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The Drosophila immune response against a natural pathogen, the parasitic wasp.

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Doctor of Philosophy

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Abstract

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Drosophila melanogaster has long been a valuable model of innate immunity. The Drosophila humoral innate immune response against opportunistic pathogens has been deeply investigated, and this work has led to a detailed map of innate immunity pathways including Toll, Imd, Jak-Stat, and JNK. However, the Drosophila cellular innate immune response remains to be characterized. Drosophila are hosts to parasitic wasps, which can infect a majority of individuals in natural populations and induce a strong cellular immune response. Thus, we study the Drosophila immune response against parasitic wasps to learn more about the genetics of cellular innate immunity. Additionally, because parasitic wasps are common natural pathogens of Drosophila, we also study the evolution of genes important in the fruitfly immune response against wasps to gain insight into the selective pressures imposed on Drosophila by wasps.

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Chapter 1: The Drosophila immune response against natural pathogens.

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 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 protein alleles, were a "match" for the Avr proteins, or Avr protein 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) Figure adapted from (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 anti-microbial 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 likely 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 as 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 anti-viral 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 yeast-like 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.

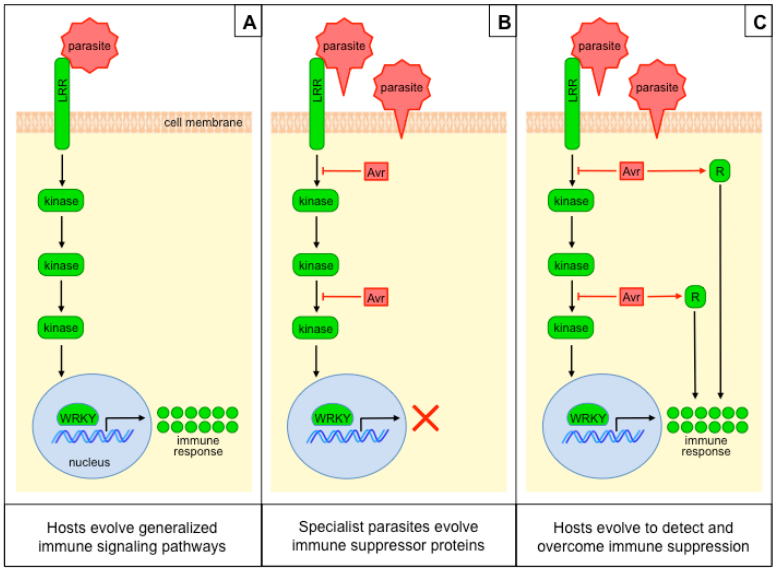

Figure 1

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).

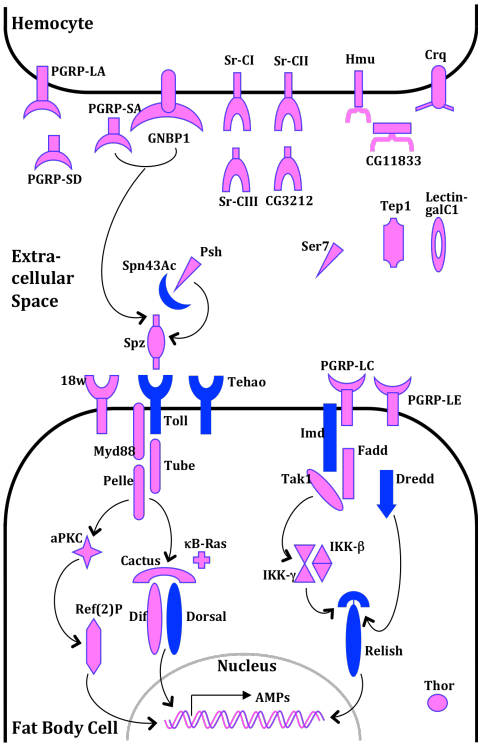

Figure 2

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).

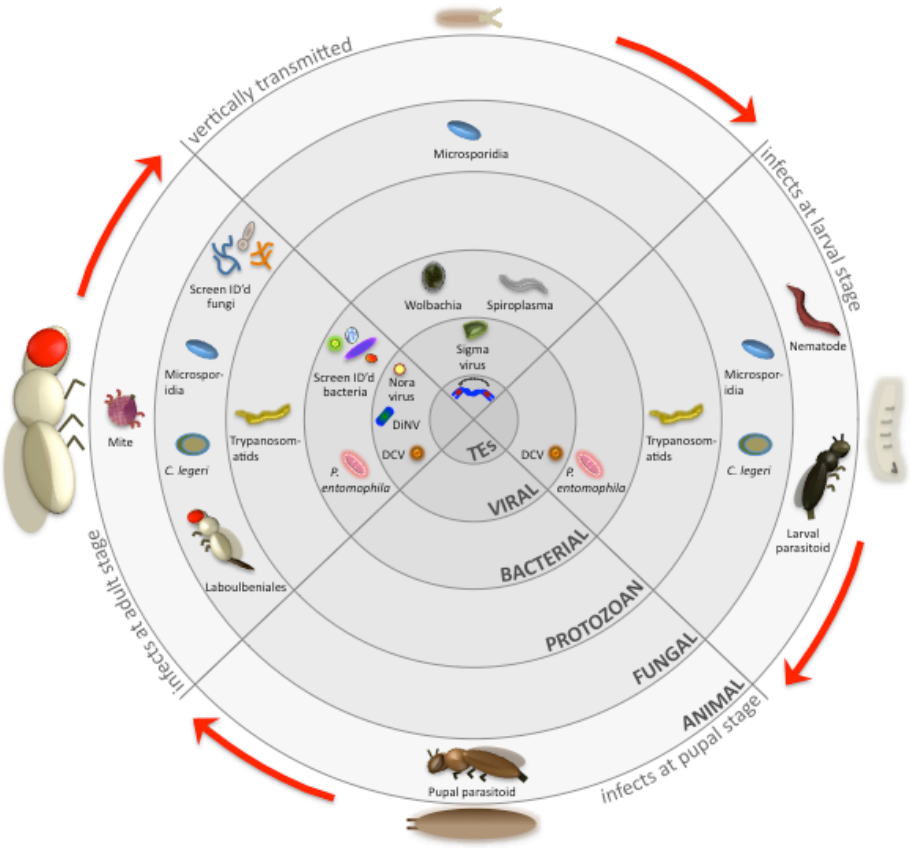

Figure 3

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 identified but are relatively uncharacterized and not included here.

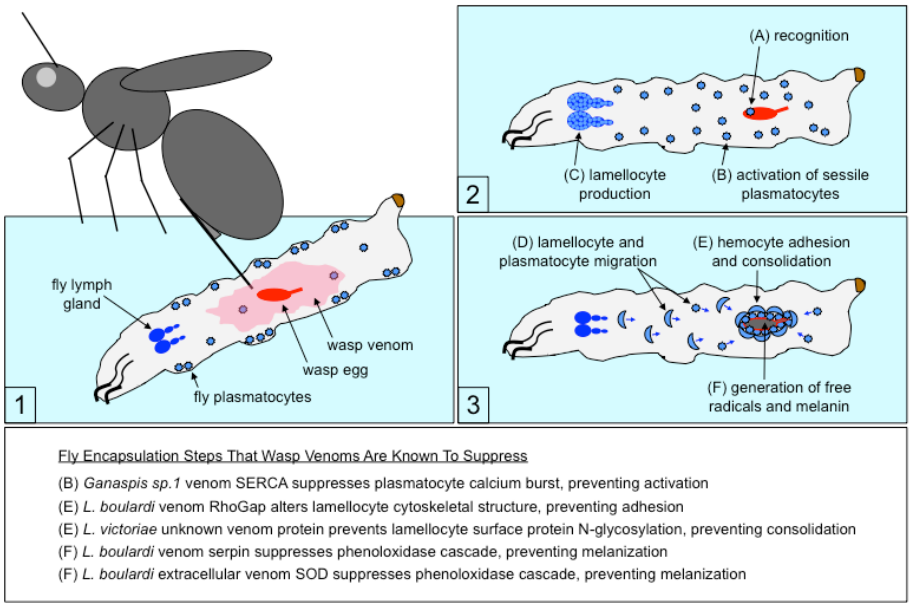

Figure 4

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.

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Chapter 2: Evolution and expression of *lectin-24A*.

Over the past 15 years, *D. melanogaster* has served as a valuable model system for the molecular genetics of innate immunity (Lemaitre, Hoffmann 2007). *D. melanogaster* is especially useful for understanding innate immune systems of other insects, such as insect vectors of human disease, agricultural pests, and crop pollinators (Schneider, Shahabuddin 2000; Evans et al. 2006). Innate immunity can be divided into two main components, the humoral response and the cellular response. The Drosophila humoral response has been intensely studied for its role in combating bacterial and fungal infections, but may also be responsible for aspects of macroparasite killing. It is governed by the fat body, which controls release of immune active extracellular proteins such as antimicrobial peptides and complement-like proteins (*e.g.* thioester-containing proteins) into the hemolymph (Lemaitre, Hoffmann 2007). Two major humoral immune response pathways operating in the fat body are the NF-κB pathways Toll and Imd, to which the JAK/STAT and JNK pathways appear to play complementary roles (Boutros, Agaisse, Perrimon 2002). There is some evidence of Toll pathway specificity for infection by gram(+) bacteria and fungi and Imd pathway specificity for infection by gram(-) bacteria, but this distinction is not absolute and crosstalk between these and other pathways appears common (Lemaitre, Hoffmann 2007).

The Drosophila cellular response is mediated by the lymph gland (the hematopoietic organ) and the hemocytes, of which there are three types. The plasmatocytes represent ~95% of the standing hemocytes, act as sentinels of infection, and are responsible for phagocytosis. The crystal cells make up the remaining 5% of the standing hemocyte

population. They are responsible for generating melanin and associated free radicals, which are important in coagulation, wound healing, and pathogen killing. The lamellocytes are large flattened hemocytes responsible for encapsulating macroparasites such as parasitic wasp eggs, and their production is induced in response to infection. Lamellocytes are derived from pro-hemocytes in the larval lymph gland, but also may develop directly from circulating plasmatocytes (Rizki 1957; Honti et al. 2010). The Toll pathway plays a major role in hematopoiesis, while the JAK/STAT pathway appears to be important for the development of lamellocytes (Sorrentino, Melk, Govind 2004).

We have decided to focus on the molecular biology and evolution of genes potentially involved in Drosophila's cellular immune response against parasitic wasps. Several wasp species from multiple Hymenopteran families attack Drosophila larvae and pupae in nature, including generalists and numerous specialists of particular Drosophila species and species groups. The larval parasites lay single eggs in their hosts that, if allowed to hatch, begin to consume internal fly tissues. Successful infections are always lethal, with the young wasps eclosing from fly pupal cases. Wasps are one of the most prevalent parasites of Drosophila in nature, infecting upwards of 50% of individuals in some natural fly populations (Carton 1986; Janssen et al. 1987; Fleury et al. 2004).

Wasp eggs elicit a strong cellular encapsulation response and can be killed by resistant flies. The current model for the steps involved in encapsulation is as follows (Carton, Nappi 1997): (1) Following receptor binding to the wasp egg, circulating hemocytes contact the wasp egg and lyse, releasing signaling factors. (2) This signal causes

activation of nearby hemocytes and potentiates hematopoiesis in the lymph gland, leading to the production of lamellocytes. (3) The lamellocytes migrate towards, and then attach and spread around the wasp egg. (4) Finally, the inner cells of the capsule surrounding the wasp egg lyse and release reactive oxygen species and an impermeable layer of melanin, resulting in death of the parasite. Encapsulation of wasp eggs is functionally similar to vertebrate granuloma formation (McKerrow, Jeong, Beckstead 1985), although little attempt has been made to establish mechanistic homology. Although many Drosophila genetic pathways including Toll and JAK/STAT have been shown to be involved in the encapsulation response (Sorrentino, Melk, Govind 2004; Zettervall et al. 2004), the genetic bases for many aspects of the encapsulation response, *e.g.* recognition, signaling between hemocytes and the lymph gland, and the encapsulation killing mechanism, remain relatively poorly characterized.

It remains an extremely interesting question as to what kind of innate immune receptors animals might use to detect other animals. It is relatively straight-forward for animal hosts to recognize bacteria and fungi as pathogens because of the distinct cell wall and cell membrane epitopes they carry, but how does a fly recognize a parasite that is much more similar to itself, such as a parasitic wasp? To date, two whole-genome gene expression studies have been conducted on wasp-attacked flies to identify novel genes involved in Drosophila's immune response against the wasps (Wertheim et al. 2005; Schlenke et al. 2007). In both of these studies, one using the Figitid wasp *Leptopilina boulardi* and one using the Braconid *Asobara tabida,* a C-type lectin named *lectin-24A* (Theopold et al. 1999) was more than seven-fold upregulated following wasp attack.

lectin-24A was also found upregulated in larvae from multiple mutant fly strains that produce melanotic aggregates of hemocytes (Bettencourt et al. 2004; Zettervall et al. 2004; Walker et al. 2011).

Lectins are sugar-binding proteins that can distinguish very specific sugar moieties, and as such have long been considered ideal candidates for specific recognition receptors in host innate immune systems. Perhaps the best-characterized immune lectin is the mannose binding lectin of the vertebrate complement cascade (Turner 1996), although many other lectins have known roles as opsonins and attack proteins in the immune systems of vertebrates and other organisms (as reviewed in (Marques, Barracco 2000; Cambi, Koopman, Figdor 2005; Willment, Brown 2008)). Thus, it was seen as a surprise that no lectins were indentified in early microarray studies of Drosophila infected with bacteria and fungi. However, two different C-type lectins were shown to aid in the Drosophila encapsulation reaction against agarose beads *in vitro* (Ao, Ling, Yu 2007), suggesting such proteins may act specifically in the cellular immune response against macroparasites. Together with the microarray and melanotic nodule mutant studies, these data suggest *lectin-24A* might play an important role in melanotic capsule formation, and perhaps as a pattern recognition receptor for wasp eggs.

In this study, we test whether *lectin-24A* is a general stress response, wound response, or immune response gene, or whether it plays a specific role in the response to attack by parasitic wasps. Furthermore, we characterize the tissue specificity of its expression following wasp attack, to better understand its potential mechanistic role in the anti-wasp

immune response. Finally, immune genes are expected to evolve rapidly and adaptively over time in order to keep pace with constantly evolving pathogen-mediated selection pressures, and Drosophila immune genes are no exception (Schlenke, Begun 2003; Schlenke, Begun 2005; Jiggins, Kim 2006; Sackton et al. 2007; Lazzaro 2008). We undertake population genetic and molecular evolution analyses of the *lectin-24A* locus to determine whether it also shows a history of rapid and adaptive evolution.

Results

Expression analysis

We measured expression levels of *lectin-24A* along with two known Drosophila immune genes, *Drosomycin* and *Diptericin*, which are antimicrobial peptides commonly used to gage activation of the two immunity signaling pathways Toll and Imd, respectively. In previous studies, *Drosomycin* and/or *Diptericin* were found upregulated after wasp attack at times ranging from 12-48 hours post-infection (Coustau et al. 1996; Nicolas, Nappi, Lemaitre 1996; Schlenke et al. 2007). We found that expression of all three genes significantly increased in whole *D. melanogaster* larvae attacked by *L. boulardi* wasps at the 2-5 hours post-infection timepoint, compared to unattacked flies (Figure 1, Supplemental Material S2(a)). *lectin-24A* was upregulated 32 fold at this timepoint, and *Drosomycin* and *Diptericin* were upregulated 81 and 38 fold, respectively, although the two antimicrobial peptide genes showed much greater variation in fold change than *lectin-24A*. Thus, wasp infection potentially activates both the Toll and Imd pathways. At the 9-12 hour post-infection timepoint, *lectin-24A* remained significantly upregulated by wasp attack, but upregulation of the two antimicrobial peptides dropped to lower, non-significant levels.

Different regulatory trends are seen in response to piercing with a sterile needle (which presumably mimics the cuticular injury caused by wasp oviposition), or piercing with septic needles dipped in gram(+) and gram(-) bacterial cultures. *D. melanogaster* larvae significantly down-regulate *lectin-24A* 3-5 fold at the early timepoint following sterile and septic injury with gram(+) and gram(-) bacteria. Pierced larvae then show modest, non-significant upregulation in the 2-6 fold range at the later timepoint following gram(+) and gram(-) injury, and significant upregulation following sterile injury, although these levels of upregulation are significantly lower than that reached by *lectin-24A* following wasp attack at the corresponding timepoint (Figure 1(a), Supplemental Material S2(a)). Thus, the *lectin-24A* response to wasp infection is very different from that to sterile or septic injury.

At the early timepoint, expression patterns for *Drosomycin* and *Diptericin* following sterile injury, gram(+) injury, and gram(-) injury were noticeably different than that of *lectin-24A*, either showing no change in expression level (*Drosomycin)* or non-significant upregulation (*Diptericin*) (Figure 1(b)(c)). Expression of *Drosomycin* and *Diptericin* at the later timepoint following sterile and septic injuries showed a trend of non-significant upregulation similar to that of *lectin-24A* following sterile and septic injuries. No significant differences in expression were observed between sterile injury, gram(+) bacterial infection, or gram(-) bacterial infection for any of the three genes, suggesting the fly larvae do not distinguish between the three treatments at these timepoints

(Supplemental Material S2(a)). Altogether, these data show that *lectin-24A* is regulated in a different manner than genes that are known targets of the Toll and Imd pathways.

We next investigated tissue specificity of *lectin-24A* expression following wasp attack in two tissues important for hemolymph immunity (fat body, hemocytes) and two control tissues (gut, body wall). The constitutive expression level of *lectin-24A* was significantly greater in the fat body than the other three tissues at both timepoints (Figure 2, Supplemental Material S2(a)). Furthermore, *lectin-24A* expression was significantly upregulated approximately 9 and 16 fold in the fat body following wasp attack at 2-5 hours and 9-12 hours post-attack. Expression of *lectin-24A* in the hemocytes, gut, and body wall also significantly increased following wasp attack, excluding the 9-12 hour timepoint in hemocytes (Figure 2, Supplementary Material S2(b)), but the overall levels of *lectin-24A* transcript (standardized by *alphaTub84B*) in these tissues still averaged approximately 40 times less than *lectin-24A* levels found in the fat body. These data indicate that the fat body, the most important humoral immunity organ, is the major site of both constitutive and wasp attack-induced *lectin-24A* production.

Species range and gene structure

The coding region of *D. melanogaster lectin-24A* is 846 bp (282 aa) long, with the lectin domain located at amino acids 169-280. The gene has no other characterized domains and also contains no introns, similar to other Drosophila C-type lectins. We used BLAST (specifically, tblastx) to search for orthologs of the *D. melanogaster lectin-24A* sequence in the nucleotide collection of Genbank. *lectin-24A* was present in only *D. melanogaster*

and its *D. simulans* sister group (including *D. simulans* and *D. sechellia*). Because the third member of the simulans group, *D. mauritiana*, has not been genome-sequenced, we tested and confirmed by PCR and sequencing that *D. mauritiana* also has a *lectin-24A* ortholog (Genbank #, Supplementary Material S3(a)). However, no *lectin-24A* ortholog was found in other genome-sequenced members of the melanogaster group (*D. yakuba*, *D. erecta, D. ananassae,* Supplementary Material S3(b)), in any of the five other genome-sequenced Drosophila species, or in any other organism. BLAST also fails to identify close homologs to *lectin-24A* in the *D. melanogaster* genome. Although both the non-lectin and lectin domains of *lectin-24A* BLAST to other *D. melanogaster* lectins (e.g., *lectin-24Db*, and *lectin-28C*, respectively), the sequence homology in both cases is quite poor (Supplementary Material S3(c)(d)).

In *D. melanogaster*, the gene *CG2818* is immediately upstream of *lectin-24A*, and the gene *Shaw* is immediately downstream, with *lectin-24A* in reverse orientation relative to the flanking genes. There is very little intergenic sequence between the transcript sequences of these three genes, as the 3' transcript end of *lectin-24A* overlaps the 3' transcript end of *CG2818* by 11 bp, and the 5' transcript start of *lectin-24A* is only 414 bp away from the 5' transcript start of *Shaw*. Orthologs of *CG2818* and *Shaw* are found physically adjacent to one another, but with little intervening sequence, across the melanogaster group of the genus Drosophila (Supplementary Material S3(b)), suggesting that *lectin-24A* arose from an insertion in the common ancestor of *D. melanogaster* and *D. simulans*.

We sequenced *lectin-24A* in California population samples of *D. melanogaster* and *D. simulans* and from more ancestral population samples from Africa (Genbank #). In these *D. melanogaster* strains, the consensus open reading frame (ORF) length is 282 aa (as in the genome sequenced strain), but in *D. simulans* the consensus ORF length is 291 aa (as in the genome sequences of *D. simulans* and *D. sechellia*). This is due to a difference in the position of the stop codon between these two species caused by an insertion in *D. melanogaster* relative to *D. simulans* at the 3' end of the coding sequence.

Interestingly, ORF length variation also exists within the African population samples of both *D. melanogaster* and *D. simulans* and in the single *D. mauritiana* allele we sequenced, due to multiple independent mutations (Supplementary Material S3(a), S4(a)). Six of ten *D. melanogaster* strains from Malawi had one of two different premature stop codons that fall within the lectin domain, resulting in truncation of *lectin-24A* and of the lectin domain itself. The first of these early stop codon variants, found in 2 strains, was generated by a point mutation resulting in a 29 aa truncation of the 3' end of *lectin-24A*, and the loss of 27 of the 112 amino acids from the lectin domain. The second early stop codon variant, found in 4 strains, was generated by an out-of-frame 169 bp deletion within the lectin domain, in combination with a short insertion, that formed a new stop codon that results in a 66 aa truncation of the 3' end of *lectin-24A*, and the loss of 64 of the 112 amino acids from the lectin domain. There appears to be an excess of shared nonsynonymous mutations upstream of the stop codons in the two *D. melanogaster* premature stop codon variants (Supplementary Material S4(b)), suggesting that the premature stop codons were independently selected for in this divergent haplotype

background. Also, one of nine *D. simulans* strains from Zimbabwe had a premature stop codon located upstream of the lectin domain, resulting in a severe truncation of *lectin-24A* (Supplementary Material S4(a)(c)). This early stop codon resulted from a 1 bp deletion, and shortens the ORF to 75 aa. Finally, the *D. mauritiana* strain we sequenced had a premature stop codon compared to the consensus lengths of other species, truncating the ORF to 103 aa (Supplementary Material S3(a), S4(a)).

Polymorphism analysis

We tested for unusual haplotype structure at the *lectin-24A* locus of the four population samples by comparing observed haplotype diversity (Hd) (Nei 1987) to a distribution of haplotype diversities generated by neutral coalescence simulation. Unlike the other samples, the California *D. simulans* population sample showed significantly low Hd, yielding only two haplotypes from the eight strains sequenced (Table 1). One distinct haplotype was found in one of eight strains (cal sim 1), while the other haplotype was found in seven of eight strains (Figure 3). The cal sim 1 haplotype is very similar to those of some African *D. simulans* strains, while the other California alleles have a divergent haplotype that is quite distinct from any African strain (Supplementary Material S4(c)).

