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Review

Immune properties of invertebrate phenoloxidases☆

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ABSTRACT

Melanin production from different types of phenoloxidases (POs) confers immunity from a variety of pathogens ranging from viruses and microorganisms to parasites. The arthropod proPO expresses a variety of activities including cytokine, opsonin and microbiocidal activities independent of and even without melanin production. Proteolytic processing of proPO and its activating enzyme gives rise to several peptide fragments with a variety of separate activities in a process reminiscent of vertebrate complement system activation although proPO bears no sequence similarity to vertebrate complement factors. Pathogens influence proPO activation and thereby what types of immune effects that will be produced. An increasing number of specialised pathogens – from parasites to viruses – have been identified who can synthesise compounds specifically aimed at the proPO-system. In invertebrates outside the arthropods phylogenetically unrelated POs are participating in melanization reactions obviously aimed at intruders and/or aberrant tissues.

Infections and tissue damage are commonly seen accompanied by melanization in many invertebrates. A flurry of recent findings has highlighted the importance of melanization reactions and associated activities as key contributors to immunity in invertebrates, in particular arthropods such as insects and crustaceans. By the use of carefully designed infection experiments, mutant analyses and RNA interference some earlier doubts of the crucial role for phenoloxidase (PO) in providing resistance to microbial organisms, parasites and viral infections have been fully overcome. Although the resistance towards pathogens in many cases is linked to melanin production or the toxic reaction intermediates of the melanogenesis some intriguing evidence obtained in several insects and crustaceans strongly suggest that some prophenolxidase (proPO) gene products confer host immunity even without a visible melanization. In line with this, recent detailed data demonstrate that crustacean proPO upon limited proteolysis gives rise to peptide fragments exhibiting cytokine, opsonic as well as microbial agglutinating and killing activities lending credit to the view that the arthropod proPO-system fulfils many of the duties that the complement system carries out in vertebrates and that even the proPO-protein itself exhibits some striking functional similarities to the vertebrate complement proteins in spite of lacking structural similarity to such proteins. In this review we will emphasise the immunological contribution of the melanization reaction and associated activities by the proPO activating system. We will begin with some general characteristics of invertebrate POs followed by a discussion of the activation cascade of arthropod proPOs that begins with pattern recognition and/or tissue damage that initiates a complex proteolytic cascade ending with processing the proPO. Finally, we will consider the different biological activities that arise by the processing of the proPO protein and compare them to the vertebrate complement.

1. Invertebrate phenoloxidases

Melanin is produced from low molecular weight aromatic compounds such as tyrosine by a combination of enzymatic and non-enzymatic reactions. The enzymatic conversion (hydroxylation) of monophenols and the subsequent oxidation of the ortho-phenols produced are both carried out by POs. For more specialised reviews of the chemical reaction mechanisms involved in melanin formation the reader is referred to e.g. Nappi and Christensen (2005) and a more general discussion of various type 3 copper proteins and their phylogeny (to which phenoloxidases belong) can be found in Aguilera et al. (2013). In vertebrates this enzyme, a tyrosinase, is a membrane-bound enzyme acting in a confined membrane-enclosed compartment, the melanosome (D'Alba and Shawkey, 2019). Melanosome-bound tyrosinases have been characterised in some invertebrates such as cuttlefish (Palumbo et al., 2000) or *Ciona* (Racioppi et al., 2019). However, in several invertebrates, such as insects, crustaceans and other arthropods,

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corresponding PO-activities are instead carried out by soluble proteins lacking an ER-localisation pro-peptide (Cerenius and Söderhäll, 2004). Arthropod POs are well-studied from an immunological viewpoint whereas the immunological role of melanization in other invertebrates is less investigated. However, the PO-activity of tunicate morula cells in rejection reactions towards foreign tissue explants (Franchi et al., 2019) or melanization reactions in corals (Bailey et al., 2019; Palmer et al., 2011) molluscs (Coaglio et al., 2018 and reviewed in Luna-Acosta et al., 2017) or in annelids (reviewed in Prochazkova et al., 2020) towards pathogens suggest a role for the melanization reaction beyond the arthropods. A recent trancriptome analysis after grafting operations in the mantle of the pearl mussel *Hyriopsis cuminglii* is one additional example that clearly supports a role for mollusc tyrosinases in immune reactions (Shen et al., 2020).

Obviously due to these differences in cellular localisation, regulation and activation of PO activities between arthropods and many non-arthropod invertebrates, control of melanization in the latter must at least partly occur along other paths than the well-investigated arthropod PO-activation system.

1.1. Laccases

To comply with the present use in the immunological literature and to distinguish them from the vertebrate membrane-bound tyrosinases the term PO will be used for the cytosolic invertebrate enzymes with tyrosinase (EC 1.14.18.1) activities. It should be noticed that technically catechol oxidase (EC 1.10.3.1) and laccase (EC 1.10.3.2) are phenoloxidases as well. Experimental evidence for the use of the latter two in immune reactions is so far relatively limited although laccases are crucial for the sclerotisation of the exoskeleton and thereby contributing to the protection of the animal especially against fungi as shown e.g. by a laccase-2 knockdown in the beetle *Tribolium castaneum* (Hayakawa et al., 2018). However, some recent data seem to indicate a more direct immune role for laccase; Shi et al. (2017) showed by laccase-knockdown in the shrimp *Litopenaeus vannamei* that the animals become more susceptible to different bacterial infections and Quinn et al. (2020) and Luna-Acosta et al. (2011) have demonstrated by in vitro experiments that laccase and catechol oxidase reactive products from a gastropod exhibit antimicrobial activity. In two *Biomphalaria* species, which are vectors for *Schistosoma mansoni*, laccase activities were registered in the hemolymph of uninfected snails. Upon infection with the parasite a gradual decrease in this laccase activity was recorded during a 4-week period (Le Clech et al., 2016). Clearly the possible role in immunity of laccases and catechol oxidases needs further scrutiny. Of note, an interesting parallel between laccases and PO in insects is their connection to Toll signalling. In silkworm Spätzle 3 and Toll 8 is crucial for the laccase regulation for cuticle pigmentation patterning in lepidopteran larvae, whereas Spätzle 1 and Toll 1 are used for immunological purposes in the proPO-cascade in the same insect (KonDo et al., 2017). Although it is commonly assumed that vertebrates do not use POs for immunological purposes there are data suggestive of such a role. Human laccaseC1 protein localises to the ER of the macrophage after the cell has been stimulated by bacteria (Huang et al., 2019). This localisation is necessary for several downstream stress-mediated processes after pattern recognition events. However, no PO-activity by the laccase is apparently required for these events to take place (Huang et al., 2019).

1.2. Hemocyanin

Type 3 copper proteins to which both tyrosinases and hemocyanins (as well as tyrosinase-related proteins and catechol oxidases) belong possess a conserved site with 2 copper atoms, each coordinated by three histidine residues. The copper atoms serve as O_2 carriers or are transferring electrons in the reactions of the phenolic substrate for hemocyanins and POs, respectively. Structural data and modelling suggest that access into the copper is more restricted in hemocyanin since some crucial amino acids are hindering the relatively bulky phenolics to reach them although it is well established that hemocyanins from many different sources can be converted into enzymatically active POs in vitro by a variety of different treatments and hereby produce antimicrobial activities (for reviews see Decker and Jaenicke, 2004; Coates and Nairn, 2014). It seems likely that these in vitro treatments serve to shift the position of an active site blocker (Li et al., 2009) residue such as the phenylalanine 371 in the crustacean *Panulirus japonicus* hemocyanin (Masuda et al., 2020) that is denying access to the copper to more bulkier substrates. This Phe residue is not present in crustacean POs; its absence and the presence of conserved glutamic acid and asparagine acid residues and a conserved water molecule around the Cu have been suggested to contribute to the enzyme activity of arthropodian PO (Masuda et al., 2020; Hu et al., 2016). The immunological relevance of hemocyanin-to-PO conversions remains to firmly establish in many cases. In a few cases endogenous substances with capacity to induce PO-activity in the corresponding hemocyanin (Nagai et al., 2001) have been identified although their physiological significance remains to clarify. However, the possible immunological role of hemocyanin goes beyond its PO-activity, proteolytic processing of the protein may produce antimicrobial substances such as the crayfish-derived astacidin 1 (Lee et al., 2003) and several shrimp hemocyanin-derived peptides (Destoumieix-Garzon et al., 2001 and reviewed in Tassanakajon et al., 2018).

2. The number and distribution of invertebrate PO genes vary

The number of *proPO*-genes in different arthropods varies greatly from one to more than 10 in some insects such as mosquitoes. A few arthropods are even lacking them, possibly hemocyanins or laccases may instead fulfil their immunological role. By increasing the numbers some species may obtain adaptability letting different proPO genes be under separate regulation and tissue localisation. A clear example of such a partial division of function and localisation has been unravelled in *Drosophila melanogaster*. In this species two PO-genes, *PPO1* and *PPO2*, are expressed in crystal cells whereas *PPO3* expression is localised to the lamellocytes (Dudzic et al., 2015). The latter gene was suggested to have arisen recently in the melanogaster group by a gene duplication of *PPO2* providing means more or less exclusively aimed at larger parasites, in particular parasitoid wasps. An additional division of labour between the PPO gene products is suggested by the fact that the PPO2 protein is stored in large protein inclusions in the cell and released by cell rupture whereas the PPO1 protein may be released continuously into the hemolymph by an undetermined mechanism (Dudzic et al., 2019). Of note, the amounts of different PPOs may vary even within what appear to be a homogenous hemocyte type by morphological criteria. In the mosquito *A. gambiae* for example there are different subpopulations of granulocytes, some of which contain high amounts of PPO6 protein whereas other possess much lower concentrations (Severo et al., 2018). The study by Severo et al. (2018) also suggests that some granulocytes can transfer PPO6 (their in vitro study with isolated granulocyte sub-populations demonstrated transfer of PPO6 transcripts between these sub-populations) to other granulocytes via micro-vesicles (exosomes) thereby enhancing the melanization potential of some hemocyte populations and indicating a route via which different hemocytes may cooperate during immune reactions.

In another insect *Nipponaphis monzeni* a soldier specific PO-gene has undergone an accelerated evolution compared to the other PO-genes of this species, presumably an adaptation to its apparent specialised role in coagulation (see section below on immunological effects) of the body fluid of self-sacrificing soldier nymphs (Kutsukake et al., 2019). Indirectly some functional diversion is suggested among the 9 PPO genes in *Anopheles gambiae* mosquitoes since silencing 3 of them, *PPO2,3* or *9* of them increased *Plasmodium berghei* survival in the insect host whereas, in contrast, knock-downs of three other genes, *PPO4,5* or *6* did not affect parasite survival (Kwon and Smith, 2019).

Although their role in immunity as indicated above is less clear it is noteworthy that in some mussels the number of PO (tyrosinase) genes has undergone an enormous expansion with some species harbouring more than 20 PO-genes (Aguilera et al., 2014).

3. Pattern recognition in the activation of the proPO-activation cascade

3.1. β-1,3 glucan-binding proteins

Interest into the proPO system grew considerably once it was demonstrated that β-1,3 glucans, a polysaccharide characteristic of fungal cell walls and a few other organisms had the capacity to specifically and in very low concentrations initiate its activation in crustaceans (Unestam and Söderhäll, 1977). It soon become evident that β-1,3 glucans triggered proPO-activation in insects as well and several proteins capable of binding β-1,3 glucans and thereafter mediate proPO-activation were isolated and characterised biochemically in insects, first by Söderhäll et al. (1988) and shortly thereafter by Ochiai and Ashida (1988) and later also in crustaceans (Duvic and Söderhäll, 1990) to be followed by the cloning and structural characterisation of a glucan-binding protein (BGBP) from freshwater crayfish (Cerenius et al., 1994). BGBP, with dual functions as lipid carrier and pattern recognition protein, can agglutinate fungal cells (Hall et al., 1995; Goncalves et al., 2012) and will after binding to the β-1,3-glucans both initiate proPO-activation and through binding to a hemocyte receptor act as an opsonin and as a promotor of hemocyte degranulation (Duvic and Söderhäll (1992). BGBP seems to be unique for crustaceans but extensive studies have revealed the wide distribution of a family of proteins with capacity to bind both β-1,3 glucans and bacterial cell wall products such as LPS followed by activation of the proPO-cascade are present in crustaceans (Lee et al., 2000; Sritunyalucksana et al., 2002) as well as in insects (Ochiai and Ashida, 2000; Ma and Kanost, 2000). These proteins, variously often called lipopolysaccharide and glucan-binding proteins (LGBPs) or β-glucan recognition proteins βGRPs or gram-negative bacteria binding-proteins (GNBPs) belong to members of a small family of ca 50–60 kD proteins with some structural similarity to glucanases (Lee et al., 1996), although the glucanase-like domain apparently is not involved in the binding of the glucans (Takashashi et al., 2009). LGBPs have also been found in other invertebrates e.g. annelids (Beschin et al., 1998) and molluscs (reviewed by Wang et al., 2018). LGBPs are likely to trigger many other immune reactions such as the expression of genes for antimicrobial peptides as shown in fruit flies (Kim et al., 2000), *Manduca sexta* βGRP3 was shown by use of the recombinant protein to agglutinate and retard growth of *Bacillus cereus* (Rao et al., 2014). Purified shrimp LGBP, although capable of binding bacteria and activate the proPO-system, does not inhibit the growth of the bacterial pathogen *Vibrio parahaemolyticus* (Phupet et al., 2018). βGRPs from different insect species differ with respect to which conformation variants of β-1,3 glucans they bind to (Adachi et al., 2019) which, is suggesting the possibility that they may have evolved to recognise different pathogens. There is often a small family of different LGBP/βGRP genes in many species but the functional significance of this diversity within a single species remains to a large extent to investigate. There are also at least a few examples of peptidoglycan-binding proteins (PGRPs see below) that are capable of binding β-1,3 glucans and activate the proPO-system (Lee et al., 2004; Zhao et al., 2018).

