Antimicrobial peptides: a key component of honey bee innate immunity

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Running head:

"Bee AMPs"

Summary (English)

Honey bee immune responses are composed of a complex suit of individual immune mechanisms and special types of behavioral adaptations. The main focus of this paper is innate immunity in the honey bee, and specifically, the role and function of antimicrobial peptides (AMPs). Insect innate immunity constitutes evolutionary conserved defense strategies that provide immediate responses against invading pathogens. It consists of the three levels of resistance: physical barriers as the first line of defense, cell-mediated immunity and cell-free humoral immunity, a complex network of intracellular signaling pathways leading to activation of a variety of humoral factors. Among those, AMPs are recognized as key components of humoral immunity in many types of organisms. The two basic mechanisms of action are: 1) the generation of leaks into prokaryotic membranes, and 2) either inhibition of bacterial proteins translation or folding.

Recently, four families of AMPs (i.e. apidaecins, abaecin, hymenoptaecin and defensins) have been described in the honey bee. One of the defensins, defensin1, was originally isolated from royal jelly, and therefore named royalisin. In addition, several bioactive peptides (e.g., apamin and melittin) were identified in bee venom. The expression of bee antimicrobial peptides is regulated mainly by two intracellular signaling pathways Toll and Imd/JNK. However, the extent of gene expression and peptides synthesis is affected by a number of different biotic and abiotic factors. In this review paper we attempted to discuss factors involved in activation of the honey bee AMPs and their role in bee resistance to microbial pathogens and the environmental stress, such as exposure to pesticides. We also discuss recent knowledge on the molecular regulation of bee AMPs. Although recent advances in genomics produced a new understanding of bee immunity in general, the exact mechanisms of gene regulation within each of the immune signaling pathways and complex network of these signaling pathways still awaits further investigations.

Summary (Spanish)

Las respuestas inmunitarias de la abeja doméstica están compuestas de un complejo conjunto de mecanismos inmunitarios individuales y unos tipos especiales de adaptacion conductual. El enfoque principal de este artículo es la inmunidad innata de la abeja doméstica, especialmente el rol y la función de los péptidos antimicrobianos (AMPs). La inmunidad innata de los insectos constituye la estrategia de defensa que se ha conservado durante la evolución y que proporcionan las respuestas inmediatas a la invasión de los patógenos. Consiste de tres niveles

de resistencia: barerras físicas como la primera línea de defensa, inmunidad celular e inmunidad humoral, y una red compleja de la seňalización intracelular que induce la activación de una variedad de factores humorales. Los AMPs son reconocidos como componentes claves de la inmunidad humoral en muchos tipos de organismos. Los dos mecanismos básicos de su acción son: 1) producción de roturas en las membranas procariotas, y 2) inhibición de la traducción de las proteínas o de su plegamiento en las bacterias.

Recentemente, cuatro famílias de AMPs (apidacinas, abaecina, himenoptaecina y defensina) fueron descritas en la abeja doméstica. Una de las defensinas, Def1, habia sido originalmente aislada de la jalea real, y fue denominado royalisina. Además, varios péptidos bioactivos (apamina y melitina) fueron identificados en el veneno de la abeja. La expresión de los AMPs es regulada por las principales vías intracelulares de seňalización de Toll y Imd/JNK. No obstante, el nivel de la expresión de los genes y la produción de los péptidos está influenciado por varios factores bióticos y abióticos. En este artículo de revisión se discutirán los factores involucrados en la activación de los AMPs de la abeja doméstica y su rol en la resistencia a los patógenos microbiales y el estres medioambiental, como la exposición a los pesticides. También se discutirán los conocimientos actuales de la regulación molecular de la expresión de los AMPs de la abeja. Aunque los avances recientes de la genómica resultaron en nuevos conocimientos acerca de la inmunidad de la abeja en general, los mecanismos exactos de la regulación de los genes dentro de cada una de las vías de seňalización inmunitaria y la red compleja que modula estas vías de seňalización espera investigación adicional.

Keywords

Honey bee, *Apis mellifera*, innate immunity, antimicrobial peptides, apidaecins, abaecin, hymenoptaecin, defensins, royalisin, jelleine, viruses, *Nosema, Varroa destructor*

Introduction

Recent losses of honey bee populations threaten to cripple worldwide production of the agricultural crops. Although the primary cause of these losses has not yet been identified, latest research indicates that combined effects of biotic and abiotic stressors could be at fault(van Engelsdorp *et al.*, 2009). Among all suspected targets, the most likely effect of these stressors is a compromised health of the honey bee. Colony collapse can be triggered by the destabilization of silent viral infections by parasitic mite infestation and resulting immunosuppression (Nazzi *et al.*, 2012) Therefore, focusing research on the honey bee immune mechanisms capable of dealing with these stressors may help to improve strength and productivity of honey bee colonies.

Social insects, including the honey bee, developed a wide array of integrated mechanisms protecting them from biotic and abiotic stressors (e.g., diseases, parasites, predators, and pesticides). Collective defenses or "social immunity" play an important role at the colony level, but not necessarily protecting its individual members (Wilson-Rich *et al.*, 2009, Le Conte *et al*., 2011). Individual bees can be sacrificed for the "good of the colony". For example, hygienic colonies are able to decrease the infection pressure as diseased larvae are removed from the colony to prevent dispersal of the disease among colony members (reviewed by Panasiuk *et al*., 2010; Invernizzi *et al.*, 2011).