Low Hd at a locus can be explained by various demographic forces operating on a population, or by the selective sweep of a beneficial allele. Demographic forces, however, are expected to affect the whole genome, whereas selection is usually locus-specific. We compared Hd of *lectin-24A* in the California *D. simulans* population sample

to the haplotype diversities of 68 other genes located across the genome from the same eight California *D. simulans* strains (Figure 4). Immunity and non-immunity genes are indicated separately as it was previously found that immune genes have significantly lower Hd than non-immune genes (Schlenke, Begun 2003). We found that the Hd of *lectin-24A* is lower than 67 of the other 68 genes analyzed (2nd percentile), and that the only gene with similarly low Hd is the immune gene *Hemomucin*. Thus, low Hd observed at *lectin-24A* in the California *D. simulans* population is likely the result of a selective sweep.

Selection skews haplotype structure at a target locus but also at loci linked to the selected locus. Thus, determining the physical span of reduced Hd to the flanks of *lectin-24A* in the California *D. simulans* population sample can help to narrow the list of genes that were potentially selection targets. We sequenced genomic regions flanking *lectin-24A* by approximately 2 kb, 5 kb, 15 kb, and 25 kb upstream and downstream and calculated Hd at those loci (Figure 5, Table 1). The region of reduced Hd appears centered on *lectin-24A* and is approximately 10 kb long, as Hd increases to approximately normal values further to either side. This 10 kb region contains 2 full and 2 partial genes other than *lectin-24A* (*cutlet*, *CG31955*, *CG2818*, and *Shaw*).

Three other partially independent population genetic descriptors also show a pattern of non-neutral polymorphism structure centered on the *lectin-24A* locus (Table 1). Tajima's D, a measure of the allele frequency distribution (Tajima 1989), was significantly low at *lectin-24A* but not at flanking loci, indicating an excess of rare alleles. Fay and Wu's H, a

measure of the frequency distribution of derived alleles (Fay, Wu 2000), was significantly low at *lectin-24A* and one flanking locus, indicating an excess of high frequency derived polymorphisms. ZnS is a measure of linkage disequilibrium, the degree to which alleles at different sites co-occur on haplotypes (Kelly 1997). ZnS was significantly high at *lectin-24A* and the two immediately flanking loci. Although the larger number of segregating sites at the *lectin-24A* locus disproportionately increases the power of significance tests at this locus, the absolute value of each statistic for the *lectin-24A* locus is greater than or equal to the same value from every flanking locus. The direction of each of these skews is consistent with the effects of a strong, recent selective sweep at the *lectin-24A* locus.

Divergence analysis

We compared the ratio of nonsynonymous to synonymous substitutions per site (dN/dS) at *lectin-24A* to other genes in the genome using the *D. melanogaster* and *D. simulans* genome sequences. Because *lectin-24A* is only found in the *D. melanogaster* and *D. simulans* lineages, no outgroup sequence is available to polarize substitutions to one or the other of the *D. melanogaster* and *D. simulans* lineages. The dN/dS value of 0.878 is significantly high (98th percentile) compared to the distribution of dN/dS values from every other shared gene in this species pair, which averages at 0.151 (Begun et al. 2007) (Figure 6). Similar analysis on *lectin-24A* using our own *D. melanogaster* and *D. simulans* strains (excluding the early termination codon strains) yields a dN/dS of 0.806 over the full coding region, 0.691 for the non-lectin domain region specifically, and 1.018 for the lectin domain. High dN/dS values can be caused by

the recurrent fixation of beneficial nonsynonymous mutations by selection, but may also indicate relaxed functional constraint at a locus if dN/dS is less than or equal to 1.0.

One method for distinguishing adaptive evolution from relaxed functional constraint is the McDonald-Kreitman test, which compares the ratio of nonsynonymous to synonymous differences between species to that same ratio within species (McDonald, Kreitman 1991). For genes evolving neutrally under varying degrees of functional constraint, these ratios are expected to be equal. For a gene evolving adaptively, however, beneficial nonsynonymous mutations are expected to sweep to fixation very fast, contributing little to nonsynonymous polymorphism but accumulating as nonsynonymous substitutions. We performed multiple McDonald-Kreitman tests using different combinations of our *D. melanogaster* and *D. simulans* population samples (Table 2). The *D. simulans* population samples consistently yielded highly significant results in the direction of excess nonsynonymous substitutions, while the *D. melanogaster* population samples trended in the same direction but did not reach statistical significance. For the analysis that includes polymorphism from all population samples, if we assume that it is only the nonsynonymous fixations causing the deviation from our expectation of equal nonsynonymous to synonymous ratios (Smith, Eyre-Walker 2002), we can infer that approximately 33 of the 51 nonsynonymous differences between *D. melanogaster* and *D. simulans lectin-24A* sequences were fixed as the result of positive selection rather than genetic drift. The nonsynonymous fixations are distributed relatively equally between the non-lectin and lectin domains of *lectin-24A* (Table 2).

Discussion

Unlike plant and vertebrate systems, most studies on Drosophila immunity have utilized pathogens that are not known to infect Drosophila in nature. While artificial infection of Drosophila with non-natural pathogens has been a powerful tool for uncovering basic aspects of the immune system, it is possible that essential parts of the immune system have been overlooked because they mediate specific responses against infection strategies of specialist parasites. In this paper, we have focused on a candidate Drosophila immune gene with potential specificity for infections by parasitic wasps, which are one of the most important groups of specialist Drosophila pathogens in nature.

Expression of *lectin-24A* was previously shown to significantly increase in Drosophila larvae after attack by parasitic wasps from two different families (Wertheim et al. 2005; Schlenke et al. 2007). Our first goal was to confirm *lectin-24A* induction following attack by the parasitic wasp *L. boulardi* using qRT-PCR. We indeed found a 32-42 fold increase in whole larvae *lectin-24A* transcript levels at both timepoints post-infection. The two antimicrobial peptide genes *Drosomycin* (often used to measure the activation of the Toll pathway) and *Diptericin* (often used to measure the activation of the Imd pathway) (Lemaitre, Hoffmann 2007), were also upregulated following wasp attack, although their expression levels began declining at the later timepoint. These data suggest wasp infection induces a general immune response shortly after infection that potentially includes activation of both the Toll and Imd pathways.

It was previously shown that sterile and septic injuries of adult flies result in induction of both *Drosomycin* and *Diptericin* at early timepoints following treatment, regardless of the bacterial type used (Lemaitre, Reichhart, Hoffmann 1997). The response to septic injury begins to show specificity at later timepoints past 6-12 hours post-treatment, *i.e.* *Drosomycin* stays induced following gram(+) bacterial infection, and *Diptericin* stays induced following gram(-) bacterial infection. A study using fly larvae also found a common induction of antimicrobial peptides at early timepoints following either sterile or septic injury (Bettencourt et al. 2004). Similarly, we found little difference in the upregulation of *Drosomycin* or *Diptericin* across sterile and septic injury treatments in fly larvae in our relatively early timepoint trials (Figure 1). Both genes were upregulated following injury, but *Drosomycin* was not upregulated until the 9 hour timepoint, and both genes showed a large amount of variance in upregulation across replicates that caused non-significant results. In contrast, *lectin-24A* was significantly downregulated by sterile and septic injuries at the early timepoint, before being modestly upregulated 2-6 fold at the later timepoint, indicating *lectin-24A* is part of a different immune regulatory network than *Drosomycin* and *Diptericin*.

We found that *lectin-24A* transcript was made at significantly higher abundance in the fat body, the main humoral immunity secretory organ, than in other tissues. The Toll, Imd, JAK/STAT, and JNK pathways are known to influence fat body production of immune proteins (Boutros, Agaisse, Perrimon 2002; Delaney et al. 2006; Lemaitre, Hoffmann 2007), and thus would seem to be good candidates for inducing *lectin-24A* expression. Given that *Drosomycin* and *Diptericin* expression levels can be used to measure the

relative activation of the Toll and Imd pathways, respectively (Lemaitre, Hoffmann 2007), and that they show expression patterns different from *lectin-24A* following injury, we find it unlikely that Toll or Imd are the primary pathways responsible for *lectin-24A* induction. Interestingly, however, both *Drosomycin* and *Diptericin* were significantly upregulated following wasp attack. These two genes may be responding to the cuticle injuries made by wasp ovipositors, but their expression may also be enhanced by a wasp infection-specific activation of JAK/STAT, JNK, or other pathways that undergo crosstalk with Toll and Imd (*e.g.* Zettervall et al. 2004).

Altogether, our *lectin-24A* expression analyses are consistent with numerous other transcriptomic and proteomic studies using assorted Drosophila life stages, tissues, and pathogens for infection. For example, *lectin-24A* was not found upregulated in microarray studies on adult *D. melanogaster* infected with bacterial, fungal, viral, and microsporidian pathogens (De Gregorio et al. 2001; Irving et al. 2001; Roxstrom-Lindquist, Terenius, Faye 2004; Dostert et al. 2005; Carpenter et al. 2009), in larvae infected with bacteria (Vodovar et al. 2005), or in Drosophila hemocyte-like S2 and mbn2 cells treated with lipopolysaccharide or bacteria (Boutros, Agaisse, Perrimon 2002; Johansson, Metzendorf, Soderhall 2005). Nor were Lectin-24A protein levels increased in larval or adult flies infected with bacteria, fungi, or lipopolysaccharide (Levy, Bulet, Ehret-Sabatier 2004; Vierstraete et al. 2004a, 2004b) or in mbn2 cells treated with lipopolysaccharide (Loseva, Engstrom 2004). Thus, *lectin-24A* shows a distinct, wasp attack-specific expression pattern and cannot be categorized as a general stress response, wound response, or immune response gene.

It is inferred that the Lectin-24A protein is secreted because it carries a secretion signal sequence. Given the ability of lectins to recognize specific cell-surface sugar moieties, it is particularly interesting to consider whether Lectin-24A might act as the initial immune recognition protein for wasp eggs. Induction of *lectin-24A* in the fat body two and nine hours post-infection does not immediately suggest a primary recognition role, as some recognition of attack must have occurred in the hemocoel prior to the induction of *lectin-24A* expression. However, it is possible that constitutively produced Lectin-24A may be responsible for recognizing wasp eggs and initiating a response that includes a positive feedback loop of self-induction, for example if more Lectin-24 protein aids in opsonizing the entire wasp egg surface. Furthermore, it is possible that flies might recognize and respond to some other aspect of the wasp attack, such as the wound caused by the wasp ovipositor or the wasp venom and its effects, before expressing molecules that can recognize wasp eggs. Alternatively, because *lectin-24A* expression is induced in response to two different wasps from different Hymenopteran families (Wertheim et al. 2005; Schlenke et al. 2007), and is also upregulated in mutant Drosophila strains that constitutively produce melanotic aggregates of hemocytes (Bettencourt et al. 2004; Zettervall et al. 2004; Walker et al. 2011), Lectin-24A may instead be a general melanotic encapsulation response gene, for example acting to facilitate the hemocyte-hemocyte interactions necessary for capsule formation. Further study of Lectin-24A's molecular function will be required to tease apart any role Lectin-24A plays in the anti-wasp immune response, be it in recognition or some other function.

We cannot rule out the possibility that genes we find upregulated after wasp attack, including *lectin-24A*, are beneficial to the wasps and may even be purposefully induced by the wasps themselves. It has long been known that parasitic wasp venoms can manipulate many aspects of their hosts' physiology (Vinson, Iwantsch 1980), and the wasp strains used in previous microarray studies and in this study are highly successful at evading and/or suppressing the immune response of *D. melanogaster* (Rizki, Rizki 1990; Eslin et al. 1996; Labrosse et al. 2003). Evidence in support of this hypothesis are the number of naturally segregating early termination codons in *lectin-24A* that might deprive the wasps of whatever potential benefit they receive from the full-length protein, as well as the fact that a fly strain artificially selected for resistance against the wasp *A. tabida* had significantly reduced constitutive *lectin-24A* expression compared to a control, unselected strain (Wertheim et al. 2011). However, we find it unlikely that wasps benefit from *lectin-24A* induction for the following three reasons: (1) given most Drosophila species don't require *lectin-24A*, it seems unlikely that the majority of *D. melanogaster* and *D. simulans* strains would continue to carry a gene that benefits one of their most common types of pathogens; (2) it seems unlikely that two wasps from different families (*A. tabida* and *L. boulardi*) could have evolved the same *lectin-24A* induction strategy, especially given that *L. heterotoma* (a close relative to *L. boulardi*) does not cause *lectin-24A* induction in infected hosts; (3) given that *A. tabida* and *L. boulardi* have European and worldwide ranges, respectively, it is surprising that early termination codons are only segregating in African fly populations. Thus, we continue to favor the hypothesis that Lectin-24A is an anti-wasp immune protein.

Surprisingly, no obvious homolog of *lectin-24* was found outside of the *D. melanogaster* and *D. simulans* sister clade, despite the fact that the genes immediately flanking *lectin-24A* upstream and downstream are present in tandem across the melanogaster group of the genus Drosophila. In previous work, *lectin-24A* was predicted to have originated via DNA-based duplication and not by an RNA-based insertion, because there is no evidence of a poly(A) tail or direct repeats flanking *lectin-24A* (Chen, Zhang, Long 2010). *De novo* evolution from standing DNA sequence is also an unlikely explanation because the DNA sequence that became *lectin-24A* seems to have been an insertion unique to the genome of the common ancestor of the *D. melanogaster* and *D. simulans* lineages.

It was suggested that the parental gene of *lectin-24A* was either *lectin-28C* (Zhou et al. 2008) or *lectin-24Db* (Chen, Zhang, Long 2010), the two *D. melanogaster* lectins that produced the best BLAST hits to *lectin-24A's* lectin domain and non-lectin domain, respectively. However, because full-length *lectin-24A* does not BLAST with high confidence to any specific lectin in the *D. melanogaster* genome, it must have evolved very rapidly from its parental sequence(s). Furthermore, none of the 40 other *D. melanogaster* C-type lectin domain-containing genes (as annotated in FlyBase), nor any gene immediately flanking *lectin-24A*, were as strongly or consistently upregulated following *L. boulardi* attack or as strongly or consistently downregulated following attack by the highly immune suppressive wasp *L. heterotoma* (Supplementary Material S5) (Schlenke et al. 2007), suggesting *lectin-24A* regulatory elements have also rapidly evolved. Rapid evolution of newly duplicated genes is expected, as gene redundancy results in relaxed selection on the new gene and the potential for accumulation of

otherwise deleterious nonsynonymous mutations (as reviewed in (Long et al. 2003)). Such alterations can cause pseudogenization, subfunctionalization (when a new gene specializes on a subset of the functions of its parental gene), or neofunctionalization (when a new gene develops a novel function) of a young gene. Given *lectin-24A*'s apparently unique role in melanotic encapsulation, it appears that neofunctionalization is contributing to the adaptive evolution of *lectin-24A*.

Some of the naturally segregating, premature termination codon *lectin-24A* haplotypes may represent a more advanced state of neofunctionalization or possibly pseudogenization. It is highly unlikely that the early termination mutations are deleterious alleles because of the relatively high frequency of haplotypes that have them, the fact that four unique mutations in three species contribute to this pool, and the fact that all such mutations are geographically localized to the African region (*D. mauritiana* is endemic to the Mauritius Islands). It is more likely that the truncated proteins perform some beneficial function, or that a null allele of *lectin-24A* is harmless or even beneficial under certain conditions in African fly populations. Interestingly, the melanogaster subgroup of the genus Drosophila (which includes *D. melanogaster* and the *D. simulans* clade) originated in Africa (Lemeunier 1986), and the diversity of Drosophila parasitic wasps that infect members of the subgroup appears to be highest there (Allemand et al. 2002).

A variety of evidence supports the idea that *lectin-24A* has evolved adaptively, especially in the *D. simulans* lineage. Haplotype structure in the California *D. simulans* population

is highly unusual, with one diverged invariant haplotype present in seven of eight strains, and a second, quite distinct African-like haplotype present in one of eight strains. Haplotype diversity is significantly low when compared to neutrally simulated data or to data from other genes from the same population sample, and extends only a very short distance around *lectin-24A*. A similar non-neutral pattern is observed for other kinds of population genetic descriptors, including Tajima's D, Fay and Wu's H, and linkage disequilibrium. These analyses suggest the common *lectin-24A* haplotype (or a haplotype from one of four closely linked genes) has been the target of a recent selective sweep, having increased in frequency in the population so rapidly and so recently that no recombinants or new mutations are observed. Furthermore, the dN/dS value for *lectin-24A* between *D. melanogaster* and *D. simulans* is in the top 1.46% of all genes in the genome, and McDonald-Kreitman analyses reveal a tremendous excess of nonsynonymous fixations within and outside the *lectin-24A* lectin domain. Altogether, *lectin-24A* polymorphism and divergence statistics suggest this recently acquired gene has evolved (and is evolving) novel function.

Previous work has shown that Drosophila immune genes as a class evolve more rapidly and adaptively than other genes in the genome (Schlenke, Begun 2003; Schlenke, Begun 2005; Jiggins, Kim 2006; Sackton et al. 2007; Lazzaro 2008). Furthermore, a number of immune genes described in *D. melanogaster*, such as *Hemese* and the drosomycins, are relatively newly arisen, being limited to the melanogaster species group (Sackton et al. 2007). These data suggest fly hosts adapt to their pathogen environments using a combination of *de novo* gene origination and standing gene evolution, and *lectin-24A*

appears to encompass both these methods of immune adaptation. If wasp venom proteins evolve to target and impair specific fly immune proteins, and if Lectin-24A showed novel anti-wasp function that wasps were not yet able to counteract, *lectin-24A* origination and adaptation may have been (and may continue to be) part of a cyclic arms race between Drosophila and parasitic wasps. However, given our limited understanding of the biological function of Lectin-24A, coevolution with wasps is only one potential explanation for the adaptive evolution of *lectin-24A*.

In conclusion, *lectin-24A* is a new gene that is evolving rapidly and adaptively, and that has a unique expression pattern of upregulation following wasp attack but downregulation immediately following wounding or bacterial infection. These data, together with the facts that *lectin-24A* has a secretion signal sequence and a sugar-binding lectin domain, suggest it plays some role in recognition of extracellularly exposed sugars during the fly immune response against parasitic wasps, although at what stage of the response is unclear. It will be interesting to further dissect the regulatory network governing *lectin-24A* expression and to uncover the functional role of Lectin-24A in fly-wasp interactions in the future.

Materials and Methods

Gene expression analysis

All aspects of the fly and wasp rearing were conducted in a 24-25 degrees C incubator with a 12:12 light cycle. For gene expression analyses following wasp infection, we used *D. melanogaster* strain Oregon R and the relatively virulent *L. boulardi* strain Lb17

(Schlenke et al. 2007). Flies were allowed to lay eggs for 3 hours, and batches of 60 larvae from these egg lays were later moved onto 35 mm petri dishes containing standard Drosophila medium. 72 hours after the egg lay period, 10 experienced female wasps were placed in each of the dishes for a 2 hour attack time. 2 and 9 hours post-attack, fly larvae were dissected or flash frozen for expression timepoint analyses. Due to the 2 hour attack time and a 1 hour handling time, these larvae had developed between 2-5 and 9-12 hours post-attack, respectively. Note that it is possible that some fly larvae may not be attacked by wasps in the given time, however, we expect the infection rate to be greater than 90% under these conditions given past results (Schlenke et al. 2007). Ten larvae per dish were used for whole body expression analysis, and another 10 larvae were dissected for individual tissue expression analyses. For the dissected larvae, the fat body, gut, and body wall (cuticle plus associated muscle) tissues were separated, and were only used if a wasp egg was found during the dissection. Dissected tissues were immediately placed into Trizol (Invitrogen), while whole larvae were placed into 1.5 mL tubes and frozen in liquid nitrogen for future processing. The remaining 40 larvae per dish were used for hemocyte analyses by draining larval hemolymph onto a metal rod that was immediately submerged into Trizol.

For gene expression analyses following sterile and septic injuries, the same larval rearing conditions were used. 72 hours post-egg lay, 20 Oregon R larvae were each pierced with a 0.1 mm diameter stainless steel needle (Fine Science Tools) dipped in sterile LB broth, *Enterococcus faecalis* gram(+) bacterial culture grown overnight and diluted to OD_{600} = 1.0, or *Escherichia coli* gram(-) bacterial culture grown overnight and diluted to OD_{600} =

1.0. Following injury, larvae were placed on moist Kimwipes inside a 35 mm petri dish, then later transferred to plates containing standard Drosophila medium. At 2 and 9 hours post-injury, 10 of the larvae were flash frozen in 1.5 mL tubes in liquid nitrogen.

Total RNA extraction for all samples was done using Trizol following the Invitrogen recommended protocol. cDNA was synthesized using the Qiagen Quantitect Reverse Transcription Kit. Each cDNA sample was used as a template for semi-quantitative (comparative Ct) real time PCR using Applied Biosystems Power SYBR Green Master Mix. Each sample was run in triplicate to account for within sample variance, and any significant outliers within a sample triplicate were discarded. *alphaTub84B* (which was not differentially regulated following wasp attack (Schlenke et al. 2007)) was used as a reference gene to control for differences in total cDNA amounts across samples. Intron spanning primers used for *alphaTub84B* are as follows: 5'-ACACTTCCAATAAAAACTCAATATGC-3', 5'-CCGTGCTCCAAGCAGTAGA-3'. Primers used for *lectin-24A* (which does not contain introns) are as follows: 5'-CGAGTGGGGTCCTGGTGAAC-3', 5'-GAAACGCATCGCTCTTGGTC-3'. Primers used for *Drosomycin* and *Diptericin*, antimicrobial peptides regulated by the Toll and Imd pathways, respectively, were modified from (Ayres, Schneider 2009) as follows: *Drosomycin* 5'-GTACTTGTTCGCCCTCTTCG-3', 5'-CTTGCACACACGACGACAG-3', and *Diptericin* 5'-ACCGCAGTACCCACTCAATC-3', 5'-CCCAAGTGCTGTCCATATCC-3'. Melting curves for PCR products were checked to ensure that no off-target loci were amplified by any primer pair. All expression experiments were done in 4 biological replicates, and un-treated control larvae or larval

tissues were included for each replicate (except for the gram(+) treated samples which were compared to 2 un-treated replicates).

Relative quantification (RQ, also known as delta delta CT) data was collected to represent the fold change of each gene following treatment relative to un-treated control samples. Most gene expression data is presented as log2 transformation of RQ data (log2(RQ)), except in the case of tissue-specific expression of *lectin-24A*, in which the abundance of *lectin-24A* relative to the reference gene (values known as delta CT) is used for data presentation. Statistical analysis was performed on log2 transformation of relative abundance values (log2(delta CT)) when testing if a gene is differentially regulated following treatment or differentially regulated between different tissues, and on log2(RQ) values when testing if a gene is differentially regulated following one treatment relative to another (as suggested in (Rieu, Powers 2009)).