3.2. Peptidoglycan binding proteins

Peptidoglycans may activate the proPO-cascade through peptidoglycan recognition proteins, PGRPs, a family of proteins widely distributed in the animal kingdom. They are structurally related to T7 lysozyme albeit some of them lack its amidase activity. Several PGRP family members participate in recognising peptidoglycan of both the diaminopimelic acid (DAP)-type and the Lys-type to initiate different immune reactions such as proPO-activation or synthesis of antimicrobial peptides. Activation of the proPO-system by a PGRP-mediated reaction was first demonstrated in silk worm (Yoshida et al., 1996) but is apparently common in insects, e.g. in *D. melanogaster* Takehana et al. (2002); *Tenebrio molitor* (Park et al., 2007); *Ostrinia furnacalis* (Sun et al., 2014); *Helicoverpa armigera* Li et al., (2015); *Antheraea pernyi* Zhao et al. (2018). Most insect species investigated possess several *PGRP*-genes that differ both structurally and functionally. In several insect species one PGRP protein, PGRP-SA has been identified in a complex with GNBP1 to mediate the proteolytic activation by Lys-type peptidoglycan of the proPO-system and the Toll-ligand Spätzle, as first demonstrated biochemically in the beetle *T. molitor* (Kim et al., 2008). In Drosophila PGRP-LE will mediate proPO-acivation by DAP-containing peptidoglycan (Takehana et al., 2002). At least some crustacean groups however lack PGRP but a complex consisting of an LGBP and two SPHs appear to fulfil a corresponding role of mediating peptidoglycan-triggered proPO activation (Liu et al., 2011). Also, a QM-protein has been demonstrated using a recombinant such protein to bind peptdoglycans and promote phagocytosis and proPO-activation in the shrimp *Penaeus monodon* (Udompetcharaporn et al., 2014). It is uncertain however whether the QM-protein is localised to the hemolymph in vivo and therefore in position to function as an extracellular pattern recognition protein. An interesting example of PRR activity among crustaceans is another large SPH, the masquerade-like protein (Huang et al., 2000) that upon binding bacterial cell walls is proteolytically cleaved into several fragments expressing different activities such as binding to hemocytes and bacterial cells hereby promoting removal of bacteria from circulation and one fragment functioning as a cell adhesion factor (Lee and Söderhäll 2001). Also, C-type lectins mediate proPO-activation e.g. the *Manduca sexta* immunolectins (Yu et al., 1999), the shrimp Fc and the crustacean mannose-binding lectin (MBL)-like protein (Wu et al., 2012). The latter is a collectin-like protein mediating LPS-induced activation of the proPO-system, i.e. a protein which both functionally and structurally has some resemblance to mannose-binding lectin associated serine protease (MASP), the initiator of the lectin pathway of vertebrate complement. An interesting observation is that the presence of high amount of a mannan binding lectin in the granular hemocytes of the crayfish *P. leniusculus* may play a role in regulating the proPO-system since a hemocyte lysate supernatant (HLS) prepared at 100 mM Ca^{2+} could become activated when the concentration of LPS was increased up to 3 mg/ml, this may indicate that Pl-MBL acts as a scavenger for LPS to prevent spreading of LPS in the hemolymph to avoid further activation of the proPO-system (Wu et al., 2013).

3.3. Thiolester-proteins

In mosquitoes thiolester-proteins (TEPs) have been shown to influence the melanization reaction. It has been suggested, primarily by a genetical approach, that in *A. gambiae* TEP1 (Levashina et al., 2001) binds to foreign surfaces and interacts with a clip serine proteinase, SPCLIP1, in a chain that via two downstream clip proteases homologues, CLIPA8 followed by CLIPA28, terminates in proPO cleavage by an uncharacterised protease (El Moussawi et al., 2019). The presence of SPCLIP1 appears to stimulate the recruitment of the TEP to the microbial surface whereas an SPH, CLIPA2 negatively regulates TEP1 binding. In the hemolymph TEP1 circulates in a complex together with two leucine-repeat proteins (Povelones et al., 2009). Different TEP1 alleles however confers different degrees of melanization and resistance towards *Plasmodium berghei* and melanization requires proteolytic processing of TEP1 (Blandin et al., 2009; Volohonsky et al., 2017). Several components in the mosquito pathway to proPO activation remain to identify, although recently one such the CLIP-domain protease C9 was characterised and found to act downstream of the SPHs (Sousa et al., 2020). Also, in the fruit fly several TEP proteins have been shown to influence the degree of melanization when the fly is experimentally infected with the bacterium *Photorhabdus* (Shokal and Eleftherianos,

2017) and in crayfish a tissue-specific Pl-TEP is involved in defence against bacteria in the gills and intestines (Wu et al., 2012).

4. Release and activation of proPO in the extracellular milieu

Arthropod POs in general are synthesised as inactive proenzymes that become active by an elaborate chain of events brought about by pattern recognition as described above or by tissue damage, e.g. by danger signalling. In several insects and crustaceans a chain of associated proteinases and regulating proteins has been identified that bring about the initiation of the proPO cascade. Several of these factors are shared with the pathway that bring about the proteolytic activation of the Toll ligand Spätzle (Kan et al., 2008; Dudzic et al., 2019) and hereby trigger gene expression of many genes for antimicrobial peptides. Thus, there is a considerable amount of cross-talk between the pathways resulting in the melanization reaction and in activation of Toll.

4.1. Release of proPO from hemocytes

Most POs whose site of synthesis has been determined are produced in specific sub-sets of hemocytes that depending on species may be called granulocytes, oenocytoids or crystal cells. The enzyme is in most cases released in its pro-form either by cell rupture after activation of the c-Jun N-terminal kinase (JNK) pathway as seen in *Drosophila* oenocytoids (Bidla et al., 2007; Dudzic et al., 2015) or possibly by other still not characterised mechanisms of continuous release. One recent study in the insect *Acyrtosiphon pisum* suggests that the JNK-signalling may also influence proPO gene expression as RNAi of this pathway decreases expression of both proPO-genes in this species (Ma et al., 2020). This suggestion requires elaboration since the effects of JNK knockdown on PO activities on the other hand was minimal.

Data from a crustacean implicates a mechanism reminiscent of inflammasome-activation involving a caspase-1-like enzyme in the release of PO from granular hemocytes into the extracellular milieu (Jearaphunt et al., 2014). This caspase-1 enzyme can also modulate the activities of the released extracellular PO; cleavage by the caspase of proPO produces PO fragments without melanising activity but with capacity to agglutinate bacteria (Jearaphunt et al., 2014). Extracellular release of proPO may be brought about by cues such as microbial products or tissue damage (danger signalling). The enzyme gains its activity after a limited highly regulated proteolysis of the proPO (for details see e.g. review by Lu et al., 2014, Cerenius et al., 2008), often the result of a chain of events involving several proteinases, pattern recognition proteins and regulatory proteins. In crustaceans is the prophenoloxidase activating enzyme (ppA) and other components of the proPO-activating system (but not proPO!) are localised into granules that are released upon pattern recognition events in the plasma (Sriacharoen et al., 2005; Jearaphunt et al., 2014) (Fig. 1). The use of an extracellular PO in many invertebrates is likely to have contributed to relatively elaborate mechanisms for controlling both the proPO-activation and the PO-activity once it becomes activated in these animals.

4.2. Proteolytic activation of proPO

ProPO is activated by limited proteolysis, in most cases so far investigated by clip-containing serine proteinases. In a few insect species the chain of events from pattern recognition to proPO cleavage has been worked out (for a review of proteolytic activation of proPO in insects see Lu et al., 2014). They consist of upstream pattern recognition proteins (se previous section), a chain of proteinases activating each other terminating with one or several prophenoloxidase activating enzymes (PPAEs or ppAs). In several instances the most upstream proteinase in the activation cascade is a modular proteinase containing some structural motifs resembling vertebrate complement initiating serine proteinase MASP or the complement component C1q as seen in the *Tenebrio molitor* modular serine protease (MSP) Park et al. (2007); *D. melanogaster* ModSP (Buchon et al., 2009); and *M. sexta* HP14 (Ji et al., 2004). These modular proteinases interact with pattern recognition proteins bound to e.g. a microbial cell wall component and as a result the proteinase becomes auto-activated as was first shown in *T. molitor* with MSP, Lys-PG, PGRP-SA and GNBP1 (Kan et al., 2008; Roh et al., 2009) and later in *M. sexta* with β1,3-glucans, βGRP2 and HP-14 (Takahashi et al., 2015). In addition, serine proteinase homologues (lacking the serine residue required for proteolytic activity) regulate activation of the system, e.g. being necessary in some systems to achieve melanization activity by the proteolytically processed PO. Both the serine proteases and the SPH contain one or several clip domains in the pro-region (reviewed in Kanost and Jiang, 2015). Unfolding of these cascades have made it clear that there is a large overlap between the extracellular part of the Toll signalling giving rise to AMP gene expression and the proPO cascade in that both pathways share several components and in many cases are activated simultaneously (Kan et al., 2008). An alternative way to achieve proPO-activation is through tissue damage, it is commonly observed in many invertebrates that injuries quickly lead to melanization of the damaged area. The signal(s) leading to this activation are mostly unknown but in *D. melanogaster* the protease Hayan (independently of the PGRP-SA/GNBP → ModSP → Grass pathway that mediates activation of PPO1 and pro-Spätzle via pattern recognition of peptidoglycan) is mediating activation of both PPO1 and PPO2 at the site of injury (Dudzic et al., 2019; Nam et al., 2012). It was suggested that H_2O_2 or other reactive oxygen species at the site of injury constituted a signal that affected other cells in the body (Nam et al., 2012). Reactive oxygen species may also regulate the hematopoietic formation of more proPO-producing granular hemocytes as in crustaceans (Junkunlo et al., 2016) and *PO*3-containing lamellocytes in fruit flies (Owusu-Ansah and Banerjee, 2009). Besides Hayan a phylogenetically closely related proteinase, Persephone is

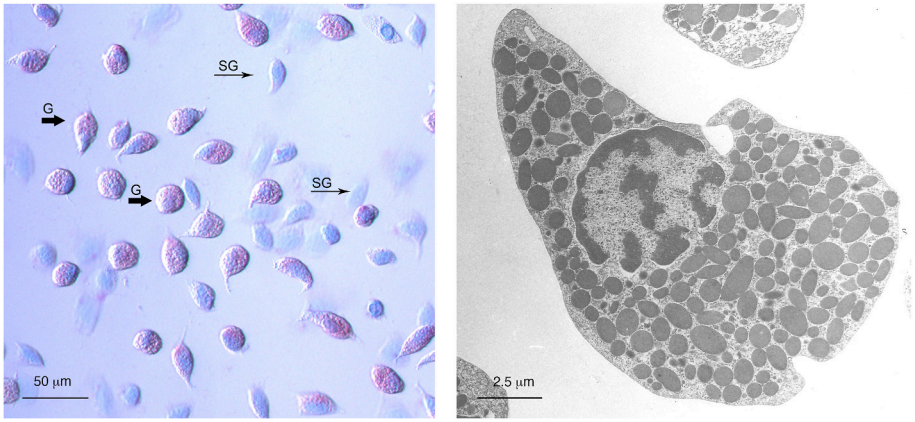

Fig. 1. Left shows a mixture of semigranular (SG, thin arrows) and granular cells (G, thick arrows) under the light microscope and stained with May-Grünwald Giemsa staining. Notice that granular cells are stained more avidly compared to semigranular cells. Photo Irene Söderhäll. Right shows a transmission electron micrograph of a granular cell which contains most of the components of the proPO-system. The black stained vesicles contain the proPO-system components except proPO which is in the cytosol of the granular cell. Photo Oleg Chaga.

activated in particular by microbial proteinases to mediate activation of the Toll-ligand as well as of melanization in *D. melanogaster* (Issa et al., 2018; Dudzic et al., 2019). As demonstrated in freshwater crayfish different bacteria may influence the outcome of the extracellular processing of proPO; an *Aeromonas hydrophila* infection led to a higher proportion of ppA-cleaved PO and a low amount of caspase-1-cleaved PO and thus higher PO-activity compared to an *E. coli* injection that produced proportionately more caspase cleaved PO (Jearaphunt et al., 2014). This result suggests that infections may trigger different proteolytic events of proPO-system components that may adjust what type of immune effectors that will appear. Results obtained with recombinant *D. melanogaster* PPO3 protein may point to similar conclusions; depending on how the recombinant protein was activated different amounts of melanization intermediates appeared (Wu et al., 2018). Together data so far appear to suggest that there are multiple ways to activate the proPO-system resulting in a variety of immune effectors produced.

