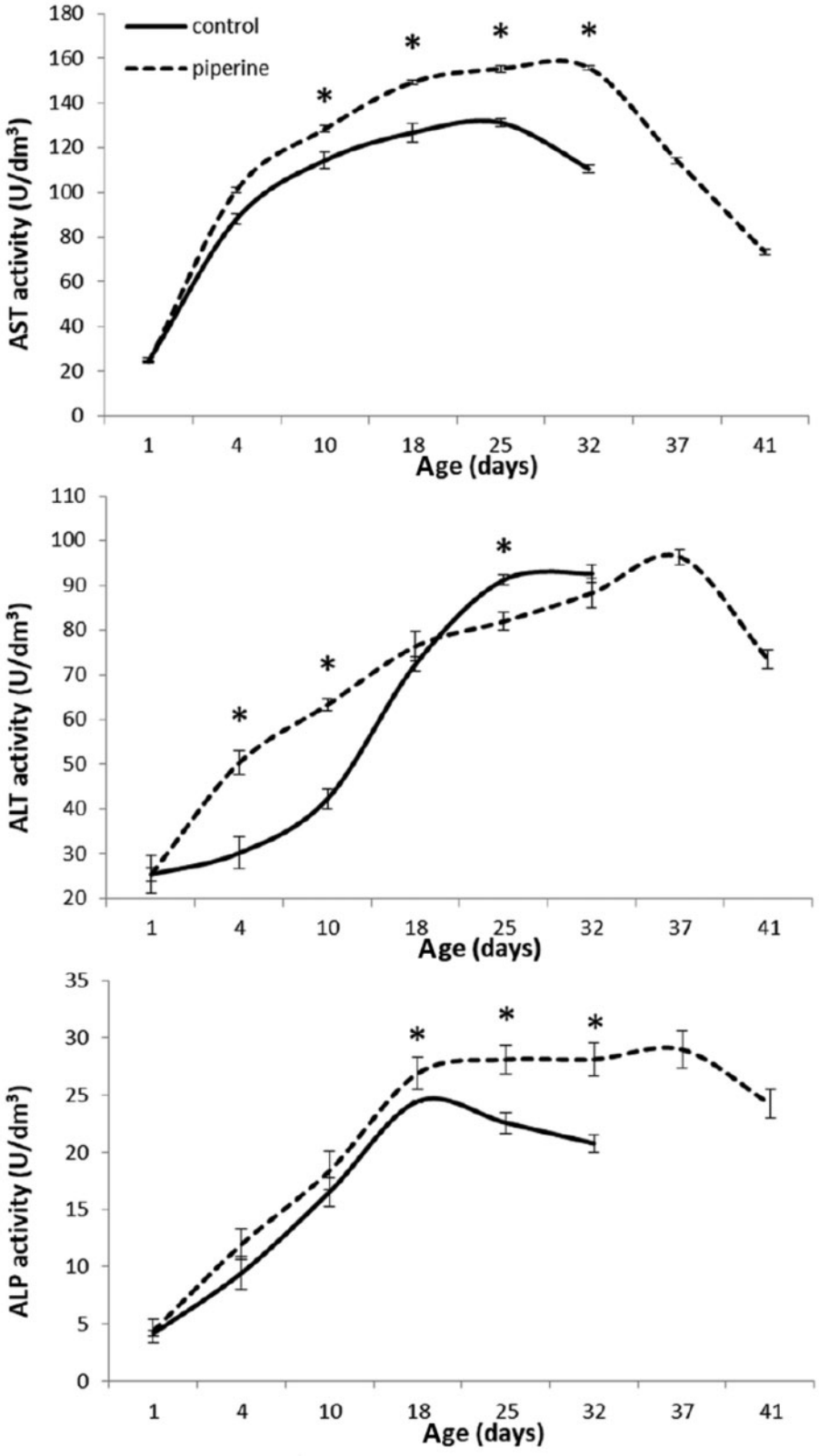

Fig. 5. Mean activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase (ALP) in the haemolymph of the workers in the control and piperine-treated groups (means ± S.E.; $N = 5–10$ pooled samples [9–15 workers in each pooled sample] in each sampling, in each group). The asterisks indicate significant differences ($P \leqslant 0.01$) between the group averages within a given apian age and for each of the individual enzymes.