Molecular Evolution

California *D. melanogaster* and *D. simulans* sequence data are from sets of eight highly inbred lines made from field-caught inseminated females collected in Winters, California. African *D. melanogaster* and *D. simulans* sequence data are from sets of ten and nine isofemale lines collected in Malawi and Zimbabwe, respectively. For the subset of African *D. melanogaster* strains found to be heterozygous at *lectin-24A*, these strains were crossed to *D. melanogaster* deficiency strain 5330 (Bloomington stock center, deficiency Df(2L)ed1) to generate individuals hemizygous for *lectin-24A* for use in sequencing.

PCR primers were designed to amplify an approximately 1900 bp region that includes the full coding sequence of *lectin-24A* plus the presumed 5' regulatory region (bp 3716293-3718252). For the California *D. simulans* population sample, we also designed PCR primers to amplify approximately 500-700 bp regions flanking *lectin-24A* at various distances. PCR products were sent to Beckman Coulter Genomics for purification and Sanger sequencing, using four internal primers for *lectin-24A* itself, and the PCR primers for flanking loci. Sequences for all primers used in the sequence analyses are provided (Supplementary Material S1).

Sequence data were edited using Lasergene software and population genetic and molecular evolution analyses were run in DnaSP version 5.10.01 (Librado, Rozas 2009). For the divergence and Fay and Wu's H statistics, which require an outgroup sequence, we used the genome sequenced *D. melanogaster* strain as an outgroup for the *D. simulans* sequences, and the *D. simulans* consensus genome sequence as an outgroup for the *D. melanogaster* sequences. Significance of some population genetic statistics for various population samples and loci was calculated by comparing the observed values to those obtained from 10,000 neutral coalescence simulations. Simulated data were generated in DnaSP by using the observed number of segregating sites from each sample and under the conservative assumption of no recombination. Fly strains found to have early stop codons relative to the *D. melanogaster* genome sequence were not included in McDonald-Kreitman or dN/dS analyses for two reasons: (1) the possibility that sequence downstream and potentially upstream of the early termination codons may be under

relaxed functional constraint, and (2) the large deletion responsible for one early termination codon causes a large portion of the *lectin-24A* coding sequence, including part of the lectin domain, to be lost from the DnaSP analyses. Furthermore, comparisons between *D. melanogaster* and *D. simulans* coding sequences used coordinates for the consensus *D. melanogaster* open reading frame rather than the longer *D. simulans* consensus open reading frame.

Supplementary Material

primer name primer sequence (5'-3') description
lectin24A 2715f tgcgagatcccagtcaggtaacat forward PCR primer for lectin-24A
lectin24A 5057r ctggggattttggggagtctggtc reverse PCR primer for lectin-24A
lectin24A 2876f cctgcacgccatcatctacgacaa sequencing primer for lectin-24A
lectin24A 3113f gaaacgaggagcagcaggcataga sequencing primer for lectin-24A
lectin24A 3113bf aactggctagatgccatactgg sequencing primer for lectin-24A
lectin24A 3650f ttccggaggctgcagacacaaag sequencing primer for lectin-24A
lectin24A 3650bf ttccggaggctgcagacacaaa sequencing primer for lectin-24A
lectin24A 3650cf aggtgacctcccattcg sequencing primer for lectin-24A
lectin24A 3650df ctgagaaatagccatccatttg sequencing primer for lectin-24A
lectin24A 4915r gggggcgcctccttccacta sequencing primer for lectin-24A
1-2Dlectin24Af gaaatggccgcagaaatcaaag forward PCR primer for 2 kb downstream of lectin-24A
1-2Dlectin24Ar acaaacccaacgctcagtctaagg reverse PCR primer for 2 kb downstream of lectin-24A
1-2Ulectin24Af actcgggagcggctattttgtcg forward PCR primer for 2 kb upstream of lectin-24A
1-2Ulectin24Ar ctcaggttcatgggcgtcactttg reverse PCR primer for 2 kb upstream of lectin-24A
5Dlectin24Af agccgcgcatttggtccttgtt forward PCR primer for 5 kb downstream of lectin-24A
5Dlectin24Ar cccacttgttcggcgatgagataa reverse PCR primer for 5 kb downstream of lectin-24A
5Ulectin24Af tgccgccctctccagtcac forward PCR primer for 5 kb upstream of lectin-24A
5Ulectin24Ar gaacgcccaaaggtccac reverse PCR primer for 5 kb upstream of lectin-24A
15Dlectin24Af ttagcccggcaatcagagtttc forward PCR primer for 15 kb downstream of lectin-24A
15Dlectin24Ar ttttcgcgtaattgtcaggttgtc reverse PCR primer for 15 kb downstream of lectin-24A
15Ulectin24Af gataaccgcgcagaaccgtaag forward PCR primer for 15 kb upstream of lectin-24A
15Ulectin24Ar ccccgcacatctgacattttg reverse PCR primer for 15 kb upstream of lectin-24A
25Dlectin24Af tcttcgcatttcgcatacccacac forward PCR primer for 25 kb downstream of lectin-24A
25Dlectin24Ar gccgagcgaaaagcgaagataata reverse PCR primer for 25 kb downstream of lectin-24A
25Ulectin24Af cggaaggcgcaggagcat forward PCR primer for 25 kb upstream of lectin-24A
25Ulectin24Ar tatcttttcgcaatctaact reverse PCR primer for 25 kb upstream of lectin-24A

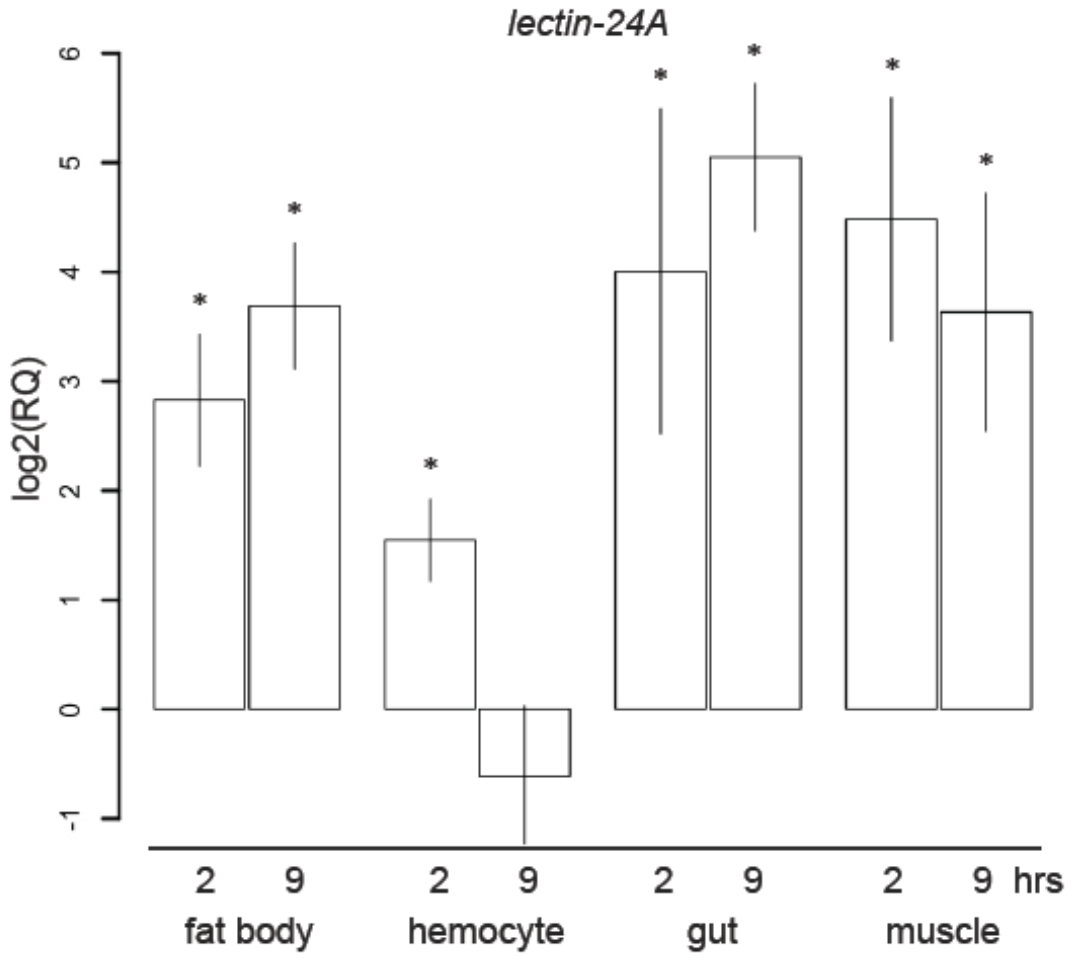
Supplementary Material S1: Primers used for molecular evolution analyses.

(a)

test used	data analyzed	*lectin-24A*	*Drosomycin*	*Diptericin*
2-tailed paired t-test	gene regulation 2 hours after wasp attack v control untreated	0.0033*	0.0328*	0.0499*
2-tailed paired t-test	gene regulation 9 hours after wasp attack v control untreated	0.0095*	0.0954	0.4599
2-tailed paired t-test	gene regulation 2 hours after poking with sterile needle v control untreated	0.0450*	0.7950	0.3240
2-tailed paired t-test	gene regulation 9 hours after poking with sterile needle v control untreated	0.0349*	0.0778	0.2519
2-tailed t-test, unequal n	gene regulation 2 hours after poking with septic (gram+) needle v control untreated	0.0284*	0.6908	0.2011
2-tailed t-test, unequal n	gene regulation 9 hours after poking with septic (gram+) needle v control untreated	0.2864	0.1683	0.0502
2-tailed paired t-test	gene regulation 2 hours after poking with septic (gram-) needle v control untreated	0.0318*	0.9254	0.5489
2-tailed paired t-test	gene regulation 9 hours after poking with septic (gram-) needle v control untreated	0.4049	0.0903	0.1138
2-tailed t-test, equal n	gene regulation 2 hours following wasp attack v 2 hours after poking with sterile needle	0.0002*	0.0247*	0.4068
2-tailed t-test, equal n	gene regulation 9 hours following wasp attack v 9 hours after poking with sterile needle	0.0426*	0.3965	0.6786
2-tailed t-test, equal n	gene regulation 2 hours following wasp attack v 2 hours after poking with septic (gram+) needle	0.0005*	0.0094*	0.4282
2-tailed t-test, equal n	gene regulation 9 hours following wasp attack v 9 hours after poking with septic (gram+) needle	0.0044*	0.1879	0.8213
2-tailed t-test, equal n	gene regulation 2 hours following wasp attack v 2 hours after poking with septic (gram-) needle	0.0002*	0.0168*	0.3290
2-tailed t-test, equal n	gene regulation 9 hours following wasp attack v 9 hours after poking with septic (gram-) needle	0.0352*	0.3005	0.4842
2-tailed t-test, equal n	gene regulation 2 hours following poking with sterile needle v 2 hours after poking with septic (gram+) needle	0.4888	0.5713	0.7355
2-tailed t-test, equal n	gene regulation 9 hours following poking with sterile needle v 9 hours after poking with septic (gram+) needle	0.1165	0.4182	0.7220
2-tailed t-test, equal n	gene regulation 2 hours following poking with sterile needle v 2 hours after poking with septic (gram-) needle	0.4502	0.7896	0.8163
2-tailed t-test, equal n	gene regulation 9 hours following poking with sterile needle v 9 hours after poking with septic (gram-) needle	0.4025	0.7629	0.7786
2-tailed t-test, equal n	gene regulation 2 hours following poking with septic (gram+) needle v 2 hours after poking with septic (gram-) needle	0.9670	0.7774	0.5687
2-tailed t-test, equal n	gene regulation 9 hours following poking with septic (gram+) needle v 9 hours after poking with septic (gram-) needle	0.9370	0.6229	0.3924
1-tailed paired t-test	*lectin-24A* upregulation in fat body 2 hours after wasp attack v control untreated	0.0091*		
1-tailed paired t-test	*lectin-24A* upregulation in fat body 9 hours after wasp attack v control untreated	0.0039*		
1-tailed paired t-test	*lectin-24A* upregulation in hemocytes 2 hours after wasp attack v control untreated	0.0128*		
1-tailed paired t-test	*lectin-24A* upregulation in hemocytes 9 hours after wasp attack v control untreated	0.7932		
1-tailed paired t-test	*lectin-24A* upregulation in gut 2 hours after wasp attack v control untreated	0.0370*		
1-tailed paired t-test	*lectin-24A* upregulation in gut 9 hours after wasp attack v control untreated	0.0025*		
1-tailed paired t-test	*lectin-24A* upregulation in body wall 2 hours after wasp attack v control untreated	0.0136*		
1-tailed paired t-test	*lectin-24A* upregulation in body wall 9 hours after wasp attack v control untreated	0.0222*		
2-tailed t-test, equal n	*lectin-24A* abundance in unattacked fatbody v unattacked hemocyte, 2 hour timepoint	0.0060*		
2-tailed t-test, equal n	*lectin-24A* abundance in unattacked fatbody v unattacked gut, 2 hour timepoint	0.0004*		
2-tailed t-test, equal n	*lectin-24A* abundance in unattacked fatbody v unattacked muscle, 2 hour timepoint	0.0078*		
2-tailed t-test, equal n	*lectin-24A* abundance in attacked fatbody v attacked hemocyte, 2 hour timepoint	0.0005*		
2-tailed t-test, equal n	*lectin-24A* abundance in attacked fatbody v attacked gut, 2 hour timepoint	0.0076*		
2-tailed t-test, equal n	*lectin-24A* abundance in attacked fatbody v attacked muscle, 2 hour timepoint	0.0031*		
2-tailed t-test, equal n	*lectin-24A* abundance in unattacked fatbody v unattacked hemocyte, 9 hour timepoint	0.0061*		
2-tailed t-test, equal n	*lectin-24A* abundance in unattacked fatbody v unattacked gut, 9 hour timepoint	0.0001*		
2-tailed t-test, equal n	*lectin-24A* abundance in unattacked fatbody v unattacked muscle, 9 hour timepoint	0.0008*		
2-tailed t-test, equal n	*lectin-24A* abundance in attacked fatbody v attacked hemocyte, 9 hour timepoint	0.0000*		
2-tailed t-test, equal n	*lectin-24A* abundance in attacked fatbody v attacked gut, 9 hour timepoint	0.0020*		
2-tailed t-test, equal n	*lectin-24A* abundance in attacked fatbody v attacked muscle, 9 hour timepoint	0.0047*		

(a)

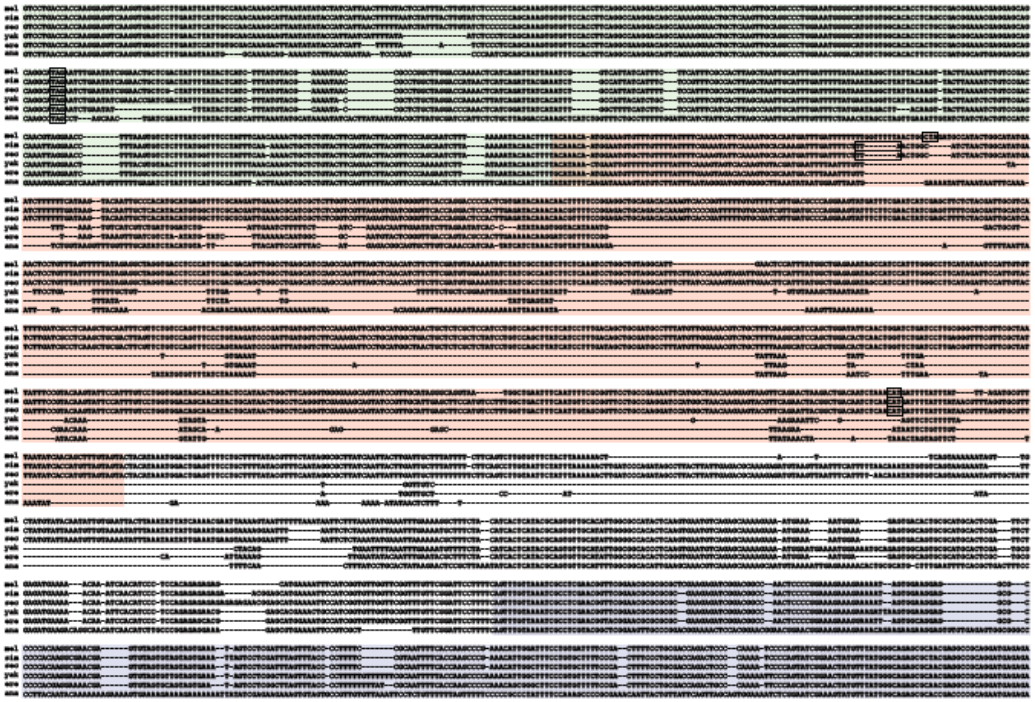
(b)



Supplementary Material S2: (a) Statistical tests and corresponding *p*-values from *lectin-24A*, *Drosomycin*, and *Diptericin* expression analyses, * $p<0.05$. (b) log2(Relative Quantification) of *lectin-24A* 2-5 and 9-12 hours following wasp attack in the fat body, hemocytes, gut, and body wall muscle, relative to unattacked tissues. Error bars +/- SEM. Significance values judged by comparison of wasp-attacked tissue averages to unattacked tissue averages, * $p<0.05$.

(a)

(b)



(c)

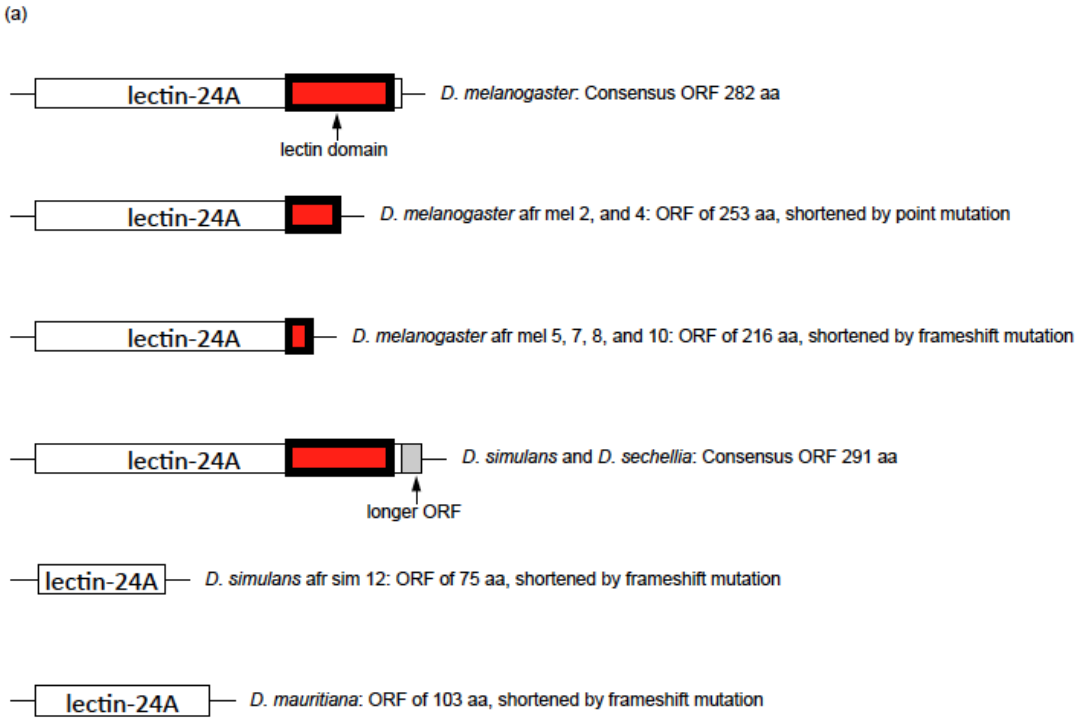
```
 Score = 44.6 bits (91),  Expect = 2e-04
 Identities = 15/50 (30%), Positives = 28/50 (56%), Gaps = 0/50 (0%)
 Frame = +1/+2

Query  91   PALCNGYCFPTLKPVMEYVAIHQDKWNTCTEILANEARKDQIQLNIQLDA  240
            P  C  +C   L+P+++++  HQ++WNT   +  NE +    ++  QL A
Sbjct  137  PNQCGEFCLSVLQPLLDHIVKHQEQWNTSEALWLNETQGKLDRIQTQLAA  286


 Score = 27.2 bits (53),  Expect = 2e-04
 Identities = 12/43 (28%), Positives = 25/43 (58%), Gaps = 0/43 (0%)
 Frame = +1/+2

Query  289  EKLDRMEREQFAMHESLETINRYLTVKLDRTKLQLEAIKNTMD  417
            ++LDRME  Q  + ESL+ +   L  +L + + Q + + + ++
Sbjct  338  DRLDRMEHLQTTLQESLKKMPAELDARLMKMENQQKTLGDQLE  466
```

(d)

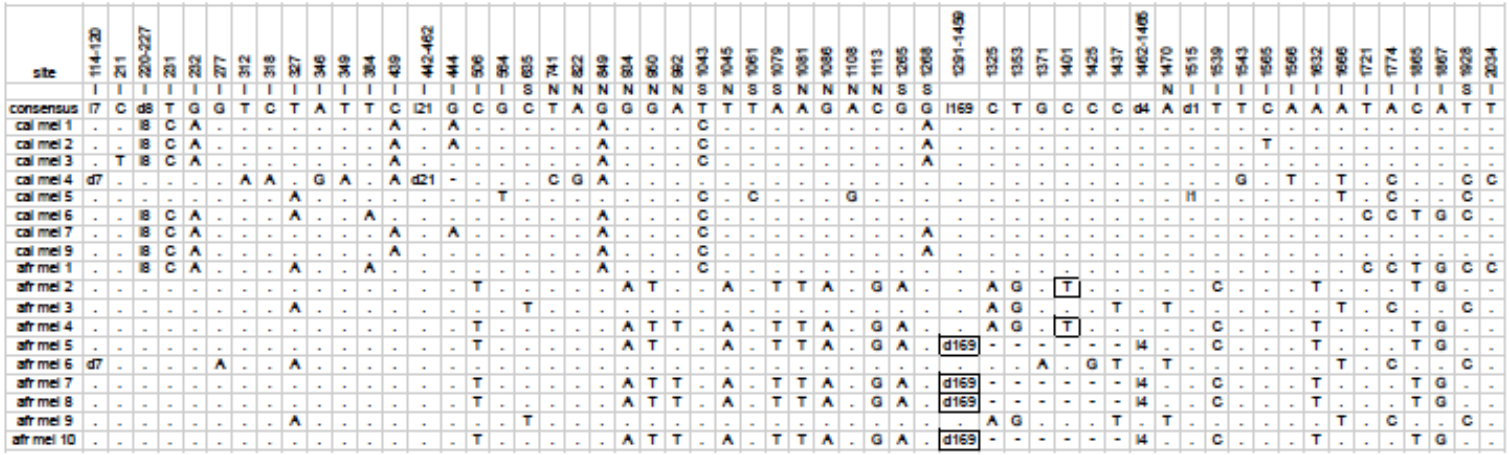
```
 Score =  115 bits (246),  Expect = 2e-23
 Identities = 44/111 (40%), Positives = 73/111 (66%), Gaps = 0/111 (0%)
 Frame = +1/+1

Query  1    YFYIEEDVELNWLDAQAKCRRMGGHLASIKTKQEFDAIVEKLDDSKSYFLGVNENTKTGD  180
            Y +IE+ V+ NW  A + C++MGG+LASI  + +F+AIV +L    +Y +G+++  + G
Sbjct  439  YLHIEDIVQQNWTSALSACQKMGGNLASIINEADFNAIVSQLSKDNTYMIGISDLAEKGV  618

Query  181  FVSAASGKSCLYHEWGPGEPHHNNDQERCVSILRKLMHVGNCTYEKRFICQ  333
            F+S +SGK   + +W PGEP + +  +RCVSI    M V +CT + ++IC+
Sbjct  619  FISVSSGKRAPFLKWNPGEPLYEHVDQRCVSIHNGGMWVASCTSDFKYICE  771
```

Supplementary Material S3: (a) Edited sequence alignment by ClustalW of *lectin-24A* and flanking regions from *D. melanogaster* (mel), *D. simulans* (sim), *D. sechellia* (sec), and *D. mauritiana* (mau). (b) Unedited sequence alignment by ClustalW of *lectin-24A* and flanking regions from *D. melanogaster* (mel), *D. simulans* (sim), *D. sechellia* (sec), *D. yakuba* (yak), *D. erecta* (ere), and *D. ananassae* (ana). Coding regions, untranslated regions, and introns (as annotated in FlyBase) are highlighted in red for *lectin-24A*, green for *CG2818*, and blue for *Shaw*. Start and stop codons are boxed. Note that the *lectin-24A* open reading frame is in reverse orientation. (c) tblastx output of the *D.*

melanogaster lectins that produced the best hit to the non-lectin domain portion of *lectin-24A* (bp 3717225-3717728 from FlyBase), *lectin-24Db*, and (d) the lectin domain of *lectin-24A* (bp 3716889-3717224), *lectin-28C*.