At the individual level, invaders are confronted by a complex network of cellular and humoral immune reactions, too complex to be discussed in details within this paper. Therefore, here we focus on the neutralizing effects of humoral immunity, and specifically, activity of the antimicrobial peptides (AMPs). AMPs have become increasingly the focus of research investigations, including their structure, biological activity, and potential use in clinical applications as substitutes for classical antibiotics and antifungal compounds (reviewed in Giuliani *et al.*, 2007). Evans *et al.* (2006) describe differences in expression in four families of AMPs as a reaction on injections of specific stimulus into bee bodies (saline buffer, *Escherichia coli*, *Paenibacillus larvae*). However, there are many aspects of the AMPs' expression, regulation and activity that are still unclear and require future investigations. This review summarizes current knowledge of honey bee's AMPs, their biological roles and molecular mechanisms involved in defenses against pathogens.

Antimicrobial peptides as part of honey bee innate immunity

Our understanding of insects' innate immunity is rapidly changing with a new research showing that invertebrates' immunity may have more in common with innate and some elements of adaptive immunity of vertebrates that previously thought. In contrast to earlier assumptions that insects lack immune memory, recent studies have shown that insects are able to activate long-lived and pathogen-specific immune responses against microbial pathogens (Rodrigues *et al*., 2010). The sequencing of the honey bee genome (Honey Bee Genome Sequencing Consortium, 2006) allowed the first global analysis of the honey bee immune components and development of the first model of the immune-related intracellular signaling in the honey bee (e.g., Toll, Imd, JNK and JAK/STAT) (Evans *et al*., 2006).

Insect humoral factors are secreted by the fat body and the haemocytes. Some of these factors are constitutively produced and always present in the haemolymph (e.g., lectins), other are produced in different levels depending on the sex and life stage (e.g. phenoloxidase), or are inducible (e.g., thioester-containing proteins and antimicrobial peptides) and secreted in response to microbial infections or septic wounding (Turner 1994, Evans *et al*., 2006, Laughton *et al*., 2011). The analysis of Honey Bee genome showed that it encodes a single *prophenoloxidase* (proPO) (GB18313), three *lysozymes* (GB10231, GB15106, GB19988), C-type lectin 5 (NP_001229926) and predicted endoplasmic reticulum lectin 1-like protein (XP_394479) (Evans *et al*., 2006).

Prophenoloxidase (pro-PO) is a zymogen of phenoloxidase (PO) (E.C.1.14.18.1). Pro-PO cascade is activated by lipopolysacharides (LPS) and peptidoglycans from bacterial cell walls or by β-1,3-glucans from fungi. The products of PO reaction are highly reactive and toxic quinones which polymerize to melanin (Cerenius and Söderhäll, 2004). Pro-PO is produced in haemocytes and the product of PO activity is involved in the stimulation of phagocytosis, encapsulation (Cerenius *et al.*, 2008). The optimum temperature for PO enzyme activity is 20 °C, while the temperature in bee nest is about 34.5 °C what is very interesting. PO is not a fundamental physiological factor in immune defense. The PO activity stays stable for higher temperatures (Zufelato *et al*., 2004). PO activity is not detectable in the first two days of brood development, but significantly increases later in the development (Chan *et al*., 2009). It was clearly detected in crude haemolymph from fourth- to fifth-instar honey bee larvae infected with bacterial pathogen, *P. larvae* (Chan *et al.*, 2009). There is also a significant difference in PO activity between young adult bees and old foragers. Phenoloxidase activity increases early in adult life and reaches a plateau by the end of the first week (Laughton *et al.*,

2011; Bull *et al.*, 2012). Cellular immunity, assessed as the haemocyte number, is dramatically reduced in adult workers, queens and drones, whereas PO activity shows caste- and age-dependent dynamics (Schmid *et al*., 2008; Wilson-Rich *et al*., 2008). This might be explained by evolutionary changes, representing trade-off individual energy-demanding cellular immunity with more effective PO defense in foragers and possibly a complex hygienic behavior (Schmid *et al*., 2008; Wilson-Rich *et al*., 2008, Laughton *et al.*, 2011).

It seems that beekeeping itself as an environmental factor significantly influences basal bee immunocompetence. In Australia, mite- and European foulbrood-free country, house bees had significantly higher level of PO activity and encapsulation responses compared to wild colonies (Lowe *et al*., 2011). It was suggested that bee colonies kept in husbandry are less stressed, and therefore have more resources available to maintain the energy expenditure of up-regulated immune defenses. The increased use of synthetic chemicals in agricultural systems, as well as commercial beekeeping, is dramatically changing the environmental conditions potentially effecting honey bee health and longevity. Some of these chemicals approved for in-hive applications (e.g., for control of *Varroa*-mite) or used in other agro systems, are known to affect bee immunocompetence (Gregorc *et al.*, 2012; reviewed by James and Xu, 2012). Bees are also relatively vulnerable to sublethal concentrations of pesticides and pesticides residues accumulated in hive stores and bee products, such as honey, pollen and wax. The presence of pesticide residues in bee products showed potential impact on bee immune responses (Chauzat *et al*., 2006; Johnson *et al*., 2010; James and Xu, 2012). The long-term application of thymol and coumaphos down-regulated JNK pathway, however, with no apparent impact on the expression of bee AMPs. Similarly, neither τ-fluvanilate nor formic acid effected levels of antimicrobial peptides (Boncristiani *et al*., 2012). This is consistent with results reported by Silverman (2003) showing that the immune activation of the JNK pathway is not required for antimicrobial peptide gene induction. However, a recent study in *Drosophila* showed that AMPs can also be induced under non-infectious stress, such as aseptic wounding and high- low body temperature. Tsuzuki and colleagues (2012) demonstrated that growth-blocking peptide (GBP)-JNK signaling regulates stressor-induced AMP expression in *Drosophila*, which did not require Toll and Imd pathway-related genes. Exposure of bee colonies to miticides (e.g., coumaphos, τ-fluvanilate, amitraz and flumethrin) had significant effects on the expression of bee antimicrobial peptides defensin, abaecin and hymenoptaecin. The expression of hymenoptaecin showed an increase after exposure to

flumethrin, in contrast, coumaphos down-regulated the expression of hymenoptaecin and abaecin (Garrido *et al*., 2013).

