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Review article

Changes in the expression of antimicrobial peptide genes in honey bees (*Apis mellifera*) under the influence of various pathogens

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ABSTRACT. Honey bees play an invaluable role in the ecosystem as plant pollinators. The dramatic decline in honey bee population has prompted a search for its underlying causes and effective remedy methods. Insects produce antimicrobial peptides (AMPs) which play an important role in the induced humoral immune response. The presence of apidaecin, abaecin, hymenoptaecin and defensin has been confirmed in honey bees. The expression of genes encoding these proteins is activated via two signalling pathways: toll pathway for apidaecins and defensins, and Imd pathway for abaecin and hymenoptaecin. Bee pathogens and parasites affect the expression of AMP genes, thus stimulating or inhibiting immune responses. The main causative agents of the global decline in bee populations include *Varroa destructor*, deformed wing virus (DWV), neonicotinoids, and microsporidian parasites of the genus *Nosema*. Researchers are divided over the influence of these pathogens and insecticides on the expression of AMP genes in insects. There is evidence to suggest that the age of honey bee plays an important role, and that peptides should be identified solely in the context of specific age groups. There is also a general scarcity of research into the long-term effects of immunosuppressive factors. The influence of various pathogens on the immune system of bees should be investigated to better understand their mutual relationships and to develop effective bee protection methods.

Keywords: antimicrobial peptides, honey bee, pathogens, colony collapse disorder, immunity, immunosupression

Introduction

Recent years have witnessed a rapid decline in the number of bee colonies around the world [1]. The current health status of bees indicates that effective measures are needed to protect bee populations [2]. However, not all factors responsible for fatal diseases in honey bees have been identified [3]. The leading causes of the colony collapse disorder (CCD) [4] include *Varroa destructor* invasions [5,6], microsporidian parasites of the genus *Nosema* [3], deformed wing virus (DWV) [7–9], acute bee paralysis virus (ABPV) [10], neonicotinoid poisoning [10] and lately, *Lotmaria passim* protozoa [11,12]. A single causative agent of CCD cannot be identified, and the undiscovered effects of other microorganisms cannot be ruled out [13].

Similarly to other eukaryotes, honey bees (*Apis mellifera*) have highly effective defence mechanisms that minimize the harmful impacts of microbial pathogens [14]. These mechanisms include innate and acquired immunity. The components of the innate immunity system are present in the body regardless of the occurrence of pathogens. They include anatomical structures that act as barriers to infectious agents, cell-mediated immunity that supports defence processes such as phagocytosis, melanisation of pathogens, polypeptides and haemolymph proteins such as polyphenol oxidase, lysozyme and pectin [14]. Acquired immunity, represented by antimicrobial peptides (AMPs), is activated in response to an ongoing infectious process. Antimicrobial peptides are not synthesized or occur in the form of inactive precursors when infectious agents capable of