(b)



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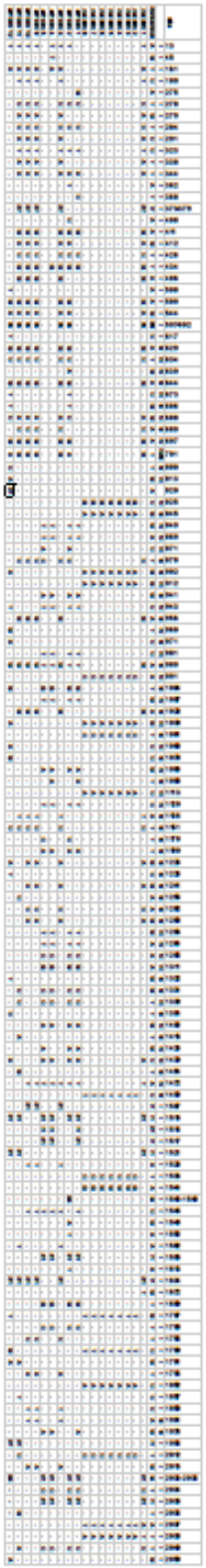
	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	I	ω	N	N	N	N	N	N	ω	N	ω	ω	N	N	N	N	ω	ω								N	I	I	I	I	I	I	I	I	I	I	I	ω	I
consensus	l7	C	d8	T	G	G	T	C	T	A	T	T	C	l21	G	C	G	C	T	A	G	G	G	A	T	T	T	A	A	G	A	C	G	G	l169	C	T	G	C	C	C	d4	A	d1	T	T	C	A	A	A	T	A	C	A	T	T
cal mel 1	.	.	i8	C	A	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cal mel 2	.	.	i8	C	A	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cal mel 3	.	T	i8	C	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cal mel 4	d7	.	.	.	.	.	A	A	.	G	A	.	A	d21	-	.	.	.	C	G	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	T	.	T	.	C	.	.	C	C
cal mel 5	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	C	.	C	.	.	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	i1	.	.	.	.	.	T	.	C	.	.	C	.
cal mel 6	.	.	i8	C	A	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	T	G	C	.
cal mel 7	.	.	i8	C	A	.	.	.	.	.	.	.	A	.	A	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
cal mel 9	.	.	i8	C	A	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
afr mel 1	.	.	i8	C	A	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	C	T	G	C	C
afr mel 2	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	T	.	.	A	.	T	T	A	.	G	A	.	.	A	G	.	T	.	.	.	.	.	C	.	.	.	T	.	.	.	T	G	.	.
afr mel 3	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	T	.	T	.	.	.	.	.	.	T	.	C	.	.	C	.
afr mel 4	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	T	T	.	A	.	T	T	A	.	G	A	.	.	A	G	.	T	.	.	.	T	G	.	.	.	.	C	.	.	.	T	G	.	.
afr mel 5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	T	.	.	A	.	T	T	A	.	G	A	.	d169	-	-	-	-	-	-	i4	.	.	C	.	.	.	T	.	.	.	T	G	.	.
afr mel 6	d7	.	.	.	.	A	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	G	T	.	T	.	.	.	.	.	.	T	.	C	.	.	C	.
afr mel 7	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	T	T	.	A	.	T	T	A	.	G	A	.	d169	-	-	-	-	-	-	i4	.	.	C	.	.	.	T	.	.	.	T	G	.	.
afr mel 8	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	T	T	.	A	.	T	T	A	.	G	A	.	d169	-	-	-	-	-	-	i4	.	.	C	.	.	.	T	.	.	.	T	G	.	.
afr mel 9	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	G	.	.	.	T	.	T	.	.	.	.	.	.	T	.	C	.	.	C	.
afr mel 10	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	A	T	T	.	A	.	T	T	A	.	G	A	.	d169	-	-	-	-	-	-	i4	.	.	C	.	.	.	T	.	.	.	T	G	.	.



Supplementary Material S4: (a) Diagram of open reading frame (ORF) length of *lectin-24A* from *D. melanogaster* and *D. simulans* strains from California (cal) and Africa (afr),

from the *D. sechellia* sequence from FlyBase, and from our *D. mauritiana* strain. The non-lectin domain portion of *lectin-24A* is represented in white and the C-type lectin domain is represented in red, with the extended ORF regions of *D. simulans* and *D. sechellia* in gray. (b) *lectin-24A* polymorphism tables from California and Africa *D. melanogaster* strains and (c) *D. simulans* strains. Site number represents the bp position of a polymorphism. N, S, and I represent nonsynonymous substitutions, synonymous substitutions, and intergenic regions, respectively. Coding sites with indel polymorphisms are not scored as nonsynonymous or synonymous. Strains matching the consensus sequence at a polymorphic site contain a dot (.). Dashes (-) represent deletions. i and d represent insertion and deletion, respectively, followed by the number of base pairs affected. Indel polymorphisms are displayed as one polymorphic site with the length and placement of the indel noted by the site range. Any polymorphism falling within an indel is included as an individual polymorphic site. Sites boxed in black represent the point mutation or frameshift mutation that cause premature stop codons in some strains. Strains containing the frameshift mutations have no unique polymorphisms between the frameshift mutation and their premature stop codons.

(a)

CG identifier	gene name	Lb-5	Lb-12	Lb-24	Lh-5	Lh-12	Lh-24
CG1084	Cont	0.94	0.78	**0.81**	1.00	0.92	0.94
CG1500	fw	0.99	1.32	0.95	1.26	1.00	1.21
CG1652	lectin-46Cb	1.03	0.97	1.03	1.03	1.01	1.03
CG1656	lectin-46Ca	0.98	0.96	1.06	0.99	0.94	1.06
CG2826	lectin-21Ca	0.97	0.96	1.02	1.01	1.05	0.97
CG2958	lectin-24Db	**0.67**	0.78	**0.75**	0.77	0.86	0.84
CG3244		0.82	0.97	0.97	0.99	0.95	1.05
CG3410	lectin-24A	**15.35**	**11.70**	**3.60**	0.70	0.36	0.44
CG3921		1.02	0.92	0.97	0.94	1.01	0.93
CG4115		0.82	2.82	1.61	1.17	0.93	1.09
CG4145	Cg25C	0.79	0.81	1.10	0.95	0.95	1.07
CG6014		0.83	1.08	**0.65**	0.76	1.09	0.65
CG6055		0.63	4.44	1.29	1.20	0.71	1.16
CG7049		1.05	0.86	0.97	1.03	0.90	1.08
CG7106	lectin-28C	1.39	1.17	0.81	0.96	0.98	0.40
CG7763		1.15	0.78	**0.37**	1.04	0.68	**0.37**
CG8343		1.01	1.04	1.09	1.01	1.11	1.05
CG8647		0.92	1.00	1.21	1.03	1.11	1.00
CG9095		1.14	**5.77**	0.88	1.23	0.64	0.85
CG9134		0.82	**1.60**	1.12	1.20	0.99	1.13
CG9138	SP1070	1.18	1.25	0.96	1.12	0.89	1.01
CG9976	Lectin-galC1	1.30	0.91	1.08	1.03	0.92	1.19
CG9978		1.01	0.96	1.32	0.89	0.96	1.14
CG11211		1.06	1.06	1.13	1.02	1.14	0.97
CG12111		0.99	0.98	1.00	0.99	1.05	0.99
CG13086		1.13	1.02	1.21	1.15	1.10	1.02
CG13587	sphinx	1.04	0.99	1.01	0.99	1.11	1.05
CG13686	lectin-21Cb	1.02	1.02	1.12	1.02	1.02	1.09
CG14500		1.76	1.02	1.61	1.32	1.05	1.40
CG14866		1.04	**8.28**	1.22	1.42	0.92	1.15
CG15358		0.92	1.14	0.95	1.18	0.94	1.20
CG15378	lectin-22C	0.80	1.23	0.99	1.12	0.95	1.03
CG15765		0.90	0.85	1.09	0.80	0.82	1.05
CG15818		1.12	0.93	**0.66**	**1.29**	1.00	0.76
CG16834	lectin-33A	0.71	1.08	1.05	0.81	1.01	0.99
CG16858	vkg	**0.76**	**0.81**	1.12	**0.86**	0.97	0.96
CG17011	lectin-30A	1.11	**1.04**	1.04	1.02	1.07	0.97
CG17797	Acp29AB	0.95	0.87	0.94	0.96	0.90	0.95
CG17799	lectin-29Ca	0.95	1.06	1.02	0.91	1.02	1.09
CG33171		**0.61**	0.86	1.15	1.01	0.97	1.00

(b)

CG identifier	gene name	Lb-5	Lb-12	Lb-24	Lh-5	Lh-12	Lh-24
CG33122	cutlet	0.92	0.84	0.96	0.91	1.03	0.95
CG31955		1.03	0.82	1.00	0.88	1.00	1.01
CG2818		1.03	0.86	0.98	1.05	0.97	1.12
CG2822	Shaw (CG2822-RA)	1.08	0.85	0.99	0.99	0.92	1.03
CG2822	Shaw (CG2822-RB)	1.35	0.86	0.92	0.99	0.92	0.87
CG10019		0.89	0.88	0.99	0.90	0.92	0.98
CG31959		1.03	0.98	1.12	1.00	0.95	0.98

Supplementary Material S5: (a) Fold expression changes for all FlyBase-annotated C-type lectin domain-containing genes 5, 12, and 24 hours following *L. boulardi* (Lb) and *L. heterotoma* (Lh) attack (adapted from (Schlenke et al. 2007)). (b) Fold expression changes following wasp attack for the three genes immediately upstream and downstream of *lectin-24A*. Numbers in bold identify significantly differentially regulated genes.

Table 1: Population genetic statistics for *lectin-24A* and flanking loci.[a]

	S[b]	π[b]	theta-W[b]	Taj D	*p*-value[c]	Fay & Wu's H	*p*-value[c]	Hd	*p*-value[c]	ZnS	*p*-value[c]	div
25 kb upstream	14	0.01	0.01	-0.31	0.40	-3.00	0.11	0.75	0.12	0.41	0.38	0.05
15 kb upstream	17	0.01	0.01	1.47	0.96	1.43	0.52	0.79	0.09	0.46	0.28	0.05
5 kb upstream	21	0.01	0.01	0.01	0.53	-2.07	0.21	0.79	0.06	0.33	0.60	0.03
2 kb upstream	5	0.00	0.00	-1.60	0.09	-4.07	0.02*	0.25	0.02*	1.00	0.00*	0.04
lectin-24A cal sim	56	0.01	0.01	-1.90	0.00*	-47.79	0.00*	0.25	0.00*	1.00	0.00*	0.09
lectin-24A afr sim	101	0.02	0.02	0.88	0.85	5.19	0.47	0.97	0.18	0.29	0.69	0.08
2 kb downstream	4	0.00	0.00	-1.53	0.12	-1.07	0.14	0.25	0.03*	1.00	0.00*	0.06
5 kb downstream	31	0.02	0.02	-0.35	0.38	-2.21	0.22	0.75	0.02*	0.34	0.58	0.04
15 kb downstream	17	0.01	0.01	0.83	0.83	2.21	0.76	0.75	0.09	0.40	0.40	0.04
25 kb downstream	30	0.02	0.02	-0.34	0.39	-3.43	0.18	0.75	0.02*	0.31	0.66	0.07
lectin-24A cal mel	30	0.00	0.01	-0.98	0.19	-2.57	0.21	0.89	0.15	0.30	0.71	0.08
lectin-24A afr mel	28	0.01	0.01	0.93	0.86	-1.24	0.26	0.82	0.07	0.51	0.15	0.08

[a] flanking loci sequenced from the California *D. simulans* (cal sim) population

[b] three measures of heterozygosity are presented: S is the number of segregating sites in the sample, π is the average number of pairwise difference between strains per bp, and theta-W is Watterson's theta (Watterson 1975)

[c] *p*-values determined from coalescent simulations, * $p<0.05$

Table 2: McDonald-Kreitman analyses for *lectin-24A*.[a]

	synonymous	nonsynonymous	*p*-value[c]
fixations[b]	14	51	-
all mel & all sim polymorphisms	30	39	0.0097*
all mel polymorphisms	4	10	0.726
california mel polymorphisms	3	4	0.344
africa mel polymorphisms	2	7	1
all sim polymorphisms	28	29	0.0021*
california sim polymorphisms	15	12	0.0026*
africa sim polymorphisms	24	24	0.0023*
fixations, non-lectin domain[d]	9	29	-
all mel & all sim polymorphisms	19	24	0.064
fixations, lectin domain[d]	5	22	-
all mel & all sim polymorphisms	11	15	0.077

[a] only full open reading frame strains included in analyses

[b] the number of fixed differences between the *D. melanogaster* (mel) and *D. simulans* (sim) population samples are compared to the number of polymorphisms from a variety of populations and species

[c] significance determined by two-tailed Fisher's exact test, * $p<0.05$

[d] the number of fixed differences between the *D. melanogaster* (mel) and *D. simulans* (sim) population samples in portions of *lectin-24A* are compared to the number of polymorphisms from the same portion

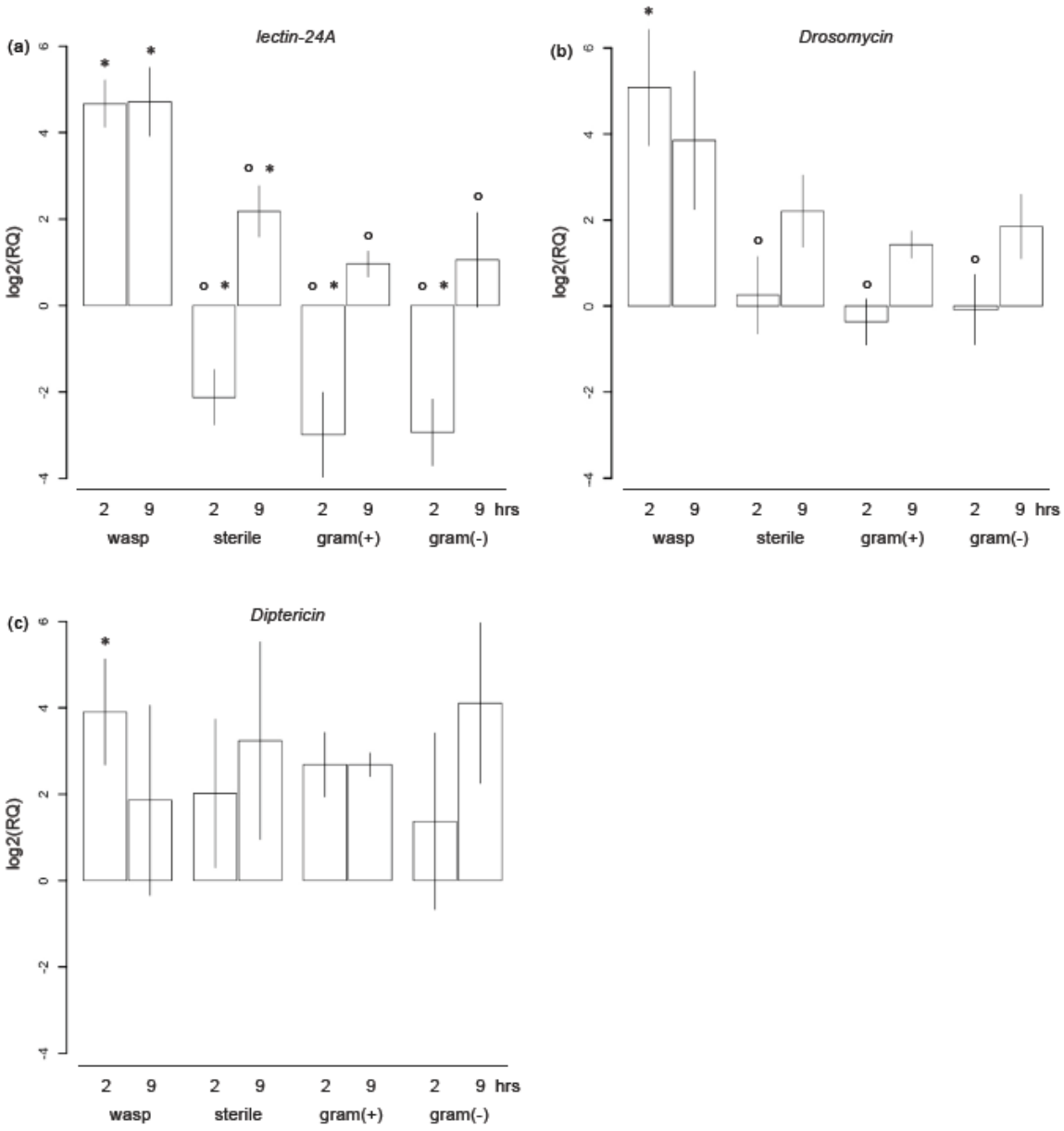


Figure 1: Gene expression following immune challenge. log2(Relative Quantification) of (a) *lectin-24A*, (b) *Drosomycin*, and (c) *Diptericin* relative to un-treated larvae 2-5 and 9-12 hours after wasp attack, sterile injury, septic injury with the gram(+) bacteria *E.*

faecalis, or septic injury with the gram(-) bacteria *E. coli*. Error bars +/- SEM. Significance values judged by comparison of treated averages to un-treated averages, * $p<0.05$. Significance values across treatments judged by comparison of treated averages to wasp attack averages at the same timepoint, ° $p<0.05$.

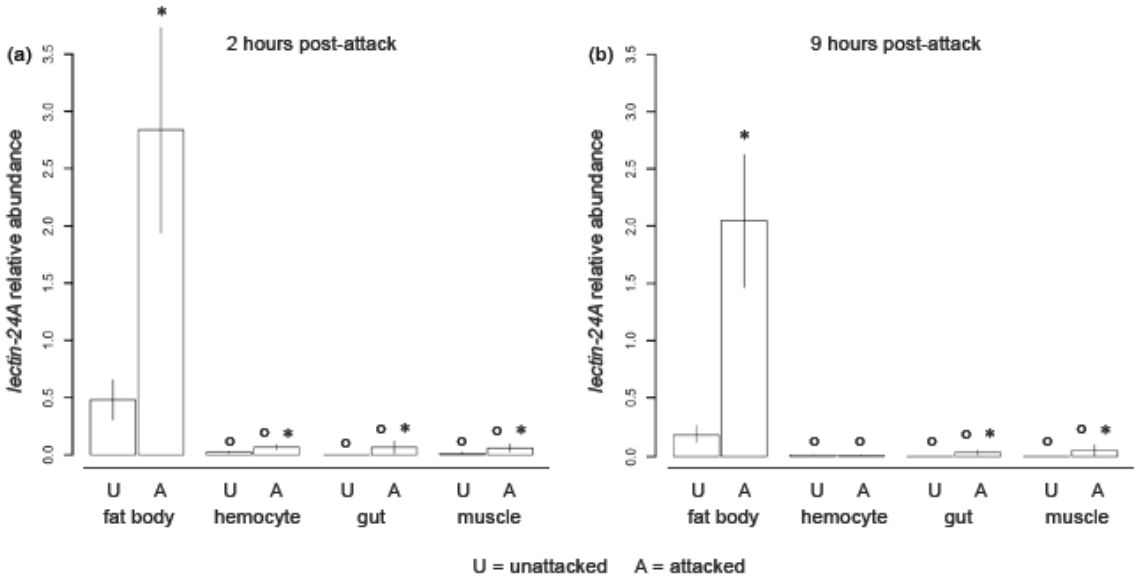


Figure 2: Tissue-specific expression of *lectin-24A*. *lectin-24A* expression levels relative to *alphaTub84B* in fat bodies, hemocytes, guts, and body wall muscles in unattacked (U) and attacked (A) larval tissues (a) 2-5 hours following wasp attack, and (b) 9-12 hours following wasp attack. Error bars +/- SEM. Significance values judged by comparison of wasp-attacked tissue averages to unattacked tissue averages, * $p<0.05$. Significance values across treatments judged by comparison of *lectin-24A* abundance in fat body to *lectin-24A* abundance in other tissues at the same timepoint and under the same condition, ° $p<0.05$.

