

MINI REVIEW

Phenoloxidase: a key component of the insect immune system

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Abstract

The innate immune system in insects is composed of a large variety of specific and non-specific responses that are activated in response to the presence of foreign agents. One important element in such responses is the enzyme phenoloxidase (PO). Here, we review recent progress in PO research and discuss new applications in the emerging field of ecological immunology. Phenoloxidase produces indole groups, which are subsequently polymerized to melanin. The enzymatic reactions in turn produce a set of intermediate products such as quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates, which are important during defense against bacterial (gram+ and -), fungal, and viral agents. Phenoloxidase requires a complex system of activation and inhibition that involves various cell types, PO zymogens, inhibitor enzymes, and signaling molecules. Finally, research in evolutionary ecology has studied the costs of PO in terms of resource use and pleiotropic relations with other key traits and functions. These studies indicate that PO is a costly trait, whose production and maintenance have fitness costs for hosts. Phenoloxidase does not seem to be an indicator of resistance but rather of host condition. Finally, we put forward some basic directions for future investigation of PO aimed at explaining its activating system, its substrates, its coordination with other immune components to fight off pathogens, and variation in PO in relation to gender, life stages, seasonality, and across different host species.

Introduction

Recent advances in invertebrate immunology have documented a complex array of host defenses. These defenses include phagocytosis, melanization (i.e., synthesis and deposition of melanin around the pathogen), synthesis of extracellular matrix, adhesion cells, recognition molecules, reactive intermediates of oxygen and nitrogen, proapoptotic molecules, pro-inflammatory cytokines, and antimicrobial peptides (Nappi & Vass, 2001; Tunaz et al., 2003; Bulet et al., 2004; Nappi & Christensen, 2005). Within this broad range of immune responses, an important immune component used by arthropods is melanogenesis (Asada et al., 1999; Eleftherianos and Revenis 2011; Amparyup et al., 2009).

Enzymatic and non-enzymatic reactions play an important role in melanogenesis. This process is responsible for encapsulating multicellular pathogens, repairing

tissues, and defending against other pathogens such as bacteria (gram+ and -), fungi, and even viral agents (Boman, 1986; Ashida & Brey, 1997; Nappi & Christensen, 2005). Both intermediate products (quinones, diphenols, superoxide, hydrogen peroxide, and reactive nitrogen intermediates) and their activating enzymes [phenoloxidase (PO) and dopa decarboxylase] play important roles. Among the most important enzymes is PO. This enzyme is responsible for the activation of melanogenesis in invertebrates (Sussman, 1949; Wyatt, 1961). Furthermore, recent research has documented PO as an important tool used against several pathogens (Cerenius & Söderhäll, 2004).

Invertebrate PO requires a complex system of activation and inhibition. Activation and inhibition involve different cell types (hemocytes, plasmatocytes, and crystal cells), PO zymogens (i.e., the inactive form of PO: proPO), proPO inhibitor enzymes (serpins), signaling molecules (peptidoglycan, membrane lipids, and viral protein segments), and even PO itself. The role of PO in the immune system has

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been subject to review in the past (Ashida & Brey, 1997; Söderhäll & Cerenius, 1998; Sugumaran, 2002; Cerenius & Söderhäll, 2004; Kanost & Gorman, 2008). Nevertheless, as there is constant discovery and modification of components involved in this complex system, our aim is to integrate old and new information. Our review mainly focuses on (1) an integration of information regarding PO participation in melanogenesis along with regulatory mechanisms of the PO-activating system, and (2) present and future applications of PO knowledge in the field of ecological immunology.

Our review is divided into three sections. In the first section, we discuss the role of PO in melanogenesis. Here, we describe the general properties of melanin, discuss the role of melanin in invertebrate immunity, and review the site of melanin synthesis and the regulatory mechanisms that control melanin action. The second section focuses on the PO enzyme as a key component in the invertebrate immune system, its chemistry and physical properties, the types and structures of invertebrate POs, the site of PO synthesis, the regulatory genes, and the components that promote or inhibit the PO system. Finally, the last section describes new applications of the PO system in the emerging field of ecological immunology.

PO and melanogenesis

The main role of PO in melanogenesis is to convert phenols to quinones, which subsequently polymerize to form melanin (Figure 1; Söderhäll & Cerenius, 1998). The most commonly described melanization pathway is the melanization process against parasitoids eggs in fruit flies (reviewed in Nappi & Vass, 1993; Figure 2). Melanin is formed from the amino acid phenylalanine, which first is hydroxylated to tyrosine by phenylalanine hydroxylase. Tyrosine is then hydroxylated by the active PO to produce DOPA. DOPA is then oxidized to dopaquinone and which is immediately converted to dopachrome by a spontaneous and non-enzymatic reaction. A non-enzymatic structural rearrangement of dopachrome, followed by decarboxylation generates 5-6 dihydroxyindole (DHI). Dihydroxyindole is then oxidized by PO to form 5-6 indolequinones. Finally, indolequinones are polymerized to eumelanin or, in the presence of thiol compounds, pheomelanins (Nappi & Christensen, 2005). In addition, DHI can be produced by an alternative pathway where DOPA decarboxylase removes CO_2 from DOPA to form dopamine (Figure 3; Beerntsen et al., 2000; Christensen et al., 2005).