(including NF-κB, AP-2, Akt, MAPK and other pathways) (Reddy *et al.*, 2014). Additionally, protein status is a key factor influencing the life-span of honey bees. Life-span is one of the key elements to determine the dynamics and health of bee colonies. Bees with higher protein concentrations were found to be less susceptible to infections than bees with lower protein concentrations in the haemolymph. This implies that piperine may be used to improve the health of bee colonies.

Piperine is used as a supplement in the formation of a self-microemulsifying drug delivery system (SMEDDS). This alkaloid

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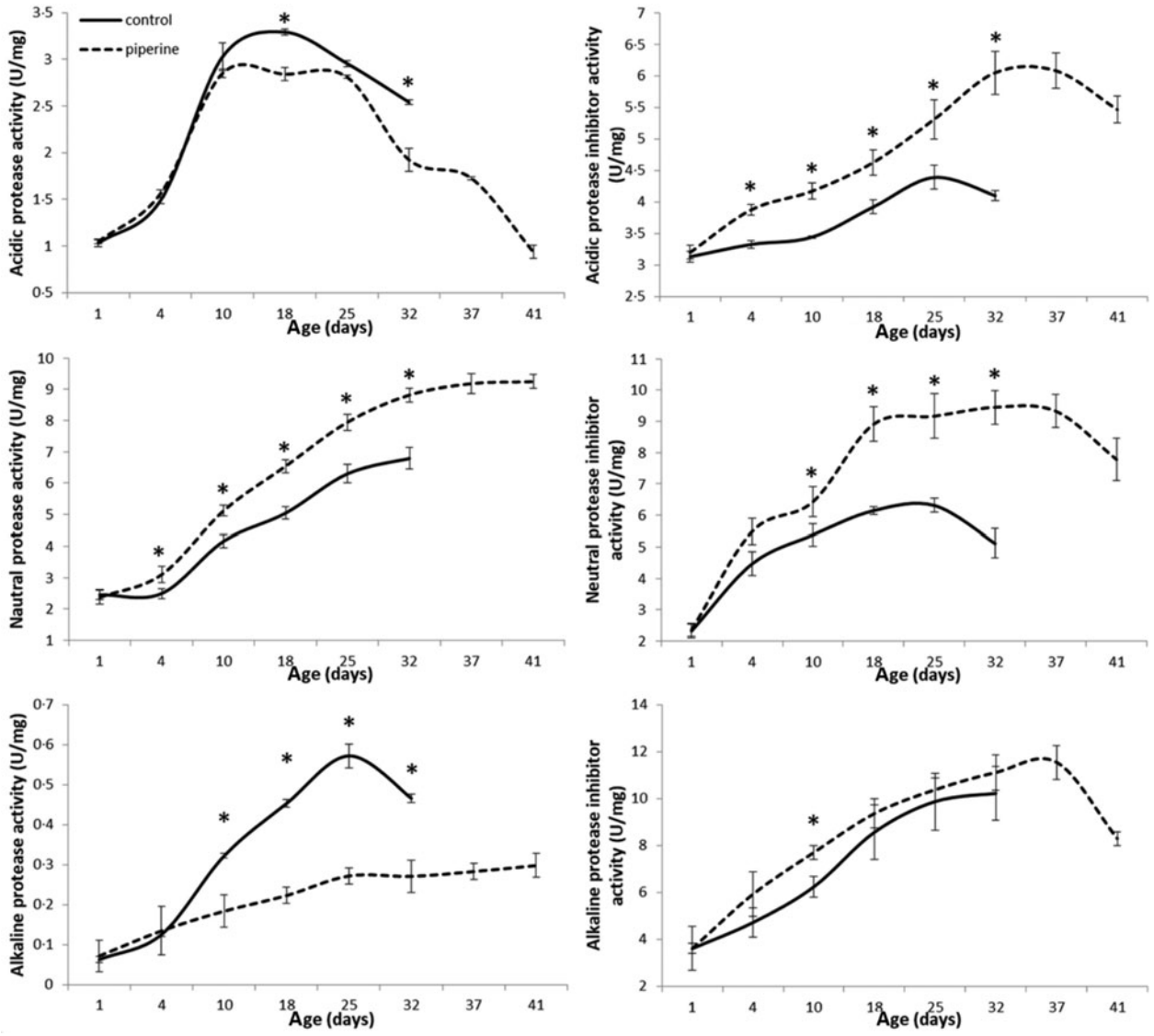

Fig. 6. Mean activities of proteases and protease inhibitors (U/mg) in the haemolymph of the workers in the control and piperine-treated groups (means ± s.e.; N = 5–10 pooled samples [9–15 workers in each pooled sample] in each sampling, in each group). The asterisks indicate significant differences ($P \leqslant 0.01$) between the group averages within a given apian age and for each of the individual enzymes.

