Insect immunology and hematopoiesis

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Abstract:

Insects combat infection by mounting powerful immune responses that are mediated by hemocytes, the fat body, the midgut, the salivary glands and other tissues. Foreign organisms that have entered the body of an insect are recognized by the immune system when pathogen-associated molecular patterns bind host-derived pattern recognition receptors. This, in turn, activates immune signaling pathways that amplify the immune response, induce the production of factors with antimicrobial activity, and activate effector pathways. Among the immune signaling pathways are the Toll, Imd, Jak/Stat, JNK, and insulin pathways. Activation of these and other pathways leads to pathogen killing via phagocytosis, melanization, cellular encapsulation, nodulation, lysis, RNAi-mediated virus destruction, autophagy and apoptosis. This review details these and other aspects of immunity in insects, and discusses how the immune and circulatory systems have co-adapted to combat infection, how hemocyte replication and differentiation takes place (hematopoiesis), how an infection prepares an insect for a subsequent infection (immune priming), how environmental factors such as temperature and the age of the insect impact the immune response, and how social immunity protects entire groups. Finally, this review highlights some underexplored areas in the field of insect immunobiology.

Keywords:

Insecta; pattern recognition receptor; immune signaling; hemocyte; pathogen; immunity

1. Introduction

Insects interact with a wide array of pathogens. Many of these pathogens seek to invade and colonize the insects they come in contact with, and in many cases, successful colonization leads to detrimental effects to the host. A diversity of pathogenic organisms can infect insects, including viruses, bacteria, fungi, protozoans, nematodes, and even other insects (Mahy, 2004; Pennacchio and Strand, 2006; Vega and Kaya, 2012). Some of these interactions are general whereas others are highly specific. For example, the fungus *Beauveria bassiana* is a facultative pathogen that infects insects from numerous taxonomic orders whereas the *Plasmodium* sp. that cause human malaria are obligate pathogens that only infect – and are only transmitted by – select species of anopheline mosquitoes (Manguin et al., 2008; Ortiz-Urquiza et al., 2015).

To reduce the probability of infection, insects have evolved physical barriers that keep pathogens from entering their main body cavity, or hemocoel. The most encompassing physical barrier that prevents the entry of pathogens is the cuticle (Lundgren and Jurat-Fuentes, 2012; Siva-Jothy et al., 2005). This chitinous, hydrophobic material forms the exoskeleton of the insect, and also lines the foregut, the hindgut, and the tracheal system. Pathogens that enter the body through the cuticle do so through natural wounds, or by the enzymatic digestion of this material. Pathogens also enter the body via ingestion. Following ingestion, pathogens are immediately subjected to antagonistic barriers, such as cibarial or pharyingial armatures, enzymes of the digestive system, inhospitable pH, and the endogenous microbiota (Cirimotich et al., 2011; Lundgren and Jurat-Fuentes, 2012; McGreevy et al., 1978; Siva-Jothy et al., 2005). Ingested pathogens that seek to exit the digestive tract and gain entry into the hemocoel must also traverse the cellular epithelium of the midgut, and in some cases, a non-cellular and chitinous peritrophic matrix (Kato et al., 2008; Kuraishi et al., 2011; Weiss et al., 2014).

Although entering the hemocoel is a formidable enterprise, many organisms have evolved mechanisms to efficiently accomplish this. Some fungi enter the hemocoel by

enzymatically degrading the cuticle (Pedrini et al., 2007), and pathogens that are routinely ingested have evolved innovative strategies to exit this intestinal compartment. For example, the bacterium, *Bacillus thuringiensis*, produces Cry toxins that destroy the epithelial cells of the digestive tract, the protozoan parasites that cause malaria utilize their apical complex to penetrate the cells of the midgut, and filarial nematodes physically burrow out of the intestinal space (Christensen and Sutherland, 1984; Roberts et al., 2013; Vachon et al., 2012). Some pathogens elect to not leave the gut. For example, *Trypanosoma cruzi* and *Yersinia pestis*, which in humans cause Chagas' disease and plague, respectively, remain within the digestive tract of their kissing bug and flea hosts (Roberts et al., 2013).

Pathogens that come to reside in the midgut, the hemocoel or the internal organs, elicit immune responses that have evolved to eliminate or control infections. These immune responses range from cellular events such as phagocytosis, to humoral events that include lysis and melanization. This review presents an overview of these and other immune processes, and how the primary immune cells of insects, the hemocytes, are produced and replenished.

2. Anatomy of the insect immune system

Once pathogens gain entry into the body of an insect they come to reside in the midgut, the hemocoel, or other cells and organs. The recognition of pathogens as both foreign and dangerous induces immune responses that are driven by multiple types of immune cells and tissues (Figure 1).

The primary immune cells in insects are the hemocytes (Hillyer and Strand, 2014; Strand, 2008). These cells, which are present in the hemocoel, drive cellular immune processes such as phagocytosis, and produce humoral immune factors that lead to pathogen killing via lysis or melanization. Hemocyte populations can be divided using two, non-exclusive criteria: their spatial state and their functional properties. From a spatial perspective, hemocytes either circulate with the hemolymph, in which case they are called circulating hemocytes, or are

attached to tissues, in which case they are called sessile hemocytes (Hillyer and Strand, 2014; Strand, 2008). The distinction between circulating and sessile hemocytes is strictly spatial, and sessile hemocytes can release from their point of attachment and enter circulation, and circulating hemocytes can attach to tissues and become sessile (Babcock et al., 2008; King and Hillyer, 2012; Markus et al., 2009; Sigle and Hillyer, 2016). From a functional perspective, most insects have several sub-populations of hemocytes that are morphologically and functionally distinct, but the nomenclature for these cell populations is not normalized across Insecta. For example, lepidopteran larvae contain four distinct types of hemocytes: plasmatocytes that are involved in capsule formation, granulocytes that drive phagocytosis, oenocytoids that produce enzymes involved in the melanization cascade (e.g., phenoloxidase), and spherule cells whose immune function remains unclear (Lavine and Strand, 2002; Strand, 2008). Mosquitoes, on the other hand, have phagocytic granulocytes, phenoloxidase-producing oenocytoids, and prohemocytes (Castillo et al., 2006; Hillyer et al., 2003a; Hillyer and Strand, 2014). As a final example, the fruit fly, *Drosophila melanogaster*, has phagocytic plasmatocytes, phenoloxidase-producing crystal cells, and encapsulating lamellocytes (Honti et al., 2014).

The fat body, the midgut and the salivary glands are also important drivers of the insect immune response. The fat body, which is composed of loosely associated cells that are rich in lipids and glycogen, lines the integument of the hemocoel (Costa-Leonardo et al., 2013; Larsen, 1976; Martins et al., 2011). Besides functioning in energy storage and the synthesis of the vitellogenin precursors required for the production of eggs, the fat body produces and secretes antimicrobial peptides with lytic activity as well as additional components of the humoral immune response (Aggarwal and Silverman, 2008; Hillyer, 2010; Wang et al., 2014a). The midgut, an organ that primarily functions in digestion and absorption of nutrients, extends almost the entire length of the abdomen and produces nitric oxide synthase and other lytic factors that kill pathogens that are either in the lumen of the gut or are attempting to enter the hemocoel by traversing this epithelium (Buchon et al., 2009; Gupta et al., 2009; Lehane et al., 1997; Lim et

al., 2005; Luckhart et al., 1998). The salivary glands, an organ primarily involved in the initial stages of feeding, are usually located in the anterior of the thorax and produce factors that impact the viability of infecting microorganisms (Ferrandon et al., 1998; Pinto et al., 2008).

These immune tissues, although physically and morphologically independent, interact during the course of an infection. For example, factors produced by the fat body induce immune activity in hemocytes (Schmid et al., 2014). Conversely, hemocyte-produced factors are required for immune activity in the fat body (Brennan et al., 2007; Shia et al., 2009). Humoral immune factors that are produced by hemocytes are also transported to the gut where they exert their lytic and melanizing activity (Fraiture et al., 2009), and midgut-produced factors can activate immune activity in the fat body (Wu et al., 2012).

3. Pathogen recognition

The initiation of an immune response requires that the insect recognize an invading agent. During an infection, such recognition usually occurs when pathogen-associated molecular patterns (PAMPs, also known as microbe-associated molecular patterns or MAMPs) bind to host-derived pattern recognition receptors (PPRs). PRRs recognize conserved motifs that are present in microbes but are absent in insects. Examples of PAMPs are bacterial peptidoglycans and fungal β-1,3 glucans.

PRRs are conceptually divided into evolutionarily conserved protein families. These protein families exhibit remarkable diversity, which is likely a consequence of the drastic differences in the ecology of members of the class Insecta. Among the different classes of PRRs are the peptidoglycan recognition proteins (PGRP), the immunoglobulin domain proteins, the fibrinogen-related proteins (FREP), the thioester-containing proteins, the β-1,3 glucan recognition proteins (also known as the Gram(-) binding proteins), the galectins, the C-type lectins, the leucine-rich repeat containing proteins (LRR), down syndrome cell adhesion molecule (DSCAM), and the Nimrod proteins (Das et al., 2009; Palmer and Jiggins, 2015;

Zhang et al., 2015). Many PRR protein families have expanded or contracted in different taxa (Figure 2). For example, there are 7, 8, 13, 6, 14, 4, 4, and 2 members of the PGRP protein family in *Anopheles gambiae*, *Aedes aegypti*, *Drosophila melanogaster*, *Tribolium castaneum*, *Manduca sexta*, *Apis mellifera*, *Bombus terrestris*, and *Megachile rotundata*, respectively (Barribeau et al., 2015; Evans et al., 2006; Waterhouse et al., 2007; Zhang et al., 2015), and the genome of the pea aphid, *Acyrthosiphon pisum*, does not encode any PGRPs (Gerardo et al., 2010).

Along with the genetic diversity of PRRs, their activity varies widely. For example, some members of the Nimrod gene family encode cell surface receptors with multiple transmembrane domains whereas others encode secreted proteins, and different members of this same protein family also recognize different combinations of pathogens (Estevez-Lao and Hillyer, 2014; Kocks et al., 2005; Kurucz et al., 2007; Zsamboki et al., 2013). Furthermore, some PRRs directly elicit immune effector processes such as phagocytosis and melanization, others activate intracellular signaling pathways that activate the transcription of immune effector genes, and yet others activate both effector and signaling pathways (Choe et al., 2002; Levashina et al., 2001). Finally, some proteins that are often considered PRRs, such as members of the LRR protein family, may not directly interact with PAMPs. Instead they regulate immune responses via the direct interaction with other host proteins (Fraiture et al., 2009).

4. Immune signaling

Pathogen recognition induces the activation of signal transduction pathways that (1) amplify immune responses, (2) induce the production of factors with antimicrobial activity, and (3) potentiate effector mechanisms. The three most characterized immune signaling pathways in insects are the Toll pathway, the Imd pathway, and the Jak/Stat pathway (Figures 2, 3).

