Supplementing with vitamin C the diet of honeybees (*Apis mellifera carnica*) parasitized with *Varroa destructor*: effects on antioxidative status

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SUMMARY

We studied a total of eight developmental stages of capped brood and newly emerged workers of *Apis mellifera carnica* colonies naturally parasitized with *Varroa destructor*. During winter and early spring four colonies were fed syrup containing 1·8 mg vitamin C kg^{-1} (ascorbic acid group; group AA) while four colonies were fed syrup without the vitamin C (control group C). Selected elements of the antioxidative system were analysed including total antioxidant status (TAS), glutathione content and antioxidative enzyme activities (superoxide dismutase, catalase, peroxidase and glutathione *S*-transferase). Body weight, protein content and indices of infestation were also determined. The prevalence (8·11%) and intensity (1·15 parasite per bee) of the infestation were lower in group AA compared with group C (11·3% and 1·21, respectively). Changes in the indicators of antioxidative stress were evidence for the strengthening of the antioxidative system in the brood by administration of vitamin C. In freshly emerged worker bees of group AA, despite the infestation, protein content, TAS, and the activity of all antioxidative enzymes had significantly higher values in relation to group C.

Key words: honeybee, *Varroa destructor*, varrosis, ascorbic acid, oxidative stress.

INTRODUCTION

Antioxidant levels and the activity of antioxidative enzymes, which prevent cell damage caused by oxidation, are important factors determining the good health of an organism (Clarkson and Thompson, 2000). Excessive oxidative reactions (oxidative stress) are the basis or result of many diseases including parasitosis (Hadaś and Stankiewicz, 1998; Mishra, 2007; Sorci and Faivre, 2009; Halliwell, 2011). In honeybees, the symptoms of oxidative stress have been observed in drone prepupae (PP) parasitized with *Varroa destructor* (Lipiński and Żółtowska, 2005).

Varroa destructor (Acari: Varroidae) is a haematophagous mite and one of the most damaging pests of honeybees. The condition caused by varroa mite – varrosis – considerably decreases the productivity of beekeeping (Murilhas, 2002; Van Engelsdorp *et al*. 2009). The mite parasitizes all of the stages of the capped brood and adult bees. The infestation negatively influences the immunity and longevity of bees (Gliński and Jarosz, 1988, 1992; Yang and Cox-Foster, 2005). The following symptoms have been observed during varrosis: body weight reduction, decrease in fertility, negative changes in the immune system and deformations of wings and limbs (Sammataro *et al*. 2000; Yang and Cox-Foster, 2005). Varrosis is often accompanied by dangerous bacterial, viral and fungal infections (Francis *et al*. 2013). In most cases, untreated varrosis leads to the collapse of the whole colony within 3–4 years (Stevenson *et al*. 2005). Commonly used acaricides are becoming less and less effective, as the parasite has developed resistance against them (Lipiński and Szubstarski, 2007; Maggi *et al*. 2011). These compounds also affect the quality of bee products and their safety for consumers (Karazafiris *et al*. 2008; Wu *et al*. 2011). Therefore, the search for effective and safe ways of controlling varrosis is critical (Rosenkranz *et al*. 2010).

Prevention methods alleviating the effects of oxidative stress can support the protective forces of an organism. One of these methods may be administration of exogenous compounds with antioxidative characteristics (Clarkson and Thompson, 2000; Berger, 2005; Mishra, 2007) such as vitamin C (ascorbic acid, AA), a natural antioxidant. Oral application of AA has no lethal effect on *Apis mellifera carnica* worker bees (Harz *et al*. 2010). The results of our previous study showed that supplementation of the diet of wintering honeybees with

