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REVIEW

Immunity of the greater wax moth *Galleria mellonella*

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Abstract Investigation of insect immune mechanisms provides important information concerning innate immunity, which in many aspects is conserved in animals. This is one of the reasons why insects serve as model organisms to study virulence mechanisms of human pathogens. From the evolutionary point of view, we also learn a lot about host-pathogen interaction and adaptation of organisms to conditions of life. Additionally, insect-derived antibacterial and antifungal peptides and proteins are considered for their potential to be applied as alternatives to antibiotics. While *Drosophila melanogaster* is used to study the genetic aspect of insect immunity, *Galleria mellonella* serves as a good model for biochemical research. Given the size of

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the insect, it is possible to obtain easily hemolymph and other tissues as a source of many immune-relevant polypeptides. The presented review article summarises our knowledge concerning *Galleria mellonella* immunity. The best-characterized immune-related proteins and peptides are recalled and their short characteristic is given. Some other proteins identified at the mRNA level are also mentioned. The infectious routes used by *Galleria* natural pathogens such *as Bacillus thuringiensis* and *Beauveria bassiana* are also described in the context of host-pathogen interaction. Finally, the plasticity of *G. mellonella* immune response influenced by abiotic and biotic factors is described.

Key words *Beauveria bassiana*; *Bacillus thuringiensis*; defence proteins and peptides; *Galleria mellonella*; insect immunity

Introduction

According to the Red Queen theory formulated by evolutionary biologist Leigh Van Valen (Van Valen, 1973), continuous change of a given organism is required for its sustenance in the changing ecosphere. In the context of host-parasite interaction, this means that both insects and their pathogens must constantly improve their defence and virulence mechanisms, respectively, in order to survive. This antagonist co-evolution also known as "arms race" has led to the emergence of wealth interaction strategies between the infected host and pathogen (Dawkins *et al.*, 1979). Insects possess anatomical and physiological barriers that protect them against invasion of microorganisms. The insect body cover is composed of a single layer of epithelium (epidermis), which rests on the basal membrane. The epithelium is involved in the structure of the cuticle, which is impregnated with chitin. This hardened insect body cover protects against mechanical injury and infection (Moussian, 2010). Similarly, the insect trachea possesses chitin padding which hardens with age. Additionally, the low humidity and lack of nutrients inside the trachea create the unfavourable conditions for colonisation by microorganisms. Insects are prevented against infection *via* the oral route by the structure of the gut. The foregut and hindgut have a lining of chitin. Additionally, the biochemical conditions in the gut, such as pH and digestive enzymes, are not friendly for development of intruders. Due to the phenomenon of antibiosis and competition, the intestinal microflora contributes in a significant way to reduction of the population of microorganisms that enter the gastrointestinal tract with food (Gliński & Kostro, 2004). When the physiological barriers are broken, insects switch on the immune response. In contrast to mammals, insects possess only innate defence mechanisms, relying only on germline-encoded factors in the recognition and infection

clearance processes. Acquired immune response, which uses somatic gene rearrangement to develop immunological T- and B-cell and antibody-based immune memory, is absent in insects (Fearon *et al.*, 1997). The innate immune response comprises cellular and humoral branches. The former is based on insect blood cells - hemocytes, which can engulf intruders in the phagocytosis process or capture them in multicellular structures called nodules or capsules (Lavine & Strand, 2002). The humoral branch involves the synthesis of defence molecules. Among them, there are reactive intermediates of oxygen and nitrogen and antimicrobial peptides (AMPs) with molecular weight between 10 kDa and 4 kDa possessing antibacterial and/or antifungal properties (Bogdan *et al.*, 2000; Vass & Nappi, 2001; Casanova-Torres *et al.*, 2013). Some of them can be synthesised by different tissues constitutively, while others may appear in the hemolymph in response to infection. Most of the defence peptides present in the insect hemolymph are produced in the fat body. The mode of action of AMPs involves, in most cases, destabilisation of cellular membranes by creating peptide- or peptide/ lipid-lined pores in barrel-stave and torroidal models, respectively. They can also solubilise membranes to form micelles in a carpet-like model. Additionally, antimicrobial peptides may differently interfere with the potential of cellular membranes. It is worth pointing out that some defence molecules can get inside the cell and interfere with physiological processes such as replication, transcription, and translation. Modes of defence peptide action can be found in the latest review articles (Nguyen *et al.*, 2011; Scocci *et al.*, 2011; van der Weerden *et al.*, 2013). The main features of antimicrobial peptides are: (i) selective toxicity, which means that they act against infecting microorganisms without disturbing the body of the host, (ii) the time of their action is shorter than the doubling time of infecting microorganism, (iii) the broad spectrum of activity allows them to act against a group of microorganisms, (iv)

they do not develop bacterial resistance (after Matsuzaki, 2001). In the best-known insect model- *Drosophila melanogaster*, it has been shown that two main pathways, Toll and Imd, regulate the expression of antimicrobial peptides in response to Gram-positive bacterial/fungal and Gram-negative bacterial infection, respectively. Both of them lead to activation of a homologue of the NF-κB transcription factor, Dif or Relish (Aggarwal & Silverman, 2008; Hetru & Hoffmann, 2009; Silverman *et al.*, 2009). Insect humoral response also includes complex enzymatic cascades that regulate melanisation of hemolymph. Melanin is synthesised during the hemolymph coagulation process at the injury site and, in some insects, in a process of nodule and capsule formation.

The defence mechanisms used by insects are summarised in Figure 1. Cellular and humoral branches of an immune response interact with each other to ensure best protection to insects. Many humoral factors regulate the hemocyte function and *vice versa*: hemocytes synthesise and secrete many humoral molecules to the hemolymph, such as defence peptides and stress proteins (Grizanova *et al.*, 2014).

