ISJ 7: 198-210, 2010 ISSN 1824-307X

REVIEW

# Cellular immunity and pathogen strategies in combative interactions involving *Drosophila* hosts and their endoparasitic wasps

**AJ Nappi**

*Department of Biology, Loyola University Chicago, Chicago, Illinois, USA*

*Accepted September 17, 2010*

## Abstract

Various cellular innate immune responses protect invertebrates from attack by eukaryotic pathogens. In insects, assessments of the factor(s) causing, or contributing to, pathogen mortality have long considered as toxic components certain molecules associated with enzyme-mediated melanogenesis. In *Drosophila* hosts, observations that have prompted additional or alternative considerations are those that document either the survival of certain endoparasitic wasps despite melanotic encapsulation, or the destruction of the parasite with no evidence of this type of host response. Investigations of the production of some reactive intermediates of oxygen and nitrogen during infection provide a basis for proposing that these molecules constitute important elements of the immune arsenal of *Drosophila*. Studies of the target specificity of virulence factors injected by female wasps during infection that suppress the host immune response will likely facilitate identification of the toxic host molecules, and contribute to a more detailed understanding of the cell-signaling pathways that regulate their synthesis.

**Key Words**: cellular innate immunity; reactive intermediates of oxygen; nitric oxide; melanization; encapsulation; *Drosophila*

## Introduction

Because of competition for some of the same limited metabolic resources, the outcome of the combative interaction between host and pathogen depends in large part on the effectiveness of apposing physiological, biochemical, and behavioral responses. In destroying pathogens, vertebrate hosts benefit from the collaborative interactions of two distinct, but not entirely separate, immune systems; adaptive and innate. The adaptive immune system produces an almost limitless repertoire of pathogen-specific responses, enabled in large part by considerable genetic plasticity that produces specific cell surface receptors, immunoglobulins, and cells possessing immune memory that rapidly initiate and enhance subsequent responses to the same antigen. Insects and other invertebrates rely exclusively on innate immune responses, which many authors regard as the first line of defense. These responses are considered to be dependent on constitutive (i.e., germ-line encoded) and dedicated cell membrane-bound pattern recognition receptors with limited responsiveness to invariant molecular motifs of certain pathogens (Pal and Wu, 2009). Some of the invertebrate innate immune effector responses elicited by prokaryotic infections include phagocytosis, hemolymph coagulation, the synthesis of pro-inflammatory cytokines, antimicrobial peptides, reactive intermediates of oxygen (ROI) and nitrogen (RNI), and stress-related proteins (Nappi and Vass, 2001; Beutler, 2004; Malagoli and Ottaviani, 2007; Malagoli *et al.*, 2007, 2010; Becker *et al.*, 2010). Eukaryotic pathogens succumb to an encapsulation response that is mediated in large part by macrophage-like blood cells (hemocytes). In insects and other arthropods, hemocyte-mediated encapsulations characteristically are accompanied by the synthesis of melanin, with intermediates such as quinones and semiquinones considered potentially toxic to invading pathogens (Nappi and Ottavani, 2000a; Nappi and Christensen, 2005; Poirie *et al.*, 2009) (Fig. 1). In both adaptive and innate systems, the binding of foreign elements to cell-surface receptors leads to the activation of signal transduction pathways, the transcription of immune genes, and the generation of reactive cells and various toxic molecules. As counter strategies, some pathogens produce virulence factors that actively suppress immune responses, while others

*Corresponding author*:
Anthony J Nappi
Department of Biology,
Loyola University Chicago,
Chicago, Illinois, USA
E-mail: anappi@luc.edu

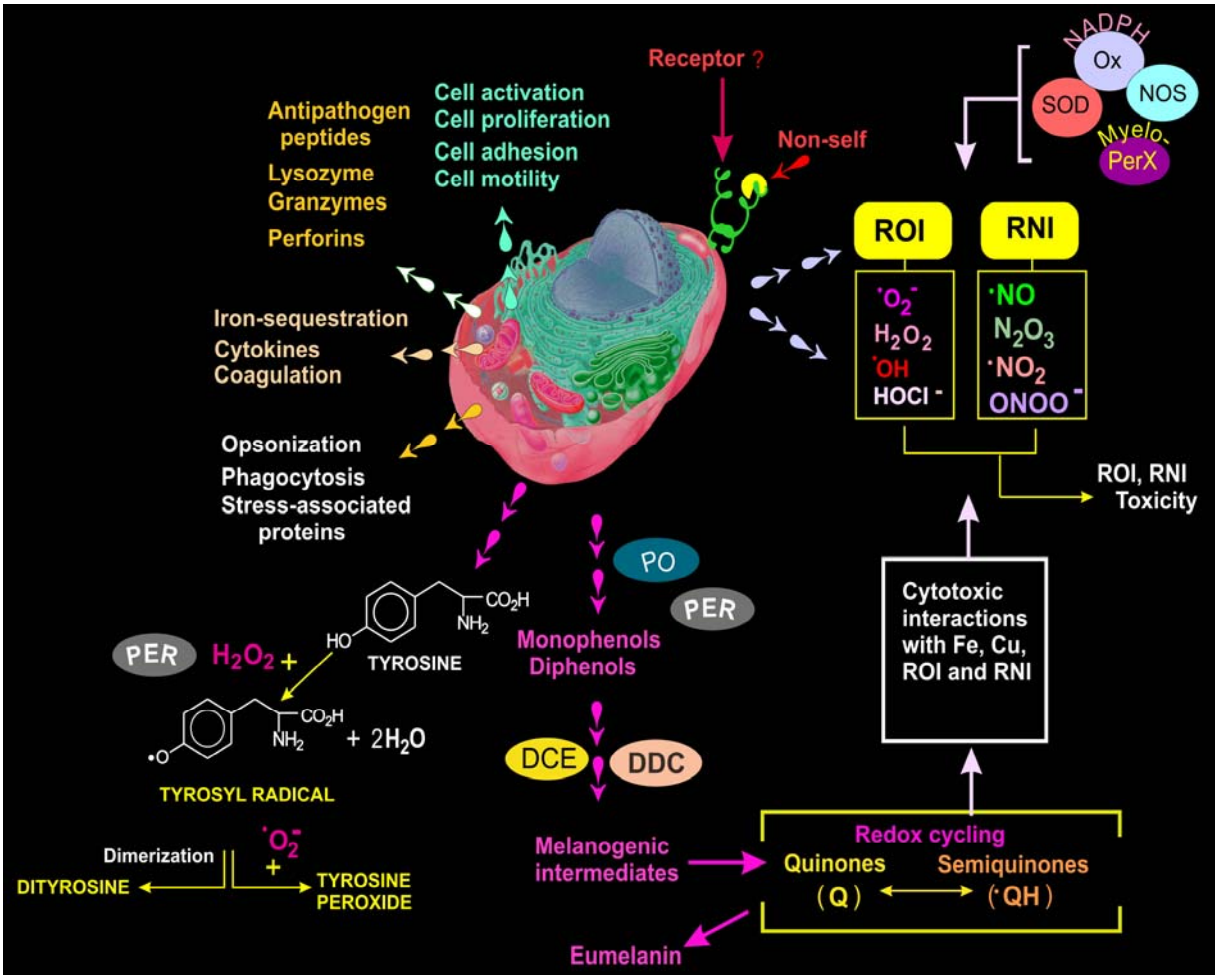


**Fig. 1** Overview of some toxic molecules manifested in the innate immune responses of various invertebrates. Non-self recognition may involve plasma membrane receptors independently functioning, or cooperatively engaging non-self binding molecules in the host's hemolymph. Melanotic encapsulation, which is a common manifestation of the defense reaction made by arthropods infected with eukaryotic pathogens, involves activation of one or more of the following enzymes; dopa decarboxylase (DDC), dopachrome conversion enzyme (DCE), phenylalanine hydroxylase (PAH), and phenoloxidase (PO). Enzymes capable of generating reactive intermediates of oxygen (ROI) and nitrogen (RNI) include myeloperoxidase (Myelo-Px), NADPH oxidase (NADPH Ox), nitric oxide synthase (NOS), and superoxide dismutase (SOD). Melanogenic intermediates such as quinones and semiquinones can react with ROI, RNI and the active centers of certain metaloenzymes to contribute additional toxic molecules.

passively avoid detection, either by molecular mimicry or by finding sanctuary within host tissues. The focus of this review concerns some unresolved aspects of the cellular innate immune responses of *Drosophila* against certain endoparasitic wasps, including the nature of the toxic molecules generated during infection, as well as the mechanisms employed by pathogens to circumvent these potentially damaging molecules.

