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Short Communication

# EXPRESSION AND ACTIVITY OF LYSOZYME IN *APIS MELLIFERA CARNICA* BROOD INFESTED WITH *VARROA DESTRUCTOR*

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**Abstract**

***Varroa destructor* is a parasitic mite that attacks the honey bee, and previous studies have suggested that parasitosis caused by this mite is accompanied by immunosuppresion in the host. In this study, the effect of mite infestation on the expression of the lysozyme-1 (lys-1) gene and lysozyme activity in *Apis mellifera carnica* was determined. The experiment was carried out on the five developmental stages of honey bee workers and drones. Developmental and gender-related differences in gene expression and lysozyme activity were observed in a *Varroa destructor*-infested brood. The relative expression of the lys-1 gene increased in a infested worker brood and decreased in a drone brood except for P3 pupae. In the final stage of development, the lys-1 gene expression was significantly lower in infested newly emerged workers and drones. Changes in the relative expression of the lys-1 gene in infested individuals was poorly manifested at the level of enzyme activity, whereas at the two final stages of development (P5 and I) there was a positive correlation between relative *lys-1* expression and lysozyme activity in infested bees of both genders (r=0.988, r=0.999, respectively). The results of this study indicate that *V. destructor* influences the lysozyme-linked immune response in bees.**

**Keywords: development, drones, gender-related differences, immune system, workers**

## INTRODUCTION

The parasitic mite *Varroa destructor,* a vector of dangerous pathogens for bees (*Apis mellifera carnica*), is one of the causative agents of the steady decline in honey bee populations observed in recent years (Bernardi & Venturino, 2016). The immune system of bees is activated in response to mite infestation (Evans et al., 2006). Lysozyme, apart of the innate immune system, hydrolyzes the β-1,4-glycosidic linkage of murein-peptidoglycan, a cell wall component in bacteria. Two classes of lysozymes, the c-type and the i-type, have been identified in insects (Beckert et al., 2016). Honey bees have three lysozyme genes, two encoding c-type lysozymes and one encoding i-type lysozyme. Only the c-type lys-1 gene is up-regulated in infested bees (Evans et al., 2006). The expression of immune-related genes varies across bee species, races and genders (Khongphinitbunjong et al., 2015; Zaobidna, Żółtowska, & Łopieńska-Biernat, 2015). Therefore, the aim of this study was to determine how natural infestation with *V. destructor* affects the expression of the lys-1 gene and lysozyme activity during the development of worker and drone broods.

## MATERIAL AND METHODS

The experiment was carried out on five developmental stages of worker and drone brood which had been parasitized by two female *V. destructor* and were free from infestation. The brood was isolated from combs collected from two *A. m. carnica* colonies which had been naturally infested with *V. destructor* and then was divided into five developmental stages: 5-day-old larvae (L5), prepupae (PP), pupae with red (P3) and dark eyes (P5), and imago (I), based on morphological features. From the infested and non-infested (control) groups of both

colonies, samples for genetic analyses (n = 5 for each stage) and enzymatic analyses (n = 10 for each stage) were collected randomly from a pool of individuals of the same gender and at the same developmental stage. The samples were stored at -72°C until needed.

Genetic analyses were performed by Zaobidna et al. (2015). The primers sense 5'-GGAGGC-GAGGATTCTGACTCAATG-3' and antisense 5'-TGTTGCATATCCCTCCGCTGTG-3' were used for the relative quantification of *lysozyme-1* (*lys – 1*, GB10231). Quantitative real-time PCR was performed using SYBRGreen PCR-MIX Taq™ (A&A Biotechnology). Fold changes in target genes, normalized to *rp49* (GB47227, primers sense 5'-CGTCATATGTTGCCAACTGGT-3' and antisense 5'-TTGAGCACGTTCAACAATGG-3') and relative to expression levels in the corresponding stages in non-infested bees, were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Pfaffl, 2001). Bee extracts for enzymatic analyses were prepared as described by Zaobidna et al. (2015). Lysozyme-like activity was determined using the method proposed by Azambuja et al. (1991).