5. Inhibitors and regulators of proPO-activation or activity

5.1. Proteinase inhibitors

Given the toxicity and reactivity of several components produced during the melanization reaction (Nappi and Christensen, 2005) strict control of the activation as well as the activity of the PO enzyme itself is of paramount importance. In several arthropods regulatory proteinase inhibitors to the different proteolytic steps in the proPO-system activation have been identified and characterised. Many of these are serpins, a superfamily whose members inhibit serine proteinases by a specific mechanism that involves cleavage of the target protease at a specific bond, the scissile bond. Thus, some pathogens have evolved specific serpins with a specific capacity to prevent host melanization. One example is the baculovirus encoded Hesp018 serpin (Ardisson-Araujo et al., 2015) another the parasitoid venom LbSPINY (Colinet et al., 2009), see also Table 1. A few examples of serpins and their target proteinases that are inhibiting the proPO-cascade (and sometimes the Toll pathway as well) known in some detail are: *D. melanogaster* SPN27A, an inhibitor of both melanization and extracellular Toll signalling (De Gregorio et al., 2002; Ligoxygakis et al., 2002) and it specifically inhibits Sp7 (also called MP2) (An et al., 2013) and SPN28D inhibits melanization although through an unidentified proteinase target (Scherfer et al., 2008). In *T. molitor* SPN40 inhibits modular serine proteinase (MSP), SPN55 inhibits spätzle-activating enzyme (SAE), SPN48 inhibits spätzle-processing enzyme (SPE) (Jiang et al., 2009) and as a result both melanization and Toll pathway is blocked by either

Table 1
Examples of interference by pathogen-derived factors on host proPO-system.

Pathogen	Host	Pathogen factor(s)	Short description of interaction
Aspergillus niger	*Galleria mellonella*	α-1,3-glucan	The glucan is binding proPO-activating complex (Stączek et al., 2020).
Baculovirus	*Helicoverpa armigera*	unknown	Virus by increasing serpin-5,-9 expression prevents proPO-system activation by the clip domain proteases cSP4,6 (Yuan et al., 2017).
Baculovirus	*Tricoplusia ni*	Hesp018	Virally encoded serpin inhibiting host melanisation (Ardisson-Araujo et al., 2015).
Beauveria bassiana	*Anopheles stephensi*	milR1	An RNA that initially during infection is highly expressed and via binding to Argonaute 1 silences Spätzle and thus Toll activation, but will by subsequently lowering its expression avoid to trigger expression of the proPO-activator CLIPB9 in the haemocoel during later phases of infection (Cui et al., 2019).
Beauveria bassiana	*Galleria mellonella*	oosporein	A bibenzoquinone derivate that inhibits PO-activity (Feng et al., 2015).
Cotesia rubecula	*Pieris rapae*	Vn50 (SPH homologue)	Inhibitor of host proPO-activation. In a *M. sexta* model Vn50 down-regulates proPO-activation by PAP-1, SPH-1,-2 (Asgari et al., 2003; Zhang et al., 2004).
Isaria javanica	*Aedes aegypti*	Uncharacterized	Strain specific subversion of the melanisation response via reduced PPO3 and PPO expression and compromised Toll pathway (Ramirez et al. 2018).
Leptopilina boulardi	*Drosophila yakuba*	LbSPNY	Parasitoid venom serpin that inhibits proPO-activation (Colinet et al., 2009).
Metarhizium robertsii	*Galleria mellonella* *Drosophila melanogaster*	MrMP4	Metalloproteinase preventing PO activities by proPO-cleavage at other sites than positions used by proPO-activating serine proteinases (Huang et al., 2020).
Metarhizium robertsii	*Bombyx mori*	*Dtx S1, Dtx S2*	*Destruxin peptides that prevent haemolymph melanisation probably via prevention of proPO activation (Wang et al., 2012).*
Microplitis demolitor bracovirus	*Manduca sexta,* and other lepidopteran	Egf1.0, Egf1.5	Virally encoded inhibitors of proPO-activating proteinases 1,2 (Beck & Strand, 2007; Lu et al., 2008, 2010).
Nasonia vitripennis	*Musca domestica*	NvSPPI	Small pacifastin-type protease inhibitor that prevents host proPO-activation (Qian et al., 2017).
Nasonia vitripennis	*Musca domestica*	NvKSPI-1,2	Two Kazal type inhibitors preventing proPO-activation (Qian et al., 2015).
Nasonia vitripennis	Boettcherisca peregrina	*nasonin-3*	Defensin-like peptide that inhibits PO-activity (Tian et al., 2010).
Nosema bombycis	*Bombyx mori*	Serpin 6 (NbSPN6)	NbSPN6 will target the prophenoloxidase activating enzyme (Bao et al., 2019).
Photorhabdus luminescens	*Manduca sexta*	(E)-1,3-dihydroxy-2-(isopropyl)-5-(2-phenylethenyl)benzene	Secreted hydroxystilbene compound with capacity to inhibit activated PO (Eleftherianos et al., 2007).
Photorhabdus temperata	*Galleria mellonella*	phthalic acid	Inhibits PO activity and nodule formation (Ullah et al., 2014).
Pseudomonas aeruginosa	*Bombyx mori*	Elastase B (*lasB*)	Infections by *ΔlasB* mutants are causing much higher PO activities than infections by the wild type (Ma et al., 2019).
Scleroderma guani	*Tenebrio molitor*	SguaSPH	A serine protease homologue interfering with host proPO-activation (Wu et al., 2020).
Scleroderma guani	*Ostrinia furnacalis*	venom superoxide	One parasitoid superoxide, SguaSOD3, suppresses host melanisation as demonstrated with dismutase recombinant protein (Liu et al., 2018).
Venturia canescens	Lepidopterans	Serine protease	Endoparasitoid inhibits melanisation in calyx fluid (Beck et al., 2000).
White spot syndrome virus	*Penaeus monodon*	WSSV164 protein	The viral protein suppresses PmPO2 and PmPO2 activities (Sangsuriya et al., 2018).
White spot syndrome virus	*Pacifastacus leniusculus*	WSSV28 protein	VP 28 Interacts with a host clip serine protease, PlcSP, leading to a compromised melanisation response (Guo et al., 2017).
White spot syndrome virus	*Penaeus monodon*	WSSV453 protein	Inhibits proPO-activation by preventing PPAE2 to become enzymatically active and thereby activate both PmPO1 and PmPO2. WSSV453 does not interfere with already activated PPAE2 (Sutthangkul et al., 2015, 2017).
Wigglesworthia	*Drosophila melanogaster, Glossina morsitans*	unknown, perhaps bacterial odorants	Obp expression indirectly regulate melanisation via *lozenge* expression and hematopoiesis of crystal cells, a major proPO source (Benoit et al., 2017).
Xenorhabdus nematophila, Photorhabdus luminiscens	*Spodoptera frugiperda, Galleria mellonella*	Rhabduscin gene cluster (e.g. rhabduscin)	Products of the gene cluster are secreted and will inhibit PO activity (Nunez-Valdez et al., 2019, Crawford et al., 2012).

inhibitor. In *M. sexta* serpins 1 J, 3, 4, 6 are all reported to inhibit different proPO-cascade proteases (reviewed in Kanost et al., 2004), *M. sexta* serpin 12 is an inhibitor of HP14 and thereby both proPO and Toll activation (Wang et al., 2020a) and serpin 7 an inhibitor of the PAP-3 in the same species (Suwanchaichinda et al., 2013). A large number of other serpins are known to inhibit melanization in many insect and crustacean species, e.g. serpin 32 in *B. mori* (Wang et al., 2019) and many other (reviewed in Meekins et al., 2017; Tassanakajon et al., 2018). Other types of proteinase inhibitors besides conventional serpins also regulate melanization, an interesting example is the crustacean high molecular weight inhibitor pacifastin, founding member of a relatively newly discovered class of proteinase inhibitors and an inhibitor of the crustacean proPO-activating enzyme itself (Liang et al., 1997). Pacifastins, characterised by multiple domains possessing six cysteine residues in a conserved pattern (Breugelmans et al., 2009) are present widely in arthropods and some other phyla as well although less in known about their possible function in immunity except for crustaeans. One functionally and structurally characterised insect pacifastin is *Nosema vitripennis* small pacifastin protease inhibitor, NvSSPI (Qian et al., 2017). NvSSPI is present in the venom of this parasitic wasp and becomes injected into the insect hosts whereupon it inhibits proPO-activation and thus aid parasite establishment (Table 1).

5.2. Melanization inhibition proteins

Both in insects (Zhao et al., 2005) and in crustaceans (Söderhäll et al., 2009) melanization inhibition proteins (MIPs) without any apparent homology to serpins or other proteinases have been characterised. These proteins negatively regulate the melanization reactions by reactions so far uncharacterised. Both proteins contain an Asp-rich domain that by mutational analysis in the crayfish *P. leniusculus* was found to be necessary for its inhibitory activity (Söderhäll et al., 2009). A recombinant shrimp MIP was able to inhibit PO-activity in vitro using crude hemolymph as source of proPO and LPS (Noothuan et al., 2017). Possibly MIPs or other so far uncharacterised factors may prevent the formation of reactive quinones without restricting factors with other immunologic activities to arise from the proPO protein.

5.3. Amyloid proteins

Amyloid proteins are well known to spatially regulate the deposition of melanin in vertebrate melanosomes (reviewed in Bissig et al., 2016) but have so far received a relatively restricted attention in invertebrate melanogenesis. However, a protein apparently homologous to p102 is known to participate in the melanization reaction of the morula cells in the ascidian *Botryllus schlosseri* (Franchi et al., 2018). A finding by Di Prisco et al. (2016) is suggestive of a role for an amyloid protein in arthropod melanization as well. Deformed wing virus is reducing the capacity of melanotic encapsulation in honey bee, after the virus was transferred there by its symbiont the bee parasitic mite *Varroa destructor*. The virus facilitates mite survival by suppressing the bee immunity measured as melanization and encapsulation capacities. The authors observed that Amel\102, a transcript related to similar proteins found associated with melanin in both insects (Falabella et al., 2012; Di Lelio et al., 2014) and ascidians (Franchi et al., 2019), was downregulated in the presence of the virus. Lower levels of Amel\102 was accompanied by a reduced capacity to encapsulate and melanize foreign objects (Di Prisco et al., 2016).

6. Immunologic effects of proPO gene products

An increasing amount of data is demonstrating the importance of the proPO-system in host defence against a variety of pathogens. Consequently, a large number of host mechanisms to avoid the effects of this system has evolved during numerous host-pathogens encounters. Table 1 contains more than 25 compounds produced by different invertebrate pathogens to counter the effects of the melanization cascade. Its diverse content, ranging from viruses, bacteria, fungi, parasites with respect to pathogens producing them and the variety of molecules employed by the pathogens ranging from low molecular weight compounds to microRNAs and proteins is a good illustration of the immunologic potential of the proPO-system.

6.1. Effects against bacteria and parasites

An active PO will produce toxic intermediates and short-lived radicals (Nappi and Christensen, 2005). The melanin itself, although chemically inert, is shielding the pathogen or the animals' own damaged tissues and creates a physical and perhaps a chemical barrier to the rest of the host tissues. Early knock-down experiments directed towards proPO genes by the use of RNAi showed that in crayfish proPO expression contributed significantly to the resistance of the animals against *Aeromonas hydrophila* (Liu et al., 2007) and similar experiments in shrimp demonstrated increased resistance towards *Vibrio harveyi* (Amparyup et al., 2009). In an insect, *Manduca sexta*, a pathogenic bacterium, *Photorhabdus luminescens*, was shown to specifically target the proPO-system by secreted hydoxystilbenes in order to establish an infection (Eleftherianos et al., 2007) and another *Photorhabdus* strain was demonstrated to make phthalic acid, a compound with capacity to inhibit PO-activity (Ullah et al., 2014). As shown in Table 1 several other pathogenic bacteria have evolved means to interfere with the host proPO-system; *Xenorhabdus nematophila* and the above-mentioned *P. luminescens* are producing rhabduscin and related products capable of inhibiting PO (Nunez-Valdez et al., 2019; Crawford et al., 2012). A *Pseudomonas aeruginosa* strain pathogenic on silk worm is synthesising an elastase whose experimental deletion is causing much higher PO-activities and reduced virulence in the insect (Ma et al., 2019).

In the mosquito *Anopheles gambiae* there are 9 PPO genes making it more challenging to ascertain the role of POs in immunity. Silencing *PPO2*, *PPO3* or *PPO9* individually each increased the survival of the *Plasmodium berghei* oocysts (Kwon and Smith, 2019). The PPOs seem not to effect infection rates but more long term survival as no effects on parasite numbers were seen 2 days post infection but was evident 8 days post infection. Silencing of *PPO5,*6 or *8* was without effect. However, targeting some of the PPO genes had side effects on the expression of some of the PPO genes making it difficult to interpret the contribution of each PPO gene in resistance in each single case. More recently, analyses of mutants in *Drosophila melanogaste*r have significantly broadened our understanding on how POs contribute to resistance towards both bacteria and parasites. In the fly three genes, PPO1,2,3, each has specific but often overlapping roles in the immunity. A double mutant defective for both PPO1 and 2 is more susceptible towards a *Staphylococcus aureus* infection than each single PPO null mutant (Dudzic et al., 2019). A triple PPO1-3 mutant was more susceptible to infections by the nematode *Steinernema carpocapsae* harbouring its symbiotic bacterium *Xenorhabdus nematophila* than any double or single PPO mutant (Cooper et al., 2019). However, when using axenic nematodes a compensatory activity by remaining PPOs were often seen in single and double PPO mutants.