initiating an immune response are not present in the body [15]. The known AMPs in bees include apidaecins [16], abaecin [17], hymenoptaecin [18] and defensins [19,20]. Apidaecins are small, proline-rich peptides that contain 18 amino acids. The conserved C-terminus of the peptide is responsible for its antibacterial properties, and the molecule is deactivated when other amino acid residues are substituted into the region. The N-terminus is a variable region, and modifications in the amino acid sequence lead to changes in the peptide's spectrum of activity, thus enabling the peptide to adapt to specific pathogens [21,22]. Four apidaecin isoforms have been identified, and three of them have been detected *in vivo* in *A. mellifera*: apidaecin 1a, 1b and 2. The expression of apidaecin genes increases shortly after infection when the toll signalling pathway is activated [23]. Gene expression is largely determined by the health and nutritional status of bees [16,23]. Gram-negative bacteria trigger the strongest immune response. Owing to their structure, apidaecin molecules are able to cross bacterial cell walls, block the synthesis of bacterial proteins, disrupt the function of bacterial ATP-ases and impair metabolism in bacterial cells [24]. Abaecin is also a proline-rich peptide which is composed of 33-34 amino acids [17]. The expression of abaecin genes increases after infection, both in brood and adult individuals. Abaecin targets mostly Gram-positive bacteria, and to a lesser degree, Gram-negative bacteria, and it provides complementary antibacterial defence together with apidaecins [25]. The activation of the Imd signalling pathway affects gene expression, and the peptide's effectiveness increases in environment with high ionic strength [17]. Similarly to apidaecins, abaecin does not exert lytic effects on bacterial cell walls, but it impairs metabolic function upon entering the bacterial cell. Research has demonstrated that the potency of abaecin is heritable, and this peptide can be used as a potential marker for selecting bee colonies with a higher level of resistance [26]. Hymenoptaecin is a glycine-rich peptide composed of 33 amino acids [18], and it targets both Gram-positive and Gram-negative bacteria. The expression of hymenoptaecin genes is upregulated at a slower rate in comparison with other AMPs, and it is controlled by the Imd pathway. Hymenoptaecin is activated in response to multiple pathogens. This AMP lyses bacterial cell membranes and creates channels in bacterial cell walls through which ions and metabolites escape, which disrupts the function of bacterial cells. Hymenoptaecin increases the permeability of bacterial cell membranes, thus intensifying the activity of proline-rich peptides, in particular abaecin [27]. In contrast to honey bees which have one hymenoptaecin homolog, eastern honey bees (*A. cerana*) contain twelve homologs, which implies that this AMP plays a much greater role in the immune system of this species [28]. Defensins are cysteine-rich peptides composed of 51 amino acids. The expression of defensin genes is activated by the toll pathway. Defensin 1 occurs in three isoforms: one of which is found in the haemolymph and two – in royal jelly. Defensin 1 is an important anti-bacterial component of honey, and it is involved in the formation of social immunity in bee colonies. In turn, defensin 2 promotes individual immunity, and it is produced in fat bodies and the haemolymph [29]. Defensins target mainly Gram-positive bacteria and fungi. They damage bacterial cell membranes and walls, and deprive cells of vital metabolites [30].

Honey bees are social insects, which makes them particularly susceptible to the rapid spread of microbial pathogens and parasites. Infections and pathogen invasions affect most, if not all colony members due to small hive area, contact with other bees and trophallaxis. Bees make frequent flights and cross long distances in search of food, which also increases the risk of contact between the insects and pathogens [31].

The aim of this article was to review the literature analysing the impact of selected pathogens on the production and activity of the above AMPs in honey bees, a species that could be used as a model organism in studies of infections in insects.

Varroa destructor

Antimicrobial peptides in bees are difficult to analyse during *V. destructor* invasion, mainly due the absence of a standardized monitoring method [32] as well as considerable exposure to environmental pathogens in bee colonies. Two research models are generally used. In the first model, peptide levels are determined in adult bees or brood that are sampled directly from the hive, without interfering with their immune status. Such studies are performed to analyse gene expression in response to infections caused by mites and environmental pathogens [33–36]. In the second model, saprophytic microorganisms are administered shortly before