Pathogens develop mechanisms that allow them to pass or break insect defence mechanisms. Among them, there are strategies to force anatomical and physiological barriers. They secrete various compounds, for example, enzymes digesting host tissues. Moreover, injured cuticle can constitute a gate of infection for a broader spectrum of microbes. Intruders try to avoid recognition by insect immune mechanisms. They hide the immune elicitors (PAMPs- pathogen associated molecular patterns), change the composition of the cell wall to be more resistant to insect defence molecules, and colonise places with limited access for hemocytes (Vallet-Gelly *et al.*, 2008). Finally, they produce and secrete many virulence factors, which can inhibit the expression or activity of insect defence molecules. Among them, there are proteases, which after

secretion to insect hemolymph, digest insect hemolymph proteins including antimicrobial polypeptides. On the other hand, virulence factors secreted by pathogens can stimulate insect immune response (Altincicek *et al.*, 2007; Griesch *et al.*, 2000).

Galleria mellonella

G. mellonella (Lepidoptera, Pyrilidae) lives in most cases in beehives, inside bee nests, and feeds with wax and pollen (Fig. 2A). It can be considered a pest causing bee- or, more seldom, bumblebee- or wasp-galleriosis. Their life cycle is approximately 7–8 weeks: after emergence from the egg, larvae undergo 6 larval stages before reaching the last instar. This takes ca. 5–6 weeks at 25–28°C. Then prepupae and pupae are formed and, after additional two weeks, adult moths appear. This bee moth has been a good model to study insect immune response and virulence factors of many pathogens, including human pathogens such as *Pseudomonas aeruginosa*, *Enterococcus faecalis*, *Staphylococcus aureus*, *Candida albicans*, *Fusarium oxysporum*, and *Aspergillus fumigatus* (Gibreel & Upton, 2013; Gomez-Lopez *et al.*, 2014; Koch *et al.*, 2014; Munoz-Gomez *et al.*, 2014; Vaz *et al.*, 2015; Maekawa *et al.*, 2015). Their virulence factors can be studied first on the insect model, which is easier, cheaper, and more ethically acceptable, before testing on mammalian organisms (Arvanitis *et al.*, 2013; Cook & McArthur, 2013; Junquirella, 2012). On the other hand, *G. mellonella* is a good model to study its interaction with natural insect pathogens like *Bacillus thuringiensis*, *Beauveria bassiana*, which are not pathogenic for healthy humans, and for this reason, can be used in agriculture for production of bioinsecticides (Ortiz-Urquiza *et al.*, 2015; Ruiu, 2015). *G. mellonella* is a cheap and relatively easily culturable model organism. The larvae are large enough (2 cm long, 250 mg weight before pupation) to be easily

injected, to obtain hemolymph and hemocytes, and to isolate other organs for further analysis (Ramarao *et al.*, 2012). Its disadvantage as a model organism is that its genome is not fully sequenced and there are no methods of creating mutant strains.

G. mellonella hemocytes can differentiate into four types of hemolymph cells, which are presented in Figure 2B. The most abundant granulocytes and plasmatocytes with adherent properties take place in phagocytosis, encapsulation, and nodulation processes; non-adhesive spherule cells transport cuticle components, and oenocytoids carry phenoloxidase precursors (Lavine & Strand, 2002; Sass *et al.*, 1994). The hemolymph of *G. mellonella* is rich in many proteins synthesised by different tissues: mainly the fat body and hemocytes. Among them, there are proteins with immune function. Some of them are constitutively present in the hemolymph, although their amount may change after immune challenge and some of them may appear there in response to infection. Although the entire genome of *Galleria* is unravelled, some broad range studies concerning transcripts that are regulated by infection have been performed (Seitz *et al.*, 2003; Vogel *et al.*, 2011). Many homologues of *D. melanogaster* immune-related peptides and proteins have been identified in *G. mellonella*. For example, sequences putatively encoding Peptydoglycan Recognising Proteins (PGRPs), Gram-negative binding proteins (GNBPs), and *β*-1,3-glucan recognition proteins (*β*-GRPs) functioning as PRR (pattern recognition receptors) have been also found in *G. mellonella*. Transcripts for the members of the Toll and IMD pathway have also been found as well as other proteins and peptides with defence function (Vogel *et al.*, 2011). Li *et al.* (2002) identified components of *G. mellonella* hemolymph clotting.

Galleria mellonella immune-relevant proteins and peptides

There is an increasing number of reports concerning identification of *G. mellonella* immune-relevant peptides and proteins. Below I will shortly describe some of them, whose role in the immune response of the greater wax moth is best known.

Apolipophorin III (apoLp-III)

Apolipophorin III has been indentified in *G. mellonella* both at the gene and protein level (Niere *et al.*, 1999, 2001). This 18-kDa protein is an exchangeable component of the lipophorin complex involving also apolipophorin I and II. The complex is responsible for lipid transport to flight muscles supplying them with an energy source. It seems that apoLp-III belongs to the group of so-called moonlighting proteins (multifunctional proteins, Jeffery, 1999). Apart from transporting lipids to flight muscles, it is also engaged in many aspects of *G. mellonella* immunity. It acts in synergy with other immune-related proteins. Among them, there are proteins permanently present in the hemolymph and these secreted in response to infection. Among them, there is the lysozyme mentioned below (Halwani & Dunphy, 1999). Its muramidase activity has been shown to increase in the presence of apoLp-III (Zdybicka-Barabas *et al.*, 2013). Furthermore, it stimulates the activity of antimicrobial peptides (Park *et al.*, 2005a). There are also reports that apoLp-III itself possesses defence activity and regulates prophenolooxidase activity (Zdybicka-Barabas & Cytryńska, 2011; Zdybicka-Barabas *et al.*, 2014). Moreover, apoLp-III is also considered as a PRR (pattern recognition receptor). It binds to the bacterial cell wall components, such as lipopolysaccharide (LPS) of Gram-negative bacteria, lipoteichoic acids of Gram-positive bacteria, and fungal β-1,3-glucan. This feature implies its

participation in opsonisation and detoxification of non-self-components (Halwani *et al.*, 2000; Whitten *et al.*, 2004; Leon *et al.*, 2006; Ma *et al.*, 2006). The former function allows adherent hemocytes to efficiently recognise and engulf intruding bacteria or fungi. Additionally, apoLp-III from *G. mellonella* binds to nucleic acids released from damaged cells and tissues. These apoLp-III - nucleic acid aggregates stimulate insect defence response acting as a danger signal, or a damage-associated molecular pattern - DAMP (Altincicek *et al.*, 2008). The amount of apoLp- III in the hemolymph is a result of a compromise between the demand for storage proteins and immunological needs (Adamo *et al.*, 2007).