Function and mechanism of action of antimicrobial peptides

More than a thousand of antimicrobial peptides have been already described across all kingdoms (Bulet *et al.,* 2004; Wimley and Hristova, 2011) with more than 200 AMPs found in insects (reviewed by Li *et al*., 2012). Some insect genomes encode for a large number of AMPs (e.g., *Drosophila* and *Anopheles*) however only a reduced number of AMPs was detected in honey bee genome (Evans *et al.*, 2006). Among them, seven antimicrobial peptides were detected in bee haemolymph coded by a total of 19 cDNAs (Table 1): apidaecins 1a, 1b and 2 (deposited as UniProt entries P35581, Q06601, Q06602), abaecin (P15450), defensin 1 (P17722), defensin 2 (Q5MQL3) and hymenoptaecin (Q10416). In addition, different isoform of defensin-1, royalisin, and jelleins-1, 2 and 4 (O18330) were found in royal jelly (Casteels *et al*., 1989, 1990, 1993; Fujiwara *et al*., 1990; Andreu and Rivas, 1998; Bíliková *et al*., 2001; Klaudiny *et al*., 2005). Different peptides secreted into bee haemolymph have complementary function by targeting microbial cells for destruction (Casteels *et al*., 1989, 1990, 1993, Casteels and Tempst, 1994; Otvos, 2000b; Fontana *et al*., 2004). For the most part they show a broad spectrum activity against bacteria, protozoa, and fungi. However, some of them may preferentially target one type of microbes over the others. For example, apidaecins and hymenoptaecin show higher efficiency against G- bacteria (Casteels *et al*., 1989, 1993), whereas abaecin is less active against most G- bacteria than apidaecin. Interestingly, abaecin displays the lowest minimum inhibitory concentration for *Xanthomonas campestris* (G-), a plant pathogen totally resistant to apidaecin (Casteels *et al*, 1990). Haemolymph defensin-1 is effective against both G+ and G- bacteria, and fungi (Klaudiny *et al.*, 2005), while royalisin is more effective against G+ bacteria including *P. larvae*, the causative agent of the American foulbrood (Fujiwara *et al*., 1990). Apisimin peptide found in honey bee royal jelly has not been confirmed to exert antimicrobial activity, similarly to jellein-3 (Bíliková *et al*., 2002; Fontana *et al*., 2004).

Antimicrobial peptides belong to a diverse group of molecules with a variety of antimicrobial activities as recently reviewed by Wimley and Hristova (2011). Most of them are cationic or amphipathic peptides that allow them to interact with and disrupt negatively charged lipid membranes containing lipopolysaccharides (LPS). The interaction of peptides and membrane components usually results in the formation of channels which enable the leakage of small

ions and essential metabolites, and in some instances even the penetration of large molecules like peptides and small proteins with fatal effects for the bacteria (Hancock, 1997; Shen *et al*., 2010). Another mechanism of peptide action is mediated by the interaction between antimicrobial peptides and inner proteins, DNA, RNA or microbial cell compartments (Maróti *et al.*, 2011).

Short proline-rich peptides (PRPs) are able to pass through plasmatic membrane and specifically bind to bacterial DnaK, a 70 kDa heat shock protein (Otvos *et al*., 2000a). PRPs permeate bacterial membrane without lytic effect (Benincasa *et al*., 2009), bind to DnaK protein and interact nonspecifically with chaperonin GroEL (Otvos, 2000b; Kragol *et al*., 2001; Scocchi *et al*., 2009). Binding to DnaK and inhibition of DnaK-dependent protein folding correlates with peptide antibacterial activity (reviewed in Markossian *et al.*, 2004).

Apidaecins permeate bacterial lipid bilayer without lytic effect to bacteria and are able to bind to GroEL-GroES complex (Piantavigna *et al.*, 2009; Zhou *et al*., 2011). It was determined that toxicity of apidaecins to bacteria depends on sequential interaction with diverse molecular targets but is devoid of any pore-forming activity (Castle *et al.*, 1999). The observed mechanism of apidaecins interaction with *E. coli* cells included irreversible permeate/transporter uptake and specific dose-dependent inhibition of protein synthesis. The conserved C-end of the peptide is required for successful binding to the receptor molecule in bacterial membrane. Importantly, any single substitution in the C-end results in almost complete loss of antimicrobial activity (Czihal and Hoffmann, 2009). Shen *et al.* (2010) experimented with synthetic proline-rich peptide PP30, which showed activity against G+ and G- bacteria, but not fungi.

The antimicrobial peptides selectively target prokaryotic cells (Chopra *et al*., 1997) due to a lower membrane potential compared to eukaryotic cells; that activity relates to lower content of cholesterol and anionic lipids in bacterial membranes (Hancock, 1997). Wimley and Hristova (2011) proposed two basic hypotheses why eukaryotic cells are resistant to antimicrobial peptides: 1) AMPs do not recognize eukaryotic cells; 2) AMPs selectively recognize prokaryotic cells; peptides bind specifically to their membranes and lytic effect of prokaryotic cells depends on the concentration and amount of a peptide.

Apidaecins

Apidaecins are small proline-rich peptides composed of 18 aminoacids. A multi-peptide precursor molecule contains a number of short peptides generated by processing of a single

precursor protein. Repeats are joined by two processing sequences. The C- end of bee apidaecin is highly conserved whereas the N-end part is variable. However, modification of the N-end produced only a small effect on the antimicrobial activity against G- bacteria. The removal of leucine residue from the C-end of the 1b isoform resulted in loss of approximately 50% of antimicrobial activity compared to the activity level of a native peptide (Dutta *et al*., 2008).