The Toll pathway is an evolutionarily conserved signaling pathway that, in insects, functions in both development and immunity (Cao et al., 2015; Clayton et al., 2014; Evans et al.,

2006; Kingsolver et al., 2013; Lemaitre and Hoffmann, 2007; Lindsay and Wasserman, 2014; Sim et al., 2014; Xu and Cherry, 2014). In the realm of immunity, the Toll pathway is initiated when PRRs activate the extracellular cytokine, Spätzle, which binds the cellular receptor Toll, leading to intracellular events involving Myd88, Pelle, Tube, and others. This signal transduction results in the nuclear translocation of NF-κB transcription factors (e.g., Dif/Dorsal and Rel1), which activate the transcription of antimicrobial peptides and other immune effector genes. The Toll pathway is primarily effective in combating Gram(+) bacteria, viruses, fungi, and in the case of mosquitoes, some plasmodia (*Plasmodium berghei*).

In a conceptually similar manner, the Imd pathway is activated when a PAMP binds the extracellular receptor PGPR-LC (Cao et al., 2015; Clayton et al., 2014; Evans et al., 2006; Kingsolver et al., 2013; Kleino and Silverman, 2014; Lemaitre and Hoffmann, 2007; Sim et al., 2014; Xu and Cherry, 2014). This induces intracellular signaling via Imd, Fadd, Dredd, and others, which leads to the nuclear translocation of NF-κB transcription factors (e.g., Relish and Rel2) and the transcriptional activation of antimicrobial peptides and other immune effector genes. This immune pathway is primarily effective in combating Gram(-) bacteria, viruses, and in the case of mosquitoes, some plasmodia (*Plasmodium falciparum*).

Similar to the Toll pathway, the Jak/Stat pathway is involved in both development and immunity (Cao et al., 2015; Clayton et al., 2014; Evans et al., 2006; Kingsolver et al., 2013; Myllymaki and Ramet, 2014; Sim et al., 2014; Xu and Cherry, 2014). During an infection, the Jak/Stat pathway becomes activated when the extracellular cytokine Unpaired (Upd) binds the cellular receptor Domeless (Dome). Domeless is phosphorylated by Hopscotch (Hop), which recruits Stat. Stat then dimerizes and is translocated to the nucleus, and this activates the transcription of antimicrobial genes such as nitric oxide synthase. The Jak/Stat pathway is involved in the antibacterial, antiviral, and in the case of mosquitoes, the anti-Plasmodium response.

The Toll, Imd and Jak/Stat pathways are tightly regulated. Their activation is initiated by the binding of PAMPs by PRRs, but intracellular negative regulators function to keep these pathways in check. Specifically, Cactus, Caspar, and Socs/Pias are intracellular signaling molecules that negatively regulate, or repress, the Toll, Imd and Jak/Stat pathways, respectively (Clayton et al., 2014; Kleino and Silverman, 2014; Lindsay and Wasserman, 2014; Myllymaki and Ramet, 2014).

Other signal transduction pathways are also involved in immunity but have received less attention. For example, components of the Imd pathway can activate the JNK pathway, which leads to, among other things, the transcriptional activation of antimicrobial genes (Boutros et al., 2002; Wojda et al., 2004). Also, the insulin/insulin growth factor-1 signaling pathway is involved in the anti-*Plasmodium* response of mosquitoes (Pakpour et al., 2014), and RNA interference pathways (RNAi), which are discussed in greater detail in section 5.5, are essential components of the antiviral response. These pathways, although highly conserved, are not present in all insects. For example, three hemimetabolous insects – the pea aphid, the body louse, and the kissing bug – lack specific components of the IMD pathway, such as Imd and Fadd (Gerardo et al., 2010; Kim et al., 2011; Mesquita et al., 2015). Interestingly, although neither Imd, Fadd, Dredd, Caspar, Ird5 nor Kenny are encoded in the genome of the kissing bug, *Rhodnius prolixus*, the Imd pathway of this insect remains active and inducible (Mesquita et al., 2015).

5. Immune effector mechanisms

Pathogen death is accomplished via multiple effector mechanisms that can be broadly divided into phagocytosis, melanization, encapsulation, nodulation, lysis, RNAi-mediated virus destruction, autophagy, and apoptosis (Figure 4). These mechanisms are not mutually exclusive. For example, (1) pathogens that have been melanized in the hemocoel are often phagocytosed (Hillyer et al., 2003a, b), (2) lytic and melanizing components can be present on the surface of the same pathogens (Hillyer and Christensen, 2005), (3) lysed pathogens are at

times melanized (Osta et al., 2004; Volz et al., 2006), and (4) encapsulation and nodulation in most cases involves the aggregation of hemocytes and melanization (Pech and Strand, 1996; Ratcliffe and Gagen, 1977).

5.1 Phagocytosis

Phagocytosis is an evolutionarily conserved cellular immune process that is used by both vertebrate and invertebrate animals for the destruction of small foreign organisms. Phagocytosis is initiated when a foreign object is recognized and bound by proteins in the plasma membrane of the phagocyte. The foreign object is then internalized into a membrane-delimited phagosome, the phagosome fuses with a lysosome, and hydrolytic enzymes digest the particle. From a spatial perspective, phagocytosis is carried out by both circulating and sessile hemocytes (Hillyer and Strand, 2014). From a functional perspective, the hemocytes that phagocytose pathogens are the granulocytes of Lepidoptera, Hemiptera and mosquitoes, and the plasmatocytes of fruit flies (Hillyer et al., 2003a, b; Hillyer and Strand, 2014; Honti et al., 2014; Laughton et al., 2011; Strand, 2008). As a percentage of the overall hemocyte population, the majority of hemocytes are phagocytic.

Phagocytosis is a rapid response. In mosquitoes, circulating and sessile hemocytes begin to phagocytose pathogens within seconds of their introduction (Hillyer et al., 2003b; King and Hillyer, 2012; Sigle and Hillyer, 2016). In mosquitoes and the tobacco hornworm, some hemocytes can phagocytose hundreds of bacteria at any given time (Dean et al., 2004; Hillyer et al., 2005). Furthermore, unlike melanization, which produces melanotic capsules that remain in the hemocoel for the lifetime of the insect, phagocytosis is a renewable response whereby hemocytes repeatedly internalize and degrade pathogens. Perhaps for this reason smaller arthropods rely more heavily on phagocytosis than larger ones for the killing of small foreign organisms (Oliver et al., 2011). It is also likely that, in a manner similar to what occurs in

vertebrates, the process of phagocytosis activates other components of the insect immune response.

Phagocytosis is initiated when a cell-surface PRR or a humoral PRR binds a PAMP. PRRs that have been empirically shown to be involved in phagocytosis include thioester-containing proteins, Nimrod proteins, DSCAM, β-integrins, and PGRPs (Dong et al., 2006; Kurucz et al., 2007; Levashina et al., 2001; Mamali et al., 2009; Ramet et al., 2002). Different PRRs have different specificities. For example, *D. melanogaster* PGRP-LC mediates the phagocytosis of *E. coli* but not *S. aureus* (Ramet et al., 2002), and NimC1 mediates the phagocytosis of *S. aureus* and to a lesser extent *E. coli* (Kurucz et al., 2007). The intracellular signaling pathways that drive or enhance phagocytosis remain poorly understood, but in mosquitoes the cell death abnormal 2 (CED2)/CED5 and CED6 pathways regulate the internalization of bacteria. Specifically, the CED6 pathways drives phagocytosis that is mediated by TEP1, TEP3, LRIM1 and LRP1, whereas the CED2/CED5 pathway drives phagocytosis that is initiated by TEP4 and BINT2 (Moita et al., 2005).

5.2 Melanization

Melanization is an enzymatic process used by insects for cuticle hardening, egg chorion tanning, wound healing, and immunity (Cerenius et al., 2008; Christensen et al., 2005; Nappi and Christensen, 2005). In the realm of immunity, melanization is an immune effector mechanism involved in the killing of bacteria, fungi, protozoan parasites, nematode worms, and the eggs of parasitoid wasps. When this process also involves the aggregation of hemocytes, it is known as nodulation or encapsulation (see sections 5.3 and 5.4). Melanization involves a series of reactions that includes the conversion of tyrosine to melanin precursors and the cross-linking of proteins to form a layer of melanin that surrounds and sequesters an invading pathogen. Melanization is phenotypically manifested as a darkened proteinaceous capsule that surrounds the invading pathogen, and the death of the pathogen presumably occurs via either

oxidative damage or via starvation, as the foreign agent becomes isolated from the nutrient-rich hemolymph (Cerenius et al., 2008; Christensen et al., 2005; Nappi and Christensen, 2005). Melanization also assists in the clearing of dead or dying pathogens (Osta et al., 2004; Volz et al., 2006).

The process of melanization involves the coordinated interaction of pattern recognition receptors, serine proteases, serine protease inhibitors, and the enzymes that drive the production of melanin (Figure 5). This process begins when PRRs, such as β-1,3 glucan recognition proteins, C-type lectins and Gram-negative binding proteins, recognize PAMPs (Matskevich et al., 2010; Wang et al., 2014b; Wang et al., 2005). This initiates a serine protease cascade that culminates in the activation of pro-phenoloxidase activating enzymes that cleave the zymogen pro-phenoloxidase to its active form: phenoloxidase (An et al., 2011; An et al., 2009). Phenoloxidase initiates the production of melanin by hydroxylating tyrosine to form dopa, dopa is then oxidized by phenoloxidase to form dopaquinone, and this is converted to dopachrome (Christensen et al., 2005; Nappi and Christensen, 2005; Zhao et al., 1995). Dopachrome conversion enzyme converts dopachrome to 5,6-dihydroxyindole, phenoloxidase oxidizes it into indole-5,6-quinone, which is cross-linked with hemolymph proteins to form melanotic capsules. In a complementary pathway, dopa decarboxylase hydroxylates dopa to form dopamine, which is converted to melanin by phenoloxidase and other enzymes. The rate-limiting substrate in the melanization pathway is tyrosine. When additional tyrosine is needed to melanize large or numerous pathogens, endogenous production of tyrosine is accomplished by the hydroxylation of phenylalanine by phenylalanine hydroxylase.

Many of the enzymes and PRRs that drive the melanization response are produced by hemocytes, with oenocytoids (crystal cells) being the major producers of pro-phenoloxidase (Ashida et al., 1988; Hillyer et al., 2003a). Small pathogens that become melanized, such as bacteria, are often phagocytosed, and larger pathogens are often encapsulated by layers of hemocytes (Hillyer et al., 2003a, b; Pech and Strand, 1995, 1996). Because the enzymatic

process that leads to melanization produces reactive oxygen species, these reactions are tightly regulated. Serine protease inhibitors, also known as Serpins, and other factors such as some C-type lectins, inhibit the activation of the phenoloxidase cascade (An et al., 2011; Osta et al., 2004). Finally, some of the proteins involved in melanization are members of protein families. For example, most insect genomes encode multiple phenoloxidases that have partially overlapping functions. For example, *D. melanogaster* PPO1 and PPO2 are involved in the antibacterial response whereas PPO2 and PPO3 are involved in the encapsulation response (Dudzic et al., 2015). Likewise, *Armigeres subalbatus* Pro-PO I is involved in the melanization of filarial worms, Pro-PO III is involved in cuticle formation, and Pro-PO V is involved in both parasite melanization and egg chorion tanning (Shiao et al., 2001; Tsao et al., 2015; Tsao et al., 2009).

