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Insights From Natural Host-Parasite Interactions: The Drosophila Model

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Abstract

Immune responses against opportunistic pathogens have been extensively studied in Drosophila, leading to a detailed map of the genetics behind innate immunity networks including the Toll, Imd, Jak-Stat, and JNK pathways. However, immune mechanisms of other organisms, particularly plants, have primarily been investigated using natural pathogens. It was the use of natural pathogens in plant research that revealed the plant R/Avr system, a specialized immune response derived from antagonistic coevolution between plant immune proteins and their natural pathogens' virulence proteins. Thus, we recommend that researchers begin to use natural Drosophila pathogens to identify novel immune mechanisms that may have arisen through antagonistic coevolution with common natural pathogens. In this review, we address the benefits of using natural pathogens in research, describe the known natural pathogens of Drosophila, and discuss exciting prospects for future research on select natural pathogens of Drosophila.

Keywords

Drosophila immunity; natural pathogens; coevolution

1. Introduction

Understanding human immunity against parasites and how parasites circumvent human immune mechanisms is of obvious importance to human well-being. The same is true for multiple other host-parasite systems. We rely on healthy agricultural plants, livestock, and pollinators for our food supply, and we often rely on parasites (or parasite virulence mechanisms) to protect us from agricultural pests and from vectors of human disease. However, for both technical and ethical reasons we often cannot perform large-scale controlled infection experiments, or genetically manipulate hosts, in the focal host-parasite systems. Some of the most powerful molecular genetic tools for elucidating host immunity and parasite virulence mechanisms are only available in "model" systems such as the mouse, the fruit fly *Drosophila melanogaster*, and the thale cress *Arabidopsis thaliana*.

The model system approach has proven extremely valuable for understanding common kinds of host immune mechanisms. Much of what we know about acquired immunity - the

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interplay between MHC, T-cells, B-cells, and antibodies - is due to studies in mouse (Parham, 2009). Likewise, much of what we know about innate immunity in invertebrates and even to some extent in vertebrates - e.g. the role of Toll/NF-kappaB pathways in immune gene upregulation - is due to studies in fruit flies (Lemaitre and Hoffmann, 2007). Finally, much of what we know about innate immunity in plants - e.g. the role of LRR/WRKY pathways in immune gene upregulation - is due to studies in a small number of plant species including thale cress (Asai et al., 2002; Spoel and Dong, 2012).

Given the importance of model systems to our understanding of immunity, it is surprising that very little is known about the natural parasites of those model hosts. Most immunity studies in model hosts have not made use of the natural parasites of those model hosts, but rather have used more generalist parasites that cause some pathology in a variety of hosts, or specialist parasites of focal hosts. This is often due to our ignorance of the natural parasites of model host species, or to a belief that we can understand pathogenesis in focal host systems best by using the same parasites in a model host system. In many cases the non-natural parasites are also made to infect model hosts in a non-natural way, for example by direct injection into the blood stream or body cavity. Thus, it is interesting that much of what we know about immune systems is based on how hosts respond to parasites and infection modes they rarely if ever have encountered in nature during their evolutionary history. Does it matter?

Hosts and parasites are thought to engage in antagonistic coevolution, where a newly evolved parasite virulence mechanism is negated over time by a newly evolved host immune mechanism and vice versa (Dawkins and Krebs, 1979). If we don’t study natural host-parasite pairs, will we uncover specialized immune mechanisms, and will this affect the identification of defense and virulence mechanisms of clinical importance? How can we hope to understand host-parasite coevolution? In this review we argue that use of non-natural parasites in immunity studies biases our understanding of immunity to those immune mechanisms suited to combating opportunistic or generalist parasites. While this approach has yielded tremendous benefits, more specialized immune mechanisms that have evolved to combat more specialized parasites may exist and may have been overlooked. We focus on the natural parasites of *D. melanogaster* as a potential tool for uncovering more specialized host immune mechanisms and parasite virulence strategies, and the genetic basis for host-parasite antagonistic coevolution.

2. Specificity in Natural Host-Parasite Interactions: The Plant R/Avr System

For obvious reasons, some of the most intensely studied natural host-parasite systems are the interactions between agricultural crop plants and their parasites. Long before any plant immune signaling pathways were fleshed out, a remarkable consensus emerged about the genetic bases for resistance and virulence in natural plant-parasite systems. Plant genomes were discovered to encode R proteins (resistance proteins) that interacted with parasite Avr proteins (avirulence proteins) (Figure 1). If host R proteins, or R proteins alleles, were a "match" for the Avr proteins, or Avr proteins alleles, of the parasite, the plant host would be resistant to the parasite. It was found that individual plant species encoded numerous R genes and R gene alleles, that parasites usually encoded multiple Avr genes, and that the plant host only needed to make one match to be resistant (Flor, 1971). It wasn’t until much more recently that the true nature of the R-Avr interactions was worked out.

Plants have receptor proteins (often leucine-rich receptors, LRRs) that recognize parasites and activate cytoplasmic signaling cascades. This results in activation of a WRKY domain transcription factor that up-regulates antimicrobial effector proteins used to control the infection (Nurnberger et al., 2004). To circumvent this generic host immunity, specialist

plant parasites have evolved virulence proteins that disrupt particular proteins in the plant immune signaling pathways. To overcome these parasite virulence mechanisms, plant hosts have counter-evolved specialized resistance proteins (R proteins) that recognize the parasite virulence proteins or the effects of parasite virulence proteins (DeYoung and Innes, 2006; Dodds et al., 2006; Jones and Dangl, 2006), and that activate downstream immune responses independent of the original immune signaling pathways (Figure 1) (Chisholm et al., 2006). Thus, when a plant R protein is a match, parasite virulence proteins end up becoming avirulence (Avr) proteins.

This amazing history of antagonistic coevolution between plant R genes and parasite Avr genes may never have been discovered if plant immune systems were studied using non-natural parasites lacking specialized Avr genes. Following this logic, in other host systems studied using non-natural parasites, we may as yet have only uncovered generalized immune mechanisms akin to the LRR/WRKY pathway of plants shown in Figure 1A. Although such generalized immune mechanisms are extremely important to understand, non-natural host-parasite pairings may tell us little about how specialist parasites suppress host immunity (Figure 1B) or about any secondary immune mechanisms hosts deploy against specialist parasites (Figure 1C).

3. Examples of the Benefits of Natural Host-Parasite Systems

Thus, an important decision faced by immunologists is the selection of natural or non-natural host-parasite pairings in empirical infection studies (Bem et al., 2011). When investigating a disease, does one study the progression of that disease in a non-natural host, or study the progression of a homologous parasite in its natural host? The decision to use a natural or non-natural host-parasite pairing always depends on the nature of the system and the project goals, but it may not always be clear ahead of time which is the ideal choice. Below, we discuss examples in which natural host-parasite pairings yield more relevant insights into host-parasite interactions, from both vertebrate and invertebrate systems.

Vertebrates

The first step required for a successful infection is the ability of pathogens to gain access to host tissues. Guinea pigs and humans are natural hosts of *Listeria monocytogenes* and have an isoform of the receptor E-cadherin that interacts with the bacteria and allows its passage across the intestinal barrier. Mouse genomes do not encode the same E-cadherin isoform (Lecuit et al., 1999), meaning studies using the guinea pig host are often more relevant to human listeriosis than the more obvious mouse model system. Scientists can sometimes overcome problems of parasite internalization into hosts using artificial infection methods such as direct injection, as long as downstream virulence ability is unrelated to the process of internalization.

Given a non-natural parasite is able to access a host, it may still find the host environment unsuitable for development, or it may quickly succumb to general host immune responses. For example, infection of a murid herpesvirus in a non-natural host, *Mus musculus*, failed to support disease transmission and evoked different responses from those mounted by natural hosts (Francois et al., 2010; Hughes et al., 2012, 2011, 2010), prompting a return to the use of a natural host capable of disease transmission (Knowles et al., 2012). Likewise, infection by the human respiratory syncytial virus (RSV) is often modeled in the mouse. Unlike in humans, there are an absence of outward symptoms of RSV infection in certain mouse strains. A comparison of the mouse response to RSV and one of its natural pneumonia viruses (PVM, the closest relative to RSV) revealed different molecular components behind the more extensive pathogenesis of the mouse-specific virus (Domachowske et al., 2000), suggesting that using a naturally infectious mouse pneumonia virus in mice could provide

more thorough mechanistic insight into the human immune response against RSV (Dyer et al., 2012).

