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Re-evaluation of insect melanogenesis research: Views from the dark side

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SUMMARY

Melanins (eumelanin and pheomelanin) are synthesised in insects for several purposes including cuticle sclerotisation and color patterning, clot formation, organogenesis and innate immunity. Traditional views of insect immunity detail the storage of pro-phenoloxidases inside specialized blood cells (hemocytes) and their release upon recognition of foreign bodies. Activated phenoloxidases convert monophenols into reactive quinones in a two-step enzymatic reaction, and until recently, the mechanism of tyrosine hydroxylation remained a mystery. Herein, we present our interpretations of these enzyme-substrate complexes. The resultant melanins are deposited onto the surface of microbes to immobilise, agglutinate and suffocate them. Phenoloxidase activity and melanin production are not limited to the blood (hemolymph) or cuticle, as recent evidence points to more diverse, sophisticated interactions in the gut and with the resident symbionts. This review offers insight into the somewhat neglected areas of insect melanogenesis research, particularly in innate immunity, its role in beneficial

insects such as pollinators, the functional versatility of phenoloxidases and the limitations of common experimental approaches that may impede progress inadvertently.

Keywords: phenoloxidase; innate immunity; hemocytes; gut melanisation; molecular scaffolds

Running title: Re-evaluation of insect melanogenesis research

INTRODUCTION

Pathogen challenge or trauma from abiotic factors (e.g. temperature shock, UV damage, overcrowding) trigger a highly distinctive feature of insect innate immunity: the recruitment of phenoloxidases (POs) to initiate the biogenesis of melanin. The subsequent blackening of insect blood (hemolymph), caused by the gross accumulation of melanic polymers, has been debated for many years due to its apparent inefficiency and toxicity to the host (reviewed by Cerenius et al., 2008; for an overview of insect immunity see Hillyer, 2016; Butt et al., 2016). Nevertheless, PO activities and melanin production are vital to many physiological processes, including cuticle sclerotisation (tanning) post ecdysis, hemostasis and wound repair, wing and eye pigmentation, as well as gut homeostasis.

The insect cuticle is hardened for muscle attachment and made impervious to pathogens and parasites via the independent outputs of the sclerotinogenic and melanogenic pathways. At the cuticle, $_{L}$-tyrosine is converted into $_{L}$-DOPA and dopamine via hydroxylase and decarboxylase activities, respectively (Figure 1). Either of these two catecholamines can undergo melanogenesis, whereas dopamine alone is metabolised independently by laccases (amongst other enzymes) into colorless (dehydro-*N*-acetyldopamine) and brown (dehydro-*N*-*β*-alanyldopamine) biopolymers used to 'glue' the newly formed cuticle together (Sugumaran and Semensi, 1991; Ricketts and Sugumaran, 1994; Sugumaran and Barek, 2016). Concurrently, $_{L}$-tyrosine is converted directly into DOPAquinone via extracellular PO activity and ultimately produces black melanin (5,6-Dihydroxyindole; DHI) (Figure 1). The quinone groups of melanin are cross-linked with existing proteins (e.g. sclerotin) in the terminal polymerisation step. Immunity-related melanogenesis is facilitated by soluble POs (*o*-diphenoloxidase) in the hemolymph, whereas cuticular melanisation (i.e. sclerotisation) is performed by laccases (*p*-diphenoloxidase) (Arakane et al., 2005). The varied pigmentation patterns displayed by insects, and the underlying color palette available, are dependent on the presence of particular amino acids (e.g. tyrosine, aspartate, cysteine) and the plethora of enzymes involved in their processing

(reviewed by Kronforst et al., 2012). Color patterning is indispensible in crypsis (camouflage and mimicry), mate selection and recognition, aposematismatic warning signals, and even thermoregulation.

The availability of sophisticated molecular and bioinformatics toolkits have enabled researchers to disentangle the complex relationships between POs, melanins and host defenses. Using such resources, we have interrogated the resolved crystal structures of the available insect PPOs and deduced that monophenol de-protonation by a conserved water molecule prior to hydroxylation and subsequent oxidation into quinone is a conserved mechanism across insects, plants and microbes. This review also highlights recent developments relating to the gut-melanin-symbiont axis and a division of labor between different POs. We additionally examine the importance of melanization in beneficial insects such as the beleagured honeybee, and re-visit evidence suggesting that POs form activation complexes and associate with molecular scaffolds (amyloids).

1. STRUCTURE-FUNCTION DYNAMICS OF INSECT (PRO) PHENOLOXIDASES

Insect proPhenoloxidases (PPOs) are metallated zymogens belonging to the family of type III copper proteins, which includes arthropod and mollusc hemocyanins, as well as microbial, vertebrate and plant oxidoreductases. Such proteins are identifiable by their distinct dicupric active sites (CuA and CuB) where each copper atom is supported by three highly conserved histidines residues, thus enabling the binding of oxygen as peroxide in a side-on bridging (μ–η^2:η^2) orientation (Solomon et al., 2014). Insect phenoloxidases (POs) share high degrees of sequence homology with arthropod hemocyanins (up to 40%), whereas microbial, human and plant tyrosinases are more closely related to mollusc hemocyanins (Kawabata et al., 1995). Insect PPOs can be found as either homo-dimers such as PPO8 from *Anopheles gambiae* (Hu et al., 2016) or hetero-dimers (~160 kDa) such as PPO1/2 (isozymes) from *Manduca sexta* and *Bombyx mori* (Li et al., 2009; Figure 2). Irrespective of the species' origin, PO monomers (70–80 kDa) consist of three domains: (I) an *N*-terminal domain, (II) a central, α-helical domain housing the copper centre, and (III) a multi-stranded, antiparallel β-barrel (*C*-terminal) domain (Kanteev et al., 2015). A pro-region containing the proteolytic site for trypsin-like serine proteases usually precedes the *N*-terminus of insect PPO (Figure 2). Serine proteases coordinate several insect defense responses (including antimicrobial peptide generation) and modulate the proPO activation cascade (Cerenius et al. 2010). Specific serine proteases, known as PPO-activating proteins (PAPs), cut the polypeptide at the carboxyl end of arginine to yield active PO (see below). Human tyrosinase shares little homology with insect POs – an exception being the invariant histidine residues found in the hydrophobic pocket of a 4-alpha helix bundle. Instead of a pro-region, human tyrosinase has an *N*-terminal signal peptide and a transmembrane domain, which guide the

protein through the secretory pathway and to the cytosolic membrane of the melanosomes (Olivares and Solano, 2009).

The term phenoloxidase (PO) is often used interchangeably across the literature and subsumes three distinct enzymes: tyrosinase (EC. 1.14.18.1), catecholoxidase (EC. 1.10.3.1) and laccase (EC 1.10.3.2). Tyrosinase (TY) is responsible for the *ortho*-hydroxylation of monophenols (e.g. $_{L}$-tyrosine) into *ortho*-diphenols (e.g. $_{L}$-DOPA) and their subsequent two-electron oxidation to *ortho*-quinones (e.g. $_{L}$-DOPAquinone), whereas catecholoxidase (CO) performs the second reaction only (Decker et al., 2007). DOPAquinone undergoes auto-catalysis to form DOPAchrome, followed by the auto-polymerisation of 5,6-dihydroxyindole (DHI) into blackish-brown **eumelanin**. Notably, the presence and functional significance of sulfurous (yellow) **pheomelanin** in insects has been overlooked for some time (see Figure 3). Melanoid polymers are deposited directly onto the surface of microbes to immobilise, agglutinate and suffocate the cells. Simultaneously, a suite of antimicrobial oxidising (•OH, O_2^-) and nitrosative ($ONOO^-$) species are produced as by-products (Zhao et al., 2007). In humans, eumelanin is composed of 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Sugumaran et al., 1999; Sugumaran and Barek, 2016), which has not been detected in any insect studied to date. Whilst insect POs and human TYs are able to convert a broad range of $_{L}$-isomers of phenolic substrates into melanoid products, information regarding the presence of $_{D}$-isomers in insect hemolymph is lacking. That said, several studies report that PO and TY have a distinct preference for $_{L}$-isomers (e.g. tyramine, dopamine, 4-methylcatechol) *in vitro* (Sugumaran and Semensi, 1991; Chang, 2009).

In contrast to binuclear TY and CO, laccase uses both a mononuclear type I copper site and a trinuclear copper cluster to catalyse the single electron oxidation of *ortho*- and *para*- isomers of diphenols (reviewed by Dittmer and Kanost, 2010; Solomon et al., 2014). Therefore, laccase should be referred to as a multicopper oxidase (MCO) rather than a PO *sensu stricto*. Until recently, the reason behind the enzymatic discrimination between TY and CO was a mystery, compounded by their almost identical physio-chemical modalities *in vitro* (Solem et al., 2016). Interrogation of the resolved crystal structures of several type III copper proteins, namely *Bacillus megaterium* TY with $_{L}$-DOPA and tyrosine bound (PDB-4P6S/4P6R; Goldfeder et al., 2014) and *Vitis vinifera* PO-g with tyrosol bound (PDB-2P3X; Solem et al., 2016), revealed that a conserved water (HOH) molecule appears to facilitate the ortho-hydroxylation of monophenols, supported by glutamate and asparagine (Kanteev et al., 2015). As $_{L}$-tyrosine enters the dicopper centre and passes over the activated water molecule it is de-protonated into a phenolate intermediate, and the liberated hydrogen combines with water to form hydronium (H_3O). Phenolate is then directed towards CuA where it interacts with the bound peroxide to form catecholate. Catecholate is oxidised and released as *o*-quinone alongside H_2O. Using a series of *V. vinifera* PO mutants, Solem et al. (2016) confirmed that both glutamate and asparagine are

necessary for *ortho*-hydroxylase activity. If asparagine does not immediately follow H1 of CuB (Figure 4) or is mutated to glycine, then only diphenolase activity is possible.

Previously, Li et al. (2009) suggested that glutamate-395 of *M. sexta* PPO2 served as a general base for de-protonation of monophenols due to its close proximity (~3 Å) to the placeholder residue, phenylalanine-88 (Figure 4). Upon PO activation, $_{L}$-tyrosine would occupy the position of the displaced phenylalanine as it enters the active site to be turned over and released as a product. However, after careful re-evaluation of the PO crystal structures from *M. sexta* (PDB-3HHS) and *A. gambiae* (PDB-4YZW; Hu et al., 2016), we propose an alternative mechanism of insect PO activity in agreement with Goldfeder et al. (2014) and Solem et al. (2016). A conserved water molecule (HOH700) bound by glutamate-353 and asparagine-369 of *M. sexta* PO2 is responsible for removing hydrogen from the hydroxyl group of monophenols, and likewise, glutamate-364 and asparagine-380 of *A. gambiae* PO8 (Figure 4). Furthermore, the key active site residues (E and N) are located in all insect POs surveyed here, including the single PO found in *Apis mellifera*. Conventional amino acid sequence alignments of human, mouse, insect, plant and microbial tyrosinases were not sufficient to reveal the importance of E and N within the copper active sites (data not shown). Only after performing structural alignments of the available crystal structures (and mapping of the residues) did the conserved positions and geometries of these critical amino acids in insect POs (and human tyrosinase) become apparent.

Despite possessing homologous active sites, the structural hierarchy and heterogeneity of the various type III copper proteins present a variety of issues for substrate accessibility (Decker et al., 2007). The common denominator in all structural investigations is that POs (TY and CO) need to undergo structural rearrangements in and around the dicopper centre prior to the admittance of substrates (i.e. activation). This is regardless of the induction mechanism – protease-mediated, intermolecular complex formation (see below), partial unfolding, or a combination of these. Selected proteases (e.g. trypsin), polar solvents and detergents have been used in vitro to characterise the biophysical and biochemical properties of POs pre- and post- activation (Kanteev et al., 2015; Coates and Decker, 2017). Intact *M. sexta* PPO2 is mostly negatively charged and this may explain why the cationic detergent, cetylpyridinium chloride (CPC), is capable of binding to and activating the enzyme. A conserved 'placeholder' residue, usually with aromatic or aliphatic chemistry, blocks the entrance to the dicopper centre. Upon activation, this placeholder residue is dislodged thereby opening the active site to accommodate bulky phenolic substrates (Cong et al., 2009). Examples of such placeholder residues in insects include Phe85/88 in *M. sexta* PPO1/2 and Phe99 in *A. gambiae* PPO8 (Li et al., 2009; Hu et al., 2016; Figure 4).

2. PHENOLOXIDASE ACTIVATION COMPLEXES AND MOLECULAR SCAFFOLDS

The canonical view of PPO activation in insects follows a series of well-defined events. First, invading microbes and their exoplasmic sugar-lipid moieties (PAMPs) are intercepted by soluble and/or hemocyte-bound receptors (PRPs) residing in the hemolymph. These warning signals are transduced intracellularly, leading to the initiation of a multi-step proteolytic cascade that converts PPO zymogens into active POs. Specialized hemocytes containing the immune enzyme, namely crystal cells or oenocytoids, rupture to deposit PPOs at particular locations, e.g. damaged cuticle, the surface of encapsulated parasitoid wasp eggs and nodulated microbes (reviewed by Cerenius et al., 2010).