5.3 Cellular encapsulation

Encapsulation is a cellular immune response used against pathogens that are too large to be phagocytosed. This response is commonly employed by dipteran and lepidopteran larvae in response to infection with the eggs of parasitoid wasps. In Lepidoptera, encapsulation is initiated when granulocytes attach to form a layer of cells that surrounds a pathogen, and this cell adhesion occurs in a manner that is dependent on the binding of integrins to specific sites defined by an Arg-Gly-Asp (RGD) sequence (Pech and Strand, 1995, 1996). The layer of granulocytes is then surrounded by several layers of plasmatocytes, and this is followed by the binding of additional granulocytes. A similar process occurs in *Drosophila*, except that the cells involved are plasmatocytes and lamellocytes (Russo et al., 1996). Depending on the pathogen and the insect, the capsule may become melanized.

5.4 Nodulation

Nodulation involves the coordinated adherence of hemocytes in a manner that surrounds large aggregates of bacteria, and this is usually followed by melanization (Ratcliffe and Gagen, 1977; Satyavathi et al., 2014). In this immune process, granulocytes adhere to each other and form layers that surround dense bacterial aggregates. The granulocytes release their contents, which encases the bacteria in a flocculent material. Plasmatocytes then aggregate around the surface of the nodule, and in most cases the entire structure becomes melanized. The molecular mechanisms behind this immune process remain poorly understood, but nodulation relies on eicosanoid-based signaling and the extracellular matrix-like protein, Noduler (Carton et al., 2002; Gandhe et al., 2007).

5.5 Lysis

Pathogen killing via lysis refers to death due to the immune-based disruption of the cellular membrane. From a practical perspective, entomologists often attribute pathogen death to lysis when an immune-based reduction in infection intensity is detected, but this reduction is not accompanied by an easily observable immune phenotype. That is, observing lysis is difficult whereas phagocytosis is observed because pathogens are seen inside of hemocytes, and melanization, cellular encapsulation, and nodulation are easily observed because large cellular aggregates, dense and dark melanin deposits, or both are formed.

Some of the earliest studied insect factors that induce pathogen death via lysis are the antimicrobial peptides, also known as AMPs. AMPs are secreted proteins that are usually between 2 and 20 kDa in mass. These peptides and proteins were initially identified for their antimicrobial activity in vitro (Hoffmann and Hoffmann, 1990; Steiner et al., 1981; Vizioli et al., 2001). Based on their structural and sequence characteristics, most AMPs can be grouped into four categories, with many insect species producing multiple members of each (He et al., 2015; Imler, 2014; Yi et al., 2014). The Defensin and Defensin-like peptides include Defensin, Drosomycin, Holitricin, Sapecin and others. These peptides are rich in cysteines and contain

cysteine disulfide bonds. Members of this type of AMP, which have been identified in Diptera, Hymenoptera, Hemiptera, Coleoptera and Lepidoptera, have activity against Gram(+) bacteria, but others also combat Gram(-) bacteria and fungi. The Cecropin and Cecropin-like peptides, including the Moricins, are α-helical peptides that have been identified in Diptera, Coleoptera, and Lepidoptera. These peptides have activity against Gram(+) and Gram(-) bacteria, as well as fungi. The Attacins and Gloverins are glycine-rich peptides. Attacins, which have been identified in Diptera and Lepidoptera, are mainly active against Gram(-) bacteria, whereas the Gloverins, which have been identified only in Lepidoptera, have activity against Gram(+) bacteria, Gram(-) bacteria, or fungi. The Lebocins (Lebocin, Drosocin, Metchnikowin, and others) are proline-rich peptides that have been identified in Hemiptera, Hymenoptera, Lepidoptera, and Diptera, and are active against Gram(+) bacteria, Gram(-) bacteria, and some fungi. Other AMPs cannot be grouped in any of the above categories, yet are important components of the insect immune response. For example, Gambicin is produced by mosquitoes and possesses activity against Gram(+) bacteria, Gram(-) bacteria, fungi, and *Plasmodium* parasites (Vizioli et al., 2001). The production of antimicrobial peptides is governed by immune signaling pathways. In *D. melanogaster*, for example, activation of the Toll pathway induces the transcription of Drosomycin whereas activation of the Imd pathway induces the transcription of Diptericin (Lemaitre and Hoffmann, 2007).

Lysozymes are another family of proteins with lytic activity. Lysozymes hydrolyze the β-1,4-glycosidic linkage between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan present in the cell wall of bacteria. Most insect genomes encode multiple lysozymes; the genomes of *Drosophila melanogaster*, *Anopheles gambiae*, *Manduca sexta*, *Tribolium castaneum*, *Apis mellifera*, *Acyrthosiphon pisum*, *Bombus terrestris*, and *Megachile rotundata* encode 14, 8, 6, 4, 3, 3, 1 and 1 lysozymes, respectively (Barribeau et al., 2015; Evans et al., 2006; Gerardo et al., 2010; He et al., 2015; Waterhouse et al., 2007; Zou et al., 2007). Unlike AMPs, which are produced in response to an immune insult, lysozymes are

usually present in low, constitutive levels and are transcriptionally upregulated following infection. Although lysozymes are classically active in the lytic antibacterial response (Elmogy et al., 2015; Kwon et al., 2014b), some also have anti-*Plasmodium* and anti-fungal activity (Kajla et al., 2011; Sowa-Jasilek et al., 2014), and can activate the phenoloxidase-based melanization pathway (Li and Paskewitz, 2006; Rao et al., 2010). In addition to being involved in immunity, some insect lysozymes are expressed in the gut and function in digestion (Ito et al., 1995; Ursic-Bedoya et al., 2008).

The function of reactive oxygen species in immune responses is arguably best known for the oxidative burst that leads to the destruction of organisms that have been internalized by phagocytes (Slauch, 2011). In addition to their function in this cellular response, reactive oxygen species and reactive nitrogen species effect lytic activity in the extracellular environment. For example, dual oxidase, which generates hydrogen peroxidase, regulates the antibacterial response in the intestinal space of fruit flies (Ha et al., 2005), and nitric oxide synthase, which produces nitric oxide via the oxidation of L-Arginine to L-Citrulline (Hughes, 2008), drives anti-*Plasmodium* activity in the gut of mosquitoes (Gupta et al., 2009; Luckhart et al., 1998). Reactive species are also involved in the antimicrobial response in the hemocoel. For example, nitric oxide, which functions as both a messenger molecule and an immune effector, is produced by hemocytes and is an important component of the antibacterial response (Hillyer and Estevez-Lao, 2010; Nappi et al., 2000).

5.6 RNA interference

RNA interference, or RNAi, is an RNA-based mechanism for gene silencing. From an experimental perspective, scientists have taken advantage of the insect's own RNAi machinery to study the function of insect genes, and it has been proposed that RNAi could be used for the management of insect pests (Scott et al., 2013; Yu et al., 2013). Nevertheless, one of the

natural functions of RNAi is to protect the organism from viral infection (Blair and Olson, 2014; Bronkhorst and van Rij, 2014; Karlikow et al., 2014; Sim et al., 2014).

The primary RNAi pathway that participates in the antiviral response is the small interfering RNA pathway (siRNA, or exo-siRNA for exogenous siRNA), whereby viral double stranded RNA (dsRNA) is cleaved by a ribonuclease, called Dicer-2 (Dcr2), that forms a complex with its cofactor R2D2 (Figure 6). This cleavage produces viral-derived small interfering RNAs (siRNAs) that are usually 21 nucleotides in length, and these siRNAs are loaded into pre-RNA induced silencing complexes (RISC) that include Argonaute-2 (Ago2). The siRNA in a RISC complex is unwound, one strand is discarded, and the other binds complementary viral RNA, which triggers its destruction by Ago2. The binding of viral RNA by Dcr2 also activates the transcription of *Vago*, which is a cysteine-rich polypeptide that negatively controls virus replication (Deddouche et al., 2008). In mosquitoes, the transcriptional activation of *Vago* is mediated by an NF-κB transcription factor associated with the Imd pathway (Rel2), and in turn, Vago activates the Jak-Stat pathway (Paradkar et al., 2012).

In addition to the siRNA pathway, the PIWI-associated RNA pathway (piRNA) also functions in the RNAi-mediated antiviral defense of mosquitoes (Blair and Olson, 2014; Bronkhorst and van Rij, 2014). piRNAs are 25-30 nucleotides in length and associate with the PIWI clade of Argonaute proteins. The piRNA pathway operates in a manner that is independent of Dicer.

5.7 Autophagy

Autophagy is an evolutionary conserved process utilized for the degradation of intracellular materials. In both vertebrate and invertebrate animals, autophagy is employed for the elimination of intracellular pathogens such as bacteria and viruses (Lamiable and Imler, 2014; Moy and Cherry, 2013; Shibutani et al., 2015; Yano and Kurata, 2011). At the molecular level, autophagy is initiated via the interaction of Toll-7 in the plasma membrane with viral

factors, which triggers the downregulation of the phosphatidylinositol 3-kinase (PI3K)-Akt pathway, thus inactivating the negative regulator of autophagy, target of rapamycin (TOR). Inactivation of TOR activates a complex containing the protein kinase Atg1 and the phosphoprotein Atg13 and this leads to the nucleation of the autophagosomal membrane via a complex that contains Atg14, Vps15 and Vps34. Following nucleation, the autophagosomal membrane elongates in a process that is dependent on both an Atg5/Atg12 complex and the conjugation of phosphatidylethanolamine to Atg8. The elongated membrane then closes to form an intracellular autophagosome, which then fuses with lysozomes, triggering the degradation of the engulfed contents. In *Drosophila*, autophagy is employed in the immune response against vesicular stomatitis virus and Rift Valley fever virus (Moy et al., 2014; Shelly et al., 2009). Autophagy, however, also enhances infection with Sindbis virus (Patel and Hardy, 2012).

5.8 Apoptosis

Apoptosis is a form of programmed cell death. This process is critical during the development of both vertebrate and invertebrate animals (Suzanne and Steller, 2013). At the molecular level, the caspase, Dronc, and the adaptor protein, Ark, form a complex. Dronc activates effector caspases such as Drice and Dcp1, and these caspases cleave proteins in a manner that eventually leads to programmed cell death (Cao et al., 2015). In Lepidoptera, apoptosis is involved in the response against baculoviruses (Clem, 2007). In mosquitoes, this process appears to be involved in the response against West Nile Virus and Sindbis virus (Vaidyanathan and Scott, 2006; Wang et al., 2012), and in the fruit fly, apoptosis protects against *Drosophila* C virus in a manner that is dependent on the phagocytosis of virus-infected apoptotic cells (Nainu et al., 2015). Some immune factors (e.g., Imd and Inhibitor of apoptosis protein 2, or IAP2) influence both the Imd and apoptosis pathways (Georgel et al., 2001; Gesellchen et al., 2005).