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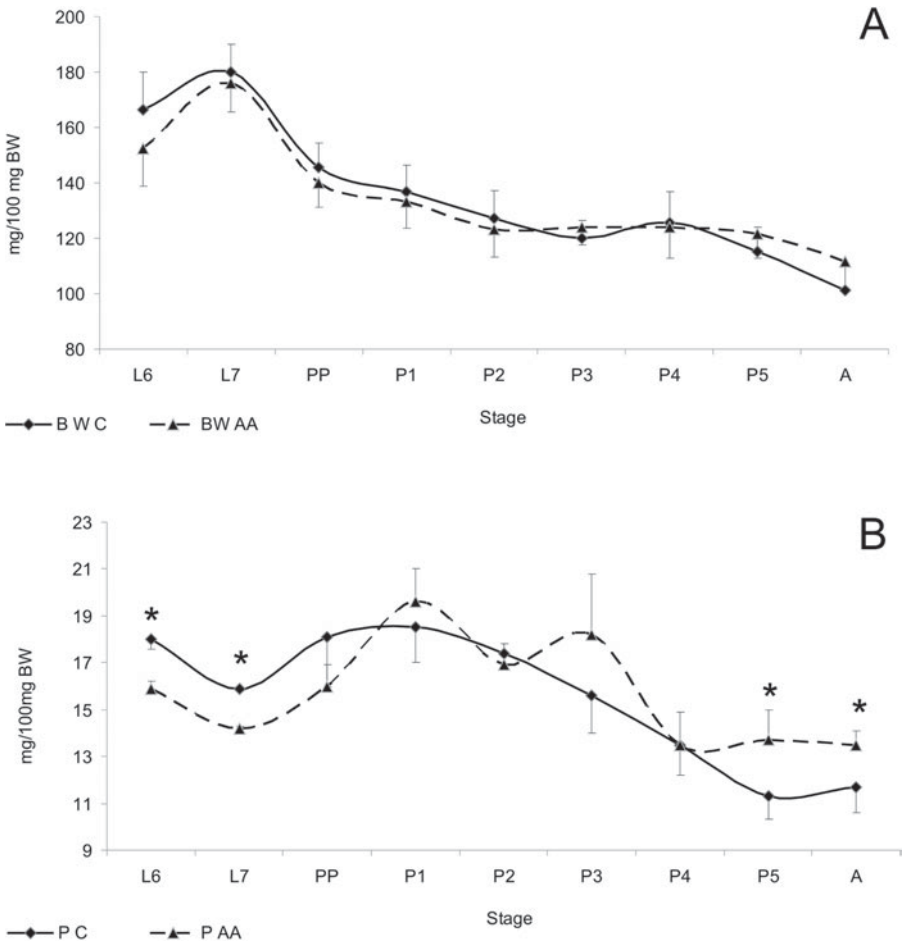

Fig. 1. Body weight (A) and protein content (B) in the developmental stages of parasitized honeybee worker broods (mean ± S.D.). C, group without supplementation with vitamin C; AA, group after supplementation with vitamin C (ascorbic acid); L, larval stages; PP, prepupae; P, pupae; A, newly emerged workers. *Significant difference between means of groups C and AA ($P < 0{\cdot}05$).

AA increased the efficacy of their antioxidative system. As a result, the winter losses in colonies receiving AA was 33% lower compared with those not receiving AA (Farjan *et al*. 2012). AA in the used doses did not show any harmful effects, and even positively influenced carbohydrate metabolism, particularly in the youngest larval stages of workers (Farjan, unpublished data). Moreover, AA is relatively cheap and a safe diet supplement (Harz *et al*. 2010), and its application in beekeeping is very simple.

The main goal of this study was to validate the use of ascorbic acid (vitamin C) administered during autumn and early spring to honeybees exposed to varroa mites by comparison of changes in the antioxidative status of parasitized broods.

MATERIALS AND METHODS

The material originated from eight colonies headed by sister queens of *Apis mellifera* derived from Carnolian honeybees naturally parasitized with *V. destructor* situated 20 km from Olsztyn, Poland. The field experiment was carried out at the same time (from September 2007 to May 2008) and the same way as described by Farjan *et al*. (2012). Briefly, four of the eight colonies were fed with a sugar–water (3:1) syrup supplemented with vitamin C (ascorbic acid, Biofactor; Poland, group AA) at a concentration of $1{\cdot}8\ \mathrm{mg\ kg^{-1}}$ syrup. The remaining four colonies received pure syrup as a control (group C). Feeding began in September 2007 to enable bees to hoard the winter stores (13 litres per colony) and a second feeding took place on 1 March 2008.