Some evidence suggests that monomeric PO may not be the terminal step in the PPO activation cascade. In fact, POs were found to interact with various macromolecules such as serine protease homologues (SPHs) in the lepidopterans *M. sexta* (Gupta et al., 2005) and *B. mori* (Clark and Strand, 2013), and membrane phospholipids (Bidla et al., 2009) in *D. melanogaster*, thereby forming '**activation complexes**'. The enzymatic properties of monomeric POs are recorded routinely in the presence of diphenolic substrates (dopamine, $_{L}$-DOPA) *in vitro* due to the convenience of dopachrome detection via spectroscopy (c.a. 470 nm) and the poor rate of catalysis observed when using tyrosine (low V_{max}; Chase et al., 2000). The latter observation is in tension with numerous studies where high levels of tyrosine compared to catecholamines were detected in the hemolymph (Hopkins et al., 1998; Clark and Strand, 2013), implying tyrosine is the natural substrate. Li et al (2009) observed a strong shift in electrostatic surface potential of *M. sexta* PPO2 models after simulated cleavage by PPO-activating proteins (PAPs). They postulated that PAP-induced proteolysis at arginine 51 enables the now positively charged region of PO to interact with the negatively charged clip domain of the non-catalytic SPH. Gupta et al. (2005) demonstrated clearly that both PAP and SPH are essential for 'switching on' PO, and in doing so, a high molecular weight oligomer assembles.

More recently, Clark and Strand (2013) removed a 670 kDa complex containing PO and SPH1 from the hemolymph of *B. mori*. Upon addition of substrate, HPLC-eluted fractions of monomeric PO showed little activity. Conversely, fractions containing the large PO-containing protein complex melanised almost instantly. SPHs, PAPs and POs from various insect species (e.g. *Holotrichia diomphalia, Tenebrio molitor*) have been shown to interact directly with microbial ligands, carbohydrates and immune-lectins (Kwon et al., 2000; Yu et al., 2003; Kan et al., 2008). In *Tenebrio*, a Spätzle-processing enzyme combines PPO (79 kDa) and SPH1 into a melanin producing complex with anti-septic properties (Kan et al., 2008). These biochemical properties may contribute to localising the PO activities (and their toxic by-products) – such that PO-containing complexes 'stick' to pathogens or wounds. In support of this, Bidla *et al.* (2007 and 2009) observed an increase in *D. melanogaster* PO activity in the presence of ruptured crystal cells and dying (perhaps senescent)

hemocytes. Further still, PO activity increased significantly in the presence of the cytoplasmic membrane phospholipid, phosphatidylserine. The authors proposed a model in which PO is released from crystal cells during injury and forms complexes with cellular fragments and is activated by PS. A clear advantage of this would be the protection of PO from serpin-mediated inhibition inside the developing clot (Bidla et al., 2009). Damage-associated molecular patterns (DAMPs) like inner membrane phospholipids and apoptotic bodies have also been shown to activate another type III copper protein, namely hemocyanin-derived phenoloxidase (Coates et al., 2011 and 2013).

The deleterious effects of toxic by-products from the PO cascade are well documented (e.g. Nappi and Christensen, 2005; Zhao et al., 2007) so insects must strictly limit their dissemination and target melanin deposition only to where it is needed. Mammals evolved melanosome organelles to compartmentalize the process of melanin synthesis, but insects have developed alternative strategies. Interestingly, both containment systems appear to have **amyloid proteins** in common. Amyloids are aggregates of protein fibrils that adopt a characteristic cross-beta-sheet quaternary structure. Melanosome amyloids accelerate the polymerization of small-molecule quinone precursors of melanin, by concentrating and sequestering them and helping to orientate the molecules optimally. The amyloid acts as a kind of molecular scaffold to enhance the rate of melanin accumulation. An additional benefit of sequestration is to protect the melanosome from the toxicity of by-products (reviewed by Fowler et al., 2007).

A similar, extracellular role for functional insect amyloid proteins was first indicated by Falabella et al. (2012) who identified a highly represented gene called *102* in the cDNA library from bracovirus-infected *Heliothis virescens* larval hemocytes (Lepidoptera). The P102 protein has 86% amino acid identity with a XenDoU family venom protein from *Lonomia obliqua* (Lepidoptera) (Falabella et al., 2012). Immunostaining showed that P102 is highly expressed in granular cells and especially spherulocytes, bundled as fibrils into cisternae surrounding the nucleus. When purified from hemocytes, these amyloids enhance the rate of hemolymph melanin formation from $_{L}$-DOPA *in vitro*, and *in vivo* they are exocytosed from hemocytes to coat the surface of injected microbeads, where they become associated with large deposits of melanic polymers (Falabella et al., 2012). Although the salient features of XenDoU proteins are RNA binding and hydrolytic activities, an analysis of 102 from *Trichoplusia ni* (Lepidoptera) indicates it lacks some essential residues in the catalytic site for classic XendoU endoribonuclease (Pascale et al., 2014). This might be a feature specific to the Lepidoptera due to a gene duplication event that separated two functional XendoU subfamilies; other non-lepidopteran insects are predicted to have retained a conserved, active XendoU domain (Pascale et al., 2014). To confirm the link between P102 and amyloid fibrils, both Falabella et al. (2012) and Pascale et al. (2014) expressed various lepidopteran *102*-like genes in *Drosophila* S2 cells; this resulted in dense clusters of membrane-bound amyloid fibrils, whereas control S2 cells transformed with XendoU did not (Pascale et al., 2014). Similar observations of amyloid in hemocyte cisternae

have been noted from larvae of other Lepidoptera: in the granular cells of wasp-parasitized *Heliothis virescens* (Grimaldi et al., 2012), in *Trichoplusia ni* (Pascale et al., 2014), and in *Spodoptera littoralis* (Di Lelio et al., 2014). Knockdown of *S. littoralis* P102 *Sl* by RNAi interferes with both the cellular encapsulation and melanization of injected beads (Di Lelio et al., 2014).

These authors collectively posit that, much as in the mammalian melanocyte, amyloids provide a site-specific molecular scaffold with which to sequester and concentrate melanin precursors, to anchor melanin, and to attach hemocytes during encapsulation. The amyloid might also make the encapsulated intruder sticky enough to adhere to the hemocoel walls. A common histology dye used to diagnose amyloid formation is the benzothiazole salt, Thiofalvin T. The dye binds preferentially to ordered β-sheet structures, orientating itself parallel to the fibre axis (perhaps using charged nitrogen from thiazole to form hydrogen bonds with exposed hydroxyl groups of amyloid fibrils or neighbouring structures) (Khurana et al., 2005; Falabella et al., 2012). Due to the chemical similarities between the benzothiazole of Thiofalvin T and the indole of $_{L}$-DOPAchrome (i.e. they are both aromatic heterocyclic compounds), it is postulated that quinone derivatives may combine with amyloids in a similar fashion. Furthermore, melanin accumulation on the surface of the amyloid plaques may be due to ionic and hydrophobic interactions. Fowler et al. (2006) argue that an amyloid formed by the protein PmeL17 is a non-pathological scaffold used to accelerate the polymerisation of melanin in mammalian tissue. Therefore, the formation of a functional amyloid-scaffold that coordinates melanin-related defenses in insects may in fact be a conserved feature of homeostasis in metazoans.

3. HEMOCYTE ROLES IN MELANIZATION

Hemocytes, the blood cells of insects, play a central role in melanogenesis as the source and vehicles of PPO. Many hemocyte types are recognised, with variable nomenclature between insect species. As with vertebrate blood cells, the role varies with each hemocyte type, and they may be active under different physiological conditions or at a different stage in ontogeny. Some hemocytes phagocytose non-self particles, others mediate the nodulation of bacteria or encapsulation of larger foreign objects, while others manufacture, store and eventually release PPO at the site of infection (reviewed by Browne et al., 2013).

In recent years, we have learnt more about how hemocytes store and release particular PPOs, and that the roles of hemocytes in melanogenesis are not as simple as thought previously. The hemocytes of mosquitoes, *Drosophila* and a handful of important Lepidoptera have consistently received most intense scrutiny. In many instances it is not clear which hemocyte types have been investigated, and it is important to note that both hemocytes circulating in the hemolymph and sessile hemocyte

populations attached to the surface of organs or tissues can detect non-self and contribute to the immune response.

Key recent advances in our understanding of hemocyte PPO storage have been made with *Drosophila* crystal cells. These are specialized larval hemocytes representing about 5% of the hemocyte population (reviewed by Honti et al., 2014). These fragile cells have characteristically crystalline cytoplasmic inclusions, and it has long been assumed, but only recently demonstrated with the use of PPO loss-of-function mutants, that the crystals contain PPO. Binggeli et al. (2014) analysed the morphology of crystal cells in PPO-deficient mutant larval *Drosophila*, and found cytoplasmic crystals persisting in both wildtype and PPO1 mutants, but no crystals in PPO2 mutants and PPO1/2 double mutants. Furthermore, PPO2-specific antibodies stained the crystals in wild type larvae while anti-PPO1 antibodies did not. This indicates that PPO2 is either the major component of the crystals or is necessary for their formation. Interestingly however, wildtype *Drosophila* crystal cells are not completely devoid of PPO1, because both PPO1 and PPO2 disappear from the hemolymph of larvae with genetic ablation of crystal cells (Binggeli *et al*., 2014). The authors concluded that PPO2 is stored in the crystals (for release upon cell rupture) while PPO1 is putatively released into the hemolymph for acute-phase PO responses to injury or infection. This sequential model of PPO release by crystal cells raises intriguing questions about the structure of PPO1: unlike PPO2, is it dissolved in the cytosol and is it secreted? Similar questions have been raised about possible exocytosis of mosquito PPO6 from granulocytes (see below). The accepted dogma is that, unlike their vertebrate counterparts, all insect PPOs lack N-terminal signal peptides and thus require rupture of hemocytes for their release upon immunological insult.

In *Drosophila*, crystal cells are attracted to the site of a primary clot to help form a scab. The nature of the attraction signals involved remains unknown (reviewed by Wang et al., 2014), although melanization at clotting wounds is locally enhanced through activity of apoptotic markers such as phosphatidylserine (Bidla et al., 2009). In clot formation, crystal cell rupture and subsequent melanization require JNK pathway activation, small GTPases and Eiger (the *Drosophila* equivalent of TNF). Factors such as Eiger, produced by both crystal cells and plasmatocytes, can also trigger melanization independently of microbial PAMPs. However, the molecular signal that activates the JNK pathways when crystal cells rupture is still not clear (Bidla et al., 2007).

Crystal cell studies are conspicuously focussed on larval melanogenesis since there are few crystal cells in adult flies, and the paradigm is that crystal cell hematopoiesis is a phenomenon limited to embryos and larvae. Thus far, one study has challenged this view by suggesting that a potential hematopoietic site exists in dorsal abdominal cell clusters of adult *Drosophila* (Ghosh et al., 2015). Although this study awaits corroboration from other researchers, the authors claimed that these clusters contain progenitors of plasmatocytes and crystal cells, which under the control of Notch

signalling give rise to functional hemocytes. A more widely acknowledged phenomenon is the flexibility of hemocyte cell lineages in the later larval stages of *Drosophila*, which allows plasmatocytes to trans-differentiate into functional crystal cells (e.g. Leitão and Sucena, 2015). This transformation, too, is under the control of Notch.

Overall, our knowledge of which insect PPO genes are expressed in which hemocyte type is currently very patchy, especially in insects with a large number of PPO genes such as the mosquitoes (Figure 5). The picture is simpler and more resolved for *D. melanogaster,* which possesses three PPO genes (all located on the 2nd chromosome): PPO1, PPO2 and PPO3, although until recently it was not clear whether the crystal cell is also the site of PPO3 synthesis. Binggeli et al. (2014) and Dudzic et al. (2015) created a series of single, double and triple mutations for all three *Drosophila* PPO genes to clarify the roles and hemocyte specificity of each enzyme; in the latter study PPO3 knockout mutants were created using the CRISPR-Cas9 system. The researchers found that although PPO1 and PPO2 are the major players in *Drosophila,* PPO3 appears to have evolved to deal with specific threats linked to parasitoid wasps like *Leptopilina boulardi.* Parasitoid (braconid or ichneumon) wasps are prominent members of the Hymenoptera, and play important ecological roles in regulating populations of lepidopteran, coleopteran and dipteran insect species by parasitising their larvae. Many host / parasitoid relationships are species-specific. Adult parasitoids oviposit into the host's body cavity, an action that also introduces a cocktail of venom, viruses, and even behaviour-modifying and immunomodulatory secretions (reviewed in Moreau and Asgari, 2015). *Drosophila* PPO3 is expressed in lamellocytes, a cell type rarely found in healthy naive insects, but which is derived directly from plasmatocytes and from larval lymph gland after parasitization (reviewed by Vlisidou and Wood, 2015). These large cells are specialized for a role in encapsulation (of parasitoid eggs) and attach during the latter stages of the capsule formation. Intriguingly, although PPO1 and PPO2 both require proteolytic cleavage to achieve an active form, this is not a requirement for PPO3 (Nam et al., 2008 and Chen et al., 2012).