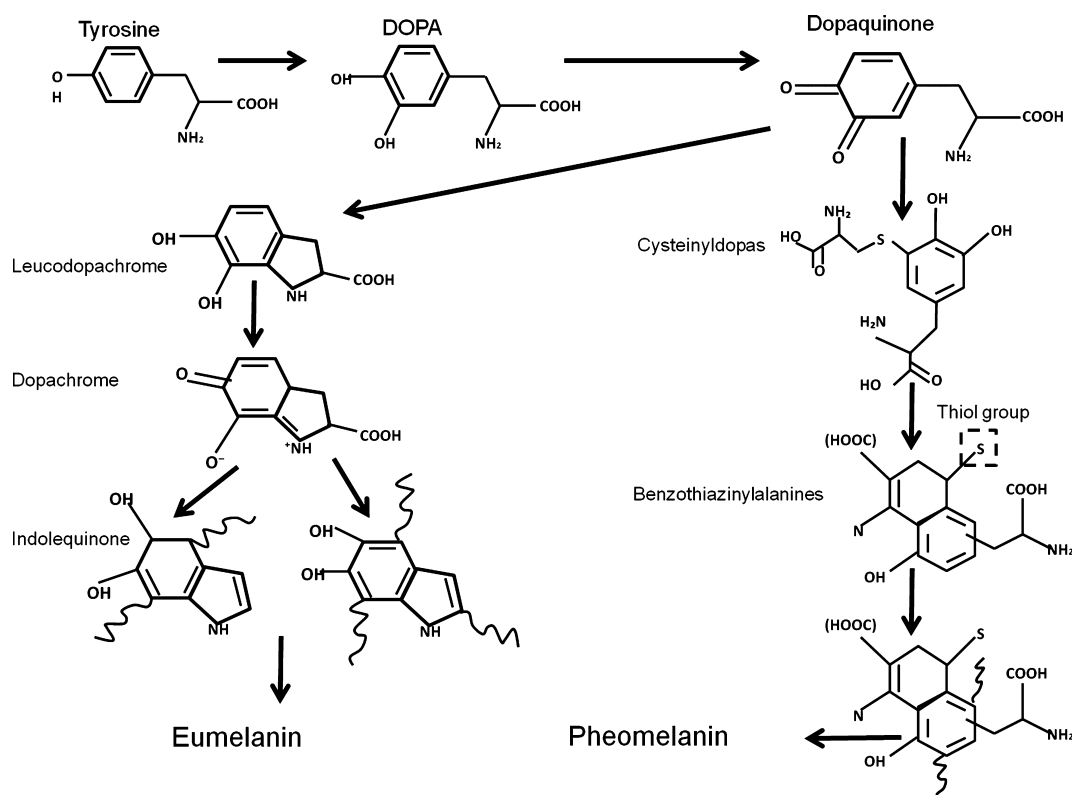


Figure 1 Types of melanins and their precursors. Left: Eumelanin by dopachrome. Right: Pheomelanin by benzothiazine.

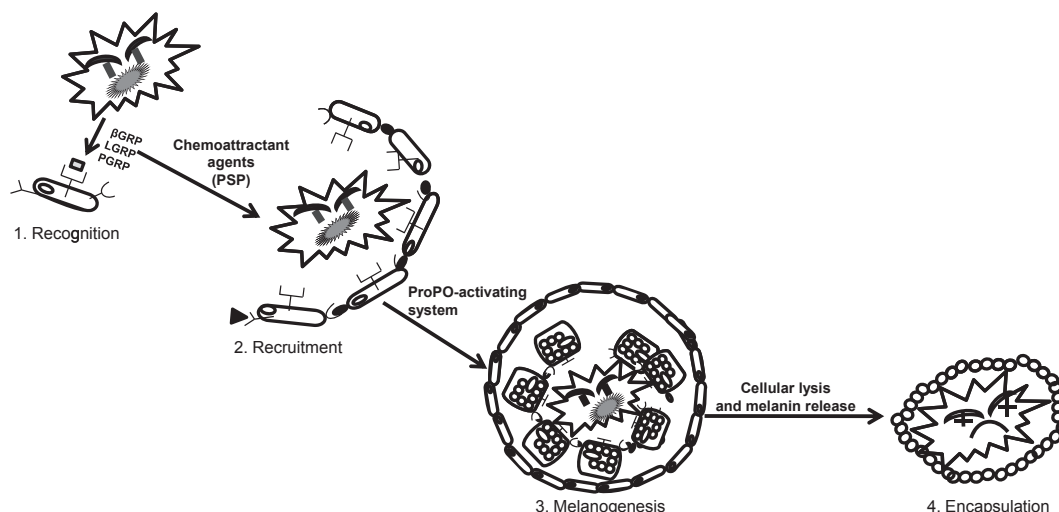


Figure 2 Melanization includes four steps: (1) the recognition of pathogens through specific molecules [e.g., lipopolysaccharides (LPSs), LGRP, PGRP]; (2) the recruitment of hemocytes to surround the pathogen by chemoattractant proteins (e.g., plasmatocyte spreading peptide, PSP); (3) melanogenesis within hemocytes; (4) melanin release and pathogen encapsulation.