6.2. Antifungal effects

Melanization seems to be a general and effective weapon against pathogenic fungi in arthropods such as insects (for a treatise of the ecological implications the reader is referred to Gonzalez-Santoyo and Cordoba-Aguilar, 2012). Also, pathogenic oomycotan parasites, a group of organisms with a fungal-like life style may be effectively controlled by the melanization reaction as demonstrated in a comparison by parasite-resistant and susceptible freshwater crayfish species (Söderhäll and Ajaxon, 1982; Cerenius et al., 2003). In the mosquito *A. gambiae* will knock-down of TEP1 and CLIPA8 genes, key regulators of the melanization response in this insect, greatly facilitate the establishment of a

Beauveria bassiana infection, an important fungal pathogen on insects and a potential biocontrol agent (Yassine et al., 2012). In a related mosquito species this pathogen was recently demonstrated to affect the Toll response and the melanization reaction via silencing RNA that was released via exosomes (microvesicles) from the pathogen to be taken up by host cells (Cui et al., 2019). This specialised pathogen controls timing of the silencing RNA in order not to stimulate synthesis of proPO activating enzyme, CLIPBP9, in the hemocoel of the insect. In the mosquito *A. aegypti* an infection with *B. bassiana* results in increased expression of the *PPO3* gene (Wang et al., 2015). Wang et al. (2017) followed up this finding by injecting recombinant *A. aegypti* PPO3 protein into the hemocoel of the mosquito, a treatment that was found to increase the survival of fungus-infected animals. In *D. melanogastor* fungi can trigger a melanization reaction that appears effective against several types of fungi (Matskevich et al., 2010) and the double PO1/PO2 mutant mentioned above was found to be susceptible towards four different tested fungi (Binggeli et al., 2014). Several specialised pathogenic fungi have developed means to specifically interfere with the proPO-system and are thereby preventing the host to launch a fully effective immune response. Several examples of host-derived compounds used by parasitic fungi and other pathogens to interfere with proPO-activation or PO-activity are listed in Table 1. One example of such an interference is demonstrated by the specialised entomopathogenic fungus, *Metharizium robertsii* that secrets a family of apparently dedicated metalloproteinases with capacity to proteolytically degrade insect proPO in a way that does not result in activation of the proPO (Huang et al., 2020). When a number of *Isaria* species, differing in pathogenicity towards *A. aegypti* mosquitoes, were tested with respect to PO activities and PO gene expression the most pathogenic fungi were able to reduce PO-activities without apparent effects on gene expression (Ramirez et al., 2018). The mechanism for this fungal inhibition of the mosquito defence is not known but some other fungal pathogens such as *B. bassiana* have been shown both to synthesise effective PO-inhibitors such as the bibenzoquinone oosporein (Feng et al., 2015) and to produce a microRNA with capacity to prevent Toll activation and modulate proPO-activation (Cui et al., 2019) whereas *Aspergillus niger* is making cell wall components that are binding the proPO-activating complex (Stączek et al., 2020).

6.3. Antiviral effects

Although the precise mechanism(s) remains to establish the melanization reaction has strong effects against some viruses e.g. against several DNA and RNA viruses in the tobacco budworm (Ourth and Renis, 1993; Shelby and Popham, 2006), against arboviruses in mosquitoes (Rodriguez-Andres et al., 2012) and baculovirus in cotton bollworm (Yuan et al., 2017; Wang et al., 2020b). One possible mediator of this antiviral effect is the melanization intermediate 5,6-hyroxyindole as suggested by experiments performed in vitro with baculovirus and lambda phages (Zhao et al., 2011). A growing list of examples of viruses interfering with the proPO-system has emerged during the last years (Table 1) lending credit to the view that the system has antiviral capacity. An early example of this is the bracovirus-encoded Egf1.0 protein, an inhibitor of PAP1 and 2 in *Manduca sexta* (Lu et al., 2008). Several viruses prevent PO activation by interfering with host PO-activating proteinases (Table 1). In crustaceans the devastating white spot syndrome virus (WSSV) is suppressing PO-activity in shrimp (Sutthangkul et al., 2015) and crayfish (Jiravanichpaisal et al., 2006). Two viral proteins were found to interact with the proPO-system, WSSV453 to the ProPO-actvating enzyme PmPPAE2 thereby preventing PO-activation (Sutthangkul et al., 2017) and WSSV164 which is reducing PO-activity by binding to both PO1 and PO2 (Sangsuriya et al., 2018). In crayfish a clip domain containing serine proteinase was found to bind to WSSV through VP28 and it was shown that this proteinase was necessary for WSSV internalisation to the hematopoietic cells (Guo et al., 2017). The WSSV is also targeting gene expression of another protease in the proPO-cascade, PmPPAE3, via up-regulating a host microRNA, miR-315 (Jaree et al., 2018). It seems very likely that once investigated many further examples of viruses interfering with the host proPO-system will be detected. In silk worm (*Bombyx mori*) nucleopolyhedrovirus infectivity is increased by knock-downs of host serpin-2, an inhibitor of the proteolytic activation of proPO in this insect (Toufeeq et al., 2019). In control silk worms serpin-2 gene expression is enhanced by the nucleopolyhedrovirus leading to a suppressed melanization in this insect (Toufeeq et al., 2019). Another virus that actively prevents melanization is bollworm, *Helicoverpa armigera,* nucleopolyhedrovirus that was found to reduce host PPO2 expression by more than 18-fold (Xing et al., 2017) strongly suggesting that this gene is of crucial importance in anti-viral defence.

6.4. Effects in the gut and outside the body

The importance of the PO-activity stretches beyond the boundaries of the body itself; the melanization reaction helps to disinfect faeces and thereby the plant matter upon which the animal feeds as exemplified in PO residing in gut in several insect species (Shao et al., 2012). The moulting fluids and the exuviae may contain PO and other proPO-system components to protect the insect from outside attack when its outer tissues are as most vulnerable during and shortly after ecdysis (Zhang et al., 2014, 2017). Another example is detoxification of harmful plant phenolics by PO in the gut (Wu et al., 2015). Their importance was demonstrated by the fact that insects with PO deletion mutants were killed by plant phenolics in the feed (Wu et al., 2015). An interesting use of PO was recently demonstrated in the social aphid *Nipponaphis monzeni*. This insect forms large galls on its plant host containing large numbers of the aphid. If the gall is broken specialised soldiers from the aphid colony seal the injured gall by releasing large volumes of body fluid that quickly coagulate and seal the injury. The insect has undergone large adaptations to achieve this remarkable process, its soldiers body cavity possesses large globular cells highly enriched in PO, the PO substrate tyrosine and a unique repeat containing protein (RCP) obviously serving as a scaffold in the clotting/melanization reaction (Kutsukake et al., 2020). It deserves consideration as hemolymph in other insects, e.g. fruit flies, after coagulation or clotting the clot becomes melanized although other proteins than the RCP such as fondue is involved in this case (Lindgren et al., 2008; Theopold et al., 2004).

7. Prophenoloxidase, a central component in an invertebrate immune system that is functionally analogous to vertebrate complement

Progress in invertebrate immunity has pinpointed some striking functional similarities between the melanization cascade and vertebrate complement. In arthropods in particular, the presence of modular serine proteinases at the top of the cascade, thiolester proteins, lectins, a chain of zymogenic proteinases resulting in the production of degranulating opsonising factors are functionally similar to complement proteins in spite of the apparent absence of complement factors in their genomes. A word of caution is necessary here, the complete composition of the melanization cascades is not fully deciphered in any species and there are apparently significant differences even within, for example the arthropods, with respect to which factors constitute such a cascade.

To this can now be added the fact that as shown in crustaceans the proPO itself by limited proteolysis is producing biologically active peptides promoting hemocyte differentiation and also with microbiocidal activities (Fig. 2). Proteolytic processing both of proPO and the proPO-activating enzyme gives rise to an N-terminal peptide fragment with several immunogenic activities such as opsonic, agglutinin, bactericidal activities and to promote hemocyte DNA synthesis and differentiation (Sirikharin et al., 2020; Jearaphunt et al., 2014; Wang et al., 2001). Interestingly, also the C3a peptide of vertebrate complement possesses antimicrobial activities against both Gram positive and negative bacteria (Nordahl et al., 2004). A recombinant peptide,

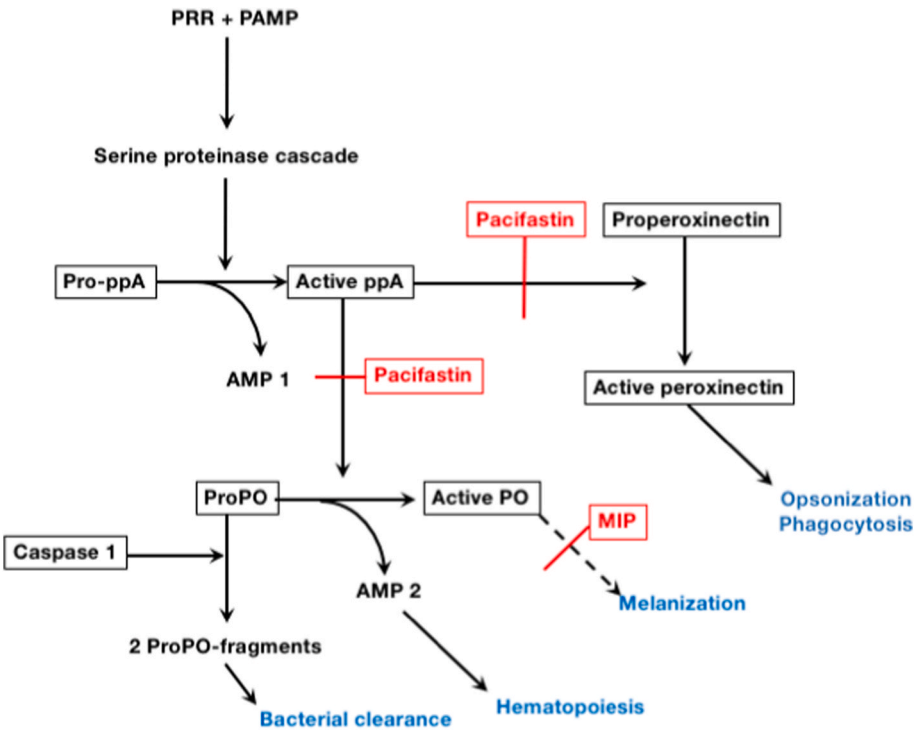

Fig. 2. The prophenoloxidase system as deciphered in crustaceans. In blue different biological activities generated by the system and in red factors that inhibit activation or the activity of phenoloxidase. Pattern recognition proteins (PRRs) will initiate a serine proteinase cascade that generates among other factors phenoloxidase (PO), peroxinectin, cytokine-like factors and proPO fragments with strong agglutinating capacities. AMP, antimicrobial peptide; MIP, melanization inhibiting protein; ppA, prophenoloxidase activating enzyme; PAMP, pathogen-associated molecular patterns.

corresponding to the proPO N-terminal part in *P. leniusculus* as produced by ppA cleavage, exhibits a strong agglutinating activity towards both Gram positive and negative bacteria and experiments with *E. coli* demonstrated that this peptide possesses a bactericidal activity and is causing cell wall damage (Jearaphunt et al., 2014). It is not known whether this peptide as vertebrate complement has a membrane perturbing effect as well. More recent data show that the proPO N-terminal has cytokine activities being active in inducing hemocyte differentiation and perhaps also hemocyte mobilisation as well (Sirikharin et al., 2020). The effects of this peptide can be seen in the hematopoietic tissue e.g. increased cell flattening and in the circulation by increased numbers of granular hemocytes in the hemolymph. It induces differentiation of granular hemocytes (the cell type producing most of the proPO in crustaceans) as well as DNA-synthesis to increase the hemocyte number. The exposure to the peptide will trigger an oxidative burst, an event known to promote hematopoiesis (Noonin et al., 2012). Different proteases may give rise to a variety of peptide products from the proPO protein which can express different activities (Jearaphunt et al., 2014) and pathogens may produce proteases aimed at cleaving the proPO at other sites than the ones creating the active PO as exemplified by the insect pathogenic fungus *Metharzium robertsii* M35 metalloproteases (Huang et al., 2020 and Table 1). The proteolytic cascade that ultimately results in proPO activation will, at least in crustaceans, also process the myeloperoxidase homologue pro-peroxinectin into peroxinectin (Lin et al., 2007). Hereby cell adhesion and opsonic activities are generated. Furthermore, the N-terminal cleavage product after cleavage of the proform of the *P. leniusculus* proPO-activating enzyme possesses antimicrobial activities (Wang et al., 2001), by recombinant techniques some of these peptides have been shown to agglutinate bacteria. Furthermore, recombinant proteolytic fragments from *D. melanogaster* and *B. mori* PO bind to fungal spores (Zhang et al., 2017). Although highly speculative at this stage the possibility exists that PO itself may express an endopeptidase activity. Both mushroom and apple tyrosinases can cleave a protein substrate, a reaction that is dependent of the histidine residue in the PO active site (Biundo et al., 2020). The reaction as measured in vitro is far too slow for likely being of physiological importance, but the possibility of a proteinase activity inherent with the PO deserves further studies.