Although parasites often show attenuated virulence in non-natural hosts, parasites sometimes cause extreme pathologies in non-natural hosts, presumably because they encode virulence mechanisms that the host is not adapted to resist. For example, natural hosts of simian immunodeficiency virus (SIV) display non-progressive infections and do not develop immunodeficiency, whereas non-natural primate hosts cannot control SIV progression. Genetic analyses have uncovered differences in the molecular underpinnings of the natural and non-natural host responses (Bosinger et al., 2012). These differences were found to be clinically relevant, as a group of human immunodeficiency virus (HIV)-infected humans that display a non-progressive immune reaction to HIV possess transcriptional responses to infection that more closely mirror those of natural (non-progressive) hosts of SIV (Rotger et al., 2011). Further investigation of the mechanistic ways a host controls a non-progressive infection could advance clinical developments in HIV treatment (Sodora et al., 2009).

Finally, trials for treatments of disease, like vaccines, only make sense in a naturally infectious system, because any reduction of disease spread can only be studied in a host that can actually become infected. For example, the mouse and mouse pox virus may provide a more suitable system for development of a new human smallpox vaccine than use of human smallpox itself in mouse hosts, given that human smallpox does not efficiently replicate within or spread between mice (Fang et al., 2006).

Invertebrates

Like with vertebrate hosts, parasites paired with non-natural invertebrate hosts often show attenuated virulence. For example, the use of non-natural mosquito-malaria pairings contributed to initial discord over the effect of plasmodium infection on mosquito viability. A meta-analysis of past studies found that decreased vector survival was more often found in pairings that do not occur in nature (Ferguson and Read, 2002). *Anopheles gambiae* mounted considerably different immune reactions against a plasmodium it encounters in nature (the human parasite *Plasmodium falciparum*) than against the rodent parasite *Plasmodium berghei* (Boete, 2005; Cohuet et al., 2006; Dong et al., 2006; Michel et al., 2006; Tahar et al., 2002). This work led to an increased focus on natural mosquito-plasmodium pairings in experimental studies (Tripet, 2009).

Multiple accounts of immune priming whereby a previously infected host demonstrates an enhanced capacity to respond to re-infection, have now been reported from invertebrate systems (Itami et al., 1989; Kurtz and Franz, 2003; McTaggart et al., 2012; Tidbury et al., 2011; Wu et al., 2002). Interestingly, in studies that compared priming against natural and non-natural parasites, hosts showed stronger priming responses against natural parasites than against parasites not known to infect the hosts in nature (Pope et al., 2011; Roth et al., 2009). These studies suggest that priming may be a secondary type of immune mechanism adapted specifically for the specialist parasites that suppress the initial host immune mechanisms.

Finally, a dynamic process of host-parasite coevolution in nature, where new host resistance and parasite virulence alleles arise and spread through populations, might be expected to cause intra-population variation in host susceptibility to natural parasites. In a genome-wide study searching for fruit fly alleles associated with resistance to viral infections, resistance variation was found to be much higher against natural viral parasites than against viruses that do not infect *D. melanogaster* in nature (Magwire et al., 2012). Resistance to Drosophila C Virus (DCV) and a *D. melanogaster*-specific Sigma virus was associated with a few SNPs of large effect while there were no SNPs significantly associated with resistance to the non-

natural Flock House Virus (FHV) or a *D. affinis*-specific Sigma virus. Interestingly, each SNP significantly associated with viral resistance was associated with resistance to only one virus, showing a degree of specificity in *D. melanogaster* immunity against different viral species.

4. Drosophila as a Model for Innate Immunity

D. melanogaster is a genetic model organism that offers ease of use and unparalleled tools for genetic and molecular characterization of biological processes. As a complex animal, *D. melanogaster* possesses the majority of molecular pathways and protein types that humans possess, although often with fewer overlapping and redundant functions than the multi-gene families of vertebrates (Adams et al., 2000). Interest in *D. melanogaster* as a model for understanding the genetic basis of innate immunity has built over the last 20 years and led to the award of the Nobel Prize in Physiology or Medicine to Jules Hoffmann in 2011. His work and that of others outlined the fruit fly humoral response against non-natural bacterial and fungal parasites (Lemaitre and Hoffmann, 2007). In this antimicrobial response, secreted or membrane-bound receptors recognize microbial antigens and initiate signaling cascades in fruit fly immune cells (mainly in the fat body and hemocytes). NF-kappaB transcription factors are activated and move into the nucleus where they upregulate antimicrobial peptides, which are then secreted to attack the extracellular microbes. Two major signaling pathways work jointly in antimicrobial defense, the Toll pathway and the Imd pathway (De Gregorio et al., 2002), and the Jak-Stat and JNK pathways seem to play complementary roles (Boutros et al., 2002). Many questions about Drosophila microbial immunity remain to be answered, such as tissue-specific immune responses, the interactions between different tissues during a systemic immune response, and the nature of the interplay between the Toll, Imd, Jak-Stat, and JNK pathways within and between these tissues. If *D. melanogaster* can still teach us much about general immune responses against non-natural bacterial and fungal infections, it is clear we know almost nothing about natural Drosophila parasite virulence mechanisms or any secondary immune mechanisms flies utilize against these parasites.

5. Evidence of Arms-Race Coevolution in Drosophila Immune Genes

Like all hosts, fruit flies are infected by a combination of generalist, specialist, and opportunistic parasites. We consider generalist parasites to be those parasites that naturally infect and overcome the immune responses of diverse hosts, while specialist parasites only have this ability in a relatively small subset of potential hosts. All else being equal, a generalist strategy should be preferred, so the existence of specialist parasites suggests there is likely some drawback to generalism, such as costly deployment of multiple virulence mechanisms, increased toxicity to host health, or lower infection success in any one host species. Opportunistic parasites are those that are ill-equipped to naturally infect a host under normal conditions, but that occasionally gain access and harm hosts due to host injury or weakened host immunity. Hosts have immune mechanisms to resist all three types of parasites, but different kinds of immune responses are expected to evolve in different ways. Basic immune mechanisms designed to repel opportunistic parasites will likely show few signs of recurrent adaptation, given that opportunistic parasites do not live in particular hosts frequently enough to select for suppressive virulence mechanisms. Generalist parasites will select for host immune response adaptation, but the strength of selection will likely be weaker than for specialist parasites, assuming hosts are infected more frequently by particular specialists than by particular generalists. Therefore, arms-race coevolution, where a new parasite virulence capability selects for a new host immune capability which selects for a new parasite virulence capability, etc, will most like occur between specialized parasites and their hosts. Furthermore, if generalist and specialist parasites suppress host

immunity using different kinds of virulence mechanisms, host-parasite coevolution can only be fully understood when both types of parasites are studied.

Comparing orthologous gene sequences within and between species can provide clues to the kinds of selective pressures that have acted on genes in the past, and *D. melanogaster* has been a hotbed for development of population genetic and molecular evolution methodology. Numerous analyses of Drosophila immunity genes, especially of the Toll and Imd signaling pathways, have led to some broad generalities about fly immune system evolution. Immune genes evolve more rapidly and adaptively (i.e., show a bigger excess of non-synonymous substitutions) than other kinds of Drosophila genes, and it is the immune recognition and signaling genes, not effector genes, that show the most evidence of adaptive evolution (Lazzaro, 2008; Lazzaro and Clark, 2003; Obbard et al., 2009; Sackton et al., 2007; Schlenke and Begun, 2003). In the *D. melanogaster* species group, immune signaling proteins in the Toll and Imd pathways show especially strong signals of adaptive evolution (Figure 2) (Sackton et al., 2007; Schlenke and Begun, 2003). These results are interpreted to mean that the natural parasites of Drosophila circumvent the Drosophila immune system by avoiding recognition (e.g. by evolving novel surface antigens) or suppressing recognition (e.g. using proteins that block expression or function of recognition proteins), or by evolving virulence proteins that interfere with components of conserved signaling cascades. A number of examples of parasite virulence proteins able to suppress aspects of host innate immune systems, including signaling through Toll/NF-kappaB pathways, now exist, supporting the Drosophila immune system population genetic and molecular evolution inferences (Revilla et al., 1998; Schesser et al., 1998).