Insect metalloproteinase inhibitor (IMPI)

The insect metalloproteinase inhibitor – IMPI has been found in *G. mellonella*, as the first, and so far, the only animal specific inhibitor of microbial metalloproteinases. Its gene encodes two protein products, one – inhibiting microbial metalloproteinases and the other one – putatively inhibiting matrix metalloproteinases (MMPs) during metamorphosis (IMPI-1 and IMPI-2, respectively). The IMPI-1 isolated from *G. mellonella* hemolymph is a glycosylated, heat-stable peptide with molecular weight 8.6 kDa, containing five intermolecular disulphide bonds. It appears in the hemolymph in response to injected bacterial or fungal elicitors and inhibits zinc-containing metalloproteinases (Clermont *et al.*, 2004; Wedde *et al.*, 1998; 2007). Thermolysin-like zinc metalloproteinases are produced by all groups of known insect pathogens as virulence factors. Their strong level of virulence could be reflected by the fact that injection of thermolysin in the amount of 1 μg per larvae is lethal for *G. mellonella* larvae (Vilcinskas & Wedde, 2002). This inhibitor is synthesised, together with

antimicrobial peptides (AMPs), to protect them against digestion by metalloproteinases secreted by the invading intruder. Interestingly, injection of thermolysin into *G. mellonella* hemocel positively regulates the expression of the IMPI gene. The regulation of IMPI expression is a good example of a mutual interaction between the host and the invading pathogen. Thermolysin-like proteases secreted by the intruder in the body of the infected insect degrade a large number of hemolymph proteins of the host, creating peptidic fragments, so-called protfrags, which stimulate the insect immune response (Altincicek *et al.*, 2007). This mechanism could be considered as a "danger signal", which besides the self/non-self-model recognises the infection (Griesh *et al.*, 2000; Vilcinskas & Wedde, 2002).

Other protease inhibitors

The *G. mellonella* transcriptom comprises inhibitors of serine proteases, such as ISPI-1, 2 and 3 (Vogel *et al.*, 2011). They were purified from *G. mellonella* hemolymph and their molecular mass was between 6.3 to 9.2 kDa. They inhibit proteases Pr1 and Pr2 of the entomopathogenic fungus *Metarhisium anisopliae*. ISPI-2 represents a Kunitz-type inhibitor. More information concerning insect protease inhibitors can be found in an excellent review by Vilcinskas and Wedde (2002).

Lysozyme

Unlike in the case of *D. melanogaster*, *G. mellonella* lysozyme, besides its digestive function in the gut, possesses defence properties. Lysozyme purified from *G. mellonella* hemolymph was shown to be a ca. 14-kDa protein, containing 121 amino acids (directly submitted by Weise, UniProtKB/Swiss-Prot: P82174.2). It is related to

type c (c-chicken) lysozymes (Hultmark, 1996). It is a muramidase that cleaves the *β*-1.4-glycosidic linkage between C1 of N-acetylomuramid acid and C4 of N-acetylglucosamine in bacterial peptidoglycan. Moreover, it may act in a non-enzymatic way as a cationic defence peptide. Additionally, its antifungal properties have also been reported (Sowa-Jasiłek *et al.*, 2014), although its mode of action in this case is unravelled. Lysozyme, which is present in non-stimulated larvae, constitutes a first line of humoral defence, creating a hostile environment for intruding microorganisms. It has been shown that its amount increases after immune challenge. Additionally, it co-operates with other proteins that are present permanently in the hemolymph e.g. anionic peptide-2 and apolipophorin III (Zdybicka-Barabas *et al.*, 2012, 2013). Analysis of the *G. mellonella* transcriptome revealed four c-type lysozyme homologues and one i-type (invertebrate) lysozyme with unknown function (Vogel *et al.*, 2011).

Prophenoloxidase

Prophenoloxidase was one of the first immune-related molecules found in *G. mellonella*. It was purified from this insect in 1995 (Kopacek *et al.*, 1995). Recently the enzyme was characterized by Demir *et al.* (2012). This is a proenzyme present in unchallenged *G. mellonella* hemocytes mainly in oenocytoides (Schmit *et al.*, 1977). After infection recognition the enzyme is released from oenocytoides and activated by limited proteolysis by cascades of serine proteases. The prophenoloxidase complex includes also inhibitors of serine proteases - serpins which prevent from hyperactivation of the enzyme. This tight control of phenoloxidase activity is very important due to high cytotoxicity of intermediate products: dihydroxyphenylalanine (DOPA), quinons and free radicals which could damage host cells. The active phenoloxidase is copper-

containing enzyme converting tyrosine to already mentioned dihydroxyphenylalanine (DOPA) and oxidation of phenolic substances to quinones and further to melanin (Aschida, 1990; Cerenius *et al.*, 2008; Kanost & Gorman, 2008). This reaction leads to protein cross-linking and generates products which are themself toxic for invading microorganisms but also stimulate defence activity of other antimicrobial molecules (Bidla *et al.*, 2009; Dubovskiy *et al.*, 2013a). On the other hand it was shown that defence molecules in *G. mellonella* can regulate the activity of phenol oxidase. For example it was shown that apolipophorin III and Gm protein-24 stimulate activation of prophenoloxidase cascade (Park *et al.*, 2005a), while lysozyme, anionic peptide-2, Gm defensis and proline-rich peptide 1 decreased phenol oxidase activity (Zdybicka-Barabas *et al.*, 2014). Melanisation takes part during wound healing, sclerolisation and hardening of cuticle. It was shown that prophenol-activating cascade and coagulation system cooperate during the formation of hemolymph clot (Li *et al.*, 2002). Melanisation often accompanies entrapping of parasites and microbes in capsules or nodules as a part of cellular immune response (Hoffmann *et al.*, 1996). The prophenoloxidase system and melanisation reaction is a good example of cooperation between humoral and cellular branches of *G. mellonella* immunity. Humoral factors such as enzymes controlling the activity of phenol oxidase influence melanin synthesis which is involved in separation of foreign bodies in structures assembled by hemolymph cells. Activation of phenol oxidase occurs not only after recognition of non-self components. As already mentioned virulence factors secreted by pathogenic organisms such as thermolysin-like metalloproteinases digest insect hemolymph proteins resulting in the formation of protein fragments called protfrags. These peptides may stimulate expression of mentioned IMPI and defence peptides but also they cause activation of prophenoloxidase system (Altincicek *et al.*, 2007).

