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REVIEW

Encapsulation and nodulation in insects

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Abstract

Evolution of the insect immune system led to the creation of a comprehensive cellular defense system, not only involving phagocytosis, but also encapsulation and nodulation (both often referred to as capsule formation) allowing the isolation and neutralization of invading pathogens and parasites. Such reactions are closely related to the anatomical and physiological characteristics in insects with their external skeleton and open circulatory blood system. Encapsulation and nodulation are most important defense mechanisms in insects, as they allow targeting of the immune response to the site of damage to quickly destroy the intruder. Host penetration results in both the production of damage-associated molecular patterns (DAMPs) and to the presence of pathogen-associated molecular patterns (PAMPs) in the hemolymph. Subsequent signal induction occurs by host pattern recognition receptors (PRRs) and other systems. Capsule formation results from aggregation and partial disruption of the hemocytes on the target surface resulting in melanization by the proPO cascade. Reactive oxygen (ROS) and nitrogen (RNS) species are emitted during melanogenesis and targeted against the invader. As a result, the intruder is not only isolated within the capsule but also destroyed. Insects have a number of systems (serpins, antioxidants), aimed at the regulation of melanogenesis and inactivation of toxic products resulting from melanization. All these complex mechanisms allow rapid and effective detection, isolation and destruction of invaders with minimal damage to the insect.

Key Words: insect immunity; hemocytes; encapsulation; nodulation; ROS; recognition; phenoloxidase; PRRs; DAMPs

Introduction

Insects have an open blood circulatory system in which hemolymph is enclosed in the body cavity or hemocel and the organs and tissues systems are bathed with hemolymph. The open circulatory system provides some benefits for immune reactivity. For example, immunomediators and hemocytes (blood cells) can be more rapidly disseminated and provide a faster immune response. Consequently, selection should favor the evolution of the rapid and efficient localization and neutralization of invaders (Kraaijeveld *et al*., 1998; Dubovskiy *et al*., 2013a). The open architecture, however, does facilitate the more rapid invasion by infectious agents throughout the host. Included in the main fast reactions of the insect cellular defense strategies are phagocytosis, nodulation and encapsulation. Phagocytosis refers to the engulfment of small numbers of microbial targets, like bacteria or yeast, by an individual hemocyte. Nodulation and encapsulation are more effective innate immune responses against large numbers of pathogens or metazoan parasites in insects, leading to sequestration of the invader together with the biopolymers, melanin and sclerotin, and proteins. Encapsulation refers to multiple hemocytes binding to larger invaders, like protozoans, nematodes and parasitoids (eggs and larvae), that cannot be phagocytized by a single cell. The binding of multiple hemocytes to aggregations of bacteria, fungi and protozoans is also sometimes called nodulation (Ratcliffe and Gagen, 1977; Garcia *et al*., 2007; Satyavathi *et al.,* 2014) (Fig. 1).

The process of encapsulation/nodulation is known to begin within the first minutes after hemolymph penetration by the foreign object (Gagen

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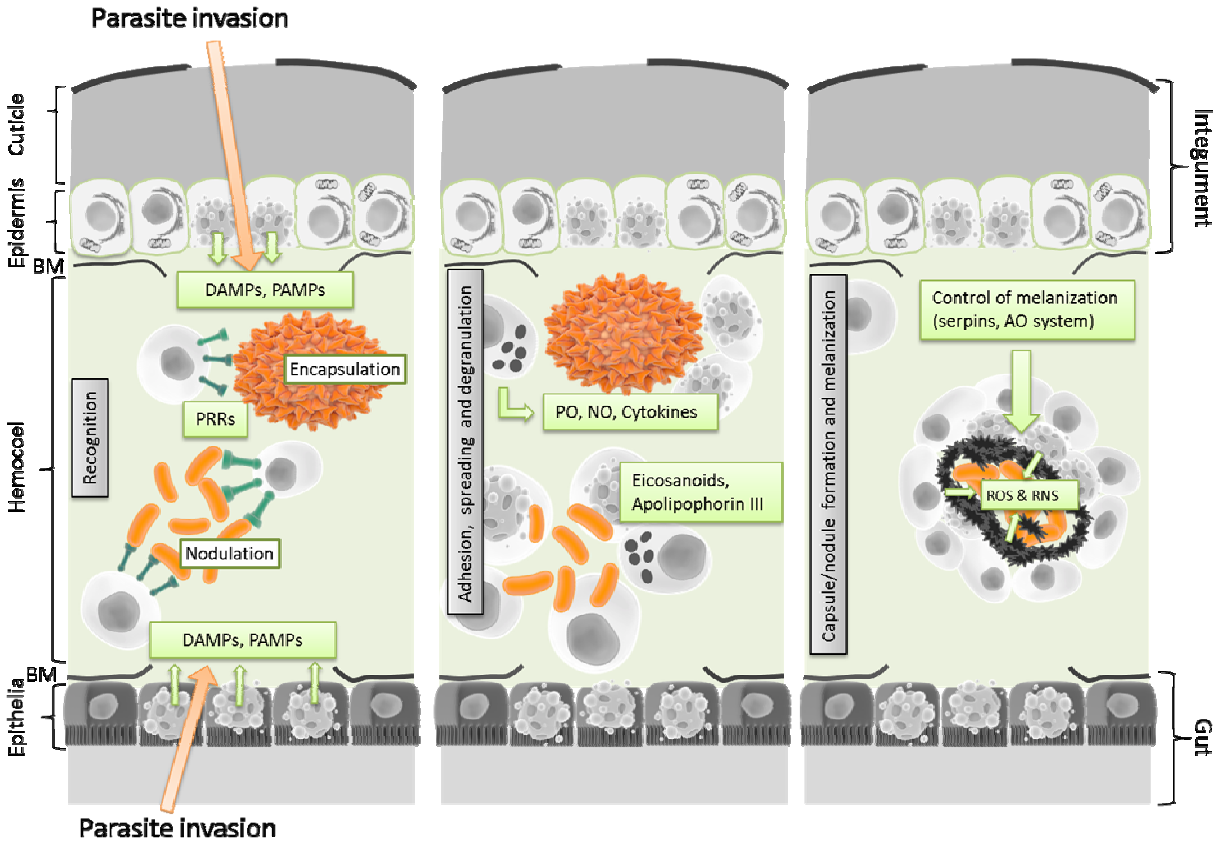

Fig. 1 Schematic capsule/nodule formation in insects. PRRs (pattern recognition receptors), DAMPs (damage-associated molecular patterns), PAMPs (pathogen-associated molecular patterns), NO (nitric oxide), ROS (reactive oxygen species), RNS (reactive nitrogen species), PO (phenoloxidase), AO (antioxidant), BM (basement membrane).