### *Drosophila*-parasitic wasp interactions

The availability of well-defined resistant and susceptible species and strains of *Drosophila*, together with both virulent and avirulent lines of endoparasitic wasps (parasitoids), provide exceptionally good models for investigating not only the genetic and biochemical components of insect cellular innate immunity, but also the varied processes by which parasitoids deal with such reactions (Fig. 2). *Leptopilina boulardi* and *L. heterotoma* are two closely related wasp species that parasitize larvae of *Drosophila* with varying degrees of success (Vass and Nappi, 2000; Dubuffet *et al.*, 2007, 2008, 2009). Eggs of avirulent wasps characteristically provoke a rapid hemocyte-mediated melanotic encapsulation response when introduced into the hemocoel of resistant *Drosophila*,

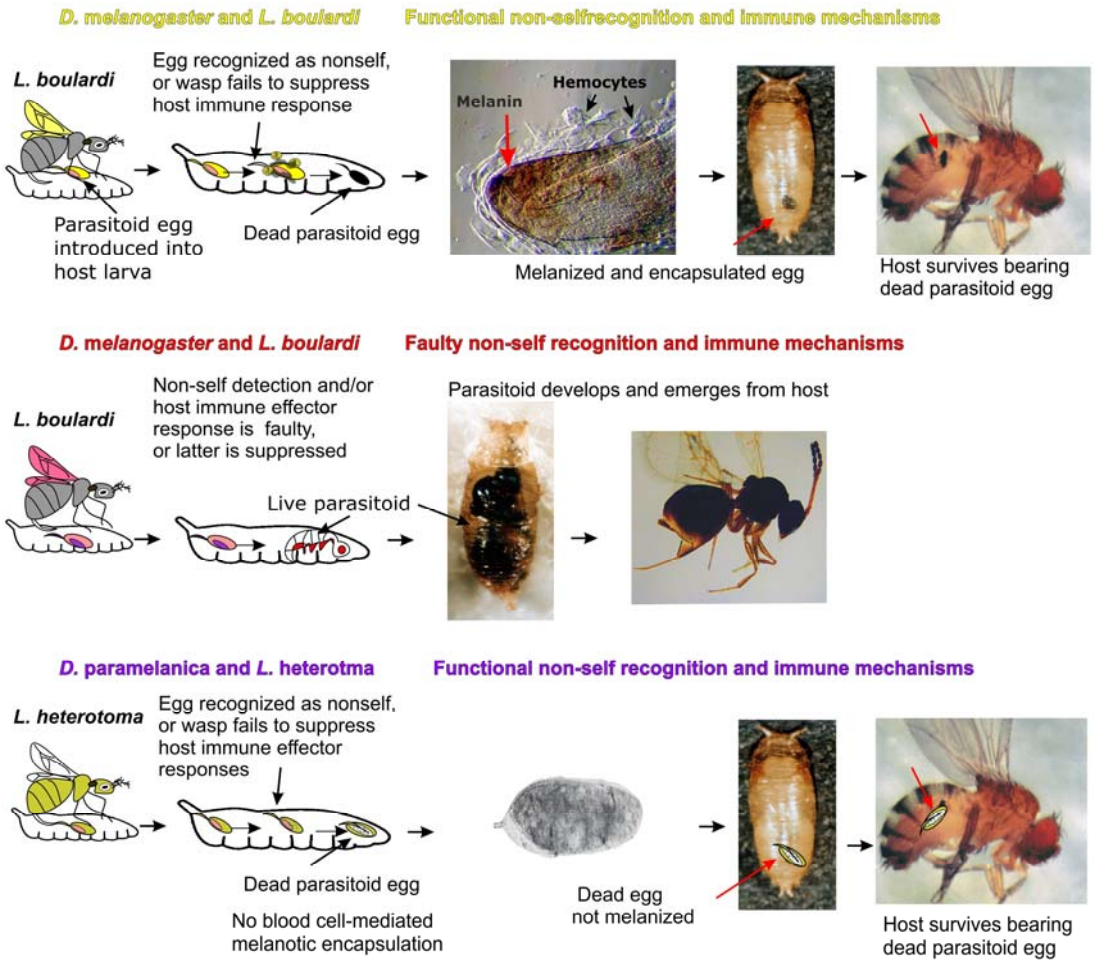


**Fig. 2** *Leptopilina boulardi* and *L. heterotoma* parasitize larvae of *Drosophila*. Genetically resistant hosts exhibit a typical hemocyte-mediated melanotic encapsulation response. Susceptible host have a faulty non-self recognition mechanism or fail to produce effective toxic responses. Immune suppressive factors (ISF) in the venom of virulent wasps block host cellular immune response. An atypical host response exhibited by *D. paramelanica* readily destroys *L. heterotoma*, but the response does not involve melanotic encapsulation.

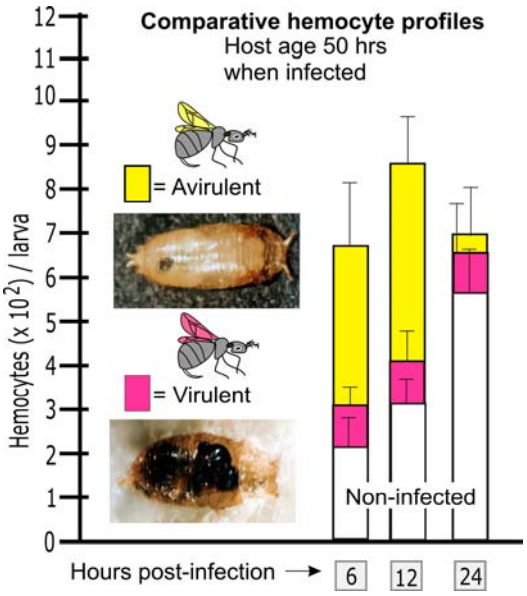


**Fig. 3** Comparative hemocyte profiles illustrating involvement of these cells in the host response, and the immune suppressive effects manifested by virulent wasps.