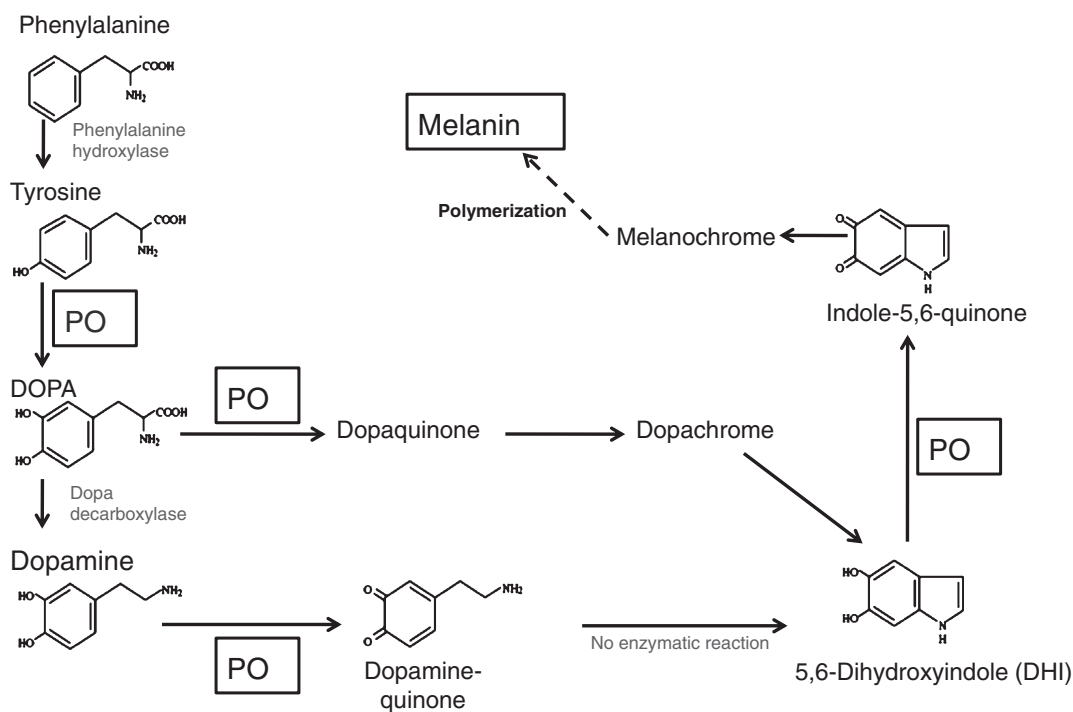


Figure 3 Phenoloxidase role in melanin synthesis. Phenoloxidase participates in the formation of DOPA from Tyrosine (Tyr). PO also convert DOPA to dopaquinone, and 5,6-dihydroxyindole (DHI) to indole-5,6-quinone. Note the alternative way to obtain DHI from dopamine, in which PO is also involved.

Melanins are a group of pigment-forming chemical compounds that are widely distributed in nature. They are found in plants, animals, and protists. The most common

melanin is eumelanin which is formed by a dihydroxyndol polymer which comes from dopaquinone (dopachrome), and produces a dark brown or blackish color (Napolitano

et al., 2000). Another common type of melanin is pheomelanin, which is formed by the polymerization of bezothiazine and produces reddish or brownish colors (Figure 1; Napolitano et al., 2000).

Melanin fills many roles in invertebrates besides pigmentation. Several studies have shown that mechanical injury or the presence of macropathogens (e.g., nematodes or parasitoids) results in melanin deposition around the damaged tissue or foreign object. This process is commonly known as melanization (Ashida & Brey, 1997; Sugumaran, 2002; Cerenius & Söderhäll, 2004; Kanost et al., 2004; Nappi & Christensen, 2005). In arthropods, melanization starts when a foreign target is located by hemocytes via a signaling cascade involving lipopolysaccharides, peptidoglycans, glucans, and their recognition proteins - the beta-1,3-glucan recognition protein (β GRP) (Figure 2; reviewed by Strand, 2008). Hemocytes surround the foreign body and release chemoattractant proteins (e.g., plasmatocyte spreading peptide or PSP). These proteins attract plasmatocytes (Lavine & Strand, 2002), which form a multicellular plasmatocyte wall (Wood et al., 2006). The internal layers of the plasmatocyte wall increase and become thick and dark due to melanin production. Melanin is eventually deposited onto the foreign target. The melanin capsule prevents the growth and reproduction of the pathogen and eventually leads to its death, mainly by starvation (Gillespie et al., 1997).

The role of the PO activating system in invertebrate immunity

ProPO and PO, structures and types

Phenoloxidase is a member of the tyrosinase group, whose main function is to oxidize phenols. Tyrosinase proteins from different species are diverse in terms of their structural properties, distribution, and cellular location. It has even been suggested that there is no one common tyrosinase protein structure (Mayer, 2006). All tyrosinases, however, have in common a binuclear type 3 copper center within their active site. This copper center is surrounded by three histidine residues (Jaenicke & Decker, 2003).

Substrate binding in PO occurs at a site containing a series of hydrophobic amino acids with benzene rings (Nappi & Christensen, 2005). The copper center reacts with dioxygen (O_2) and a highly chemically reactive intermediate is obtained by oxidizing the substrate.

Phenoloxidases are expressed as inactive zymogens (proPOs) in all insects and are converted to active PO when required. ProPOs are polypeptides that contain two copper atoms per protein molecule, with a total weight of 50–60 and 70–80 kDa in their active and inac-

tive forms, respectively (Ashida & Brey, 1997). In arthropods, the proPO amino acid sequences are homologous with hemocyanins (an oxygen-transport protein in invertebrates) and hexamerins (storage proteins). ProPO and hemocyanin contain two well-conserved copper atoms that form an oxygen-binding site, whereas hexamerin lacks copper atoms and therefore does not recruit oxygen. Recent studies support the idea that POs are the ancestral enzyme, that hemocyanins evolved secondarily, and finally that hexamerins diverged from hemocyanins (Decker & Terwilliger, 2000; Burmester, 2001; Burmester, 2002).