**Statistical analysis**

Data were expressed as mean values with standard deviation, and the results were analyzed statistically with the use of Statistica 12 software (StatSoft Inc.). Normality was checked with the Kolmogorov–Smirnov test and the homogeneity of variance with Levene's test. The statistical significance of differences between samples collected at various developmental stages was analyzed by one-way analysis of variance (ANOVA) and Tukey's test. The results were considered to be statistically significant at $p \leq 0.05$. Pearson's correlation coefficient between the activity of lysozyme and the expression of *lys-1* in various developmental stages of *A. m. carnica* was calculated.

## RESULTS AND DISCUSSION

The lys-1 gene expression was significantly higher in the *V. destructor*-infested worker brood than in the non-infested brood, and the highest expression was noted in P5 pupae (30.4-fold, $p<0.0002$) (Fig. 1A). Similar results had been reported by Khongphinitbunjong et al. (2015) in infested Italian bee pupae. Ryabov et al. (2014) had demonstrated lower lys-1 gene expression in the P3 pupae of western honeybee workers infested with *V. destructor* and infected with deformed wing virus (DWV). The observed differences could have resulted from the mixed infection since DWV causes immunosuppression in mite-infested bees (Di Prisco et al., 2016).

Different trends were observed in the drone brood. The lys-1 gene expression of the remained unchanged in larvae, increased expression (relative to control samples) was noted only in P3 pupae (1.6-fold, $p<0.0002$) and decreased expression levels were found in the remaining pupae (Fig. 1A). The expression of the lys-1 gene was significantly lower in infested newly emerged workers and drones (0.1-fold and 0.5-fold, respectively, $p<0.0002$), which corroborates the findings of Yang and Cox-Foster (2005). Decreased lys-2 gene expression was also noted in the prepupae and imago of Carniolan honey bees exposed to pesticides (Cizelj et al., 2016). The above effects may be strengthened, making *V. destructor*-infested bee colonies more susceptible to bacterial and viral infections.

Lysozyme activity which was approximately two-fold higher in workers than in drones (Fig. 1B). At the P5 developmental stage, lysozyme activity was 4-fold higher in infested females than in males. In the analyzed period, lysozyme activity was lowest in infested P5 workers and remained at a stable level in drones at all developmental stages (Fig. 1B). In infested individuals, changes in lysozyme mRNA expression (Fig.1A) were less likely to manifest at the level of enzyme activity (Fig. 1B). A positive correlation between *lys-1* expression and activity was noted in infested bees of both genders at the final two stages of development (P5 and I) at $r=0.988$ and $r=0.999$, respectively. Significant gender-related differences in *lys-1* expression and lysozyme activity were observed in bees at all developmental stages (Fig. 1A, B). In comparison with workers, drones were char-