In non-drosophilid insects, non-phagocytic oenocytoids are functionally analogous to crystal cells, but do not exhibit obvious crystalline cytoplasmic inclusions. Despite many efforts, there is paucity in the literature regarding the fundamental differences between crystal cells and oenocytoids. The roles of oenocytoids and granular cells in nodule formation have been reviewed recently by Dubovskiy et al. (2016) and will not be addressed here. The cytosol of oenoytoids has been shown to contain PPO mRNA and protein (e.g. PPO1 in *Bombyx mori*; Kato et al., 2014; Tokura et al., 2014). In some insects including the Lepidoptera, oenocytoids lyse upon immune challenge to release PPO and other components of the PO cascade, but in other insects, such as mosquitoes, it is still unclear exactly how PPO is released from these cells.

Granular cells (granulocytes) are phagocytic hemocytes that often internalize melanized bacteria and other particles; they are the equivalent of *Drosophila* plasmatocytes. In mosquitoes, granular cells occupy 90% of the hemocyte population and can be isolated and enriched by inducing the phagocytosis of magnetic beads (Smith et al., 2016). A proteomic analysis of enriched proteins specifically associated with these cells (Smith et al., 2016) indicated PPO2 and PPO4 clustered with phagocytic activity. Not only does this suggest that melanization is important in phagocytosis, but also that some mosquito PPOs can be expressed in granular cells.

Bryant and Michel (2016) provided evidence for mosquito granulocyte synthesis of PPO6 triggered by a bloodmeal. Adult female *A. gambiae* hemocyte PPO6 enzyme concentrations peaked one day after a blood meal (but not after feeding on sugar solution). All *A. gambiae* hemocytes exhibited detectable levels of PPO6 immunostaining, manifesting as granule-like punctate signals, with a small hemocyte subpopulation exhibiting consistently very intense staining (presumably oenocytoids). Although there was no deliberate attempt to identify the different hemocyte types in the study, assuming that the anti-PPO6 antibody was sufficiently specific, we can deduce that most of the cells observed with moderate PPO6 staining were granulocytes. It is tempting to imagine this could be a pre-emptive strategy to protect the insect from parasite invasion.

A recently-described immune phenomenon in mosquitoes relies on sessile periostial hemocytes attached to the periostium region of the heart (i.e. flanking the valves) to sequester and concentrate circulating microbes and pathogens (King and Hillyer, 2012; League and Hillyer, 2016; Sigle and Hillyer, 2016). This builds on previous observations that pathogens accumulate in discrete foci along the surface of the mosquito heart during infection (Hillyer et al., 2001). Using intravital imaging and microdissection techniques, King and Hillyer (2012) showed that during bacterial and malarial infections, hemocytes migrate to the periostial regions where they bind to the musculature and each other, and engage in the rapid phagocytosis and melanization of certain pathogens including *Plasmodium* sporozoites. The population of sessile periostial cells is permanently elevated thereafter, although the situation is dynamic with cells constantly arriving and leaving.

A similar phenomenon, with stronger melanization, occurs when using soluble PAMPs as elicitors. The location of periostial hemocytes coincides with the site of the fastest hemolymph flow (Sigle and Hillyer, 2016). The authors point out that this is probably a common occurrence, and indeed there are already accounts of hemocytes on, or in, the heart of other insects including *D. melanogaster* (Diptera), *Baculum extradentatum* (Phasmida) and *Galleria mellonella* (Lepidoptera), and the periostial accumulation of pathogens and melanin has been detected in both the Lepidoptera and

Diptera (reviewed in Sigle and Hillyer, 2016). The accumulation of melanin at these sites may be due to phagocytosis of microbes that have already been melanized in the hemolymph, but it is also possible that local melanization occurs. It will be interesting to uncover the identity of the hemocyte types in these periostial clusters.

4. PO ACTIVITY ASSOCIATED WITH THE INSECT GUT

In insects, ingested food is stored and partially digested in the foregut, whereas the midgut is the primary site of digestion and absorption of nutrients. During feces formation the hindgut absorbs water and salts to balance the hemolymph osmotic pressure. The gut is a particular site of interest due to its contact with external abiotic and biotic factors and the need to regulate its microflora and fauna. Although some PO activity and melanization in the gut clearly originate from hemocytes, there is increasing evidence that the gut itself expresses PPO genes *in situ*, and is the location of gut-specific melanogenesis (Figure 6).

Melanin precursors have long been posited to originate in the hemocytes and infiltrate the gut tissues by crossing the basal lamina, particularly in the context of melanotic encapsulation of parasites that breach the gut epithelium (reviewed in Whitten et al., 2006). More recent studies have indicated PO activity in the lumen of the gut, such as in the cricket *Gryllus bimaculatus* (Joseph, 2014). When the cricket midgut was removed, washed and incubated *in vitro* with the appropriate substrates, PO activity was undetectable in either the epithelium or the lumenal contents and the zymogen PPO could not be detected in the lumen. This strongly suggests that PPO enters the midgut from a hemocytic origin, transported via the hemolymph, and then is activated by gut trypsin behaving as a serine protease (Joseph, 2014). The gut contents visibly darken from green to black as they travel towards the hindgut - a phenomenon not limited to crickets. Why do insects need PO activity in their gut lumen? The most obvious answer is as protection against microbial overpopulation, particularly in insect species that might feed on substrates contaminated by their own feces, but also conceivably to protect the gut microbiota or to prevent host toxic shock-like responses to microbial overgrowth in the host insect.

While the cricket study above linked hemocytes to gut PO activity, other studies have identified the gut itself as the source of endogenous PPO synthesis and PO activity. In herbivores such as the silkworm *Bombyx mori* whose larvae feed on mulberry leaves in dense populations, PPO is transcribed and expressed by hindgut epidermal cells to induce fecal melanization (Shao et al., 2012). The ultimate effect is that melanization turns the food debris from green to black in the hindgut and in so doing destroys the majority of microorganisms in the feces. To support these observations guts were stained for PO activity using a dopamine substrate and ethanol to activate PPO; this activity was

suppressible by the PO-specific inhibitor phenylthiourea (PTU). When PTU was administered to live larvae, the feces reverted to a green color with a high bacterial population. The authors also performed *in situ* hybridization and immunostaining to demonstrate that mRNA and protein of both *B. mori* PPO genes (PPO1 and PPO2) localize to epidermal cells in the hindgut, but not nearby structures such as the Malpighian tubules, or midgut. The contribution of other enzymes such as laccase or peroxidase was excluded via enzymatic assays using enzyme-specific substrates and inhibitors, as well as Western blots.

In their study of silkworm larvae, Shao et al. (2012) injected fluorescently-labeled beads as bait for phagocytic hemocytes, which acted as a tool to visualize these cells. Finding no fluorescent signal in the hindgut, the authors deduced that hemocytes do not co-localize with melanisation activity that arises endogenously from gut cells in this region. However, it is possible that melanin could quench fluorescence, and it should also be noted that PPO-generating oenocytoids are non-phagocytic and so would not be visualized by this method. Shao et al. (2012) ruled out plasma infiltration of the gut by probing hemolymph, hindgut and gut contents for the plasma protein lysozyme: of these samples, only the hemolymph yielded a positive lysozyme signal by Western blot analysis. Pulpitel et al. (2015) used immuno-stimulatory molecules to trigger insect gut PPO expression. Hemocoelic injection of bacterial LPS, but not sterile saline, triggered up-regulated PPO mRNA expression in all regions of the adult *Locusta migratoria* gut and especially in the foregut (Pulpitel et al., 2015). Although hemocyte contamination of these tissues could not be discounted, their observations are likely to reflect endogenous expression since hemolymph samples did not evidence a concomitant increase in PPO expression.

Bacteria similarly trigger insect gut melanization. Natural (oral) infections with the *Drosophila* pathogen *Pseudomononas entomophila,* for example, trigger melanisation in the area around the larval midgut / foregut junction (Vodovar et al., 2005). The distinct localization of such responses is nicely illustrated by a phenomenon that has been known - but remained unexplained - for 70 years: the highly prevalent and very specifically-located darkened “scab” which appears in the gut of several European bee species. The scab develops shortly after eclosion, thickens with age, and occurs only on the luminal surface of the pylorus region dividing the midgut and hindgut. Engel et al. (2015) found that the scabs correlated with the presence of the gut γ-proteobacterium *Frischella perrara*, which densely colonizes the edges of the scab structure. Scabs are absent from bees eclosing under sterile conditions and from bees naturally lacking *F. perrara*, but present in naturally-infected hive bees as well as those deliberately infected in the lab (Emery et al., 2017). Although low populations of *F. perrara* inhabit other gut regions, the pylorus appears to represent a special niche for the bacterium; the dense population here triggers host immune responses including the upregulation of tyrosine hydroxylase and DOPA decarboxylase genes, and the multicopper oxidase MCO1 (Emery et al., 2017). Other components of the immune system are also activated (e.g. antimicrobial peptides),

leading the authors to suggest that this could represent a case of immune priming, aimed in particular at protecting newly-emerged adult bees from more extensive infections of the hindgut region. This may hinder other pathogens, but only keeps *F. perrara* in check, since it is still possible to recover some viable *F. perrara* from the scab, and may explain why scabs continue to build as the bee ages. Although it is often described as an opportunistic pathogen, it is still unclear whether *F. perrara* directly impinges on the growth and health of honeybees.

The Shao et al. (2012) study mentioned above is also interesting because it looked beyond silk moths to guts from a diverse range of insect orders. The researchers applied PO activity staining assays to guts from termite, *Drosophila*, mosquito, cricket, flour beetle, Asian corn borers and cotton bollworm, and noted blackened melanic coloration in the foregut and hindgut of all these species. However, it is not reported whether this activity was susceptible to PTU inhibition. When larval *Tribolium castaneum* (red flour beetle) PPO2 was knocked down by RNAi alone or in combination with PPO1, foregut and hindgut melanization was greatly diminished (Shao et al., 2012). Taken together, these studies are tantalizing glimpses of a potentially highly conserved strategy to keep gut microbes in check. There are also some interesting parallels with the phenomenon of brown body formation and autotomy in annelids (Valembois et al., 1992), clearly deserving of further study.

Melanization is also evident in the gut under stress conditions. When the *Drosophila* hindgut is put under dietary salt stress and deprived of protective stress-activated protein kinases, the enterocytes become disorganized, undergo JNK-mediated apoptosis and melanize. During this process, Hemese (a hemocyte marker) cannot be detected at or within the hindgut epithelium, which indicates that the hindgut melanization is independent of hemocyte involvement (Seisenbacher et al., 2011). In this situation, melanin probably fulfills a wound-healing role to mitigate the loss of apoptotic enterocytes, similar to that seen during *Plasmodium* invasions of the mosquito gut (Shiao et al., 2006).

A parallel role for gut PO activity is emerging in the context of **detoxification of plant secondary metabolites** that evolved as protection against insect herbivory (Figure 6). Sometimes this role is delegated to beneficial microbes; for example, several insects are known to benefit from specialized gut symbionts that detoxify metabolites like caffeine or carotenoids (e.g. Ceja-Navarro et al., 2015), and leaf-cutter ants employ laccase from their so-called '*fungal gardens*' to detoxify polyphenolic assemblages in ingested leaf matter (De Fine Licht et al., 2013). Other insects perform detoxification roles for themselves. Insects fed toxic plant diets spiked with excess $_{L}$-DOPA exhibit foregut PO activity that converts phenolics into non-toxic intermediates (Wu et al., 2015). This has been observed in the silkworm (*B. mori*), the cotton bollworm (*Helicoverpa armigera*) and *Drosophila.* In *Drosophila*, PPO1 and PPO2 were detectable in the foregut lumen by immunoblotting. In the silkworm foregut, PPO1 is released into the lumen where it binds to toxic food particles and is activated by an as-yet unidentified serine protease. The PPO activity is unaffected by antibiotics and

so is unlikely to have arisen as an immune response to gut bacteria (Wu et al., 2015). The active PO then oxidizes the phenolics to non-toxic intermediates that travel to the hindgut (possibly indirectly: via the hemolymph and then Malpighian tubules) where they are oxidized further into melanized particles. Silkworm PPO1 mRNA and protein were detected in the foregut, but only trace amounts of PPO2. Plasma contamination was ruled out due to the absence of detectable lysozyme, but it is unclear whether sessile hemocytes were attached to the gut.