and Ratcliffe, 1976; Dubovskii *et al*., 2010). Depending on the insect species and properties of the target, capsules may be continually formed over 2 - 24 h (Carton *et al*., 2008). In most cases on the next day after the penetration by the invader, the capsule is clearly visible, but is considered fully complete only after 72 h (Ratcliffe and Gagen, 1977). These processes are complex mechanisms that include a wide range of cellular and humoral immune reactions. Recent research has shown the contribution of signals associated with the wound and damage (damage associated molecular patterns, DAMPs) generated during mechanical or enzymatic action to the insect by invading parasites (Altincicek and Vilcinskas, 2006; Abreu-Blanco *et al.,* 2011; Krautz *et al.,* 2014). Insect cellular and humoral pattern-recognition receptors (PRRs) are able to recognize invaders and initiate hemocyte adhesion to the parasite (Strand, 2008). After contact with invaders, hemocytes begin to spread and this leads to the formation of an overlapping sheath around a target. These processes together trigger signaling pathways that produce several activators of immunity (Marmaras and Lampropoulou, 2009).

An important stage of encapsulation/nodulation is hemocyte degranulation, often destroying the cells, and releasing prophenoloxidase (proPO) and activators of cell aggregation. The proPO cascade takes part in the melanization of hemocytes attached to the surface of the invader (Chain and Anderson, 1982; Takahashi and Enomoto, 1987; Pech and Strand, 2000). Phenoloxidase (PO), as an inactive proenzyme prophenoloxidase (proPO), is contained in the cuticle and hemolymph of insects (Ashida and Brey, 1995; Kopacek *et al*., 1995; Sugumaran, 2002). Most reports indicate that proPO is synthesized predominantly by hemocytes, especially in granular cells and oenocytoides (Iwama and Ashida, 1986; Ribeiro and Brehelin, 2006; Williams, 2007). Cell-free melanotic capsules are, however, also found in a range of insects, primarily the Diptera (Carton and Nappi, 1997; Gorman and Paskewitz, 2001). During melanization of the nodules and capsules, some reactive oxygen (ROS) and nitrogen (RNS) species, including o-semiquinone (Slepneva *et al.,* 2003), hydrogen peroxide (Nappi and Vass, 1998; Komarov *et al*., 2006; Dubovskii *et al*., 2010), superoxide anion (Nappi *et al.,* 1995; Whitten and Ratcliffe, 1999; Glupov *et al.,* 2001) and nitric oxide (Nappi *et al*., 2000) are generated. These reactive molecules can both enhance the melanization and take part in destruction of the intruder. Once a capsule has formed, the encapsulated parasite commonly dies (Walters and Ratcliffe, 1996).

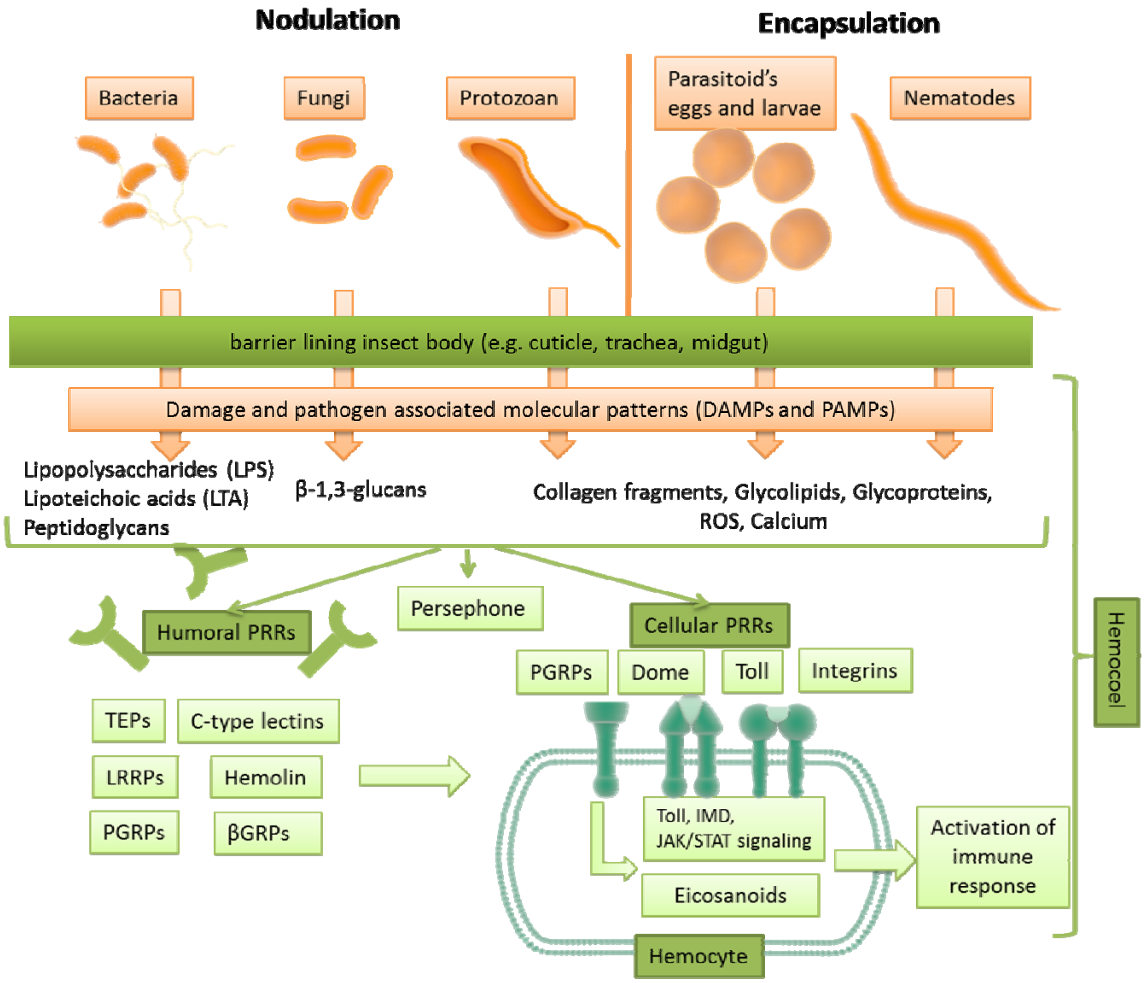

Fig. 2 Recognition of foreign targets by insects pattern-recognition receptors in hemolymph. Pattern-recognition receptors (PRRs), Peptidoglycan recognition proteins (PGRPs), β-1,3-Glucanase related proteins (βGRPs), Thioester proteins (TEPs), Leucine-rich repeat proteins (LRRPs), Hemolin and other immunoglobulins.