6. Immune responses and hemolymph circulation

The hemocoel is a dynamic environment where hemolymph is continuously propelled to all the regions of the body by a series of autonomous pumps. The primary pump is the dorsal vessel, which is a muscular tube that extends the length of the body along the dorsal midline of the insect and is divided into a heart in the abdomen and an aorta in the thorax (Ejaz and Lange, 2008; Glenn et al., 2010; League et al., 2015; Wasserthal, 2007). In most adult insects, the heart periodically alternates between propelling hemolymph toward the head (anterograde direction) and propelling hemolymph toward the posterior of the abdomen (retrograde direction) (Gerould, 1933; Glenn et al., 2010; League et al., 2015; Wasserthal, 2007). During anterograde contractions, hemolymph enters the heart via valves, called ostia, that are located in each abdominal segment, and exits the vessel through an excurrent opening located near the head (Glenn et al., 2010; League et al., 2015). During retrograde contractions, hemolymph enters the heart through a pair of ostia located at the thoraco-abdominal junction and exits through excurrent openings located at the terminal abdominal segment. Once in the hemocoel, hemolymph flows toward the ostia and re-enters the heart (Andereck et al., 2010; Glenn et al., 2010; Lee and Socha, 2009). Insects also possess a series of accessory pulsatile organs, or auxiliary hearts, that propel hemolymph into the appendages (e.g., antennae, wings, and some reproductive structures) and other areas of the body that would otherwise experience little flow (Andereck et al., 2010; Boppana and Hillyer, 2014; Hustert et al., 2014; Lehmacher et al., 2009). The above description details what generally occurs in adult stage Diptera, but there are variations to this plan. For example, the heart of immature insects and some adult insects only contracts anterograde, some insects contain segmental vessels that extend laterally from the heart, and the location, number and function of the ostia varies between species and life stages (Ejaz and Lange, 2008; Gerould, 1933; Hertel and Pass, 2002; League et al., 2015).

Pathogens, circulating hemocytes and humoral immune factors present in the hemocoel move throughout the body with the flow of hemolymph, and sessile hemocytes, fat body and all internal organs are exposed to a steady stream of this fluid (Babcock et al., 2008; Hillyer et al., 2007; King and Hillyer, 2012; Paulson and Grimstad, 1989). Immune processes such as phagocytosis, melanization and lysis occur both in circulation and at sessile locations (Borges et al., 2008; Hillyer et al., 2003a, b; King and Hillyer, 2013; Kwon et al., 2014a), and in response to injury, circulating hemocytes adhere to wounds and initiate a healing response that usually involves melanization (Babcock et al., 2008; Krautz et al., 2014; Lai et al., 2001; Rowley and Ratcliffe, 1978). The distribution of circulating and sessile hemocytes can dynamically change, including the attachment of circulating hemocytes to sessile tissues and the release of sessile hemocytes into circulation (Babcock et al., 2008; King and Hillyer, 2012, 2013; Markus et al., 2009; Sigle and Hillyer, 2016).

Many insect immune responses take place as hemocytes passively flow with the hemolymph. Others, however, actively take advantage of circulatory currents (Figure 7). This co-adaptation of the circulatory and immune systems has been most comprehensively demonstrated in mosquitoes, where these flies possess a resident population of sessile hemocytes – called periostial hemocytes – that are positioned in the extracardiac regions that are immediately adjacent to the ostia (King and Hillyer, 2012). Upon infection, periostial hemocytes rapidly begin to phagocytose pathogens at these locations of high hemolymph flow, and as this occurs, additional hemocytes arrive at the heart, adhere to the periostial hemocytes and the cardiac-associated musculature, and amplify this phagocytic response. The periostial regions of the heart are the sessile areas of the body that contain the highest density of hemocytes, and once periostial hemocyte aggregation is initiated, elevated numbers of hemocytes remain at these locations for the lifetime of the insect (King and Hillyer, 2012, 2013). Along the length of the heart, hemocyte aggregation is asymmetric, with the majority of hemocytes congregating at the periostial regions that receive the most hemolymph flow, thus

placing immune cells at the locations of the body where they are most likely to encounter, and kill, circulating pathogens (Sigle and Hillyer, 2016). The aggregation of pathogens on the surface of the heart has been observed in Diptera and Lepidoptera (Bao et al., 2011; Elrod-Erickson et al., 2000; Hillyer et al., 2007; King and Hillyer, 2012, 2013; Pereira et al., 2015; Sigle and Hillyer, 2016), and hemocytes in or on the heart have been detected in Diptera, Lepidoptera, and Orthoptera (da Silva et al., 2012; Ghosh et al., 2015; King and Hillyer, 2012, 2013; Pereira et al., 2015; Sigle and Hillyer, 2016). Interactions between the immune and circulatory systems extend past the insects and into other members of Pancrustacea (Hillyer, 2015).

7. Hematopoiesis

Hemocytes are the primary immune cells of insects, and the production of these cells occurs during all life stages. Hematopoiesis, and more specifically hematopoiesis during the embryonic and immature stages, has been most comprehensively described for the model insect, *D. melanogaster*. In the embryo of the fruit fly, hemocytes are produced when cells from the procephalic mesoderm differentiate into plasmatocytes and crystal cells, and this is mediated by the GATA transcription factor, Serpent (Holz et al., 2003; Honti et al., 2014; Wood and Jacinto, 2007). The differentiation of plasmatocytes, which are the vast majority of the hemocytes, occurs as the mesodermal cells disperse throughout the embryo in a developmentally programmed manner, and requires the activity of Glial cells missing (Gcm) and Glial cells missing 2 (Gcm2). The differentiation of crystal cells involves non-dispersing mesodermal cells that congregate around the proventriculus, and requires the transcription factor Lozenge and Serrate/Notch signaling. In the larva, additional hemocytes are produced within a hematopoietic organ called the lymph gland, which is derived from the cardiogenic mesoderm (Holz et al., 2003; Honti et al., 2014; Jung et al., 2005). The lymph gland is anatomically divided into primary and secondary lobes that are positioned along the anterior

portion of the dorsal vessel. The primary lobes are further divided into a posterior signaling center, an inner medullary zone and an outer cortical zone, with the posterior signaling center and the inner medullary zone containing prohemocytes at different stages of differentiation and the cortical zone containing plasmatocytes and crystal cells (Jung et al., 2005). Hemocyte differentiation in the primary lobes of the lymph gland involves the InR/TOR, Jak/Stat, and other pathways (Honti et al., 2014; Wood and Jacinto, 2007), and mature hemocytes are released from the lymph gland during a larval infection and shortly prior to pupation, at which point the lymph gland begins a degeneration process that is completed around the time of eclosion (Lanot et al., 2001). The secondary lobes contain prohemocytes that survive into adulthood, at which point they give rise to hematopoietic hubs that flank the anterior portion of the heart (Ghosh et al., 2015). In the larval stages, patches of sessile hemocytes that are attached to the integument, as well as circulating hemocytes, produce additional hemocytes by undergoing mitosis and cellular differentiation (Lanot et al., 2001; Leitao and Sucena, 2015; Markus et al., 2009).

In non-drosophilid Diptera, the process of hematopoiesis has also been examined in mosquitoes. Unlike for *D. melanogaster*, where hematopoiesis has primarily been scrutinized during the larval stages, hemocyte proliferation in mosquitoes has been exclusively assessed in the adult life stage, and this is likely because it is this stage that transmits disease-causing pathogens to humans and animals. In adults from both major families of mosquitoes, the Culicinae and the Anophelinae, imbibing blood induces the replication of hemocytes, and this replication is controlled by insulin signaling and Ras-MAPK signaling (Bryant and Michel, 2014; Castillo et al., 2011). Blood feeding-induced hemocyte proliferation is transient, as by 36 h post feeding the mitotic rate of hemocytes returns to near baseline levels (Bryant and Michel, 2016). During the course of some infections, the number of hemocytes in the hemocoel also increases, and this is at least in part due to the mitosis of circulating hemocytes (Christensen et al., 1989; Coggins et al., 2012; King and Hillyer, 2013).

In immature Lepidoptera, hemocyte proliferation and differentiation also relies on embryonically-derived hemocytes as well as hemocytes produced in compact, hematopoietic organs that are positioned in the mesothorax and the metathorax, near the imaginal wing discs (Akai and Sato, 1971; Gardiner and Strand, 2000; Monpeyssin and Beaulaton, 1978; Nardi, 2004; Nardi et al., 2003). Much like in *Drosophila*, hemocyte production occurs via both the mitosis of embryonic hemocytes, and via the differentiation of cells within the hematopoietic organs (Gardiner and Strand, 2000; Mangalika et al., 2010; Nakahara et al., 2003; Tan et al., 2013). During the late larval stages, these hematopoietic organs release mature hemocytes into circulation and then degenerate around the time of metamorphosis. The molecular basis of hemocyte replication in Lepidoptera is not well understood, but it involves insulin signaling and Noduler-based activation of p38 mitogen-activated protein kinase (Nakahara et al., 2006; Satyavathi et al., 2015).

Hemocyte replication in other insect orders has not been studied in great detail. However, hematopoietic organs have been described in Orthoptera, and for one member of this group (*Locusta migratoria*) it was shown that an immune challenge during the adult stage induces an increase in hemocyte numbers, with this increase being primarily due to the mitosis of circulating hemocytes (Duressa et al., 2015). The proliferation of hemocytes has also been reported in Blattodea and Hemiptera, although the spatial and molecular mechanics of these mitotic events have not been elucidated (Feir and O'Connor, 1965; Tauber, 1940). Finally, although hemocytes are divided into functionally distinct populations, experiments in both Diptera and Lepidoptera show, or suggest, that a mature hemocyte can give rise to a different functional morphotype (e.g., plasmatocytes giving rise to crystal cells), demonstrating the plasticity of the hemocyte lineages (Honti et al., 2010; Leitao and Sucena, 2015; Nakahara et al., 2003).

8. Immune priming

The adaptive immune response of vertebrate animals relies on somatic hypermutation and V(D)J recombination to create highly diverse repertoires of antibodies and T cell receptors that specifically recognize invaders (Murphy, 2011). This process also allows for immune memory, where exposure to a given pathogen induces the development of cells that are specifically tuned to that pathogen. The presence of these memory cells allows the organism to mount a more rapid and powerful immune response when it is re-exposed to the same pathogen or a highly related one. This type of adaptive immune response, as classically defined for the vertebrate immune system, does not occur in insects.