site													
13	—	A	T	G	G	G	G	G	G	G	G	G	G
161	—	T	A	C	C	C	C	C	C	C	C	C	C
189	—	C	T	T	T	T	T	T	T	T	T	T	T
278	—	T	C	C	C	C	C	C	C	C	C	C	C
279	—	G	A	A	A	A	A	A	A	A	A	A	A
284	—	T	C	C	C	C	C	C	C	C	C	C	C
291	—	C	A	A	A	A	A	A	A	A	A	A	A
323	—	C	T	T	T	T	T	T	T	T	T	T	T
336	—	T	A	A	A	A	A	A	A	A	A	A	A
344	—	T	A	d7	d7	d7	d7	d7	d7	d7	d7	d7	d7
373-379	—	i7	G	A	A	A	A	A	A	A	A	A	A
411	—	A	G	G	G	G	G	G	G	G	G	G	G
412	—	G	A	A	A	A	A	A	A	A	A	A	A
426	—	T	C	C	C	C	C	C	C	C	C	C	C
434	—	T	G	G	G	G	G	G	G	G	G	G	G
486	—	A	G	G	G	G	G	G	G	G	G	G	G
509	—	A	G	G	G	G	G	G	G	G	G	G	G
544	—	G	A	i6	i6	i6	i6	i6	i6	i6	i6	i6	i6
597-602	—	9B	A	G	G	G	G	G	G	G	G	G	G
629	S	A	T	C	C	C	C	C	C	C	C	C	C
634	N	T	A	G	G	G	G	G	G	G	G	G	G
644	S	A	G	G	G	G	G	G	G	G	G	G	G
688	N	G	A	A	A	A	A	A	A	A	A	A	A
689	N	A	C	G	G	G	G	G	G	G	G	G	G
697	N	A	G	G	G	G	G	G	G	G	G	G	G
701	N	T	G	C	C	C	C	C	C	C	C	C	C
825	N	G	A	G	G	G	G	G	G	G	G	G	G
845	S	A	T	C	C	C	C	C	C	C	C	C	C
878	S	T	A	G	G	G	G	G	G	G	G	G	G
902	S	A	A	G	G	G	G	G	G	G	G	G	G
912	N	A	A	G	G	G	G	G	G	G	G	G	G
956	S	A	A	G	G	G	G	G	G	G	G	G	G
989	S	A	A	G	G	G	G	G	G	G	G	G	G
991	N	C	A	A	A	A	A	A	A	A	A	A	A
1028	S	A	A	G	G	G	G	G	G	G	G	G	G
1030	N	A	A	G	G	G	G	G	G	G	G	G	G
1061	S	C	A	T	T	T	T	T	T	T	T	T	T
1113	N	A	A	C	C	C	C	C	C	C	C	C	C
1136	S	A	A	T	T	T	T	T	T	T	T	T	T
1151	S	T	A	C	C	C	C	C	C	C	C	C	C
1223	S	G	A	A	A	A	A	A	A	A	A	A	A
1241	S	G	A	A	A	A	A	A	A	A	A	A	A
1262	S	A	C	A	A	A	A	A	A	A	A	A	A
1268	S	G	C	A	A	A	A	A	A	A	A	A	A
1430	N	C	A	T	T	T	T	T	T	T	T	T	T
1472	N	A	T	C	C	C	C	C	C	C	C	C	C
1499	S	T	C	d1	d1	d1	d1	d1	d1	d1	d1	d1	d1
1515	—	i1	d1	i1	i1	i1	i1	i1	i1	i1	i1	i1	i1
1521	—	i1	d1	T	T	T	T	T	T	T	T	T	T
1536	—	C	T	A	A	A	A	A	A	A	A	A	A
1544	—	G	A	G	G	G	G	G	G	G	G	G	G
1634	—	i1	d1	i1	i1	i1	i1	i1	i1	i1	i1	i1	i1
1672	—	G	T	G	G	G	G	G	G	G	G	G	G
1707	—	T	G	C	C	C	C	C	C	C	C	C	C
1780	—	T	C	T	T	T	T	T	T	T	T	T	T
1852	—	A	C	C	C	C	C	C	C	C	C	C	C
2012	—	C	T	T	T	T	T	T	T	T	T	T	T
2033	—	i1	d1	i1	i1	i1	i1	i1	i1	i1	i1	i1	i1
2034	—	T	C	T	T	T	T	T	T	T	T	T	T
2035	—	i1	d1	i1	i1	i1	i1	i1	i1	i1	i1	i1	i1
2037	—	T	A	T	T	T	T	T	T	T	T	T	T
2038	—	A	T	A	A	A	A	A	A	A	A	A	A
2040	—	T	C	T	T	T	T	T	T	T	T	T	T

Figure 3: *lectin-24A* polymorphism table for the California *D. simulans* population. Site number represents the position of a polymorphism. N, S, and I represent nonsynonymous substitutions, synonymous substitutions, or intergenic regions, respectively. Strains matching the consensus sequence at polymorphic site contain a dot (.). i and d represent insertion and deletion, respectively, followed by the number of base pairs affected. Indel polymorphisms are displayed as one polymorphic site with the length and placement of the indel noted by the site range.

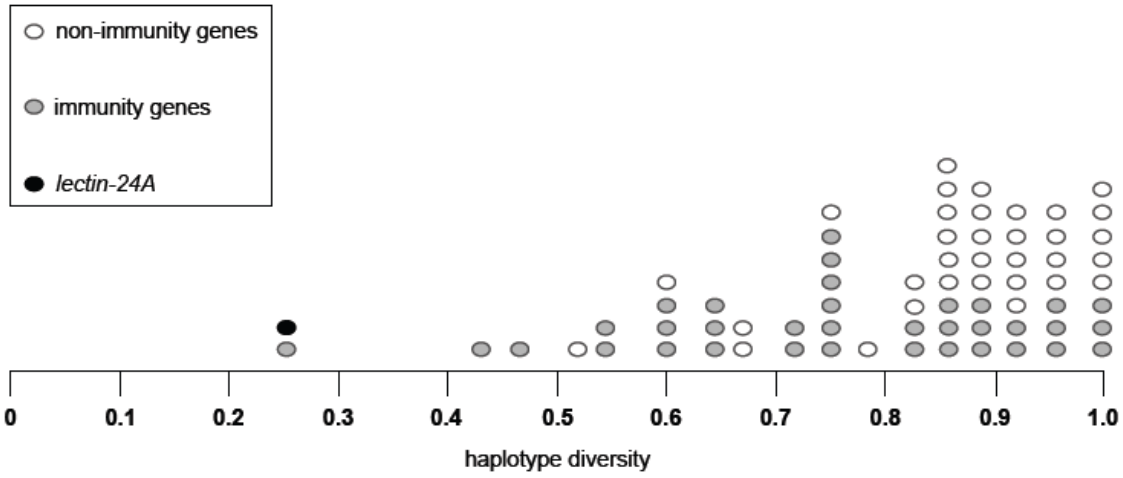


Figure 4: Haplotype diversity of *lectin-24A* and 68 other genes from the California *D. simulans* population (Schlenke, Begun 2003).

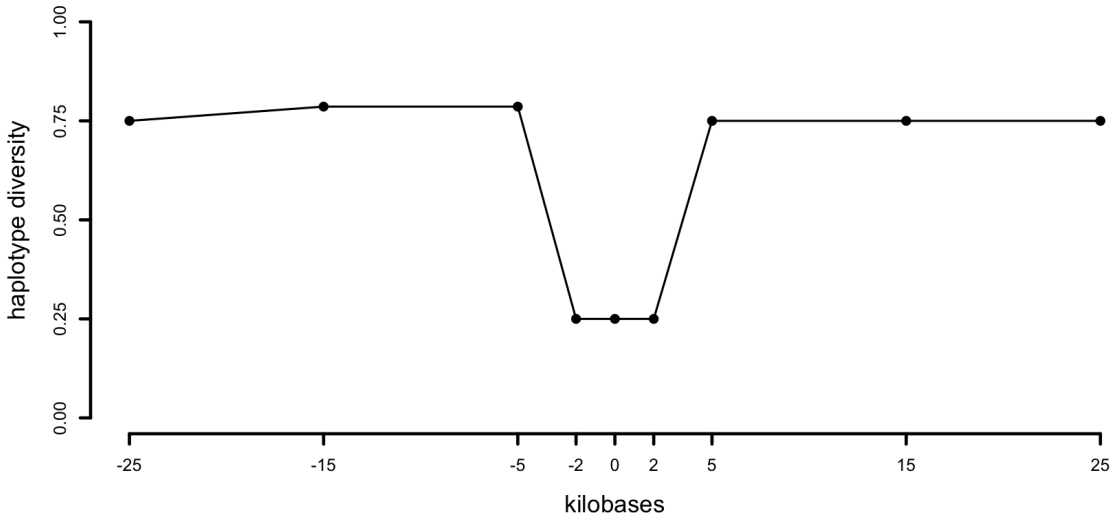


Figure 5: Haplotype diversity of *lectin-24A* and flanking loci from the California *D. simulans* population. *lectin-24A* is located at 0 kb, negative values represent regions upstream of *lectin-24A*, positive values represent regions downstream of *lectin-24A*.

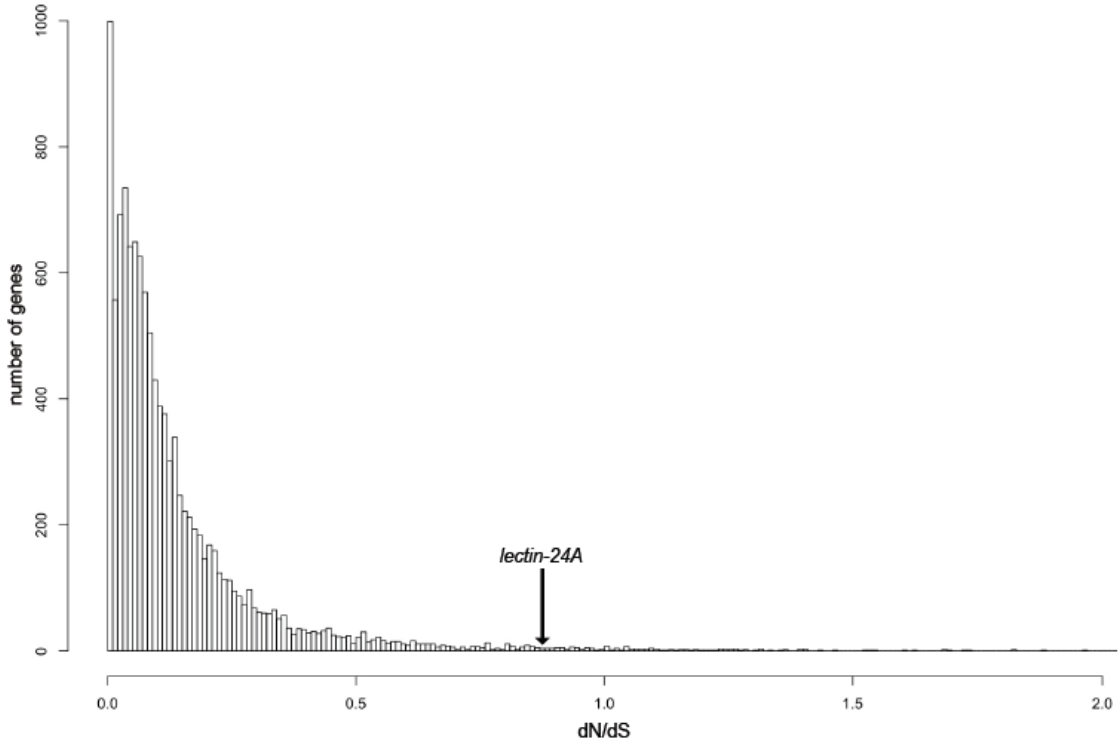


Figure 6: dN/dS for every gene in the genome shared between *D. melanogaster* and *D. simulans* (Begun et al. 2007). An arrow marks the dN/dS value for *lectin-24A*. The 23 genes with dN/dS > 2 were excluded from the graph.

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Chapter 3: *lectin-24A's* role in the Drosophila anti-wasp immune response.

Drosophila encounter a diverse range of pathogens in nature (Keebaugh, Schlenke 2013), and can combat those pathogens with an innate immune response that shares homology to that of other organisms, including humans. A deep understanding of the fruitfly's defense mechanisms against natural pathogens has the potential to inform translational studies on innate immunity, and provide insight into Drosophila's coevolutionary battle against the virulence mechanisms of commonly encountered threats. To build upon what is already known about Drosophila innate immunity, and to learn more about ecologically relevant immune strategies of the fruitfly, we study the immune response mounted by Drosophila against their common natural pathogen, the parasitic wasp.

Drosophila innate immunity can be broken down into two major components; humoral immunity and cellular immunity (Lemaitre, Hoffmann 2007). The humoral immune response is largely controlled by the Toll and Imd pathways, with input from Jak/Stat and Jnk pathways, all of which are active in the fat body (Boutros, Agaisse, Perrimon 2002). Microparasites can induce the humoral immune response, and are targeted by antimicrobial peptides secreted into the hemocoel by the fat body. The cellular immune response is mediated by hemocytes of which there are four types; plasmatocytes, podocytes, lamellocytes, and crystal cells (Rizki 1957). Microparasites can also induce the cellular immune response, resulting in their clearance via phagocytosis. Furthermore, parasitoid wasps can induce a strong cellular immune response in fruitflies.

Parasitoid wasps infect Drosophila larvae by ovipositing eggs into the Drosophila hemocoel. In a successful wasp infection, one wasp will proceed in hatching within and consuming larval tissues, ultimately eclosing from the Drosophila pupal case. However, if the fly larva mounts an effective immune response against wasps, the fly will kill the wasp at its egg stage by forming a multilayered hemocytic capsule around the wasp egg that becomes melanized in a process referred to as melanotic encapsulation (Carton, Nappi 1997). This hemocytic capsule is built by a primary layer of plasmatocytes, which serve as the initial responders to wasp infection. The plasmatocyte response is supplemented with lamellocytes, made from the lymph gland and circulating plasmatocytes (Markus et al. 2009) (Stofanko, Kwon, Badenhorst 2010) (Lanot et al. 2001), which build upon the growing capsule. This capsule eventually becomes melanized supposedly by crystal cells, which possess melanization machinery. Podocytes, which are intermediaries between plasmatocytes and lamellocytes, are also likely involved in the encapsulation process.

Previous studies have shown that hemocyte differentiation and/or numbers, and their cellular adhesions are sensitive to altered levels of Jak/Stat, Toll, and Jnk pathway components, respectively, which in turn negatively affect Drosophila's anti-wasp response (Sorrentino, Melk, Govind 2004; Williams et al. 2006). Additionally, phenoloxidase production, hemocyte shape and spreading, and N-glycosylation, is also important for Drosophila's anti-wasp immune reaction (Rizki, Rizki 1990; Williams, Ando, Hultmark 2005; Williams et al. 2006; Mortimer et al. 2012). Together, these data support that the cellular response induced following parasitoid attack is highly complex.

As such, key players in this immune response remain unknown, although microarray analyses of wasp attacked larvae identified many novel immune candidates to study (Wertheim et al. 2005; Schlenke et al. 2007).

lectin-24A, a predicted secreted C-type lectin (Theopold et al. 1999), was significantly upregulated in both microarray studies, one using a Braconid *Asobara tabida*, and the other using a Figitid *Leptopilina boulardi*. C-type (Ca^{2+}-dependent) lectins are a family of carbohydrate-binding proteins, and C-type lectin-like domain-containing proteins have been identified in all sequenced metazoans, and even outside Metazoa (Zelensky, Gready 2005). Because of their capacity to bind specific sugar moieties, lectins are often thought of as primary candidate immune recognition proteins. A well-studied example is the mannose-binding lectin in the vertebrate complement cascade (Turner 1996), and numerous examples of lectins functioning in vertebrate and invertebrate immunity abound (Marques, Barracco 2000; Cambi, Koopman, Figdor 2005; Zelensky, Gready 2005; Willment, Brown 2008). Of note, two different Drosophila C-type lectins have been shown to promote bead encapsulation *in vitro* (Ao, Ling, Yu 2007), insect C-type lectins have been connected to phenoloxidase reactions (Chen et al. 1995; Yu, Gan, Kanost 1999; Yu, Kanost 2000), and *lectin-24A* itself was significantly upregulated in mutant fly larvae that produce melanized hemocytic masses (Bettencourt et al. 2004; Walker et al. 2011). Of particular importance to this study, N-glycosylation sites, which lectins potentially have affinity for, were shown to be produced on the surface of lamellocytes when encapsulating parasitoid eggs, and to be necessary for effective capsule consolidation (Mortimer et al. 2012). Together, these data suggest that *lectin-24A*

could be involved in the initial recognition, hemocytic capsule construction, or melanization that occurs following wasp infection, therefore, we chose to investigate if *lectin-24A's* significant upregulation post-wasp attack underlies it's role in Dosophila's immune response against wasp eggs.

In a primary study, we showed that *lectin-24A* is most highly abundant in the fat body and exhibits a wasp-specific transcriptional response (Keebaugh, Schlenke 2012), a finding supported by the absence of differential transcription or translation of *lectin-24A* post-immune challenge across multiple other pathogens infecting different Drosophila life stages (De Gregorio et al. 2001; Irving et al. 2001; Vierstraete et al. 2003; Levy, Bulet, Ehret-Sabatier 2004; Roxstrom-Lindquist, Terenius, Faye 2004; Vierstraete et al. 2004; Dostert et al. 2005; Vodovar et al. 2005; Carpenter et al. 2009). We also detected signs of recent and recurrent rapid evolution of *lectin-24A*, which we hypothesized were due to a coevolutionary battle with parasitoids, which commonly infect natural Drosophila populations (Carton 1986; Janssen et al. 1987; Fleury et al. 2004; Keebaugh, Schlenke 2012). This evolutionary trend is seen in multiple other Drosophila immune proteins (Lazzaro, Clark 2003; Schlenke, Begun 2003; Sackton et al. 2007; Lazzaro 2008; Obbard et al. 2009). Here, we test if *lectin-24A* is essential for an anti-wasp immune response, if *lectin-24A* is regulated by a know immunity pathway in Drosophila, and we test the range of wasp species that induce *lectin-24A* expression following attack.

Results and discussion

lectin-24A deletion strain shows defective anti-wasp immune response.

To test if *lectin-24A* is important in Drosophila's immune response against parasitoid wasps, we attacked *lectin-24A* deletion strain larvae with LcNet, a wasp that is avirulent in most wild-type *D. melanogaster* strains (Mortimer et al. 2012). The *lectin-24A* deletion strain was significantly worse at encapsulating LcNet compared to controls (w^{1118}: $p<0.001$) (Fig 1a). Furthermore, deletion flies were less successful at eclosing post-LcNet attack ($p=0.005$) (Fig 1b). Melanized particles were made in all parasitized larvae, suggesting that the defect in encapsulation displayed by *lectin-24A*-deletion larvae does not result from the inability to melanize, rather there was a drop in the ability to effectively encapsulate LcNet eggs. It should be noted that *lectin-24A*-deletion larvae and w^{1118} controls eclose at the same rates when unattacked, thus, the drop in proportion fly eclosion following wasp attack in *lectin-24A* deletion larvae is not due to a general fitness defect (Supplemental Fig 1a). Additionally, insertion strains of the two genes immediately flanking *lectin-24A* (that are partially deleted in *lectin-24A*-deletion larvae) did not show a defect in their encapsulation of LcNet eggs, or survival post-LcNet attack (Supplemental Fig 1b,c). These data suggest that *lectin-24A* is important in Drosophila's immune response against parasitoid wasps. Experiments testing for the effect of knocking-down *lectin-24A* ubiquitously demonstrated no drop in encapsulation or eclosion ability post-LcNet attack, however, we also found that *lectin-24A* was still approximately 19-fold upregulated post-wasp attack in RNAi larvae (data not shown). Therefore, using the Gal4-UAS system to knock-down *lectin-24A* might not be effective post-wasp attack.

lectin-24A deletion strain shows defective lamellocyte differentiation post-LcNet attack.

To determine if the drop in LcNet encapsulation of the *lectin-24A*-deletion strain results from an inadequate hemocyte response following wasp attack, we measured hemoctye counts in unattacked and LcNet-attacked deletion and control larvae. Between *lectin-24A*-deletion and control strains, each hemocyte type responded to attack in the same way (Fig 2a,b). Specifically, plasmatocyte, podocyte, lamellocyte, and crystal cell numbers increased following LcNet attack ($p=0.014$, $p=0.003$, $p<0.001$, $p=0.029$, respectively). However, within attack, the deletion strain produced significantly more plasmatocytes ($p=0.012$), and less lamellocytes post-LcNet attack compared to w^{1118} controls ($p=0.040$). Because plasmatocytes differentiate into lamellocytes post-parasitism (Markus et al. 2009) (Stofanko, Kwon, Badenhorst 2010) (Lanot et al. 2001), it could be that *lectin-24A*-deletion larvae are defective in lamellocyte differentiation. Furthermore, because lamellocytes are a major factor in the formation of hemocyte capsules, it follows that *lectin-24A*-deletion strains are less successful at encapsulating LcNet eggs because of an insufficient lamellocyte count following parasitization.

To see if the drop in lamellocyte levels of the *lectin-24A*-deletion strain underlies an inability to properly respond to parasitization, or results from an inability to produce lamellocytes from prohemocytes, or for hemocytes to differentiate into lamellocytes, we pierced deletion and control larvae with sterile needles to mimic wasp ovipositor injury. There was no significant difference in the hemocyte counts between the two strains

following piercing (Fig 2c). Together, these data suggest that *lectin-24A*-deletion strains have an inadequate lamellocyte response post-wasp attack, specifically, although it should be noted that lamellocyte levels across both strains are not as strongly induced following piercing. All statistics for hemocytes counts are listed in Supplemental Table 1.

lectin-24A-reporter flies show activity in the fat body following attack by multiple parasitoid species.

To determine if *lectin-24A* expression is induced following attack by multiple or select parasitoid wasp species, we generated *lectin-24A*-reporter flies. *lectin-24A* expression in the fat body was detected only in wasp-attacked larvae, or in late 3rd instar larvae in portions of tissue directly surrounding the gonads. Thus, it was straightforward to determine which wasp species induced *lectin-24A* expression in the fat body.

lectin-24A was induced following attack by 25 of the 27 larval parasitoid strains used in this study (Fig 3). Specifically, every wasp except for strains GxHaw and LhSw induced *lectin-24A* expression. This suggests that *lectin-24A* is induced following attack by a broad range of specialist and generalist parasitoids across multiple families that may not commonly attack *D. melanogaster* in the wild. Furthermore, *D. melanogaster's* immune competence against the wasp strains that induce *lectin-24A* varies from high susceptibility to high resistance, and the two wasp strains that do not induce *lectin-24A* expression range from complete to partial virulence in *D. melanogaster* (unpublished results). To determine if *lectin-24A* is a general stress response gene, we fed larvae 25-

mM paraquat and checked fat bodies for reporter activity. No reporter activity was detected (Fig 3). We also pierced *lectin-24A*-reporter larvae with sterile needles to mimic wasp-ovipositor injury, and needles dipped in Lb17 venom extract to expose larvae to venom proteins. No reporter activity was detected (Fig 3). Together, these data suggest that some general aspect of parasitoid attack besides cuticle injury or venom protein exposure must induce *lectin-24A* expression, and perhaps GxHaw and LhSw possess venom proteins that inhibit *lectin-24A* expression. Of note, other wasp strains of the same species as GxHaw and LhSw were shown to induce *lectin-24A* expression, thus the lack *lectin-24A* induction by these two strains is not species-specific.

lectin-24A-reporter flies show activity when Toll, Jak/Stat, or Imd pathway components are overexpressed.

lectin-24A-reporter flies were crossed to fly strains possessing genetic alleles or heat-shock constructs that overexpress immune pathway components to uncover which, if any, classical immune pathway regulates *lectin-24A*. *lectin-24A*-reporter flies crossed to hop^{Tum} and Toll10B fly strains, whose genomes contain alleles that activate the Jak/Stat and Toll pathway, respectively. *lectin-24A*-reporter activity was detected in the fat body of larval offspring. Larval progeny from a *lectin-24A*-reporter x hs-DJnk cross did not show increased *lectin-24A* levels post-heat-shock. Furthermore, *lectin-24A*-reporter x UAS-hep.CA (a leaky construct, used here without Gal4-driven expression) did not show *lectin-24A* upregulation (Fig 3).