Thus, encapsulation/nodulation are similar but complicated multifactorial defense reactions and are often referred to together, subsequently, as encapsulation. The complex biochemical and molecular factors involved in neutralization of invaders and localization in these events are discussed in more detail below.

Wounding and damage-associated signals

The penetration of parasites into the insect hemocel is related to the process of wounding and infringement of the integrity of barrier tissues. Several natural infection models with various parasites have described wounding of the integument as part of the infection process (Schmidt *et al.,* 2001; Wertheim *et al*., 2005; Hallem *et al*., 2007; Arefin *et al.,* 2014). Some pathogens/parasites can invade the hemolymph via the gut. For example, bacteria, such as *Bacillus* spp. (Raymond *et al*., 2010; Dubovskiy *et al*., 2016), and some protozoans, including *Plasmodium* and *Trypanosoma rangeli* (Garcia *et al.,* 2007; Vega-Rodriguez *et al.,* 2014), can cross the gut epithelium during infection, while nematodes invade via both the integument and the gut by mechanically damaging tissues with their mouth parts (Eleftherianos *et al.,* 2010). Trematodes cercariae, likewise, penetrate the cuticle or gut tissues and encyst in a variety of aquatic intermediate hosts, usually insects (Fryer and Bayne, 1996; Brivio *et al*., 2005). The massed infections by entomopathogenic fungi, especially *Metarhizium* and *Beauveria,* also lead to considerable damage of the integument and destruction of the epidermal cell integrity (Dubovskiy *et al*., 2013a; Butt *et al.,* 2016). All these invading parasites result in the release of a number of molecules associated with damage - DAMPs (Fig. 2).

The initial wound reaction and damage-associated signals will undoubtedly influence the subsequent processes of encapsulation and nodulation of invaders. A crucial early wound response is the recruitment of host blood cells attracted by the danger signals released by the

DAMPs (Krautz *et al.*, 2014). This process is similar to the mammalian inflammatory reaction. Hydrogen peroxide (H_2O_2) has recently been identified as the earliest wound attractant in *Drosophila* embryos (Moreira *et al.,* 2010), and H_2O_2 generation has been found in early stages of encapsulation in wax moth larvae (Dubovskii *et al.,* 2010). H_2O_2 synthesis can be activated by a calcium burst as a result of calcium release from the damaged tissue (Razzell *et al.,* 2013). The H_2O_2 can be generated during activation of a Dual oxidase (DUOX), nicotinamide adenine dinucleotide phosphate (NADP) -oxidase, and as a result, the first hemocytes are recruited to the wound site within minutes (Razzell *et al*., 2013). Similar to the mammalian immune system, it has been shown in *G. mellonella* that nucleic acids naturally released by damaged tissues and by activated oenocytoids can induce hemocytes to form net-like structures, initiating hemocyte aggregation and melanization (Altincicek *et al.*, 2008).

Various parasitic metabolites may also be involved in DAMPs generation, for example, microbal thermolysine protease can produce collagen fragments which may demonstrate functions of damage signals in wax moths (Altincicek *et al.,* 2009; Berisha *et al.,* 2013). The loss of collagen from the wound site is also commonly associated with proteinases activity, which are important virulence factors for fungi (St Leger *et al.,* 1994) and bacteria associated with entomopathogenic nematodes (Cabral *et al*., 2004). One detector of proteolytic activity is the *Drosophila* serine protease, Persephone, which can be triggered by virulent proteases produced by entomopathigenic fungi or bacteria (El Chamy *et al.,* 2008; Ming *et al.,* 2014).

Another early event in the insect response to pathogen/parasite invasion is clot formation at the wound site with cellular components, such as PO, hemolectin and possibly transglutaminase in the hemolymph, contributing to this process (Goto *et al.,* 2003; Johansson *et al*., 2005; Bidla *et al.,* 2005; Lesch *et al.,* 2007). Humoral factors, including lipophorin, some hexamerins, and factor Fondue, have also been described as clotting factors in *Drosophila* (Karlsson *et al.,* 2004; Scherfer *et al.,* 2004; Scherfer *et al*., 2006). One necessary condition for clot formation is an emission of Ca^{2+} ions into the surrounding area (Willott *et al.,* 2002; Dushay, 2009; Kryukova *et al.,* 2013).

Recognition of invaders

During assessment of the literature on recognition processes in insects, it was evident that much information is still incomplete, hindering a totally comprehensive overview. Basically, damage at the cuticle or epithelium occurs, invasion of pathogens/parasites into the hemocel takes place followed by recognition by PRRs of PAMPS on the surface or released by these invaders. This results in activation of the appropriate IMD, Toll, or JAK/STAT pathways, and, eventually, through complex signal cascades, transcription of the immune genes (Fig. 2).

Both encapsulation and nodulation depend upon recognition of the invader as foreign and activation of different signaling cascades (Fig. 2). In the case of microbial pathogens, hemocytes and fat body produce receptors, mediators, regulators and effectors during the recognition stage of innate immunity. The receptor proteins (PRRs) recognize conserved pathogen-associated molecular patterns (PAMPs) of microbes (*e.g.,* peptidoglycans, lipopolysaccharide (LPS), lipoteichoic acid (LTA), and β-1,3-glucan) (Yu *et al*., 2002; Pal and Wu, 2009).

In lepidopterans, hemolin (48 kDa plasma protein), peptidoglycan recognition proteins (PGRPs), β-1,3-glucan recognition proteins (βGRPs), Gram-negative bacteria binding proteins (GNBs) (family of 55 kDa plasma proteins), and C-type lectins are PRRs (Jiang *et al.,* 2010; Zhu *et al*., 2010; Zhang *et al*., 2015). In other insects, especially in *Drosophila,* the PGRPs, βGRPs, C-type lectins, galectins, leucine-rich repeat proteins (LRRPs), Nimrods, fibrinogen-related proteins, thioester proteins (TEPs), hemocytins, Dscam, and Reeler may recognize pathogens or parasites (Wang *et al*., 2005; Pal and Wu, 2009; Yassine and Osta, 2010; Estevez-Lao and Hillyer, 2014). For mosquitoes, LRRPs, fibrinogen-related proteins and C-type lectins act as PRRs related to recognition of *Plasmodium* (Cirimotich *et al.,* 2010). Interestingly, the PRRs of *Anopheles gambiae* against *Plasmodium* demonstrate similarity to those involved in bacteria recognition (Blandin *et al*., 2004; Dong *et al*., 2006; Dong and Dimopoulos, 2009; Sandiford *et al.,* 2015).

Genome analyses have uncovered putative PRR genes in other model insect species, including *Tenebrio molitor* (Zhu *et al.,* 2013), *Apis mellifera* (Evans *et al*., 2006) and *Tribolium castaneum* (Zou *et al*., 2007; Altincicek *et al.,* 2013). However, genomic data need experimental confirmation of PRR functioning with biochemical and immunological approaches.