Insects, however, employ other tactics to protect themselves from re-infection. Studies on a diverse group of insects have shown that a sub-lethal infection can provide partial or full protection from a subsequent infection (Masri and Cremer, 2014). Protection is sometimes specific to the pathogen already encountered (Pham et al., 2007; Roth et al., 2009; Sadd and Schmid-Hempel, 2006). Most often, however, protection is conferred against other pathogens as well, or protection from re-infection with the same pathogen is clearly established but the specificity of the protection remains unknown (Contreras-Garduno et al., 2015; Moret and Siva-Jothy, 2003; Rodrigues et al., 2010). The length of the protection may last the lifetime of the insect (although most of these studies have been performed in short-lived insects), or may last only for only a few days (Moret and Siva-Jothy, 2003; Pham et al., 2007). In addition, protection from infection may be conferred to the offspring (Moret and Schmid-Hempel, 2001; Trauer-Kizilelma and Hilker, 2015), may be passed along to the nestmates (Traniello et al., 2002; Ugelvig and Cremer, 2007), and may persist across molts (Bargielowski and Koella, 2009; Thomas and Rudolf, 2010).

The mechanisms underlying immune priming in insects are poorly understood. It has been hypothesized that immune priming occurs because the insect is inherently able to activate immune responses more rapidly during a second exposure, or because pathogens or pathogen components are retained within the insect, which maintains the animal in a heightened state of

immune alertness (Masri and Cremer, 2014). Although immune priming has been demonstrated in multiple insect orders, it is not a universal feature of the insect immune system, nor is it universal for all infection types (Gonzalez-Tokman et al., 2010; Reber and Chapuisat, 2012).

9. Environmental factors and immune responses

The strength and composition of an immune response is impacted by factors that are independent of the nature of the infection. One of these factors is temperature. Insects are poikilotherms, and thus, their temperature fluctuates with the temperature of the environment. The environmental temperature during an infection, or even the temperature experienced earlier in life, can affect the anti-pathogen response. Temperature affects the immune response of mosquitoes, but these changes are complex and do not simply scale with temperature (Murdock et al., 2012). For example, temperatures that favor the expression of nitric oxide synthase are less favorable for phenoloxidase-based melanization, and vice versa. These complexities have also been observed in crickets, where elevated temperatures are advantageous when fighting some types of pathogens but not others (Adamo and Lovett, 2011). Within a species, the strength of the immune response varies with both temperature and genotype. For example, *D. melanogaster* that were collected in tropical regions of the world were less capable of mounting immune responses when placed at a low temperature when compared to similarly treated flies that had been originally collected in temperate locations (Lazzaro et al., 2008). Finally, temperature-associated stress can affect the strength of immune responses mounted later in life, as reported for the deer ked, *Lipoptena cervi* (Kaunisto et al., 2015).

In addition to temperature, other external factors influence immune responses. The availability of resources and the composition of those resources affects the strength of insect immune responses (Boots, 2011; Cotter et al., 2011). Likewise, ambient humidity and the circadian clock also impact the strength and composition of an immune response (Lee and

Edery, 2008; Mostafa et al., 2005). For example, the time of day when a fruit fly is infected influences the probability of survival, and this is at least in part due to the modulation of phagocytosis by the circadian clock protein, Timeless (Lee and Edery, 2008; Stone et al., 2012).

10. Insect age and immune responses

The age of an insect impacts the response to infection. In adult insects, the general observation is one of immunosenescence, where the strength of the immune response decreases with age. Such observations have been made in Diptera, Hymenoptera, Lepidoptera, Coleoptera, and Odonata. Often, senescence refers to a decrease in the potency of a response that involves encapsulation, nodulation or melanization (Daukste et al., 2012; Park et al., 2011; Prasai and Karlsson, 2012; Robb and Forbes, 2006), or refers to a decrease in the number of hemocytes or their phagocytic activity (Hillyer et al., 2005; Horn et al., 2014; Mackenzie et al., 2011; Park et al., 2011; Pigeault et al., 2015; Schmid et al., 2008). This senescence often results in increased infection intensities in older insects, which may be accompanied by either increased (Roberts and Hughes, 2014) or decreased (Hillyer et al., 2005) survival. Seemingly conflicting with the concept of immunosenescence, reports on insect vectors have demonstrated that older flies are less capable of becoming infected with – and transmitting – malaria parasites, filarial nematodes, or the parasites that cause human African trypanosomiasis (Aksoy et al., 2014; Ariani et al., 2015; Pigeault et al., 2015), indicating that the relationship between immunity, tolerance, and aging is complex.

11. Social immunity

The anti-pathogen response of insects has been most intensely studied at the level of the individual, with most of this work focusing on internal factors that protect an organism from an infectious agent. In addition to these individual responses, several groups of insects mount collective, anti-pathogen defenses, and these defenses are referred to as social immunity.

Investigations into social immunity initially focused on eusocial insects, and defined this process as the collective action or altruistic behaviors of insects that result in the avoidance, control or elimination of parasitic infections (Cremer et al., 2007). Subsequent studies broadened this definition to include non-eusocial insects, and defined social immunity as (1) immune responses that have been selected to increase the fitness of a challenged individual and one or more recipient (Cotter and Kilner, 2010), or (2) collective and personal mechanisms that are maintained at least in part by the anti-pathogen defense they provide to other members of the group (Meunier, 2015). Examples of social immunity include, but are not limited to, avoiding contaminated habitats (Rozen et al., 2008), collecting antimicrobial substances (Simone-Finstrom and Spivak, 2010), depositing materials with antimicrobial properties on the nest or brood (Baracchi et al., 2012; Reavey et al., 2014), removing infected or dead individuals (Diez et al., 2012), allogrooming (Walker and Hughes, 2009), social fever (Starks et al., 2000), transgenerational immune priming (Moret and Schmid-Hempel, 2001; Rosengaus et al., 2013; Trauer-Kizilelma and Hilker, 2015), and immune priming of nestmates (Traniello et al., 2002; Ugelvig and Cremer, 2007).

12. Concluding remarks

Over the past few decades it has become exceedingly clear that the insect immune system is composed of a complex network of components that synergistically form barriers that prevent or fight infection. Multiple tissues, led by the hemocytes, work in concert to combat invading pathogens, and the molecular underpinnings of immune processes are becoming well understood. However, many aspects of the insect immune response remain unknown or underexplored, and the remainder of this section highlights a few of these areas.

Insects are the most speciose group of animals, with thirty orders (give or take a few, depending on the analysis) that contain approximately one million taxonomically described species (Gullan and Cranston, 2014; Misof et al., 2014; Trautwein et al., 2012). Yet, our

understanding of insect immunity comes primarily from studies conducted in Diptera and Lepidoptera, which are closely related holometabolous insect orders. The immune systems of hemimetabolous insects, with the exception of some members of Hemiptera, have gone largely ignored. Similarly, with some notable exceptions (Park et al., 2010; Zou et al., 2007), comparatively little is known about the immune system of the most speciose group of insects, the Coleoptera (beetles), which contains over one third of the described insect species. Even within the Diptera, which is the taxonomic group that has received the most attention, taxon coverage has been sparse. This group contains over 150,000 species that are classified into 130 families (Yeates and Wiegmann, 1999), yet most of what we know about the immune system of dipterans has focused on two families: Drosophilidae (fruit flies) and Culicidae (mosquitoes). Even within the Culicidae, which contains approximately 3,500 described species (Reidenbach et al., 2009), most studies gave concentrated on three species: *Anopheles gambiae*, *Aedes aegypti*, and to a lesser extent, *Culex quinquefasciatus*. Thus, the field of insect immunity is fairly mature in some taxa but largely underexplored in others.

Within a taxonomic group, studies on insect immunity have often shown a developmental bias. In *D. melanogaster*, studies pertaining to hematopoiesis have almost exclusively focused on the larval stages (Honti et al., 2014). Studies in Lepidoptera have also mainly focused on the larvae (Jiang et al., 2010), whereas studies on mosquitoes have focused almost exclusively in the adult life stage (Clayton et al., 2014; Hillyer, 2010). The ecology of an insect species often varies dramatically between life stages (e.g., from aquatic to aerial, or from subterranean to above ground), and these changes, together with the reproductive status of the insect, are expected to impact pathogen-associated immune responses.

Many questions also remain pertaining to how insects with greatly different life histories fight infection. For example, immunity-based studies conducted on Diptera and Lepidoptera have focused on species with relatively short lifespans. Fruit flies, mosquitoes, the silkworm, and the tobacco hornworm, to name a few, all have life cycles that can be completed in a few

weeks, although diapause can extend this timeline. In contrast, some insects live longer than a year, and in the extreme case of periodical cicadas, these insects live thirteen or seventeen years prior to reaching sexual maturity (Sota et al., 2013). Whether there are significant differences in the manner in which long-lived insects combat infection, relative to their shorter-lived counterparts, remains a mystery.

Hemocyte classification and biology has often been a controversial topic. Hemocytes have been examined by light microscopy for many insect species, and most initial studies have relied exclusively on morphology to create hemocyte classification nomenclatures. Subsequent studies at the functional, cytochemical or molecular levels have validated those morphological classifications or revised them, with revisions usually resulting in a reduction in the number of accepted cell types (Hillyer and Strand, 2014; Strand, 2008). Even when the nomenclature systems are properly validated, a lack of communication between investigators working on different species has led to cells that are functionally and morphologically similar being named differently (Hillyer and Christensen, 2002; Strand, 2008). Within a species, different nomenclatures continue to exist, as has been reported, for example, for the pea aphid (Laughton et al., 2011; Schmitz et al., 2012). In attempts to circumvent this problem, some investigators have proposed that the names of hemocyte sub-types be normalized (Ribeiro and Brehelin, 2006), but such a normalized terminology has not been adopted, perhaps because real differences indeed exist due to the 400 million year evolutionary history of the class Insecta. The field of hemocyte biology is ripe with questions, but initial studies on any insect should seek to balance structure and function, while being mindful of the nomenclatures used in related taxa.

Finally, our understanding of the molecular basis of immunity is advancing at a rapid pace. This has been facilitated by the continuous development and accessibility of next generation sequencing technologies, genomic approaches, and both RNAi- and transgenic-based methods to modify gene expression. A continued pursuit of the molecular basis of

immune effectors, pathways, and their cross-talk, will continue to inform not only on how insects combat infection, but will also inform on how the immune system of animals has evolved.

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Figure legends:

Figure 1. Anatomy of the insect immune system. The insect body cavity, called the hemocoel, is a fluid and dynamic space that houses tissues with immune activity. The primary immune cells are the hemocytes. Hemocytes are found in circulation (circulating hemocytes) and attached to tissues (sessile hemocytes), where they phagocytose, encapsulate and nodulate pathogens, and produce humoral immune factors. The fat body, the midgut, the salivary glands, and other tissues produce numerous humoral immune factors with, among other things, lytic and melanizing activity. For a description of how hemolymph circulation impacts immune responses, and the activity of periostial hemocytes, see figure 7.