It was observed in several of the investigations described earlier (Dudzic et al., 2019; Kwon and Smith, 2019) that although the proPO-gene was required to achieve an immunological effect (as suggested by data obtained from null mutants of proPO-genes), its melanin formation is not necessarily required for the immune effect. In such cases other effects by parts of the PO protein or MIP activities towards the active enzyme may provide an explanation. Regardless how these effects beyond melanization were produced one should not overlook the fact that although the melanization reaction itself confers an immune effect other factors or, indeed, parts of the proPO molecule and other molecules of the proPO-system can give rise to important immune factors.

8. Conclusions

Melanin production from different types of phenoloxidases confer immunity from a variety of pathogens ranging from viruses and microorganisms to parasites.

The arthropod (insect and crustacean) prophenoloxidase expresses a variety of activities including such influencing hemocyte proliferation and differentiation, opsonins and microbiocidal activities independent of and even without melanin production.

Proteolytic processing of prophenoloxidase and its activating enzyme gives rise to several peptide fragments with a variety of separate activities in a process highly reminiscent of vertebrate complement system activation in spite of being structurally unrelated to complement.

Pathogens influence prophenoloxidase activation and thereby what types of immune effects that will be produced. An increasing number of specialised pathogens – from parasites to viruses – have been identified who can synthesise compounds specifically aimed at the prophenoloxidase system.

In other invertebrates outside the arthropods phylogenetically unrelated phenoloxidases are participating in melanization reactions obviously aimed at intruders and/or aberrant tissues.

References

Adachi, Y., Ishii, M., Kanno, T., Tetsui, J., Ishibashi, K., Yamanaka, D., Miura, N., Ohno, N., 2019. N-terminal (13)-β-D-glucan recognition proteins from insects recognize the difference in ultra-structures of (13)-β-D-glucan. Int. J. Mol. Sci. https://doi.org/10.3390/ijms20143498.

Aguilera, F., McDougall, C., Degnan, B.M., 2013. Origin, evolution and classification of type-3 copper proteins: lineage-specific gene expansions and losses across the Metazoa. BMC Evol. Biol. 13, 96.

Aguilera, F., McDougall, C., Degnan, B.M., 2014. Evolution of the tyrosinase gene family in bivalve molluscs: independent expansion of the mantle gene repertoire. Acta Biomater. 10, 3855–3865.

Amparyup, P., Charoensapsri, W., Tassanakajon, A., 2009. Two prophenoloxidases are important for the survival of *Vibrio harveyi* challenged shrimp *Penaeus monodon*. Dev. Comp. Immunol. 33, 247–256. https://doi.org/10.1016/j.dci.2008.09.003.

An, C., Zhang, M., Chu, Y., Zhao, Z., 2013. Serine protease MP2 activates prophenoloxidase in the melanization immune response of Drosophila melanogaster. PloS One 8 (11), e79533. https://doi.org/10.1371/journal.pone.0079533.

Ardisson-Araujo, D., Rohrmann, G.F., Ribeiro, B.M., Clem, R.J., 2015. Functional characterization of hesp018, a baculovirus-encoded serpin gene. J. Gen. Virol. 96, 1150–1160. https://doi.org/10.1099/vir.0.000041.

Asgari, S., Zhang, G.M., Zareie, R., Schmidt, O., 2003. A serine proteinase homolog venom protein from an endoparasitoid wasp inhibits melanization of the host hemolymph. Insect Biochem. Mol. Biol. 33, 1017–1024.

Bailey, G.F., Bilsky, A.M., Rowland, M.B., Poole, A.Z., 2019. Characterization and expression of tyrosinase-like genes in the anemone *Exaiptasia pallida* as a function of health and symbiotic state. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2019.103459.

Bao, J., Liu, L., An, Y., Ran, M., Ni, W., Chen, J., Wei, J., Li, T., Pan, G., Zhou, Z., 2019. *Nosema bombycis* suppresses host hemolymph melanization through secreted serpin 6 inhibiting the prophenoloxidase activation cascade. J. Invertebr. Pathol. 168, 107260. https://doi.org/10.1016/j.jip.2019.107260, 2019 Nov.

Beck, M.H., Strand, M.R., 2007. A novel polydnavirus protein inhibits the insect prophenoloxidase activation pathway. Proc. Natl. Acad. Sci. U.S.A. 104, 19267–19272. https://doi.org/10.1073/pnas.0708056104.

Beck, M., Theopold, U., Schmidt, O., 2000. Evidence for serine protease inhibitor activity in the ovarian calyx fluid of the endoparasitoid *Venturia canescens*. J. Insect Physiol. 46, 1275–1283. https://doi.org/10.1016/S0022-1910(00)00048-2.

Benoit, J.B., Vigneron, A., Broderick, N.A., Wu, Y., Sun, J.S., Carlson, J.R., Aksoy, S., Weiss, B.L., 2017. Symbiont-induced odorant binding proteins mediate insect host hematopoiesis. Elife 6, e19535. https://doi.org/10.7554/eLife.19535, 2017 Jan 12.

Beschin, A., Bilej, M., Hanssens, F., Raymakers, J., Van Dyck, E., Revets, H., Brys, L., Gomez, J., De Baetselier, P., Timmermans, M., 1998. Identification and cloning of a glucan- and lipopolysaccharide-binding protein from *Eisenia foetida*. J. Biol. Chem. 273, 22762–22770.

Bidla, G., Dushay, M.S., Theopold, U., 2007. Crystal cell rupture after injury in Drosophila requires the JNK pathway, small GTPases and the TNF homolog Eiger. J. Cell Sci. 120, 1209–1215.

Binggeli, O., Neyen, C., Poidevin, M., Lemaitre, B., 2014. Prophenoloxidase is required for survival to microbial infections in Drosophila. PLoS Pathog., e1004067

Bissig, C., Rochin, L., van Niel, G., 2016. PMEL amyloid fibril formation: the bright steps of pigmentation. Int. J. Mol. Sci. 17, 1438. https://doi.org/10.3390/ijms17091438.

Biundo, A., Braunschmid, V., Pretzler, M., Kampatsikas, I., Darnhofer, B., Birner-Gruenberger, R.T., Rompel, A., Guebitz, G.M., 2020. Polyphenoloxidases exhibit promiscuous proteolytic activity. Communications Chemistry 3, 62. https://doi.org/10.1038/s42004-020-0305-2.

Blandin, S.A., Wang-Sattler, R., Lamacchia, M., Gagneur, J., Lycett, G., Ning, Y., Levashina, E.A., Steinmetz, L.M., 2009. Dissecting the genetic basis of resistance to malaria parasites in *Anopheles gambiae*. Science 326, 147–150.

Breugelmans, B., Simonet, G., van Hoef, V., van Soest, S., van den Broeck, J., 2009. Pacifastin-related peptides: structural and functional characteristics of a family of serine peptidase inhibitors. Peptides 30, 622–632. https://doi.org/10.1016/j.peptides.2008.07.026.

Buchon, N., Poidevin, M., Kwon, H.-M., Guillou, A., Sottas, V., Lee, B.L., Lemaitre, B., 2009. A single modular serine protease integrates signals from pattern-recognition receptors upstream of the *Drosophila* Toll pathway. Proc. Nat. Acad. Sci. USA. 106, 12442–12447. https://doi.org/10.1073/pnas.0901924106.

Cerenius, L., Söderhäll, K., 2004. The prophenoloxidase-activating system in invertebrates. Immunol. Rev. 198, 116–126.

Cerenius, L., Liang, Z., Duvic, B., Keyser, P., Hellman, U., Palva, E.T., Iwanaga, S., Söderhäll, K., 1994. Structure and biological activity of a 1,3-β-D-glucan-binding protein in crustacean blood. J. Biol. Chem. 269, 29462–29467.

Cerenius, L., Bangyeekhun, E., Keyser, P., Söderhäll, I., Söderhäll, K., 2003. Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. Cell Microbiol. 5, 353–357. https://doi.org/10.1046/j.1462-5822.2003.00282.x.

Cerenius, L., Lee, B.L., Söderhäll, K., 2008. The proPO-system: pros and cons for its role in invertebrate immunity. Trends Immunol. 6, 263–271.

Coaglio, A.L., Ferreira, M.A.N.D., dos Santos Lima, W., Pereira, C.A.J., 2018. Identification of a phenoloxidase- and melanin-dependent defence mechanism in *Achatina fulica* infected with *Angiostrongylus vasorum*. Parasites *V*ectors 11, **1**13. https://doi.org/10.1186/s13071-018-2710-2, 2018.

Coates, C.J., Nairn, J., 2014. Diverse immune functions of hemocyanins. Dev. Comp. Immunol. 45, 43–55.

Colinet, M., Dubbuffet, A., Cazes, D., Moreau, S., Drezen, J.-M., Poirié, M., 2009. A serpin from the parasitoid wasp Leptopilina boulardi targets the Drosophila phenoloxidase cascade. Dev. Comp. Immunol. 33, 681–689. https://doi.org/10.1016/j.dci.2008.11.013.

Cooper, D., Wuebbolt, C., Heryanto, C., Eleftherianos, I., 2019. The prophenoloxidase system in *Drosophila* participates in the antinematode immune response. Mol. Immunol. 109, 88–98.

Crawford, J.M., Portmann, C., Zhang, X., Roeffaers, M.B., Clardy, J., 2012. Small molecule perimeter defense in entomopathogenic bacteria. Proc. Natl. Acad. Sci. U.S. A. 109, 10821–10826. https://doi.org/10.1073/pnas.1201160109.

Cui, C., Wang, Y., Liu, J., Zhao, J., Sun, P., Wang, S., 2019. A fungal pathogen deploys a small silencing RNA that attenuates mosquito immunity and facilitates infection. Nat. Commun. 10, 4298.

De Gregorio, E., Han, S.J., Lee, W.J., Baek, M.J., Osaki, T., Kawabata, S., Lee, B.L., Iwanaga, S., Lemaitre, B., Brey, P.T., 2002. An immune-responsive Serpin regulates the melanization cascade in *Drosophila*. Dev. Cell 3, 581–592. https://doi.org/10.1016/s1534-5807(02)00267-8.

Decker, H., Jeanicke, E., 2004. Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanin. Dev. Comp. Immunol. 28, 673–687.

Destoumieix-Garzon, D., Salunier, D., Garnier, J., Jouffrey, C., Bulet, P., Bachere, E., 2001. Antifungal peptides are generated from the C-terminus of shrimp hemocyanin in response to microbial challenge. J. Biol. Chem. 276, 47070–47077.

Di Lelio, I., Varricchio, P., Di Prisco, G., Marinelli, A., Lasco, V., Caccia, S., Casartelli, M., Giordana, B., Rao, R., Gigliotti, S., Pennachio, F., 2014. Functional analysis of an immune gene of *Spodoptera littoralis* by RNAi. J. Insect Physiol. 64, 90–97.

Di Prisco, G., Annoscia, D., Margiotta, M., Ferrara, R., Varricchio, P., Zanni, V., Caprio, E., Nazzi, F., Pannacchio, F., 2016. A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health. Proc. Nat. Acad. Sci. USA 113, 3203–3208. https://doi.org/10.1073/pnas.1523515113.

Dudzic, J.P., Kondo, S., Ueda, R., Bergman, C.M., Lemaitre, B., 2015. Drosophila innate immunity: regional and functional specialization of prophenoloxidases. BMC Biol. https://doi.org/10.1186/s1215-015-0199-6.

Dudzic, J.P., Hanson, M.A., Iatsenko, I., Kondo, S., Lemaitre, B., 2019. More than black or white: melanization and Toll share regulatory serine proteases in *Drosophila*. Cell Rep. 27, 1050–1061. https://doi.org/10.1016/j.celrep.2019.03.101.

Duvic, B., Söderhäll, K., 1990. Purification and characterization of a β-1,3-glucan binding protein from the plasma of the crayfish *Pacifastacus leniusculus*. J. Biol. Chem. 265, 9327–9332.

Duvic, B., Söderhäll, K., 1992. Purification and characterization of a β-1,3-glucan binding protein receptor from blood cells of the crayfish *Pacifastacus leniusculus*. Eur. J. Biochem. 207, 223–228.

D'Alba, L., Shawkey, M.D., 2019. Melanosomes: biogenesis, properties, and evolution of an ancient organelle. Physiol. Rev. 99, 1–19. https://doi.org/10.1152/physrev.00059.2017.

El Moussawi, L., Nakhleh, J., Kamareddine, L., Osta, M.A., 2019. The mosquito melanisation response requires hierarchical activation of non-catalytic clip domain serine protease homologs. PLoS Pathog. 15 (11), e1008194.

Eleftherianos, I., Boundy, S., Joyce, S.A., Aslam, S., Marshall, J.W., Cox, R.J., Simpson, T. J., Clarke, D.J., ffrench-Constant, R.H., Reynolds, S.E., 2007. An antibiotic produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. Proc. Nat. Acad. of Sci. USA 104, 2419–2424. https://doi.org/10.1073/pnas.0610525104.

Falabella, P., Riviello, L., Pascale, M., Lelio, I.D., Tettamanti, G., Grimaldi, A., Iannone, C., Monti, M., Pucci, P., Tamburro, A.M., Deeguileor, M., Gigliotti, S., Pennacchio, F., 2012. Functional amyloids in insect immune response. Insect Biochem. Mol. Biol. 42, 203–211. https://doi.org/10.1016/j.ibmb.2011.11.011.