Following recognition, the Toll, Imd, and JAK/STAT pathways are the three main signaling pathways responsible for activation of immune responses in insects (Lemaitre and Hoffmann, 2007; Stokes *et al.,* 2015) (Fig. 2). Each pathway participates in recognition of invaders, and induces the transcription of a number of specific immune-related genes. These genes encode peptides and proteins, which can both target the invader for degradation or act as signaling molecules to induce and enhance the innate immune response such as encapsulation and nodulation (Lemaitre *et al.,* 1996; Marmaras and Lampropoulou, 2009; Myllymaki and Ramet, 2014).

The Toll pathway is responsible for the detection of Gram-positive bacteria and fungi, whereas the Imd pathway is required for responses to Gram-negative bacteria and DAMPs (Lindsay and Wasserman, 2014; Myllymaki *et al*., 2014). The JAK/STAT pathway is activated by fungal and viral infections (Agaisse and Perrimon, 2004) (Fig. 2).

In comparison with microbes and protozoans, the recognition of nematodes, parasitoids and xenobiotic transplants is less well understood. One factor potentially involved in recognition of these targets is the integrity of the basement membrane (BM), an extracellular matrix surrounding most

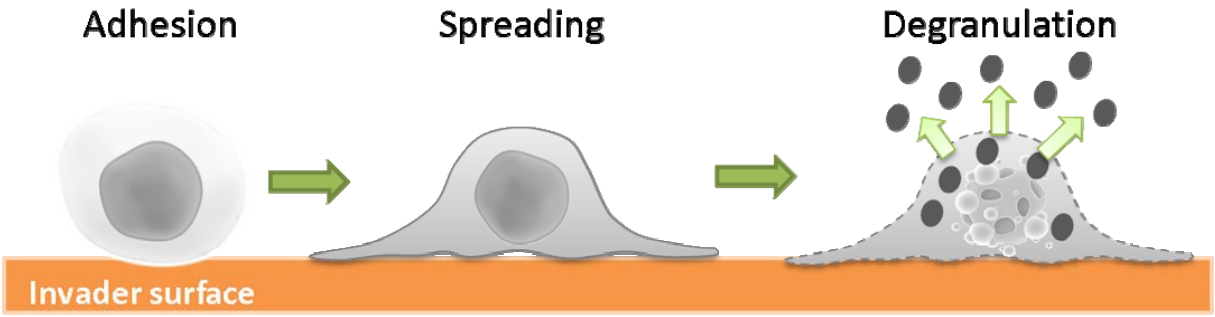

Fig. 3 Hemocytes adhesion, spreading and degranulation during encapsulation of parasites.

tissues. The BM of insects consists of many components including collagen IV, laminin and some proteoglycans (*e.g.,* perlecan and nidogen) (Gullberg *et al.,* 1994; Yurchenco, 2011). The hemocytes take part in the production, and regeneration of the BM to which they normally weakly attach (Ball *et al.,* 1987; Nardi *et al.,* 2001). Moreover, the termination of encapsulation occurs when hemocytes produce a BM-like layer surrounding the capsule, with encapsulated invader isolated from the immune system (Grimstone *et al*., 1967; Pech and Strand, 1996; Liu *et al.,* 1998). Interestingly, differences in the BM contents increase with phylogenetic distance between species, and hemocytes tend to encapsulate quicker transplants from more distant species (Lackie, 1988). Also, insects usually fail to encapsulate tissues transplanted from individuals from the same species unless there is physical or enzymatic damage to the surface (Rizki and Rizki, 1980, 1983). Research on *Drosophila* has shown that the BM component laminin is crucial in BM structural maintenance and preventing self-tissue autoimmunity (Kim and Choe, 2014). Moreover, Sephadex beads coated with laminin are less-avidly encapsulated in the mosquito hemocel (Warburg *et al*., 2007). Thus, the dissimilarity of surface components of parasitoids, nematodes, artificial targets (like nylon, sephadex or latex) to the insects' BM components may help to control recognition of invaders by hemocytes. The recognition of the molecular architecture of the BM is mediated by lectins through specific carbohydrate binding motifs (Vijayan and Chandra, 1999; Fang *et al*., 2010). For example, the eggs and larvae of the parasitoid, *Venturia canescens,* are identified inside the hemocel as “foreign” since they contain Gal-specific glycomodifications on the surface (Castro *et al.,* 1987; Schmidt, 2008). Among the C-type lectins, mannose-binding lectins are involved in innate immune defense as PRRs in both vertebrates and insects and trigger pro-inflammatory signaling cascades (Wilson *et al*., 1999; Turner, 2003; Malagoli *et al.,* 2010).

In addition, different lipid-containing compounds (glycolipids or lipoproteins) in the hemolymph could increase cellular immune responses (Whitten *et al.,* 2004). In the case of parasitoid eggs deposited inside the hemocel, the reaction products from oxidative cross-linking of chorion proteins (Li, 1994) or oxidation induced melanization during egg oviposition through the integument, may alter host lipid particles (Schmidt *et al*., 2010). This could cause local coagulation reactions on the egg surface leading to hemocyte attachments and pro-coagulant deposition on foreign surfaces (Schmidt *et al*., 2010).

Hemocyte adhesion, spreading and degranulation

Cellular immune reactions of insects involve hematopoietic tissue, pericardial cells, and fixed and free-circulating hemocytes (Hoffmann, 1995; Strand and Johnson, 1996; Lavine and Strand, 2002). The contribution of hemocytes to immunity-related defenses is the major known function for these insect cells (Gillespie *et al*., 1997). There are five to six main types of hemocytes identified for insects: prohemocytes, plasmatocytes, granular cells, oenocytoids and spherule cells (Price and Ratcliffe, 1974; Luckhart *et al.,* 1992; Fenoglio *et al*., 1993; Joshi and Lambdin, 1996; Hernandez *et al*., 1999; Lavine and Strand, 2002). In contrast, in *Drosophila* only three main types of hemocytes are recognized: plasmatocytes, crystal cells and lamellocytes (Meister and Lagueux, 2003; Meister, 2004; Ribeiro and Brehelin, 2006). The ratio of the hemocytic types can differ depending upon the stage and species of the insect.