Figure 2. Gene counts for select genes and gene families in *Anopheles gambiae* (Order: Diptera), *Aedes aegypti* (Diptera), *Drosophila melanogaster* (Diptera), *Pediculus humanus humanus* (Siphonaptera), *Manduca sexta* (Lepidoptera), *Tribolium castaneum* (Coleoptera), *Apis mellifera* (Hymenoptera), and *Acyrthosiphon pisum* (Hemiptera). The gene number data was collected from (Cao et al., 2015; Chevignon et al., 2015; Evans et al., 2006; Gerardo et al., 2010; He et al., 2015; Kim et al., 2011; Sackton et al., 2007; Waterhouse et al., 2007; Zhang et al., 2015; Zou et al., 2007), with additional searches in https://www.vectorbase.org and http://metazoa.ensembl.org. The evolutionary relationships between the species, depicted at the top of the figure, are based on (Trautwein et al., 2012). Gene numbers can vary slightly depending on the analysis. Abbreviations: PRR, pattern recognition receptor; Toll (vertical orientation), Toll pathway; Imd (vertical orientation), Imd pathway; Effector, effector peptides and proteins; PGRP, peptidoglycan recognition protein; FREP, fibrinogen-related protein; βGRP, β-1,3 glucan recognition protein; GNBP, Gram(-) binding protein; CTL, C-type lectin; PPO, pro-phenoloxidase.

Figure 3. Simplified diagram illustrating key players in the Toll, Imd, and Jak/Stat pathways, using mosquitoes as the example. For a description of the pathways, see section 4.

Figure 4. Immune effector mechanisms of insects. Insects kill pathogens via phagocytosis (section 5.1), melanization (section 5.2), cellular encapsulation (section 5.3), nodulation (section 5.4), lysis (section 5.5), RNA interference (section 5.6), autophagy (section 5.7), and apoptosis (section 5.8).

Figure 5. Biochemical pathway of phenoloxidase-based melanization. For a description of the pathway, see section 5.2. Abbreviations: PRR, pattern recognition receptor; βGRP, β-1,3 glucan recognition protein; CTL, C-type lectin; GNBP, Gram(-) binding protein; PPAE, phenoloxidase activating enzyme; PAH, phenylalanine hydroxylase; PO, phenoloxidase; DDC, dopa decarboxylase; DCE, dopachrome conversion enzyme.

Figure 6. Small interfering RNA pathway. For a description of this RNAi pathway, see section 5.6.

Figure 7. Co-adaptation of the insect circulatory and immune systems, as exemplified in mosquitoes. Hemocytes exist in two states: in circulation and in sessile form. In naïve mosquitoes (top), some of the sessile hemocytes are aggregated around the ostia (valves) of the heart, and are called periostial hemocytes. Hemolymph flow is swift in the regions surrounding the ostia (the periostial regions), and the majority of hemolymph enters the heart through the ostia located in abdominal segments 4, 5, and 6. Upon infection (bottom), circulating hemocytes undergo mitosis and increase in number, and many hemocytes migrate to, and aggregate in, the periostial regions of the heart where they phagocytose pathogens. The periostial regions are the only location of the body where sessile hemocytes increase in

number in response to infection. Infection does not significantly alter the proportion of hemolymph that flows through each periostial region, and periostial hemocytes – including their immune activity – preferentially aggregate around the ostia that experience the most hemolymph flow. Figure from Sigle and Hillyer (Sigle and Hillyer, 2016); reproduced with permission.

Graphical Abstract

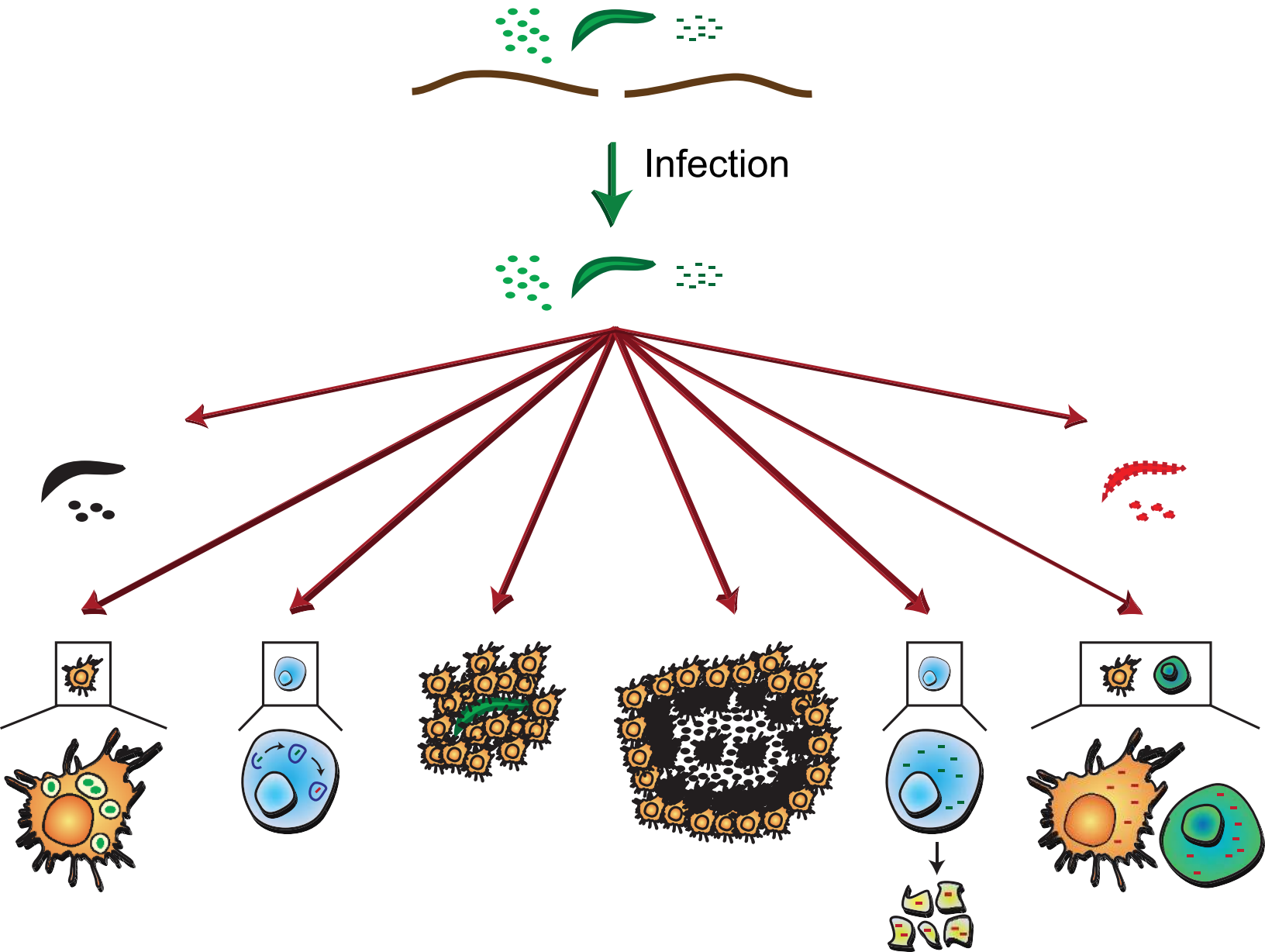
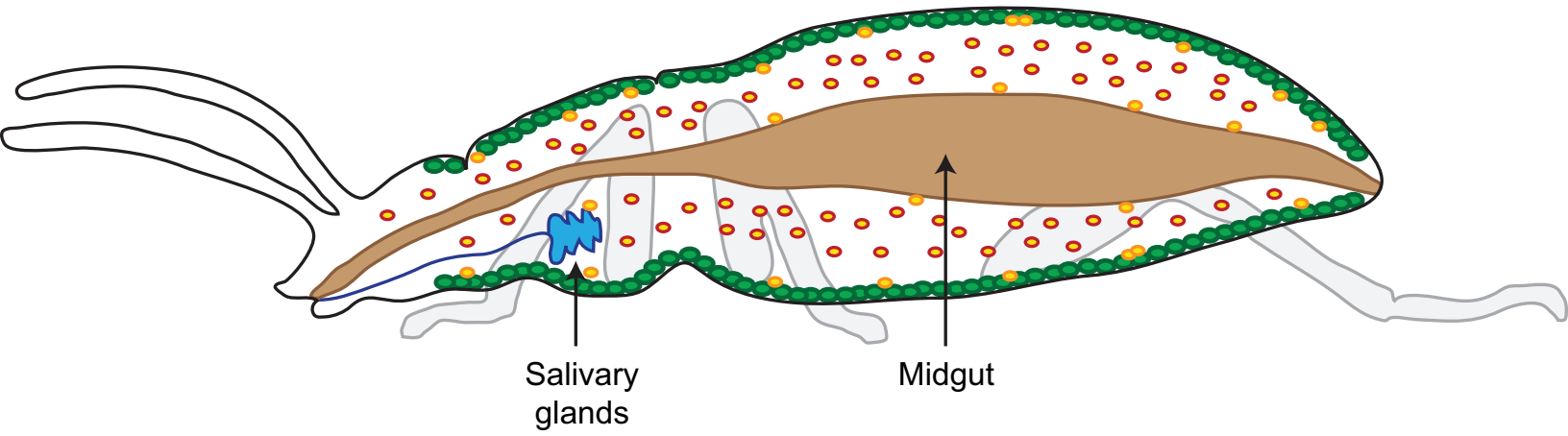


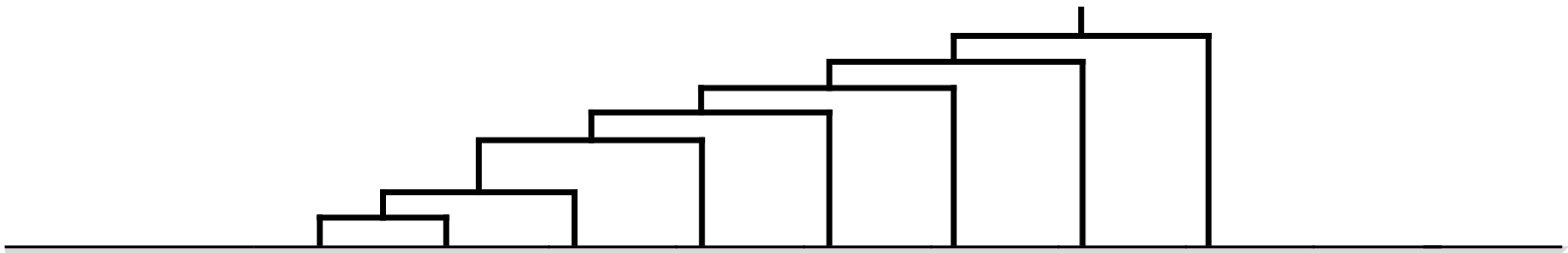
Figure 1



Legend: Circulating Hemocytes; Sessile Hemocytes; Fat body

Dorsal
Anterior — Posterior
Ventral

Figure 2



		Anopheles gambiae	Aedes aegypti	Drosophila melanogaster	Pediculus humanus	Manduca sexta	Tribolium castaneum	Apis mellifera	Acyrthosiphon pisum
PRR	PGRP	7	8	13	1	14	6	4	0
	FREP	61	37	14	2	4	7	2	
	Tep	13	8	6	3	3	4	4	
	βGRP/GNBP	7	7	3	0	5	3	2	2
	Galectin	10	12	6	3	4	3	2	2
	CTL	25	39	34	9	34	16	12	5
	DSCAM	1	1	1	1	1		1	1
Toll	Spätzle	6	9	6	3	8	7	2	10
	Toll	10	12	9	6	16	9	5	7
	MyD88	1	1	1	1	1	1	1	1
	Tube	1	1	1	1	1	1	1	1
	Pelle	1	1	1	1	1	1	1	1
	TRAF2	1	1	1	1	1	1	1	1
	Cactus	1	1	1	1	1	1	3	1
	Dif/Dorsal	1	2	2	1	5	2	2	2
IMD	Imd	1	1	1	0	1	1	1	0
	Dredd	1	1	1	1	1	1	1	0
	Tak1	1	1	1	1	1	1	1	1
	FADD	1	1	1	0	1	1	1	1
	IKK	1	1	2	1	2	3	2	2
	Relish	1	1	1	1	1	2	1	0
Effector	PPO	9	10	3	1	2	3	1	2
	Defensin	5	4	1	2	24	4	2	0
	Cecropin	4	10	5	0	15	3	0	0
	Lysozyme	8	7	13	1	6	4	3	3
	CLIP protease	54	68	45	6	54	30	18	
	Serpin	17	23	29	16	34	31	7	