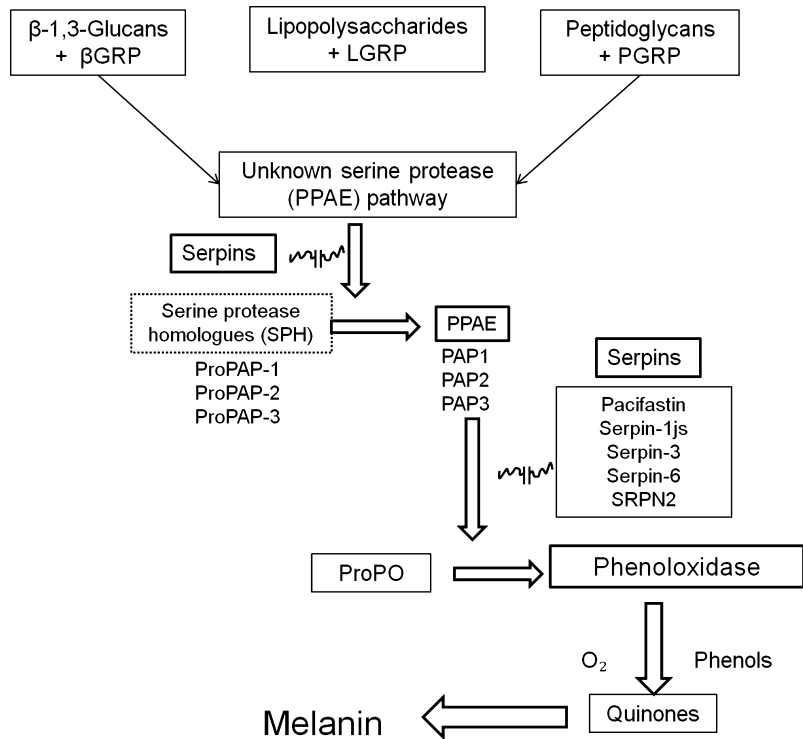
Gene expression of proPO

Researchers have found two or more proPO genes in most insect species, although there are a few cases, such as the honeybee, with only one proPO gene (Evans et al., 2006). ProPOs are sometimes highly conserved. For example, *Drosophila melanogaster* Meigen and *Tribolium castaneum* (Herbst) share two of three proPO genes (Waterhouse et al., 2007). In other groups, there has been a radiation of the proPO gene family – for example, mosquitoes have nine proPO genes in *Anopheles gambiae* Giles and 10 in *Aedes aegypti* (L.) (Waterhouse et al., 2007). It is not clear why PO is highly conserved in some clades and diversified in others. One possibility is that a higher number of pathogen species may lead to increased specialization, via a higher gene number in host species (Waterhouse et al., 2007). A second possibility is that a coevolutionary arms race between hosts and pathogens may lead the former to evolve specific regulator mechanisms. In mosquitoes, for example, the same genes that are involved in the melanization process against *Plasmodium berghei* Vincke and Lips, do not offer any protection against *Plasmodium falciparum* Welch (Cohuet et al., 2006; Michel et al., 2006). One last explanation is that there is differential expression of proPO in relation to distinct insect life-history stages, so that different genes are involved in the same process, but at different ontogenetic times (Li et al., 2005). Thus, if life history stages are different between host species, different gene numbers may appear.

The site of PO synthesis

Synthesis of proPO occurs mostly in hemocytes (Figure 4; Cerenius & Söderhäll, 2004) with species-specific variation in relation to hemocyte type. In lepidopterans, a type of hemocyte, known as oenocytoids, functions as the site of proPO synthesis (Jiang et al., 1997). In *D. melanogaster*, crystal cells, another class of hemocytes present in larval stage, provide a site for proPO synthesis (Rizki et al., 1985; Williams, 2007). In mosquitoes, proPO is synthesized in granular hemocytes and oenocytoids (Hillyer et al., 2003;

Figure 4 General model for proPO activation system in insects. The system starts when hemolymph recognition proteins (β GRP, LGRP, or PGRP) bind to β -1,3-glucans, lipopolysaccharides, or peptidoglycans situated on the surface of the pathogen. The interaction will activate an unknown serine protease pathway. The activation of a serine protease pathway leads to prophenoloxidase activation and active PO forms. Phenoloxidase catalyzes the oxidation of phenols to form quinones, which then are polymerized to obtain melanin. A regulatory mechanism is present, consisting of serine protease inhibitors (serpins) such as pacifastin, serpin-1, and serpin-3.



Castillo et al., 2006). The fat body, however, is definitively not a place where proPO synthesis takes place.

ProPO secretion

Arthropod proPOs lack a peptide signal for their secretion; therefore the presence of proPO in hemolymph probably results from hemocyte lysis (Kanost & Gorman, 2008). The hemocytes in which proPO synthesis takes place (oenocytoids, crystal cells, and granular hemocytes) vary in robustness. Some sites are extremely fragile, and hemocyte lysis occurs immediately after an injury or infection. In these sites, secretion can occur at a low rate even in the absence of injury or infection, perhaps to maintain standard levels of proPO in hemolymph. As a result, it is difficult to evaluate baseline levels of proPO in insects that have fragile oenocytoids (Ashida & Brey, 1997).

In lepidopterans, proPO mostly appears in hemolymph. In other groups such as locusts and cockroaches, proPO appears mainly stored in hemocytes until a pathogen induces its release (Brehélin et al., 1989; Durrant et al., 1993).

ProPO-activating system in invertebrates

ProPO can be activated by several factors. In the laboratory, activation of proPO in hemolymph can occur via detergents, fatty acids, or alcohols. Researchers have activated proPO purified from *D. melanogaster* by 2-propanol

(Asada, 1998). ProPO from *Sarcophaga bullata* Parker may be activated by the cationic detergent cetyl pyridinium chloride (Xie et al., 2007). In nature, insect proPOs can be activated by amphiphilic lipids such as lysolecithin and perhaps by the presence of damaged cells (Sugumaran & Nellaippan, 1991). Other natural proPO activators are β -1,3-glucans, lipopolysaccharides, and peptidoglycans. Natural proPO activators have been identified in several insect pathogens such as gram+ bacteria, gram- bacteria, and fungi (Cerenius & Söderhäll, 2004). All of these activation agents could trigger a conformational change in the protein, resulting in an accessible binding site for the substrate (Kanost & Gorman, 2008). Experiments with proPO purified from silkworm identified the presence of a serine protease cascade as an intermediate step between natural proPO activators and the production of active proPO (Ashida et al., 1974; Ashida & Dohke, 1980). Serine proteases are hydrolases with a serine amino acid in their active center (Hedstrom, 2002). Hydrolases include trypsin, chymotrypsin, and subtilisin. Serine proteases cut the polypeptide chain in the carboxyl side of specific amino acids, resulting in peptide bond degradation (Hedstrom, 2002). Studies in the laboratory have generally used chymotrypsin as a proPO activator. This activator has become a useful experimental tool to assay both PO in the hemolymph and total PO in the whole insect (Sugumaran et al., 1985; Saul & Sugumaran, 1987; Adamo, 2004).