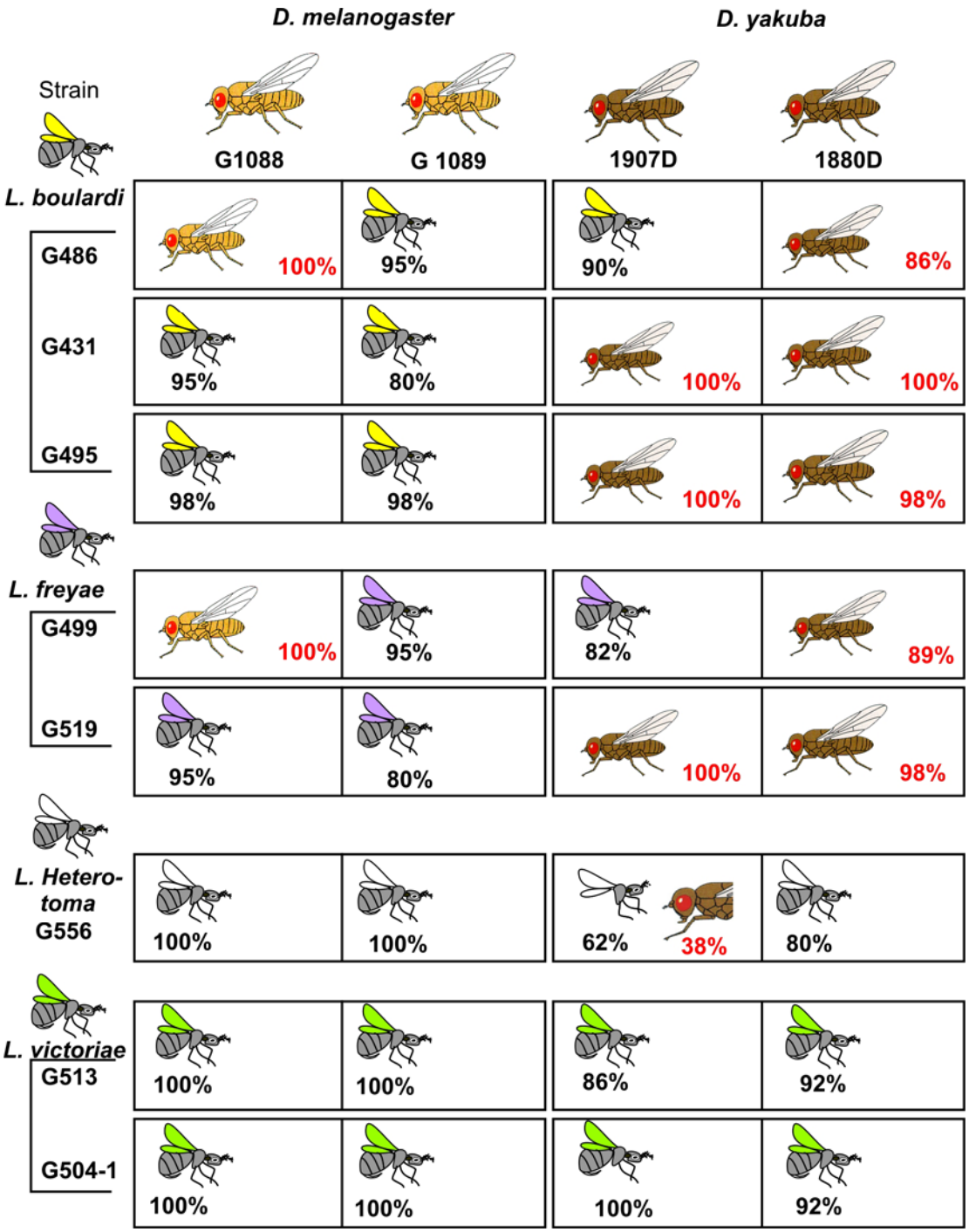


**Fig 4** Genetic complexity exhibited by the varying degrees of immune reactivity and parasite survival in different species and stains of *Drosophila* and *Leptopilina* (Courtesy S Dupas, M Poirié, Y Carton, Laboratoire Evolution, Génomes et Spéciation, CNRS, Gif-sur-Yvette cedex, France).

whereas the eggs of virulent wasps survive host defenses. Melanin typically appears at the site of infection, generally just before or at about the same time hemocytes are observed adhering to the surface of the dead parasitoid.

The *Drosophila* encapsulation response involves the collaborative activities of three types of hemocyte: plasmatocytes, lamellocytes and crystal cells. Comparative examinations of hemocyte profiles show significantly elevated numbers of hemocytes in resistant or immune competent hosts (Fig 3). During infection, plasmatocytes and lamellocytes show precocious increases in numbers as they participate in the formation of the cellular

components of the capsule, while crystal cells, which represent an important source of melanin precursors, decline in number (Nappi and Streams, 1969). Some of the immune-activated hemocytes participating in the encapsulation response appear to be recruited from those already in circulation at the time of infection, while others are mobilized from hematopoietic glands (i.e., lymph glands) (Lanot *et al.*, 2001; Sorrentino *et al.*, 2002; Meister and Lagueux, 2003; Meister, 2004; Carton *et al.*, 2005, 2008; Crozatier and Meister, 2007; Honti *et al.*, 2010) and/or a subepidermal population of normally sessile cells ( Krzemien *et al.*, 2007; Markus *et al.*, 2009).

The genetic complexity of the *Drosophila*-wasp associations is illustrated by the varied outcomes of the combative interactions made by different species and strains of both host are parasite (Fig 4). The gene for host resistance, which is associated with the second chromosome, is specific for each species of wasp. Reciprocal chromosome exchange between resistant and susceptible host strains virtually completely reverses immune competence in each recipient. Immune competence is later restored following reciprocal return of the chromosome (Fig. 5). During oviposition, wasp venom also is introduced into the host hemocel. Virus-like particles (i.e., immune suppressive factors, ISF) in the venom protect the wasp egg from encapsulation, either by lysing host hemocytes, or interfering with essential transcriptional responses so as to abrogate or diminish host responses. Unlike ISF of virulent wasps, those present in the venom of avirulent species and strains exhibit little or no immune suppressive effect (Labrosse *et al.*, 2003; Kohler *et al.*, 2007). In experiments involving double infections, first by avirulent wasps followed by a virulent strain of *L. boulard*, ISF from virulent parasitoids were found capable of also protecting avirulent wasps, provided the interval between infections is 12 hrs or less (Fig. 6). Also, the ability of *Leptopilina* spp. to immune suppress depends on her egg-laying experience. During the latter part of the ovipositional period of *L. boulardi*, eggs introduced into *D. melanogaster* larvae are more susceptible to melanotic encapsulation than are eggs laid earlier (Fig. 7). The decrease in immune suppression presumably correlates with a corresponding depletion of ISF (Vass and Nappi, 1998). Wasps with prior ovipositional experience not only lack or have a diminished capacity to immune suppress, but they also infect far fewer hosts than females with no prior ovipositional experience. If such ovipositional restraint retains eggs that would otherwise be encapsulated, selection pressure in host populations for evolving specific immune reactivity would be reduced.

**Melanization and associated toxic molecules**

In immune reactive insects, melanin appears in many cases to occur concurrently with early capsule formation, an observation that has long been viewed as evidence that the proteinase cascade leading to activation of one or more enzymes involved in

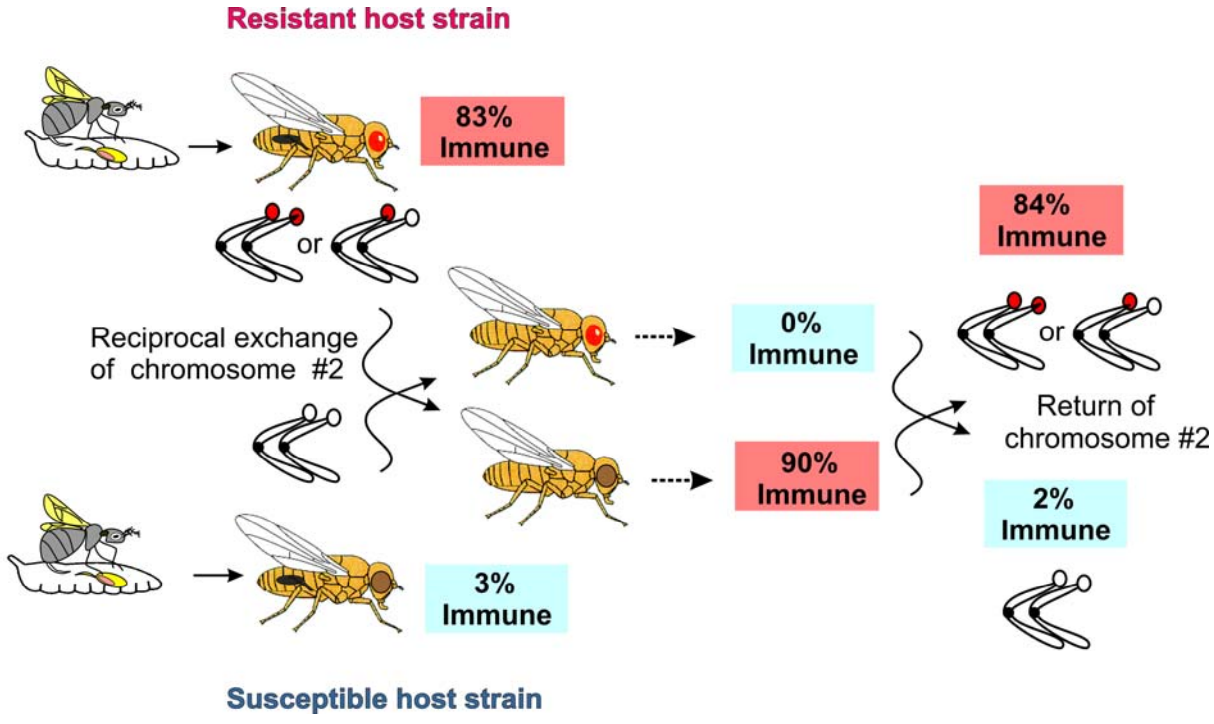


**Fig. 5** Reciprocal exchange of resistant and non-resistant gene reverses the immune capacity of the recipient, which can be restored in subsequent exchanges (Carton and Nappi, 1997).