Antimicrobial peptides (AMPs)

Cecropins Cecropins are linear, amphipathic peptides with α-helical structure. A cecropin-like peptide has been purified from the hemolymph of immune-stimulated *G. mellonella* larvae (Kim *et al.*, 2004). The analysis of its cDNA and protein has revealed that it is synthesised as a prepropeptide with a putative 22-aa signal peptide and an additional 4-residue propeptide. The 37-residue peptide has molecular weight of 4.16 kDa. It shares similarity to cecropins A and B from *Hialophora cecropia* (Boman *et al.*, 1989), *Hyphantria cunea* (Park *et al.*, 1997), and *Bombyx morii* (Yamano, 1994). This cationic peptide is active against Gram-positive and Gram-negative bacteria. Four different cecropins have been identified in the *Galleria* trascriptome dataset. These also included D-type cecropin, which was further purified from *G. mellonella* hemolymph. This 4.2-kDa peptide was active against Gram-positive and Gram-negative bacteria and against filamentous fungi *Aspergillus niger* (Cytryńska *et al.*, 2007)

Gallerimycin The peptide encoding a defensin-like, cystein-rich peptide named by authors gallerimycin was identified in 2003 (Schumann *et al.*, 2003). Its deduced amino acid sequence exhibits similarities with the antifungal peptide drosomycin from *D. melanogaster* and heliomycin from *Heliothis virescens*. The recombinant preprotein possesses 76 amino acids and, when tagged with a V-5 epitope and His to allow purification, has molecular weight of 11.6 kDa. A recombinant protein exhibits activity against entomopathogenic fungus *Metarhizium anisopliae*. On the other hand, it was not active against yeast *Saccharomyces cerevisiae* or bacteria tested (*Micrococcus luteus, Bacillus subtilis*) (Schumann *et al.*, 2003).

Galiomicin This peptide was identified as *G. mellonella* defensin by Lee *et al.* (2004). Its cDNA consists of 622 nucleotides and contains an open reading frame of 216 nucleotides, corresponding to a preprotein with 72 residues. A mature protein contains 43 residues and has a molecular weight 4.7 kDa. Typically of insect defensins it contains six cysteine residues, which form three intramolecular disulphide linkages. It shows 90.7% identity to heliomycin. This defence peptide shows activity against two filamentous fungi and yeast but exhibits no antibacterial activity.

Moricins and gloverins Moricin-like peptides and gloverins are intriguingly restricted to Lepidoptera. The former were firstly found in *Bombyx morii* (Yi *et al.*, 2013). In *G. mellonella*, there are eight genes encoding seven different moricin-like peptides (two mature transcripts are identical). They are particularly active against filamentous fungi but also, to a certain extent, against yeast, Gram-positive and Gram-negative bacteria (Brown *et al.*, 2008). Moricins belong to amphipathic α-helical antimicrobial peptides. Gloverins are basic, heat-stable proteins enriched with glycine residues but lacking cysteines. They interact with LPS and inhibit the formation of bacterial outer membrane. Gloverins have first been found in the silk moth *Hyalophora gloveri* (Axen *et al.*, 1997). Among immune-induced *G. mellonella* transcripts, five members of the gloverin family have been identified (Vogel *et al.*, 2011).

Other peptides and proteins Many antimicrobial peptides have been purified from immune-stimulated *G. mellonella* hemolymph. Their *in vitro* activity has been tested against Gram-positive and Gram-negative bacteria, as well as against yeast and filamentous fungi. The repertoire of defence molecules depends on the type of the immune elicitor, showing specificity of antimicrobial peptides synthesis against different kinds of microorganisms. Many of these peptides are known only at the peptide level and their transcripts need to be identified. They include apolipophoricin,

anionic peptide-1, cecropin D-like peptide, heliocin-like peptide (Cytryńska *et al.*, 2007; Brown *et al.*, 2009; Mak *et al.*, 2010).

Table 1 shows a short summary of the above-mentioned *G. mellonella* defence peptides and proteins. Additionally, it contains some information about other selected immune-related peptide and proteins, whose mRNA sequences are deposited in the GenBank.

Natural pathogens *of Galleria mellonella*

G. mellonella can be infected by different kinds of bacteria, fungi, and viruses. All pathogens can be divided into generalists, specialists, and opportunists. The first ones naturally infect diverse hosts, while specialists infect only a small subset of insects. In turn, opportunistic parasites can occasionally gain access to an injured or weakened insect body, where they are repelled by basic, unspecialised immune mechanisms. On the contrary, specialised parasites will select for more specialised host defence adaptation than generalised parasites (Keebaugh & Schlenke, 2014). During growth, they produce virulence factors, which inhibit or destroy immune relevant peptides and proteins. If the infection is strong, insects die as a common result of toxins secreted by the pathogen and the mechanical injury of the insect body. Here, I will shortly mention two of them, Gram-positive bacteria *Bacillus thuringiensis* and the filamentous fungus *Beauveria bassiana,* as an example of their interaction with *G. mellonella* larvae.