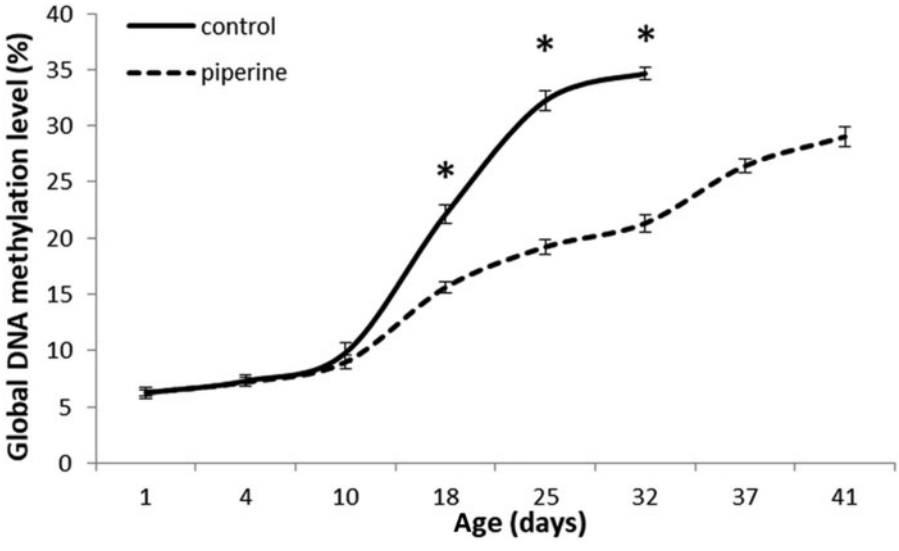

Fig. 7. Mean global DNA methylation levels (%) in the workers from the control and piperine-treated groups (average ± s.e.; $N = 10$ workers in each sampling, in each group). The asterisks indicate significant differences ($P \leqslant 0.01$) for comparisons between the group averages within a given apian age.

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increases the bioavailability of other natural compounds, such as curcumin. The combination of piperine with curcumin makes drugs more stable and shows anti-colitis activity (Li *et al*., 2015). The synergistic effect of combined piperine and curcumin treatment has been shown to attenuate the morphological, histopathological, biochemical, apoptotic and proliferative changes in rat liver and serum in contrast to treatment with curcumin only (Patial *et al*., 2015). Since curcumin combined with piperine produces much higher positive results, the accuracy of this assumption for honeybees should be checked. Piperine can also operate as a factor increasing the bioavailability of CoQ10 in human plasma in oral administration (Badmaev *et al*., 2000). In another publication, Strachecka *et al*. (2014*b*) also indicated that CoQ10 has an unexpectedly strong positive impact on honey bee health. Considering these findings, the combination of piperine with curcumin and CoQ10 in honey bees can contribute to achieving good results in terms of their health and longevity.

The positive effects of piperine on the organism probably stems from the fact that piperine activates a family of vanilloid receptor – related transient receptor potential (TRPV) receptors, which are ion channels located on the cell plasma membrane (McNamara *et al*., 2005). Only two kinds of TRPV receptors have been discovered in insects (*Drosophila melanogaster* Meigen). The current results indicate the positive effects of piperine on apians, which means that this insect could also have TRPV receptors on cell membranes. Looking for TRPV receptors and substances that interact with them in honey bees should be included in setting out future research directions.

The data obtained in the current work can contribute to and encourage further studies of the usage of natural agents to improve the health and condition of honeybees in efficient and safe ways, as well as contributing to combining the bio-stimulators in experimental animal diet and testing their joint effects. Moreover, their influence on *Nosema* spp. and bacterial infections in honeybees should be tested.

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Author contributions. The research was designed by AS (70%) (Team leader). The technical part of the experiment was conducted by AS. All the chapters were written by MS (10%), AŁ (10%) and AS. The abstract was written by AS. The statistical analyses and figures were made by AS in consultation with MG (5%) and RŚ (5%). The editing of the manuscript was done by AŁ and MS under the supervision of AS. All the authors accepted the final version of the manuscript.

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Conflicts of interest. No competing interests were declared.

Ethical standards. No specific permits were required to conduct this investigation.

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