It is interesting that the majority of the above studies indicate a regional localization to the fore and hindgut, but limited involvement of the midgut. Perhaps this reflects suboptimal midgut conditions such as very high pH (as in Lepidoptera) that could inhibit the PO cascade, or it may be connected to the different embryonic origin of the gut regions: while the midgut develops from endodermal cells, the fore and hind-guts are derived from embryonic ectoderm. The latter are lined with a chitinous exoskeleton that is shed during moulting while the midgut is lined (in most insects) by a secreted peritrophic matrix (reviewed by Engel and Moran, 2013). There may be a requirement to protect symbionts that precludes excessive PO activity in the midgut. The regional localisation of immune responses in the gut, and how this relates to pathogens and resident microbiota, deserves to be a focus for future investigations.

It is a shame that many studies of insect melanotic immunity do not specifically examine gut melanization. Having seen some convincing evidence of endogenous expression of gut PPOs, an obvious question is how - and by what triggers - PPO could be released from the gut epithelium. Considering the dynamic and complex environment of the gut, the exact composition and structure of the PO cascade components is probably critical, and has no obvious parallels among higher animals. These gut-specific molecules need to be hardier than those acting at physiological pH (in the hemolymph), considering they could encounter digestive proteases, extremes of pH, sudden temperature fluctuations (in hematophagous insects), toxins, or inhibitory factors produced by gut bacteria or pathogens; factors which may be localised to specific regions of the gut. Interestingly, Lu et al. (2014) demonstrated that the stability of *Drosophila* PPO1 is conditional on the formation of two di-sulphide bonds at the C-terminus of the native structure. Expression of recombinant PPO mutants lacking one or both of these bonds led to a decrease in PO activity and antibacterial activity, and an increase in protein susceptibility to thermal-induced denaturation. A single mutation to cysteine596 (i.e. disruption of one disulphide bond) had a negative impact on enzymatic activities at pH 5 and >pH 8.5. Disulphide bonds are known to improve stability and protein folding (sometimes making them rigid), however, the presence of two disulphide bonds in *Drosophila* PPO1 may enable it to withstand the harsh environment of the gut, whilst remaining functionally intact.

5. BACTERIA AND OTHER MICROBES VERSUS MELANOGENIC COMPETENCE

The previous section touched on melanotic activity in the insect gut triggered by bacteria. Relatively few immunological studies have focused on infections (or intoxications) via the gut, and fewer still have interrogated the role of symbionts, commensals and other resident microbes in these interactions. An exciting development in recent years is the recognition that symbiotic gut bacteria are critical in insect hematopoiesis, and particularly in dipteran crystal cell development.

Dipteran embryonic hematopoiesis initially relies on the GATA family factor serpent (Srp) for progenitor hemocyte maturation. In both the embryo and the larval lymph gland, Lozenge (Lz; a RUNX family transcription factor) directs the prohemocytes towards a crystal cell fate (e.g. Waltzer et al., 2003). The larvae of the viviparus tseste fly (*Glossina morsitans morsitans*) develop *in utero*, during which they receive two symbionts from maternal milk gland secretions: *Sodalis glossinidius* and *Wigglesworthia*. It has been appreciated for several years that an aposymbiotic larva (Gmm^{Apo}) derived from a symbiont-cured (antibiotic-treated) mother will develop into a severely immunocompromised adult lacking all hemocyte types and incapable of melanization. These individuals have low expression of Srp and Lz (Weiss et al., 2011). Using transcriptomics comparing tsetse gene expression in Gmm^{Apo} and Gmm^{WT} larvae, Benoit et al. (2017) have identified an *odorant binding protein* gene (*obp6*) that is 22 times more highly transcribed in the latter. Tsetse larvae that develop in the presence of reduced obp6 transcript abundance (Gmm^{OBP6-}) fail to express Lz and thus fail to generate crystal cells. Their phenotype can be rescued by restoring *Wigglesworthia* (but not *Sodalis*) to the symbiont-cured mother tsetse. Transgenerational RNAi targeting *obp6* results in crystal cell ablation in siOBP6 larvae, although phagocytic hemocytes are unaffected - which strongly suggests that obp6 is expressed during larvagenesis but only controls crystal cell maturation (Benoit et al., 2017). Despite normal populations of functional phagocytic hemocytes, siOBP6 adult offspring nevertheless succumb to normally sub-lethal challenges with *E. coli,* and even sterile injury causes exceptionally high mortality consistent with a failure of the melanotic wound healing process. Benoit et al. (2017) also identified the site of obp6 expression as the larval gut (indeed, it is localised to the specialized bacteriocytes harboring *Wigglesworthia*).

A parallel phenomenon occurs in another dipteran, *D. melanogaster*. Benoit et al. (2017) show that the expression of *Lz* and *obp28a* (orthologous to the tsetse *obp6*) was compromised in axenically reared *Drosophila* larvae, which was attributable to a lack of (still to be identified) gut bacteria. Just like the tsetse flies, most adult *Drosophila* from an RNAi line (obp28a knockdowns) can neither melanize nor survive sterile injury. It seems, therefore, that odorant binding protein expression, triggered by the presence of enteric symbiotic bacteria, is an evolutionarily conserved mechanism for hematopoiesis of crystal cells in Diptera.

While some bacteria guide the development of the immune system, others suppress it either directly or via toxins. The nematode symbiont but insect pathogen *Photorhabdus temperata* M1021 secretes 1,2-benzenedicarboxylic acid (phthalic acid, PA) which dose-dependently inhibits PO activity and nodule formation in larvae of the lepidopteran *Galleria mellonella* (Ullah et al., 2014). Similar chemicals interact directly with insect PO (*Pieris rapae* larvae) to inhibit the enzyme non-competitively in the presence of $_{L}$-DOPA (Xue et al., 2007). Phthalic acid also has antioxidant properties that could help ameliorate oxidative stress resulting from the melanization cascade - this may be protective to the nematode parasite when it invades an insect. Phthalic acid adds to a growing list of secondary metabolites produced by *Photorhabdus* spp. that act as toxins to a range of insects and which interfere with melanization (reviewed in Hu et al., 2000). Such compounds have obvious potential as biocontrol agents.

With some bacterial toxins, the outcome and tissue specificity may depend on the exact insect-bacterium species combination. The most studied relationships are those between *Bacillus thuringiensis* (Bt) toxins and lepidopteran hosts. In *Spodoptera exigua*, sublethal intoxication with Vip3A toxin triggers over-expression of PPO activating enzyme (PPAE) and the majority of the insect's serpin gene repertoire (Bel et al., 2013). Conversely, Crava et al. (2015) found that gut-confined sublethal exposure to Vip3Aa or Cry1Ca fails to trigger hemolymph (i.e. systemic) PO activity in this insect (unfortunately the authors did not look for gut melanization). Some insect strains that are refractory to Bt exhibit permanently induced melanization in the hemolymph, e.g. Bt-tolerant strains of *Ephestia kuehniella* (Rahman et al., 2004) and Cry1Ac resistant strains of *H. armigera* (Ma et al., 2005), which must surely impart a fitness cost to the insect.

There is also an unusual report of melanization specifically in fat body tissue (Akhouayri et al., 2013). Chronic melanotic lesions form in the fat body cells of *Anopheles gambiae* larvae, pupae and adults in response to a dominant vertically transmitted commensal gut bacterium, *Elizabethkingia meningoseptica*. This is significant because adult co-infection with *Plasmodium* seems to have a synergistically negative impact on mosquito survival. The fat body is a vital organ in general physiology and immune defenses, and melanotic damage in the tissue also leads to a decreased ability to heal wounds.

Deserving of special mention are pollinators and especially honeybees, which are receiving closer scrutiny due to their exposure to multiple stressors, including pesticides and pathogens. Colony Collapse Disorder (CCD) has caused worrying honeybee losses recently (Oldroyd, 2007). Not surprisingly, the impact of pesticides is under examination, especially neonicotinoids that tend to contaminate pollen and nectar due to their system action on plants. Several studies indicate sub-lethal pesticide effects that weaken bee immunity, including melanization. This renders bees more susceptible to other stressors such as parasites (e.g. varroa mite) and pathogens (e.g. deformed wing

virus, DMV). The neonicotinoids thiacloprid and imidacloprid dramatically inhibit melanotic encapsulation, hemocyte numbers and antimicrobial activity (Brandt et al., 2016). Both the neonicotinoid clothianidin and DMV negatively modulate the honeybee NF-kB pathway (Di Prisco et al., 2013 & 2016). NF-kB activation is needed for Toll-mediated immune responses - including encapsulation - against fungi, viruses and Gram-positive bacteria, and it is also required for expression of Amel\102, which forms amyloids (see the section on amyloid, above, for details). Immunocompetence fluctuates seasonally in honeybees, with a winter down-regulation of cellular immunity and melanization genes PPO, PPOa (PPO activator), eater (a phagocytic receptor), and glucose dehydrogenase, which generates free radicals by interacting with quinones derived from the PO cascade (Steinmann et al., 2015). If, as suspected, melanization turns out to be an effective defense against honeybee viruses (e.g. Mazzei et al., 2016), then the recent *Varroa* and DWV outbreaks could leave honeybees insufficiently protected from viruses during the winter.

In a search for protective countermeasures, the potential use of pre- and pro-biotics to boost the immunity of beleaguered honeybee colonies is at an experimental stage, but this practice has precedents in aquaculture to support fish and shellfish immunity (Akhter et al., 2015). However, Ptaszyńska et al. (2016) presented a cautionary tale in their assessment of *Lactobacillus rhamnosus* (a commercial probiotic) on the survival rates of honeybees. The authors were surprised that their probiotic treated bees were more susceptible to infection and had shorter lifespans. In bees infected with the microsporidian gut pathogen *Nosema ceranae* (a common affliction of honeybees worldwide), the effect of ingesting the probiotic was dramatic: a 20-fold loss of PO activity. Administration of the prebiotic inulin had an innocuous effect but failed to confer any immune priming (Ptaszyńska et al., 2016). In administering probiotics it is apparently vital to choose carefully; an incompatible bacterium might exclude or displace the normal gut microbiota and synergistically augment the immunosuppressive activity of pathogens. Feeding a sugar solution containing 0.5% β-glucan to newly emerged honeybees has been a more encouraging approach (Mazzei et al., 2016). This immunostimulatory fungal cell component boosted melanization, which in DWV-infected bees overcame virus-induced immunosuppression and helped to limit viral loads. However, it was noted that the correct dose was important to avoid a negative impact on bee survival.

Larvae of the waxmoth, *Galleria mellonella,* exposed to varying doses of fungus (*Aspergillus fumigatus;* Fallon et al., 2011) and/or β-glucan (Mowlds et al., 2010) induced changes in cellular and humoral responses including PO activity and PO expression, which directly correlated in magnitude and duration to the dose or pathogenicity of the challenge. Importantly, sub-lethal doses of *A. fumigatus*, or an optimized dose of β-glucan, can confer immune priming effects on the insect that protect it from subsequent, normally lethal doses of fungi or yeast. This priming has an impressive longevity lasting several days (Mowlds et al., 2010).

CONCLUSIONS

The selected studies dissected by this review illustrate exciting nuances in insect melanogenesis and some previously unrecognized overlap with mammalian systems. Thanks to the availability of PO crystal structures, we now have better insight into tyrosine hydroxylation mechanisms. The gut is emerging as an active player in insect melanogenesis that goes beyond immunity, but due to the complexity and species-specific properties of the gut environment, challenges lie ahead in teasing out the various roles of host and microbe factors. Finally, we have noted a small focus-shift in recent publications, from *Drosophila* and pest insects, to include more non-model and beneficial species (especially pollinators). However, the balance between holo- and hemi-metabolous insects still leans heavily towards the former. We would welcome studies that address this imbalance, particularly with the availability of exciting new molecular tools such as symbiont-mediated RNAi (Whitten and Dyson, 2017), CRISPR-Cas9 (e.g. Chen et al., 2016) and bioinformatic resources like the 5,000 arthropod genomes initiative (i5k; http://i5k.github.io/).

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REFERENCES

Abrudan, J., Ramalho-Ortigão, M., O'Neil, S., Stayback, G., Wadsworth, M., Bernard, M., *et al.* (2013) The characterization of the *Phlebotomus papatasi* transcriptome, *Insect Mol Biol*, 22 (2), pp. 211–232. DOI:10.1111/imb.12015.

Akhouayri, I. G., Habtewold, T. and Christophides, G. K. (2013) Melanotic pathology and vertical transmission of the gut commensal *Elizabethkingia meningoseptica* in the major malaria vector *Anopheles gambiae*, *PLoS ONE*, 8 (10), pp. e77619.

Akhter, N., Wu, B., Memon, A. M. and Mohsin, M. (2015) Probiotics and prebiotics associated with aquaculture: A review, *Fish & Shellfish Immunology*, 45 (2), pp. 733–741. DOI:http://dx.doi.org/10.1016/j.fsi.2015.05.038.