Next, we analyzed *lectin-24A*-reporter activity in crosses involving immune pathway components expressed in the fat body under the control of a *C833*-Gal4 driver. Specifically, *lectin-24A*-reporter;*C833*-Gal4 flies were crossed to UAS-Rel, -Rac1, -Pak, -bskDN, -Dorsal, and –Dif. UAS-Dorsal, -Dif, and –Rel all increased reporter activity in the fat body, however, UAS-Rac1 and –Pak did not (Fig 3). Lb17 attack induced reporter activity in the presence of UAS-driven bskDN (Fig 3). All together, these data suggest *lectin-24A* is downstream of Toll, Jak/Stat, and Imd pathways, but not the Jnk pathway.

Finally, alleles of different Imd and Toll pathway components were crossed into *lectin-24A*-reporter fly backgrounds. Of these crosses, *lectin-24A*-reporter x Rel[E20] and Rel[E23] showed *lectin-24A*-reporter activity post Lb17 attack. However, *lectin-24A*-reporter x spz[rm7]/TM6C (screened against tubby, and hereafter referred to only as spz[rm7]) and Tak1[179] offspring larvae did not show reporter activity post Lb17 attack (Fig 3). To verify these findings, semi-quantitative PCR was performed on imd[10191], Rel[E20], Rel[E23], Tak1[179], and spz[rm7] larvae following Lb17 attack at 9-12 and/or 2-5 hours. Both Imd pathway mutant strains, imd[10191], Rel[E20], showed significantly less *lectin-24A* upregulation following wasp attack compared to background controls (OreR and Rel[E23], respectively). Tak1[179], and spz[rm7] larvae do not have background control strains, but their levels of *lectin-24A* upregulation were roughly on the same level as pathway mutants, and control strains, respectively (Fig 4, Supplemental Table 2). Interestingly, absolute *lectin-24A* levels were extremely low in imd[10191] and Tak1[179] flies compared to all other strains, and significantly lower than controls (all:

$p<0.001$), while Rel[E20] flies had significantly higher *lectin-24A* levels than controls (all: $p<0.009$) (Table 1, Supplemental Table 2).

Together, these data suggest that *lectin-24A* is regulated by immune pathways, Toll, Jak/Stat, and Imd, all of which are active in the fat body upon immune challenge (Lemaitre, Hoffmann 2007). Perhaps this regulation occurs via crosstalk between the different pathways, or perhaps *lectin-24A* is downstream of all three pathways. In the case of spz[rm7] larvae, *lectin-24A* upregulation was detected in semi-quantitative PCR, but not in reporter larvae. Thus, the semi-quantitative PCR must be more sensitive than reporter larvae at detecting increases in *lectin-24A* expression.

Analysis of Lectin-24A glycan binding affinity.

Recombinant his-tagged Lectin-24A was tested against version 5.1 of the Center for Functional Genomics (CFG) glycan array to determine glycan binding affinity of this C-type lectin. However, the his-tagged protein did not bind to the CFG glycan array (data not shown), meaning either Lectin-24A does not bind to any glycans used in the assay, or that the his-Lectin-24A does not retain normal Lectin-24A binding function, potentially because of improper modification of Lectin-24A when made in the *e. coli* cells used for the expression of recombinant his-Lectin-24A.

Materials and methods

Insect strains

Bloomington Drosophila deficiency stocks numbered 7789 (Df(2L)Exel7018) and 9604 (Df(2L)BSC171) are viable *in trans*. Three genes, CG2818, *lectin-24A*, and *Shaw* are the only genes physically deleted in this *trans* heterozygous strain, henceforth referred to as the *lectin-24A*-deletion strain. w^{1118} is considered the background control for 7789 and 9604, and therefore, is considered the background control for the *lectin-24A*-deletion strain. The *lectin-24A*-deletion strain was used in encapsulation, eclosion, and hemocyte count experiments. It should be reported that, of the genes deleted in the *lectin-24A*-deletion strain, only *lectin-24A* is significantly differentially regulated post-wasp attack (Schlenke et al. 2007). Furthermore, to account for the effect of CG2818-deletion, Bloomington stock number 13830, which contains a P{y[+mDint2] w[BR.E.BR]=SUPor-P} insertion in CG2818's 3' UTR, was also phenotyped for encapsulation and eclosion ability. Similarly, Bloomington stock 22786, which contains a Mi{ET1} insertion in a *Shaw* intron, was pehnotyped for encapsulation and eclosion ability.

For gene expression studies, various strains were used that were mutant for different Imd or Toll pathway components. Specifically, imd pathway mutants used were *imd* null imd[10191] (OreR background) (Pham et al. 2007), *Relish* null Rel[E20] (non-mutant background Rel[E23]) (Hedengren et al. 1999), and *Tak1* null Tak1[179] (Bloomington 26275, no background control)(Delaney et al. 2006). The Toll pathway *spz* mutant used was spz[rm7]/TM6C (a gift from Bruno Lemaitre, background unknown, described in (Lemaitre et al. 1996)). This strain contains a strong larval marker (TM6C) that we screened against in expression analyses.

A *lectin-24A*-reporter fly strain was created by PCR-cloning approximately 2 kb of DNA sequence upstream of *lectin-24A* from *D. melanogaster* strain OreR, which was used in our previous study on *lectin-24A* (Keebaugh, Schlenke 2012). Primers used were 5'-GCATCAACGCGTGAAACGTCCTGACAGCCGAAATG-3', 5'-GCATCAGCGGCCGCAATGGACTGAGTTTTCTGCT-3'. PCR was performed with iProof High-Fidelity DNA Polymerase (BIO-RAD), and fragment was cloned using StrataClone PCR Cloning kit. The OreR fragment was then subcloned into pRedRabbit (Housden, Millen, Bray 2012). *lectin-24A-RedRabbit* plasmid DNA was purified using Qiagen Maxi Prep kit and sent to Rainbow Transgenics for injection into Bloomington phiC31 strain 9752 (PBac{y^+-attP-3B}). Multiple transgenic strains were generated, and only one strain was selected for use in this report. A second transgenic strain was tested to verify that the *lectin-24A*-reporter activity was not unique to the strain used throughout this study.

lectin-24A-reporter flies were crossed to 3rd chromosome heat-shock-construct hs-DJnk flies (a gift from Y. Tony Ip)(Sluss et al. 1996), hop^{Tum}, Toll10B strain (Bloomington 30914) to check for reporter activity when activating Toll, Jak/Stat, or Jnk pathway components. The *C833*-Gal4 driver was crossed into the *lectin-24A*-reporter fly background for use in experiments using UAS-transgenes: P{EPgy2}puc^{EY09772} (Bloomington), UAS-Rel (Bloomington), -Rac1 (Bloomington 6293), -Pak (Bloomington), -bskDN (Bloomington), and UAS-Dorsal and –Dif (gifts from Y. Tony Ip) to check for reporter activity when overexpressing different immune pathway

components, or when expressing dominant negative forms of immune pathway components. Note that UAS-hep.CA (Bloomington) was only analyzed when crossed to *lectin-24A*-reporter flies without *C833*-Gal4 drivers because the construct is leaky (Woolner, Jacinto, Martin 2005), and expression of hep.CA under the control of *C833*-Gal4 results in greatly diminished growth. Furthermore, Tak1[179], Rel[E20], Rel[E23], and spz[rm7]/TM6C were crossed into *lectin-24A*-reporter fly background for analysis of reporter activity when different Imd and Toll pathway components are mutant.

27 Drosophila parasitoid wasp strains collected from around the world were used for infection trials on *lectin-24A*-reporter flies. Strain LbG486 was provided by D. Hultmark, strains LcNet, AjJap, ApIndo, and AcIC were provided by J. van Alphen, strain GxUg was provided by J. Pool, and strain AtFr was provided by B. Wertheim. All other strains used were collected by the Schlenke lab. All wasp species were grown on *D. melanogaster* strain Canton S, except for *L. clavipes*, *A. tabida*, and *Aphaereta*, which were maintained on *D. virilis*.

All fly and wasp experiments took place in a 24-25°C incubator on a 12:12 light cycle unless otherwise noted.

Imaging of *lectin-24A* reporter

lectin-24A-reporter flies or crosses involving *lectin-24A*-reporter flies were allowed to lay eggs on molasses plates supplemented with yeast paste for 72 hours. For experiments checking for reporter activity following wasp attack, thirty late second instar larvae were

picked from egg lay plates and transferred to standard Drosophila medium in 35 mm diameter Petri dishes. Following transfer, female wasps from individual wasp strains were added to food plates for a 2 hour attack time. Specifically, 3 LgCam, 4 Lh14, LvHaw, GxUg, AjJap, ApIndo, AtSw, LhSw, AtFr, LvPi, GxUnk, LvUnk, GxHaw, LgSA, or ByNiag, 5 Lb17, G1F1, AcIc, LbFr, LbKen, or LbG486, 8 AphAtl, 10 LcAtl or LcNet, or 15 G2, G3, or G4 were used for attacks. Following attack, larvae were dissected in Ringer's solution and imaged (TRITC and brightfield) on diagnostic slides (Tekdon, Inc.) every 24 hours until pupation. For experiments involving pierced *lectin-24A*-reporter larvae, second instar larvae were immobilized on double sided tape and pierced with a 0.1-mm-diameter stainless steel needle (Fine Science Tools), placed on standard food, and imaged every 24 hours until pupaion. For experiments checking for reporter activity following heat-shock, or resulting from UAS-driven, or allelic immune pathway gene expression, early second instar larvae were transferred to 35mm standard food plates and either imaged every 24 hours until pupation, or heat-shocked at 37°C for one hour and imaged every 24 hours until pupation. For experiments feeding *lectin-24A*-reporter larvae hydrogen peroxide or paraquat, 1st to 3rd instar larvae were transferred to instant Drosophila medium containing 25-mM paraquat plus one drop of blue food coloring and placed in a room temperature fume hood covered in foil. Larvae with blue guts (that fed on prepared food) were imaged every 24 hours until pupation.

Recombinant Lecin-24A and antibody production

The *lectin-24A* open reading frame, minus the start codon, from OreR was PCR cloned using the StrataClone PCR Cloning kit. Primers used were 5'-

TAGCTGAGGGATCCTTTAGATTGTCAGTCTTA-3', 5'-GCATCTAGGGGCCCCTAGATGCCATACTGGCA-3'. This fragment was then subcloned into pGEX-2T (Amersham). Expression of GST-Lectin-24A was robust in BL21 and Shuffle cells (NEB), insoluble in all conditions tested (temperatures ranging from 4 to 37°C, IPTG concentrations from 0.05 mM to 0.5 mM, expression periods from 15 minutes to overnight), and was resistant to urea solubilization. Insolubility of recombinant protein using *Escherichia coli* expression systems is not uncommon (Oliveira, Teixeira, Domingues 2013). Therefore, inclusion bodies containing insoluble GST-Lectin-24A were purified and sent to Pocono Rabbit Farm & Lab for antibody production in rabbits. Meanwhile, pGEX-2T-Lectin-24A was sent to Neo Bioscience for subcloning and expression trials. His-Lectin-24A was generated after removing the predicted secretion signal, resolubilization of expressed protein in 8M urea, and refolding in 50 mM Tris, 1 mM GSH, 0.1 mM GSSG, pH 8.5. To aid in antibody production, soluble His-Lectin-24A was sent to Pocono for injections, and for affinity purification using a Ni-column.

Gene expression analysis

Relative-quantification analyses was performed as in (Keebaugh, Schlenke 2012) using the same experimental set-up and primers. Briefly, each *D. melanogaster* immune pathway mutant strain was attacked with the relatively virulent *L. boulardi* strain Lb17 (Schlenke et al. 2007). Flies were allowed to lay eggs for 3 hours, and 20 or 30 larvae from these egg lays were later moved onto 35 mm petri dishes containing standard Drosophila medium. Seventy-two hours after egg lay, 4 (paired with 20 larvae) or 5

(paired with 30 larvae) experienced female wasps were placed in each of the dishes for a 2 h attack time. Two and nine hours post-attack, 5 fly larvae were dissected, and if 4 or more were attacked, then the remaining larvae were flash frozen for expression timepoint analyses. Because of the 2 h attack time and a 1 h handling time, these larvae had developed between 2–5 and 9–12 h post-attack, respectively.

Total RNA extraction was done using Trizol following Invitrogen's recommended protocol. cDNA was synthesized using Qiagen Quantitect Reverse Transcription kit. Each cDNA sample was used as a template for semiquantitative (comparative Ct) real-time polymerase chain reaction using Applied Biosystems Power SYBR Green Master Mix. Each sample was run in triplicate, and any significant outliers within a sample triplicate were discarded. Intron spanning primers used for *alpha-Tub84B* are as follows: 5#-ACACTTCCAATAAAAACTCAATATGC-3#, 5#-CCGTGCTCCAAGCAGTAGA-3#. Primers used for *lectin-24A* (which is intronless) are as follows: 5#-CGAGTGGGGTCCTGGTGAAC-3#, 5#-GAAACGCATCGCTCTTGGTC-3#. Melting curves were checked to ensure that off-target loci were not amplified by primer pairs. All expression experiments were done in four biological replicates, and untreated control larvae were included for each replicate. Note that imd10191 (in OreR background) transcript levels are compared to OreR transcript levels from (Keebaugh, Schlenke 2012). Samples from Rel[E20] and Rel[E23], both in the same background, were collected at the same time. Tak1[179] and Spz[rm7]/TM6C do not have background control strains, and, therefore, are not compared statistically to another strain. Additionally, these strains were only analyzed at the 2-5 hour timepoint. Furthermore, Spz[rm7]/TM6C larvae were

screened against tubby balancers, and only non-tubby (homozygous Spz[rm7]) larvae were used in analyses.

Relative quantification (RQ or delta delta CT) represents the fold change of *lectin-24A* following wasp attack relative to unattacked control samples. Two tailed t-tests on log2 transformation of RQ values were used to judge RQ differences between strains within a treatment. Statistical analyses (2-tailed paired t-tests) were performed on log2 transformation of relative abundance values (the abundance of *lectin-24A* relative to the reference gene, known as delta CT) to judge the differences in *lectin-24A* abundance between unattacked and attacked samples. Two tailed t-tests were used to analyze differences in log2 transformations of *lectin-24A* relative abundance levels between control and mutant strains within treatments.

Resistance trials

Egg lay and attack protocols for resistance trials were performed as reported in (Mortimer et al. 2012). Flies were allowed to lay eggs onto molasses medium with yeast paste. 72 hours after the egg lay period, second instar fly larvae were collected for infection trials. 40 fly larvae were transferred to 35 mm Petri dishes containing standard Drosophila medium and three female LcNet were placed into the Petri dish and allowed to attack fly larvae for approximately 72 hours. For encapsulation experiments, fly larvae were dissected to measure the proportion fly success, calculated as the number of attacked larvae with only encapsulated wasp eggs divided by the total number of attacked larvae dissected. For eclosion experiments, larvae were transferred to food vials and allowed to

eclose as either flies or wasps for approximately six weeks. Eclosed flies were examined or dissected to look for encapsulated wasps and counted as unattacked if no sign of melanization was found, or attacked if any sign of melanization was found. Unattacked were flies discarded from analysis. Any lethality was not counted as fly success.

Generalized linear models were used to examine differences between fly strains in proportion of wasp–attacked fly larvae that encapsulated all wasp eggs laid inside them. If needed, stepdown pairwise comparisons between fly strains were assessed using Tukey's honestly significant difference test. For each fly strain, N = 3 or more replicates of 40 exposed larvae, approximately 30 of which were dissected, except for eclosion trials in which larvae were allowed to eclose.

Hemocyte counts

Flies were allowed to lay eggs for 24 hours on molasses medium with yeast paste. For hemocyte counts post-wasp attack, 40 second instar larvae were transferred to 35 mm diameter Petri dishes containing Drosophila cornmeal medium and exposed to 4 LcNet females for a 24 hour attack period. Unattacked hemocyte count larvae were treated the same way except for the addition of wasps. For counts following sterile needle piercing, early 3rd instar larvae were cleaned in Ringer's solution, dried, and placed on double sided tape to prevent movement during which time their cuticle was pierced with a 0.125 mm tungsten carbide needle (Fine Science Tools). Following piercing, larvae were transferred to 35 mm diameter Petri dishes containing standard Drosophila medium. 24 hours following wasp attack or piercing, 5 size matched larvae were bled into 20ul of 1X

PBS 0.01% phenylthiourea (PTU). Solution containing bled hemolymph was pippetted into Incyto C-Chip DHC-N01 hemocytometer chips and hemocytes were counted from sixteen 0.25X0.25X0.1 mm squares and normalized to per larva values. Each experiment was replicated three to five times for each strain and treatment. Waiting 24 hours post-LcNet attack is sufficient for the encapsulation or melanization reaction in strains used, and the lamellocyte reaction post parasitoid attack is robust during this time (Lanot et al. 2001). Because more hemolymph is lost, and because higher rates of mortality result from needle piercings compared to wasp attack, larvae were aged approximately 24 hours older in piercing experiments to improve the chance of survival through hemocyte count experiments.

GLMs with quasi–Poisson error distributions and log link functions were used to examine the interactive and additive effects of fly strain and attack treatment on the numbers of circulating hemocytes (plasmatocytes, podocytes, lamellocytes, crystal cells) within unattacked and attacked fly larvae. For each fly strain, N = 5 replicates of 5 pooled larvae. Starting from the full model H = S + T + (S x T) + E, where H is hemocyte count, S is fly strain, T is attack treatment, S x T is the interaction between S and T, and E is error, the effects of removing individual terms were assessed sequentially. The interaction term was evaluated first and the main effects of S and T were determined if there was no significant interaction. Hemocyte counts were also performed on N = 3 replicates of 5 pooled larvae that were poked with a sterile needle. Differences between strains in counts and proportions of each hemocyte type following sterile pokes were assessed using quasi–Poisson and quasi–binomial GLMs respectively.

Acknowledgements

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Fig 1

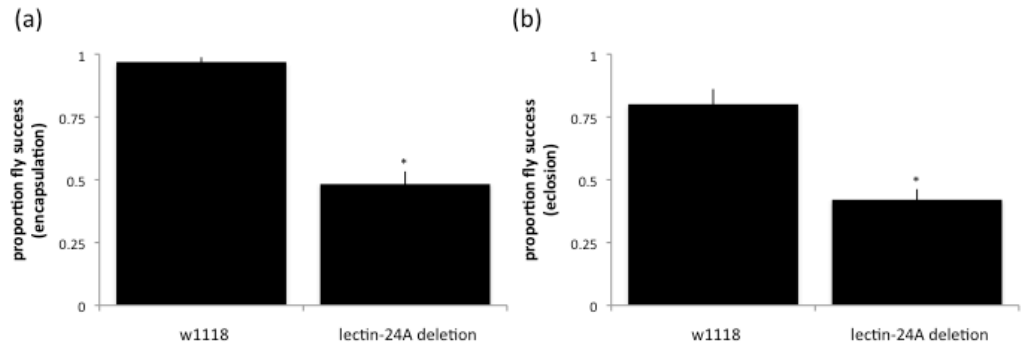
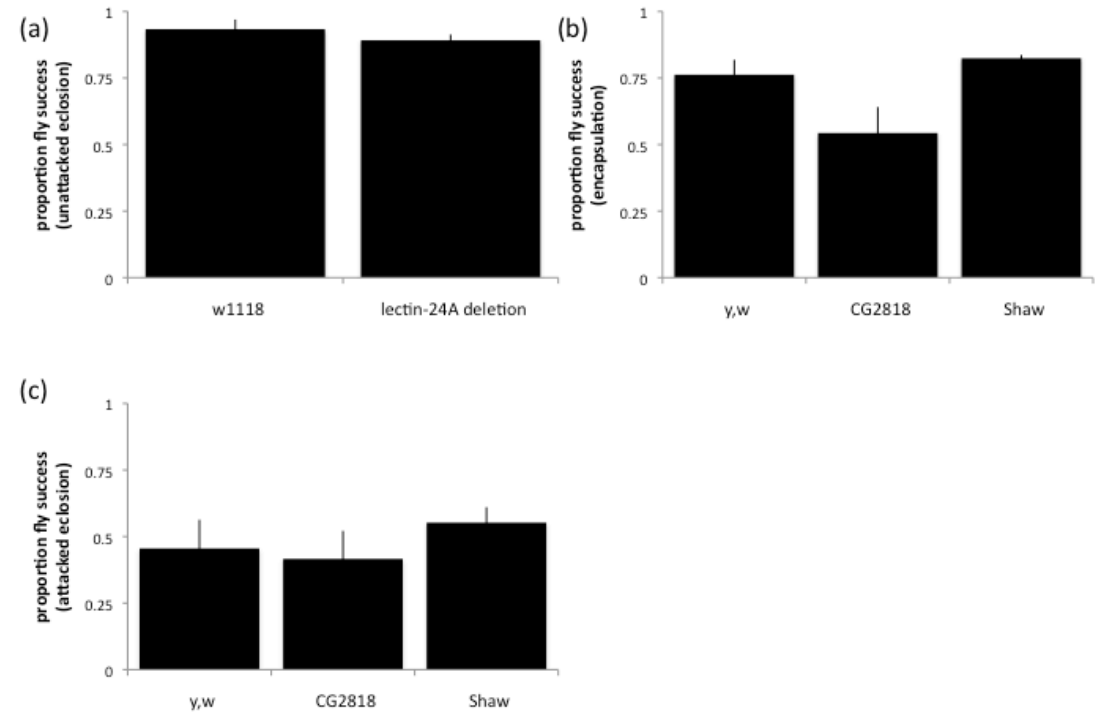


Fig 1: Proportion fly success measured by (a) encapsulation of LcNet eggs and (b) eclosion post-LcNet attack. Bars represent replicate averages +/- SEM.

Supplemental Fig 1



Supplemental Fig 1: Proportion fly success measured by (a) eclosion of unattacked flies, (b) encapsulation of LcNet eggs, and (c) eclosion post-LcNet attack. Bars represent replicate averages +/- SEM.