Some questions regarding Drosophila immune gene evolution remain unanswered, such as what are the natural parasites that actually selected for rapid and adaptive Drosophila immune protein evolution? What are the interacting immunity and virulence protein pairs that are driving the arms race between hosts and parasites? Are there differences between generalist and specialist parasites in terms of the virulence mechanisms and selection pressures they impose on host immunity? Rapid evolution of Toll and Imd pathway genes and other genes can apparently provide flies some protection against parasites, but could flies have also evolved secondary immune mechanisms similar to the R genes of plants for use against specialist parasites? Use of natural parasites in Drosophila immunity studies could lead to the identification of novel virulence proteins specialized to suppress Drosophila immunity, as well as any specialized immune mechanisms the flies employ.

6. The Natural Parasites of Drosophila

Drosophila are host to a range of parasites in nature including representatives of most major parasite groups (Figure 3):

TEs

Transposable elements (TEs) are mobile genetic parasites that multiply in host genomes by the "copy and paste" mechanism of retrotransposons (requiring reverse transcriptase and endonuclease) or by the "cut and paste" mechanism of DNA TEs (requiring transposase). The cut and paste mechanism causes transposon duplications if the transposition happens during S phase of the cell cycle when the "donor" site has already been replicated, but the "target" site has not. TEs are obligate parasites that are usually transmitted vertically from parent to offspring, but may occasionally be transmitted horizontally via vectors or other unknown mechanisms (Silva et al., 2004). Besides the assumed metabolic cost to the host of replicating, transcribing, and translating TE sequences, uncontrolled TE duplication causes fitness effects due to chromosomal double strand breaks, insertions in functional host genetic elements, and an increased rate of chromosomal dysgenesis in host genomes. *D.*

melanogaster is the natural host to at least 90 TE families, with many other unique TE families found in other Drosophila lineages (Kaminker et al., 2002). Fruit flies keep TE numbers under control using RNA interference (RNAi) mechanisms, including the germline PIWI system that is functionally analogous to the prokaryotic CRISPR system (Senti and Brennecke, 2010).

Viruses

Like TEs, viruses are mobile genetic parasites that use host transcription and translation machinery to duplicate, but unlike TEs they often exist in an extra-chromosomal state in host cell cytoplasm where they are protected by a protein coat. Viruses are obligate parasites that can be transmitted horizontally when in lytic phase or vertically when they have incorporated themselves as proviruses into host genomes in lysogenic phase. Besides the assumed metabolic cost to the host of replicating, transcribing, and translating viral sequences, viruses can cause substantial pathology to the host by lysing infected host cells. *D. melanogaster* is the natural host to at least four viral species, including the RNA viruses Sigma, Drosophila C, and Nora, and the DNA virus DiNV (Brun and Plus, 1980; Fleuriet, 1981; Habayeb et al., 2006; Kapun et al., 2010; Thomas-Orillard, 1988; Unckless, 2011). Other viruses have been identified in lab and natural populations of Drosophila but are relatively uncharacterized (Brun and Plus, 1980; Plus et al., 1976; Plus et al., 1975a; Plus et al., 1975b; Plus and Duthoit, 1969). Fruit flies resist viral infections using RNAi mechanisms, which silence viral gene transcripts in a sequence-specific manner via small interfering RNAs (siRNAs) and RNAi pathway machinery, and by autophagy, whereby autophagosomes collect cytoplasmic material to be degraded and recycled (Galiana-Arnoux et al., 2006; Ghildiyal and Zamore, 2009; Kemp et al., 2013; Shelly et al., 2009; van Rij et al., 2006; Zambon et al., 2006).

Prokaryotes

Eubacterial parasites reproduce by fission and can live outside of or within host cells. They are not always obligate parasites and can be transmitted either horizontally or vertically. Fitness affects arise from the fact that bacteria consume host nutrients, often leading to host cell and tissue necrosis. *D. melanogaster* is the natural host to hundreds of bacterial species (Chandler et al., 2011; Corby-Harris et al., 2007), including the vertically transmitted intracellular parasite *Wolbachia* and the dramatically genome-reduced, vertically transmitted Spiroplasma parasites (Haselkorn et al., 2009; Riegler et al., 2005). However, for most of these bacterial species it remains unclear whether they are parasites versus symbionts, obligate versus facultative parasites, or specialist versus generalist parasites. Fruit fly immune responses against bacteria include the humoral production of antimicrobial peptides by conserved innate immune signaling pathways such as Toll and Imd, as well as phagocytosis of extracellular bacteria by circulating hemocytes (Lemaitre and Hoffmann, 2007).

Protists

Protozoan parasites are a diverse group of motile protists (unicellular eukaryotes) that often have complex life histories, such as different life stages (e.g. trophozoites versus cysts), a developmental progression through different host tissues (e.g. malaria-causing Plasmodium have liver and blood stages), and/or a cyclical progression of host species (e.g. insect-vectored trypanosomatids causing human disease). Protozoans usually reproduce asexually via mitosis and cytokinesis, are usually transmitted horizontally, and are usually obligate parasites. There can be intracellular and extracellular life stages, with intracellular forms causing host cell death and extracellular forms consuming host nutrients. *D. melanogaster* is the natural host to only one known protozoan parasite, trypanosomatids. Multiple trypanosomatid species naturally colonize fruit fly guts, consume food in the gut, and are

passed back into the environment via feces, but their pathogenic effects in flies are unclear (Chatton and Alilaire, 1908; Corwin, 1962; Rowton and Mcghee, 1978; Wilfert et al., 2011). Fruit fly immune responses against trypanosomatids are poorly characterized, but production of antimicrobial peptides and an oxidative burst in the gut characterizes anti-trypanosomatid immune responses of other insects (Boulanger et al., 2002, 2001; Hu and Aksoy, 2005; MacLeod et al., 2007; Munks et al., 2005).

Plants

Plant parasites are ectoparasitic and mostly infect other plants. There are no known plant parasites of Drosophila.

Fungi

Unicellular fungal parasites have life histories similar to different bacterial parasite groups, and the fly immune responses against such unicellular fungal parasites are also similar. *D. melanogaster* is the natural host to numerous unicellular fungal species (Chandler et al., 2012), including intracellular vertically transmitted microsporidians (Futerman et al., 2006), and the intracellular yeast-like fungus *Coccidiascus legeri*, which lives in fly intestinal epithelial cells and sometimes develops in concert with trypanosomatids (Ebbert et al., 2003; Lushbaugh et al., 1976). Like for bacteria, it remains unclear whether most of these unicellular fungal species are parasites versus symbionts, obligate versus facultative parasites, or specialist versus generalist parasites. Fungal parasites typically grow as thin thread-like structures termed hyphae, which can have specialized structures (e.g. haustoria) for penetrating host cells and consuming host cell nutrients. Most multicellular fungal parasites reproduce by generating fruiting bodies that release spores into the environment, which horizontally infect new hosts following ingestion or by boring through the host cuticle. Some Drosophila lineages (e.g. the obscura group) act as host to specialized multicellular fungal parasites from the ascomycete order Laboulbeniales, which forms fruiting bodies on the dorsal abdominal cuticles of adult flies (Starmer and Weir, 2001). No other multicellular fungal parasites are known from Drosophila, and immune responses against such parasites are uncharacterized.

