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Original Article

EXPRESSION OF THE PROPHENOLOXIDASE GENE AND PHENOLOXIDASE ACTIVITY, DURING THE DEVELOPMENT OF *APIS MELLIFERA* BROOD INFECTED WITH *VARROA DESTRUCTOR*

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Abstract

The pathogenesis of varroasis has not been fully explained despite intensive research. Earlier studies suggested that parasitic infections caused by *Varroa destructor* mites were accompanied by immunosuppression in the host organism. The objective of this study was to analyse the influence of varroasis on one of the immune pathway in *Apis mellifera* measured by the expression of the prophenoloxidase (*proPO*) gene and the enzymatic activity of this gene's product, phenoloxidase (EC 1.14.18.1). An evaluation was done of five developmental stages of honey bee workers and drones. The relative expression of *proPO* decreased in infected individuals. The only exceptions were worker prepupae (PP) and drone pupae with brown eyes and dark brown thorax (P5) where propo gene expression was 1.8-fold and 1.5-fold higher, respectively, than in the control. Phenoloxidase (PO) activity was 2.8-fold higher in infected pp workers and 2-fold higher in p5 drones in comparison with uninfected bees. Phenoloxidase activity was reduced in the remaining developmental stages of infected workers and drones. The relative expression of *proPO* was positively correlated with the relative PO activity in both workers (r = 0.988) and drones (r = 0.996). The results of the study indicate that *V. destructor* significantly influences the phenoloxidase-dependent immune pathway in honey bees.

Keywords: ***Apis mellifera*, phenoloxidase activity, prophenoloxidase gen expression, *Varroa destructor*.**

INTRODUCTION

The honey bee (*Apis mellifera*) is an economically important pollinator whose population has been dramatically reduced in the past decades (Genersch, 2010). There are several causes for the decline of the bee populations, including exposure to agricultural chemicals such as pesticides (Krupke et al., 2012). Bee breeding in large apiaries of thousands of colonies can contribute to the spread of viral, bacterial, fungal, and parasitic diseases (Wilson-Rich et al., 2008; Laughton et al., 2011). The most widespread parasitic disease in bee colonies is caused by the ectoparasite *Varroa destructor*, a vector of dangerous pathogens for bees (vanEngelsdorp et al., 2009; Rosenkranz et al., 2010). By feeding on the hemolymph, *V. destructor* weakens the bees and depletes their food reserves, which has particularly serious consequences for wintering insects. The parasite also induces immunosuppression in the host organism. Some studies have demonstrated that immunosuppression is closely linked with varroasis (Gregory et al., 2005; Yang and Cox-Foster, 2005; Aronstein et al., 2012). The expression of antimicrobial peptides and enzymes involved in immune responses was lower in bees infected with *Varroa* than in the control insects (Yang and Cox-Foster, 2005; Aronstein et al., 2012). The major mechanisms of the immune response are induction of antimicrobial peptides and the activation of phenoloxidase cascade (PO, EC 1.14.18.1). The enzyme's protective role in insects was reviewed by Sugumaran (2002). He emphasised phenoloxidase's involvement in melanogenesis and in cuticle sclerotisation. These two processes are responsible for the development of the first physical barrier that makes the body resistant to water, the formation of attachments for muscles and other organs, as well as substances secreted by the cuticle and subcuticular glands, such as hydrocarbons,

waxes, and proteolytic enzymes which provide bees with antimicrobial protection (Strachecka et al., 2010; 2014). Phenoloxidase mediates the nodulation or encapsulation of foreign organisms. This action prevents the development and spread of pathogens in the host's body. The enzyme also participates in wound healing, which minimises the loss of hemolymph and exerts cytotoxic effects on pathogens in the wound area with the involvement of quinoid precursors (Ashida and Yamazaki, 1990).

In arthropods, PO is synthesised as an inactive prophenoloxidase (ProPO) precursor, which is activated by proteolysis (Sugumaran, 2002). Bodily injury or exposure to bacterial polysaccharides activates serine proteases which induce the specific activation of ProPO. The ProPO activation pathway functions as a system for non-self recognition of pathogens and parasites (Ashida and Yamazaki, 1990). Changes in proPO gene expression were observed in a laboratory when G+ and G- bacteria and bacterial lipopolysaccharides (LPS) were injected into invertebrates (Leclerc et al., 2006). The expression of genes encoding enzymes that regulate the ProPO activation cascade was studied extensively. The resulting knowledge will be used to control dangerous insects that pose a sanitation risk (Cerenius and Söderhöl, 2004; Leclerc et al., 2006; Rodriguez-Andres et al., 2012).