The Gram-positive bacterium *B. thuringiensis*, motile by means of peritrichous flagella, is widely distributed in the world and can be found in soil. It belongs to the *Bacillus cereus* group. There are two ways, in which this bacterium may infect *G. mellonella*. Spores or, vegetative cells, which may be taken up by the larvae by

ingestion, cause gut perforation and then reach the hemocel causing septicaemia (Schunemann *et al.*, 2014; Vachon *et al.*, 2012). In addition, the microorganism can get directly into the body cavity *via* injured cuticle. In the first case, injected bacteria have to force the insect gut to gain access to the hemolymph. At the stationary growth phase, *B. thuringiensis* produces spores, which are accompanied by parasporal crystals containing plasmid-encoded insecticidal toxins: Cry- (crystal, also called delta endotoxins) and Cyt- (cytolytic). The diversity of the Cry and Cyt toxins and their mode of action have been a subject of many articles (Bravo *et al.*, 2007; 2011; Bulushova *et al.*, 2011; Chengchen *et al.*, 2014; Palma *et al.*, 2014) and will not be described here in details. Briefly, crystals are dissolved in the gut and Cry toxins are activated by limited proteolysis. Afterwards, they bind to receptor proteins in the midgut membrane. Among proteins that are capable of binding *Bacillus* toxins, there are amidopeptidase N and cadherin. Attached toxins form pores in the epithelial cells of the midgut. It is worth mentioning that different *B. thuringiensis* strains produce different toxins, which act specifically against particular insect species. This specificity concerns mostly Cry toxins, while Cyt toxins are less specific and less toxic. They contain domains that are rich in hydrophobic amino acids to incorporate into the lipid layer of the gut epithelium. This non-specific binding to lipids causes disorders of membrane permeability leading to cell lysis. This effect of bacterial toxins - toxaemia can be lethal for many insect species. For other insects' lethality, including *G. mellonella*, the presence of bacterial cells accompanying crystals is necessary (Heimpel & Anguis, 1959). *Via* a perforated gut, bacterial cells enter the larval hemocel. As mentioned, *B. thuringiensis* cells may directly get into the body cavity *via* injured cuticle. An injury may occur in the case of a high insect density, which often causes "chewing up" of the insect larvae. Both routes lead to penetration of bacterial cells into

the hemolymph, where the bacteria multiply intensively and contribute to the development of the so-called septicaemia. While they are in the hemolymph, they secrete many virulence factors, which allow them to proliferate despite the fact that the insect activates defence mechanisms. During the infection process, *B. thuringiensis* produces phospholipases, proteases, cytotoxins, and other components, which break the host defence barriers. For example, mostly at the stationary growth phase, it secretes zinc-metalloproteinases. These enzymes digest antimicrobial peptides of the infected host (Dalhammar & Steiner, 1984). Indeed, *G. mellonella* antibacterial activity was abolished after thermolysin treatment both *in vivo* and *in vitro*. Similarly, apolipophorin III appeared to be susceptible to such degradation (Wojda & Taszłow, 2013; Taszłow & Wojda, 2015). On the other hand, thermolysin did not decrease lysozyme activity in the hemolymph of infected *G. mellonella* larvae. Inside the hemocel, *Bacillus* is able to change the properties of its cell wall to become more resistant to insect defence peptides. One of the mechanisms to do so is alanylation of teichoic acids. This modification neutralises the negative charge of acids and reduces binding of lysozyme. The proteins engaged in alanylation are encoded by the *dlt* operon. It has been shown that a *dlt* null mutant of *Bacillus cereus* was less virulent than the wild type when injected into the hemocel of *G. mellonella* and *Spodoptera litoralis* (Abi Khattar, 2009). Bacteria belonging to the *Bacillus cereus* group secrete factors involved in iron acquisition, such as IlsA. Its gene is expressed in insect hemocel. It is involved in iron uptake from ferritin. Mutant bacterial strains devoid of this virulence factor exhibit decreased virulence toward *G. mellonella* (Daou *et al.*, 2009; Segond *et al.*, 2014). Many virulence factors of *B. thuringiensis* are controlled by a pleiotropic regulator PlcR. This regulon is activated at the onset of the stationary phase (Salamitou *et al.*, 2000).

The entomopathogenic fungus *Beauveria bassiana* is a worldwide, facultative saprophyte that grows in the soil. It can form a mycelium but also different types of single cells. Among them, there are aerial conidia, blastospores, and submerged conidia, which are produced on solid media, nutrient-rich, and nutrient-limited liquid media, respectively (Holder & Keyhani, 2005). They differ in the morphology and biochemical properties but all are able to attach to insect cuticle and begin the infectious process. First, there is adsorption followed by adhesion of a fungal propagule to insect cuticle. Then, the propagule germinates and forms an appressorium – a flattened cell, from which a minute infection peg grows. Then, the fungus grows across the insect. During these steps, the invading fungus efficiently evades host defences and secretes enzymes degrading the insect cuticle. Among them, there are proteases, esterases, lipases, and chitinases. Degradation of the insect tissues together with physical pressure of the penetration peg on the host body cover allows the invading fungus to enter the hemocel. The fungus activates necessary signalling pathways to sense the host environment (Chen *et al.*, 2014). *Beauveria* catabolises and uses nutrients taken up from damaged host tissues. Finally, elongated hyphae reach the hemolymph. Inside the hemocel, *B. bassiana* grows in the form of yeast-like cells or blastospores. These cells have a much thinner cell wall inside the insect host than cells growing *in vitro* because of down-regulation of chitin and glucan synthases (Tartar *et al.*, 2005). They also lack galactose residues. These changes are meant to reduce the number of pathogen-associated molecular patterns (PAMPs). Additionally, *B. bassiana* secretes bioactive secondary metabolites such as beauvericin, bassianolide, and oosporein. They have insecticidal properties but also inhibit the growth of other organisms. Damaged insect tissues such as the fat body are not able to perform their

defence function, e.g. production and secretion insect defence peptides and proteins to the hemolymph.