Feng, P., Shang, Y., Cen, K., Wang, C., 2015. Oosporein biosynthesis and virulence aid in fungus. Proc. Nat. Acad. Sci. USA 112, 11365–11370. https://doi.org/10.1073/pnas.1503200112.

Franchi, N., Balarin, L., Personato, A., Cima, F., Grimaldi, A., Girardello, R., de Eguileor, M., 2019. Functional amyloidogenesis in immunocytes from the colonial ascidian *Botryllus schlosseri*: evolutionary perspective. Dev. Comp. Immunol. 90, 108–120. https://doi.org/10.1016/j.dci.2018.09.010.

Goncalves, P., Vernal, J., Rosa, R.D., Yepiz-Plascencia, G., de Souza, C.R.B., Barracco, M. A., Perazzolo, L.M., 2012. Evidence for a novel biological role for the multifunctional β -1,3-glucan binding protein in shrimp. Mol. Immunol. 51, 363–367.

Gonzalez-Santoyo, I., Cordoba-Aguilar, A., 2012. Phenoloxidase: a key component of the insect immune system. Entomol. Exp. Appl. 142, 1–16.

Guo, E., Korkut, G.G., Jaree, P., Söderhäll, I., Söderhäll, K., 2017. *Pacifastcus leniusculus* serine protease interacts with WSSV. Fish Shellfish Immunol. 68, 211–219. https://doi.org/10.1016/j.fsi.2017.07.026.

Hall, M., Vanheusden, M.C., Söderhäll, K., 1995. Identification of the major lipoproteins in crayfish haemolymph as proteins involved in immune recognition and clotting. Biochem. Biophys. Res. Commun. 216, 939–946.

Hayakawa, Y., Sawada, M., Sekii, M., Sirasoonthorn, P., Shiga, S., Kamiya, K., Minakuchi, C., Miura, K., 2018. Involvement of *laccase2* and *yellow*-e genes in antifungal host defense of the model beetle. *Tribolium castaneum*. J. Invert. Pathol. 151, 41–49.

Hu, Y., Wang, Y., Deng, J., Jiang, H., 2016. The structure of prophenoloxidase (PPO) from *Anopheles gambiae* provides new insights into the mechanism of PPO activation. BMC Biol. 14, 2. https://doi.org/10.1186/s12915-015-0225-2, 2016.

Huang, T.S., Wang, H., Lee, S.Y., Johansson, M.W., Söderhäll, K., Cerenius, L., 2000. A cell adhesion protein from the crayfish *Pacifastacus leniusculus*, a serine proteinase homologue similar to *Drosophila* masquerade. J. Biol. Chem. 275, 9996–10001.

Huang, C., Hedi, M., Ranjan, K., Abraham, C., 2019. LACC1 required for NOD2-induced, ER stress-mediated innate immune outcomes in human macrophages and *LACC1* risk variants modulate these outcomes. Cell Rep. 29, 4525–4539.

Huang, A., Lu, M., Ling, E., Ping Li, P., Wang, C., 2020. A M35 family metalloprotease is required for fungal virulence against insects by inactivating host prophenoloxidases and beyond. Virulence 11 (1), 222–237. https://doi.org/10.1080/21505594.2020.1731126.

Issa, N., Guillaumot, N., Lauret, E., Matt, N., Schaeffer-Reiss, C., Van Dorsselaer, A., Reichhart, J.M., Veillard, F., 2018. The circulating protease Persephone is an immune sensor for microbial proteolytic activities upstream of the *Drosophila* Toll pathway. Mol. Cell. 69, 539–550. https://doi.org/10.1016/j.molcel.2018.01.029.

Jaree, P., Wongdontri, C., Somboonwiwat, K., 2018. White spot syndrome virus-induced shrimp miR-315 attenuates prophenoloxidase activation via *PPAE3* gene suppression. Front. Immunol. 9, 2184. https://doi.org/10.3389/fimmu.2018.02184.

Jearaphunt, M., Noonin, C., Jiravanichpaisal, P., Nakamura, S., Tassanakajon, A., Söderhäll, I., Söderhäll, K., 2014. Caspase-1-like regulation of the proPO-system and role of ppA and caspase-1-like cleaved peptides from proPO in innate immunity. PLoS Pathog. 10 (4), e1004059 https://doi.org/10.1371/journal.ppat.1004059.

Ji, C., Wang, Y., Guo, X., Hartson, S., Jiang, H., 2004. A pattern recognition serine proteinase triggers the prophenoloxidase activation cascade in the tobacco hornworm, *Manduca sexta*. J. Biol. Chem. 279, 34101–34106.

Jiang, R., Kim, E.-H., Gong, J.-H., Kwon, H.-M., Kim, C.-M., Ryu, K.-H., Park, J.-W., Kurokawa, K., Zhang, J., Gubb, D., Lee, B.-L., 2009. Three pairs of protease-serpin complexes cooperatively regulate the insect innate immune responses. J. Biol. Chem. 284, 35652–35658. https://doi.org/10.1074/jbc.M109.071001.

Jiravanichpaisal, P., Söderhäll, I., Söderhäll, K., 2006. Characterization of white spot syndrome virus replication in in vitro-cultured haematopoietic stem cells of freshwater crayfish, Pacifastacus leniusculus. J. Gen. Virol. 87, 847–854.

Junkunlo, K., Söderhäll, K., Söderhäll, I., Noonin, C., 2016. Reactive oxygen species affect transglutaminase activity and regulate hematopoiesis in a crustacean. J. Biol. Chem. 291, 17593–17601. https://doi.org/10.1074/jbc.M116.741348, 2016.

Kan, H., Kim, C.H., Kwon, H.M., Park, J.W., Roh, K.B., Lee, H., Park, B.J., Zhang, R., Zhang, J., Söderhäll, K., Ha, N.C., Lee, B.L., 2008. Molecular control of phenoloxidase-induced melanin synthesis in an insect. J. Biol. Chem. 283, 25316–25323. https://doi.org/10.1074/jbc.M804364200.

Kanost, M.R., Jiang, H., 2015. Clip-serine proteases as immune factors in insect hemolymph. Curr. Op. Insect Sci. 11, 47–55.

Kanost, M.R., Jiang, H., Yu, X.Q., 2004. Innate immune responses of a lepidopteran insect, *Manduca sexta*. Immunol. Rev. 198, 97–105.

Kim, Y., Ryu, J., Han, S., Choi, K., Nam, K., Jang, I., Lemaitre, B., Brey, P.T., Lee, W.J., 2000. Gram-negative bacteria-binding protein, a pattern recognition receptor for lipopolysaccharide and β-1,3-glucan that mediates the signaling for the induction of innate immune genes in *Drosophila melanogaster* cells. J. Biol. Chem. 275, 32721–32727.

Kim, C.H., Kim, S.J., Kan, H., Kwon, H.M., Roh, K.B., Jiang, R., Yang, Y., Park, J.W., Lee, H.H., Ha, N.C., Kang, H.J., Nonaka, M., Söderhäll, K., Lee, B.L., 2008. A three-step proteolytic cascade mediates the activation of the peptidoglycan-induced toll pathway in an insect. J. Biol. Chem. 283, 7599–7607. https://doi.org/10.1074/jbc.M710216200.

KonDo, Y., Yoda, S., Mizoguchi, T., Ando, T., Yamaguchi, J., Yamamoto, K., Banno, Y., Fujiwara, H., 2017. Toll ligand Spätzle3 controls melanization in the stripe pattern formation in caterpillars. Proc. Natl. Acad. Sci. U.S.A. 114, 8336–8341.

Kutsukake, M., Moriyama, M., Shigenobu, S., Meng, X.-Y., Nikoh, N., Noda, C., Kobayashi, S., Fukatsu, T., 2019. Exaggeration and co-option of innate immunity for social defense. Proc. Natl. Acad. Sci. U.S.A. 116, 8950–8959.

Kwon, H., Smith, R.C., 2019. Chemical depletion of phagocytic immune cells in *Anopheles gambiae* reveals dual roles of mosquito hemocytes in anti-*Plasmodium* immunity. Proc. Nat. Acad. Sci. USA 116, 14119–14128. https://doi.org/10.1073/pnas.1900147116.

Le Clech, W., Anderson, T.J.C., Chevalier, F.D., 2016. Characterization of hemolymph phenoloxidase activity in two *Biomphalaria* snail species and impact of *Schistosoma mansoni* infection. Parasites Vectors 9, 32. https://doi.org/10.1186/s13071-016-1319-6.

Lee, S.Y., Söderhäll, K., 2001. Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*. J. Immunol. 166, 7319–7326.

Lee, W.J., Lee, J.D., Kravchenko, V.V., Ulevitch, R.J., Brey, P.T., 1996. Purifaction and cloning of an inducible gram-negative bacteria-binding protein from the silk-worm, *Bombyx mori*. Proc. Nat. Acad. Sci. USA. 93, 7888–7893.

Lee, S.Y., Wang, R., Söderhäll, K., 2000. A lipopolysaccharide- and β-1,3-glucan binding protein from the freshwater crayfish *Pacifastacus leniusculus*. J. Biol. Chem. 275, 1337–1343.

Lee, S.Y., Lee, B.L., Söderhäll, K., 2003. Processing of an antimicrobial peptide from hemocyanin of the freshwater crayfish *Pacifastacus leniusculus*. J. Biol. Chem. 278, 7927–7933.

Lee, M.H., Osaki, T., Lee, J.Y., Baek, M.J., Zhang, R., Park, J.W., Kawabata, S., Söderhäll, K., Lee, B.L., 2004. Peptidoglycan recognition proteins involved in 1,3-beta-D-glucan-dependent prophenoloxidase activation system of insect. J. Biol. Chem. 279, 3218–3227. https://doi.org/10.1074/jbc.M309821200.

Levashina, E.A., Moita, L.F., Blandin, S., Vriend, G., Lagueux, M., Kafatos, F.C., 2001. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. Cell 104, 709–718.

Li, Y., Wang, Y., Jiang, H., Deng, J., 2009. Crystal structure of *Manduca sexta* prophenoloxidase provides insights into the mechanism of type 3 copper enzymes. Proc. Nat. Acad. Sci USA. 106, 17002–17006.

Li, Y.-P., Xia, M., Li, L., Song, C.-X., Wang, J.-L., Liu, X.-S., 2015. Molecular characterization of a peptidoglycan recognition protein from the cotton bollworm, Helicoverpa armigera and its role in the prophenoloxidase activation pathway. Mol. Immunol. 65, 123–132.

Liang, Z., Sottrup-Jensen, L., Aspán, A., Hall, M., Söderhäll, K., 1997. Pacifastin, a novel 155-kDa heterodimeric proteinase inhibitor containing a unique transferrin chain. Proc. Nat. Acad. Sci. USA 94, 6682–6687. https://doi.org/10.1073/pnas.94.13.6682.

Ligoxygakis, P., Pelte, N., Ji, C., Leclerc, V., Duvic, B., Belvin, M., Jiang, H., Hoffmann, J. A., Reichhart, J.M., 2002. A serpin mutant links Toll activation to melanization in the host defence of Drosophila. EMBO J. 2, 6330–6337. https://doi.org/10.1093/emboj/cdf661.

Lin, X., Cerenius, L., Lee, B.L., Söderhäll, K., 2007. Purification of properoxinectin, a myeloperoxidase homologue and its activation to a cell adhesion molecule. Biochim. Biophys. Acta 1770, 87–93. https://doi.org/10.1016/j.bbagen.2006.06.018.

Lindgren, M., Riazi, R., Lesch, C., Wilhelmsson, C., Theopold, U., Dushay, M.S., 2008. Fondue and transglutaminase in the Drosophila larval clot. J. Insect Physiol. 54, 586–592. https://doi.org/10.1016/j.jinsphys.2007.12.008.

Liu, H., Jiravanichpaisal, P., Cerenius, C., Lee, B.L., Söderhäll, I., Söderhäll, K., 2007. Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* infection in a crustacean. *Pacifastacus leniusculus*. J. Biol. Chem. 282, 33593–33598.

Liu, H., Wu, C., Matsuda, Y., Kawabata, S-i., Lee, B.L., Söderhäll, K., Söderhäll, I., 2011. Peptidoglycan activation of the proPO-system without a peptidoglycan receptor protein (PGRP)? Dev. Comp. Immunol. 35, 51–61.

Liu, N.Y., Huang, J.M., Ren, X.M., Xu, Z.W., Yan, N.S., Zhu, J.Y., 2018. Superoxide dismutase from venom of the ectoparasitoid Scleroderma guani inhibits melanization of hemolymph. Arch. Insect Biochem. Physiol. 99 (3), e21503 https://doi.org/10.1002/arch.21503, 2018 Nov.

Lu, Z., Beck, M.H., Wang, Y., Jiang, H., Strand, M.R., 2008. The viral protein Egf1.0 is a dual activity inhibitor of prophenoloxidase-activating proteinases 1 and 3 from *Manduca sexta*. J. Biol. Chem. 283, 21325–21333. https://doi.org/10.1074/jbc.M801593200.

Lu, Z., Beck, M.H., Strand, M.R., 2010. Egf1.5 is a second phenoloxidase cascade inhibitor encoded by *Microplitis demolitor* bracovirus. Insect Biochem. Mol. Biol. 40, 497–505. https://doi.org/10.1016/j.ibmb.2010.04.009.