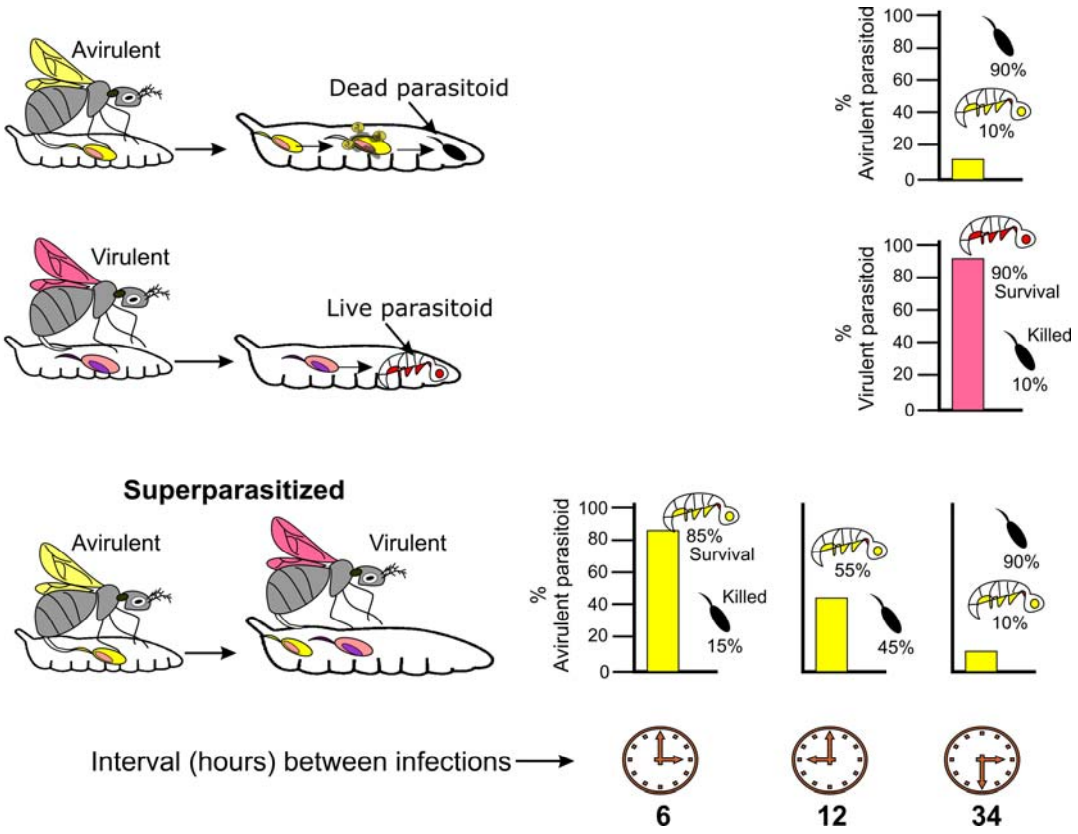


**Fig. 6** In superparasitized hosts, immune suppressive factors from virulent parasitoids also protect avirulent wasps provided the interval between infections is 12 hrs or less.

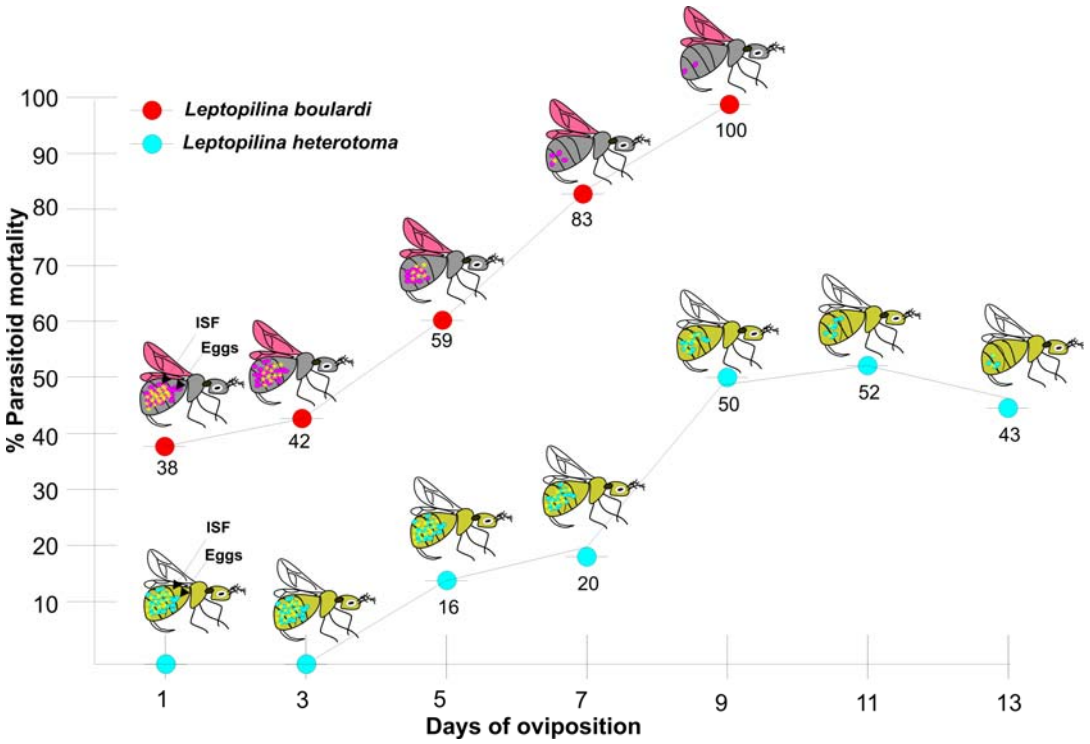


**Fig. 7** The effects of prolonged oviposition on the diminishing capacity of ISF from *L. boulardi* (Vass and Nappi, 1998) and *L. heterotoma* (Streams, 1968) to suppress the immune response of D. melanogaster. Wasp eggs introduced into hosts by females during the latter part of their ovipositional period are more susceptible to destruction than eggs laid earlier. The increase in parasitoid mortality is believed to result from a decline in ISF.

catechol metabolism and pigment synthesis forms toxic molecules that target and destroy foreign organisms (Nappi and Vass, 2001; Sugumaran, 2002; Nappi and Christensen, 2005; Sideri *et al.*, 2008; An *et al.*, 2009; Bidla *et al.*, 2009; Nappi *et al.*, 2009). Support for this proposal derives in large part from studies showing diminished immune responsiveness when components of the enzyme-regulated melanin pathway are experimentally inhibited. Reservations about such an interpretation concern the questionable specificity and excessive levels of agents injected into the host to inhibit and thereby demonstrate involvement of melanin intermediates in immune reactions.

Frequently overlooked in studies assessing the role of melanin in insect immunity is the initial enzyme-mediated reaction involving the hydroxylation of L-phenylalanine to L-tyrosine, a reaction catalyzed by phenylalanine hydroxylase (PAH). Ensuing oxidations of L-tyrosine and/or L-DOPA, which can be catalyzed either by phenoloxidase (PO; Terland *et al.*, 2006), or peroxidase (PER; Kasraee, 2002; Okun, 1996), generate dopaquinoine, a reactive intermediate essential for the formation of eumelanin, a brownish-black pigment, and, in the presence of sufficient levels of thio compounds, pheomelanin, a reddish-brown pigment. Precursors of both pigments possess cytoprotective and cytotoxic properties, given their capacity to scavenge potentially toxic organic and inorganic cations and free-radical species, engage in metal-binding and sequestering responses, initiate redox reactions, cross-link proteins and mediate detoxification processes. To date, only eumelanin has been identified as the pigment type formed in the encapsulation response of *D. melanogaster* (Nappi *et al.*, 1992). Following the formation of dopaquinone, a series of enzyme-regulated and/or spontaneous oxidoreductions occur yielding dopachrome and