After recognition of invader, the hemocytes attach and start to spread (Fig. 3). The next stages of the cellular immune response involve hemocyte destruction (degranulation) that results in discharge of effector molecules and immunomediators. The processes of nodule formation and encapsulation are similar, forming multicellular clumps of hemocytes with large number of bacteria or other entrapped foreign invaders. Nodulation begins when the number of the target cells exceeds the level that hemocytes can phagocytize, while encapsulation occurs when the parasite is too large to be engulfed by a single cell (Fig. 1). The hemocytes and targets form conglomerates, increasing in size as further hemocytes attach. At the later stages, melanization may occur, usually commencing around the entrapped invaders (Ratcliffe and Gagen, 1977). Nodule formation is the one of the most effective ways to isolate bacterial or fungal infections (Satyavathi *et al*., 2014) and some protozoans (Garcia *et al*., 2012). The order in which hemocytes attach onto the surface of foreign body often

depends on the insect Order. Plasmatocytes and granular cells are usually the first responders to invaders. Aggregation of granular cells followed by degranulation is typical for the lepidopterans unlike the dipterans that are characterized initially by the spreading of plasmatocytes or a purely humoral encapsulation response (Vey and Gotz, 1976; Lavine and Strand, 2002). Granular cell degranulation and breakdown in the surrounding space leads to the accumulation of coagulogen around the foreign invaders (Dushay, 2009).

Discharge of hemocyte cytoplasm and granule contents (degranulation) is a necessary process during capsule and nodule formation, and is followed by the release of proPO and calcium ions (Marmaras *et al*., 1996; Dushay, 2009). Cell transformation, capsule formation, hemolymph clotting, and the release of calcium ions are some of the most important factors in the initial steps of cellular immunity (Willott *et al.,* 2002; Kryukova *et al.,* 2013). Degranulation of the granular cells along with calcium emission induce synthesis of nitric oxide (NO) by NO synthase (Semenova *et al*., 2014). Nitric oxide plays mediating and cytotoxic roles in the insect immune system especially during nodule and capsule formation (Faraldo *et al*., 2005). Tissue- and time-specific alterations in NO production were documented in *Rhodnius prolixus* during *Trypanosoma* infection (Whitten *et al*., 2001, 2007). *Plasmodium* infection in the mosquito, *Anopheles stephensi,* induces significant expression of nitric oxide synthase and as a result, the inflammatory levels of NO in the midgut affect parasite development (Lim *et al*., 2005). The augmented production of NO also occurs in *Drosophila melanogaster* during hemocyte-mediated melanotic encapsulation of the parasitoid *Leptopilina boulardi* (Nappi *et al*., 2000). During hemocytes degranulation and initiation of the proPO activation system, a crucial role is also played by ROS in signaling and enhancement of melanization to destroy parasites (Nappi and Vass, 1993; *Kumar et al.,* 2003; Komarov *et al*., 2005; Komarov *et al.,* 2006; Dubovskii *et al.,* 2010) (see in details of capsule melanization section).

Apart from the action of NO and ROS in immune activation (see above), there are number of other mediator molecules, that are crucial for development of capsules (Fig. 1). Prostaglandins (PGs) and other eicosanoids mediate cellular immune reactions to different challenges in insects (Stanley *et al.,* 2012). These molecules are metabolites of arachidonic acid (AA) and two other C20 polyunsaturated fatty acids. Phospholipase A_2 catalyzes the hydrolysis of AA from cellular membrane phospholipids (Burke and Dennis, 2009). Free AA is a substrate for cyclooxygenases and lipoxygenases that convert AA into PGs and other bioactive molecules (Stanley, 2000, 2005). Activation of eicosanoid synthesis is induced after hemocyte interaction with the PRRs of an invader and induction of Phospholipase A_2, which ultimately leads to PGs biosynthesis (Fig. 2). The PGs are exported from the cell, where they can interact with specific G-protein coupled receptors on the cell that produced the PGs or on nearby cells (Shrestha and Kim, 2009; Stanley *et al*., 2012). In many insect species, eicosanoids are critically important for spreading, aggregation and nodulation after bacterial invasion by *Serratia marcescens* (Miller *et al.,* 1994, 1996; Jurenka *et al*., 1997; Stanley-Samuelson *et al.,* 1997; Miller *et al*., 1999; Tunaz *et al.,* 2003; Schmid *et al.,* 2008). Evidences for eicosanoid participation in cellular immune reactions has been widely reported in insects from seven orders during invasion by parasitoids (Carton *et al*., 2002) and nematodes (Park and Kim, 2000; Park and Stanley, 2006), infections by protozoan (Garcia *et al.,* 2004) and fungi (Dean *et al*., 2002; Lord *et al*., 2002; Tunaz, 2006). Moreover, eicosanoids are involved in wax moth proPO activation that is important for capsule melanization (Mandato *et al*., 1997).

Immunocytochemical methods have also detected molecules similar to mammalian cytokines in insects that can affect several immune reactions, including phagocytosis, cytotoxicity, cell motility and chemotaxis (Ottaviani *et al.,* 2004). Based on molecular and functional studies, the Spätzle and Upd3 cytokines from *D. melanogaster* (Malagoli *et al*., 2010; Vanha-Aho *et al*., 2016) and the hemocyte chemotactic peptide (HCP) from the moth, *Pseudaletia separate*, were isolated (Nakatogawa *et al.,* 2009). Spätzle is involved in the Toll pathway and may be similar to mammalian interleukin 1 (Brightbill and Modlin, 2000). HCP has similarities with another group of signaling molecules from the ENF family - the hemocyte-spreading factor (Nakatogawa *et al*., 2009). ENF peptides are a family of insect cytokines containing 23 - 25 amino acids (Kamimura, 2012). Involvement of the ENF-peptide in triggering of the plasmatocyte spreading has been detected in some species of insects (Pech and Strand, 2000; Kamimura *et al.,* 2001; Eleftherianos *et al.,* 2009). Plasmatocyte-spreading peptide (PSP) which has been found in *Pseudoplusia* stimulates adhesion and spreading of plasmatocytes on the invader's surface (Clark *et al.,* 1997). PSP is combined with specific receptors causing activation of the cytoplasmic adhesive proteins initially including the integrins. Integrins are transmembrane receptor proteins actively taking part not only in the recognition of foreign invaders but also controlling the spreading capacity of the plasmatocytes (Lavine and Strand, 2002, 2003). Those proteins can work as PRRs and as cytokines that regulate adhesion of the plasmatocytes (Pech and Strand, 2000; Nakahara *et al*., 2003). One of the most studied proteins in the ENF family is the growth-blocking peptide (GBP). Like most ENF family peptides, GBP is polyfunctional. Active GBP changes the plasmatocytes from a nonadhesive state to an adhesive state, after which the cells immediately begin to adhere to one another or to foreign surfaces (Oda *et al*., 2010; Tsuzuki *et al*., 2014).

In some species of insects Apolipophorin-III are involved in the encapsulation process (Whitten et al., 2004), as well as DOPA decarboxylase (DDC) (Sideri *et al*., 2008). Furthermore, during the study of genes involved in nodule formation following the injection of the bacteria *E. coli* and *B. subtilis*, two protein mediators, Noduler (Gandhe *et al.,* 2007) and Reeler1 (Bao *et al.,* 2011) have been identified.