Analysis of honey bee cDNA showed the relative occurrence of 3 isotypes of apidaecins mRNA. Apidaecin type 73 (Q06602, UniProtKB) is cleaved into 3 peptides: apidaecin, apidaecin 1a and apidaecin 1b. Apidaecin type 22 (P35581, UniProtKB) is composed only of apidaecin 1a and apidaecin 1b. Apidaecin type 14 (Q06601, UniProtKB) consist of apidaecin 2, apidaecin 1a and apidaecin 1b (Table 2). There are four natural forms of apidaecin which differs in amino acid sequence. Only apidaecins 1a, 1b and 2 peptides were detected in bees *in vivo* (Casteels *et al*., 1989; Casteels-Josson *et al*., 1993, Li *et al*., 2006). In contrast, 13 different cDNAs encoding 4 apidaecins were found in Asian honey bee *Apis cerana* (Xu *et. al*., 2009).

The up-regulation of apidaecin expression in response to bacterial infection of adult bees can be detected early post infection. However, the highest concentration in haemolymph (360 µg per ml) is reached by 36 hour post inoculation with *E. coli* (Casteels-Josson *et al*., 1993). The average concentration of all three isoforms (apidaecin 1a, 1b and 2) in immune bee haemolymph is about 50 nmol (100 µg) per ml. The level of apidaecin expression likely depends on health and nutritional status of bee colony (Casteels *et al*., 1989, Casteels-Josson *et al*., 1993).

Apidaecin-like peptides are also found in other insects, but they showed different antimicrobial specificity compared to bee apidaecins (Casteels and Tempst, 1994; Casteels *et al*., 1994; Li *et al*., 2006).

Abaecin

Abaecin (P15450) is another antimicrobial peptide which belongs to the prolin-rich family of peptides. Abaecin precursor contains 19 amino acids signal peptide which is cleaved after peptide activation, resulting in the mature peptide of 33-34 amino acids. Abaecin-1 (NM_001011617) was described in the Western honey bee (*Apis mellifera*). Xu and colleagues (2009) identified 11 cDNAs encoding 2 different abaecin peptides (AcAb1 and AcAb2) in Asian bee, *Apis cerana*. Abaecin2 (U15954, UniProtKB) lacks Gln at position 9 of

abaecin-1 and contains Ile at position 7 instead of Val found in abaecin-1 primary sequence of Western honey bee (Xu *et al*., 2009). The bee abaecin signal sequence is highly homologous to signal sequence of drosocine in *Drosophila melanogaster* (Casteels-Josson *et al*., 1994).

Abaecin inhibits growth of *G+ bacteria* (Casteels *et al.*, 1990). The abaecin precursor was found both in adult bees and in bee brood haemolymph (Casteels *et al*., 1990; Casteels-Josson *et al*., 1994). Expression and abundance of abaecin is rapidly up-regulated in response to bacterial infection (Casteels *et al.*, 1993; Evans, 2004; Randolt *et al.*, 2008), there is a time-dependent increase in expression of this peptide in first-instar larvae after *P. larvae* spores exposure (Evans, 2004). Abaecin-like peptides were also found in bumblebee haemolymph. Unlike bee peptide, the bumblebee forms are *O*-glycosylated (Hara and Yukamawa, 1995). Studies on the heritability of abaecin showed moderate heritability of abaecin expressions and it was suggested the level of abaecin expression could be a useful marker for selection of disease-resistant bee colonies (Decanini *et al*., 2007).

Defensins

The occurrence, role and expression of defensins in honey bees were recently reviewed by Ilyasov *et al.* (2012). Two different defensin genes were found in the honey bee genome, *defensin-1* and *defensin-2* (Evans *et al.*, 2006). First honey bee defensin was isolated from royal jelly, and therefore named royalisin (Fujiwara *et al*., 1990). However, sequence analysis showed that this is one of the *defensin-1* isoforms. Second *defensin-1* isoform was found in bee haemolymph by Casteels-Josson *et al.* (1994). Kwakman and colleagues (2010, 2011) have discovered defensin-1 as a key antimicrobial compound of honey. Both peptides contain 51 amino acids and belong to a cysteine-rich peptide family, with 6 cysteine residues forming three disulfide bonds. Mass spectrometry analysis of deefensin-1 showed differences between molecular mass of haemolymph defensin (MW = 5519.03 Da) that contains amidated C-and defensin-1 previously known as royalisin (theoretical MW = 5526.66 Da). The difference in molecular mass is caused by arginine to tyrosine substitution in royalisin at position 50 of C-end (Casteels-Josson *et al*., 1994; Casteels, 1998). The *defensin-1* gene encodes royalisin as a peptide from royal jelly and haemolymph's peptide defensin (P17722, UniProtKB) (Klaudiny *et al*., 2005). The second *defensin-2* gene (Q5MQL3, UniProtKB) was identified by Klaudiny *et al*. (2005) and showed 55.8 % identity with *defensin-1*. *Defensin-2* was highly expressed in the honey bee in response to LPS injections (Richard *et al*., 2008), whereas *defensin-1* was up-regulated upon bacterial infection (Richard *et al*., 2012, Aronstein and Salivar, 2005).

Interestingly, homologues of both *defensin-1* and *defensin-2* were identified in cDNA of Asian bee, *A. cerana* (Xu *et al*., 2009).