On 10 May 2008, honeycomb sections were collected from all the colonies. Sealed broods were carefully isolated from honeycomb cells. Simultaneously, the material was evaluated in parasitological terms. All capped cells of the honeycombs were opened, isolated from the brood and the cells' interiors were searched for the presence of the parasite. The brood was divided, based on morphological features (Jay, 1962, 1963) in eight stages of development: 6 days old (L6); cocoon-spinning larvae (L7); prepupae (PP); pupae with white eyes (P1); pupae with pale-pink eyes (P2); pupae with pink eyes (P3); pupae with brown eyes and yellow thorax (P4);

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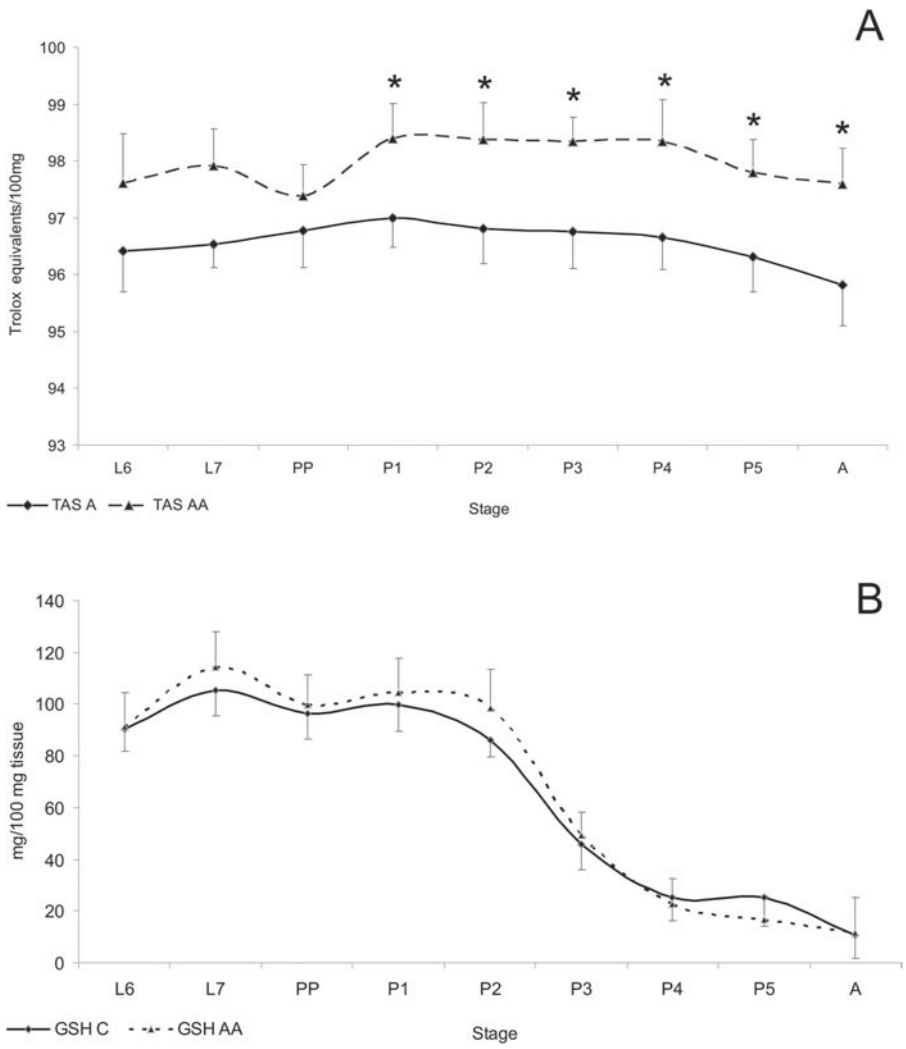

Fig. 2. Total antioxidant status (TAS) (A) and glutathione (GSH) (B) content in developmental stages of parasitized honeybee worker broods (mean ± S.D.). C, group without supplementation with vitamin C; AA, group after supplementation with vitamin C (ascorbic acid); L, larval stages; PP, prepupae; P, pupae, A, newly emerged workers. *Significant difference between means of groups C and AA ($P < 0{\cdot}05$).

pupae with black eyes and dark thorax (P5). Freshly emerged workers (A) were also examined. The samples ($n = 15$) were collected for each stage consisting of three individuals in each sample. The samples were rinsed in 0·9% NaCl, carefully dried on filter paper, and weighed. The material was stored at −70 °C until further analysis.