Arakane, Y., Muthukrishnan, S., Beeman, R. W., Kanost, M. R., and Kramer, K. J. (2005) Laccase 2 is the phenoloxidase gene required for beetle cuticle tanning, *Proc Natl Acad Sci U S A*, *102* (32), pp. 11337-11342. DOI:10.1073/pnas.0504982102.

Arensburger, P., Megy, K., Waterhouse, R. M., Abrudan, J., Amedeo, P., Antelo, B., *et al.* (2010) Sequencing of *Culex quinquefasciatus* establishes a platform for mosquito comparative genomics, *Science*, 330 (6000), pp. 86 LP-88.

Arp, A. P., Hunter, W. B. and Pelz-Stelinski, K. S. (2016) Annotation of the Asian Citrus Psyllid Genome Reveals a Reduced Innate Immune System, *Frontiers in Physiology*, 7, pp. 570. DOI:10.3389/fphys.2016.00570.

Bel, Y., Jakubowska, A. K., Costa, J., Herrero, S. and Escriche, B. (2013) Comprehensive analysis of gene expression profiles of the beet armyworm *Spodoptera exigua* larvae challenged with *Bacillus thuringiensis* Vip3Aa toxin, *PLoS ONE*, 8 (12), pp. e81927. DOI:0.1371%2Fjournal.pone.0081927.

Benoit, J. B., Vigneron, A., Broderick, N. A., Wu, Y., Sun, J. S., Carlson, J. R., Aksoy, S. and Weiss, B. L. (2017) Symbiont-induced odorant binding proteins mediate insect host hematopoiesis, *eLife*. DOI:10.7554/eLife.19535.

Bidla, G., Dushay, M. S. and 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. DOI:10.1242/jcs.03420.

Bidla, G., Hauling, T., Dushay, M. S. and Theopold, U. (2009) Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors, *J Innate Immun*, 1 (4), pp. 301–308. DOI:10.1159/000168009.

Binggeli, O., Neyen, C., Poidevin, M. and Lemaitre, B. (2014) Prophenoloxidase activation is required for survival to microbial infections in *Drosophila*, *PLoS Pathog*, 10. DOI:10.1371/journal.ppat.1004067.

Brandt, A., Gorenflo, A., Siede, R., Meixner, M. and Büchler, R. (2016) The neonicotinoids thiacloprid, imidacloprid, and clothianidin affect the immunocompetence of honey bees (*Apis mellifera* L.), *J Insect Physiol*, 86, pp. 40–47. DOI:http://dx.doi.org/10.1016/j.jinsphys.2016.01.001.

Browne, N., Heelan, M. and Kavanagh, K. (2013) An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes, *Virulence*, 4 (7), pp. 597–603. DOI:10.4161/viru.25906.

Bryant, W. B. and Michel, K. (2016) *Anopheles gambiae* hemocytes exhibit transient states of activation, *Dev Comp Immunol*, 55, pp. 119–129. DOI:http://dx.doi.org/10.1016/j.dci.2015.10.020.

Butt, T. M., Coates, C. J., Dubovskiy, I. M., and Ratcliffe, N. A. (2016). Chapter Nine-Entomopathogenic Fungi: New Insights into Host–Pathogen Interactions. *Advances in genetics*, *94*, 307-364. DOI: 10.1016/bs.adgen.2016.01.006.

Ceja-Navarro, J. A., Vega, F. E., Karaoz, U., Hao, Z., Jenkins, S., Lim, H. C., *et al.* (2015) Gut microbiota mediate caffeine detoxification in the primary insect pest of coffee, *Nature comm*, 6, pp. 7618.

Cerenius, L., Lee, B. L. and Söderhäll, K. (2008) The proPO-system: pros and cons for its role in invertebrate immunity, *Trends in Immunology*, 29 (6), pp. 263–271.

Cerenius, L., Kawabata, S., Lee, B. L., Nonaka, M. and Söderhäll, K. (2010) Proteolytic cascades and their involvement in invertebrate immunity, *Trends Biochem Sci*, 35. DOI:10.1016/j.tibs.2010.04.006.

Chang, T. S. (2009). An updated review of tyrosinase inhibitors. *Int J Mol Sci. 10* pp. 2440-2475.

Chase, M. R., Raina, K., Bruno, J. and Sugumaran, M. (2000) Purification, characterization and molecular cloning of prophenoloxidases from *Sarcophaga bullata*, *Insect Biochem Mol Biol*, 30 (10), pp. 953–967.

Chen, L., Wang, G., Zhu, Y.-N., Xiang, H., Wang, W. (2016) Advances and perspectives in the application of CRISPR/Cas9 in insects, *Zool Res*, 37 (4), pp. 136–43. DOI:10.13918/j.issn.2095-8137.2016.4.220.

Chen, Y., Liu, F., Yang, B., Lu, A., Wang, S., Wang, J., *et al.* (2012) Specific amino acids affecting *Drosophila melanogaster* prophenoloxidase activity *in vitro*. *Dev Comp Immunol*. 38, pp. 88-97. DOI:10.1016/j.dci.2012.04.007.

Clark, K. D. and Strand, M. R. (2013) Hemolymph melanization in the Silkmoth *Bombyx mori* involves formation of a high molecular mass complex that metabolizes tyrosine, *J Bio Chem*, 288 (20), pp. 14476–14487.

Coates, C. J., Kelly, S. M. and Nairn, J. (2011) Possible role of phosphatidylserine–hemocyanin interaction in the innate immune response of *Limulus polyphemus*, *Dev Comp Immunol*, 35 (2), pp. 155–163. DOI:10.1016/j.dci.2010.08.015.

Coates, C. J., Whalley, T., Wyman, M. and Nairn, J. (2013) A putative link between phagocytosis-induced apoptosis and hemocyanin-derived phenoloxidase activation, *Apoptosis*, 18 (11), pp. 1319–1331. DOI:10.1007/s10495-013-0891-x.

Coates, C. J. and Decker, H. (2017) Immunological properties of oxygen-transport proteins: hemoglobin, hemocyanin and hemerythrin, *Cell Mol Life Sci*. 74, pp. 293-317. DOI:10.1007/s00018-016-2326-7.

Cong, Y., Zhang, Q., Woolford, D., Schweikardt, T., Khant, H., Dougherty, M., Ludtke, S. J., Chiu, W. and Decker, H. (2017) Structural mechanism of SDS-induced enzyme activity of scorpion hemocyanin revealed by electron cryomicroscopy, *Structure*, 17 (5), pp. 749–758. DOI:10.1016/j.str.2009.03.005.

Crava, C. M., Jakubowska, A. K., Escriche, B., Herrero, S. and Bel, Y. (2015) Dissimilar regulation of antimicrobial proteins in the midgut of *Spodoptera exigua* larvae challenged with *Bacillus thuringiensis* toxins or baculovirus, *PLoS ONE*, 10 (5), pp. e0125991.

Cristino, A. S., Nunes, F. M. F., Barchuk, A. R., Aguiar-Coelho, V. M., Simões, Z. L. P. and Bitondi, M. M. G. (2010) Organization, evolution and transcriptional profile of hexamerin genes of the parasitic wasp *Nasonia vitripennis* (Hymenoptera: Pteromalidae), *Insect Mol Bio*, 19, pp. 137–146. DOI:10.1111/j.1365-2583.2009.00970.x.

Decker, H., Schweikardt, T., Nillius, D., Salzbrunn, U., Jaenicke, E. and Tuczek, F. (2007) Similar enzyme activation and catalysis in hemocyanins and tyrosinases, *Gene*, 398 (1–2), pp. 183–191. DOI:http://dx.doi.org/10.1016/j.gene.2007.02.051.

De Fine Licht, H. H., Schiøtt, M., Rogowska-Wrzesinska, A., Nygaard, S., Roepstorff, P. and Boomsma, J. J. (2013) Laccase detoxification mediates the nutritional alliance between leaf-cutting ants and fungus-garden symbionts, *Proc Natl Acad Sci U S A*, 110 (2), pp. 583–587. DOI:10.1073/pnas.1212709110 .

Di Lelio, I., Varricchio, P., Di Prisco, G., Marinelli, A., Lasco, V., Caccia, S., *et al.* (2014) Functional analysis of an immune gene of *Spodoptera littoralis* by RNAi, *J Insect Physiol*, 64, pp. 90–97. DOI:http://dx.doi.org/10.1016/j.jinsphys.2014.03.008.

Di Prisco, G., Cavaliere, V., Annoscia, D., Varricchio, P., Caprio, E., Nazzi, F., Gargiulo, G. and Pennacchio, F. (2013) Neonicotinoid clothianidin adversely affects insect immunity and promotes replication of a viral pathogen in honey bees, *Proc Natl Acad Sci U S A*, 110 (46), pp. 18466–18471. DOI:10.1073/pnas.1314923110.

Di Prisco, G., Annoscia, D., Margiotta, M., Ferrara, R., Varricchio, P., Zanni, V., Caprio, E., Nazzi, F. and Pennacchio, F. (2016) A mutualistic symbiosis between a parasitic mite and a pathogenic virus undermines honey bee immunity and health, *Proc Natl Acad Sci U S A*, 113 (12), pp. 3203–3208. DOI:10.1073/pnas.1523515113.

Dittmer, N. T. and Kanost, M. R. (2010) Insect multicopper oxidases: Diversity, properties, and physiological roles, *Insect Biochem Mol Biol*, 40 (3), pp. 179–188. DOI:http://dx.doi.org/10.1016/j.ibmb.2010.02.006.

Dubovskiy, I. ., Kryukova, N. ., V.V, G. and Ratcliffe, N. A. (2016) Encapsulation and nodulation in insects, *Invertebr Survival J*, 13, pp. 229–246.

Dudzic, J. P., Kondo, S., Ueda, R., Bergman, C. M. and Lemaitre, B. (2015) *Drosophila* innate immunity: regional and functional specialization of prophenoloxidases, *BMC Biol*, 13 (1), pp. 1–16. DOI:10.1186/s12915-015-0193-6.

Emery, O., Schmidt, K., and Engel, P. (2017) Immune system stimulation by the gut symbiont *Frischella perrara* in the honey bee (*Apis mellifera*), *Mol Ecol*, DOI:10.1111/mec.14058.

Engel, P. and Moran, N. A. (2013) The gut microbiota of insects – diversity in structure and function, *FEMS Microbiol Rev*, 37 (5), pp. 699-735. DOI:10.1111/1574-6976.12025.

Engel, P., Bartlett, K. D. and Moran, N. A. (2015) The bacterium *Frischella perrara* causes scab formation in the gut of its honeybee host, *mBio*, 6 (3), pp. e00193-15. DOI:10.1128/mBio.00193-15.

Evans, J. D. and Gundersen-Rindal, D. (2003) Beenomes to Bombyx: future directions in applied insect genomics, *Genome Biol*, 4 (3), pp. 107. DOI:10.1186/gb-2003-4-3-107.

Falabella, P., Riviello, L., Pascale, M., Di Lelio, I., Tettamanti, G., Grimaldi, A., *et al.* (2012) Functional amyloids in insect immune response, *Insect Biochem Mol Biol*, 42 (3), pp. 203–211.

Fallon, J. P., Troy, N. and Kavanagh, K. (2011) Pre-exposure of Galleria mellonella larvae to different doses of *Aspergillus fumigatus* conidia causes differential activation of cellular and humoral immune responses, *Virulence*, 2 (5), pp. 413–421. DOI:10.4161/viru.2.5.17811.

Fowler, D. M., Koulov, A. V, Balch, W. E. and Kelly, J. W. (2007) Functional amyloid – from bacteria to humans, *TIBS*, 32 (5), pp. 217–224. DOI:http://dx.doi.org/10.1016/j.tibs.2007.03.003.

Galván, I., Jorge, A., Edelaar, P. and Wakamatsu, K. (2015) Insects synthesize pheomelanin, *Pigment Cell Res*, 28 (5), pp. 599–602. DOI:10.1111/pcmr.12397.

García, J. A., Polidori, C. and Nieves-Aldrey, J. L. (2016) Pheomelanin in the secondary sexual characters of male parasitoid wasps (Hymenoptera: Pteromalidae), *Arthropod Structure & Development*, 45 (4), pp. 311–319. DOI:http://dx.doi.org/10.1016/j.asd.2016.05.001.

Gerardo, N. M., Altincicek, B., Anselme, C., Atamian, H., Barribeau, S. M. and Vos, M. (2010) Immunity and other defenses in pea aphids, *Acyrthosiphon pisum*, *Genome Biol*, 11. DOI:10.1186/gb-2010-11-2-r21.

Ghosh, S., Singh, A., Mandal, S. and Mandal, L. (2015) Active hematopoietic hubs in *Drosophila* adults generate hemocytes and contribute to immune response, *Developmental Cell*, 33 (4), pp. 478–488. DOI:http://dx.doi.org/10.1016/j.devcel.2015.03.014.