sampling. In this case, the expression of AMP genes changes in response to the infection caused by saprophytic pathogens, but it is deregulated by mite invasions [37]. It should also be noted that the expression of genes encoding proteins is highly influenced by the developmental stage and age of bees [38], and that the results should be interpreted only in the context of specific age groups where AMP gene expression can differ considerably [39]. The feeding mechanism of *V. destructor* has not been fully elucidated [39]. It is generally believed that mites feed on the haemolymph of bee larvae and imagines, but Ramsey et al. [40] demonstrated that fat body tissue is integral to mite diets. Fitness metrics were similar in mites fed haemolymph alone and in starved mites, whereas mites fed fat body tissue survived longer, had better fitness metrics, and produced more eggs. Mites feed by puncturing larval cuticles and integuments or the membranes between body segments in imagines. Wounds heal slowly, and they are aggravated by other parasites in the cell feeding from the same site [41,42]. When larval integuments were punctured artificially under laboratory conditions, wounds healed rapidly, and bacterial infections, which often occur during *V. destructor* invasion, were not observed [41]. These findings suggest that the saliva of *V. destructor* contains substances which prolong wound opening and facilitate mite feeding. The data relating to the impact of *V. destructor* invasion on the immune status of bees are inconclusive. Research indicates that parasitic invasions increase [35,43], decrease [36,44], exert a minor effect or no effect [45] on the expression of AMP genes. However, the absence of a standardized method for analysing gene expression contributes to a discrepancy in results. The age and developmental stage of insects, the immune status and the treatment applied in brood where gene expression and peptide concentrations are analysed should be taken into account when interpreting data [45]. Based on the presence of correlations between AMP genes, these peptides can be divided into groups (clusters) where the expression of genes encoding different AMPs is modulated by a given pathogen. Correlations were found between the expression of defensin and Relish genes, and apidaecin and hymenoptaecin genes. Abaecin was not correlated with any cluster [45]. According to Yang and Cox-Foster [37], *V. destructor* mites suppress the production of AMPs. Newly emerged bees from severely infested colonies were characterized by decreased expression of genes encoding hymenoptaecin, abaecin and defensin in response to infection caused by saprophytic bacteria, compared with bees that were free of *V. destructor*. These findings were partially confirmed by Gregory et al. [36] who reported a decrease in the expression of AMP genes in pupae moderately infested by mites, but did not observe immunosuppression in severely infested pupae. The above could be attributed to the achievement of an invasion threshold that initiates the immune response, or prolonged exposure to mites which leads to a more effective immune response. The results of the cited study could also have been influenced by the presence of infections caused by other pathogens. Gregorc et al. [35] noted increased expression of abaecin, hymenoptaecin and defensin 1 in larvae infested by *V. destructor*. Recent research has confirmed that *V. destructor* does not downregulate the expression of AMP genes in bees. According to Kuster et al. [33], permanent and definitive changes in gene expression are not observed 24 to 240 hours after emergence, which could suggest that mites do not exert immunosuppressive effects. The expression of abaecin, apidaecin, hymenoptaecin and defensin was periodically upregulated and decreased over time to reach the levels noted in healthy bees. Defensin was the only exception, and its expression did not change significantly over time, but was elevated 4 days after emergence in bees that were simultaneously infested with more than three mites. *Varroa destructor* mites could also exert a long-term negative effect on older bees, which can be attributed to the lower health status of older insects, rather than immunosuppression. A positive correlation was also reported between the severity of *V. destructor* invasion and DWV transcript abundance [33,34,45]. In bees, DWV transcript abundance also increase after sterile mechanical damage to body integuments, but the noted increase is much smaller than during mite invasion.

Research suggests that the adverse impact of *V. destructor* mites on honey bees is determined by the severity of the invasion. Severe invasions trigger specific immune responses and stimulate the expression of AMP genes in bees. There is evidence to indicate that *V. destructor* mites contribute to a decrease in the size of fat bodies, but it remains unknown whether the above compromises AMP production [40].

Deformed wing virus (DWV)

Honey bees are frequently infected by several viruses, and the effects exerted by individual viruses are difficult to determine [46]. According to Randolt et al. [46], *V. destructor* invasion enhances the transcription of the deformed wing virus (DWV-A, DWV-B), black queen cell virus (BQCV) and slow bee paralysis virus (SBPV). The DWV enters into mutualistic interactions with *V. destructor* [46] which also acts as a vector of DWV [10,47]. This pathogen inhibits the expression of the dorsal-1A8 gene, a member of the NF-κB family, which plays a key role in the toll signalling pathway [48–50] that regulates the expression of genes encoding AMPs: defensin 1, defensin 2 and apidaecin. Early research into DWV revealed that abaecin and defensin expression was downregulated in mixed infections, whereas the expression of hymenoptaecin was not altered [37]. An increase in DWV transcript abundance and, consequently, higher disease severity, led to a further decrease in the expression of AMP genes, in particular defensin [51]. More recent research demonstrated that similarly to *V. destructor*, the immunosuppressive effect of DWV on bees is difficult to confirm [48]. The expression of AMP genes was not modified in response to infections caused by ABPV [52,53] and the Israeli acute paralysis virus (IAPV) in *A. mellifera* [54], or the cricket paralysis virus (CrPV) in the genus *Drosophila* [55]. RNA interference has been identified as the key mechanism in viral infections in insects [56,57]. The expression of AMP genes did not change in bee larvae orally administered DWV [58]. In turn, a minor increase in the expression of defensin 1 and hymenoptaecin genes was noted in larvae injected with DWV, which most likely resulted from a defense response to mechanical injury [46]. According to Erban et al. [59], mixed *V. destructor* and DWV infections lead to competitive interactions at the molecular level rather than synergistic effects. *Varroa* activates, whereas the DWV deactivates the expression of NF-κB genes in newly emerged bees [50,59].

