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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

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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, \( \text{L-tyrosine} \) is converted into \( \text{L-DOPA} \) 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-\( N\text{-acetyldopamine} \)) and brown (dehydro-\( N\text{-β\text{-alanyldopamine} \) biopolymers used to ‘glue’ the newly formed cuticle together (Sugumaran and Semensi, 1991; Ricketts and Sugumaran, 1994; Sugumaran and Barek, 2016). Concurrently, \( \text{L-tyrosine} \) 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, aposematic 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 hydroxylolation 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 beleaguered 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 ($\mu-\eta^2:\eta^2$) 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, $\alpha$-helical domain housing the copper centre, and (III) a multi-stranded, antiparallel $\beta$-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, O2•−) 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 (H3O). 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 H2O. 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 (c.a. 470 nm) and the poor rate of catalysis observed when using tyrosine (low $V_{\text{max}}$; 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, Thiofalin 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 Thiofalin 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

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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 PPO 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 oenocytoids 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 peristomial hemocytes attached to the peristomial 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 peristomial 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 peristomial 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 peristomial 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 peristomial 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 Pseudomonas 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 viviparous tsetse 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\textsuperscript{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\textsuperscript{Apo} and Gmm\textsuperscript{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\textsuperscript{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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**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-dihydroxyphenylalanine) 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 pheomelansins, and what do they do?

In the presence of cysteine or glutathione, dopaquinone converts stoichiometrically into sulfur-containing pheomelansins (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 pheomelansins in insects, and thus far these are limited to cuticle coloration roles.

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

Is there a role for pheomelanin 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.
## Holometabolous Insects

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## Hemimetabolous Insects

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<td>Hemiptera</td>
<td>Rhodius pugalis</td>
<td>kissing bug</td>
<td>Zhang et al., 2014</td>
</tr>
</tbody>
</table>

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**Source:**
- Vectorbase (2017)
- Zhang et al., 2014
- NCBi Genbank (2016)
- Joseph, 2014
- Ap et al., 2016
- Caron et al., 2016
- Caron et al., 2016