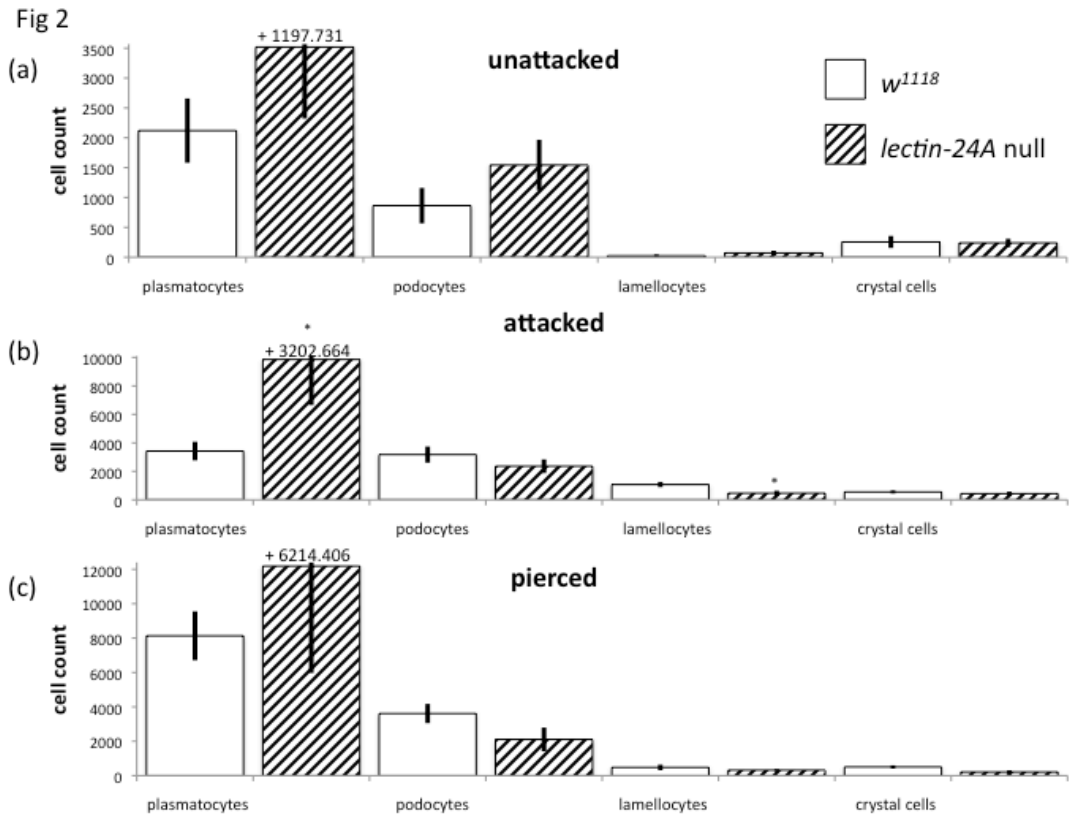


Fig 2: Hemocyte counts in (a) unattacked, (b) attacked, and (c) pierced larvae. Bars represent average counts +/- SEM.

Supplemental Table 1

plasmatocytes	p-value
following wasp attack	
no significant interaction b/w strain and treatment	0.447
lec del had significantly more plasmatocytes than w1118	*0.012
attacked had significantly more plasmatocytes than unattacked	*0.014
following piercing	
no significant effect of strain	0.528
podocytes	
no significant interaction b/w strain and treatment	0.102
no significant effect of strain	0.890
attacked had significantly more podocytes than unattacked	*0.003
following piercing	
no significant effect of strain	0.183
lamellocytes	
no significant interaction b/w strain and treatment	0.163
lec del had significantly more lamellocytes than w1118	*0.040
attacked had significantly more lamellocytes than unattacked	*p<0.001
following piercing	
no significant effect of strain	0.379
crystal cells	
no significant interaction b/w strain and treatment	0.778
no significant effect of strain	0.528
attacked had significantly more crystal cells than unattacked	*0.029
following piercing	
no significant effect of strain	0.109

Supplemental Table 1: statistical analyses of hemocyte counts (unattacked v. wasp-attacked counts and pierced counts between w^{1118} and *lectin-24A* deletion larvae). * $p<0.05$.

Fig 3

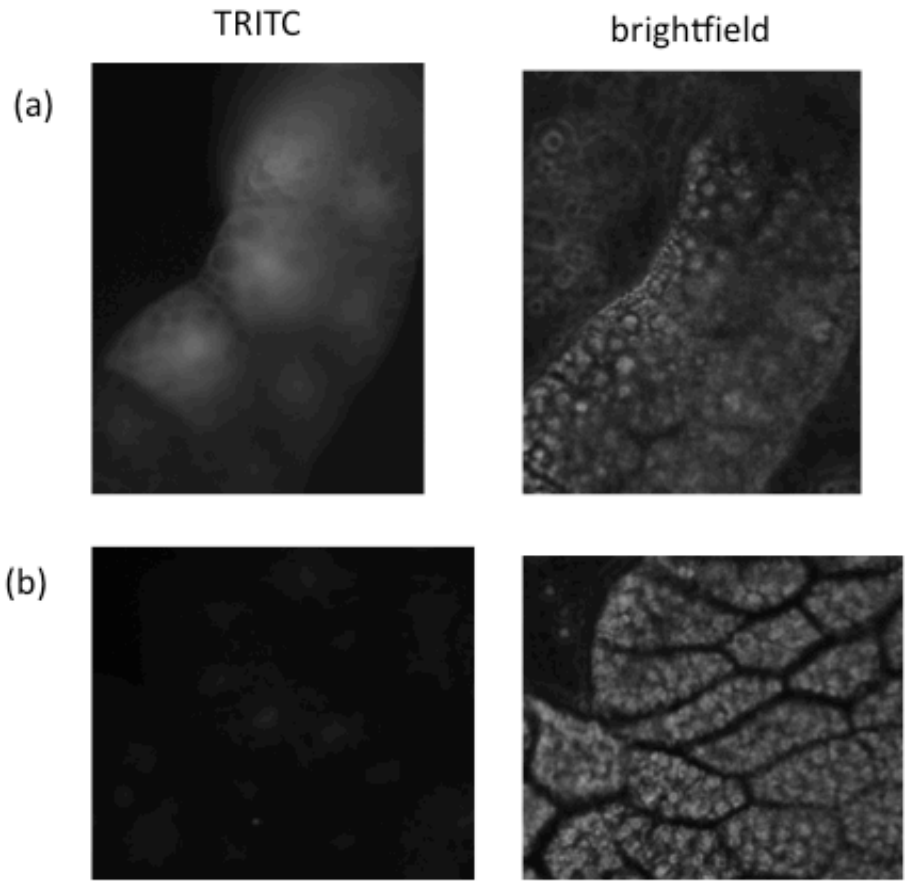


Fig 3: representative fat body when *lectin-24A* reporter activity is (a) present or (b) absent.

Fig 4

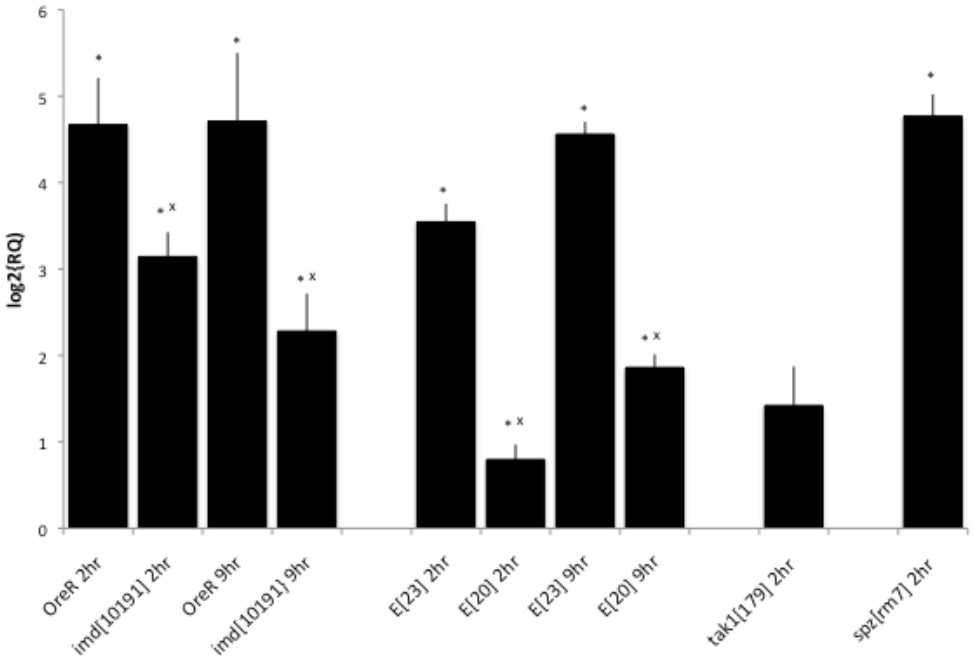


Fig 4: $\log_2$ transformation of *lectin-24A* relative quantification values across control and pathway mutant strains. Times denote time post-Lb17 attack. * $p<0.05$ when measuring significance of *lectin-24A* upregulation post attack, ^{x} $p<0.05$ when measuring significance of *lectin-24A* expression levels in attacked mutant versus control strains.

Supplemental Table 2

comparison	test used	p-value
OreR v imd[10191] 2hr post attack	2 tailed t-test, equal n	*0.047
OreR v imd[10191] 9hr post attack	2 tailed t-test, equal n	*0.036
Rel[E23] v Rel[E20] 2hr post attack	2 tailed t-test, equal n	*p<0.001
Rel[E23] v Rel[E20] 9hr post attack	2 tailed t-test, equal n	*p<0.001
lectin-24A upregulation 2hr PA OreR	2 tailed paired t-test	*0.003
lectin-24A upregulation 9hr PA OreR	2 tailed paired t-test	*0.010
lectin-24A upregulation 2hr PA imd[10191]	2 tailed paired t-test	*0.002
lectin-24A upregulation 9hr PA imd[10191]	2 tailed paired t-test	*0.013
lectin-24A upregulation 2hr PA Rel[E23]	2 tailed paired t-test	*p<0.001
lectin-24A upregulation 9hr PA Rel[E23]	2 tailed paired t-test	*p<0.001
lectin-24A upregulation 2hr PA Rel[E20]	2 tailed paired t-test	*0.020
lectin-24A upregulation 9hr PA Rel[E20]	2 tailed paired t-test	*0.001
lectin-24A upregulation 2hr PA Tak1[179]	2 tailed paired t-test	0.052
lectin-24A upregulation 2hr PA spz[rm7]	2 tailed paired t-test	*p<0.001
abundance in 2U tissues OreR v imd[10191]	2 tailed t-test, equal n	*p<0.001
abundance in 2A tissues OreR v imd[10191]	2 tailed t-test, equal n	*p<0.001
abundance in 9U tissues OreR v imd[10191]	2 tailed t-test, equal n	*p<0.001
abundance in 9A tissues OreR v imd[10191]	2 tailed t-test, equal n	*p<0.001
abundance in 2U tissues Rel[E23] v Rel[E20]	2 tailed t-test, equal n	*p<0.001
abundance in 2A tissues Rel[E23] v Rel[E20]	2 tailed t-test, equal n	*0.009
abundance in 9U tissues Rel[E23] v Rel[E20]	2 tailed t-test, equal n	*p<0.001
abundance in 9A tissues Rel[E23] v Rel[E20]	2 tailed t-test, equal n	*p<0.001

Supplemental Table 2: statistical analyses of *lectin-24A* expression levels. * $p<0.05$.

Table 1

	abundance	SEM
OreR 2hr	0.199588147	0.143061139
OreR 2hrA	12.1417864	11.27996881
OreR 9hr	0.053064101	0.027974938
OreR 9hrA	4.028756248	3.630083746
imd[10191] 2hr	3.17711E-05	3.71527E-06
imd[10191] 2hrA	0.000291298	5.52704E-05
imd[10191] 9hr	3.44176E-05	3.85573E-06
imd[10191] 9hrA	0.000173804	3.7378E-05
Rel[E20] 2hr	0.088493478	0.007873118
Rel[E20] 2hrA	0.157246936	0.021769245
Rel[E20] 9hr	0.073463127	0.004237548
Rel[E20] 9hrA	0.271028242	0.031303228
Rel[E23] 2hr	0.00478932	0.0004029
Rel[E23] 2hrA	0.059228932	0.012631058
Rel[E23] 9hr	0.002233338	0.000155742
Rel[E23] 9hrA	0.053428719	0.005781112
spz[rm7] 2hr	0.005801826	0.00060293
spz[rm7] 2hrA	0.163843806	0.029723799
Tak1[179] 2hr	0.000111403	2.48954E-05
Tak1[179] 2hrA	0.000297474	6.67231E-05

Table 1: abundance of *lectin-24A* relative to *alpha-Tub84B* in different fly strains. Abundance represents the average of 4 replicates. SEM values included.

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Chapter 4: *edl's* role in the Drosophila anti-wasp immune response.

Innate immunity encompasses a variety of primary defense mechanisms against infection that are highly conserved across the tree of life. Studies on the innate immune response of the fruitfly *Drosophila melanogaster* have revealed much about the genetics of innate immunity and have informed translational studies. For example, characterization of the role Toll-like receptors hold in innate immunity was first accomplished in a study using the Drosophila model (Lemaitre et al. 1996), and follow-up studies have identified pathogen-recognition functions of human Toll-like receptors.

In nature, Drosophila are commonly infected by parasitoid wasps (Carton 1986) (Janssen et al. 1987), which oviposit eggs directly into the larval hemocoel. In a successful parasitoid infection, one wasp succeeds in hatching and consuming Drosophila larval tissue, and eclosing from the Drosophila pupal case. This intimate relationship leads to a continual arms race between host immune proteins and pathogen virulence proteins. As such, pathogens exert huge selective forces on natural fly populations, reflected in the rapid evolution of *D. melanogaster* immune genes (Lazzaro, Clark 2003; Schlenke, Begun 2003; Sackton et al. 2007; Lazzaro 2008; Obbard et al. 2009), and host-parasite coevolution can lead to variation in parasite resistance within populations (Magwire et al. 2012). Natural variation in wasp resistance was identified in wild-caught flies, and subsequent studies mapping wasp resistance identified two distinct loci that conferred resistance against two different wasp species (Carton, Frey, Nappi 1992; Benassi, Frey, Carton 1998). These studies, along with a chromosome-wide analysis of wasp resistance across populations (Orr, Irving 1997), are the first of their kind and identified the only

naturally evolved parasitoid resistance loci in the fruitfly, providing wonderful insight into ecologically relevant defense mechanisms of Drosophila. In fact, the study of fly antiwasp immune responses offers a unique opportunity to identify novel genetics behind the innate immune response of the fruitfly (Keebaugh, Schlenke 2013).

Fly larvae respond to wasp infection by mounting a cellular encapsulation response (Carton, Nappi 1997). Cellular immunity is mediated by circulating hemocytes, of which *D. melanogaster* have four main types: plasmatocytes, which are responsibly for the phagocytosis of invading microbes and apoptotic cells, make up approximately 95% of total constitutive hemocytes; crystal cells, which are capable of melanin production that proves useful in wound healing (Galko, Krasnow 2004) and likely in pathogen killing; lamellocytes, which are differentiated from hemocyte precursors in the lymph gland (Lanot et al. 2001) and from circulating plasmatocytes (Markus et al. 2009; Stofanko, Kwon, Badenhorst 2010) following wasp infection and function in the encapsulation response; and podocytes, which are suggested to represent a transitional state as plasmatocytes differentiate into lamellocytes (Rizki 1957).

In the cellular encapsulation response, the fly recognizes the wasp egg as foreign and mounts a cellular immune response against the egg resulting in a multilayered, melanized hemocyte capsule (Carton, Nappi 1997). Plasmatocytes are sentinels of infection and bind to the wasp egg, forming the primary hemocyte layer on the wasp egg surface. Following plasmatocyte adherence, lamellocytes serve as the main bulk of the capsule as they build upon the initial plasmatocyte layer. Eventually, this multilayered hemocytic

capsule becomes melanized and kills the entrapped wasp egg (Russo et al. 1996) (Mortimer et al. 2012) (Williams, Ando, Hultmark 2005). Understanding the genes involved in this anti-wasp immune response is an active area of study (Howell et al. 2012) (Mortimer et al. 2013) (Mortimer et al. 2012) (Williams, Ando, Hultmark 2005) (Williams et al. 2006) , and microarray analyses of wasp-attacked Drosophila identified the involvement of canonical immune genes of the Toll and Jak/Stat pathways along with numerous novel anti-wasp immune candidates (Wertheim et al. 2005; Schlenke et al. 2007).

Here, we focus on the mapped wasp resistance locus, *rlb*, so named because it confers 'resistance to *Leptopilina boulardi* (strain G486) (Hita et al. 1999; Poirie et al. 2000). *rlb* harbors multiple genes but it is believed that changes in expression levels of *edl* lead to the variation in *L. boulardi* resistance. During fly development, *edl* acts to regulate the activity of the ETS domain containing transcriptional repressor *yan*/*aop* (Baker et al. 2001) (Tootle, Lee, Rebay 2003) (Vivekanand, Tootle, Rebay 2004). Yan activity is attenuated by ERK MAP Kinase phosphorylation, which results in the *edl*-dependent nuclear export of Yan (O'Neill et al. 1994) (Rebay, Rubin 1995) (Tootle, Lee, Rebay 2003).

Interestingly, *yan* is a positive regulator of hemocyte differentiation in the lymph gland (Tokusumi et al. 2011), and ectopic expression of an Edl-insensitive form of Yan (Yan^{ACT}) (Baker et al. 2001) in larval hemocytes leads to hemocyte proliferation and the ectopic differentiation of lamellocytes (Zettervall et al. 2004), and phenocopies *edl*

mutant phenotypes in developing embryos (Tootle, Lee, Rebay 2003). We have previously shown that *edl* is significantly differentially regulated following *L. boulardi* (strain Lb17) attack (Schlenke et al. 2007), and these findings suggest that Edl and Yan mediated signaling may be important for fly hematopoiesis and play a role in cellular immune response to wasp infection.

Because *edl* is differentially regulated following wasp attack, lies within *rlb*, and is putatively connected to hematopoiesis via Yan, we performed experiments to determine if *edl* is important for D. melanogaster's cellular immune response against parasitoid wasps.

Results

edl is differentially regulated in hemocytes post-wasp attack

To determine if *edl* is expressed in fruitfly immune tissues (fat body, hemocytes, lymph gland), we imaged the *edl* GFP reporter strain *CB04040*. GFP expression was detected in some hemocytes, and in the fat body, but not in the lymph glands (Supplemental Fig 1). For quantitative analysis following wasp attack, *edl* transcript levels were monitored in unattacked and attacked larvae and in individual larval tissues at two timepoints following *L. boulardi* (strain Lb17) attack as done in (Schlenke et al. 2007) (Keebaugh, Schlenke 2012). We analyzed the immune tissues showing GFP expression, the fat body and hemocytes (Fig 1), and as a control, two non-immune tissues, the gut and body wall muscles (Supplemental Table 1). While *edl* was, on average, upregulated following wasp

attack in whole larvae, it was not significantly upregulated as in (Schlenke et al. 2007), perhaps due to the differential sensitivity of the techniques used. Nor was *edl* differentially regulated in the fat body, gut, or in body wall muscles. However, *edl* levels were significantly downregulated 9-12 hours following wasp attack in hemocytes (Fig 1, Supplemental Table 1). It is not surprising that the decrease in *edl* expression levels in hemocytes is not reflected in overall larval *edl* expression levels because hemocytes contribute a small amount to whole larval transcript totals.

edl is important in melanotic encapsulation

The detected decrease in *edl* expression in immune-induced hemocytes could be critical for regulation of hemocyte proliferation or function following attack, which could factor into a proper melanotic encapsulation response. To test if potential *edl*-mutant fly strains are impaired in their ability to encapsulate wasp eggs, we attacked three different homozygous viable fly strains containing insertions within the *edl* transcript or approximately 20 base pairs upstream of *edl's* transcription start site with *L. clavipes*, which is relatively avirulent in wild-type *D. melanogaster* strains. LcNet eggs were readily encapsulated by control strains (w^{1118} and *y,w*), while the three insertion strains showed variation in their melanotic encapsulation ability (Fig 2). Melanotic capsules that were formed in *CB04040* tended to be pale brown in coloration as opposed to its background control, *y,w*, which formed darker, black melanotic capsules. Strain *EY11665*, also in a *y,w* background, produced dark melanization that often did not surround the entire wasp egg, resulting in smaller melanized particles in the larval

hemocoel. Strain *HP26541*, in w^{1118} background, very rarely formed melanotic capsules around wasp eggs, and mostly produced small melanized particles in the hemocoel.

We next tested if the visible wasp egg melanization defects of the three insertion strains affects rates of egg melanization by assaying LcNet encapsulation rate in each strain. *CB04040, EY11665*, and *HP26541* encapsulated LcNet eggs at a significantly lower level ($p = 0.00669$, $p < 0.001$, and $p < 0.0001$, respectively) than their background controls (Fig 3), suggesting that the visible defects in LcNet encapsulations shown in Fig 2 translate into low encapsulation rates, and that *edl* is important for proper wasp egg encapsulation.

EY11665 and *HP26541* are loss of function alleles of *edl*

To test if the drop in wasp egg encapsulation rates of the three *edl* locus insertion strains is due to altered *edl* expression or to background effects in each strain, we performed qPCR to measure *edl* transcript levels in each strain, followed by complementation testing among the insertion strains. Whereas *EY11665* and *HP26541* both showed decreased *edl* levels relative to control, *edl* levels were higher in *CB04040* (data not shown). This suggests that *EY11665* and *HP26541* (but not *CB04040*) are likely to be *edl* alleles. Accordingly, $edl^{EY11665}/edl^{HP26541}$ larvae encapsulated LcNet wasp eggs at a significantly lower rate than the control cross, $w^{1118}/y,w$ ($p < 0.001$), and complementation tests with *CB04040* (*CB04040*/$edl^{EY11665}$ and *CB04040*/$edl^{HP26541}$) showed no significant decrease in encapsulation ability (Fig 4). Thus, $edl^{EY11665}$ and

edl^{HP26541} fail to complement each other and their defective encapsulation of LcNet eggs is likely due to their insertion in and near *edl*. These results further suggest that *CB04040* is not an allele of *edl* and that the encapsulation defects displayed by *CB04040* are not due to loss-of-function of *edl*.

edl^{HP26541} shows decreased fly eclosion rates

To test the ecological relevance of the decreased encapsulation ability of *edl* alleles *edl*^{EY11665} and *edl*^{HP26541}, we assayed eclosion rates of flies following LcNet attack. Only strain *edl*^{HP26541} suffered significantly lower fly success ($p < 0.0001$), and significantly higher wasp success than control ($p < 0.001$) (Fig 5a,b). There was no significant difference in the proportion of fly success between *edl*^{EY11665} and *y,w*, although more wasps were successful post-attack on *y,w* larvae ($p = 0.041$) (Fig 5a,b; all statistics for resistance analyses are listed in Supplemental Table 2). It should be noted that *y,w* shows a large decrease in the proportion of fly success in eclosion experiments compared to encapsulation experiments. Thus, it is difficult to detect decreases in fly success when comparing strains in the *y,w* background. Interestingly, *edl*^{EY11665} flies that survived attack contained small melanized particles (see Fig 2), not fully encapsulated wasp eggs, suggesting that LcNet eggs can be killed without the formation of long-term, complete capsules. Previous studies that mapped *edl* in a *L. boulardi* G486-resistance locus did so by measuring the encapsulation ability between *L. boulardi*-resistant and susceptible fly strains. Thus, *edl* may be important in the encapsulation of LcNet and *L. boulardi* (strain G486) wasp eggs, but *edl* is not necessarily required fly survival following wasp attack.

edl mutants show defects in hemocyte response post-wasp attack

To determine if the wasp encapsulation ability of *edl* mutants results from a defective hemocyte response, hemocytes were counted before and after LcNet attack. There were no significant differences in plasmatocyte counts between *edl* mutants and their respective controls following wasp attack. Following attack both control and *edl* mutant larvae demonstrated increased production of podocytes (all: $p<0.0001$) and lamellocytes (all: $p<0.001$) (Fig 6a,b), but interestingly $edl^{EY11665}$ had significantly more podocytes than *y,w* ($p<0.0001$) (Fig 6b), and both $edl^{HP26541}$ and $edl^{EY11665}$ had significantly more lamellocytes than their controls ($p=0.003$ and $p=0.023$, respectively)(Fig 6a,b). These findings suggest that *edl* plays a role in the regulation of hemocyte production following wasp attack.