Neonicotinoids

Neonicotinoids such as thiamethoxam, imidacloprid and clothianidin are among the most widely used insecticides around the world. These compounds are toxic for bees, and they have been linked with the CCD [60–62]. Research conducted on worker bee larvae revealed differences in the expression of AMP genes across age groups [63]. The expression of abaecin, defensin 1 and defensin 2 genes was downregulated in six-day-old larvae from thiamethoxam treated colonies, but it was upregulated in nine-day-old larvae. In 15-day-old larvae, the expression of defensin 2 was down - regulated, whereas the expression of abaecin and defensin 1 was upregulated. Higher concentrations of thiamethoxam appear to upregulate AMP expression because in larvae administered additional doses of thiamethoxam, gene expression increased in all age groups. Contrastingly, according to Tesovnik et al. [64], thiamethoxam compromises the immune response to *V. destructor* and viruses transmitted by these mites. In honey bee larvae infested with *V. destructor*, thiamethoxam decreased the expression of abaecin and defensin 1 genes [64]. Fat bodies break down harmful compounds, and their depletion during *V. destructor* invasion enhances the adverse effects of neonicotinoids by impairing the insects' ability to eliminate these toxic substances. Imidacloprid exerts immunosuppressive effects on honey bee larvae by downregulating the expression of most AMP genes [43]. The above can probably be attributed to intensified detoxification of this pesticide, which requires considerable energy expenditure and leads to changes in the immune status of bees [65]. The only exception was lysozyme 2 whose expression increased under exposure to imidacloprid. Clothianidin also affects the immune system of bees by upregulating the expression of the *AmeΝLRR* gene – an inhibitor of NF-κB proteins. Impaired activation of transcription factors decreases the expression of AMPs, including apidaecin [62].

Nosema spp.

The influence of microsporidian parasites of the genus *Nosema* on the immune system of bees is difficult to evaluate due to the absence of a standardized research protocol. The severity of the infection (expressed by the number of spores per bee), the age and developmental stage of bees, and the duration of exposure to the pathogen (usually expressed in days post infection, dpi) exert significant effects on the immune system and the expression of AMP genes. The type of the sampled material also plays a role, and considerable differences were reported in analyses of whole bees and abdominal segments [66]. The results of studies

evaluating the influence of *Nosema* spores on bees are inconclusive. According to Li et al. [67], prolonged exposure to *N. ceranae* has immunosuppressive effects on honey bees. Gene expression is upregulated in the initial stages of infection [68], after which a gradual decrease in the expression of abaecin, apidaecin and hymenoptaecin is noted [66,69]. In a study by Chaimanee et al. [66], *N. ceranae* exerted significant immunosuppressive effects at a concentration of 10^6 spores/ml. The expression of defensin, abaecin, apidaecin and hymenoptaecin genes was considerably downregulated in the first days of infection. However, the expression of the analysed AMPs did not differ significantly from that observed in the control group at 12 dpi, which suggests that *N. ceranae* causes only transient immunosuppression. Jefferson et al. [70] reported upregulated expression of apidaecin and abaecin genes in both younger and older worker bees. Other researchers noted a positive correlation between thiamethoxam exposure and the severity of *N. ceranae* infection [63,71–73]. A positive relationship was also found between *N. ceranae* and BQCV. These pathogens often interact [24], and mixed infections increase mortality in bees [27].

In conclusions, not all of the described pathogens exert a significant influence on the expression of AMPs in bees. Severe *V. destructor* invasions stimulate the expression of AMP genes, but they can be masked by co-occurring microorganisms. The peptides that are affected by mites differ in variously aged bees. The DWV inhibits the expression of AMP genes and enters into antagonistic interactions with *V. destructor* which acts as a vector of DWV. All stress factors increase DWV transcript abundance in bees, which can probably be attributed to the strong immunosuppressive effects of varroosis. Neonicotinoids downregulate the expression of AMP genes. They influence various mechanisms, such as cell metabolism, and activate signalling pathway inhibitors. The results of studies analysing *Nosema* spp. infections in bees are ambiguous and difficult to interpret. Most research findings indicate that *Nosema* spp. exert transient immunosuppressive effects, and the expression of AMP genes eventually returns to normal levels. The mechanisms that affect the status of AMPs should be investigated in greater detail to expand our understanding of the effects exerted by pathogens on the immune system of bees.

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