Figure 3

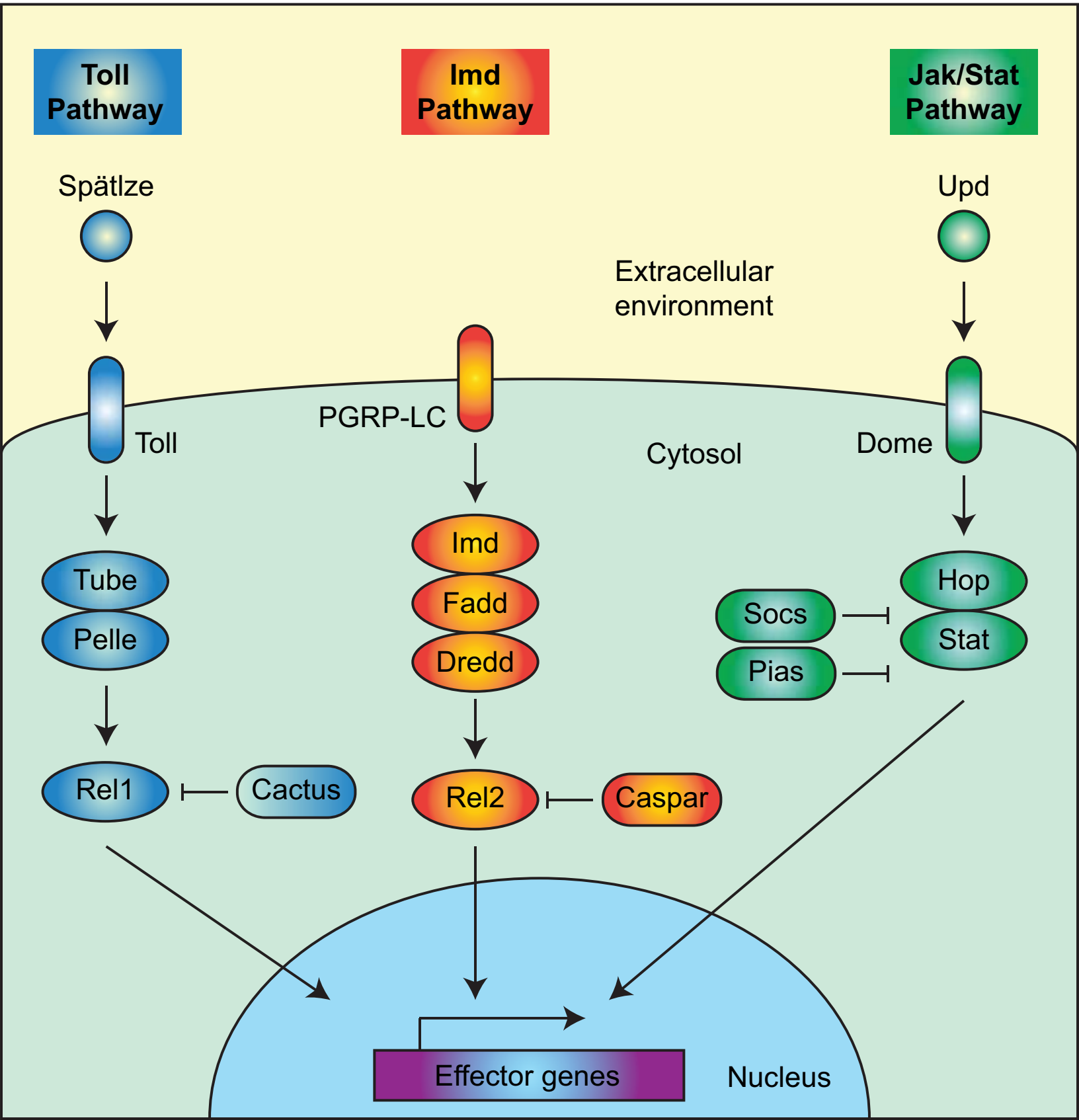

Toll Pathway
Imd Pathway
Jak/Stat Pathway
Spätlze
Upd
Extracellular environment
Toll
PGRP-LC
Dome
Cytosol
Tube
Pelle
Imd
Fadd
Dredd
Hop
Stat
Socs
Pias
Rel1
Cactus
Rel2
Caspar
Effector genes
Nucleus

Figure 4

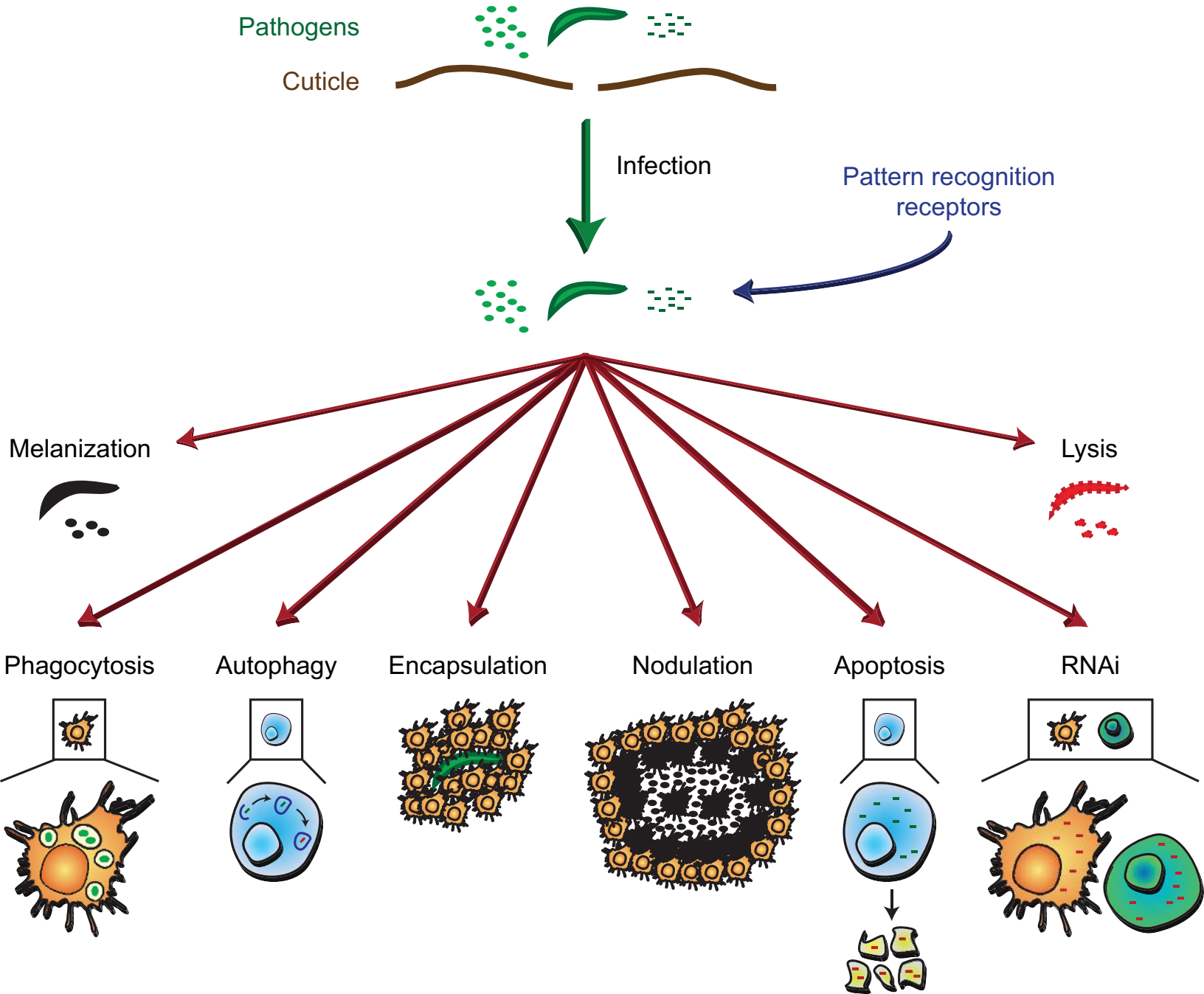

Pathogens
Cuticle
Infection
Pattern recognition receptors
Melanization
Lysis
Phagocytosis
Autophagy
Encapsulation
Nodulation
Apoptosis
RNAi