Modulation of host-pathogen interaction in *Galleria mellonella*

G. mellonella resistance changes with larval age and depends on many factors such as external temperature, hormones, and diet (Wojda *et al.*, 2004; Wojda & Jakubowicz, 2007; Kangassalo *et al.*, 2015; Krams *et al.*, 2015; Wu *et al.*, 2015a). There is also an increasing number of reports concerning the role of lipid mediators - eicosanoids in the modulation of *G. mellonella* immunity (Buyukguzel *et al.*, 2007, 2011; Stanley *et al.*, 2009). Interestingly, *G. mellonella* resistance depends on the previous experience of individuals. This may concern the previous experience of mechanical or heat stress. These factors applied before infection made *G. mellonella* more resistant to further infection (Mowlds *et al.*, 2008; Wojda *et al.*, 2009; Taszłow & Wojda, 2015; Wojda & Taszłow, 2013). Interestingly, heat shock applied before infection differently modulates particular components of *G. mellonella* immune response after infection with *B. thuringiensis*. Expression of genes encoding antimicrobial peptides was enhanced in pre-shocked animals in comparison to larvae permanently kept at optimal growth temperature, while expression of IMPI and apolipophorin III in the fat body was not affected. However, the amount of apolipophorin III was shown to be slightly higher in pre-shocked animals. Antimicrobial peptides and apolipophorin III seem to be very sensitive to digestion by bacterial proteases and this sensitivity was reduced by heat shock. On the other hand, thermolysin treatment does not inhibit lysozyme type activity (Wojda & Taszłow, 2013; Taszłow & Wojda, 2015). Stronger heat shock, when applied on already infected insects, inhibits expression of immune-induced genes, but in larvae

recovered from heat shock, the expression of apolipophorin III is higher than in infected animals not exposed to elevated temperature (Vertyporokh *et al.*, 2015).

Another exciting finding concerning the plasticity of the *G. mellonella* immune system concerns previous immune experience. This is especially important in the light of the fact that insects possess only an innate immune system which is deprived of T- and B- cells and antibodies. There are reports presenting that insects exposed to infection with low doses of microorganisms became more resistant to a next infection. In agreement with this observation is the finding of *Dscam* receptors in *D. melanogaster*, which are re-arranged after immune challenge, allowing the fly to respond more efficiently to re-infection (Cherry & Silverman, 2006; Watson *et al.*, 2005). This phenomenon is named *immune priming* or *trained immunity* (Chambers & Schneider, 2012). It has been reported that pre-exposure of *G. mellonella* to *Candida albicans* or *Saccharomyces cerevisiae* results in increased resistance of the insect to further injection with *Candida albicans*. In addition, *G. mellonella* priming was achieved after injection of immune elicitors: glucan, laminarin, LPS, or heat-killed bacteria (Bergin *et al.*, 2006; Mowlds *et al.*, 2010; Wu *et al.*, 2014, 2015b). Interestingly, immune priming can also be transgenerational. In the studies performed by Dubovskiy *et al.*, 2013b, the 25th generation of *G. mellonella* larvae under selective pressure from *B. bassiana* exhibited resistance to this pathogen. This resistance involved front-line defences – the integument, so the larvae were better protected against invasion of *Beauveria*. Similarly, the report by Freitak *et al.* (2014) shows that trans-generational immune priming of *G. mellonella* can be mediated by maternal transfer of bacteria to developing eggs.

Summary

There are many advantages of using *G. mellonella* as an insect model. The investigations are performed on different fields. One is to understand the mechanisms of the insect immune system, which has similar features to those of the innate immune system of mammals, and is not interfered by acquired immunity. Therefore, it serves as a model to study the virulence mechanisms of human pathogens. Another reason is that its hemolymph is a rich source of defence molecules that can be purified and sequenced. They are considered an alternative to antibiotics due to the fact that they do not induce resistance (Ezzati-Tabrizi *et al.*, 2013; Li *et al.*, 2014; Vilcinskas, 2011; Yeung, 2011; Yi *et al.*, 2014). Finally, we can follow the interaction of *G. mellonella* with natural insect pathogens and consider this in the light of a host-pathogen evolutionary arms race. This concerns interactions of this insect with both generalised and specialised pathogens since they induce also generalised and specialised defence mechanisms, respectively (Keebaugh & Schlenke, 2014).

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885 **Legends to Figures**

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887 **Table 1** Chosen *Galleria mellonella* immune-relevant proteins of identified mRNA.

Protein/ Accession number	Description	References
Apolipoprotein III/ AJ006975.1	Protein with multiple functions. Involved in lipid transport to flight muscles, meeting the high metabolic energy demands during flight. This protein of ca. 18 kDa is also reported to be involved in many aspects of immunity such as: acting as a Pathogen Recognition Receptor (PRR), stimulating the activity of defence peptides, and possessing antimicrobial activity itself .	Niere *et al.*, 1999, 2001; Ryan & van der Horst, 2000; Zdybicka-Barabas & Cytryńska, 2011
Cecropin/ Sequence not deposited in the GenBank but published (see the third column)	α-helical cationic antimicrobial peptide with molecular weight ca.4 kDa.	Kim *et al.*, 2004
Gallerimycin/ AF453824	Antifungal peptide. Not active against yeast and bacteria.	Schuhmann *et al.*, 2003
Galiomicin/ AY528421	Antifungal ca. 5-kDa peptide belonging to the defensin family.	Lee *et al.*, 2004
IMPI/ AY330624.1	The first inhibitor of metalloproteinases identified in insects. This protein of 8.6 kDa inhibits the activity of bacterial proteases secreted by invading microorganisms. digesting immune relevant polypeptides of the	Clermont *et al.*, 2004; Wedde *et al.*, 2007

	host.	
Moricin-like peptides/ EF564371.1 EF564370.1 EF564366.1 EF564365.1 EF564372.1 EF564369.1 EF564368.1 EF564367.1	Defence peptides intriguingly restricted to *Lepidoptera*. First found in *Bombyx mori*. In *Galleria mellonella*, there are eight genes encoding seven different peptides (two mature transcripts are identical). They are particularly active against filamentous fungi but also, to a certain extent, against yeast and bacteria.	Brown *et al.*, 2008
Gloverine/ AF394588.1	For *Galleria mellonella*, nothing but mRNA is known for this protein. First isolated from hemolymph of immunised *Hyalophora gloveri. Homologous gloverin proteins or cDNA were isolated from Lepidopteran species. They are* glycine-rich and heat-stable antibacterial proteins (~14kDa) with activity against *Escherichia coli*, Gram-positive bacteria, fungi, and viruses.	Axen *et al.*, 1997; Seitz *et al.*, 2003; Yi *et al.*, 2013
Hemolin / FJ609299.1	Although insects do not possess antibodies, hemolin is a protein from the immunoglobulin superfamily. It is known to function in *Lepidoptera* as an opsonin.	Shaik & Sehnal, 2009
Proline-rich peptide-1/ FJ 494919.1	The peptide with molecular weight ca. 4.3 kDa. Shown to possess antifungal activity. The gene was shown to be unique for moths.	Brown *et al.*, 2009.
Transferin/ AY364430.2	Recently, transferin has been implicated in the innate immune response, as its expression is up-regulated following immune challenge. It reversibly binds iron, controlling its amount in	Kelly & Kavanagh, 2011; Seitz *et al.*, 2003