Hymenoptaecin

Hymenoptaecin is a linear peptide of 93 amino acids, which belongs to glycin-rich peptide family and inhibits the growth of both G+ and G- bacteria (Casteels *et al*., 1993). It is rapidly up-regulated in response to infection in adult bees and in brood. The basal level of hymenoptaecin in haemolymph of larvae is lower compared to adult workers, drones and queens (Chan *et al*., 2006). Interestingly, exposure to ethanolic extracts of the propolis resin was reported to decrease the expression of hymenoptaecin in 7-days old bees (Simone *et al*., 2009).

Similar to bee hymenoptaecin, a larger hymenoptaecin-like antimicrobial protein has been recently described in parasitic wasp *Nasonia vitripensis* (Tian *et al*., 2010). The analysis of its full-length and truncated forms of the peptide revealed that the N-end assumes the random coil conformation and is critical for selective bacterial targeting, while glycine-rich C-end represents its biologically active unit (Gao and Zhu, 2010). The comparative analysis of *A. cerana vs. A. mellifera* AMPs showed that despite high similarity between the three AMP families (apidaecin, abaecin and defensin), Asian bees maintained much higher divergence in hymenoptaecin peptide. In contrast to only 1 hymenoptaecin peptide found in *A. mellifera*, 13 different hymenoptaecins peptides were detected in *A. cerana*, which is potentially associated with shorter history of domestication compared to Western bee (Xu *et al*., 2009)

Peptides identified in royal jelly and bee venom

Besides royalisin, an isoform of defensin mentioned above (Fujiwara *et al*., 1990), four different types of peptides called jelleins were identified in the royal jelly. Jelleins are constitutively produced by the workers and secreted into royal jelly. Jelleins- 1, 2 and 3 have antimicrobial activity against yeast, G+ and G- bacteria, whereas jellein type 4 showed no antimicrobial activity (Fontana *et al*., 2004). Romanelli *et al*. (2011) confirmed high antimicrobial activity of jelleins-1-4 against yeast, G+ and G- bacteria, whereas jellein-4 was inactive in all assay, likely due to the absence of C-end leucine residue critical for antimicrobial activity of cationic peptides.

Bee venom (apitoxin) is a complex mixture containing several peptides. Among them melittin (26 amino acids peptide) is the main component composing 40 – 60 % of the dry weight of bee venom. Other venom peptides (e.g. apamin, adolapin, MCD peptide) each present at less than one tenth the amount of melittin (de Lima and Brochetto-Braga, 2003). Baracchi and Turillazzi (2010) and Baracchi *et al*. (2011) discovered that melittin and apamin are also present on bee cuticle. Foragers and guards have higher levels of melittin and lower levels of apamin compared to young nurses (Baracchci *et al*., 2010, 2011). Melittin, an amphipathic molecule with lytic activity, showed the antimicrobial activity against G+ and G- bacterial (Fennel *et al*., 1968), whereas apamin has neurotoxic effects both in invertebrates and vertebrates (Baracchi *et al*., 2011). Recently, a group of new antimicrobial peptides, including melectin (cleptoparasitic bee *Melecta albifrons*), lasioglossins (eusocial bee *Lasioglossum laticeps*), halictins (eusocial bee *Halictus sexcinctus*) and macropin (solitary bee *Macropis fulvipes*), displaying activity against G- and G+ bacteria and fungi, has been isolated from the venom of wild eusocial and solitary bees in the Czech Republic (Slaninová *et al.*, 2011). These compounds belong to cationic amphipatic peptides and contain 12-18 amino acids.

Signaling pathways controlling expression of antimicrobial peptides

Recent studies have led to important advances in understanding the underlying molecular mechanisms for the regulation of honey bee AMPs. Although it is generally understood that expression of AMPs in the honey bee is controlled by the three intracellular signaling pathways (Toll, IMD-JNK and JAK/STAT) (Evans *et al*., 2006), there is still a wide gap in understanding the mechanisms of regulation of each of the honey bee AMP.

Modulations of the expression of antimicrobial peptides have been often studied on model organism *Drosophila melanogaster* (Diptera) (reviewed by Engström, 1999), but only a few studies has been published on bees (Hymenoptera). Based on data obtained from sequenced honey bee genome (Honeybee Genome Sequencing Consortium, 2006), Evans *et al.* (2006) compared 17 gene families of *A. gambie*, *D. melanogaster* and *A. mellifera* involved in their immune responses. They showed that honey bee genome contains only a one third of genes involved in immunity in comparison to *Anopheles* or *Drosophila*. Imd and Toll pathways are involved in the regulation of defensin in *Drosophila* (Rutschmann *et al*., 2002). Moreover, some AMPs could be regulated synergistically by both Toll and IMD pathways (Tanji *et al.*, 2007). Correlation was observed between the expression of defensin, hymenoptaecin and transcriptional factor Relish, whereas the expression of abaecin did not show any correlation

with Relish and Dorsal (Erler *et al*, 2011). In bumble bee (*Bombus ignitus*), PBS or LPS injections stimulated increased expression of four antimicrobial peptides: apidaecin, abaecin, defensin and hymenoptaecin (Choi *et al.*, 2008). The increased expression of antimicrobial peptides in response to sterile wounding was confirmed in bumble bees (Erler *et al*, 2011), showing that AMPs could be involved in response to injury. Hymenoptaecin, defensin-1 and abaecin was not detected neither in aseptically wounded bee adults nor in larvae (Randolt *et al.*, 2008). Aseptic inoculation of drone larvae by PBS tends to increase antimicrobial activity of haemolymph, but hymenoptaecin and defensin-1 abundance was not detectable on peptide level (Gätschenberger *et al.*, 2012).