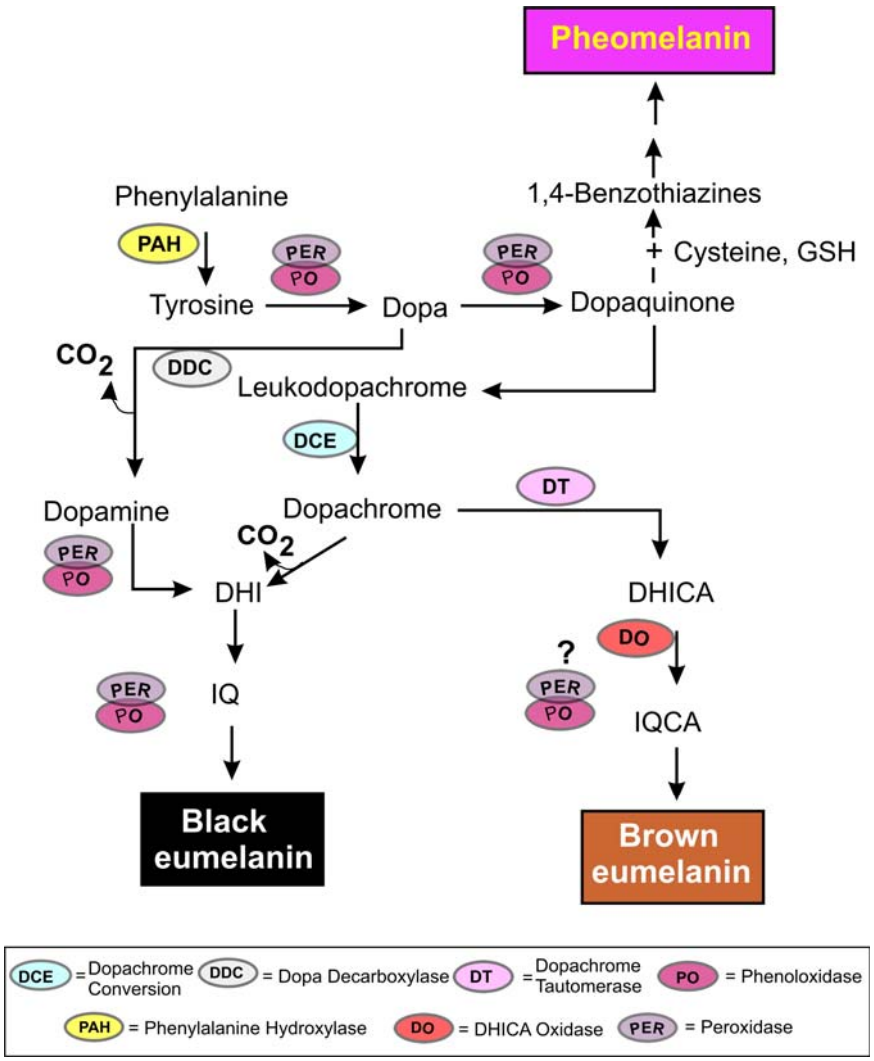


**Fig. 8** Overview of the principal pathways involved in the formation of eumelanin and pheomelanin and some their reactive intermediates, including quinones and semiquinones. Redox cycling and univalent transfers, which represent important mechanisms for generating cytotoxic molecules, also occur with DHI-derived indolequinone (IQ) and indolesemiquine (not illustrated). Insects apparently are incapable of forming DHICA.

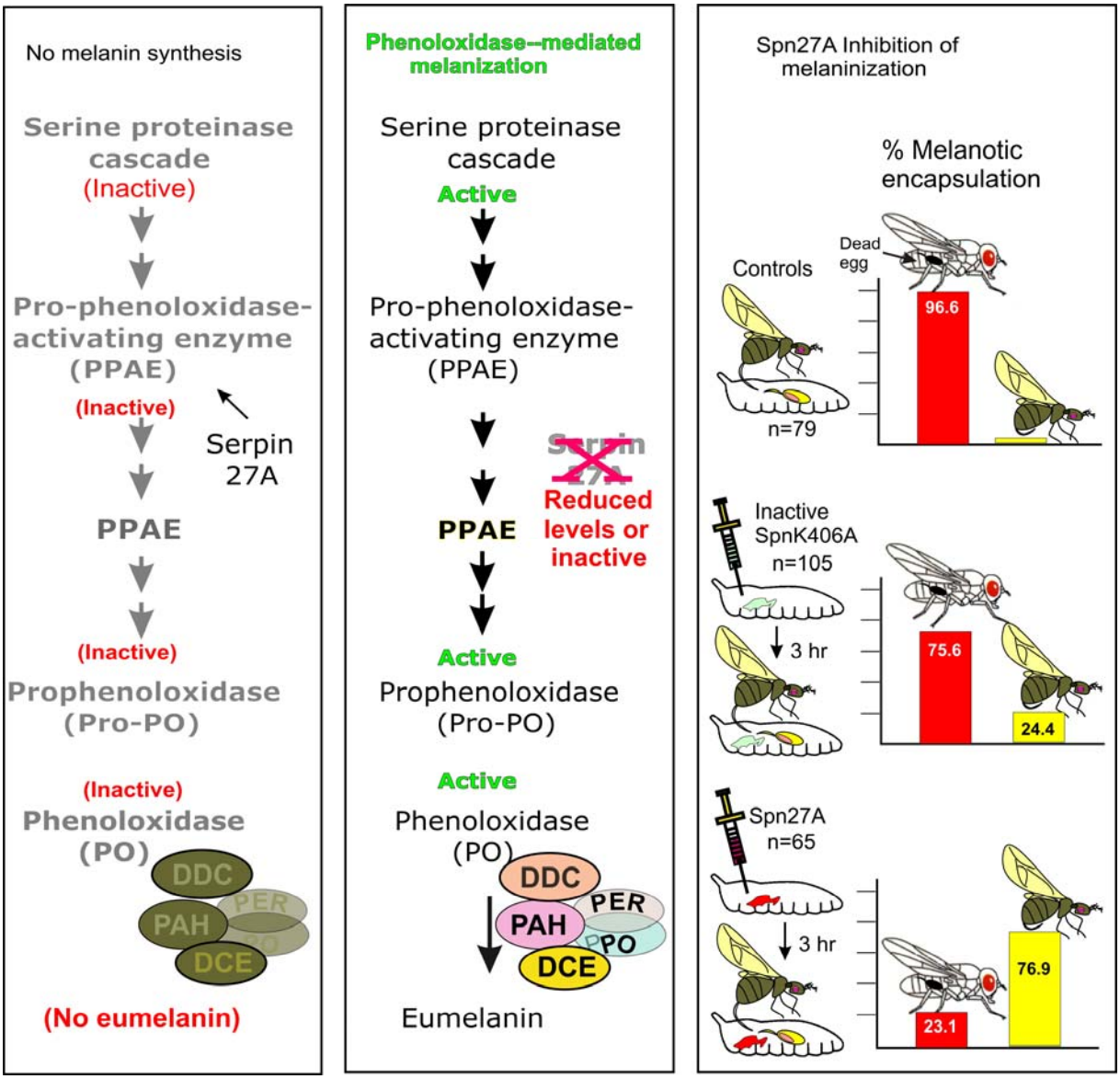


**Fig. 9** Effects of injection of Spn27A on the percentage of *D. melanogaster* hosts exhibiting a successful melanotic encapsulation of *L. boulardi*. Enzymes involved in melanin synthesis include dopa decarboxylase (DDC), dopachrome conversion enzyme (DCE), phenylalanine hydroxylase (PAH), peroxidase (PER), and phenoloxidase (PO).

additionally potentially cytotoxic eumelanin intermediates, including 5,6- dihydroxyindole (DHI), 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and their respective indole quinones (IQ, and IQCA) (Fig. 8). The dopa decarboxylase (DDC)-mediated pathway to DHI may be a principal route for production of pigment precursors in infected *Drosophila*, as the melanotic encapsulation response against eggs of *L. boulardi* is severely compromised in temperature-sensitive DDC-deficient mutants (Nappi *et al.*, 1992) Accordingly, it was recently shown that silencing the genes for DDC and Dopachrome conversion enzyme (DCE) significantly reduced melanization of foreign objects implanted in the mosquito *Anopheles gambiae* (Paskewitz and Andreev, 2008). In the medfly *Ceratitis capitata*, DDC-dependent pathways have been shown to regulate such immune functions as phagocytosis, nodulation and melanization by hemocytes (Sideri *et al.*, 2008).

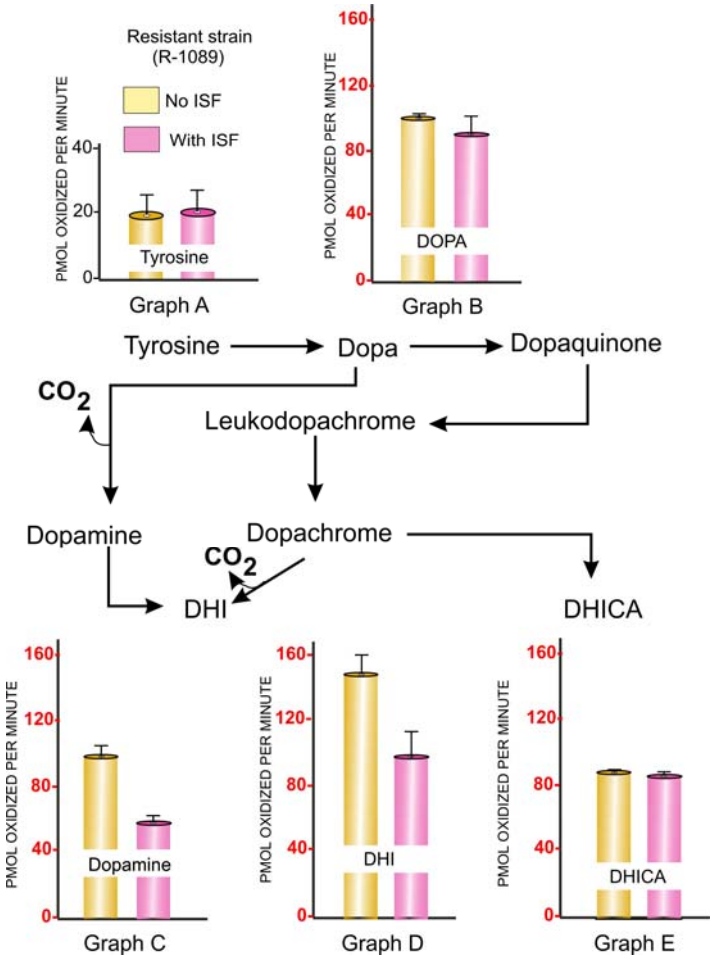


**Fig. 10** *L. boulardi* ISF diminishes the in vitro oxidations of two diphenol eumelanin precursors, dopamine and DHI. Tyrosine and the diphenols dopa and DHICA are not affected by ISF.