Animals

Animal parasites are usually horizontally transmitted, typically infect particular host tissues and life stages, and are obligate parasites. Different groups may reproduce asexually or sexually within the host or outside the host and may be endo- or ectoparasitic. Animal parasites harm their hosts by consuming nutrients in various body cavities (e.g. the bloodstream and gut) or by consuming host cells. *Drosophila melanogaster* is the natural host to a number of endo- and ectoparasitic wasp species as well as a number of ectoparasitic mite species (Carton, 1986; Polak, 2003, 1996). Endoparasitic wasps lay their eggs in fly larval or pupal body cavities, and flies respond by mounting an encapsulation response defined by hemocytes migrating towards, binding to, and consolidating around the wasp eggs, and by releasing free radicals and melanin inside the hemocyte capsule (Carton et al., 2008). This melanotic encapsulation response is functionally homologous to granuloma formation in vertebrates infected by animal parasites such as helminths, whereby macrophages, eosinophils, and other host blood cell types surround (and sometimes melanize) the large invaders (Anthony et al., 2007; Koppang et al., 2005; Mukhopadhyay et al., 2012; Richards et al., 1996; Secombes and Chappell, 1996; Swartz et al., 2004). Surviving wasp eggs complete their life cycles by eventually consuming their fly hosts. Ectoparasitic wasps and mites consume fly hemolymph (Carton et al., 2008; Polak, 2003, 1996). The wasps eventually kill their fly hosts by consuming other tissues, whereas mites may never kill their fly hosts outright. Fly immune responses against ectoparasitic wasps and mites are uncharacterized. Some Drosophila lineages (e.g. the mushroom-feeding flies)

also act as host to parasitic nematodes (Jaenike, 1992). Nematodes pierce fly larval cuticles and release offspring into the fly hemocoel, which eventually leave the body of the adult flies through the ovipositor and/or anus onto new fly food sources.

7. Insights from Natural Drosophila Infections

Only a small subset of natural Drosophila-parasite interactions have been investigated at the genetic level, but these studies have begun to provide significant insight into ecologically relevant mechanisms of innate immunity. Here we review the literature on Drosophila defense mechanisms and parasite virulence mechanisms identified through the use of natural Drosophila parasites.

TEs

Self-replicating mobile genetic elements are a source of deleterious genomic alterations in eukaryotes. Transcriptional silencing of mobile elements in the germline occurs via the PIWI-interacting RNA (piRNA) pathway. The piRNA pathway involves distinct genomic loci containing deactivated mobile element sequence clusters that get transcribed and processed into small RNAs termed piRNAs, which are then paired with the PIWI family proteins Piwi, Aub, and Ago3 (Brennecke et al., 2007). A cycle of RNA silencing is proposed to be mediated by unique protein-piRNA pairs, which target and cleave active transposon transcripts, and in turn produce more piRNAs to be partnered with PIWI family proteins and continue the silencing cycle (Senti and Brennecke, 2010). The different PIWI proteins act on specific piRNA strands (sense vs antisense) and have different RNA sequence affinity, helping promote the cyclic aspect of the proposed silencing process (Brennecke et al., 2007). A useful tool for studying piRNA defense is to overwhelm it by setting up Drosophila matings where a female is naïve to the transposable element families of her mate. Such crosses result in hybrid dysgenesis, whereby progeny suffer infertility from unrestrained novel mobile element activity (Rubin et al., 1982). Offspring generated from reciprocal crosses with naïve fathers receive some protection against hybrid dysgenesis because piRNA pathway activity is encouraged early on by the maternal deposition of PIWI proteins and piRNAs (Brennecke et al., 2008; Harris and Macdonald, 2001; Megosh et al., 2006). Studying the capture of novel transposable element sequences into piRNA clusters is an important next step in understanding the arms race between a host and its mobile genetic parasites.

Viruses

Sigma viruses are negative sense single-stranded RNA Rhabdoviruses that are common Drosophila parasites in nature. Different Sigma viruses specialize on different Drosophila species, they can be both maternally and paternally transmitted, and they can cause a decrease in host fecundity (Fleuriet, 1981). Gene expression studies of Sigma virus-infected *D. melanogaster* identified differential transcription of novel genes and pathways as well a handful of peptidoglycan recognition proteins and antimicrobial peptides involved in the Toll and Imd pathways (Carpenter et al., 2009; Tsai et al., 2008). Furthermore, genetic mapping of *D. melanogaster* loci that confer resistance to Sigma virus in natural fly populations identified *ref(2)P*, a homolog of a mammalian autophagy receptor (Longdon and Jiggins, 2012; Magwire et al., 2011; Nezis, 2012). Autophagy, the vesicularization of cell cytoplasm, was previously shown to play a role in clearing non-natural fly viral infections (Shelly et al., 2009). Association mapping also identified the genes *CHKov1* and *CHKov2* as resistance factors (Magwire et al., 2011). Two rearrangements near the ancestral *CHKov1* and *CHKov2* locus that contain partial sequences of both genes and a *Doc* transposable element insertion in the CHKov1 coding region make up one causative resistance locus, while another resistance-associated allele differs from the ancestral (susceptible) strain by

the *Doc* transposon insertion, causing a putative shortened protein. The mechanism behind increased Sigma virus resistance of flies carrying truncated CHKov1 is unclear, but this *Doc* insertion has also been implicated in fly resistance to organophosphate pesticides (Aminetzach et al., 2005). Protective alleles of both the *ref(2)P* and *CHKov* loci have swept to high frequency in natural fly populations due to positive selection, presumably as a result of viral and/or insecticide-mediated selection pressures (Bangham et al., 2007; Magwire et al., 2011).

Drosophila C Virus (DCV) is a single-stranded positive sense RNA virus transmitted by feeding at the larval or adult stage, naturally infects a range of Drosophila species (Kapun et al., 2010), and causes increased mortality (Thomas-Orillard, 1988). The Jak-Stat pathway is thought to play an important role in the Drosophila immune response against DCV, as flies mutant for *hopscotch* (the fly Jak kinase) are more susceptible to DCV infection (Dostert et al., 2005; Kemp et al., 2013). A genome-wide association study found that alleles of *pastrel* were associated with resistance to DCV and that flies with knocked-down levels of *pastrel* displayed lower survival and higher viral titers than control flies (Magwire et al., 2012). The molecular function and the role of *pastrel* in combating DCV is unknown. Although an RNAi-based immune response is important for fly survival of DCV infection (Galiana-Arnoux et al., 2006; Kemp et al., 2013; van Rij et al., 2006), the DCV genome harbors an RNAi suppressor that may upset the RNAi response by binding to long RNAs and inhibiting the production of siRNAs (Huszar and Imler, 2008; Kemp and Imler, 2009; van Rij et al., 2006). Thus, *pastrel* may be part of a more specialized secondary antiviral immune mechanism.

D. melanogaster is also naturally infected by the picorna-like RNA Nora virus, but RNAi, Toll, and Jak-STAT activity are not sufficient for immune clearance of this virus (Habayeb et al., 2009), There is as yet very little overlap in immune genes and pathways found to be important for fly immunity against Sigma, DCV, and Nora viruses, suggesting that *D. melanogaster* has evolved specialized responses against its different natural viral parasites.

Bacteria

Most bacterial immunity studies in Drosophila have infected flies via a septic needle wound through the cuticle. Flies may suffer septic cuticle wounds in nature, for example when they are attacked by cuticle-piercing animal parasites like parasitic wasps, nematodes, and mites (Carton, 1986; Houck et al., 1991; Jaenike, 1992), but most natural host contact with pathogenic bacteria likely arises from bacterial uptake through the gut, trachea, and reproductive tracts. Thus, use of *D. melanogaster* as a model system for understanding, e.g., specialized interactions between insect vectors and the human parasites they carry in their guts, may have more practical application if an oral rather than bloodstream route of infection is used.

The gram-negative entomopathogenic bacterium *Pseudomonas entomophila* was isolated from a wild-caught fly and selected for experimentation because of its strong induction of the *D. melanogaster* immune response following oral infection.

The *P. entomophila* genome encodes multiple putative virulence factors, some of which are regulated by the GacS/GacA two-component system (Haas and Defago, 2005; Rahme et al., 1995; Vodovar et al., 2006; Vodovar et al., 2005) and *pvf* gene cluster regulatory system (Vallet-Gely et al., 2010). The GacS/GacA two-component system acts post-transcriptionally via small noncoding RNAs to regulate virulence protein production, while the *pvf* cluster encodes a signaling-factor that can influence virulence gene expression independent of the Gac system. Both systems are involved in the production of the pore-forming toxin, Monalysin, which is a key player in damaging host gut cells and upsetting gut

homeostasis as part of the bacteria virulence strategy (Opota et al., 2011). Specifically, monalysin, in combination with host production of reactive oxygen species, blocks mRNA translation in infected tissues, inhibiting immune responses and epithelial renewal (Chakrabarti et al., 2012). GacS/GacA is also involved in regulating AprA, a protease secreted by *P. entomophila* that suppresses induction of Imd-regulated antimicrobial peptides in the host fly gut (Liehl et al., 2006).