The transduction of pathogen-derived trigger by cell signaling pathways leads to rapid increase in the expression of AMPs. The increased expression of antimicrobial peptides can be detected soon after microbial challenge (Aronstein *et al*., 2010; Erler *et al.*, 2011). The only functional study to directly address this question in the honey bee system was conducted by Schlüns & Crozier (2007). By using the RNAi approach to silence Relish, a nuclear factor-κB, they were able to demonstrate that abaecin and hymenoptaecin are regulated by the IMD pathway. Interestingly, *defensin-1* expression was not effected by Relish silencing, suggesting that expression of defensin-1 is probably regulated by the Toll pathway. All other studies, including the more recent by Erler and colleagues (2011) utilized microbial challenges to monitor the level of gene expression in bumble bee (Choi *et al*., 2008, Riddell *et al*., 2011) or in honey bee (Yang and Cox-Foster, 2005; Aronstein *et al.*, 2010). Using *in silico* analysis of AMPs 5'-UTR sequences, Lourenco *et al.* (2013) infer that abaecin gene is regulated by transcription factor Dorsal (Toll pathway) and Relish (IMD pathway), whereas defensin-1 is regulated by Dorsal and hymenoptaecin by Relish, indicating cross talk between immune signaling pathways. However, none of these studies could provide direct evidence for molecular signaling of a specific receptor, transcription factor or a pathway.

Aronstein and Saldivar (2005) investigated the role of a Toll-like receptor Am18w in bee immunity and found that the expression of antimicrobial peptides (apidaecin, abaecin, defensin1 and hymenoptaecin) is independent of this receptor. Yang and Cox Foster (2005) found a correlation between LPS injections and the expression of abaecin and defensin1, but not hymenoptaecin. Siede and colleagues (2012) tested immune reactions in adult bees using several deactivated pathogens and preparations of bacterial membranes (*P. larvae*, ABPV, LPS). Inoculation with bacterial derived cells triggered an increased expression of AMPs

(apidaecin, abaecin, defensin1 and hymenoptaecin), but Toll and hopscotch (JAK/STAT pathway) showed unexpectedly very small transcriptional changes. Therefore, Siede and colleagues (2012) concluded that the expression profile of Toll is probably less suited as a marker for monitoring bees' immune competence.

Modulation of antimicrobial peptides by pathogens

Stage- and tissue-dependent levels of AMPs transcripts can be detected in healthy bees (Aronstein and Saldivar, 2005), however, production of antimicrobial peptides was not detected in healthy bees of any developmental stages and the antimicrobial activity of haemolymph was not detectable by the zone-inhibition assay (Randolt *et al.,* 2008). Antimicrobial peptides were first identified in bee haemolymph after injection with G- bacteria *E. coli* (Casteels *et al*., 1989, 1990, 1993). It is now well understood that expression of antimicrobial peptides can be modulated by a number of different organisms, including G-, G+ bacteria, fungi and microsporidia (Casteels *et al*., 1989, 1990, 1993; Yang and Cox-Foster, 2005; Antunez *et al*., 2009 Aronstein *et al.,* 2010). In bees, *E.coli* challenge triggers the production of AMPs several hours post infection with the antimicrobial activity continually increasing up to two days post infection. For example, the hymenoptaecin transcript level was increased more than 100-fold six hours after the injection of *E. coli* (Kucharski and Maleszka, 2003).

In addition to live or heat killed bacteria, the antimicrobial activity of honey bee or bumble bee haemolymph is often tested using injections of purified LPS (Choi *et al*., 2008; Richard *et al*., 2008;; Erler *et al*., 2011; Laughton *et al*., 2011). Laughton and colleagues (2011) observed differences in antimicrobial activity of haemolymph between workers and drones of different ages. In response to LPS, the antimicrobial activity of haemolymph of young workers and drones increased in comparison to old workers, as a sign of immune senescence in the capacity to trigger AMP response to immune challenge (Laughton *et al*., 2011).

The expression of abaecin is significantly up-regulated in larvae 24 h post infection with *P. larvae*. Expression is age dependent, as older larvae showed higher expression of abaecin and defensin (Evans, 2004). No differences in the activation of AMPs were found between winter or summer generations of adult bees or larvae (Randolt *et al*., 2008). However, the interracial variations in expression of abaecin, hymenoptaecin and defensin-1 were observed in the immune challenge study using *A. mellifera caucasica*, *A. mellifera mellifera* and their hybrids

infected with *Bacillus thuringiensis* (Saltykova *et al.*, 2005). More rapid increase in the expression of AMPs in purebred bees suggested they had more balanced immune response compared to hybrids. Interestingly, honey bee drones are capable to maintain immune competence during all life stages, including the capacity to strongly up-regulate AMPs upon bacterial challenge (Gätschenberger *et al.*, 2012).

Honey bee responses to infection by the fungus *Ascosphaera apis* were investigated by Aronstein and colleagues (2010). Interestingly, the increased expression of abaecin and defensin-1 correlated with the increased expression of MyD88 protein, a signaling component of Toll pathway which is activated in bee larvae by fungal infection. The effects of *Nosema* spp. on the honey bee immune responses are currently at the center of many investigations (Alaux *et al*. 2010, 2011, Dussaubat *et al*. 2010, 2012, Huang *et al*. 2012). Feeding adult bees with *Nosema apis* spores induced the expression of honey bee AMPs (abaecin, defensin-1 and hymenoptaecin) several days post infection (Antunez *et al*., 2009). *Nosema ceranae* infection suppressed the expression of a large number of genes; however, tissue degeneration and cell renewal impairment induced by *Nosema* infection were the two main factors leading to bee mortality (Dussaubat *et al*., 2012). Chaimanee *et al.* (2012) observed down regulation of AMPs expression (apidaecin, abaecin, defensin and hymenoptaecin) in response to *N. ceranae* 3 and 6 days post inoculation. By the day 12, the difference in transcript levels between control and inoculated bees were not statistically significant. The immunosuppression might occur during the first fourdays of *N. ceranae* infection (Antúnez *et al.*, 2009). In contrast, *N. ceranae* positive bees were reported to have significantly higher level of apidaecin and abaecin transcripts in the midgut tissue (Jefferson *et al.*, 2013). A significant increase of abaecin transcript was demonstrated in nurses and forager bees, whereas a significant increase of apidaecin was detected in foragers. Differences in the virulence between *N. ceranae* and *N. apis* isolates is still under investigation (reviewed by Fries, 2010).