**Parasite suppression of host melanization**

Melanization in insects is controlled by a cascade of serine proteases that ultimately activates prophenoloxidase (PPO) and leads to activated phenoloxidase (PO) and pigment formation (Tang *et al.*, 2006, 2008; Scherfer *et al.*, 2008; Tang, 2009). In *Drosophila*, melanization induced by activated PO is a tightly regulated reaction sequence (Aggarwal and Silverman, 2008; Kan *et al.*, 2008) involving at least three PPO isoforms, as well as serine protease inhibitors. Two isoforms are expressed in crystal cells, the third is associated with lamellocytes (Kan *et al.*, 2008). An important regulating element in the cascade of proteolytic cleavages that converts PPO to PO is the serine protease inhibitor Serpin 27A (Spn27A), which inhibits the terminal protease prophenoloxidase-activating enzyme (PPAE) (De Gregorio *et al.*, 2002; Nappi *et al.*, 2005) (Fig. 9). Because of the critical role played by Spn27A as a negative regulator of melanogenesis, the molecule and the signaling elements mediating its activity likely represent critically important factors in determining immune reactivity against *Leptopilina.* This was established by experiments involving the introduction of Spn27A into immune competent *D. melanogaster* larvae just before infection by *L. boulardi*. In these hosts, the ability to form melanotic capsules was significantly reduced. The specificity of action of Spn27A establishes some of the components of the PO-mediated pathway in the insect's defense response against *L. boulardi* (Fig. 9). More recent comparative investigations using ISF from virulent and avirulent wasps provide additional evidence that ISF inhibits melanization in *D. yakuba* by affecting one or more steps in the cascade leading to PO activation, but not PO activity by itself (Dubuffet *et al.*, 2009). Other experiments designed to determine if venom factors from *L. boulardi* targeted the principal oxidation pathways leading to synthesis of eumelanin, sensitive electrochemical detection methods showed that venom factors diminished the oxidations of the two diphenol eumelanin precursors, dopamine and DHI, while oxidations of the monophenol tyrosine, and two other related

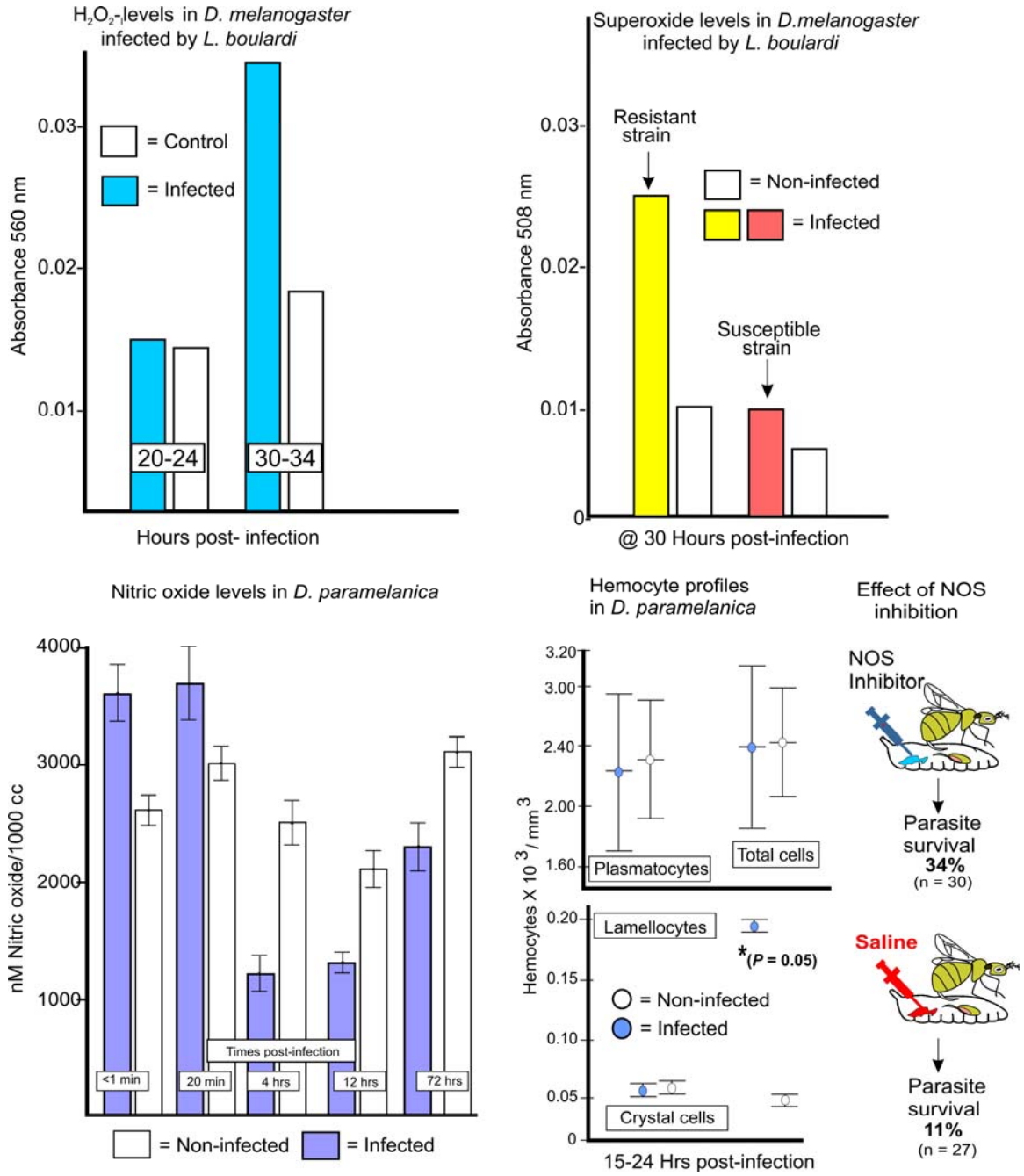


**Fig. 11** Comparative analyses of hemocytes and nitric oxide levels in infected and non-infected larvae of *D. paramelanica*, and the effects of introducing a NOS inhibitor (NG-monomethyl-L-arginine on the fate of *L. heterotoma* (Nappi *et al.*, 2009).

diphenols, dopa and DHICA, were not significantly inhibited (Kohler *et al.*, 2007) (Fig. 10). Collectively, these related studies suggest that, in addition to targeting specific hemocytes, ISF from *Leptopilina spp.* specifically suppresses the oxidation pathways synthesizing certain pigment precursors, especially the decarboxylated pigment precursors derived from DHI.

**Reactions lacking evidence of the involvement of melanization**

It is generally believed that at least some of the melanogenic enzymes and intermediate pigment products play a role in the defense reactions of insects (Cerenius and Soderhall, 2004; Christensen *et al.*, 2005; Nappi *et al.*, 2009), although this issue