Phenoloxidase is an important component of the immune system in insects. The activity levels of phenoloxidase are measured to estimate disease resistance in insects (Adamo, 2004). The activity of the prophenoloxidase gene is correlated with insect immunity against pathogens (Rodriguez-Andres et al., 2012; Roberts and Hughes, 2014). Roberts and Hughes (2014) observed a correlation between Nosema ceranae infestation and the expression of the phenoloxidase gene in honey bees. Also, several authors have investigated the changes in gene expression levels induced by varroasis in honey bees (Zhang et al., 2010; Gregorc et al., 2012; Khongphinitbunjong et al., 2015). Most studies analysed the expression of the gene encoding the PO activating enzyme (*proPOact*). Less frequently examined are the PO activity levels in bees (Gregorc et al., 2012). Some studies were experimentally performed but only on laboratory infected bee larvae (Gregorc et al., 2012; Khongphinitbunjong et al., 2015). Our study relies on a novel approach whereby the expression of the *proPO* gene and total phenoloxidase activity were measured simultaneously in five developmental stages of uninfected *A. mellifera*, and honey bees of both sexes which were naturally infected with *V. destructor*. Our experimental design was adopted to identify varroasis-related changes and to evaluate transcript levels and the active form of the gene product. The enzymatic activity of the bee hemolymph was determined in earlier studies (Zufelato et al., 2004; Laughton and Siva-Jothy, 2010). The activity of PO sampled from the entire body can be an indicator of the overall status of the immune system, including the cuticle which provides the first structural barrier of resistance (Brey et al., 1993; Grzywnowicz et al., 2009). A comparison of PO activity in males and females also supports the identification of sex-specific developmental changes in *proPO* gene expression. We supposed that infection with *V. destructor* induces changes in the status of the immune system of bees. The changes could then be measured by the expression of the *proPO* gene and PO activity. The above changes may be related to the developmental stage and sex of bees. The aim of our study was to determine the effect of a natural infection with *V. destructor* on the expression of the *proPO* gene and PO activity in developed worker and drone brood. These are factors that are critical for the development and immune response of bees.

MATERIAL AND METHODS

Biological material

The experimental material was harvested at the beginning of May 2013 in an apiary situated 15 km from Orneta (54°10'10"N; 20°17'37"E), Poland. The material was collected from two colonies of *A. mellifera*, which were naturally infected by *V. destructor* and where varroasis prevention measures had not been implemented during the two years preceding the study. Bees were obtained from the same apiary and colonies headed by sister queen bees to minimise the influence of genetic and environmental factors. Four combs with brood from each of the colonies were transported to the laboratory (within approximately 180 minutes). Sealed broods were carefully isolated from comb cells. The material was subjected to parasitological analyses (Ellis, 2001). The brood was divided based on its morphological features (Jay, 1962, 1963) into five stages of development: cocoon-spinning larvae (L5), pre-pupae (PP), pupae with red eyes (P3), pupae with brown eyes and dark-brown body (P5), and newly emerged workers and drones (I). The samples for genetic analysis (n = 5 for each stage) and enzymatic analysis (n = 15 for each stage) from uninfected individuals (the control) and individuals infected by two female *V. destructors* were collected randomly in each developmental stage. The samples were weighed, placed in Eppendorf tubes, immediately frozen (anesthetised) in liquid nitrogen, and stored at -72°C until analysis.

Genetic analysis

Total RNA Isolation and Reverse Transcription (RT)
Total RNA was extracted from the bees with the Total RNA Kit (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. The quantity and purity of isolated RNA were determined by spectrometry using the Nano Drop 1000 spectrophotometer (Thermo Scientific, Wilmington, USA). Reverse transcription was performed using the reverse transcriptase TranScriba Kit (A&A Biotechnology, Gdynia, Poland). Total RNA (2 µg) was reverse-transcribed to cDNA using oligo$(dT)_{18}$ primer. The Reverse transcription reaction was carried out at 42°C for 60 min and at 70°C for 5 min. The cDNA product with a final volume of 20 µL, was stored at -20°C until further analysis.