Capsule melanization

Melanization during encapsulation and nodulation involves phenoloxidases (PO) which can hydroxylate tyrosine (enzyme EC 1.14.18.1) and also oxidize o-diphenols to quinones (enzyme EC 1.10.3.1). PO are copper-containing oxidoreductase enzymes, oxidizing phenolic compounds (Gorman *et al.,* 2007). In initial stages of melanogenesis, peroxidases can also be involved and oxidize monophenols, aminophenols and diphenols (Nappi and Vass, 1993; Li, 1994).

PO is found in insects as its inactive zymogen form, prophenoloxidase (proPO) (Fujimoto *et al*., 1995; Cerenius *et al*., 2008). The proPO is present in hemolymph (in plasma and hemocytes) and the integument (Ashida and Brey, 1995; Dubovskiy *et al.,* 2013a). In the integument, there is a third type of PO, laccase (enzyme EC 1.10.3.2.) (Nappi and Vass, 1993; Ashida and Brey, 1998; Sugumaran and Bolton, 1998). This enzyme participates in cuticle formation by oxidizing phenylenediamines and polyphenols, but not tyrosine.

Activation of proPO in insects occurs with the help of protease cascades, prophenoloxidase activating systems (Cerenius and Soderhall, 2004) (Fig. 4). These proPO activating proteinases (PAPs) are present in the hemolymph as zymogens, and are activated in response to certain factors, including penetration by invaders (Hung and Boucias, 1996; Meister *et al*., 2000). PRRs (βGRP, PGRP, C-type lectins) bind to PAMPs and this interaction leads to activation of initiator proteases which trigger a proteases cascade resulting in conversion of proenzyme PAPs to active proteinases (Ji et al., 2004). Activated PAPs cleave proPO by limited proteolysis to form active PO (Jiang et al., 1998; Satoh *et al*., 1999; Jiang *et al*., 2003a) (Fig. 4). It has been shown that the damage signal provided by DAMPs can also trigger the proPO activating system in *Drosophila* (Bidla *et al*., 2009; Nam *et al.,* 2012).

At wound sites, activation of PO and melanin formation are observed and these occur in combination with the coagulation mechanism that "close" a wound by forming a clot (Sugumaran, 1998, 2010). PO is released from hemocytes by degranulation and deposited around wounds or encapsulated parasites. During melanization, derivatives of tyrosine, act as substrates for PO and are involved in the structure of capsules (Nappi and Vass, 1993; Carton and Nappi, 1997). At the first stage in melanogenesis, hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) occurs, then the oxidation to DOPA into DOPA-quinone (Nappi and Vass, 1993; Zhao *et al*., 1995; Nappi and Ottaviani, 2000) (Fig. 4). The processes of melanization proceed in the environment where there is a considerable quantity of thiol-containing compounds (glutation, cysteine, proteins), and it is not surprising that various intermediate products of melanogenesis can interact with SH-groups. This lead to the incorporation into melanotic capsules not only of eumelanin and pheomelanin, but also of sclerotin formed with the participation of proteins and amino acids (Nappi and Vass, 1993) (see details of the pathway on Fig. 4). Probably, various proteins (both parasite and host) can act as a matrix for polymeric reactions of oxidizing condensation of indolequinone (Carton *et al.,* 2008).

There are a number of ROS and other intermediates linked with the melanotic cascade (o-quinones, hydrogen peroxide, o-semiquinone radicals, etc.) that possess cytotoxicity and can destroy pathogenic microorganisms (Nappi and Vass, 1993; Komarov *et al*., 2009). At enzymatic oxidation of catechols, including DOPA, semiquinone radicals are formed (Kalyanaraman and Sealy, 1982; Kalyanaraman *et al.,* 1984). In research on larval *Galleria mellonella* and *Dendrolimus superans sibiricus* hemolymph melanization, using electron paramagnetic resonance (EPR) by spin traps, formation of DOPA-semiquinone radicals have been detected (Slepneva *et al*., 1999, 2003). The formation of DOPA-semiquinone in the hemolymph of *G. mellonella* is a consequence of PO activity since after the addition of phenylthiourea (specific inhibitor of PO, Ryazanova *et al.,* 2012), the EPR spectrum of DOPA-semiquinone radicals was not observed (Slepneva *et al*., 2003). o-Semiquinone intermediates of melanization, for example DOPA-semiquinone radical, can interact with molecular oxygen results in superoxide anion radical formation followed by H_2O_2 production (Nappi and Vass, 1993; Nappi *et al*., 1995; Glupov *et al*., 2001; Nappi and Christensen, 2005). The toxic properties of o-semiquinones probably play important roles in killing of parasites in the hemolymph of insects during melanization of the capsule (Dubovskii *et al.,* 2010).

The center of the fully-formed capsule is composed of the foreign invader(s) surrounded by layers of lysed blood cells, eumelanin, sclerotin and proteins. The middle layers consist of strongly flattened and partially destroyed hemocytes, and the outer layer consists of loosely attached blood cells (Lavine and Strand, 2002). The termination of encapsulation occurs when a basement membrane-like (BM-like) layer appears on the capsule's periphery (Pech and Strand, 1996; Liu *et al*., 1998). The capsule acts as the mechanical barrier, limiting the growth and development of pathogens/parasites. However, by the time the capsule is fully formed the encapsulated organisms are often dead. The destruction of the parasite and/or pathogen may be associated with asphyxia, as well as with the cytotoxic effects of the melanin (Soderhall and Ajaxon, 1982; St Leger *et al.,* 1988) and cytotoxic ROS and NO radicals formed during melanogenesis in the melanotic capsule (Slepneva *et al.,* 1999; Nappi and Ottaviani, 2000; Komarov *et al*., 2009; Dubovskii *et al.,* 2010).