The preparation of honeybee extracts and analysis were carried out in the same way as in our earlier study (Farjan *et al*. 2012). Protein content was analysed using the Bradford (1976) method. Glutathione (GSH) concentration was measured according to Ellman (1959). Total antioxidant status (TAS) was assayed with use of a Randox Laboratories Ltd kit according to the manufacturer's instructions. Results were calculated as Trolox (a water-soluble analogue of vitamin E) equivalents/100 mg fresh weight.

The activity of superoxide dismutase (SOD) was measured according to Podczasy and Wei (1988). Catalase (CAT) was determined according to Aebi (1983). Peroxidase (POX) activity was measured according to Chance and Maehly (1955), and was expressed in international units (U). Glutathione *S*-transferase (GST) was assayed by the Papadopoulos *et al*. (2004) method. All the enzymatic activities were calculated per mg protein.

Statistical analysis

Statistical significance was determined by use of the Student's *t*-test and differences between stage's means in group C and AA were considered significant when $P < 0{\cdot}05$.

RESULTS

The mean prevalence of infestation with *V. destructor* was 11·32% in group C, while in group AA it was 8·11%. The mean intensity of infestation was

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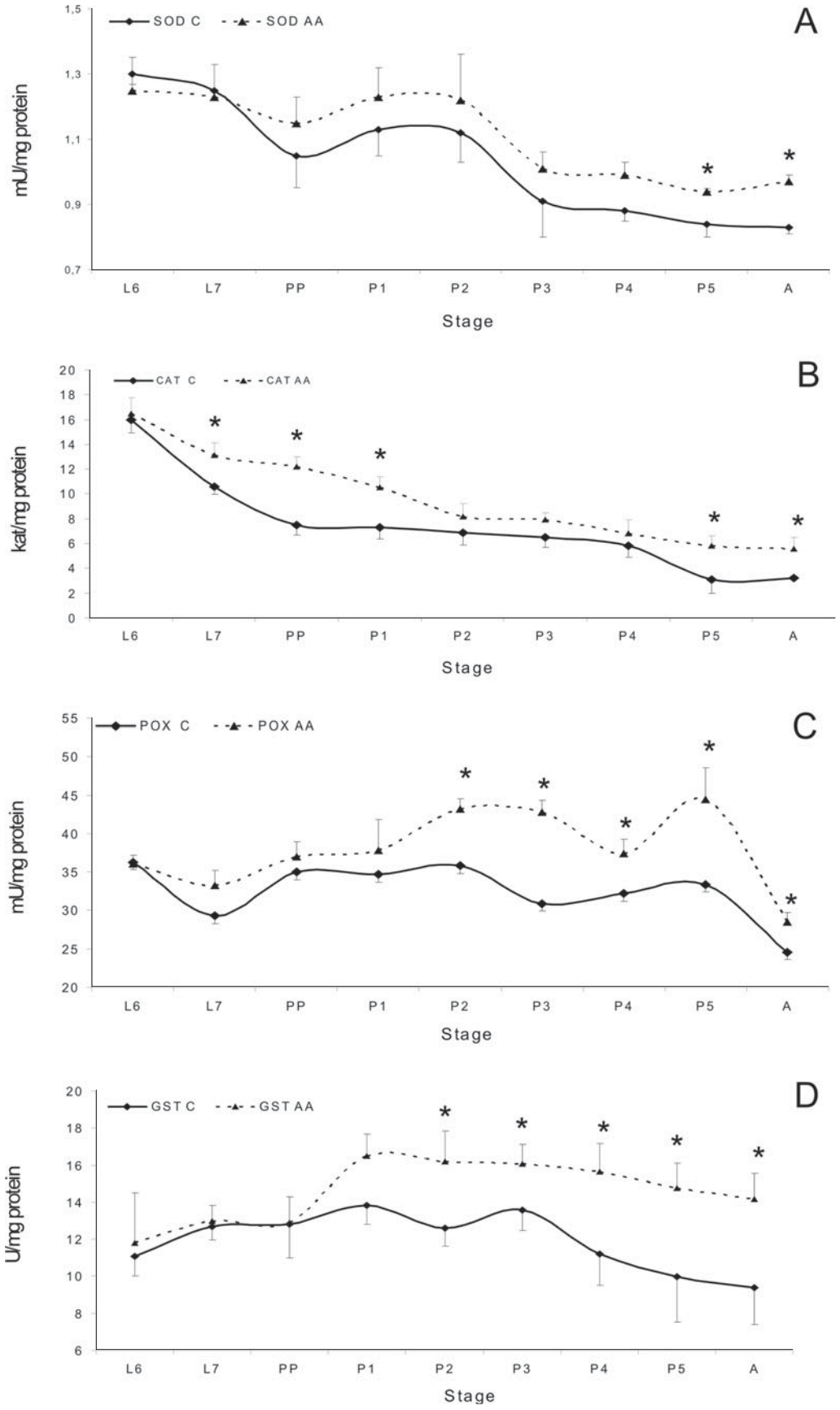