Relative Quantification of Gene Expression
Relative quantification of gene expression by real-time PCR was performed to compare *proPO* (GenBank AY242387) transcription levels in *A. mellifera* workers and drones. Fold changes in target genes, normalised to *rp49* (GenBank AF441189) and relative to expression levels in endogenous control samples (corresponding stages in uninfected workers or drones), were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method (Pfaffl, 2001). Quantitative real-time PCR was performed using the SYBRGreen B PCR-MIX Taq (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol. Twenty µL of the reaction solution contained 1 µL of the template, 10 µL of SYBRGreen B PCR-MIX Taq, 1 µL of 10 µM of each specific primer, 6.4 µL water, and 0.4 µL of ROX Reference dye II.
The specific primers used in the experiment were designed with Primer3 (http://frodo.wi.mit.edu) based on gene sequences in the GenBank database (http://www.ncbi.nlm.gov). The specific *A. mellifera proPO* primer sequences were: sense 5'-ACA GAT CCT GTA TGG ATT GC-3' and antisense 5'-TCT TGG ACG AGT AAA ACG AT-3'. *A. mellifera* ribosomal protein 49 (rp49): sense 5'-CGT CAT ATG TTG CCA ACT GGT-3' and antisense 5'-TTG AGC ACG TTC AAC AAT GG-3' was used as the control. The following thermal cycling conditions were applied: 10 min at 95°C, followed by 40 cycles of 20 s at 95°C, 20 s at 55°C, and 30 s at 72°C. All samples were tested in triplicate in the FAST7500 LightCycler (Applied Biosystems, Grand Island NY, USA). Melting curves were developed after amplification.

Enzymatic analysis

Preparation of enzymatic extracts from *A. mellifera*
Each development stage of workers and drones was homogenised separately in an ice bath for 2 minutes with PBS buffer (Contreras-Garduno et al., 2007), pH 7.0, at a 1:5 (w/v) ratio. The homogenate was centrifuged at 10,000 g for 10 minutes at 4°C. The supernatant was used in further analyses.

Phenoloxidase activity assay
The bee extract's PO activity was determined according the method recommended by Adamo (2004). Phenoloxidase assays were carried out in typical reaction mixtures in a 96 well plate containing 50 µL of *A. mellifera* extract and 70 µL of α-chymotrypsin (from bovine pancreas, Sigma Aldrich, St. Louis, USA) solution (2 mg per 1.5 mL of PBS buffer), for activating zymogen *proPO* to PO. After dark incubation at 37°C for 20 minutes, 150 µL of 5 mM L-dopamine (Sigma Aldrich, St. Louis, United States) was added as substrate to each well, immediately before the first absorbance measurement at 492 nm.
The accumulation of the melanin pigment as a product of the enzymatic reaction was measured by quantifying the absorbance of the sample. Changes in absorbance were measured continuously in the ELISA ASYS UVM 340 Microplate Reader (Biochrom, Cambridge, Great Britain). Measurements were performed at 15 minute intervals for 3 hours. The velocity of the reaction was proportional to the enzyme concentration. One PO unit (U) was defined as the amount of enzyme necessary to induce an increase in absorbance at 492 nm of 0.01 per minute. Specific activity was expressed as the number of units per mg of protein. Protein concentration was determined by Bradford (1976).

Statistical analysis

Data were expressed as mean values with standard deviation. The results were subjected to statistical analysis using Statistica 8 software (StatSoft Inc., Tulsa, Oklahoma, USA). Normality was analysed using the Kolmogorov–Smirnov test. Homogeneity of variance was investigated using Levene's test. The statistical significance of differences between samples collected at various developmental stages was analysed by one-way analysis of variance (ANOVA) and Tukey's test. The results were considered to be statistically significant at $p<0.05$.

Pearson's correlation coefficient was determined between the relative activity of PO and the relative expression of *proPO* mRNA in various developmental stages of *A. mellifera*.