Control of melanization by host

The proPO activation system produces several types of molecules that could damage the host insect if produced in excess. These include proteases that could degrade host proteins, cytotoxic quinones and ROS. The cytotoxic ROS can lead to an uncontrolled increase of lipid peroxidation and damage to DNA and protein molecules (Lyakhovich *et al*., 2006). Thus, the system is regulated under most conditions to produce a local melanization response at a specific

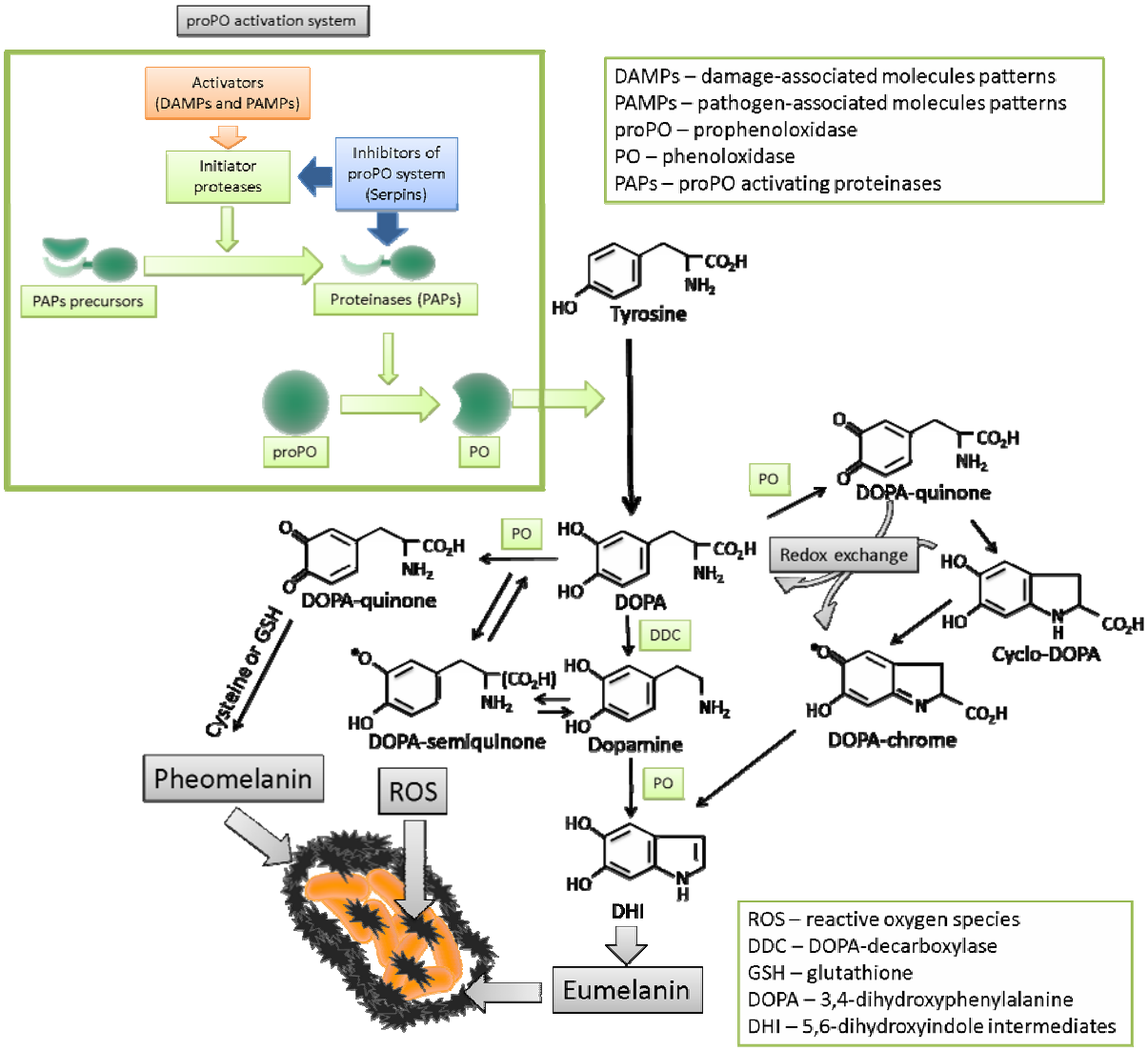

Fig. 4 Activation of proPO system and melanogenesis in insects.

site and for limited duration (Dubovskii *et al*., 2010). The serine protease cascade mediating the processing of proPO to PO is tightly controlled by protease inhibitors. In this way the reaction is maintained near the site of invasion avoiding highly reactive and detrimental oxygen intermediates (Kanost, 1999). In insects, these serine proteases inhibitors, called serpins are a family of 50 kDa proteins (Silverman *et al.,* 2001; Gettins, 2002). Several serpins from *Manduca sexta* hemolymph (serpin-1J, serpin-3, serpin-6, serpin-7) directly inhibit PAPs, the proPO activating proteases (Jiang *et al*., 2003b; Wang and Jiang, 2004; Suwanchaichinda *et al*., 2013).

Insects also have a complex of antioxidant and detoxifying enzymes whose action is involved in ROS elimination (Felton and Summers, 1995). In animals, including insects, important antioxidants include enzymatic antioxidants such as ascorbate peroxidases, superoxide dismutases, catalases, peroxidases and glutathione-S-transferase, as well as the non-enzyme antioxidants, ascorbic acid, thiols, and α-tocopherol (Felton and Summers, 1995; Dubovskiy *et al.,* 2008). Significant increases in ROS generation in hemolymph and a decrease of the enzymatic antioxidant activities have been detected in wax moth hemocytes during encapsulation of nylon monofilaments (Dubovskii *et al.,* 2010). We found the key role in maintenance of the oxidation-reduction balance in the hemolymph of wax moths during the encapsulation process is due to the non-enzyme antioxidants (thiols and ascorbates) (unpublished data). The suppression of melanization and encapsulation by antioxidants ascorbic acid also has been shown in *An. gambiae* (Kumar *et al.,* 2003) and *Aedes aegypti* mosquitoes (Li *et al*., 1994).

Evasion/modulation of nodule formation and encapsulation by parasites and pathogens

Encapsulation is a multifactorial defense reaction and many pathogens/parasites can manipulate either the cellular or humoral factors to inhibit recognition, hemocyte activation or melanization of the capsule. A commonly used method by parasites to avoid recognition by immune system is "molecular mimicry" (Schmidt and Strand, 2001; Brivio *et al*., 2005; Ludin *et al.,* 2011; Yoshino *et al.,* 2012). This is based on the parasite´s capacity to secrete on their surfaces a protective layer of proteins, glycoproteins or glycolipids, imitating host molecules, and not detected by the host immune system as foreign. In some cases, host proteins or/and glycoproteins can be absorbed and later embedding into the parasite surface (Capinera, 2008). Thus, hemomucin, a homolog of the egg and larval surface of the parasitoids *Venturia canescens* (Kinuthia *et al*., 1999) and *Macrocentrus cingulum* (Hu *et al.,* 2008), forms a special layer protected from recognition by the insect host defense system. *Plasmodium* parasites, too, are able to utilize the mosquito C-type lectins CTL4 and CTLMA2 to protect themselves from being killed and subsequently melanized (Osta *et al*., 2004). The venom of the endoparasitoid, *Pteromalus puparum,* inhibits the host immune responses by silencing the expression of the host C-type lectin gene, *Pr-CTL* (Fang *et al*., 2011). The surface coat protein, SCP3a, also protects the nematode, *Steinernema glaseri,* from being detected and eliminated by encapsulation in larvae of the beetle, *Popillia japonica* (Wang and Gaugler, 1999). In addition, *Brivio et al*. (2005), proposed immunoevasion mechanism were also caused by the mimetic properties of the body surface of *Steinernema*, due to the cuticle lipid compounds. Similar avoidance mechanisms of the host immune response are shown by the metacercariae of the *Plagiorchidae* and *Prosthogonimidae* trematodes developing in *Aeshna* dragonflies larvae, which they use as secondary intermediate hosts (Kryukova *et al*., 2005). The entomopathogenic fungus, *M. anisopliae,* also secretes a collagen-like immune evasion protein, MCL1, which is produced within minutes of the pathogen contacting the hemolymph and masks the antigenic cell wall components (β-glucans) of blastospores/hyphal bodies (Wang and St Leger, 2006).