Lu, A., Zhang, Q., Zhang, J., Yang, B., Wu, K., Xie, W., Luan, Y.-X., Ling, E., 2014. Insect prophenoloxidase: the view beyond immunity. Front. Physiol. https://doi.org/10.3389/fphys.2014.00252.

Luna-Acosta, A., Saulnier, D., Pommier, M., Haffner, P., De Decker, S., Renault, T., Thomas-Guyon, H., 2011. First evidence of a potential antibacterial activity involving a laccase-type enzyme of the prophenoloxidase system in Pacific oyster *Crassostrea gigas* hemocytes. Fish Shellfish Immunol. 31, 795–800.

Luna-Acosta, A., Breitwieser, M., Renault, T., Thomas-Guyon, T., 2017. Recent findings on phenoloxidases in bivalves. Mar. Polution Bull. 122, 5–16.

Ma, C.C., Kanost, M.R., 2000. A β -1,3-glucan recognition protein from an insect, *Manduca sexta*, agglutinates microorganisms and activates the phenoloxidase cascade. J. Biol. Chem. 275, 7505–7514.

Ma, L., Zhou, L., Lin, J., Ji, J., Wang, Y., Jiang, H., Shen, X., Lu, Z., 2019. Manipulation of the silkworm immune system by a metalloprotease from the pathogenic bacterium *Pseudomonas aeruginosa*. Dev. Comp. Immunol. 90, 176–185. https://doi.org/10.1016/j.dci.2018.09.017.

Ma, L., Liu, L., Zhao, Y., Yang, L., Chen, C., Li, Z., Lu, Z., 2020. JNK pathway plays a key role in the immune system of the pea aphid and is regulated by microRNA-184. PLoS Pathog. 16 (6), e1008627.

Masuda, T., Baba, S., Matsuo, K., Ito, S., Mikami, B., 2020. The high-resolution crystal structure of lobster hemocyanin shows its enzymatic capability as a phenoloxidase. Archiv. Biohys. Biochem. 688, 108370.

Matskevich, A.A., Quintin, J., Ferrandon, D., 2010. The Drosophila PRR GNBP3 assembles effector complexes involved in antifungal defences independently of its Toll-pathway activation function. Eur. J. Immunol. 40, 1244–1254.

Meekins, D.A., Kanost, M.R., Michel, K., 2017. Serpins in arthropod biology. Semin. Cell Dev. Biol. 62, 105–119. https://doi.org/10.1016/j.semcdb.2016.09.001.

Nagai, T., Osaki, T., Kawabata, S.-I., 2001. Functional conversion of hemocyanin to phenoloxidase by horseshoe crab antimicrobial peptides. J. Biol. Chem. 276, 27166–27170.

Nam, H.-J., Jang, I.-H., You, H., Lee, K.-A., Lee, W.-J., 2012. Genetic evidence of a redox-dependent systemic wound response via Hayan protease-phenoloxidase system in Drosophila. EMBO J. 31, 1253–1265.

Nappi, A.J., Christensen, B.M., 2005. Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. Insect Biochem. Mol. Biol. 35, 443–459.

Noonin, C., Lin, X., Jiravanichpaisal, P., Söderhäll, K., Söderhäll, I., 2012. Invertebrate hematopoiesis: an anterior proliferation center as a link between the hematopoietic tissue and the brain. Stem Cell. Dev. 21, 3173–3186. https://doi.org/10.1089/scd.2012.0077.

Noothuan, N., Amparyup, P., Tassanakajon, A., 2017. Melanization inhibition protein of Penaeus monodon acts as a negative regulator of the prophenoloxidase-activating system. Dev. Comp. Immunol. 72, 97–102. https://doi.org/10.1016/j.dci.2017.02.014.

Nordahl, E.A., Rydengård, V., Nyberg, P., Nitsche, D.P., Mörgelin, M., Malmsten, M., Björck, L., Schmidtchen, A., 2004. Activation of the complement system generates antibacterial peptides. Proc. Natl. Acad. Sci. U.S.A. 101, 16879–16884.

Nunez-Valdez, M.E., Lanois, A., Pages, S., Duvic, B., Gaudriault, S., 2019. Inhibition of *Spodoptera frugiperda* phenoloxidase activity by the product of the *Xenorhabdus* rhabduscin gene cluster. PloS One 14 (2), e0212809. https://doi.org/10.1371/journal.pone.0212809.

Ochiai, M., Ashida, M., 1988. Purification of a β−1,3-glucan recognition in the prophenoloxidase activating system from hemolymph of the silk worm, *Bombyx mori*. J. Biol. Chem. 263, 12056–12062.

Ochiai, M., Ashida, M., 2000. A pattern-recognition protein for β -1,3-glucan: the binding domain and the cDNA-cloning of β -1,3-glucan recognition protein from the silkworm *Bombyx mori*. J. Biol. Chem. 275, 4995–5002.

Ourth, D.D., Renis, H.E., 1993. Antiviral melanization reaction of Heliothis virescens hemolymph against DNA and RNA viruses in vitro. Comp. Biochem. Physiol. B 105, 719–723. https://doi.org/10.1016/0305-0491(93)90111-H.

Owusu-Ansah, E., Banerjee, U., 2009. Reactive oxygen species prime *Drosophila* haematopoietic progenitors for differentiation. Nature 461, 537–541.

Palmer, C.V., Bythell, J.C., Willis, B.L., 2011. A comparative study of phenoloxidase activity in diseased and bleached colonies of the coral Acropora millepora. Dev. Comp. Immunol. 35, 1098–1101.

Palumbo, A., Poli, A., Di Cosmo, A., d'Ischia, M., 2000. N-Methyl-D-aspartate receptor stimulation activates tyrosinase and promotes melanin synthesis in the ink gland of the cuttlefish *Sepia officinalis* through the nitric Oxide/cGMP signal transduction pathway. A novel possible role for glutamate as physiologic activator of melanogenesis. J. Biol. Chem. 275, 16885–16890. https://doi.org/10.1074/jbc.M909509199.

Park, J.-W., Kim, C.-H., Kim, J.-H., Je, B.-R., Roh, K.-B., Kim, S.-J., Lee, H.-H., Ryu, J.-H., Lim, J.-H., Oh, B.-H., Lee, W.-J., Ha, N.-C., Lee, B.-L., 2007. Clustering of peptidoglycan recognition protein-SA is required for sensing lysine-type peptidoglycan in insects. Proc. Nat. Acad. Sci. USA 104, 6602–6607. https://doi.org/10.1073/pnas.0610924104.

Phupet, B., Pitakpornpreecha, T., Baowubon, N., Runsaeng, P., Utarabhand, P., 2018. Lipopolysaccharide- and β-1,3-glucan-binding protein from *Litopenaeus vannamei*: purification, cloning and contribution in shrimp defense immunity via phenoloxidase activation. Dev. Comp. Immunol. 81, 167–179.

Povelones, M., Waterhouse, R.M., Kafatos, F.C., Christophides, G.K., 2009. Leucine-rich complex repeat protein complex activates mosquito complement in defense against *Plasmodium* parasites. Science 324, 258–261.

Prochazkova, P., Roubava, R., Dvorak, J., Navarro Pachero, N.I., Bilej, M., 2020. Pattern recognition in annelids. Dev. Comp. Immunol. https://doi.org/10.1016/j.dci.2019.103493.

Qian, C., Fang, Q., Wang, L., Ye, G.Y., 2015. Molecular cloning and functional studies of two Kazal-type serine protease inhibitors specifically expressed by *Nasonia vitripennis* venom apparatus. Toxins 7 (8), 2888–2905. https://doi.org/10.3390/toxins7082888, 2015 Aug 4.

Qian, C., Liang, D., Wang, P., Kausar, S., Wei, G., Zhu, B., Wang, L., Liu, C., 2017. Identification of a small pacifastin protease inhibitor from Nasonia vitripennis venom that inhibits humoral immunity of host (Musca domestica). Toxicon 131, 54–62. https://doi.org/10.1016/j.toxicon.2017.03.005.

Quinn, E.A., Malkin, S.H., Rowley, A.F., Coates, C.J., 2020. Laccase and catecholoxidase activities contribute to innate immunity in slipper limpets, *Crepidula fornicata*. Dev. Coxmp. Immunol. https://doi.org/10.1016/j.dci.2020.103724.

Racioppi, C., Coppola, U., Christiaen, L., Ristoratone, F., 2019. Transcriptional regulation of Rab32/38, a specific marker of pigment cell formation in Ciona robusta. Dev. Biol. 448, 111–118. https://doi.org/10.1016/j.ydbio.2018.11.013.

Ramirez, J.L., Muturi, E.J., Dunlap, C., Rooney, A.P., 2018. Strain-specific pathogenicity and subversion of phenoloxidase activity in the mosquito *Aedes aegypti* by members of the fungal entomopathogenic genus *Isaria*. Sci. Rep. 8, 9896. https://doi.org/10.1038/s41598-018-28210-6.

Rao, X.-J., Zhong, X., Lin, X.-Y., Huang, X.-H., Yu, X.-Q., 2014. Characterization of a novel *Manduca sexta* beta-1,3-glucan recognition protein (βGRP3) with multiple functions. Insect Biochem. Mol. Biol. 52, 13–22.

Rodriguez-Andres, J., Rani, S., Varjak, M., Chase-Topping, M.E., Beck, M.H., Ferguson, M.C., Schnettler, E., Fragkoudis, R., Barry, G., Merits, A., Fazakerley, J.K., Strand, M.R., Kohl, A., 2012. Phenoloxidase activity acts as a mosquito innate immune response against infection with Semliki Forest virus. PLoS Pathog. 8 (11), e1002977 https://doi.org/10.1371/journal.ppat.1002977, 2012.

Roh, K.B., Kim, C.H., Lee, H., Kwon, H.M., Park, J.W., Ryu, J.H., Kurokawa, K., Ha, N.C., Lee, W.J., Lemaitre, B., Söderhäll, K., Lee, B.L., 2009. Proteolytic cascade for the activation of the insect toll pathway induced by the fungal cell wall component. J. Biol. Chem. 284, 19474–19481. https://doi.org/10.1074/jbc.M109.007419.

Sangsuriya, P., Charoensapsri, W., Sutthangkul, J., Senapin, S., Hirono, I., Tassanakajon, A., Amparyup, P., 2018. A novel white spot syndrome virus protein WSSV164 controls prophenoloxidases, PmproPOs in shrimp melanization cascade. Dev. Comp. Immunol. 86, 109–117. https://doi.org/10.1016/j.dci.2018.05.005.

Scherfer, C., Tang, H., Kambris, Z., Lhocine, N., Hashimoto, C., Lemaitre, B., 2008. Drosophila Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation. Dev. Biol. 323, 189–196. https://doi.org/10.1016/j.ydbio.2008.08.030.

Severo, M.S., Landry, J.J.M., Lindquist, R.L., Goosmann, C., Brinkmann, V., Collier, P., Hauser, A.E., Benes, V., Henriksson, J., Teichmann, S.A., Levashina, E.A., 2018. Unbiased classification of mosquito blood cells by single-cell genomics and high-content imaging. Proc. Nat. Acad. Sci. USA. www.pnas.org/cgi/doi/10.1073/pnas.1803062115.

Shao, Q., Yang, B., Xu, Q., Li, X., Lu, Z., Wang, C., Huang, Y., Söderhäll, K., Ling, E., 2012. Hindgut innate immunity and regulation of fecal microbiota through melanization in insects. J. Biol. Chem. 287, 14270–14279. https://doi.org/10.1074/jbc.M112.354548.

Shelby, K.S., Popham, H.J.R., 2006. Plasma phenoloxidase of the larval tobacco budworm, *Heliothis virescens,* is virucidal. J. Insect Sci. 6, 1–12. https://doi.org/10.1673/2006_06_13.1.

Shen, W., Hu, Y., He, Z., Xu, S., Zhang, G., Ren, G., 2020. Histological and comparative transcriptome analyses provide insights into the immune response in pearl sac formation of *Hyriopsis*. Front. Mar. Sci. https://doi: 10.3389/fmars.2020.00256.

Shi, L., Chan, S., Li, C., Zhang, S., 2017. Identification and characterization of a laccase from Litopenaeus vannamei involved in ant-bacterial host defense. Fish Shellfish Immunol. 66, 1–10.

Shokal, U., Eleftherianos, I., 2017. Evolution and function of Thioester-containing proteins and the complement system in the innate immune response. Front. Immunol. 8, 759. https://doi.org/10.3389/fimmu.2017.00759.

Sirikharin, R., Söderhäll, K., Söderhäll, I., 2020. The N-terminal peptide generated after activation of prophenoloxidase affects crayfish hematopoiesis. Dev. Comp. Immunol. 108, 103687, 2020.

Söderhäll, K., Ajaxon, R., 1982. Effect of quinones and melanin on mycelial growth of Aphanomyces spp. and extracellular protease of Aphanomyces astaci, a parasite on crayfish. J. Invertebr. Pathol. 39, 105–109.

Söderhäll, K., Rögener, W., Söderhäll, I., Newton, R., Ratcliffe, N.A., 1988. The properties and purification of a *Blaberus craniifer* plasma protein which enhances the activation of haemocyte prophenoloxidase by a β-1,3-glucan. Insect Biochem. 18, 323–330.