Fly larvae mount a robust transcriptional response to *P. entomophila* oral infection that includes activation of the Imd, Jak-Stat, and JNK pathways, upregulation of antimicrobial peptides, production of reactive oxygen species as well as detox and stress response genes to contain the damage, and increased rates of intestinal stem cell proliferation to repair gut tissue (Buchon et al., 2009; Chakrabarti et al., 2012; Jiang et al., 2009; Vodovar et al., 2005). Notably, flies mutant for the Imd transcription factor *Relish* suffered heightened mortality compared to wildtype flies (Vodovar et al., 2005), and it is Imd expression in the gut specifically that provides protection (Liehl et al., 2006). Jak-Stat signaling and Upd cytokine expression are required for maintaining gut homeostasis (Buchon et al., 2009; Jiang et al., 2009). *P. entomophila* infection of fruit fly guts may be an ideal model to understand how hosts balance the clearance of gut parasites while maintaining equilibrium of the delicate commensal microbiota community (Ryu et al., 2008).

Infection by the maternally transmitted, intracellular, endosymbiotic bacteria *Wolbachia* naturally occurs in widespread arthropod and nematode species. In Drosophila, a well-described effect of *Wolbachia* infection is cytoplasmic incompatibility (CI). CI describes embryonic lethality resulting from mitotic defects when *Wolbachia*-infected males mate with uninfected females, a condition that selects females to gain the infection. Expression of CI is complex and varies across Drosophila species (Bourtzis et al., 1996). The mechanism behind CI is argued to result from *Wolbachia*-induced changes in the sperm pronucleus upsetting sperm development (Presgraves, 2000). A similar sperm pronucleus phenotype is found in flies mutant for the histone chaperone *Hira*, and it was shown that *Hira* transcripts are less abundant in Wolbachia infected Drosophila males, suggesting *Wolbachia*-induced alteration of Hira expression causes CI (Zheng et al., 2011a). With respect to immune resistance, microarray studies of *Wolbachia*-infected testes identified a number of upregulated genes including IMD pathway components and antimicrobial peptides (Zheng et al., 2011b), but flies do not regularly clear *Wolbachia* infections, perhaps because it has evolved to be more of a mutualist symbiont than a parasite.

Because vertically transmitted *Wolbachia* completely rely on their hosts for survival, they are selected to develop ways to increase host, and thus self, fitness. A decade-long study on the effects of *Wolbachia* infection in a *D. simulans* population found that a decrease in infected female fecundity transitioned to a fitness boost over time (Weeks et al., 2007). This boost was tied to *Wolbachia*, and not host, evolution. Furthermore, infection with certain strains of *Wolbachia* can confer resistance to natural (DCV, Nora virus) and non-natural (Flock House virus, Cricket paralysis virus) RNA viruses of *D. melanogaster* and *D. simulans* (Hedges et al., 2008; Osborne et al., 2009; Teixeira et al., 2008), as well as to the insect fungal pathogen *Beauveria bassiana* (Panteleev et al., 2007). The mechanism behind Wolbachia protective effects is unknown, but *Wolbachia*-mediated protection against DCV is independent of host RNAi machinery as siRNA pathway mutants still show increased viral resistance when infected with *Wolbachia* (Hedges et al., 2012). Wolbachia protective effects are not general across all parasites, as no protection is provided against two DNA viruses or five intra- and extracellular bacteria species (Rottschaefer and Lazzaro, 2012; Teixeira et al., 2008; Unckless, 2011; Wong et al., 2011).

Another mechanism by which hosts can limit costly bacterial infections is to avoid being infected in the first place. *D. melanogaster* are attracted to rotting fruits that contain a diversity of yeasts and bacteria that flies use as food, but rotting fruits can also contain a diversity of microbes that are potentially toxic or pathogenic if taken into the gut. Many such harmful microbes produce geosmin, a compound of unknown function that has a distinct earthy smell. Fruit flies have a dedicated olfactory circuit for recognizing geosmin odor, mediated by signaling through sensory neurons expressing the odorant receptor Or56a, which innervate the DA2 glomerulus in the antennal lobe (Stensmyr et al., 2012). Geosmin sensing leads to a strong aversion behavior, even if geosmin odor is combined with odors that flies are normally attracted to (Becher et al., 2010; Stensmyr et al., 2012). Thus, fruit flies can avoid harmful microbes from a distance due to olfactory recognition.

Wasps

Outside of transposable elements, viruses, and bacterial parasites, the only other natural Drosophila-parasite interactions studied at the genetic level are fruit fly interactions with endoparasitoid wasps that lay eggs in fly larvae. Flies mount a melanotic encapsulation response against the wasp eggs, whereby the egg is recognized as foreign, circulating plasmatocytes are activated and migrate to the wasp egg, the lymph gland (the hematopoietic organ) begins producing new specialized hemocytes termed lamellocytes, the lamellocytes form successive cellular layers on top of the plasmatocytes, the hemocytes consolidate around the wasp egg via septate junctions, and inner cells in the capsule release free radicals and melanin inside the capsule to kill the developing wasp (Figure 4) (Carton et al., 2008; Russo et al., 1996). Flies mount the same "immune" response against any large foreign object in their hemocoel, including oil droplets, beads, tissue transplants, and human hairs (Carton, 1986). Thus, the real benefit of using live wasps in infection experiments is that specialized virulence strategies for suppressing the basic encapsulation response, as well as potential specialized immune mechanisms flies use to prevent immune suppression, can be uncovered.

The genetic basis for the fly melanotic encapsulation response against wasp eggs is partially characterized (Carton et al., 2008). A cytoplasmic calcium burst in plasmatocytes activates them to begin migration towards the wasp egg (Mortimer et al., 2013), and the Toll and Ras pathways are required for de novo hemocyte proliferation in the lymph gland following infection (Sorrentino et al., 2004; Zettervall et al., 2004). The Jak-Stat and JNK pathways control differentiation of plasmatocytes and/or prohemocytes in the lymph gland into the large flattened lamellocytes responsible for outer layers of the melanotic capsule (Sorrentino et al., 2004; Zettervall et al., 2004). The transcription factor knot is specifically required in the lymph gland for lamellocyte differentiation and dispersal (Crozatier et al., 2004). Hemocyte adherence to the wasp egg requires the integrin myospheroid (Irving et al., 2005), while the cytoskeletal Rac GTPase Rac2 is required for those cells to spread over the egg (Williams et al., 2005). N-glycosylation of lamellocyte membrane proteins is required for the lamellocytes to adhere to one another and consolidate over the primary layer of plasmatocytes (Mortimer et al., 2012). Melanization of the cellular capsule surrounding the wasp egg is controlled by the phenoloxidase cascade, which is made up of several pro-enzymes that enzymatically cleave each other to make active forms. This eventually leads to the generation of melanin from the amino acid tyrosine, as well as free radicals as a side product (Nappi et al., 2009). Many gaps in our understanding of the melanotic encapsulation response remain, including the tissue and temporal specificity of immune pathway activation. Furthermore, the genetic basis for recognition of the wasp egg as foreign, signaling between the first responding hemocytes and the lymph gland, and the signal that leads activated hemocytes to the wasp egg remain open questions.