RESULTS

Analysis of *proPO* gene expression by Real-Time PCR. Significant differences were noted in *proPO* gene expression between infected and uninfected workers and drones in all stages of development (for workers: L5 $p<0.002$, PP $p<0.0002$, P3 $p<0.04$, P5 $p<0.008$, I $p<0.0002$; for all stages of drones $p<0.0002$) (Fig. 1). In comparison with the control, gene expression levels in infected individuals were lower in all the developmental stages. The only exceptions were worker PP and drone P5 stages where the amounts of the gene transcript were 1.8-fold higher and 1.5-fold higher, respectively, than for the control bees ($p<0.0002$) (Fig. 1). Those were the only developmental stages where *proPO* gene expression differed significantly from the other stages within same-sex bee groups.

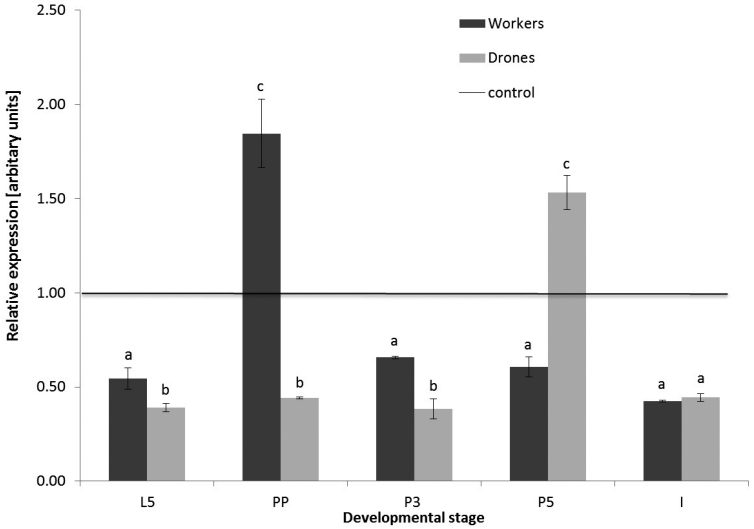

Fig. 1. Expression of the *proPO* gene in developing A. mellifera workers and drones ("L5" cocoon-spinning larvae, "PP" pre-pupae, "P3" pupae with red eyes, "P5" pupae with brown eyes and dark-brown body, "I" newly emerged bee) infected with *V. destructor*. The results were normalised relative to reference gene *rp49* and an endogenous control sample where relative quantification (RQ) = 1. Different letters at the top of the bars indicate statistically significant ($p<0.04$) differences in gene expression.

Phenoloxidase activity during the development of the control and infected honey bees

The results of the enzyme activity measurements are presented in Fig. 2. In all stages, regardless of sex, the differences in the average enzyme activity induced by varroasis were statistically significant (Fig. 2). Phenoloxidase activity was lower in infected bees than in the control bees in all developmental stages. The only exception was stage PP of the workers and P5 of the drones where PO activity was 2.8-fold higher and approximately twice higher in infected than in the control groups (Fig. 2). The trend of enzyme activity differed between females (Fig. 2A) and males (Fig. 2B). As a result, significant differences in the average enzyme activity were observed between the sexes, except stage P3 in the control groups. In uninfected individuals, PO activity levels were higher in drones than in workers until stage P3. This trend changed in successive stages. Due to this, in newly emerged workers, PO activity was approximately twice higher than in drones.

A strong positive correlation was observed between the relative activity of PO and the relative expression of *proPO* mRNA. The correlation coefficients were high in both workers and drones at $r = 0.988$ and $r = 0.996$, respectively.

DISCUSSION

The innate immunity system of insects consists of cellular and humoral responses (Osta et al., 2004). The activation of the phenoloxidase cascade is an important cell-mediated immune response (Sugumaran, 2002).