Another successful strategy, providing safe development, is destruction of the immune cells. Hemocytes can be disrupted by different mechanisms from immediate destruction to partial inactivation. Thus, protein from the venom of the parasitoid, *Pimpla hypochondriaca,* causes the death of some of the host hemocytes and a decrease in phagocytic activity and the ability to spreading in others both *in vitro*, and *in vivo* (Parkinson *et al.,* 2004; Huang *et al*., 2009). The venom of some parasitoids induces apoptotic or necrotic cell damage and immune disruption as a result. For example, components of *Nasonia vitripennis* venom causes lysis of host hemocytes due to disruption of the calcium-dependent processes in the cells. Thereby, the total number of the circulating blood cells is significantly reduced, and granular cells and plasmatocytes lose their adhesive and spreading properties, respectively, and do not participate in the processes of coagulation and melanization (Richards and Edwards, 2002; Rivers *et al*., 2002, 2005). Similar effects for the venom of the ectoparasitoid, *Eulophus pennicornis* and *Habrobracon hebetor,* have been observed (Richards, Edwards, 2002; Er *et al.,* 2011; Kryukova *et al.,* 2011). The influence of the *H. hebetor* venom on the hemocytes induces Ca^{+2} release from intracellular stores, probably via phospholipase C activation and inositol trisphosphate production, that finally leads to cell death (Kryukova *et al*., 2015). The calreticulin in the parasitic wasp venom of *Cotesia rubecula* (Zhang et al., 2006) and *Pteromalus puparum* (Wang *et al*., 2013) inhibits host hemocyte spreading behavior probably as a receptor antagonist, to prevent the encapsulation response. In *Rhodnius prolixus, Trypanosoma rangeli*, suppress Phospholipase A2 activity in the hemocytes which reduces arachidonic acid release and inhibits the biosynthesis of prostaglandins and other eicosanoids. Reducing these signals impairs hemocyte aggregation, increases mortality of the cells, and inhibits phagocytosis (Garcia *et al.,* 2004; Figueiredo *et al.,* 2008; *Genta et al.,* 2010). This inhibition seems to be specific and crucial for the development of this parasite in the vector, as *T. rangeli* commonly invades the hemocel, reaching the salivary glands after division and differentiation in this compartment (Garcia *et al*., 2009). The symbiotic bacteria, *Xenorhabdus nematophilus,* associated with the nematode *Steinernema feltiae,* are released into the hemolymph of the wax moth, *G. mellonella,* and the cricket, *Acheta domesticus,* and adhere to the surface of hemocytes to damage and destroy them (Dunphy and Webster, 1984, 1986; Dunphy *et al*., 1998; da Silva *et al.,* 2000). At the same time, *Xenorhabdus* synthesizes and releases a toxin, alpha-Xenorhabdolysin, which pathologically changes the hemocytes. The peptide destroys and blocks the potassium channels of the hemocyte plasma membrane (Ribeiro *et al.,* 2003) and probably inhibits immune-mediating eicosanoid pathways (Park and Kim, 2000). Interestingly, the entomopathigenic fungi, *Beauveria* and *Metarhizium* species, secrete a wide range of immunomodulatory metabolites including bassianin, bassiacridin, bassianolid, tenellin, oosporein, cyclosporine and destruxin (Molnar *et al*., 2010; Gibson *et al*., 2014). Some of these compounds have been detected *in vivo* and their activities have been linked with blocking of hemocyte activity via cytoskeleton alterations (Vilcinskas *et al*., 1997a, b; Kershaw *et al.,* 1999; Amiri-Besheli *et al*., 2000).

Suppression of the proPO cascade is another impressive strategy to suppress a critical stage of encapsulation*, i.e.,* melanization of the capsule. This immunosuppressive approach is broadly used by parasites, especially by parasitic wasps. Venom of parasitoids may contain analogs of serine proteases, that act as antagonists for host hemolymph proteases preventing proPO activation (Beck and Strand, 2007; Asgari and Rivers, 2011). The venom of *Leptopilina boulardi* contains a serpin LbSPNy, inactivating the serine protease in the

hemolymph of its *Drosophila yakuba* host (Colinet *et al*., 2009), while protein Vn-50 from the venom of the *Cotesia rubecula* competitively binds with proPO or with proPO activating proteases (Asgari *et al*., 2003). In the parasite, *T. rangeli,* oral infection of *R. prolixus* can suppress the proPO-activating system in the vector, but the mechanisms are still unclear (Gomes *et al.,* 2003; Castro *et al.,* 2012; Vieira *et al.,* 2015). Finally, the most effective and commonly used method by parasitoids for reduction of the phenoloxidases is by the release of polydnaviruses (PDVs) into the host. Once in the host, the PDVs reduce the synthesis of a number of key hemolymph enzymes of melanogenesis, in particular, phenoloxidase, dopachrome isomerase and DOPA decarboxylase (Shelby and Webb, 1999; Renault *et al*., 2002). Polydnaviruses of *Microplitis demolitor* also express an inhibitor of serine proteases, named Egf (Beck and Strand, 2007; Lu *et al*., 2010).

Conclusions

Encapsulation and nodule formation in insects involve complex interactions between different hemocytes types, proPO activation, as well as NO, ROS and eicosanoid generation during formation of multilayered capsules around invaders. Significantly, the evolution of resistance to multicellular parasites (Carton and Nappi, 1997; Kraaijeveld *et al*., 1998) and entomopathogenic fungi (Dubovskiy *et al.,* 2013b) are associated with the development and improvement of the encapsulation reactions of host insects against these organisms. Through coevolution, pathogens/parasites evolved a number of adaptations allowing them to escape the encapsulation response. These included the masking of the parasite surface antigens and immunosuppression (Vinson, 1990; Pennacchio and Strand, 2006; Castillo *et al*., 2011). Studying features of these processes will help to understand the structure and key principles of the defensive strategies of insects, as well as their evolutionary success due to innate immunity against invaders.

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