Goldfeder, M., Kanteev, M., Isaschar-Ovdat, S., Adir, N. and Fishman, A. (2014) Determination of tyrosinase substrate-binding modes reveals mechanistic differences between type-3 copper proteins, *Nature Comm*, 5, pp. 4505.

Grimaldi, A., Tettamanti, G., Congiu, T., Girardello, R., Malagoli, D., Falabella, P., Valvassori, R., Ottaviani, E. and de Eguileor, M. (2012) The main actors involved in parasitization of *Heliothis virescens* larva, *Cell Tissue Res*, 350 (3), pp. 491–502. DOI:10.1007/s00441-012-1503-8.

Gupta, S., Wang, Y. and Jiang, H. (2005, March) Manduca sexta prophenoloxidase (proPO) activation requires proPO-activating proteinase (PAP) and serine proteinase homologs (SPHs) simultaneously, *Insect Biochem Mol Biol*. DOI:10.1016/j.ibmb.2004.12.003.

Hillyer, J. F., Barreau, C. and Vernick, K. D. (2007) Efficiency of salivary gland invasion by malaria sporozoites is controlled by rapid sporozoite destruction in the mosquito hemocoel, *Int J Parasitol*, 37 (6), pp. 673–681. DOI:10.1016/j.ijpara.2006.12.00.

Hillyer, J. F. (2016) Insect immunology and hematopoiesis, *Dev Comp Immunol*, 58, pp. 102–118. DOI:http://dx.doi.org/10.1016/j.dci.2015.12.006.

Honti, V., Csordás, G., Kurucz, É., Márkus, R. and Andó, I. (2014) The cell-mediated immunity of *Drosophila melanogaster*: Hemocyte lineages, immune compartments, microanatomy and regulation, *Dev Comp Immunol*, 42 (1), pp. 47–56. DOI:http://dx.doi.org/10.1016/j.dci.2013.06.005.

Hopkins, T. L., Starkey, S. R. and Beckage, N. E. (1998) Tyrosine and catecholamine levels in the hemolymph of tobacco hornworm larvae, *Manduca sexta*, parasitized by the braconid wasp, *Cotesia congregata*, and in the developing parasitoids, *Arch Insect Biochem Physiol*, 38 (4), pp. 193–201. DOI:10.1002/(SICI)1520-6327(1998)38:4<193::AID-ARCH5>3.0.CO;2-S.

Hu, K. and Webster, J. M. (2000) Antibiotic production in relation to bacterial growth and nematode development in *Photorhabdus Heterorhabditis* infected *Galleria mellonella larvae, FEMS MICROBIOL LETT,* 189 (2), pp. 219–223.

Hu, Y., Wang, Y., Deng, J. and Jiang, H. (2016) The structure of a prophenoloxidase (PPO) from *Anopheles gambiae* provides new insights into the mechanism of PPO activation, *BMC Biol*, 14 (1), pp. 1–13. DOI:10.1186/s12915-015-0225-2..

Ishiwata, K., Sasaki, G., Ogawa, J., Miyata, T. and Su, Z.-H. (2011) Phylogenetic relationships among insect orders based on three nuclear protein-coding gene sequences, *Mol Phylogenet Evoltion*, 58 (2), pp. 169–180. DOI:http://dx.doi.org/10.1016/j.ympev.2010.11.001.

Ito, S. and Wakamatsu, K. (2003) Quantitative analysis of eumelanin and pheomelanin in humans, mice, and other animals: a comparative review, *Pigment Cell Res*, 16 (5), pp. 523–531. DOI:10.1034/j.1600-0749.2003.00072.x.

Joseph, W. (2014) Origins and activation of prophenoloxidases in the digestive tract of the cricket, *Gryllus bimaculatus*, *Arch Insect Biochem Physiol*, 87 (2), pp. 95–104. DOI:10.1002/arch.21183.

Kan, H., Kim, C.-H., Kwon, H.-M., Park, J.-W., Roh, K.-B., Lee, H., *et al.* (2008) Molecular control of phenoloxidase-induced melanin synthesis in an insect, *J Biol Chem* 283 (37), pp. 25316–25323.

Kanteev, M., Goldfeder, M. and Fishman, A. (2015) Structure–function correlations in tyrosinases, *Protein Sci*, 24 (9), pp. 1360–1369. DOI:10.1002/pro.2734.

Kato, Y., Yoshida, T., Miura, K., Tanaka, T., Nakamatsu, Y. and Ochiai, M. (2014) A novel type of hemocytes localizing melanization with high-spreading behavior in *Mythimna separata*, *Arch Insect Biochem Physiol*, 86 (4), pp. 220–239. DOI:10.1002/arch.21173.

Kawabata, T., Yasuhara, Y., Ochiai, M., Matsuura, S. and Ashida, M. (1995) Molecular-cloning of insect pro-phenol oxidase - a copper- containing protein homologous to arthropod hemocyanin, *Proc Natl Acad Sci U S A*, 92 (17), pp. 7774–7778.

Khurana, R., Coleman, C., Ionescu-Zanetti, C., Carter, S. A., Krishna, V., Grover, R. K., Roy, R. and Singh, S. (2005) Mechanism of thioflavin T binding to amyloid fibrils, *J Structural Biol*, 151 (3), pp. 229–238. DOI:http://dx.doi.org/10.1016/j.jsb.2005.06.006.

King, J. G. and Hillyer, J. F. (2012) Infection-induced interaction between the mosquito circulatory and immune systems, *PLoS Pathog*, 8 (11), pp. e1003058.

Kronforst, M. R., Barsh, G. S., Kopp, A., Mallet, J., Monteiro, A., Mullen, S. P., *et al*. (2012) Unraveling the thread of nature's tapestry: the genetics of diversity and convergence in animal pigmentation. *Pigment Cell Res*, *25* (4), 411-433. DOI:10.1111/j.1755-148X.2012.01014.x

Kwon, T. H., Lee, S. Y., Lee, J. H., Choi, J. S. and Lee, S. K. S. I. and B. L. (1997) Purification and characterization of prophenoloxidase from the hemolymph of coleopteran insect, *Holotrichia diomphalia* larvae, *Mol. Cells*, 7 (1), pp. 90–97.

Kwon, T. H., Kim, M. S., Choi, H. W., Joo, C. H., Cho, M. Y. and Lee, B. L. (2000) A masquerade-like serine proteinase homologue is necessary for phenoloxidase activity in the coleopteran insect, *Holotrichia diomphalia* larvae, *Eur J Biochem*, 267 (20), pp. 6188–6196. DOI:10.1046/j.1432-1327.2000.01695.x.

Latocha, M., Chodurek, E., Kurkiewicz, S., Świątkowska, L. and Wilczok, T. (2000) Pyrolytic GC-MS analysis of melanin from black, gray and yellow strains of *Drosophila melanogaster*, *J Analytical Applied Pyrolysis*, 56 (1), pp. 89–98. DOI:http://dx.doi.org/10.1016/S0165-2370(00)00082-6.

League, G. P. and Hillyer, J. F. (2016) Functional integration of the circulatory, immune, and respiratory systems in mosquito larvae: pathogen killing in the hemocyte-rich tracheal tufts, *BMC Biol*, 14 (1), pp. 78. DOI:10.1186/s12915-016-0305-y.

Leitão, A. B. and Sucena, É. (2015) *Drosophila* sessile hemocyte clusters are true hematopoietic tissues that regulate larval blood cell differentiation, *ELife*, 4:e06166. DOI:10.7554/eLife.06166.

Li, D., Liang, Y., Wang, X., Wang, L., Qi, M., Yu, Y. and Luan, Y. (2015) Transcriptomic analysis of *Musca domestica* to reveal key genes of the prophenoloxidase-activating system, *G3: Genes|Genomes|Genetics*. DOI:10.1534/g3.115.016899.

Li, Y., Wang, Y., Jiang, H. and Deng, J. (2009) Crystal structure of *Manduca sext*a prophenoloxidase provides insights into the mechanism of type 3 copper enzymes, *Proc Natl Acad Sci U S A*, 106 (40), pp. 17002–17006. DOI:10.1073/pnas.0906095106.

Liu, F., Yao, P., Guo, X., Liu, Z., Guo, X. and Xu, B. (2015) Characterization of prophenoloxidase in resisting adverse stresses in *Apis cerana cerana*, *J Environ Anal Chem*, 2 (3). DOI:10.4172/jreac.1000144.

Lourenço, A. P., Zufelato, M. S., Bitondi, M. M. G. and Simões, Z. L. P. (2005) Molecular characterization of a cDNA encoding prophenoloxidase and its expression in *Apis mellifera*, *Insect Biochem Mol Biol*, 35 (6), pp. 541–552. DOI:10.1016/j.ibmb.2005.01.013.

Lu, A., Peng, Q. and Ling, E. (2014) Formation of disulfide bonds in insect prophenoloxidase enhances immunity through improving enzyme activity and stability, *Dev Comp Immunol*, 44 (2), pp. 351–358. DOI:http://dx.doi.org/10.1016/j.dci.2014.01.011.

Ma, G., Roberts, H., Sarjan, M., Featherstone, N., Lahnstein, J., Akhurst, R. and Schmidt, O. (2005) Is the mature endotoxin Cry1Ac from *Bacillus thuringiensis* inactivated by a coagulation reaction in the gut lumen of resistant *Helicoverpa armigera* larvae?, *Insect Biochem Mol Biol*, 35 (7), pp. 729–739. DOI:http://dx.doi.org/10.1016/j.ibmb.2005.02.011.

Matetovici, I., Caljon, G. and Van Den Abbeele, J. (2016) Tsetse fly tolerance to *T. brucei* infection: transcriptome analysis of trypanosome-associated changes in the tsetse fly salivary gland, *BMC Genomics*, 17 (1), p. 971. DOI:10.1186/s12864-016-3283-0.

Mazzei, M., Fronte, B., Sagona, S., Carrozza, M. L., Forzan, M., Pizzurro, F., *et al.* (2016) Effect of 1,3-1,6 β-glucan on natural and experimental deformed wing virus infection in newly emerged honeybees (*Apis mellifera ligustica*), *PLoS ONE*, 11 (11), pp. e0166297.

Moreau, J. S. and Asgari, S. (215) Venom proteins from parasitoid wasps and their biological functions, *Toxins*, 7 (7), pp. 2385-2412. DOI:10.3390/toxins7072385

Mowlds, P., Coates, C., Renwick, J. and Kavanagh, K. (2010) Dose-dependent cellular and humoral responses in *Galleria mellonella* larvae following β-glucan inoculation, *Microbes and Infection*, 12 (2), pp. 146–153. DOI:http://dx.doi.org/10.1016/j.micinf.2009.11.004.

Nam, H-. J., Jang, I. H., Asano, T., and Lee, W-. J. (2008). Involvement of pro-phenoloxidase 3 in lamellocyte-meidated [sic] spontaneous melanization in *Drosophila*. *Mol Cells*, 26, 606-610.

Nappi, A. J. and Vass, E. (1993) Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. *Pigment Cell Res*, 6, pp. 117–126.

Nappi, A. J. and Christensen, B. M. (2005) Melanogenesis and associated cytotoxic reactions: Applications to insect innate immunity, *Insect Biochem Mol Biol*, 35 (5), pp. 443–459.

Oldroyd, B. P. (2007) What's Killing American Honey Bees?, *PLoS Biology,* 5 (6). DOI:10.1371/journal.pbio.0050168.

Olivares, C. and Solano, F. (2009) New insights into the active site structure and catalytic mechanism of tyrosinase and its related proteins. *Pigment Cell Res*, 22 (6), pp. 750-760.

Pascale, M., Laurino, S., Vogel, H., Grimaldi, A., Monné, M., Riviello, L., Tettamanti, G. and Falabella, P. (2014) The Lepidopteran endoribonuclease-U domain protein {P102} displays

dramatically reduced enzymatic activity and forms functional amyloids, *Dev Comp Immunol*, 47 (1), pp. 129–139. DOI:http://dx.doi.org/10.1016/j.dci.2014.07.009.

Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004). UCSF Chimera—a visualization system for exploratory research and analysis. *J. Comp Chem*. *25*(13), 1605-1612. DOI: 10.1002/jcc.20084.

Ptaszyńska, A. A., Borsuk, G., Zdybicka-Barabas, A., Cytryńska, M. and Małek, W. (2016) Are commercial probiotics and prebiotics effective in the treatment and prevention of honeybee nosemosis C?, *Parasitol Res*, 115 (1), pp. 397–406. DOI:10.1007/s00436-015-4761-z.

Pulpitel, T., Pernice, M., Simpson, S. J. and Ponton, F. (2015) Tissue-specific immune gene expression in the migratory locust, *Locusta Migratoria*, *Insects*, 6 (2), pp. 368–380. DOI:10.3390/insects6020368.