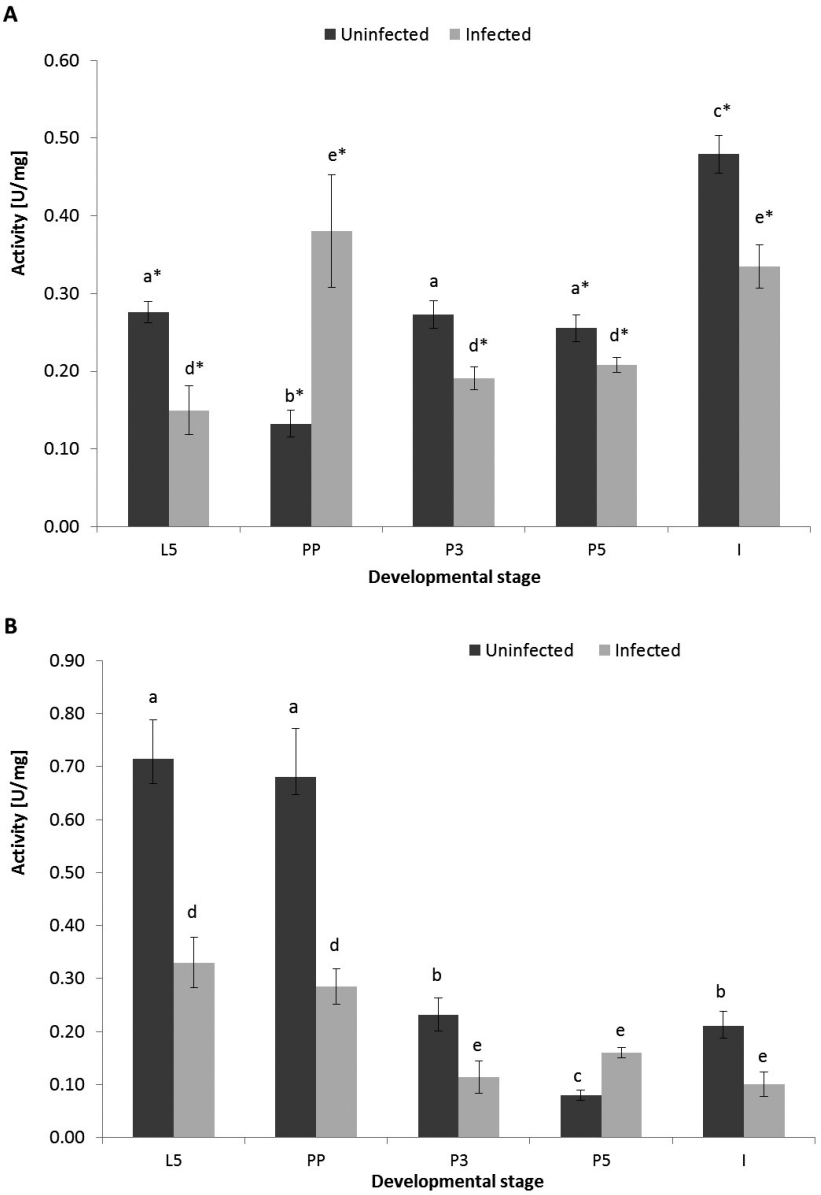

Fig. 2. Phenoloxidase activity in developmental stages ("L5" cocoon-spinning larvae, "PP" pre-pupae, "P3" pupae with red eyes, "P5" pupae with brown eyes and dark-brown body, "I" newly emerged bee) of the control and infected A. mellifera workers (A) and drones (B). Key: Different letters at the top of the bars indicate statistically significant ($p<0.003$) differences in average enzyme activity levels in the control (a-c) and infected (d-e) individuals. Symbol * in Fig. 2A denotes statistically significant ($p<0.03$) differences in average enzyme activity in a given developmental stage between the sexes.

The activity of the *proPO* gene is correlated with insect immunity against pathogens (Rodriguez-Andres et al., 2012; Roberts and Hughes, 2014). Previous studies demonstrated that the effect of *Varroa* mites on the expression of immune response genes in bees, including *proPO*-encoding genes, is determined by genotype, age, and environmental factors (Zhang et al., 2010; Lee et al., 2013; Khongphinitbunjong et al., 2015). We decided to evaluate the sensitivity and status of the immune system in developing worker and drone brood of honey bees, based on both the expression of the *proPO* gene and PO activity.

In most infected individuals, *proPO* gene expression and PO activity were significantly lower than in healthy bees. We also observed sex-related differences in PO expression induced by varroosis. In infected worker brood, the expression of the *proPO* gene increased significantly in the prepupae stage, whereas in drones, the said increase was noted later - in stage P5. Similar observations were made in drones, by Zhang et al.

(2010). Our findings suggest that significant sex-specific differences in the expression of the *proPO* gene could partially explain higher infestation rates in drones than in workers due to weaker stimulation of the phenoloxidase pathway in males in initial stages of infection (Rosenkranz et al., 2010).