Söderhäll, I., Wu, C., Novotny, M., Lee, B.L., Söderhäll, K., 2009. A novel protein acts as a negative regulator of prophenoloxidase activation and melanization in the freshwater crayfish *Pacifastacus leniusculus*. J. Biol. Chem. 284, 6301–6310. https://doi.org/10.1074/jbc.M806764200.

Sousa, G.L., Bishnoi, R., Baxter, R.H.G., Povelones, M., 2020. The CLIP-domain serine protease CLIPC9 regulates melanization downstream of SPCLIP1, CLIPA8, and CLIPA28 in the malaria vector *Anopheles gambiae*. PLoS Pathog. 16 (10), e1008985 https://doi.org/10.1371/journal.ppat.1008985.

Sriacharoen, S., Kim, J.J., Tunkijjanukij, S., Söderhäll, I., 2005. Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish. Dev. Comp. Immunol. 29, 1017–1031. https://doi.org/10.1016/j.dci.2005.03.010.

Sritunyalucksana, K., Lee, S.Y., Söderhäll, K., 2002. A β -1,3-glucan binding protein from the black tiger shrimp, *Penaeus monodon*. Dev. Comp. Immunol. 26, 237–245.

Stączek, S., Zdybicka-Barabas, A., Pleszczyńska, M., Wiater, A., Cytryńska, M., 2020. *Aspergillus niger* α-1,3-glucan acts as a virulence factor by inhibiting the insect phenoloxidase system. J. Invertebr. Pathol. 2020 https://doi.org/10.1016/j.jip.2020.107341, 171:107341.

Sun, Q., Xu, X.-X., Shoaib, F., Huang, W.-J., Zheng, Z., Wang, S., Ren, S.-X., Jin, F.-L., 2014. Molecular characterization of a short peptidoglycan recognition protein (PGRP-S) from Asian corn borer (*Ostrinia furnacalis*) and its role in triggering proPO activity. World J. Microbiol. Biotechnol. 30, 263–270. https://doi-org.ezproxy.its.uu.se/10.1007/s11274-013-1449-3.

Sutthangkul, J., Amparyup, P., Charoensapsri, W., Senapin, S., Phiwsaiya, K., Tassanakajon, A., 2015. Suppression of shrimp melanization during white spot syndrome virus infection. J. Biol. Chem. 290, 6470–64781. https://doi.org/10.1074/jbc.M114.605568.

Sutthangkul, J., Amparyup, P., Eum, J.H., Strand, M.R., Tassanakajon, A., 2017. Anti-melanization mechanism of the white spot syndrome viral protein, WSSV453, via interaction with shrimp proPO-activating enzyme. PmproPPAE2. J. Gen. Virol 98, 769–778. https://doi.org/10.1099/jgv.0.000729.

Suwanchaichinda, C., Ochieng, R., Zhuang, S., Kanost, M.R., 2013. *Manduca sexta* serpin-7, a putative regulator of hemolymph prophenoloxidase activation. Insect Biochem. Mol. Biol. 43, 555–561. https://doi.org/10.1016/j.ibmb.2013.03.015.

Takahashi, D., Dai, H., Hiromasa, Y., Krishnamoorthi, R., Kanost, M.R., 2014. Self-association of an insect β-1,3-glucan recognition protein upon binding laminarin stimulates prophenoloxidase activation as an innate immune response. J. Biol. Chem. 289, 28399–28410.

Takashashi, K., Ochiai, M., Horiuchi, M., et al., 2009. Solution structure of the silkworm BGRP/GNBP3 N-terminal domain reveals the mechanism for β -1,3-glucan specific recognition. Proc. Nat. Acad. Sci USA 106, 11679–11684.

Takehana, A., Katsuyama, T., Yano, T., Oshima, Y., Takada, H., Aigaki, T., Kurata, S., 2002. Overexpression of a pattern-recognition receptor, peptidoglycan-recognition protein-LE, activates imd/relish-mediated antibacterial defense and the prophenoloxidase cascade in Drosophila larvae. Proc. Nat. Acad. Sci. USA 99, 13705–13710.

Tassanakajon, A., Rimphanitchayakit, V., Visetnan, S., Amparyup, P., Somboonwiwat, K., Charoensapsri, W., Tang, S., 2018. Shrimp humoral responses against pathogens: antimicrobial peptides and melanization. Dev. Comp. Immunol. 80, 81–93. https://doi.org/10.1016/j.dci.2017.05.009.

Theopold, U., Schmidt, O., Söderhäll, K., Dushay, M., 2004. Coagulation in arthropods: defence wound closure and healing. Trends Immunol. 25, 289–294.

Tian, C., Wang, L., Ye, G., Zhu, S., 2010. Inhibition of melanization by a *Nasonia* defensin-like peptide: implications for host immune suppression. J. Insect Physiol. 56, 1857–1862. https://doi.org/10.1016/j.jinsphys.2010.08.004.

Toufeeq, S., Wang, J., Zhang, S.-Z., Li, B., Hu, P., Zhu, L.-B., You, L.-L., Xu, J.-P., 2019. Bmserpin 2 is involved in BmNPV infection by suppressing melanisation in Bombyx mori. Insects.

Udompetcharaporn, A., Junkulo, K., Senapin, S., Roytrakul, S., Flegel, T.W., Sritunyalucksana, K., 2014. Identification and characterization of a QM protein as a possible peptidoglycan recognition protein (PGRP) from the giant tiger shrimp Penaeus monodon. Dev. Comp. Pathol 46, 146–154.

Ullah, I., Khan, A.L., Ali, L., Khan, A.R., Waqas, M., Lee, I.J., Shin, J.H., 2014. An insecticidal compound produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition. Molecules 19, 20913–20928. https://doi.org/10.3390/molecules191220913.

Unestam, T., Söderhäll, K., 1977. Soluble fragments from fungal cell walls elicit defence reactions in crayfish. Nature 267, 45–46.

Volohonsky, G., Hopp, A.-K., Saenger, M., Soichot, J., Scholze, H., Boch, J., Blandin, S.A., Marois, E., 2017. Transgenic expression of the anti-parasitic factor TEP1 in the malaria mosquito *Anopheles gambiae*. PLoS Pathog. 13 (1), e1006113 https://doi.org/10.1371/journal.ppat.1006113.

Wang, R., Lee, S.Y., Cerenius, L., Söderhäll, K., 2001. Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. Eur. J. Biochem. 268, 895–902.

Wang, B., Kang, Q., Lu, Y., Bai, L., Wang, C., 2012. Unveiling the biosynthetic puzzle of destruxins in *Metarhizium* species. Proc. Natl. Acad. Sci. U. S. A. 109, 1287–1292. https://doi.org/10.1073/pnas.1115983109.

Wang, Y.H., Hu, Y., Xing, L.S., Jiang, H., Hu, S.N., Raikhel, A.S., Zou, Z., 2015. A critical role for CLSP2 in the modulation of antifungal immune response in mosquitoes. PLoS Pathog. 11 (6), e1004931 https://doi.org/10.1371/journal.ppat.1004931.

Wang, Y., Jiang, H., Cheng, Y., An, C., Chu, Y., Raikhel, A.S., Zou, Z., 2017. Activation of *Aedes aegypti* prophenoloxidase-3 and its role in the immune response against entomopathogenic fungi. Insect Mol. Biol. 26, 552–563. https://doi.org/10.1111/imb.12318.

Wang, W., Song, C.X., Wang, L., Song, L., 2018. Pathogen-derived carbohydrate recognition in molluscs immune defense. Int. J. Mol. Sci. https://doi.org/10.3390/ijms19030721.

Wang, L., Liu, H., Fu, H., Zhang, L., Guo, P., Xia, Q., Zhao, P., 2019. Silkworm serpin32 functions as a negative-regulator in prophenoloxidase activation. Dev. Comp. Physiol. 91, 123–131.

Wang, Y., Yang, F., Cao, X., Huang, R., Paskewitz, S., Hartson, S.D., Kanost, M.R., Jiang, H., 2020a. Inhibition of immune pathway-initiating hemolymph protease-14 by Manduca sexta serpin-12, a conserved mechanism for the regulation of melanization and Toll activation in insects. Insect Biochem. Mol. Biol. 116, 103261. https://doi.org/10.1016/j.ibmb.2019.103261.

Wang, Q., Yin, M., Yuan, C., Liu, X., Hu, Z., Zou, Z., Wang, M., 2020b. Identification of a conserved prophenoloxidase activation pathway in cotton bollworm *Helicoverpa armigera*. Front. Immunol. 11, 785. https://doi.org/10.3389/fimmu.2020.00785.

Wu, C., Noonin, C., Jiravanichpaisal, P., Söderhäll, I., Söderhäll, K., 2012. An insect TEP in a crustacean is specific for cuticular tissues and involved in intestinal defense. Insect Biochem. Mol. Biol. 42, 71–80.

Wu, C., Charoensapsri, W., Nakamura, S., Tassanakajon, A., Söderhäll, I., Söderhäll, K., 2013. An MBL-like protein may interfere with the activation of the proPO-system, an important innate immune reaction in invertebrates. Immunobiology. https://doi.org/10.1016/j.imbio.2012.02.011.

Wu, K., Zhang, J., Zhang, Q., Zhu, S., Shao, Q., Ling, E., 2015. Plant phenolics are detoxified by prophenoloxidase in the insect gut. Sci. Rep. 5, 16823. https://doi.org/10.1038/srep16823.

Wu, K., Han, F., Yan, Y., Liu, Y., Ling, E., Wang, Q., Huang, W., 2018. Effect of the insect phenoloxidase on the metabolism of L-DOPA Arch. Insect Biochem. Physiol. 98, e21457. https://doi.org/10.1002/arch.21457, 2018.

Wu, C.-Y., Huang, J.-M., Zhao, Y.-J., Xu, Z.-W., Zhu, J.-Y., 2020. Venom serine proteinase homolog of the ectoparasitoid *Scleroderma guani* impairs host phenoloxidase cascade. Toxicon 183, 29–35.

Xing, L., Yuan, C., Wang, M., Lin, Z., Shen, B., Hu, Z., Zou, Z., 2017. Dynamics of the interaction between cotton bollworm Helicoverpa armigera and nucleopolyhedrovirus as revealed by integrated transcriptomic and proteomic analyses. Mol. Cell. Proteomics 16, 1009–1028. https://doi.org/10.1074/mcp.M116.062547.

Yassine, H., Kamareddine, L., Osta, M.A., 2012. The mosquito melanization response is implicated in defense against the entomopathogenic fungus *Beauveria bassiana*. PLoS Pathog. 8 (11), e1003029 https://doi.org/10.1371/journal.ppat.1003029.

Yoshida, H., Kinoshita, K., Ashida, M., 1996. Purification of a peptidoglycan recognition protein from hemolymph of the silkworm, *Bombyx mori*. J. Biol. Chem. 271, 13854–13860.

Yu, X.Q., Gan, H., Kanost, M.R., 1999. Immulectin, an inducible C-type lectin from an insect, *Manduca sexta*, stimulates activation of plasma polyphenoloxidase. Insect Biochem. Mol. Biol. 29, 585–597.

Yuan, C., Xing, L., Wang, M., Wang, X., Yin, M., Wang, Q., Hu, Z., Zou, Z., 2017. Inhibition of melanization by serpin-5 and serpin-9 promotes baculovirus infection in cotton bollworm *Helicoverpa armigera*. PLoS Pathog. 13 (9), e1006645 https://doi.org/10.1371/journal.ppat.1006645.

Zhang, G.M., Lu, Z.Q., Jiang, H.B., Asgari, S., 2004. Negative regulation of prophenoloxidase (proPO) activation by a clip- domain serine proteinase homolog (SPH) from endoparasitoid venom. Insect Biochem. Mol. Biol. 34, 477–483.

Zhang, J., Lu, A., Kong, L., Zhang, Q., Ling, E., 2014. Functional analysis of insect molting fluid proteins on the protection and regulation of ecdysis. J. Biol. Chem. 289, 35891–35906. https://doi.org/10.1074/jbc.M114.599597.

Zhang, J., Huang, W., Yuan, C., Lu, Y., Yang, B., Wang, C.-Y., Zhang, P., Dobens, L., Zou, Z., Wang, C., Ling, E., 2017. Prophenoloxidase-mediated ex vivo immunity to delay fungal infection after insect ecdysis. Front. Immunol. 8, 1445. https://doi.org/10.3389/fimmu.2017.01445.

Zhao, M., Söderhäll, I., Park, J.W., Ma, Y.G., Osaki, T., Ha, N.C., Wu, C.F., Söderhäll, K., Lee, B.L., 2005. A novel 43-kDa protein as a negative regulatory component of phenoloxidase-induced melanin synthesis. J. Biol. Chem. 280, 24744–24751. https://doi.org/10.1074/jbc.M504173200.

Zhao, P., Lu, Z., Strand, M.R., Jiang, H., 2011. Antiviral, anti-parasitic, and cytotoxic effects of 5,6-dihydroxyindole (DHI), a reactive compound generated by phenoloxidase during insect immune response. Insect Biochem. Mol. Biol. 41, 645–652. https://doi.org/10.1016/j.ibmb.2011.04.006.

Zhao, S., Wang, X., Cai, S., Zhang, S., Luo, H., Wu, C., Zhang, R., Zhang, J., 2018. A novel peptidoglycan recognition involved in the prophenoloxidase activation system and antimicrobial peptide production in *Antheraea pernyi*. Dev. Comp. Immunol. 86, 78–85.