still remains to be clarified (Schnitger *et al.*, 2007). Reports that would appear to discredit or at least down-play the role of melanogenesis in insect immunity and prompt additional or alternative proposals are those that document parasite mortality prior to, or in the absence of, melanotic encapsulation (Tardieu and Rabasse, 1988; Henter and Via, 1995; Vernick *et al.*, 1995), and those that clearly show successful parasite development despite extensive melanotic responses (Shin *et al.*, 2003). In the *D. paramelanica–L. heterotoma* association, eggs of the endoparasite succumb with no evidence of blood cell-mediated encapsulation and no pigment reaction (Fig. 2) (Nappi and Streams, 1970; Carton *et al.*, 2008). If melanization is not a universal feature of insect cellular immunity, the destruction of some pathogen must involve host molecules other than those associated with melanogenesis, and one would expect successful parasites to have evolved specific inhibition strategies that suppress or detoxify such potentially biochemically hostile reactions. Although identity of the cytotoxic molecules remains unknown, attention has focused on ROI and RNI, given that elevated levels of some of these molecules have been found in immune responsive hosts (Luckhart and Li, 2001; Foley and O'Farrell, 2003; Novas *et al.*, 2004; Whitten *et al.*, 2007; Molina-Cruz *et al.*, 2008), including those in which hemocyte-mediated melanotic encapsulation reactions are typically formed (Nappi and Vass, 1998, 2001a, b; Nappi *et al.*, 1995, 2000b), and in wasp-infected *D. paramelanica* where parasites are destroyed but no melanotic capsules are produced (Carton *et al.*, 1992). Potentially damaging ROI and RNI can form during normal metabolism as a result of successive univalent reductions of molecular oxygen. Initially, superoxide anion is produced, with subsequent electron transfers ultimately generating highly reactive and potentially cytotoxic molecules, including the hydroxyl radical ($^{\cdot}OH^{-}$), peroxynitrite ($ONOO^{-}$) and hypochlorous acid ($HOCl^{-}$). Interestingly, melanogenic intermediates may serve to promote or augment cytotoxic activity by reacting with certain transition metal ions, ROI and RNI (Fig. 1). The univalent oxidations of redox active o-diphenols ($QH_2$) such as L-DOPA and dopamine by PO and/or PER can form semiquinones ($^{\cdot}QH^{-}$) and quinones (Q), which then can interact with ROI and RNI. Reactions involving PER and tyrosine can lead to the production of potentially injurious molecules, such as the tyrosyl radical, dityrosine, and tyrosine peroxide (Fig. 1), without producing melanin. An important issue to consider is that the production of toxic molecules in response to infection must by a tightly regulated and localized reaction in order to avoid damage to nonspecific sites within the host's open circulatory system. The binding of the copper-containing PO or the heme-containing PER to pathogens would expose the metal active sites of these enzymes, a response that would facilitate their interaction with ROI and RNI and form $^{\cdot}OH^{-}$ and other reactive molecules, and also serve to localize metal ion-mediated cytotoxicity. Because of its intrinsic coordination properties, copper can induce a more site-specific $^{\cdot}OH^{-}$ cytotoxicity to bound ligands than can iron (Berthon, 1993).

Recent studies (Carton *et al.*, 2009) support earlier reports that document the involvement of $^{\cdot}NO$ in mediating various toxic responses in *Drosophila* and other invertebrates. Nitric oxide is a well known signaling molecule associated with certain innate immune pathways. The radical serves an equally important role as a toxic effector molecule in eliminating pathogens (Nappi and Ottavani, 2000a; Nappi *et al.*, 2000b; Luckhart and Li, 2001; Dimopoulos, 2003; Han *et al.*, 2009). In *D. paramelanica* where elevated levels of $^{\cdot}NO$ are produced almost immediately following infection *by L. heterotoma*, immune capacity is diminished when a specific nitric oxide synthase (NOS) inhibitor is introduced in larvae prior to infection **(**Fig. 11). These observations suggest $^{\cdot}NO$ is involved in the host immune response, either a critical signaling molecule in recruiting hemocytes to sites of infection, or as a component of the insect's arsenal of defense, given the capacity of the radical to readily react with various ROI and RNI.

**Conclusions**

The associations between host and pathogen represent coevolved adaptations of great complexity. Insects typically manifest a unique defense response against metazoan parasites that involves hemocyte-mediated melanotic encapsulation. The use of melanin for protection from foreign insult involves a multifaceted biochemistry and an equally complex genetic regulation. An equally fascinating component of insect host-parasitoid combative relationships is the ability of some wasp species and strains to develop unmolested within otherwise immune competent hosts. Either such parasitoids evolve with passive immune evasion strategies that effectively preclude host detection, or with the capacity to actively combat and render ineffective host defenses. Despite numerous descriptive accounts of non-self responses and associated cell-signaling molecules that summon blood cells to sites of infection, much remains to be learned about the identity of the killing molecules employed by insect hosts, knowledge of which would enhance current efforts to better define immune cell-signaling pathways, and most likely contribute to a more comprehensive understanding than presently exists of receptor-mediated processes involved in detection of non-self. The mechanism employed by pathogens to suppress cellular innate immunity in insects and other invertebrate hosts may likewise contribute to our understanding of immune signaling and non-self discrimination. Studies that merely correlate host immune competence or parasite virulence with the presence or absence of melanin fail to provide substantive information about the actual cytotoxic mechanism(s) involved and how the pathogen circumvents such hostile chemistry. Future proteomic and transcriptomic studies of parasitoid ISF will likely facilitate identification of the cytotoxic molecules, the cell-signaling pathways that regulate their synthesis, and their mode of target-specific engagement with foreign organisms.

**References**


Aggarwal K, Silverman N. Positive and negative regulation of the *Drosophila* immune response. BMB Rep. 41: 267-277, 2008.

An C, Ishibashi J, Ragan EJ, Jiang H, Kanost MR. Functions of *Manduca sexta* hemolymph proteinases HP6 and HP8 in two innate immune pathways. J. Biol. Chem. 284: 19716-19726, 2009.

Becker T, Loch G, Beyer M, Zinke I, Aschenbrenner AC, Carrera P, *et al*. FOXO-dependent regulation of innate immune homeostasis. Nature 463: 369-373, 2010.

Berthon G. Is copper pro- or anti-inflammatory? A reconciling view and a novel approach for the use of copper in the control of inflammation. Agents Actions 39: 210-217, 1993.

Beutler B. Innate immunity: an overview. Mol. Immunol. 40: 845-859, 2004.

Bidla G, Hauling T, Dushay MS, Theopold U. Activation of insect phenoloxidase after injury: endogenous versus foreign elicitors. J. Innate Immunol. 1: 301-308, 2009.

Carton Y, Frey F, Nappi AJ. Inheritance of cellular immune resistance in *Drosophila melanogaster*. Heredity 69: 393-399, 1992.

Carton Y, Frey F, Nappi AJ. Parasite-induced changes in nitric oxide levels in *Drosophila paramelanica*. J. Parasitol. 95: 1134-1141, 2009.

Carton Y, Nappi AJ. *Drosophila* cellular immunity against parasitoids. Parasitol. Today 13: 218-227, 1997.

Carton Y, Nappi AJ, Poirie M. Genetics of anti-parasite resistance in invertebrates. Dev. Comp. Immunol. 29: 9-32, 2005.

Carton Y, Poirie M, Nappi AJ. Insect resistance to parasitoids. Insect Sci. 15: 67-87, 2008.

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

Christensen BM, Li J, Chen C-C, Nappi AJ. Melanization immune responses in mosquito vectors. Trends Parasitol. 21: 192-199, 2005.

Crozatier M, Meister M. *Drosophila* haematopoiesis. Cell. Microbiol. 9: 1117-1126, 2007.

De Gregorio E, Han SJ, Lee WJ, Baek MJ, Osaki T, Kawabata SI, *et al*. An immune-responsive serpin regulates the melanization cascade in *Drosophila*. Dev. Cell 3: 581-592, 2002.

Dimopoulos G. Insect immunity and its implication in mosquito-malaria interactions. Cell. Microbiol. 5: 3-14, 2003.

Dubuffet A, Colinet D, Anselme C, Dupas S, Carton Y, Poirie M. Variation of *Leptopilina boulardi* success in *Drosophila* hosts: what is inside the black box? Adv. Parasitol. 70: 147-188, 2009.

Dubuffet A, Doury G, Labrousse C, Drezen JM, Carton Y, Poirie M. Variation of success of *Leptopilina boulardi* in *Drosophila yakuba*: the mechanisms explored. Dev. Comp. Immunol. 32: 597-602, 2008.

Dubuffet A, Dupas S, Frey F, Drezen JM, Poirie M, Carton Y. Genetic interactions between the parasitoid wasp *Leptopilina boulardi* and its *Drosophila* hosts. Heredity 98: 21-27, 2007.

Foley E, O'Farrell PH. Nitric oxide contributes to induction of innate immune responses to gram-negative bacteria in *Drosophila*. Genes Dev. 17: 115-125, 2003.

Han Y, Niu M, An L, Li W. Upregulation of proinflammatory cytokines and NO production in BV-activated avian macrophage-like cell line (HD11) requires MAPK and NF-kappaB pathways. Int. Immunopharmacol. 9: 817-823, 2009.

Henter H, Via S. The potential for coevolution in a host-parsitoid system. I. Genetic variation within an aphid population in susceptibility to a parasitic wasp. Evolution 49: 427-438, 1995.

Honti V, Csordás G, Márkus R, Kurucz E, Jankovics F, Andó I. Cell lineage tracing reveals the plasticity of the hemocyte lineages and of the hematopoietic compartments in *Drosophila melanogaster*. Mol. Immunol. 47: 1997-2004, 2010.