	the hemolymph and creating an environment low in free iron, which impedes bacterial survival.	
27 kDa *Galleria mellonella* hemolymph protein / AJ575661	This gene encodes an unknown protein. The sequence deposited in the GeneBank was found to be the same with the sequence encoding the 24 kDa protein described further (see third column). This protein was shown to be involved in the activation of prophenolooxidase cascade (PPO).	Park *et al.*, 2005a
Anionic peptide -2/ JQ862476.1	Unlike most antimicrobial peptides, it is permanently present in the hemolymph of naive *G. mellonella* larvae. It acts synergistically against bacteria with lysozyme and apolipoprotein III.	Cytryńska *et al.*, 2007
Peptidoglycan recognition-like proteins A and B respectively/ AF394583 AF394587	On the basis on their similarity with others PGRPs, they may be involved in the process of infection recognition in *Galleria mellonella*.	Seitz *et al.*, 2003
Prophenoloxidase AF336289.1	After activation by limited proteolysis, phenoloxidase converts tyrosine to dihydroxyphenylalanine, chinons and subsequently to melanin. Melanisation process occurs during would chilling, and as a part of cellular immune response. In unchallenged larvae the components of phenoloxidase activating system are kept in oenocytoides and are released after recognition of infection or after injury.	Li *et al.*, 2002

Prophenoloxidase activating factor-like protein/AF394589	Protein homologous to the prophenoloxidase activating factor in *Tenebrio molitor*. Prophenoloxidase is involved in the hemolymph melanisation process.	Seitz *et al.*, 2003
Anti-fungal protein/ DI105103.1	An anti-fungal protein found to be useful for control of plant diseases caused by *Trichoderma viride, Pyricularia grisea, Fusarium oxysporum, Candida albicans, Geotrichum candidum,* and *Cryptococcosis neoformans.* A patent anti-fungal pharmaceutical composition comprises the anti-fungal protein isolated from the larvae of the wax moth *Galleria mellonella*.	Park *et al.*, 2005b

888 †Because in some cases submitted sequences are not published, the literature cited may
889 concern only the source of other information given in the table.
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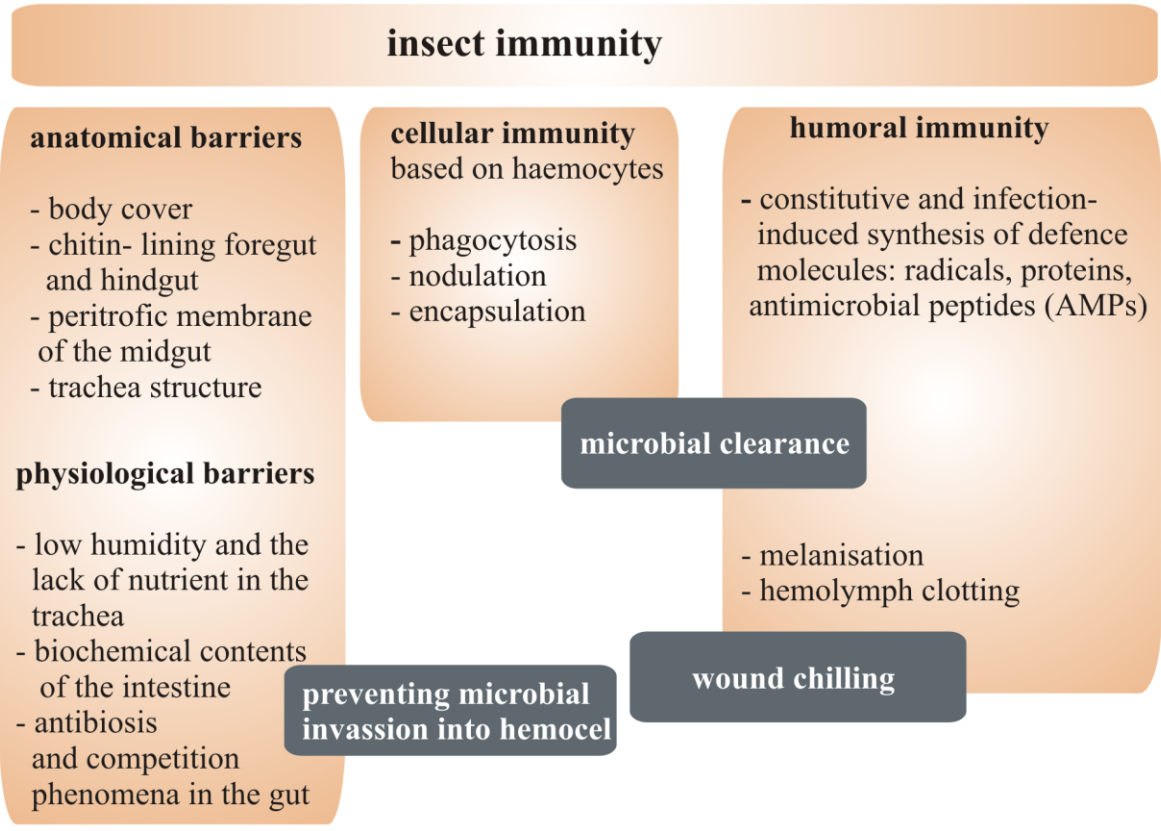
Fig. 1 Simplified scheme presenting insect immunity. Insects are protected by anatomical and physiological barriers and by cellular and humoral reactions. All defence elements are interconnected and mutually cross-regulated. Injury can cause activation of humoral and cellular mechanisms and *vice versa*, these reactions take part in wound healing. Hemocytes can be activated by humoral factors but they also secrete particles affecting humoral reactions.