Venom of the specialist wasp *Leptopilina boulardi* includes a RhoGap protein that interferes with *D. melanogaster* lamellocyte cytoskeletal structure via interaction with Rac1 and Rac2, causing cytoplasm of this specialized host cell type to bleb from opposite poles, inhibiting the encapsulation response (Colinet et al., 2007; Labrosse et al., 2005a, 2005b). *L. boulardi* venom also includes a serpin and superoxide dismutases (SOD) that disrupt the production of melanin (Colinet et al., 2011, 2009). At least one fly serpin (Spn43Ac) acts to suppress activation of this proteolytic cascade, so the wasp venom presumably mimics the inhibitory effect of the native fly serpin. SODs are antioxidant enzymes that convert superoxide to hydrogen peroxide, which is then converted to water. Although reactive oxygen species including superoxide are generated during the production of melanin, it is unclear how a SOD can prevent melanin production. Another specialist wasp, *L. victoriae*, disrupts N-glycosylation of surface proteins on Drosophila lamellocytes, which prevents the lamellocytes from adhering to one another and consolidating into a tight capsule around the wasp egg. Hemocyte-specific expression of the N-glycosylation gene *Mgat1* confers resistance to *L. victoriae*. Given that the building of protein N-glycans is a multi-step process, and that the Mgat1 protein acts at an intermediate step in this process, these data suggest the wasp venom acts immediately upstream of Mgat1, although the responsible venom protein has not yet been identified (Mortimer et al., 2012). Finally, the venom of a more generalist Figitid wasp species, *Ganaspis sp.1*, contains a SERCA calcium pump that inhibits an excitatory cytoplasmic calcium burst in *D. melanogaster* plasmatocytes, preventing them from becoming activated and migrating and adhering to the wasp egg (Mortimer et al., 2013). Genetically enhancing or diminishing the hemocyte calcium burst alters fly immunity against different wasp species, demonstrating that study of natural parasite virulence factors can lead to important discoveries about host immune systems.

Wasp virulence mechanisms are usually distinct to individual wasp species and even show variation within wasp species, indicating that interactions between wasp virulence proteins and the fly immune system are dynamic and constantly evolving (Colinet et al., 2013; Dubuffet et al., 2009; Goecks et al., 2013; Mortimer et al., 2013; Schlenke et al., 2007). Given wasp venoms are made up of dozens of proteins and that there are numerous wasp species that infect Drosophila, further characterization of these virulence proteins and the innate immune mechanisms they suppress looks to be a fertile line of research. The next step will be to determine how flies have evolved or are evolving resistance to these specialized wasp virulence proteins.

The melanotic encapsulation response is not the only defense fruit flies have against their wasp parasites; at least four immune behaviors also play an important role in preventing wasp infection or in curing fly larvae once infected. First, when wasps insert their ovipositors into the body cavity of fruit fly larvae, the larvae undergo a specialized rolling behavior to dislodge the wasp before she can lay an egg. The behavior is mediated by nocireceptors from class IV multidendritic neurons (Hwang et al., 2007). Second, infected fly larvae have been shown to use a secondary metabolite of yeasts, alcohol, as a form of medication. *D. melanogaster* larvae live in rotting fruits and have evolved tolerance of the products of fermentation they are surrounded by. Fly larvae infected by wasps actively seek out high levels of alcohol to consume because raising their hemolymph alcohol content can kill the wasp larvae living in their hemolymph in the absence of a melanotic encapsulation response (Milan et al., 2012). Third, when adult flies sense the presence of wasps in their environment, they preferentially lay their eggs in more alcoholic substrates, which both protects their offspring from being infected and enables the larvae to cure themselves if they become infected. Fly adults sense wasps by sight, causing a reduction of neuropeptide F levels in the fan-shaped body of the brain and enhanced alcohol-seeking behavior (Kacsoh et al., 2013). As a counter-defense to fly medication behavior, the *D. melanogaster* specialist wasp *Leptopilina boulardi* has evolved higher tolerance of alcohol than its generalist relative

L. heterotoma, protecting *L. boulardi* from the host medication behavior (Bouletreau and David 1981; Milan et al., 2012). Fourth, in the presence of parasitic wasps, female adult *D. melanogaster* reduce their oviposition rate, presumably in anticipation of finding non-infested oviposition sites later, or as a cost of producing stronger, more resistant offspring (Lefevre et al., 2012).

Finally, similar to *Wolbachia*-mediated immunity against viral and fungal infections, the Spiroplasma parasite/symbiont of *Drosophila hydei* has been shown to protect that fly against infection by endoparasitoid wasps (Xie et al., 2011, 2010). Wasps infect Spiroplasma-infected flies at similar rates and their eggs hatch normally, but the development of hatched wasp larvae in fly hemolymph is severely impaired. Symbiotic bacteria have now been shown to modulate host immunity in a number of natural host-parasite systems, but the genetic bases for symbiont-mediated immunity are still poorly understood. In pea aphids, which benefit from protection against parasitic wasps when harboring the bacterial symbiont *Hamiltonella defensa*, it is actually the Hamiltonella bacteriophage APSE, rather than the bacteria itself, which confers protection (Degnan et al., 2009; Degnan and Moran, 2008b; Degnan and Moran, 2008a; Moran et al., 2005; Oliver et al., 2005; van der Wilk et al., 1999).

8. Future Prospects

D. melanogaster has been and continues to be exploited for understanding conserved immune mechanisms targeted at generalist and non-natural parasites, many of which would likely be considered opportunistic if they actually infected a fly in nature. We argue here that this powerful innate immunity model system can also be exploited to uncover more specialized virulence strategies and immune mechanisms of naturally interacting parasites and hosts. Are there fruit fly immune mechanisms similar to R gene-based immunity in plants? What are the weak links in innate immune mechanisms that specialist fruit fly parasites tend to exploit?

Future research growth in natural Drosophila-parasite interactions will likely come from study of natural transposable element, viral, bacterial, fungal, trypanosomatid, and wasp parasites of flies. The transposable elements of *D. melanogaster* are well-characterized and the piRNA pathway appears to be the main host defense, but many functional aspects of the piRNA system are unclear. Only a handful of natural fly viruses have been identified and cultured, even though several other viruses were identified via microscopy from wild and lab *D. melanogaster* strains (Brun and Plus, 1980; Plus et al., 1976; Plus et al., 1975a; Plus et al., 1975b; Plus and Duthoit, 1969). Surveys of bacteria associated with *D. melanogaster* in nature have identified hundreds of bacterial species (Chandler et al., 2011; Corby-Harris et al., 2007). Some of these bacteria may be pathogenic when injected back into flies (and other insects), but in most cases it remains unclear which bacterial species would be pathogenic using a natural infection route. Surprisingly, outside of *Wolbachia* and perhaps *P. entomophila*, specialist *D. melanogaster* bacterial parasites have yet to be identified. Numerous trypanosomatid species infect Drosophila in nature (Chandler and James, 2013; Wilfert et al., 2011), but we know virtually nothing about host specificity of Drosophila trypanosomatids, or types of immune mechanisms that the flies might utilize against these protozoan parasites. Microsporidians and the yeastlike fungus *Coccidiascus legeri* are the only specialized fungal parasites known from *D. melanogaster*, but nothing is known about fly immune mechanisms against such fungal parasites. Finally, new parasitoid wasp species that successfully infect *D. melanogaster* continue to be discovered (Allemand et al., 2002; Mitsui et al., 2007; Novkovic et al., 2011), but we know almost nothing about the natural histories and natural host ranges of these wasps. We are just beginning to determine the identities of the venom cocktails specialist wasps use to circumvent the fly cellular immune

response (Colinet et al., 2013; Goecks et al., 2013; Heavner et al., 2013; Mortimer et al., 2013). These and other topics will become more important as the field of Drosophila immunity matures from being based almost solely on non-natural host-parasite interactions to more heavily based on natural interactions.

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- We discuss the benefits of using natural pathogens in research.
- We review a variety of known natural pathogens of Drosophila.
- We cover the genetics and evolution of Drosophila's immune response.