Rahman, M. M., Roberts, H. L. S., Sarjan, M., Asgari, S. and Schmidt, O. (2004) Induction and transmission of *Bacillus thuringiensis* tolerance in the flour moth *Ephestia kuehniella*, *Proc Natl Acad Sci U S A*, 101 (9), pp. 2696–2699.

Ricketts, D., and Sugumaran, M. (1994) 1, 2-dehydro-*N*-beta-alanyldopamine as a new intermediate in insect cuticular sclerotization, *J Biol Chem*, *269* (35), pp. 22217-22221.

Seisenbacher, G., Hafen, E. and Stocker, H., (2011) MK2-Dependent p38b signalling protects *Drosophila* hindgut enterocytes against JNK-induced apoptosis under chronic stress, *PLOS Genetics*, 7 (8), pp. 1–14. DOI:10.1371/journal.pgen.1002168.

Shao, Q., Yang, B., Xu, Q., Li, X., Lu, Z., Wang, C., Huang, Y., Söderhäll, K. and Ling, E. (2012) Hindgut innate immunity and regulation of fecal microbiota through melanization in insects, *J Biol Chem* 287 (17), pp. 14270–14279. DOI:10.1074/jbc.M112.354548.

Shiao, S.-H., Whitten, M. M. A., Zachary, D., Hoffmann, J. A., Levashina, E. A. L. (2006) Fz2 and Cdc42 mediate melanization and actin polymerization but are dispensable for *Plasmodium* killing in the mosquito midgut, *PLoS Pathog*, 2 (12), e133. DOI:10.1371/journal.ppat.0020133.

Sigle, L. T. and Hillyer, J. F. (2016) Mosquito hemocytes preferentially aggregate and phagocytose pathogens in the periostial regions of the heart that experience the most hemolymph flow, *Dev Comp Immunol*, 55, pp. 90–101. DOI:http://dx.doi.org/10.1016/j.dci.2015.10.018.

Smith, C. D., Zimin, A., Holt, C., Abouheif, E., Benton, R., Cash, E., *et al.* (2011) Draft genome of the globally widespread and invasive *Argentine ant* (Linepithema humile), *Proc Natl Acad Sci U S A*, 108 (14), pp. 5673–5678. DOI:10.1073/pnas.1008617108.

Smith, R. C., King, J. G., Tao, D., Zeleznik, O. A., Brando, C., Thallinger, G. G. and Dinglasan, R. R. (2016) Molecular profiling of phagocytic immune cells in anopheles gambiae reveals integral roles for hemocytes in mosquito innate immunity, *Molecular & Cellular Proteomics*, 15 (11), pp. 3373–3387. DOI:10.1074/mcp.M116.060723.

Solem, E., Tuczek, F. and Decker, H. (2016) Tyrosinase versus catechol oxidase: one asparagine makes the difference, *Angewandte Chemie International Edition*, 55 (8), pp. 2884–2888. DOI:10.1002/anie.201508534.

Solomon, E. I., Heppner, D. E., Johnston, E. M., Ginsbach, J. W., Cirera, J., Qayyum, M., *et al.* (2014) Copper active sites in biology, *Chemical Reviews*, 114 (7), pp. 3659–3853. DOI:10.1021/cr400327t.

Steinmann, N., Corona, M., Neumann, P. and Dainat, B. (2015) Overwintering is associated with reduced expression of immune genes and higher susceptibility to virus infection in honey bees, *PLoS ONE*, 10 (6), pp. e0129956. DOI:10.1371/journal.pone.0129956.

Sugumaran, M., and Semensi, V. (1991) Quinone methide as a new intermediate in eumelanin biosynthesis *J Biol Chem*, *266* (10), pp. 6073-6078.

Sugumaran, M., Duggaraju, R., Generozova, F., and Ito, S. (1999) Insect melanogenesis. II. Inability of *Manduca* phenoloxidase to act on 5, 6-dihydroxyindole-2-carboxylic acid, *Pigment Cell Res, 12* (2), pp. 118-125. DOI: 10.1111/j.1600-0749.1999.tb00751.x

Sugumaran, M. and Barek, H. (2016) Critical analysis of the melanogenic pathway in insects and higher animals, *Int J Mol Sci*, 17 (10), pp. 1753. DOI:10.3390/ijms17101753.Tanaka, H., Ishibashi, J., Fujita, K., Nakajima, Y., Sagisaka, A., Tomimoto, K., *et al.* (2008) A genome-wide analysis of genes and gene families involved in innate immunity of *Bombyx mori*, *Insect Biochem Mol Biol*, 38 (12), pp. 1087–1110. DOI:http://dx.doi.org/10.1016/j.ibmb.2008.09.001.

Tang, H. (2009) Regulation and function of the melanization reaction in *Drosophila*, *Fly (Austin)*, 3. DOI:10.4161/fly.3.1.7747.

Tokura, A., Fu, G. S., Sakamoto, M., Endo, H., Tanaka, S., Kikuta, S., Tabunoki, H. and Sato, R. (2014) Factors functioning in nodule melanization of insects and their mechanisms of accumulation in nodules, *J Insect Physiol*, 60, pp. 40–49. DOI:http://dx.doi.org/10.1016/j.jinsphys.2013.11.003.

Ullah, I., Khan, L. A., Ali, L., Khan, R. A., Waqas, M., Lee, I.-J. and Shin, J.-H. (2014) An insecticidal compound produced by an insect-pathogenic bacterium suppresses host defenses through phenoloxidase inhibition, *Molecules*, 19 (12). DOI:10.3390/molecules191220913.

Valembois, P., Lassègues, M. and Roch, P. (1992) Formation of brown bodies in the coelomic cavity of the earthworm *Eisenia fetida* andrei and attendant changes in shape and adhesive capacity of constitutive cells, *Dev Comp Immunol*, 16 (2–3), pp. 95–101. DOI:http://dx.doi.org/10.1016/0145-305X(92)90010-A.

Vlisidou, I. and Wood, W. (2015) *Drosophila* blood cells and their role in immune responses, *FEBS J*, 282 (8), pp. 1368-82. DOI:10.1111/febs.13235.

Waltzer, L., Ferjoux, G., Bataille, L. and Haenlin, M. (2003) Cooperation between the GATA and RUNX factors Serpent and Lozenge during *Drosophila* hematopoiesis, *EMBO J*, 22. DOI:10.1093/emboj/cdg622.

Wang, L., Kounatidis, I. and Ligoxygakis, P. (2014) *Drosophila* as a model to study the role of blood cells in inflammation, innate immunity and cancer, *Front Cell Inf Microbiol*, 3: p.113. DOI:10.3389/fcimb.2013.0011

Weiss, B. L., Wang, J. and Aksoy, S. (2011) Tsetse immune system maturation requires the presence of obligate symbionts in larvae, *PLoS Biology*, 9 (5), pp. e1000619.

Whitten, M. M. A., Shiao, S. H. and Levashina, E. A. (2006) Mosquito midguts and malaria: cell biology, compartmentalization and immunology, *Parasite Immunol*, 28 (4), pp. 121–30. DOI:10.1111/j.1365-3024.2006.00804.x.

Whitten, M. and Dyson, P. (217) Gene silencing in non-model insects: Overcoming hurdles using symbiotic bacteria for trauma-free sustainable delivery of RNA interference, *BioEssays*, 39 (3), p. 1600247. DOI:10.1002/bies.201600247.

Wu, K., Zhang, J., Zhang, Q., Zhu, S., Shao, Q., Clark, K. D., Liu, Y. and Ling, E. (2015) Plant phenolics are detoxified by prophenoloxidase in the insect gut, *Scientific Reports*, 5, pp. 16823. DOI:10.1038/srep16823.

Xia, X., Yu, L., Xue, M., Yu, X., Vasseur, L., Gurr, G. M., *et al.* (2015) Genome-wide characterization and expression profiling of immune genes in the diamondback moth, *Plutella xylost*ella (L.), *Scientific Reports*, 5, p. 9877. DOI:10.1038/srep09877.

Xue, C.-B., Zhang, L., Luo, W.-C., Xie, X.-Y., Jiang, L. and Xiao, T. (2007) 3D-QSAR and molecular docking studies of benzaldehyde thiosemicarbazone, benzaldehyde, benzoic acid, and their derivatives as phenoloxidase inhibitors, *Bioorg Med Chem*, 15 (5), pp. 2006–2015. DOI:http://dx.doi.org/10.1016/j.bmc.2006.12.038.

Yu, X.-Q., Jiang, H., Wang, Y. and Kanost, M. R. (2003) Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*, *Insect Biochem Mol Biol*, 33 (2), pp. 197—208. DOI:10.1016/s0965-1748(02)00191-1.

Zhang, S., Hong, F., Song, H., Wang, L., Liu, Q. and An, C. (2016) Cloning, expression, and characterization of prophenoloxidases from asian corn borer, *Ostrinia furnacalis* (Gunée), *J Immunol Res*. DOI:10.1155/2016/1781803.

Zhang, Y., Wang, X.-X., Zhang, Z.-F., Chen, N., Zhu, J.-Y., Tian, H.-G., Fan, Y.-L. and Liu, T.-X. (2016) Pea aphid *Acyrthosiphon pisum* sequesters plant-derived secondary metabolite L-DOPA for wound healing and UVA resistance, *Scientific Reports*, 6, pp. 23618.

Zhao, P., Li, J., Wang, Y. and Jiang, H. (2007) Broad-spectrum antimicrobial activity of the reactive compounds generated *in vitro* by *Manduca sexta* phenoloxidase, *Insect Biochem Mol Biol*, 37 (9), pp. 952–959. DOI:http://dx.doi.org/10.1016/j.ibmb.2007.05.001.

Zhu, J.-Y., Yang, P., Zhang, Z., Wu, G.-X. and Yang, B. (2013) Transcriptomic immune response of *Tenebrio molitor* pupae to parasitization by *Scleroderma guani*, *PLoS ONE*, 8 (1), pp. e54411. DOI:10.1371/journal.pone.0054411.

Zou, Z., Shin, S. W., Alvarez, K. S., Bian, G., Kokoza, V. and Raikhel, A. S. (2008) Mosquito RUNX4 in the immune regulation of PPO gene expression and its effect on avian malaria parasite infection, *Proc Natl Acad Sci U S A*, 105 (47), pp. 18454–18459. DOI:10.1073/pnas.0804658105.

FIGURE LEGENDS

Figure 1. A simplified schematic of the (A) melanogenic and (B) sclerotinogenic pathways in insects. During infection, phenoloxidases (released by hemocytes) in the hemolymph convert tyrosine directly into DOPAquinone via a two-step enzymatic process (hydroxylation followed by oxidation). DOPAquinone undergoes auto-catalysis to form DOPAchrome. An isomerase converts DOPAchrome into DHI, which polymerises to form black eumelanin. Concurrently (and independently), laccases in the cuticle can oxidise dopamine-derivatives (NBAD and/or NADA) into quinones. Isomerases then convert NBAD and NADA into quinone methides that will eventually form brown and colorless polymers in the cuticle, respectively. Additionally, DOPA (3,4-dihydroxphenylalanine) and dopamine can be oxidised by cuticular laccases.

Figure 2. Structural features of insect proPhenoloxidases. A) The resolved crystal structure of *Manduca sexta* PPO (PDB-3HHS) reveals a heterodimer consisting of PPO1/2. *Anopheles gambiae* PPO8 is a homo-dimer (PDB-4YZW) sharing ~41% and ~39% amino acid sequence similarities with *M. sexta* PPO1 and PPO2, respectively. **B)** The distinct regions of each phenoloxidase monomer are depicted: pro-region containing the proteolytic site (R51 - black), N-terminal domain I (orange), middle domain II containing the dicopper active site (gray) and C-terminal domain III (pink). **C)** Conservation mapping of insect phenoloxidase sequences onto *M. sexta* PPO2. Sequences from *Anopheles gambiae* (PDB – 4YZW), *Bombyx mori* (NP_001037335.1), *Drosophila melanogaster* (NP_476812.1) and *M. sexta* (PDB – 3HHS) were aligned in CLUSTALX 2.1 and mapped in UCSF Chimera using the 'sum of pairs' algorithm (Pettersen et al., 2004). The coloured scale bar indicates percentage conservation of amino acids. Inset is the dicopper active site containing the six highly (100%) conserved histidine residues (red). Each copper atom is depicted as an orange sphere.