Figure 5

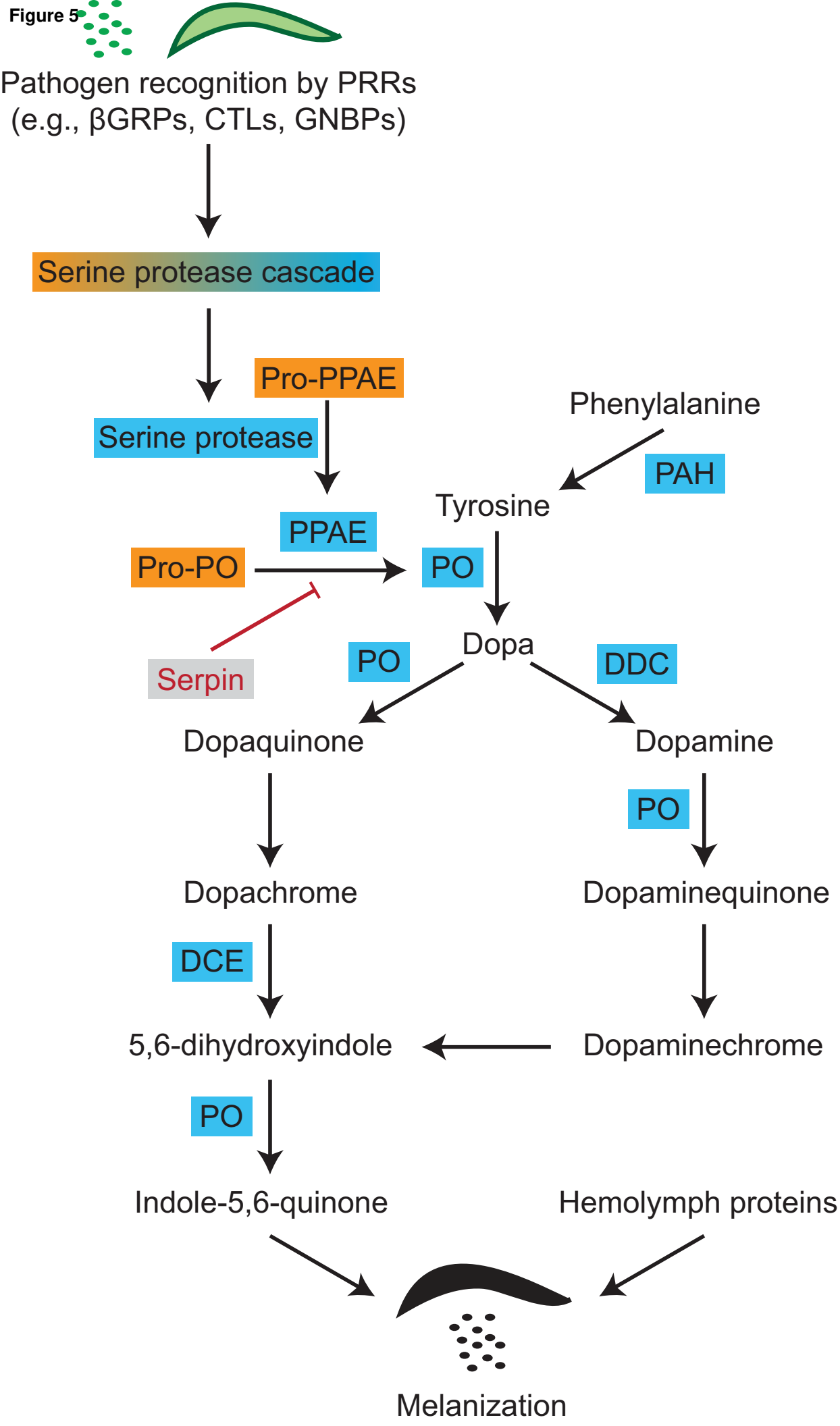

Pathogen recognition by PRRs
(e.g., βGRPs, CTLs, GNBPs)
Serine protease cascade
Pro-PPAE
Serine protease
PPAE
Pro-PO
PO
Serpin
Phenylalanine
PAH
Tyrosine
Dopa
PO
DDC
Dopaquinone
Dopamine
PO
Dopachrome
Dopaminequinone
DCE
5,6-dihydroxyindole
Dopaminechrome
PO
Indole-5,6-quinone
Hemolymph proteins
Melanization

Figure 6

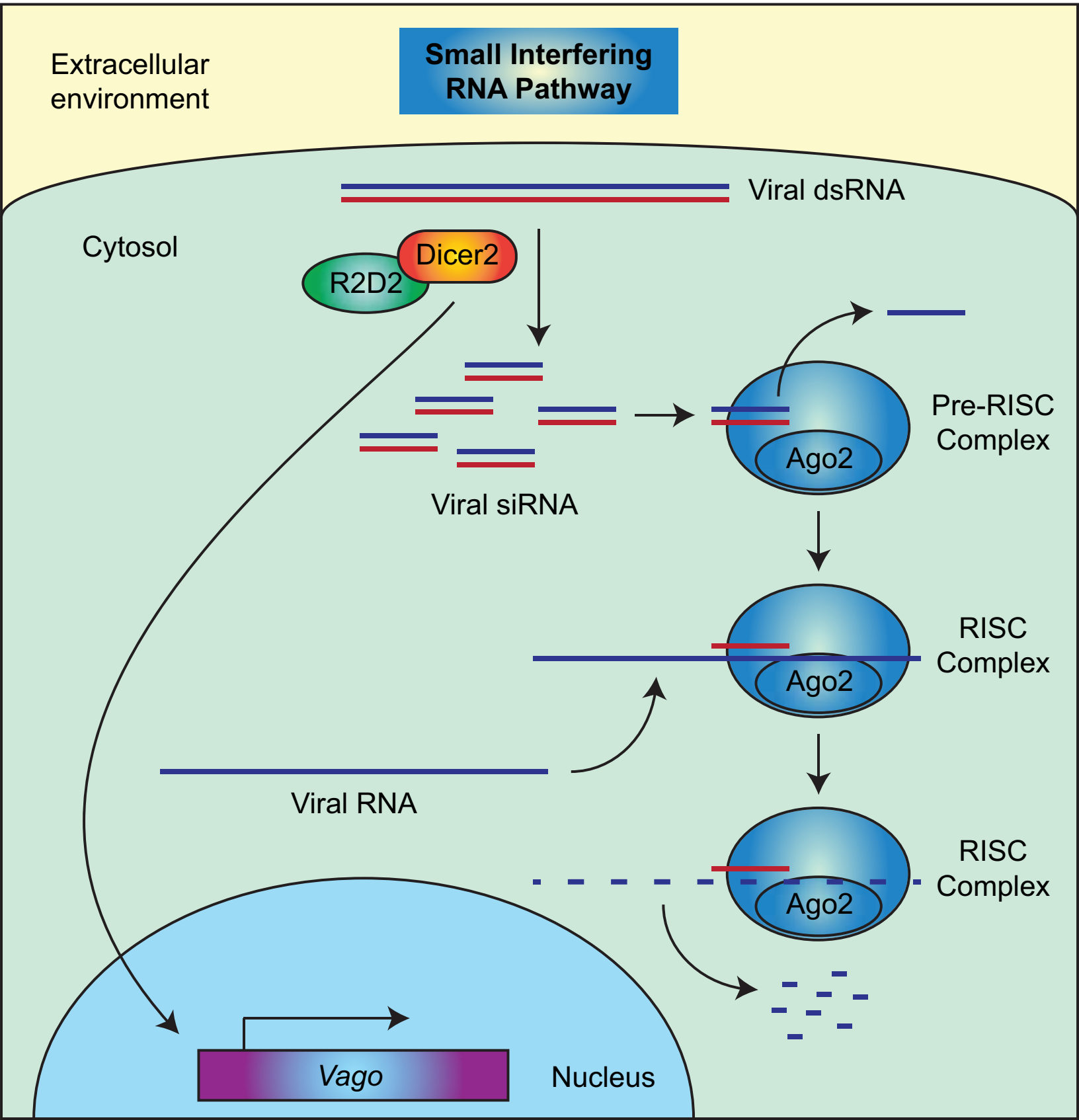
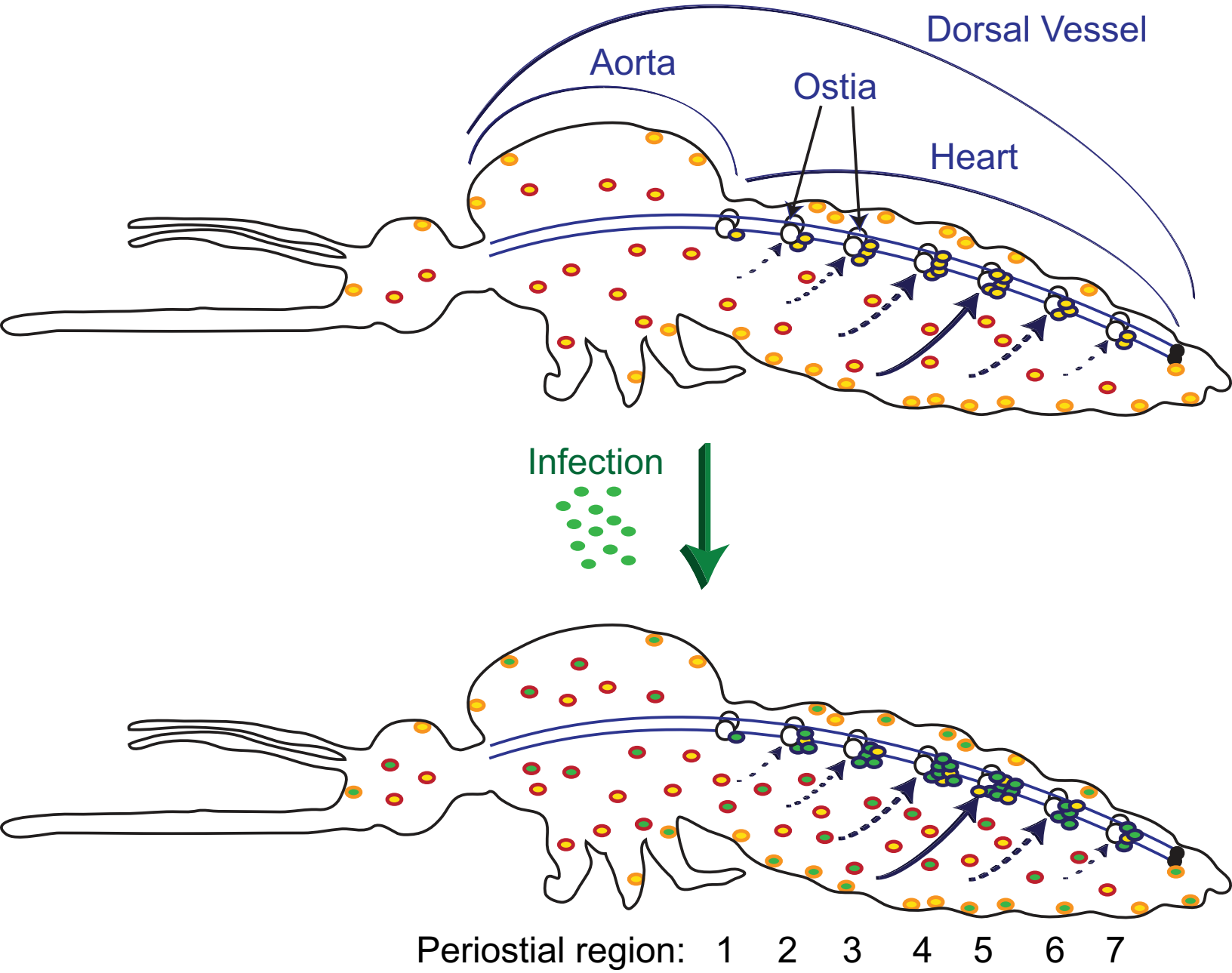

Extracellular environment
Small Interfering RNA Pathway
Viral dsRNA
Cytosol
Dicer2
R2D2
Viral siRNA
Pre-RISC Complex
Ago2
RISC Complex
Ago2
Viral RNA
RISC Complex
Ago2
Vago
Nucleus

Figure 7



Legend:

Circulating Hemocytes

Sessile Hemocytes
- Periostial
- Non-periostial

Hemocytes with phagocytosed pathogen

Intensity of hemolymph flowing into the ostia (from low to high)

Dorsal
Anterior — Posterior
Ventral