In addition to the well-characterized effects of bacterial and fungal challenges on AMPs' expression and peptide level, latest reports also point out to the AMPs role in the bee responses to viral infections. In recent study any modulation of innate immunity, including the transcription level of AMPs, was detected in bees inoculated with acute bee paralysis virus (Azzami *et al.*, 2012). Jefferson *et al.* (2013) demonstrated a linear correlation between DWV level and apidaecin transcript in the midgut epithelium, while the expression of other

AMPs (abaecin, hymenoptaecin, defensin-1, defensin-2) and peptide apisimin did not correlate with DVW. Interestingly, the transcript levels of AMPs measured in the immune challenged bees showed no significant differences between nurses and forages. However, another recent study showed apidaecin, hymenoptaecin, and abaecin exhibited significant decreased expression in virus-infected and dsRNA-injected bees (Flenniken *et al.*, 2013). In summary, effects of bee viruses on the expression and levels of AMPs are very complex and need to be addressed in future studies. These studies ought to consider the source of experimental material including vitality of a colony, presence of pathogens, age as well as genetic diversity of collected bees. Additionally, bees are usually collected from colonies living outside a laboratory so they are exposed to environmental conditions. All factors common for honey bee colonies could influence the final result of an experiment, because the results might be also modulated by many difficult-specified factors. On the other hand it is very difficult to except all possible influences of immune reaction in this eusocial insect.

Modulation of antimicrobial peptides by *Varroa*

Varroa destructor is a new parasite in western honey bee originally from eastern bee *A. cerana*. It has high impact on world beekeeping, it causes colony colapses and in addition it is a vector of several viruses (reviewed by Rosenkranz *et al.*, 2010). New evidence suggests that differences in hygienic behavior, rather than in the immune system, underlie *Varroa* tolerance in honey bees (Evans *et al*., 2010).

Early studies showed inhibition of honey bee AMPs in *Varroa*-infested bees (Yang and Cox-Foster, 2005). Navajas and colleagues (2008) detected decreased expression of candidate immune gene pUf68 and autophagic-specific gene 18 (*Atg18*) in *Varroa* parasitized bees. Yang and Cox-Foster (2007) demonstrated *Varroa's* significantly negative effect on bee longevity and body weight. The longevity was substantially reduced in bees exposed to *Varroa* and microbial pathogens. However, more recent studies indicate that *Varroa* infestation has no significant effects on suppression of honey bee humoral immunity (Navajas *et al*., 2008; Aronstein *et al*., 2012). In fact, some AMPs were somewhat up-regulated in bee colonies highly infested with *Varroa*. Another recent study supports this new hypothesis that *Varroa* parasitism coupled with viral infection significantly induces the level of abaecin, hymenoptaecin and defensin-1 transcripts (Gregorc *et al.*, 2012).

Methods for the analysis of bee antimicrobial peptides

Most studies focused on changes in the level of AMPs gene expression using quantitative PCR and its modifications (Evans, 2004; Klaudiny *et al.*, 2005, Antunez *et al.*, 2009). While gene expression profile is certainly very important for the understanding of innate immunity, it has been shown that in general the mRNA levels do not correlate well with the concentrations of corresponding peptides. These discrepancies can be explained by issues related to the regulatory mechanism of peptide biosynthesis, including mRNAs coding and processing of the bioactive peptide(s). The analysis of bee cDNA library showed that native apidaecin is produced from a long pre-propeptide composed of several (up to 12) short peptides (Casteels-Josson *et al*., 1993), whereas abaecin precursor mRNA contains only a single coding sequence for native peptide (Yoshiyama and Kimura, 2010).

Therefore, in addition to transcriptional studies, analysis of proteins in bee haemolymph and royal jelly has become an essential tool used in immune related studies. Among these, HPLC, protein sequencing, SDS-PAGE and MALDI-TOF are the most commonly methods for detection and quantification of antimicrobial peptides (Casteels *et al*., 1989, 1990, 1993; Biliková *et al*., 2002; Baracchi and Turillazzi 2010, Baracchi *et al*., 2011).

For peptide structure analysis, the haemolymph was collected, purified under strong acidic condition and analyzed by a reverse-phase HPLC method with UV-VIS detection. Edman degradation was used to determine the primary structure of peptides (Casteels *et al*., 1989, 1990, 1993). Other published methods for peptide analysis include isolation of royalisin from royal jelly using a combined low- and high- pressure liquid chromatography (Fujiwara *et al*., 1990). Royalisin and defensin were also isolated from homogenates of royal jelly, bee thoraxes and heads using polyacrylamide gel electrophoresis followed by the identification of peptides by Edman sequencing (Bachanová *et al*., 2002). Bíliková *et al.* (2002) successfully combined chromatography and electrophoresis to detect apisimin in the royal jelly.

Currently, only a limited number of studies reported the application of highly sensitive mass spectrometry or proteomics methods for the analysis of bee antimicrobial peptides. Fontana *et al.* (2004) used liquid chromatography coupled to mass spectrometric detection (LC/MS) to detect jelleines in royal jelly. Matrix assisted laser desorption ionization – time of flight (MALDI-TOF) was shown as a technique suitable for peptide detection in the bee brain tissue without purification (Boerjan *et al*., 2010). Audsley and Weaver (2006) used MALDI-TOF for the analysis of peptides from *corpora cardiaca* and *corpora allata* with initial HPLC purification of peptides. MALDI-TOF was successfully used to compare changes between two peptides, melittin and apamin, in one sample (Baracchi and Turillazzi 2010, Baracchi *et*

al., 2011). The development of quantitative proteomics and peptidomics has enabled the introduction of promising methods for studying changes in global protein and peptide level modulated by immune pathways (Chan *et al*., 2009).