899 **Fig. 2** *Galleria mellonella* larvae of different developmental stages, taken from the
900 culture reared on natural died of honeybee nest debris (A) and hemocytes seen
901 under a confocal microscope (B). P–plasmatocyte, G–granulocyte, O–oenocytoid,
902 S–spherulocyte.

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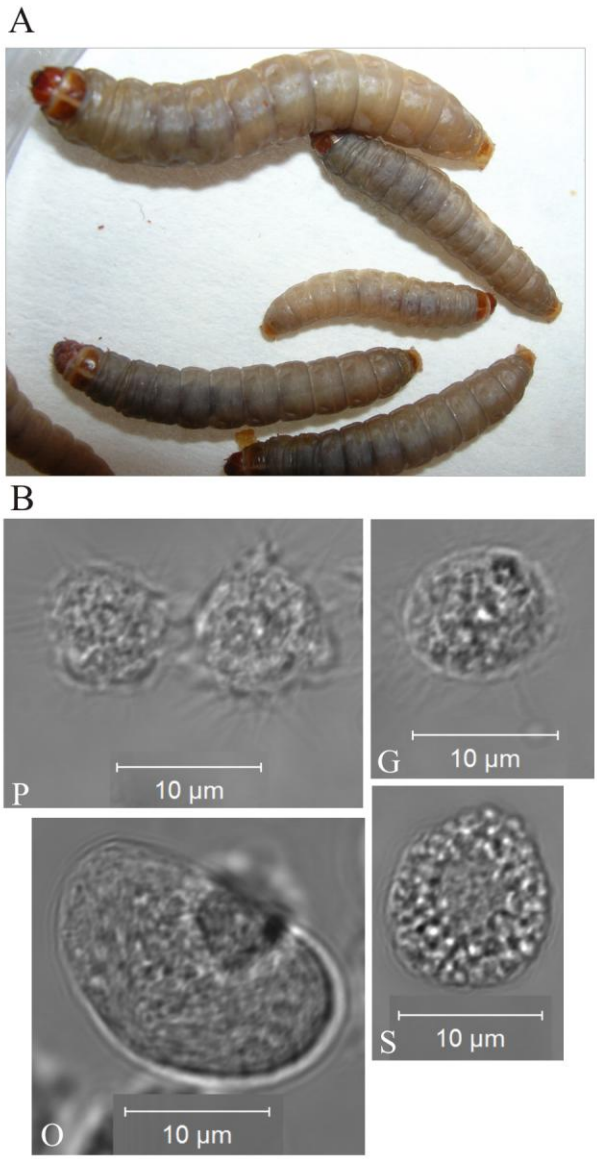

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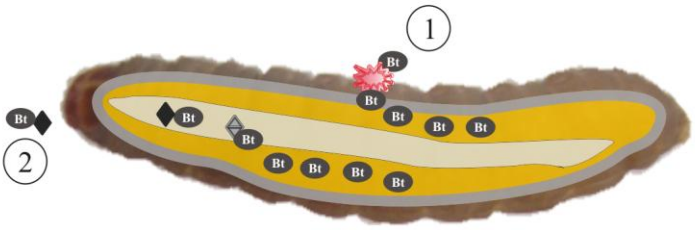
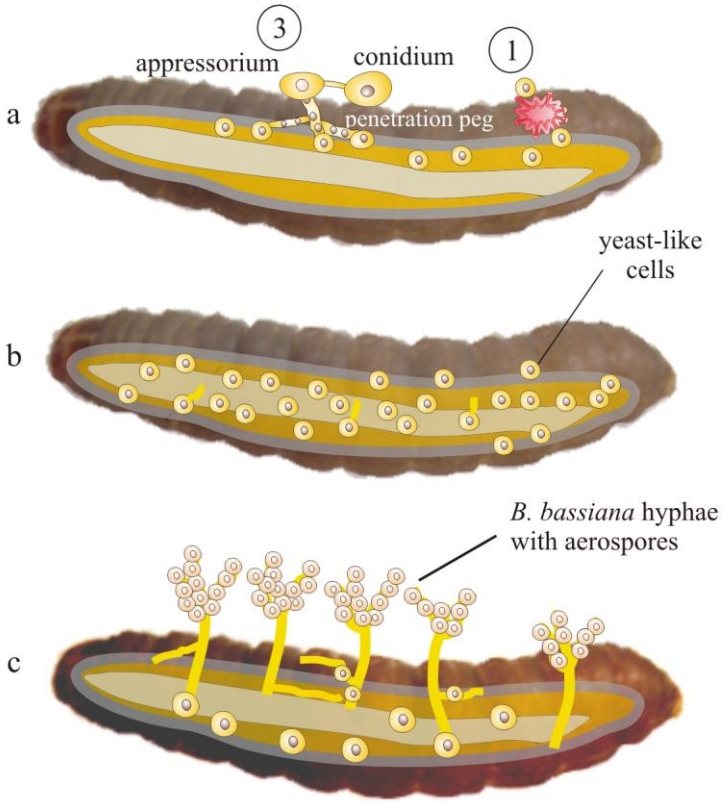
Fig. 3 Simplified model demonstrating routes of *G. mellonella* infection by *Bacillus thuringiensis* (A) and *Beauveria bassiana* (B); 1–infection *via* wounded cuticle (may be used by opportunistic pathogens); 2–oral infection and 3–infection via intact cuticle, both requiring more specific virulence mechanisms. A. *Bacillus thuringiensis* spores and toxin-containing parasporal crystals are ingested by the larvae. In the gut, crystals are solubilised and proteolytically activated toxins bind to the inner membrane of the gut. After gut perforation, bacterial cells get access to the hemolymph, where they proliferate. Bacteria can also access the hemocel directly via injured cuticle. B. After binding of the fungal propagule to the cuticle, it forms an appressorium and grows across the cuticle forming a penetration peg (penetration hyphae). During this step, the fungus secretes enzymes digesting host tissues (a). In the hemolymph, *Beauveria bassiana* grows in the form of single cells (b). The body of dead, melanised animals is overgrown with fungal hyphae producing spores (c). Both pathogens can also get access *via* injured cuticle.

A. Infection by *Bacillus thuringiensis*



B. Infection by *Beauveria bassiana*



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