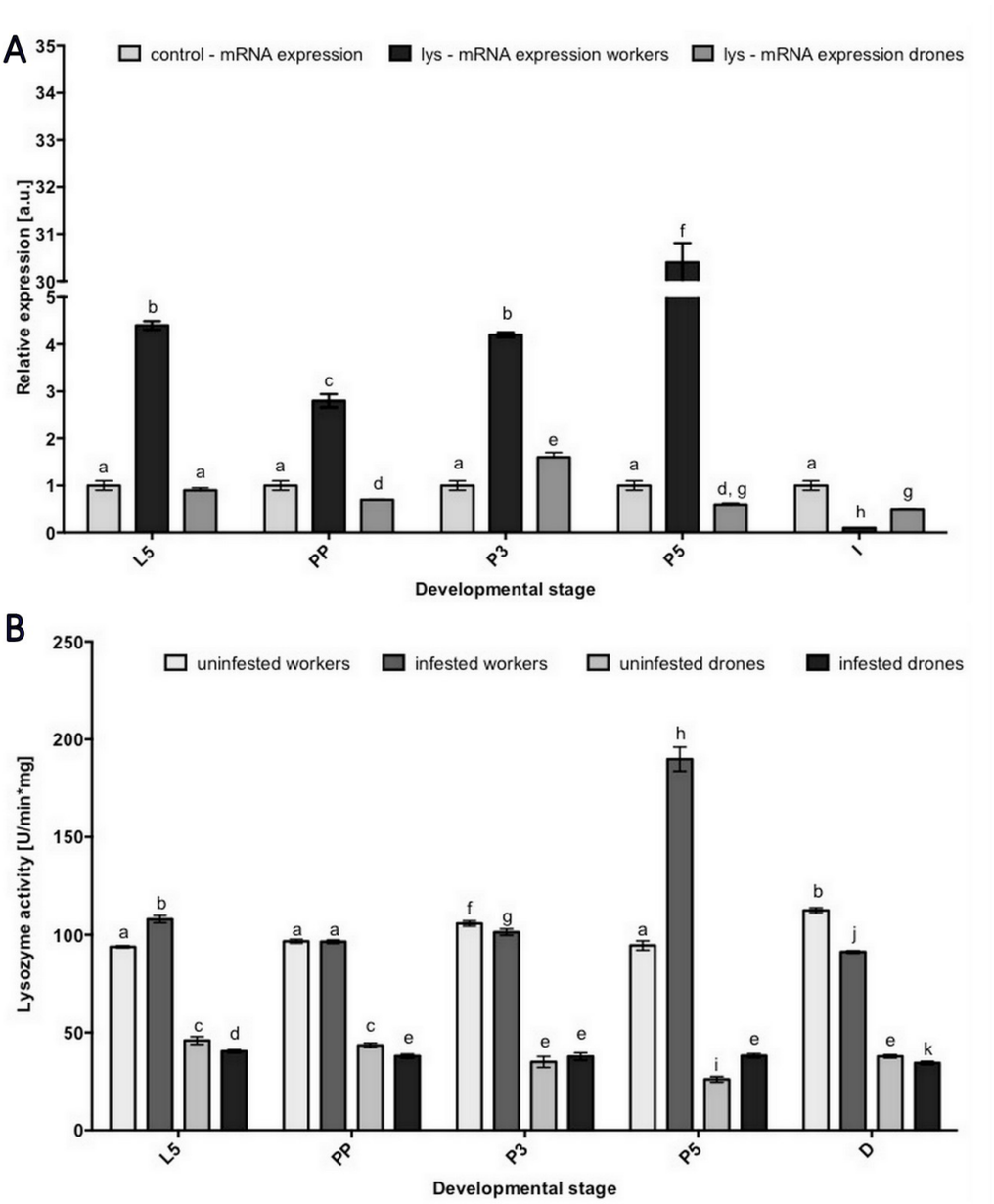


Fig. 1. Comparison of lys-1 gene (A) expression and lysozyme activity (B) during the development of *A. m. carnica* (L5 - 5-day-old larvae, PP - pre-pupae, P3 - pupae with red eyes, P5 - pupae with brown eyes and dark-brown body, I - imago) infested with *V. destructor.* Gene expression was normalized relative to reference gene rp49 and an endogenous control sample (RQ = 1). The letters (a–k) above the bars represent significant differences ($p \leq 0.05$) during the development of *A. mellifera* in gene expression and enzyme activity.

acterized by lower relative gene expression, lower lysozyme activity and weaker stimulation of the phenoloxidase pathway at initial stages of development, which could partially explain their higher susceptibility to *V. destructor* infestations (Rosenkranz, Aumeier & Ziegelmann, 2010; Zaobidna, Żółtowska, & Łopieńska-Biernat, 2015).

The results of this study confirmed that *V. destructor* induces immune suppression in the adult of honey bees. Our findings also indicate that the response of lysozyme, an important component of the innate immune system, varies depending on the developmental stage and gender of the honey bees. Further research into other components of the immune system could contribute to a better understanding of immune suppression caused by *V. destructor* in bees.

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