Future prospects for studies of bee antimicrobial peptides

Despite great efforts to better understand expression and regulation of antimicrobial peptides in honey bees, further studies are needed to acquire more insight into the function of the honey bee antimicrobial peptides and their role in humoral immunity. The use of RNA interference (RNAi) is currently replacing traditional methods in functional genomics.

This new technology will help scientists to gain a better understanding of AMPs function in the nearest future. Major advances are also expected from the utilization of proteomics and peptidomics in studies focusing on tissue-specific modulations of peptides levels. Moreover, the application of these highly sensitive analytical methods (liquid chromatography and mass spectrometry) has already led to the discovery of new immune mechanisms and components, similarly to that described by Albert *et al.* (2011). In addition to the elucidation of AMPs role in bee immunity, future research will aid to a better understanding of molecular mechanisms of their microbial activity in mammalian system and the use of AMPs and their derivatives in clinical applications (Fritsche *et al*., 2012)

Acknowledgements

We thank Joachim R. de Miranda for his valuable comments on the manuscript and Lorenzo Lamattina for his kind revision of summary in Spanish.

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Table 1
Sequences of bee antimicrobial peptides deposited in UniProt database

Peptide name	AA sequence	UniProt Entry	Gene Name	Feature identifier	Ref.
Apidaecin Ia	GNNRPVYIPQPRPPHPRI	Q06601	Apid14	PRO_0000004919	*23, 29*
		P35581	Apid22	PRO_0000004927	*23, 29*
		Q06602	Apid73	PRO_0000004945	*23, 29*
Apidaecin Ib	GNNRPVYIPQPRPPHPRL	Q06601	Apid14	PRO_0000004915	*23, 29*
		Q06601	Apid14	PRO_0000004917	*23, 29*
		P35581	Apid22	PRO_0000004921	*23, 29*
		P35581	Apid22	PRO_0000004923	*23, 29*
		P35581	Apid22	PRO_0000004925	*23, 29*
		Q06602	Apid73	PRO_0000004929	*23, 29*
		Q06602	Apid73	PRO_0000004931	*23, 29*
		Q06602	Apid73	PRO_0000004935	*23, 29*
		Q06602	Apid73	PRO_0000004939	*23, 29*
		Q06602	Apid73	PRO_0000004941	*23, 29*
		Q06602	Apid73	PRO_0000004943	*23, 29*
Apidaecin 2	GNNRPIYIPQPRPPHPRL	Q06601	Apid14	PRO_0000004911	*23, 29*
		Q06601	Apid14	PRO_0000004913	*23, 29*
Apidaecin	GNNRPVYISQPRPPHPRL	Q06602	Apid73	PRO_0000004933	*23, 29*
		Q06602	Apid73	PRO_0000004937	*23, 29*
Abaecin	YVPLPNVPQPGRRPFPTFPGQGPFNPKIKWPQGY	P15450	Abaecin	PRO_0000004946	*24, 30*
Abaecin (natural variant G52S)	YVPLPNVPQPGRRPFPTFPGQGPFNPKIKWPQSY	P15450	Abaecin	N/A	*24, 30*
Defensin-1	VTCDLLSFKGQVNDSACAANCLSLGKAGGHCEKGVCICRKTSFKDLW DKRF	P17722	Defensin-1	PRO_0000006741	*67*
Defensin-2	SVPKVVYDGPIYELRQIEEENIEPDTELMDSNEPLLPLRHRRVTCDVLS WQSKWLSINHSACAIRCLAQRRKGGSCRNGVCICRK	Q5MQL3	Defensin-2	PRO_0000394508	*67*
Hymenoptaecin	QERGSIVIQGTKEGKSRPSLDIDYKQRVYDKNGMTGDAYGGLNIRPGQ PSRQHAGFEFGKEYKNGFIKGQSEVQRGPGGRLSPYFGINGGFRF	Q10416	Hymenoptaecin	PRO_0000021475	*30*
Hymenoptaecin (natural variant P49S)	QERGSIVIQGTKEGKSRPSLDIDYKQRVYDKNGMTGDAYGGLNIRPGQ **S**SRQHAGFEFGKEYKNGFIKGQSEVQRGPGGRLSPYFGINGGFRF	Q10416	Hymenoptaecin	N/A	*30*
Jellein 1	PFKISIHL	O18330	Major royal jelly protein-1	PRO_0000224648	*51*
Jellein 2	TPFKISIHL	O18330	Major royal jelly protein-1	PRO_0000224649	*51*
Jellein 4	TPFKISIH	O18330	Major royal jelly protein-1	PRO_0000224650	*51*

1

2

Table 2
Arrangement of signal and coding sequences in apidaecin precursor (type 14)

	Positions	Amino Acids	UniProt Feature identifier
Signal peptide	1-19	19	
Propeptide	20-42	23	PRO_0000004910
Apidaecin 2	43-60	18	PRO_0000004911
Propeptide	63-70	8	PRO_0000004912
Apidaecin 2	71-88	18	PRO_0000004913
Propeptide	91-98	8	PRO_0000004914
Apideacin 1B	99-116	18	PRO_0000004915
Propeptide	119-124	6	PRO_0000004916
Apideacin 1B	125-142	18	PRO_0000004917
Propeptide	145-150	6	PRO_0000004918
Apideacin 1A	151-168	18	PRO_0000004919

1