Kan H, Kim CH, Kwon HM, Park JW, Roh KB, Lee H, *et al*. Molecular control of phenoloxidase-induced melanin synthesis in an insect. J. Biol. Chem. 283: 25316-25323, 2008.

Kohler LJ, Carton Y, Mastore M, Nappi AJ. Parasite suppression of the oxidations of eumelanin precursors in *Drosophila melanogaster*. Archiv. Insect Biochem. Physiol. 66: 64-75, 2007.

Krzemien J, Dubois L, Makki R, Meister M, Vincent A, Crozatier M. Control of blood cell homeostasis in *Drosophila* larvae by the posterior signalling centre. Nature 446: 325-328, 2007.

Labrosse C, Carton Y, Dubuffet A, Drezen JM, Poirie M. Active suppression of *D. melanogaster* immune response by long gland products of the parasitic wasp *Leptopilina boulardi*. J. Insect Physiol. 49: 513-522, 2003.

Lanot R, Zachary D, Holder F, Meister M. Postembryonic hematopoiesis in *Drosophila*. Dev. Biol. 230: 243-257, 2001.

Luckhart S, Li K. Transcriptional complexity of the *Anopheles stephensi* nitric oxide synthase gene. Insect Biochem. Mol. Biol. 31: 249-256, 2001.

Malagoli D, Conklin D, Sacchi S, Mandrioli M, Ottaviani E. A putative helical cytokine functioning in innate immune signalling in *Drosophila melanogaster*. Biochim. Biophys. Acta 1770: 974-978, 2007.

Malagoli D, Ottaviani E. Helical cytokines and invertebrate immunity: a new field of research. Scand. J. Immunol. 66: 484-485, 2007.

Malagoli D, Sacchi S, Ottaviani E. Lectins and cytokines in celomatic invertebrates: two tales with the same end. Inv. Surv. J. 7: 1-10, 2010.

Markus R, Laurinyecz B, Kurucz E, Honti V, Bajusz I, Sipos B, *et al*. Sessile hemocytes as a hematopoietic compartment in *Drosophila melanogaster*. Proc. Natl. Acad. Sci. USA 106: 4805-4809, 2009.

Meister M. Blood cells of *Drosophila*: cell lineages and role in host defence. Curr. Opin. Immunol. 16: 10-15, 2004.

Meister M, Lagueux M. *Drosophila* blood cells. Cell. Microbiol. 5: 573-580, 2003.

Molina-Cruz A, DeJong RJ, Charles B, Gupta L, Kumar S, Jaramillo-Gutierrez G, *et al*. Reactive oxygen species modulate *Anopheles gambiae* immunity against bacteria and Plasmodium. J. Biol. Chem. 283: 3217-3223, 2008.

Nappi A, Poirie M, Carton Y. The role of melanization and cytotoxic by-products in the cellular immune responses of *Drosophila* against parasitic wasps. Adv. Parasitol. 70: 99-121, 2009.

Nappi AJ, Christensen BM. Melanogenesis and associated cytotoxic reactions: Applications to insect innate immunity. Insect Biochem. Mol. Biol. 35: 443-459, 2005.

Nappi AJ, Frey F, Carton Y. *Drosophila* serpin 27A is a likely target for immune suppression of the blood cell-mediated melanotic encapsulation response. J. Insect Physiol. 51: 197-205, 2005.

Nappi AJ, Ottavani E. Cytotoxicity and cytotoxic molecules in invertebrates. BioEssays 22: 469-480, 2000.

Nappi AJ, Streams FA. Haemocytic reactions of *Drosophila melanogaster* to the parasites *Pseudocoila mellipes* and *P. bochei*. J. Insect. Physiol. 15: 1551-1566, 1969.

Nappi AJ, Streams FA. Abortive development of the cynipid parasite *Pseudocoila bochei* (Hymenoptera) in species of the *Drosophila melanica* group. Ann. Entomol. Soc. Am. 63: 321-327, 1970.

Nappi AJ, Vass E (2001). Cytotoxic reactions associated with insect immunity. In: Beck G, Sugumaran M, Cooper EL (eds), Phylogenetic perspectives on the vertebrate immune system, Kluwer Academic-Plemum**,** New York, pp 329-348, 2001.

Nappi AJ, Vass E, Carton Y, Frey F. Identification of 3,4-dihydroxyphenylalanine, 5,6-dihydroxyindole, and N-acetylarterenone during eumelanin formation in immune reactive larvae of *Drosophila melanogaster*. Arch. Insect Biochem. Physiol. 20: 181-191, 1992.

Nappi AJ, Vass E, Frey F, Carton Y. Nitric oxide involvement in *Drosophila* immunity. Nitric Oxide 4: 423-430, 2000b.

Novas A, Cao A, Barcia R, Ramos-Martinez JI. Nitric oxide release by hemocytes of the mussel *Mytilus galloprovincialis* Lmk was provoked by interleukin-2 but not by lipopolysaccharide. Int. J. Biochem. Cell Biol. 36: 390-394, 2004.

Pal S, Wu LP. Lessons from the fly: pattern recognition in *Drosophila melanogaster*. Adv. Exp. Med. Biol. 653: 162-174, 2009.

Paskewitz SM, Andreev O. Silencing the genes for dopa decarboxylase or dopachrome conversion enzyme reduces melanization of foreign targets in *Anopheles gambiae*. Comp. Biochem. Physiol. 150B: 403-408, 2008.

Scherfer C, Tang H, Kambris Z, Lhocine N, Hashimoto C, Lemaitre B. *Drosophila* Serpin-28D regulates hemolymph phenoloxidase activity and adult pigmentation. Dev. Biol. 323: 189-196, 2008.

Schnitger AK, Kafatos FC, Osta MA. The melanization reaction is not required for survival of *Anopheles gambiae* mosquitoes after bacterial infections. J. Biol. Chem. 282: 21884-21888, 2007.

Shin SW, Kokoza VA, Raikhel AS. Transgenesis and reverse genetics of mosquito innate immunity. J. Exp. Biol. 206: 3835-3843, 2003.

Sideri M, Tsakas S, Markoutsa E, Lampropoulou M, Marmaras VJ. Innate immunity in insects: surface-associated dopa decarboxylase-dependent pathways regulate phagocytosis, nodulation and melanization in medfly haemocytes. Immunology 123: 528-537, 2008.

Sorrentino RP, Carton Y, Govind S. Cellular immune response to parasite infection in the *Drosophila* lymph gland is developmentally regulated. Dev. Biol. 243: 65-80, 2002.

Streams FA. Factors affecting the susceptibility of *Pseudeucoila bochei* eggs to encapsulation by *Drosophila melanogaster*. J. Invertebr. Pathol. 12: 379-387, 1968.

Sugumaran H. Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. Pigm. Cell. Res. 15: 2-9, 2002.

Tang H. Regulation and function of the melanization reaction in *Drosophila*. Fly (Austin) 3: 105-111, 2009.

Tang H, Kambris Z, Lemaitre B, Hashimoto C. Two proteases defining a melanization cascade in the immune system of *Drosophila*. J. Biol. Chem. 281: 28097-28104, 2006.

Tang H, Kambris Z, Lemaitre B, Hashimoto C. A serpin that regulates immune melanization in the respiratory system of *Drosophila*. Dev. Cell 15: 617-626, 2008.

Tardieu I, Rabasse M. Some aspects of host immunity and physiological suitability in aphids attacked by *Aphidius colemani*. In: Anderson AFG (ed), Ecology and effectiveness of aphophaga, The Hague, Academic publishing, pp 311-315, 1988.

Vass E, Nappi AJ. Prolonged oviposition decreases the ability of the parasitoid *Leptopilina boulardi* to suppress the cellular immune response of its host *Drosophila melanogaster*. Exp. Parasitol. 89: 86-91, 1998.

Vass E, Nappi AJ. Developmental and immunological aspects of *Drosophila*-parasitoid relationships. J. Parasitol. 86: 1259-1270, 2000.

Vernick KD, Fujioka H, Seeley DC, Tandler B, Aikawa M, Miller LH. *Plasmodium gallinaceum*: a refractory mechanism of ookinete killing in the mosquito, *Anopheles gambiae*. Exp. Parasitol. 80: 583-595, 1995.

Whitten M, Sun F, Tew I, Schaub G, Soukou C, Nappi A, *et al*. Differential modulation of *Rhodnius prolixus* nitric oxide activities following challenge with *Trypanosoma rangeli*, *T. cruzi* and bacterial cell wall components. Insect Biochem. Mol. Biol. 37: 440-452, 2007.