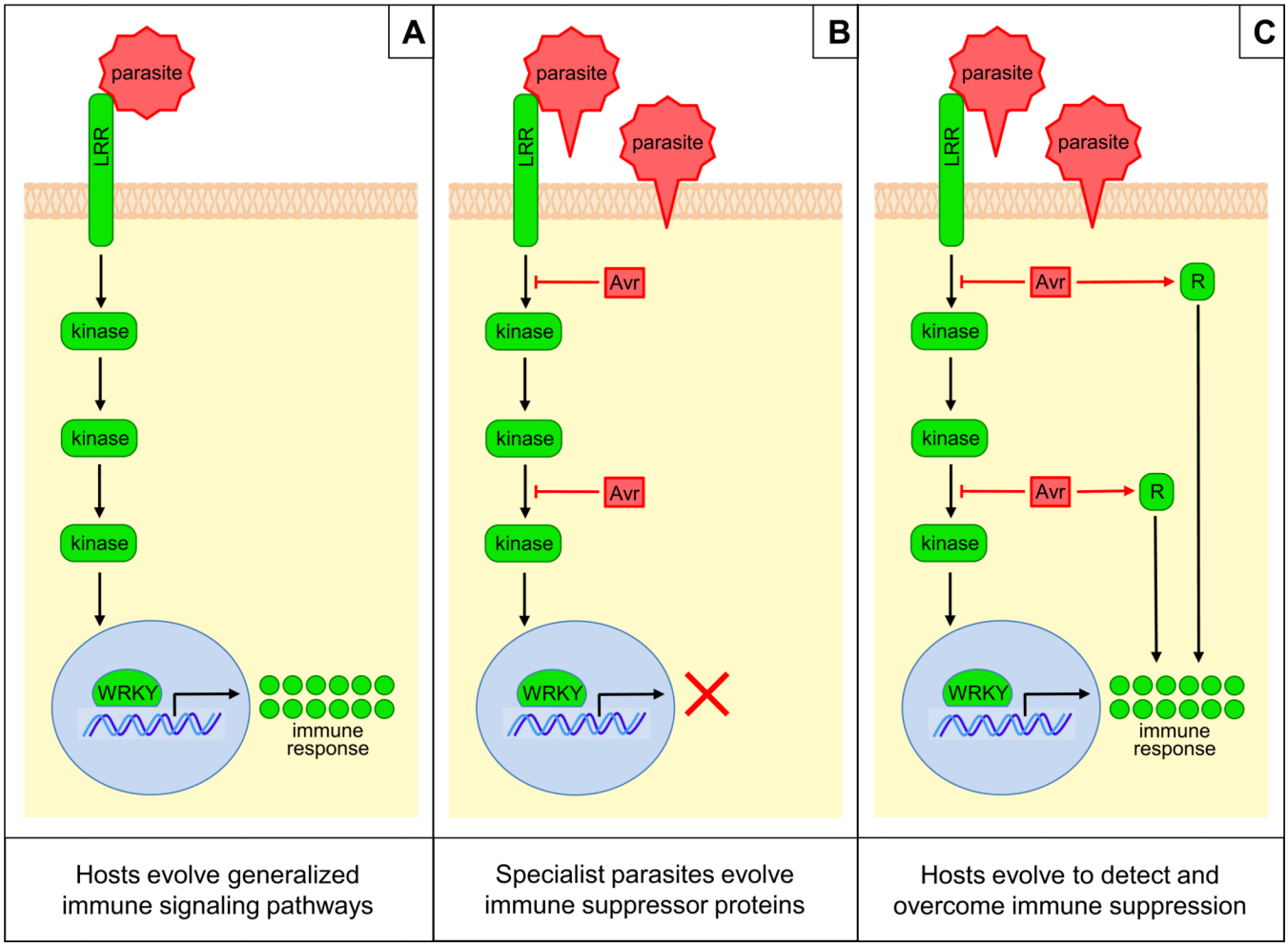

Figure 1.

A plant example of host-parasite antagonistic coevolution. In Step A, host plants evolve an anti-parasite immune response that protects them from most parasites. Specialist parasites evolve suppressive virulence mechanisms in Step B, selecting the plant hosts to counter-evolve secondary immune mechanisms in Step C. Steps B and C can then repeatedly cycle in an evolutionary "arms race". Use of non-natural parasites in infection experiments can limit our understanding of host immunity to the general types of immune responses exemplified in Step A. (Chisholm et al., 2006).

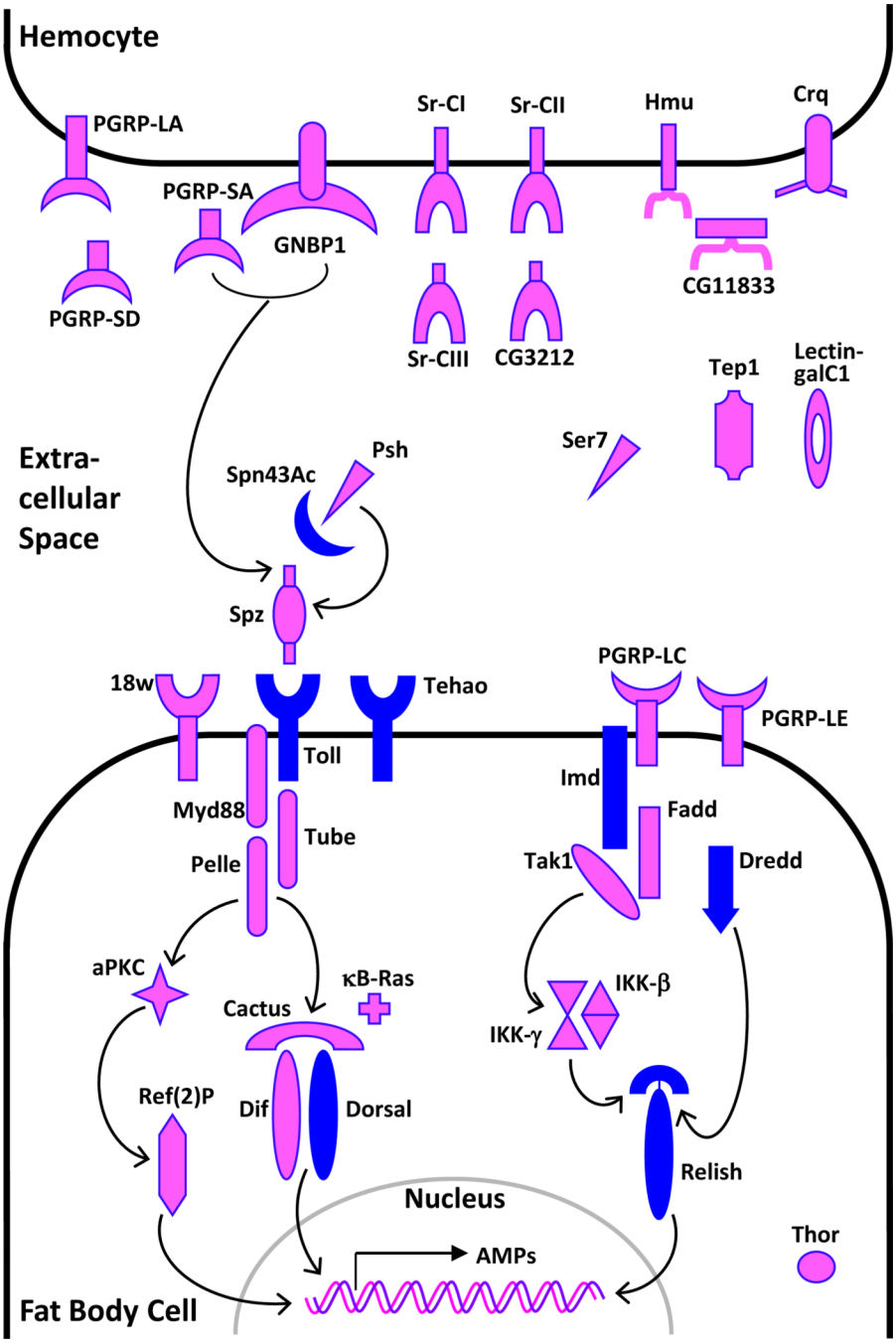

Figure 2.

Evolution of immune genes in *Drosophila simulans*. Numerous secreted and hemocyte membrane-bound antigen receptors are represented, as well as members of the Toll and Imd pathways, which control the humoral response to microbial infections in the fat body. Genes shown in blue showed significant evidence of adaptive evolution along the *D. simulans* lineage. These data suggest that the main virulence strategy of natural *D. simulans* parasites is production of secreted virulence proteins that suppress immune signaling through the Toll and Imd pathways, rather than recognition avoidance or antimicrobial peptide tolerance (antimicrobial peptide data not shown) (Schlenke and Begun, 2003).