The first proPO activating serine protease (PAP), also known as prophenoloxidase-activating enzyme (PPAE), to be purified was extracted from cuticle (Aso et al., 1985), and perhaps has a role in proPO activation upon wounding (Kanost & Gorman, 2008). In addition, PAPs have been purified in hemolymph from *Manduca sexta* L. (Jiang et al., 1998, 2003), *Bombyx mori* L. (Satoh et al., 1999), *Holotrichia diomphalia* Bates (Lee et al., 1998), and crayfish *Pacifastacus leniusculus* (Dana) (Wang et al., 2001). Three types of PAPs have been identified, PAP-1, PAP-2, and PAP-3 (Figure 4).

ProPO activating serine proteases can be found before and during an infection depending on the animal and the PAP type. For example, PAP from *B. mori* is expressed in integument, hemocytes, and salivary glands, but not in the fat body (Satoh et al., 1999). Its putative *M. sexta* ortholog, PAP-1, is expressed in the larval fat body, tracheae, and nerve tissue, and is up-regulated in the fat body and hemocytes after injection of bacteria (Zou et al., 2005). ProPO activating serine protease-2 expression was detected in the fat body and hemocytes only after *M. sexta* larvae were injected with bacteria (Jiang et al., 2003). ProPO activating serine protease-3 mRNA is present at a low level in the fat body and hemocytes of naïve *M. sexta* larvae, and its expression is significantly upregulated in those tissues after injection of bacteria (Jiang et al., 2003). At the prepupal stage, it is highly expressed in integument, the fat body, and hemocytes (Zou & Jiang, 2005). Finally, in some cases, PAPs require the presence of protein cofactors or serine protease homologs (SPH) for their activation (Figure 4; Jiang et al., 1998; Kwon et al., 2000). These SPHs have been found in genomes such as *D. melanogaster*, *A. aegypti*, *A. gambiae*, and *Apis mellifera* L. (reviewed in Zou et al., 2006; Waterhouse et al., 2007; Kanost & Gorman, 2008).

PO activity in insect hemolymph

Mammalian tyrosinases and insect POs catalyze three types of reactions in the melanogenesis process: (1) hydroxylation of a monophenol, (2) oxidation of an *o*-diphenol and (3) dehydrogenation of a dihydroxyindole (Korner & Pawelek, 1982). However, insect POs and mammalian tyrosinases have different amino acids sequences in their protein structures (van Holde et al., 2001).

The insect ability to produce multiple enzymes with similar functions was a major challenge for PO activity studies. For example, laccase can directly oxidize both *o*- and *p*-diphenols, but not monophenols (Solomon et al., 1996). Similarly, tyrosine hydroxylase can hydroxylate monophenols, but does not oxidize diphenols. Another enzyme produced by insects, peroxidase, can oxidize both monophenols and diphenols (Nappi & Vass, 1993; Okun,

1996; Vie et al., 1999). Later studies have shown that these enzymes have different substrate specificity. In addition, several chemical inhibitors can be used to isolate the enzyme activity specifically (Barrett, 1991). Although insect POs can potentially oxidize monophenol groups, there are only a few isolated POs from *S. bullata* and *M. sexta* documented with this function (Chase et al., 2000).

Phenoloxidase substrate

There are two classes of compounds that have been documented as substrates for insect POs: (1) monophenols, such as tyrosine or tyramine and (2) catechol substrates, including 1,2-dihydroxybenzene (catechol), 4-methyl catechol, DOPA, dopamine, *N*-acetyldopamine (NADA), *N*- β -alanyldopamine (NBAD), *N*-acetyl norepinephrine (NBANE), 3,4-dihydroxyphenylacetic acid (DOPAC), norepinephrine, 3,4-dihydroxymandelic acid, and 3,4-dihydroxybenzoic acid (Chase et al., 2000). Of these catechols, DOPA has been most frequently used to assay PO activity experimentally. Based on solubility and oxidative properties, dopamine, NADA, or NBAD could be the natural substrates for PO (Sugumaran, 2002). Nonetheless, tyrosine and most of the diphenols mentioned above have been detected in the hemolymph of one or more insect species, but typically in their non-substrate form. These compounds are stored in hemolymph until needed (Zhao et al., 1995).

Regulatory mechanisms of PO activity

Phenoloxidases are expressed as inactive zymogens (pro-POs) in all insects and are converted to active PO by PPAEs. Nevertheless, PO also produces several molecules such as proteases that could degrade host proteins, cytotoxic quinines, reactive oxygen and nitrogen intermediates. These molecules could harm the host if produced in excess. Therefore, the presence of regulatory mechanisms of PO activity is necessary to produce an optimal response localized to a specific place and for a specific duration (Cerenius & Söderhäll, 2004; Nappi & Christensen, 2005; Kanost & Gorman, 2008).

In insect hemolymph, several serine protease inhibitors have been identified that inhibit PO activation (Kanost & Jiang, 1996; Kanost, 1999). One such serine protease inhibitor is pacifastin, which may be obtained from the crayfish *P. leniusculus* Dana (Hergenhahn et al., 1987; Liang et al., 1997). Other groups of serine protease inhibitors are serpins (Silverman et al., 2001; Gettins, 2002). Several types of serpins (serpin-1j, serpin-3, serpin-6) have been identified in *M. sexta* (Jiang et al., 2003; Kanost, 2007). A serpin-3 ortholog has been found in *Hyphantria cunea* Drury (Park et al., 2000). Studies in *A. gambiae* have shown that

decreased expression of a serpin-3 ortholog (SRPN2) results in the formation of melanotic pseudotumors and increases lysis of ookynetes. It is likely that all of these effects are caused by excessive synthesis of melanin (Michel et al., 2005). Studies in *Musca domestica* L. also found a 4 kDa peptide as an efficient PO inhibitor. This peptide directly inhibits the PO activity rather than PPAEs (Tsukamoto et al., 1992). Finally, studies in *M. sexta* showed that dopachrome isomerase is also a PO regulator. Because dopachrome isomerase is activated by the insect's own presence of PO, this leads to down-regulation (Sugumaran et al., 2000).