Fig. 3. Activity of antioxidative enzymes in the developmental stages of parasitized honeybee worker broods (mean ± S.D.). C, group without supplementation with vitamin C; AA, group after supplementation with vitamin C (ascorbic acid); L, larval stages; PP, prepupae; P, pupae, A, newly emerged workers. (A) Superoxide dismutase (SOD); (B) catalase (CAT); (C) peroxidase (POX); (D) glutathione *S*-transferase (GST). *Significant difference between means of group C and AA ($P < 0{\cdot}05$).

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Table 1. Influence of varrosis and supplementation of diet with vitamin C on body weight, protein concentration and antioxidants of newly emerged honeybee workers (mean ± S.D.)

	Group		
		Infested with *V. destructor*	
Qualities measured	Non-infested†	C	AA
Body weight (mg)	116·72 ± 10·1	101·24 ± 11·2[a]*	111·71 ± 9·7[a]
Protein (mg 100 mg^{-1})	8·01 ± 0·41	11·74 ± 0·95[a]	13·50 ± 0·43[b]
TAS (eq 100 mg^{-1})	95·50 ± 0·87	95·82 ± 0·72[a]	97·60 ± 0·62[b]
GSH (mg 100 mg^{-1})	4·86 ± 2·51	10·65 ± 3·91[a]	11·65 ± 3·52[a]
SOD (mU mg^{-1})	1·05 ± 0·10	0·83 ± 0·06[a]	0·99 ± 0·02[b]
CAT (kat mg^{-1})	4·50 ± 0·10	3·22 ± 1·11[a]	5·64 ± 0·91[b]
POX (mU mg^{-1})	23·00 ± 2·10	24·6 ± 1·1[a]	28·53 ± 1·22[b]
GST (U mg^{-1})	8·20 ± 1·42	9·40 ± 1·99[a]	14·16 ± 1·40[b]

*Different lower-case letters indicate significant difference between means of the C and AA groups based on Student's *t*-test ($P < 0·05$). †Data from Farjan *et al*. (2012). TAS, total antioxidant status; GSH, glutathione; SOD, superoxide dismutase; CAT, catalase; POX, peroxidase; GST, glutathione *S*-transferase.

1·21 mites/one parasitized individual in group C and 1·15 in group AA.

Ascorbic acid did not significantly influence the brood body weight (Fig. 1A). However, the mean weight of parasitized freshly emerging workers from group C was about 10% lower than in group AA. In the capped larvae the level of proteins from group V was higher than in group AA. Then until the P5 stage there were no differences between the groups. The pupae of the P5 stage and freshly emerged worker bees of the AA group had a higher protein content than those of group C (Fig. 1B).

In group AA higher total antioxidative capacity was observed in comparison with group C. Differences between the means of both groups were significant, starting from the P1 stage (Fig. 2A). However GSH level was similar in both groups (Fig. 2B).

In the pupae of group AA, the activity of SOD was only slightly higher than in the brood not receiving AA. The difference was statistically significant only for the P5 and imago stages (Fig. 3A). The activity of CAT was higher in the L7, PP, P1, P5 and imago stages of the AA group than in the group without supplementation (Fig. 3B). Similar results were found for POX and GST, but significant differences were evident only from the P2 pupa stage (Fig. 3C and D).