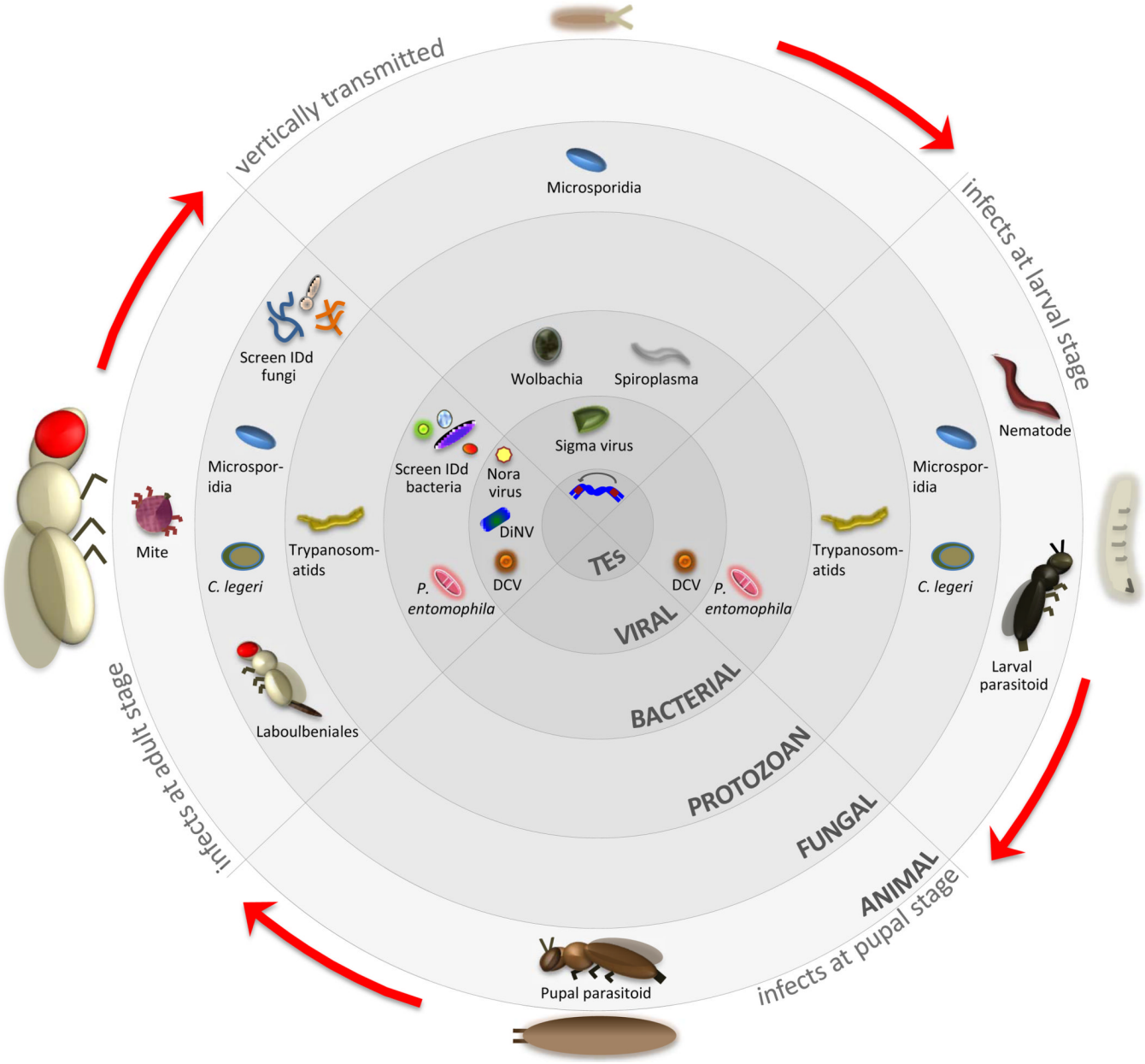

Figure 3.

The natural parasites of Drosophila. The parasites are arranged by phylogenetic group as well as by the fruit fly life stage they infect. Note that all parasites that infect fly eggs are transmitted vertically from parent flies, while all other parasites are horizontally transferred. Only parasites specifically named in the text or identified by screens are included. Other natural parasites of Drosophila have been dentified but are relatively uncharacterized and not included here.

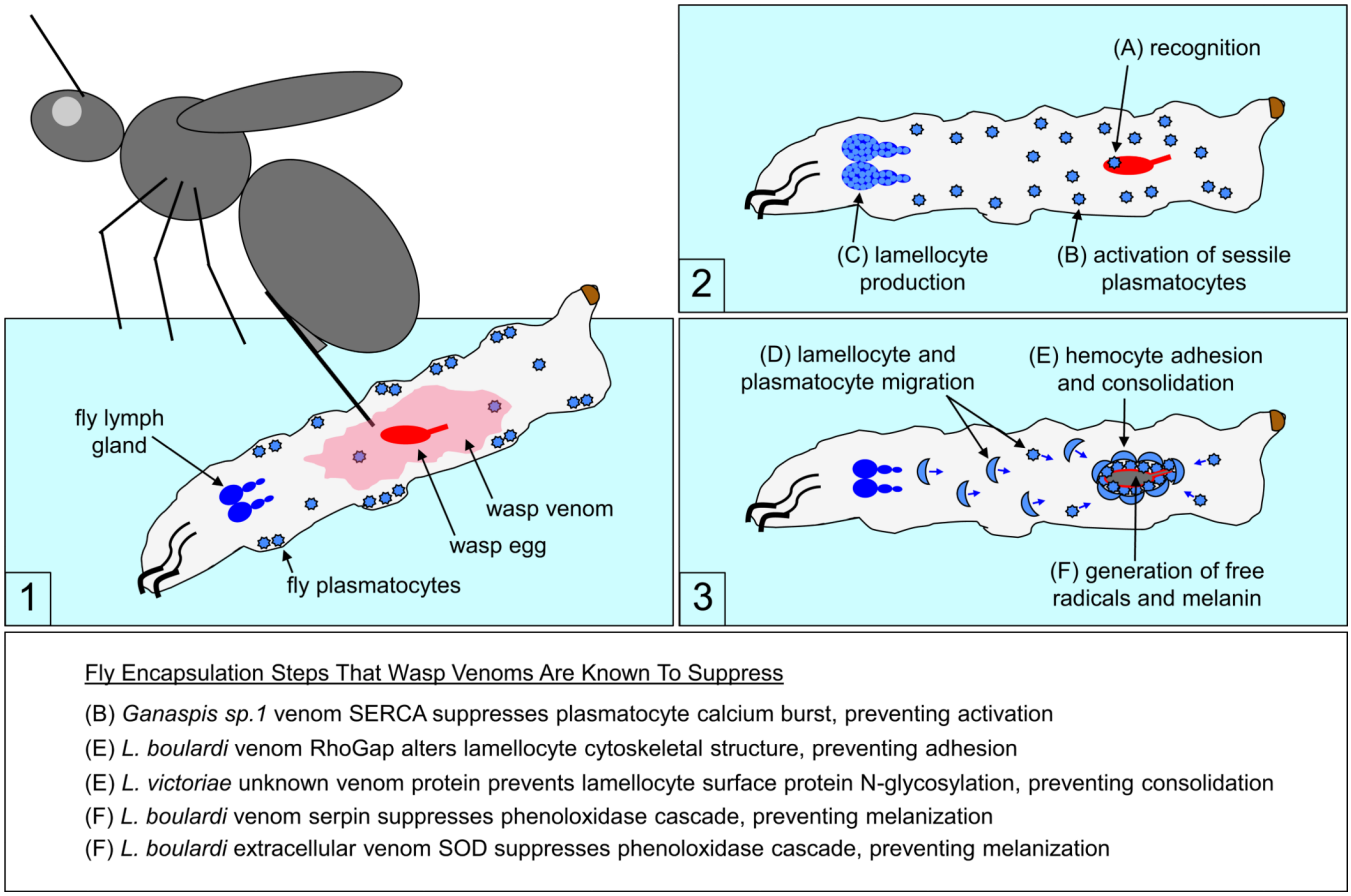

Figure 4.

Interactions between Drosophila and endoparasitoid wasps. Wasps inject an egg and venom into the body cavity of a fly larva, and the fly recognizes the egg as foreign and mounts a melanotic encapsulation response. However, wasps evolve venom proteins that have specific ways of suppressing this fly immune response.