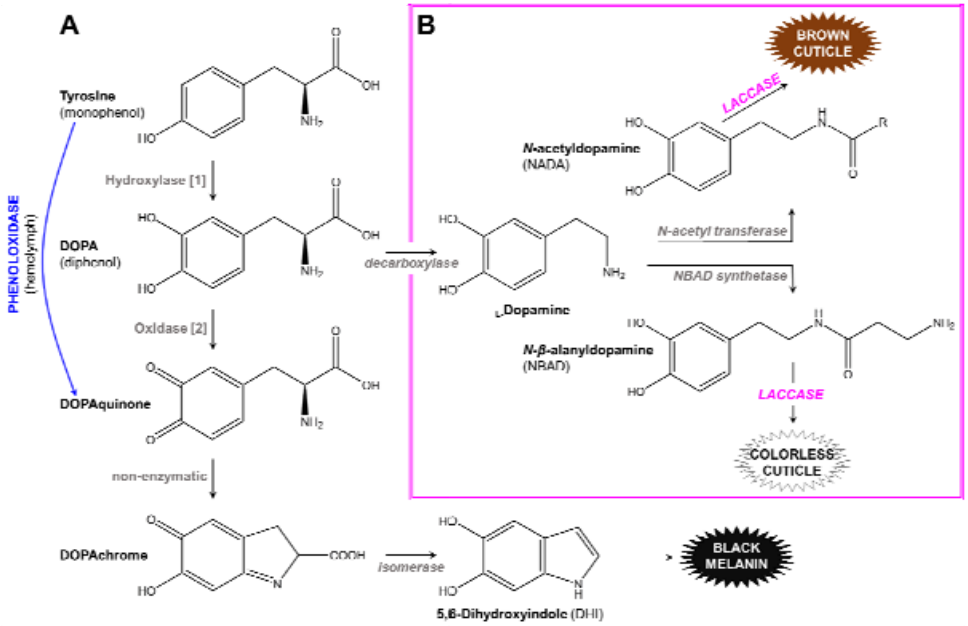
Figure 3. Box 1: Where are the insect pheomelanins, and what do they do?

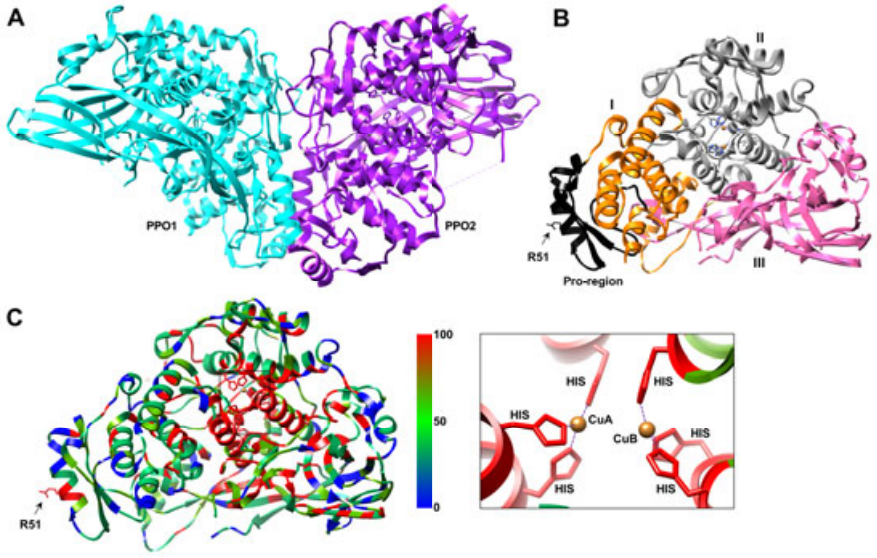
Figure 4. Monophenolase activity is facilitated by conserved amino acids in the dicopper active site of insect phenoloxidases. Secondary structural elements of (**A**) *Manduca sexta* PPO2 (PDB-3HHS) and (**B**) *Anopheles gambiae* PPO8 (PDB-4YZW) are presented as ribbons. Amino acid side chains of the six highly conserved histidines are visible, alongside those residues considered to be responsible for monophenolase activity, namely glutamate (E) and asparagine (N). **(C**) The copper centres of *M. sexta* PPO2 and *A. gambiae* PPO8 have been aligned and then superimposed onto the corresponding structural motif of the common grape (*Vitis vinifera*) PPOg (PDB-2P3X; refined by Solem *et al.,* 2016) - represented in green. In this instance, the copper atoms are depicted as black spheres and the peptide backbone has been removed for clarity. If an asparagine (N) residue does not immediately follow H1 of CuB, then only diphenoloxidase activity is observed. **(D**) Sequence alignments of the CuB sites of insect PPOs. Images were produced using UCSF Chimera (Pettersen et al., 2004).

Figure 5. PPO genes in various insect orders. PPO gene data for most insects are based on published analyses, or annotated genes retrieved from genome databases (Diptex: http://diptex.crg.es/; VectorBase: http://vectorbase.org/index.php; GenBank® www.ncbi.nlm.nih.gov/genbank/). Insects indicated in red are considered beneficial species. Selected insect orders are placed in a basic phylogenetic tree adapted from Ishiwata et al., 2011 & Evans and Gundersen-Rindal, 2003; no information should be inferred from the branch lengths. Note the relative under-representation of studies on hemimetabolous insects (those undergoing incomplete metamorphosis).

Figure 6. The insect gut: a hub for melanogenesis and factors influencing melanogenic activity. Phenoloxidase activity in the gut epithelium and lumen is derived from both endogenous and hemocytic sources in response to triggers such as microbes and PAMPs. Examples of various triggers are indicated in white boxes, and outcomes in yellow boxes. Many insects, both adult and juvenile, upregulate endogenous PPO gene expression in the fore- and hind- gut, and sometimes in highly localized regions, as in honeybees infected by *Frischella perrara*. In Diptera, the presence of gut symbionts and/or other bacteria can trigger crystal cell hematopoiesis in the lymph gland and a bloodmeal in mosquitoes can trigger upregulated hemocyte PPO synthesis. Ingested pesticides, bacterial toxins and probiotics have variable effects in modulating an insect's PPO activity, but are often suppressive; certain gut entomopathogens e.g. *Elizabethkingia meningoseptica* trigger melanotic lesion formation in fat body tissue, thereby suppressing host immunity. Many phytophagous insects employ endogenous gut PPO activity to suppress bacterial growth in the hindgut; this results in black

feces and prevents contamination of their food plants. PPOs bind to toxic plant secondary metabolites in the foregut, which are progressively oxidized (and therefore detoxified) by components of the melanization cascade.





Box 1. Where are the insect pheomelanins, and what do they do?

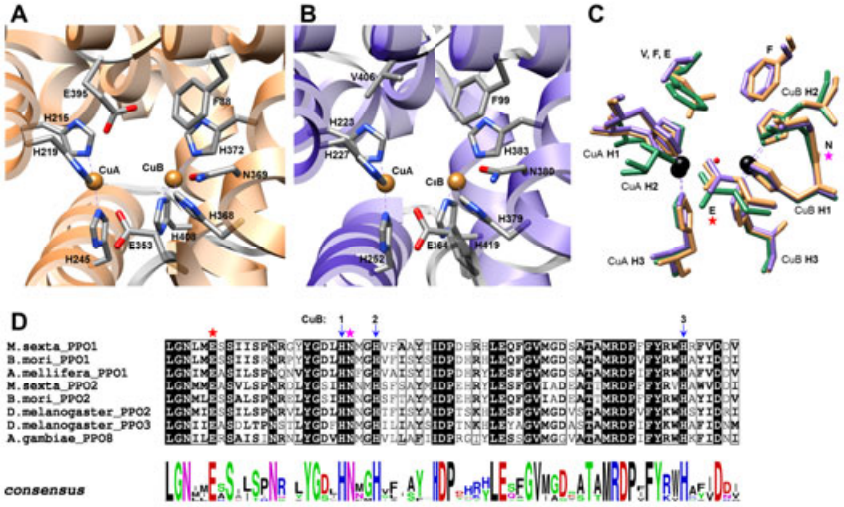
In the presence of cysteine or glutathione, dopaquinone converts stoichiometrically into sulfur-containing pheomelanins (yellow-red), rather than black eumelanin (e.g. Nappi & Christensen 2005). According to Ito and Wakamatsu (2003), up to 25% of melanin found in metazoans is pheomelanin, but there are very few observations of pheomelanins in insects, and thus far these are limited to cuticle coloration roles.

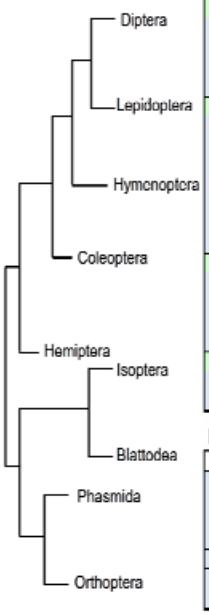
Red-yellow colored insects abound in nature, but the palette of available pigments is large - papiliochromes, ommochromes, carotenoids, pterins and flavonoids. So pheomelanins may be under-utilized in insects, or it may be that we are not looking yet.

Is there a role for pheomalanin in insect immunity? The prediction that insect pheomelanin could be incorporated together with eumelanin into a melanotic capsule was made by Nappi & Vass in 1993, but as far as we know this is still awaiting experimental confirmation. This is clearly a topic deserving greater attention.

Timeline of pheomelanin detection in insects

year	insect	tissue	method	Ref
2000	yellow (yw) *Drosophila*	whole fly	Pyrolytic GC-MS	Latocha et al., 2000
2015	grasshopper	cuticle	Raman spectroscopy & HPLC	Galván et al., 2015
2016	5 parasitoid wasp species	cuticle (leg)	Dispersive Raman spectroscopy	García et al., 2016





HOLOMETABOLOUS insects

#PPO genes	insect order	insect species	common name	source
10	Diptera	*Aedes aegypti*	mosquito	Vectorbase (Jan2017); Zou et al., 2008.
9	Diptera	*Anopheles gambiae*	mosquito	Bryant & Michel, 2016; Smith et al., 2016
9	Diptera	*Culex quinquefasciatus*	mosquito	Arensburger et al., 2010
7	Diptera	*Armigeres subalbatus*	mosquito	NCBI Genbank (Jan2017)
5?	Diptera	*Aedes albopictus*	mosquito	Vectorbase (Jan2017)
3	Diptera	*Drosophila melanogaster*	vinegar fly	e.g. Tang, 2009
3	Diptera	*Phlebotomus papatasi*	sandfly	Vectorbase (Jan2017); Abrudan et al., 2013
2	Diptera	*Anopheles stephensi*	mosquito	Vectorbase (Jan2017)
2	Diptera	*Glossina morsitans morsitans*	tsetse fly	Matetovici et al., 2016
2	Diptera	*Musca domestica*	housefly	Li et al., 2015
1?	Diptera	*Megaselia abdita*	phorid scuttle fly	Diptex (Jan2017)
4	Lepidoptera	*Ostrinia furnacalis*	Asian corn borer	Zhang et al., 2016
2	Lepidoptera	*Manduca sexta*	tobacco hornworm	e.g. Li et al., 2009
2	Lepidoptera	*Bombyx mori*	silkworm	Tanaka et al., 2008; Shao et al., 2012; Tokura et al., 2014; Wu et al., 2015
2	Lepidoptera	*Pieris rapae*	cabbage white butterfly	NCBI Genbank (24.01.17)
2	Lepidoptera	*Mythimna separata*	common armyworm	Kato et al., 2014
1	Lepidoptera	*Plutella xylostella*	diamondback moth	Xia et al., 2015
1	Lepidoptera	*Plodia interpunctella*	Indian meal moth	NCBI Genbank (Jan2017)
3	Hymenoptera	*Nasonia vitripennis*	jewel wasp (parasitoid)	Cristino, et al., 2010
1	Hymenoptera	*Apis mellifera*	honeybee	Lourenço et al., 2005
1	Hymenoptera	*Apis cerana cerana*	Asiatic honeybee	Liu et al., 2015
1?	Hymenoptera	*Solenopsis invicta*	fire ant	NCBI Genbank (Jan2017)
1	Hymenoptera	*Linepithema humile*	Argentine Ant	Smith et al., 2011
3	Coleoptera	*Tribolium castaneum*	red flour beetle	Shao et al., 2012; Zhu et al., 2013
2	Coleoptera	*Holotrichia diomphalia*	scarab beetle	Kwon et al., 1997
2	Coleoptera	*Tenebrio molitor*	grain beetle	NCBI Genbank (Jan2017)

HEMIMETABOLOUS insects

#PPO genes	insect order	insect species	common name	source
2	Hemiptera	*Nilaparvata lugens*	brown planthopper	Vectorbase (Jan2017)
2	Hemiptera	*Diaphorina citri*	Asian citrus psyllid	Arp et al., 2016
2	Hemiptera	*Acyrthosiphon pisum*	pea aphid	Gerardo et al., 2010
?	Hemiptera	*Rhodnius prolixus*	kissing bug	
1?	Isoptera	*Coptotermes formosanus*	termite	NCBI Genbank (Jan2017)
2	Orthoptera	*Locusta migratoria*	migratory locust	NCBI Genbank (Jan2017)
?	Orthoptera	*Gryllus bimaculatus*	cricket	Joseph, 2014