DISCUSSION

Although the bees from the group receiving vitamin C were still parasitized with varroa mites, the prevalence of parasitism was 30%; lower compared with the control group. In addition, the average intensity of infection was lower in group AA compared with the group not receiving AA. The values of these indicators suggest that the administration of AA to bees has a positive effect. Even a small reduction in the number of mites in the colony may be significant (Wilkinson and Smith, 2002). It appears that the decrease in the prevalence of parasitism of broods resulted from the properties of AA. Other organic acids, with similar redox properties, such as formic acid, oxalic acid and lactic acid, have been successfully used as acaricides (Rosenkranz *et al*. 2010). However, these acids have demonstrated toxic effects on bees with longer periods of oral administration (8 days), even at low doses (1000 parts per million) (Ebert *et al*. 2007). AA at the dose used in this study was not toxic for bees. On the contrary, AA administration has been found to decrease winter losses of bees and support the health of the colony (Farjan *et al*. 2012).

The results for newly emerged workers were compared with our earlier data on non-infected honeybees (Farjan *et al*. 2012) in Table 1. The combined data showed that *V. destructor* parasitism did not have a significant influence on the weight of the worker brood. The observed slight reduction in body weight in the parasitized bees was in accordance with previous reports (Bowen-Walker and Gunn, 2001; Duay *et al*. 2003; Żółtowska *et al*. 2005). Importantly, the protein content in newly emerged bees from the colonies receiving AA was significantly higher than in the control group (Fig. 1B). This can be considered a secondary benefit of the supplementation with AA of the winter diet of honeybees.

It is known that parasitic disease increases free radical production in the host (Clarkson and Thompson, 2000; Sorci and Faivre, 2009). It can result from inflammatory and non-inflammatory reactions of the host to the infestation. The data from Table 1 show that varrosis was accompanied by a slight increase in overall ability to scavenge free radicals, and significant reduction in the activity of two antioxidant enzymes. However, a considerable increase in TAS occurred in the parasitized bees

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receiving vitamin C compared with solely parasitized bees (Table 1, Fig. 2A). Among the analysed elements of the non-enzymatic antioxidative system, it was GSH that responded most intensively to the infestation stress (Table 1). This was not surprising because GSH is known to be a central element that mediates in many oxidation and reduction reactions (Ghezzi, 2011). In parasitized bees, the level of GSH was almost constant and independent of vitamin C administration (Fig. 2B). The lower activity of the two antioxidant enzymes CAT and SOD in the parasitized bees could be concerning. However, AA counteracted the decrease in SOD and CAT activity induced by the varroa mite parasitism in workers (Table 1, Fig. 3A and B). This also confirmed the validity of enriching the diet of bees exposed to varrosis with this antioxidant. The infestation did not significantly influence the activity of POX and GST (Table 1). The statistically significant increase in the activity of both enzymes was noted for the parasitized pupae and newly emerged bees of the AA group compared with the C group (Fig. 3C and D). This phenomenon is particularly important in the case of GST, because it acts not only as an antioxidative enzyme but also participates in phase II of detoxification (Kostaropoulos *et al*. 1996). In insects, this enzyme is involved in the inactivation of toxic compounds present in the environment, including pesticides (Oakeshott *et al*. 2010; Sau *et al*. 2010). This suggests that supplementation of the diet of wintering bees with vitamin C not only augments the efficiency of the antioxidative system, but also may lead to an increased ability to remove toxic and undesired metabolites which may appear during varrosis.

In conclusion, *V. destructor* parasitism has a negative influence on the antioxidative system of the developing honeybee brood. For these reasons, the supplementation of the diet of bees before the winter period with vitamin C may be recommended. The freshly emerged workers, despite the infestation, were in a better condition than those that did not receive AA (Table 1). This was confirmed by a significantly higher protein content, increase in TAS and by the elevated level of the activities of all the antioxidative enzymes studied. This phenomenon is very important because the ability of honeybees to defend against reactive oxygen species seems to be severely limited compared with other insects (Claudianos *et al*. 2006; Corona and Robinson, 2006; Oakeshott *et al*. 2010). However, the present study was a preliminary study, requiring confirmation in field trials on a broader scale.

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