Phenoloxidase in an evolutionary ecology context

Recently, evolutionary biologists and immunologists alike have studied how physiology and ecology interact to drive the evolution of immune defense systems (reviewed by Schulenburg et al., 2009). A key assumption in these studies is that there are individual differences in immune ability. Variation in immune ability may be maintained via costs that emerge either through limited resource availability and/or pleiotropic effects on non-immune related, genetically linked traits (Schmid-Hempel, 2005). In the following section, we review studies of PO activity in the context of pathogen defense, their costs and the environment*gene interaction to explain PO activity.

Just how useful is PO against pathogens?

Table 1 summarizes studies in which a variety of immune challenges were used and an association was examined between proPO and/or PO activity and indicators of individual success against any pathogen agent. We did not find broad support for a positive relationship between PO activity and successful pathogen defense (Table 1). A number of reasons can be put forward to explain this unexpected finding before accepting the idea that PO is not useful against pathogens. First, many studies failed to estimate the severity of the pathogen challenge used (e.g., failed to measure the median lethal dose LD₅₀). Thus, some studies used challenges that far from represented a real threat when too low a dose was used, or else overwhelmed any differential individual immune response and induced anti-septic shock when too high a dose was used. A second reason is that many studies did not consider that PO may be used for other functions besides immunity (i.e., egg production, pigment synthesis, molting, or sclerotization), and hence measuring total PO activity in a sample cannot be considered as a good indicator of immunocompetence. This can be solved by finding out which PO deals with immunity and then to quantify its activity. Third, PO may

only be activated for short periods of time in specific tissues and also acts in different ways depending on the immune challenge (Nappi & Christensen, 2005). As an alternative, immunoeological studies could measure proPO activators such as serine proteases, activation of proPO genes, or even proPO itself for more accurate measurements of immunity (Cerenius et al., 2008). One final explanation is that the use of dead pathogens may trigger different responses than real pathogens (e.g., Contreras-Garduño et al., 2007). This has to be properly controlled once such variation in response is known.

Resource-based costs of PO production

The cost of production and maintenance of the PO system (including the proPO-activating system) is likely to be high for two reasons. First, the main compound of the proPO-activating system – tyrosine – is obtained from phenylalanine, which can only be obtained from ingested food (Chapman, 1998). Second, melanin, a final product of proPO-activating system, is a nitrogen-rich compound, which may require substantial nitrogen or protein investment for its synthesis (Blois, 1978; Lee et al., 2008). Thus, production and maintenance of the proPO-activating system is dietary-dependent. Several laboratory studies have supported this. For example, the mealworm beetle, *Tenebrio molitor* L., and the damselfly *Hetaerina americana* Fabricius that were fed ad libitum showed higher PO values than males that were non-fed (Siva-Jothy & Thompson, 2002; Rantala et al., 2003; González-Tokman et al., 2011). Other studies have also found that a protein-based diet is needed for an optimal proPO-activating system. For example, in the Mormon cricket, *Anabrus simplex* Haldeman, animals fed a high-protein diet showed higher values of total PO activity than animals fed a high-carbohydrate diet (Srygley et al., 2009). Related results were found in the caterpillars *Spodoptera exempta* Walker and *Spodoptera littoralis* Boisduval: infected animals fed a higher protein:carbohydrate ratio had more PO activity and survived for longer than those fed a lower protein:carbohydrate ratio (Lee et al., 2006; Povey et al., 2009). A possible explanation for these results is that protein content directly affects the production of amino acids, which can be used for the synthesis of several compounds of the proPO-activating system, including PO itself (Abisgold & Simpson, 1987).

If PO production is diet-dependent, then it follows that individuals in good condition [condition defined as any trait closely linked to viability (Iwasa et al., 1991; Iwasa & Pomiankowski, 1994)] ought to have high levels of proPO and PO production. We have reviewed the existing evidence testing this prediction by filtering studies in which different sources of stress were studied in relation to their

Table 1 Phenoloxidase as an indicator of strength against pathogens

Species	Immune challenge	Relation PO/proPO activity and pathogen defense	Basis of relation	References
<i>Spodoptera exempta</i>	Contaminated diet with nuclear polyhedrosis virus (NPV)	Positive	Larvae with higher survival after an NPV infection showed higher PO activity in hemolymph	Reeson et al. (1998)
<i>Calopteryx splendens xantostoma</i>	Eugregarine parasite burden	Negative	Males with higher capacity to fight off eugregarines had lower PO levels	Siva-Jothy (2000)
<i>Gryllus texensis</i>	Inoculation with <i>Serratia marcescens</i> , <i>Serratia liquefaciens</i> , and <i>Bacillus cereus</i>	Non-significant	Total PO levels did not predict male survival after three bacterial challenges	Adamo (2004)
<i>Daphnia magna</i>	Infection with one bacterial species (<i>Pasteurina ramosa</i>) and three microsporidia species (<i>Octospora bayeri</i> , <i>Ordospora colligata</i> , <i>Glugoides intestinalis</i>)	Non-significant	PO activity in hemolymph did not predict immunocompetence in any immune-challenged subjects	Mucklow et al. (2004)
<i>Drosophila melanogaster</i>	Natural infection with fungus <i>Beauveria bassiana</i> and challenge with a needle contaminated with the yeast <i>Candida albicans</i> , the gram- bacteria <i>Agrobacterium tumefaciens</i> and <i>Escherichia coli</i> or the gram+ bacteria <i>Enterococcus faecalis</i> and <i>Staphylococcus aureus</i>	Non-significant	<i>Drosophila</i> mutants that fail to activate PO in the hemolymph following microbial challenge were as resistant to infection as wild-type flies	Leclerc et al. (2006)
<i>Hetaerina americana</i>	Inoculation with <i>S. marcescens</i>	Positive	Territorial males subjected to bacterial inoculation and nylon monofilament implants had higher PO activity and survival for longer than non-territorial males	Contreras-Garduño et al. (2007)
<i>Litopenaeus vanamei</i>	Challenge with white spot syndrome virus	Negative	Down regulation of proPO	Ai et al. (2008)
<i>Plodia interpunctella</i>	Oral inoculation and direct intrahaemocoelic injection of naturally occurring granulosis virus (PiGV)	Non-significant	No elevated hemolymph PO activity during the early stage of infection. Hemolymph PO activity increased in the susceptible (infected) larvae only at a later stage of the infection	Saejeng et al. (2010)
<i>Hetaerina vulnerata</i>	Inoculation with <i>S. marcescens</i>	Non-significant	Males subjected to bacterial inoculation had higher PO activity after 24 h, but survival was not related	González-Santoyo et al. (2010)
<i>Daphnia magna</i>	Spores of <i>P. ramosa</i>	Positive	PO levels in infected hosts did predict spore load	Pauwels et al. (2011)