In recent years, attempts have been made to explain the differences in the susceptibility of honey bees to varroosis. In addition to species-specific variations, differences were also reported between populations of the same stock (Zhang et al., 2010; Khongphinitbunjong et al., 2015). These crucial findings support the selection of stock and breeding of hybrids, that are more resistant to parasitic infections. Zhang et al. (2010) compared the expression of various genes in Asian and European bees to determine why *V. destructor* is far less pathogenic for Asian bees. The above authors studied only one developmental stage – prepupae. They noted differences in gene expression between the species: in *Apis cerana,* an increase in *proPO* gene expression was observed in response to infection, whereas no significant changes were noted in *A. mellifera*. In our study, the results were the opposite. Our results showed that the expression of the *proPO* gene increased in prepupae of *A. mellifera* workers. Those differences could be attributed to the genetic dissimilarity of the analysed bees whose taxonomic assignment to the subspecies or stock could not be precisely determined. This information is important because in a study of pupae (8 days after honeycomb sealing) of Russian and Italian honey bees, the varroosis disease induced differences in *proPO* gene expression, between races (Khongphinitbunjong et al., 2015). No changes in *proPO* gene expression were reported in Russian bees which are more resistant to infection (Rinderer et al., 1997), whereas *proPO* gene expression decreased in Italian bees (Khongphinitbunjong et al., 2015). Similar to a study of *A. m. ligustrica* (Kuster et al., 2014), we observed a decrease in *proPO* gene expression in P3 workers. A significant decrease in gene expression and PO activity, observed in stage P3 in infected bees of both sexes, suggests that the infection inhibits melanisation and sclerotisation. Those processes are intensified in stage P3 (Zufelato et al., 2004), and their inhibition could contribute to the risk of microbiological and viral infections, and the spread of pathogens. The expression of the *proPO* gene was significantly lower in infected newly emerged workers and drones than in healthy individuals. Those findings are consistent with the results reported by other authors (Yang and Cox-Foster, 2005; Aronstein et al., 2012).

Several studies revealed that *proPO* gene expression and PO activity change throughout an insect's development (Michell, 1966; Adamo et al., 2001). Laughton et al. (2011) observed an increase in PO activity in successive development stages of *A. mellifera* workers and drones. The highest levels of PO activity were noted in newly emerged individuals. In our study, the results noted in healthy individuals differed significantly from those reported by Laughton et al. (2011). The noted difference could result from the fact that PO activity was measured in whole bee-body extracts in different developmental stages and not in the hemolymph, as in earlier studies. Laughton et al. (2011) reported a significant decrease in enzyme activity in all age groups, excluding L5 workers, after bacterial lipopolysaccharide had been administered to stimulate the immune system of the studied brood. In our study, enzyme activity was also generally lower in infected brood of both sexes than in healthy individuals. The only exceptions were PP workers and P5 drones where varroosis enhanced PO activity (Fig. 2). This is consistent with the observed positive correlation between *proPO* gene expression and the activity of the enzyme encoded by that gene in infected workers and drones. The above findings confirm that there is a relationship between time-related changes in transcription intensity and enzymatic protein translation.

Low enzyme activity in infected bees, in particular in newly emerged individuals, indicates that the cellular immune response was significantly more compromised in drones than in workers. In view of our knowledge about the suppression of other resistance genes, including genes encoding antibacterial peptides (defensin-1, abaecin and hymenoptaecin) and genes encoding resistance enzymes in insects (dehydrogenase and glucose oxidase) in newly emerged individuals infected by *V. destructor* (Gregory et al., 2005; Yang and Cox-Foster 2005), this suggests that drones are less resistant to varroosis and other pathogens from inside and outside the hive, than workers.

CONCLUSION

Our results suggest that mite infections cause cellular and humoral immunosuppression in honey bees . In our study, the responses to varroasis differed across developmental stages, and not all of them involved immunosuppression. The expression of the *proPO* gene and PO activity were induced earlier in infected worker prepupae than in drone pupae with brown eyes and dark brown thorax (Fig. 1). In genetic analyses of the immune responses of honey bees

infected with *V. destructor,* the observed changes should be evaluated across different developmental stages and in both sexes.

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