effect on animal condition. These studies appear in Table 2. From this review, there is clear evidence that individuals in better condition produce higher levels of proPO

and/or PO. The fact that stressors across these studies varied so considerably suggests that proPO and PO are shaped by a suite of environmental factors.

Table 2 Relationship between phenoloxidase and individual condition

Species	Stressful source affecting condition	Relation condition-proPO/PO	Basis of relation	References
<i>Spodoptera exempta</i>	Population density	Positive	Larvae reared at high density had significantly higher PO levels	Reeson et al. (1998)
<i>Gryllus texensis</i>	Aging	Negative	Old males showed lower PO values than mature males	Adamo et al. (2001)
<i>Schistocerca gregaria</i>	Population density	No relation	Animals in <i>solitaria</i> and <i>gregaria</i> phase did not differ in PO activity	Wilson et al. (2002)
<i>Tenebrio molitor</i>	Nutritional status	Positive	Starvation reduced the PO activity	Siva-Jothy & Thompson (2002)
<i>S. exempta</i>	Population density	Positive	Animals kept under crowded conditions showed higher PO values in hemolymph, midgut, and cuticle compared to animals kept in solitary conditions	Wilson et al. (2001)
<i>Daphnia magna</i>	Nutritional status	Positive	Well-fed <i>Daphnia</i> showed higher PO activity than <i>Daphnia</i> kept at a low food level and wounding provoked a higher level of PO activity	Mucklow & Ebert (2003)
<i>T. molitor</i>	Nutritional status and attractiveness	Positive	Males in better nutritional conditions had more PO activity and were more attractive for females	Rantala et al. (2003)
<i>T. molitor</i>	Attractiveness	Positive	More attractive males had higher PO levels after an immune challenge by a nylon monofilament insertion	Sadd & Schmid-Hempel (2006)
<i>Lestes viridis</i>	Predation risk	Negative	The presence of a predator (fish) reduced PO and proPO activity in early hatched larvae	Stocks et al. (2006)
<i>Hetaerina americana</i>	Territoriality	Positive	Territorial males had higher PO activity levels than non-territorial males	Contreras-Garduño et al. (2007)
<i>Epirrita autumnata</i>	Nutritional status	Negative	Starvation during the final phase of larval development increased both the absolute and specific PO activity	Yang et al. (2007)
<i>Bombus terrestris</i>	Aging	Negative	PO activity decreased with age	Moret & Schmid-Hempel (2009)
<i>Aedes aegypti</i>	Nutritional status	No relation	No direction for males and females	Moreno-García et al. (2010)
<i>H. americana</i>	Nutritional status	Positive	Males under starvation showed lower PO values than ad libitum fed males	González-Tokman et al. (2011)

Life-history theory predicts that competition for finite resources leads to resource allocation conflicts among different functions (Stearns, 1992). Two relevant questions in this context are: (1) which functions become impaired when insects are immune challenged and (2) what physiological mechanisms mediate resource allocation in insects? As for the first question, various immunoeological studies have provided direct and indirect evidence that resources allocated to proPO and/or PO production affect a plethora of functions. Some examples of traits affected by immune function resource allocation trade-offs are production of sexual traits (e.g., Siva-Jothy, 2000; Rantala et al., 2003; Pomfret & Knell, 2006; Sadd et al., 2006), mating (Rolff & Siva-Jothy, 2004), aggressive behavior (Contreras-Garduño et al., 2009), and larval development (Cotter et al., 2004). The wide array of affected functions suggests that there are common pathways in the utilization of resources. The presence of these common pathways also suggests that in many life stages, insect hosts are subject to unavoidable risks of infection. As for the second question, juvenile hormone (JH) is a strong candidate as a physiological mechanism to regulate insect resource allocation. Juvenile hormone is a hormone produced by the corpora allata that affects a number of developmental and life history functions in insects. For example, the presence of JH favors sexual maturation, vitellogenesis, reproduction, and metabolism, but its absence leads to a reduction in lifespan, survival, diapause, stress resistance, and immunity (Flatt et al., 2005). One example that shows not only JH control but also the effects of JH on very different functions is the case of calopterygid damselflies. In these animals, experimental increase of a JH analog (methoprene) led to increased aggression during territorial behavior at the expense of PO activity in males (Contreras-Garduño et al., 2008). Similarly, the JH analog also led males to develop increased wing pigmentation, a sexual trait used to court females, at the cost of survival (Contreras-Garduño et al., 2011).

Pleiotropic effects of PO activity

Studies of narrow-sense heritability (h^2) have concluded that PO is a heritable trait ($h^2 > 0.8$) in *S. littoralis* (Cotter & Wilson, 2002; Cotter et al., 2004), in the yellow dung fly, *Scathophaga stercoraria* L. (Schwarzenbach et al., 2005; Schwarzenbach & Ward, 2007), the mealworm beetle *T. molitor* (Armitage & Siva-Jothy, 2005), and the cricket *Gryllodes sigillatus* Walker (Gershman et al., 2010a,b). This is not surprising, given the strong relationship of PO with fitness (reviewed by Schulenburg et al., 2009). However, due to this relationship, it is also expected that natural selection will erode additive genetic variation (Stearns, 1992). It has been suggested that one possible way to main-

tain such high genetic variance is via antagonistic pleiotropy between PO and other traits (Cotter et al., 2004), that is, a gene or set of genes with positive effects on one component of fitness, but a negative effect on another component (Roff, 1992). Probably, the most common example of such a genetic trade-off comes from a study of experimental selection in the yellow dung fly, *S. stercoraria* (Hosken, 2001). After 12 generations of enforced monogamy or polyandry, PO values in polyandric lines were lower than in monogamous lines. However, polyandric flies also evolved larger reproductive organs. These results indicate a potential evolutionary trade-off between investment in reproductive tissues and immunity (Hosken, 2001). More recent studies have found genetic trade-offs between PO activity and cuticular darkness (Rolff et al., 2005), development rate (Cotter et al., 2004, 2008b), survival (Schwarzenbach & Ward, 2006), and pupal mass (Klemola et al., 2007). Interestingly, antagonistic pleiotropy has been detected between PO and lysozyme-like activity, another key immune component (Moret & Siva-Jothy, 2003; Cotter et al., 2004, 2008a; Bailey & Zuk, 2008). All these pieces of evidence strongly indicate that increases in PO-based immune ability may not outweigh its benefits. Therefore, selection should favor individuals that are more prudent in their investment into PO. For example, if pathogen action is not as challenging in a certain environment, selection may act to favor a PO reduction so that fitness can be maximized. One example of a genotype*environment interaction is the negative effect on survival in *S. stercoraria* shown by Schwarzenbach & Ward (2006), which only occurs when animals are not provided with food.

Finally, PO has positive genetic correlations with functions that seem costly. For example, selection on PO activity led to larger first clutches (Schwarzenbach & Ward, 2006), pupal weight, and adult longevity (Cotter et al., 2004).

Gene*environment interaction in the study of PO

A genetic background that allows different phenotypes is likely to have an advantage in heterogeneous environments (Zhivotovsky et al., 1996). The ability of a genotype to give rise to distinct phenotypes when exposed to different environments is known as phenotypic plasticity (Roff, 1997; Nylin & Gotthard, 1998; Schlichting & Pigliucci, 1998). Given the role of environment and the genetic basis of PO, it is not surprising that this trait is phenotypically highly plastic. Phenotypic plasticity in PO production has been investigated in lepidopterans (Cotter et al., 2008a), beetles (Barnes & Siva-Jothy, 2000), and mosquitoes (Moreno-García et al., 2010). These studies indicate the extent of the variance in PO activity caused by environmental influences. For example, and contrary to what has

been determined for other insects (Siva-Jothy & Thompson, 2002; Rantala et al., 2003; González-Tokman et al., 2011), PO was not food dependent in mosquitoes (Moreno-García et al., 2010). Whether other insects also show this and whether this is an adaptive response remain to be seen.

Some remarks for future development

In the last decades, there has been growing knowledge about PO activating systems, especially regarding the components that activate or inhibit PO activity in insect immune systems. However, there are still gaps in our understanding of the PO activating system. Some of these gaps are a consequence of the intrinsic properties of PO. For example, most cells that synthesize PO are fragile hemocytes that are difficult to study under controlled conditions (i.e., in culture). Also, intermediate molecules involved in the PO activating system are rapidly activated and degraded, which makes their isolation and recognition difficult. Due to these logistical challenges, some aspects remain to be elucidated. For example, although it is known how quinones are polymerized to melanin in vitro, it remains unknown how this takes place in vivo. Also, despite the discovery that some molecules that act as protein cofactors of serine proteases (i.e., SPH1, SPH3) are involved in PO activation by gram+ bacteria or fungi, it is still unclear whether there are analog cofactors acting in the face of infections by gram- bacteria.

Another important gap is that the natural substrates of PO are unknown. Under laboratory conditions, DOPA has been used as the main PO substrate in in vitro studies, but catechols such as NADA and NBAD are also present in insect hemolymph and could be used as natural substrates of PO in insect immune response. Indeed, PO has higher affinity to NADA and NBAD than to DOPA (Asano & Ashida, 2001). Furthermore, more research is needed to determine which substrates are used by PO in response to infections with different pathogens.

Immunoecological studies are still in their infancy. A basic issue is to resolve the extent to which PO is the immune parameter against pathogens. The insect immune system is composed of a number of other components in addition to PO which have received relatively less attention. These other components, along with PO, should be examined in a more coordinated manner to form an integrated view of the evolutionary ecology of PO. One example is the detected negative genetic correlation between PO and lytic activity (Cotter et al., 2004), which constrains the host response to immune challenges. In relation to the environmental action, future studies should address PO-related questions with more realistic scenarios of natural

interaction between hosts and their pathogens to explain coevolutionary scenarios. In this respect, it remains unclear, for example, why both sexes (Moreno-García et al., 2010), different stages of the same individual (Eleftherianos & Revenis, 2011), different seasonal times of the same species (Córdoba-Aguilar et al., 2009), and different but closely related species (Córdoba-Aguilar et al., 2009 et al., 2006) show differences in PO activity. These differences may be related to differential exposure to distinct pathogens. Moreover, not only gene*environment but also gene*gene interactions of hosts and their pathogens can explain susceptibility to infection (Lazzaro & Little, 2009), which has not been studied for PO. One final issue is to relate PO activity with selection strength, which will show which PO phenotypes are favored (e.g., Rolff & Siva-Jothy, 2004). As with a number of physiological traits, little is known about the strength of natural selection, and given the importance of PO for survival, future studies should address this.

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