We also pierced larvae with a sterile needle to induce the cellular immune response (Markus et al. 2005) to determine if a wasp-induced signal is involved in the production of excess podocytes and/or lamellocytes in *edl* mutants. We found that excess numbers of lamellocytes and/or podocytes of $edl^{HP26541}$ and $edl^{EY11665}$ are not seen following piercing, suggesting that these cell types are in excess post-wasp attack due to a wasp-specific signal that is not a factor in the general induction of the cellular immune response (Supplemental Table 3; all statistics for hemocyte analyses are listed in Supplemental Table 4).

Overexpression of *edl* does not increase encapsulation ability

Because of the evidence that *edl* is expressed in the fat body, some hemocytes, and is differentially regulated in hemocytes post-wasp attack, we decided to test if overexpression of *edl* in these tissues in a susceptible fly strain would result in an increased ability to encapsulate *L. boulardi* G486. We drove expression of *edl* by crossing the UAS-site bearing enhancer-promoter strain $edl^{HP26541}$ (Rorth et al. 1998) to hemocyte (*He-Gal4*) and fat body (*C833*) drivers. No significant increase in wasp encapsulation ability was found (Supplemental Table 5), suggesting that an increase in *edl* expression in immune tissues of strain $edl^{HP26541}$ is not sufficient for rescuing G486 egg encapsulation. In fact, CB04040 displays decreased *L. clavipes* encapsulation ability despite increased levels of *edl* transcript (data not shown).

Expression of Yan^{ACT} in hemocytes does not alter encapsulation ability

In other developmental contexts, *edl* functions to negatively regulate the transcriptional repressor Yan, and expression of Yan^{ACT} in embryonic tissues phenocopies *edl* mutations (Tootle, Lee, Rebay 2003). We expressed Yan^{ACT} in hemocytes and the fat body (via the *He-Gal4* and *C833* drivers) and assayed encapsulation of *L. clavipes* eggs. Expression of Yan^{ACT} in the fat body resulted in arrested larval growth, so encapsulation rates in these larvae could not be measured. We found that Yan^{ACT} expression in hemocytes did not lead to a significant change in encapsulation success (Supplemental Table 6), suggesting that *edl* may function to regulate a novel target during the encapsulation process.

edl shows no signs of rapid evolution

Because *edl* is located in *rlb*, and susceptible and resistant *rlb* alleles are segregating in a natural population of flies, it follows that *edl* could be evolving under parasitoid wasp-induced selective pressures. We sequenced the *edl* locus in natural populations of *D. melanogaster* and *D. simulans* from ancestral and new world localities. To measure for signs of recent selective pressures, we analyzed polymorphism data to calculate the haplotype diversity, Theta-Watterson (a measure of heterozygosity), and Tajima's D (a measure of allele frequency distribution) of each population. No significant results were found in polymorphism analyses of the *edl* locus within any of the four fruitfly populations sequenced (Supplemental Table 7a). We next performed McDonald-Kreitman analysis, which compares the ratio of nonsynonymous to synonymous differences between species to that same ratio within species (McDonald, Kreitman 1991). No significant results were obtained from McDonald-Kreitman analysis of the *edl* coding sequence between species of Drosophila using *D. yakuba* as an outgroup (Supplemental Table 7b). Given *edl's* important role in regulating *yan*, it is not surprising that *edl's* coding region is not evolving rapidly. As suggested by the absence of association between coding changes and *L. boulardi* susceptibility (Hita et al. 2006), it could be that slight regulatory changes of *edl* drive the variation in *L. boulardi* resistance, and these changes would not be detected in our divergence analyses.

Conclusion

edl falls within the *rlb* locus, and is likely the resistance-causative gene (Hita et al. 2006). We show that *edl* is important for the encapsulation of another closely related Figitid species *L. clavipes* (LcNet), and in combination with earlier mapping studies (Carton, Frey, Nappi 1992; Benassi, Frey, Carton 1998; Hita et al. 2006), our findings suggest that *edl* is *rlb*.

Post-LcNet attack, there are interesting trends in the hemocyte counts of *edl* mutants. In particular, $edl^{HP26541}$ had significantly more lamellocytes than w^{1118}, and $edl^{EY11665}$ had significantly more podocytes and lamellocytes than *y,w*. This suggests that $edl^{EY11665}$ and $edl^{HP26541}$ have higher podocytes and/or lamellocytes in circulation following wasp attack because the cells either fail to adhere to wasp eggs (reflected in the low LcNet encapsulation rates of these strains), or because the strains are not capable of shutting down blood cell production and differentiation following either a general induction of the cellular immune response, or perhaps a wasp-specific-induction of the cellular immune response. To differentiate between these hypotheses, we measured hemocyte levels in larvae of each strain following sterile needle piercing (to activate the cellular immune response without the factor of wasp-specific signals) and did not find excess production of lamellocytes and/or podocytes. Thus, following wasp attack, we detect elevated counts of some cell types in *edl*-mutant strains either because of their inability to properly adhere to invading wasp eggs, their inability to react to negative feedback signals that normally serve to shut down the production of lamellocytes, or to some combination of both factors.

Our finding that *edl*-mutant strains have increased numbers of circulating lamellocytes and/or podocytes post attack is especially interesting given that Yan^{ACT} is known to phenocopy some *edl* mutant phenotypes (Tootle, Lee, Rebay 2003), that Yan^{ACT} expression in hemocytes leads to hemocyte proliferation and differentiation (Zettervall et al. 2004), and that *yan* regulates hemocyte differentiation in the lymph gland (Tokusumi et al. 2011). Perhaps *edl* acts through *yan* to regulate blood cell numbers and hemocyte differentiation, leading to the increased circulating hemocytes we detected post LcNet attack, but has a different target important for regulating wasp egg encapsulation, which is why Yan^{ACT} expression in hemocytes did not lead to a decrease in LcNet encapsulation. This idea supports the importance of the use of natural pathogens in identifying novel immune genes, and a more complete understanding of *edl*-mediated signaling in hemocytes will be important to understand fly cellular immunity.

Materials and methods

Insect strains

CB04040 contains a P{PTT-GB} insertion 20 base pairs upstream of the *edl* 5' UTR and is a FlyTrap Enhancer strain (Buszczak et al. 2007). This strain was used as an *edl*-reporter strain. $edl^{HP26541}$ and $edl^{EY11665}$ (Bloomington) contain a P{EPg} insertion approximately 20 base pairs upstream of the *edl* 5' UTR (the exact insertion location is unknown), and a P{EPgy2} insertion within the *edl* 5' UTR, respectively. Overexpression analyses were performed using $edl^{HP26541}$ and and Yan^{ACT} (Bloomington)

crossed to Gal-4 drivers *C833* (Hrdlicka et al. 2002) and *He-GAL4* (Zettervall et al. 2004). w^{1118} is used as the genetic background control for $edl^{HP26541}$ and *y,w* is used as the genetic background control for $edl^{EY11665}$ and *CB04040*. Because Yan^{ACT} is in an unknown background, w^{1118}/*y,w* flies were used as the genetic background control for Yan^{ACT} overexpression experiments in statistical analyses. Only homozygous viable insertion strains were used in our analyses because balancers negatively affect wasp resistance. All experiments were performed at 25°C.

Wasp strains used were *L. boulardi* strains Lb17 and G486, and *L. clavipes* strain LcNet. *L. boulardi* stains are maintained on *D. melanogaster* strain *Canton S*, and *L. clavipes* is maintained on *D. virilis*. G486 was provided by D. Hultmark, and *L. clavipes* was provided by J. van Alphen. Lb17 was collected in California, United States of America (Schlenke et al. 2007).

Imaging of *edl* reporter

CB04040 flies were allowed to lay eggs on molasses plates supplemented with yeast paste for 24 hours. Thirty early third instar larvae were picked from egg lay plates and transferred to standard Drosophila medium in 35 mm diameter Petri dishes. Age-matched larval tissues were dissected and imaged (brightfield and FITC) 24 hours after transfer. Lymph glands and blood cells were dissected under oil immersion, and fat bodies were dissected in Ringer's solution.

Gene expression analysis

Relative-quantification analyses using *D. melanogaster* strain *Oregon R* and Lb17 was performed as in (Keebaugh, Schlenke 2012). Briefly, flies were allowed to lay eggs for 3 hours on molasses medium with yeast paste, and 60 larvae from these egg lays were later moved onto 35 mm Petri dishes containing standard Drosophila medium. 72 hours after egg lay, 10 female wasps were placed in the Petri dishes and allowed to attack for 2 hours. 2 and 9 hours post-attack, fly larvae were dissected or flash frozen expression analyses. Because of attack duration and handling time, these larvae had developed between 2-5 and 9-12 hours post-attack. From each dish, ten larvae were used for whole body expression analysis, and another 10 were dissected for tissue-specific expression analyses from the fat body, gut, and body wall muscle. Dissected tissues were placed into Trizol (Invitrogen), and whole larvae were placed into 1.5 mL tubes and frozen in liquid nitrogen. Remaining larvae from each dish were used for hemocyte analyses by draining hemolymph onto a metal rod that was then submerged into Trizol. RNA extraction for was done using Trizol following Invitrogen's recommended protocol and cDNA was synthesized using the Qiagen Quantitect Reverse Transcription Kit. Real time PCR was performed using Applied Biosystems Power SYBR Green Master Mix and samples were run in triplicate with any significant outlier within a triplicate discarded. *alphaTub84B* was used as a reference gene. Intron spanning primers used for *alphaTub84B* are as follows: 5'-ACACTTCCAATAAAAACTCAATATGC-3', 5'-CCGTGCTCCAAGCAGTAGA-3'. Primers used for *edl* are as follows: 5'-GCCACCTTTGGACCTCAC-3', 5'-GTTATTGCCGCCACCATT-3'.

Relative quantification (known as RQ or delta delta CT) represents the fold change of edl

following wasp attack relative to unattacked control samples. Statistical analysis (2-tailed paired t-test) was performed on log2 transformation of relative abundance values (the abundance of *edl* relative to the reference gene, known as delta CT).

Resistance trials

Egg lay and attack protocols for resistance trials are as reported in (Mortimer et al. 2012). Flies were allowed to lay eggs onto molasses medium supplemented with yeast paste. 72 hours following the start of the egg lay second instar fly larvae were collected for infection trials. 40 fly larvae were moved into 35 mm Petri dishes containing standard Drosophila medium. Three female wasps were placed into the Petri dish and allowed to attack fly larvae for approximately 72 hours.

For whole larva Imaging, larvae were chilled and imaged using a Leica stereo-dissecting scope with a Moticam MIP 2.0 and Multi-Focus Pro software. For encapsulation experiments, fly larvae were dissected to determine the proportion fly success, which was calculated as the number of attacked larvae with only encapsulated wasp eggs divided by the total number of attacked larvae. For eclosion experiments, larvae were transferred to larger food vials and allowed to eclose as either flies or wasps for approximately six weeks. Eclosed flies were examined or dissected to look for encapsulated wasps and counted as unattacked if no sign of melanization was found, or attacked if any sign of melanization was found. Unattacked were flies discarded from analysis. Eclosed wasps were also tallied from each vial.

Generalized linear models (GLMs) with binomial error distributions and logit link functions were used to examine differences between fly strains in proportion of wasp–attacked fly larvae that encapsulated all wasp eggs laid inside them and were thus predicted to survive parasitism. Quasi–binomial errors were used whenever the residual deviance was higher than the residual degrees of freedom for the model with binomial errors. If needed, stepdown pairwise comparisons between fly strains were assessed from these models using Tukey's honestly significant difference test. Differences between complementation crosses were analyzed using a GLM with quasi–binomial error distribution and logit link function. Stepdown pairwise comparisons between crosses were assessed from this model using Tukey's honestly significant difference test. For each fly strain, N = 3 replicates of 40 exposed larvae, approximately 30 of which were dissected, except for eclosion trials, in which at least 5 reps of 40 larvae were allowed to eclose. Any lethality was not counted as fly success.

Fly larvae that have been attacked by wasps can (i) survive parasitism and eclose as flies, (ii) eclose as wasps, or (iii) die. Differences between fly strains in the proportions of wasp–attacked fly larvae that eclosed as flies and wasps were analyzed using GLMs with binomial error distributions and logit link functions. Quasi–binomial errors were used whenever the residual deviance was higher than the residual degrees of freedom for the model with binomial errors. For each fly strain, N = 5 replicates of between 31 and 40 wasp–exposed larvae that were allowed to eclose.

Hemocyte counts

Flies were allowed to lay eggs for 24 hours on molasses medium with yeast paste. For counts following wasp attack, 40 second instar larvae were transferred to 35 mm diameter Petri dishes containing Drosophila cornmeal medium and exposed to 4 LcNet females for 24 hours. For counts following sterile needle piercing, early 3rd instar larvae were rinsed in Ringer's solution, dried, and placed on double sided tape to prevent movement during which time their cuticle was pierced with a 0.125 mm tungsten carbide needle (Fine Science Tools). Following piercing, larvae were transferred to 35 mm diameter Petri dishes containing standard Drosophila medium. 24 hours following wasp attack or piercing, 5 size matched larvae were bled into 20ul of 1X PBS 0.01% phenylthiourea (PTU). This solution containing bled hemolymph was pipetted into Incyto C-Chip DHC-N01 hemocytometer chips and hemocytes were counted from sixteen 0.25X0.25X0.1 mm squares. Hemocyte counts were normalized to per larva values. Each treatment was replicated three times for each strain. It should be noted that waiting 24 hours post-LcNet attack is sufficient for the encapsulation or melanization reaction in all strains (similar to what is seen in Fig 2), and the lamellocyte reaction post parasitoid attack is robust during this time (Lanot et al. 2001). Additionally, because more hemolymph is lost and higher rates of mortality result from needle piercings compared to wasp attack, larvae were aged approximately 24 hours older in piercing experiments to improve the chance of surviving cuticle injury. Because expression of Yan^{ACT} in hemocytes leads to hemocyte proliferation and differentiation of lamellocytes, we present only plasmatocyte, podocyte, and lamellocyte counts. Crystal cell counts are listed in Table 8.

GLMs with quasi–Poisson error distributions and log link functions were used to examine

the interactive and additive effects of fly strain and attack treatment on the numbers of circulating hemocytes (plasmatocytes, podocytes, lamellocytes, crystal cells) within unattacked and attacked fly larvae. For each fly strain, N = 3 replicates of 5 pooled larvae. Starting from the full model H = S + T + (S x T) + E, where H is hemocyte count, S is fly strain, T is attack treatment, S x T is the interaction between S and T, and E is error, the effects of removing individual terms were assessed sequentially. The interaction term was evaluated first and the main effects of S and T were determined if there was no significant interaction. Hemocyte counts were also performed on N = 3 replicates of 5 pooled larvae that were poked with a sterile needle. Differences between strains in counts and proportions of each hemocyte type following sterile pokes were assessed using quasi–Poisson and quasi–binomial GLMs respectively.

Molecular evolution analyses

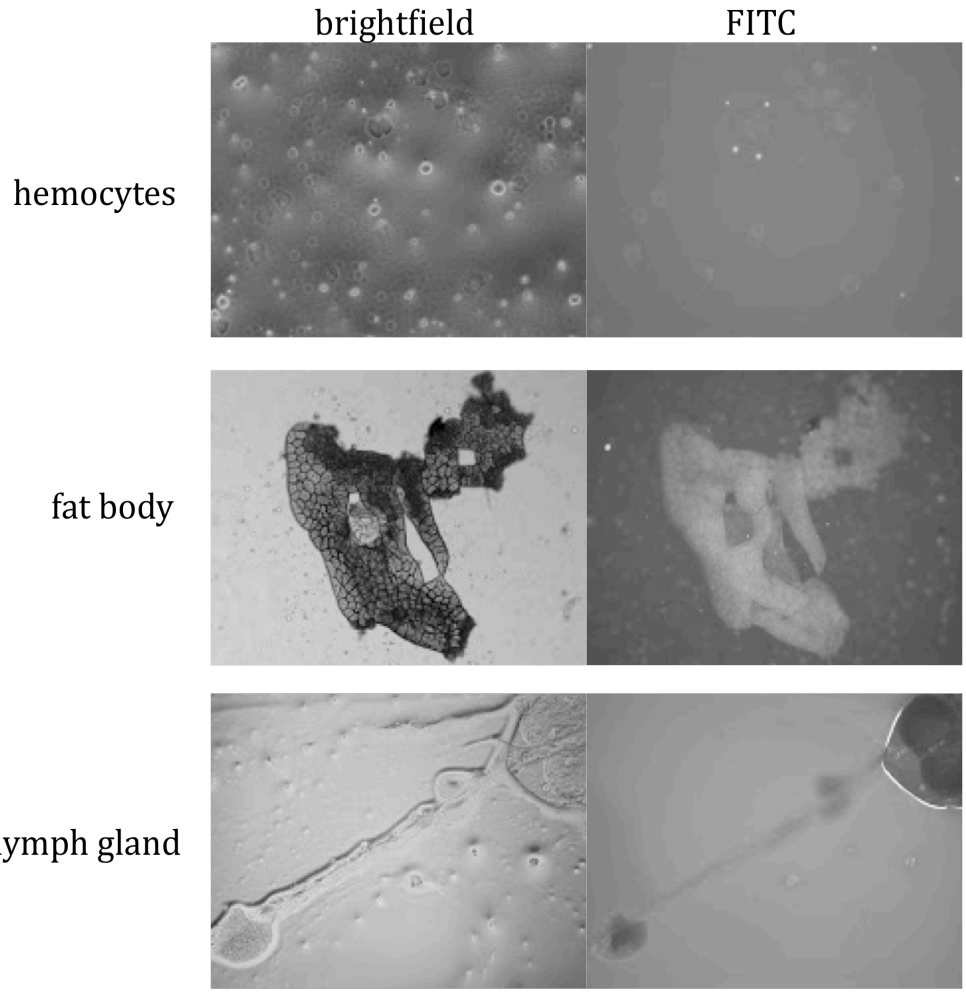
Molecular evolution analyses were performed as in (Keebaugh, Schlenke 2012). Briefly, California *D. melanogaster* and *D. simulans* sequence data are from eight inbred lines from mated field-caught females from a population in Winters, California. African *D. melanogaster* and *D. simulans* sequence data are from ten and nine isofemale lines from populations in Malawi and Zimbabwe, respectively.

PCR primers were made to amplify an approximately 2,016 bp region containing the full coding sequence of edl (534 bp) and immediate surrounding sequence. PCR products were sent to Beckman Coulter Genomics for purification and Sanger sequencing, using two internal primers. Sequences for those primers are as follows: PCR primers 5'-

CATTCCGGCAGCAAGGTCAGATTT-3', 5'-GGTTGCATTTGCCGGGAGGTTT-3', internal primers 5'-CAAATCGGCGTATGCGTGTTA-3', 5'-CTGCGGGAAATTGTGAAAGAC-3'.

Lasergene software was used to analyze sequence data and population genetic and analyses were run in DnaSP version 5.10.01 (Librado, Rozas 2009). Significance of some population genetic statistics for various population samples and loci was calculated by comparing the observed values to those obtained from 10,000 neutral coalescence simulations. Simulated data were generated in DnaSP by using the observed number of segregating sites from each sample and under the conservative assumption of no recombination. Furthermore, comparisons between *D. melanogaster* and *D. simulans* coding sequences with *D. yakuba* used coordinates for the *D. yakuba* open reading frame rather than the longer *D. melanogaster* or *D. simulans* consensus open reading frame.

Supplemental Fig 1



Supplemental Fig 1: CB04040 FITC activity in 3rd instar immune tissues, shown with paired brightfield images.

Fig 1

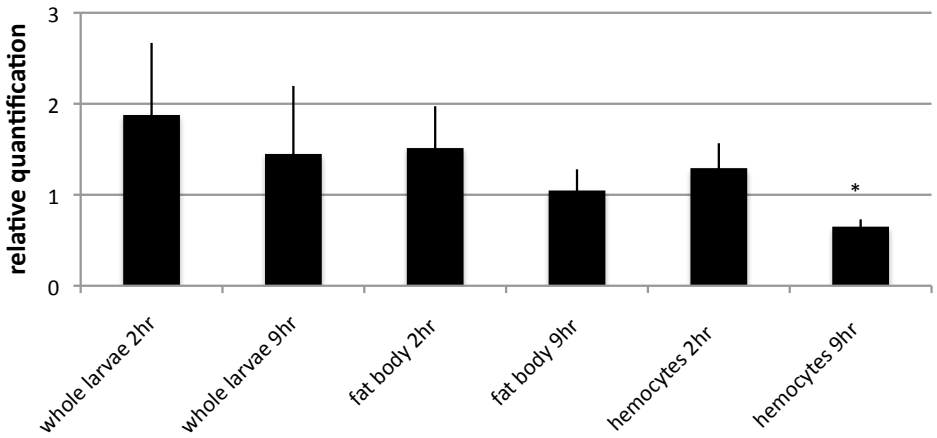


Fig 1. Relative quantification (relative to unattacked controls) of *edl* 2 and 9 hours post Lb17 wasp attack. Values above 1 mean that *edl* is upregulated, below 1 mean that *edl* is downregulated. Average of 4 reps +/- SEM. * $p<0.05$.

Supplemental Table 1

edl semi-quantitative PCR statistics 2-tailed paired t-test timepoint and tissue	relative quantification average +/- SEM	p-value
2 hour whole body	1.877 +/- 0.792	0.422
9 hour whole body	1.449 +/- 0.747	0.990
2 hour fatbody	1.513 +/- 0.460	0.483
9 hour fatbody	1.047 +/- 0.233	0.881
2 hour hemocytes	1.292 +/- 0.274	0.523
9 hour hemocytes	0.649 +/- 0.080	* 0.036
2 hour gut	4.578 +/- 2.986	0.231
9 hour gut	1.868 +/- 0.561	0.232
2 hour body wall	2.877 +/- 1.287	0.279
9 hour body wall	1.082 +/- 0.263	0.866

Supplemental Table 1: Relative quantification of *edl* (relative to unattacked controls) in individual tissues and whole larvae at 2 and 9 hours following Lb17 attack. p-values from statistical analysis of unattacked v attacked values. *$p<0.05$.

Fig 2

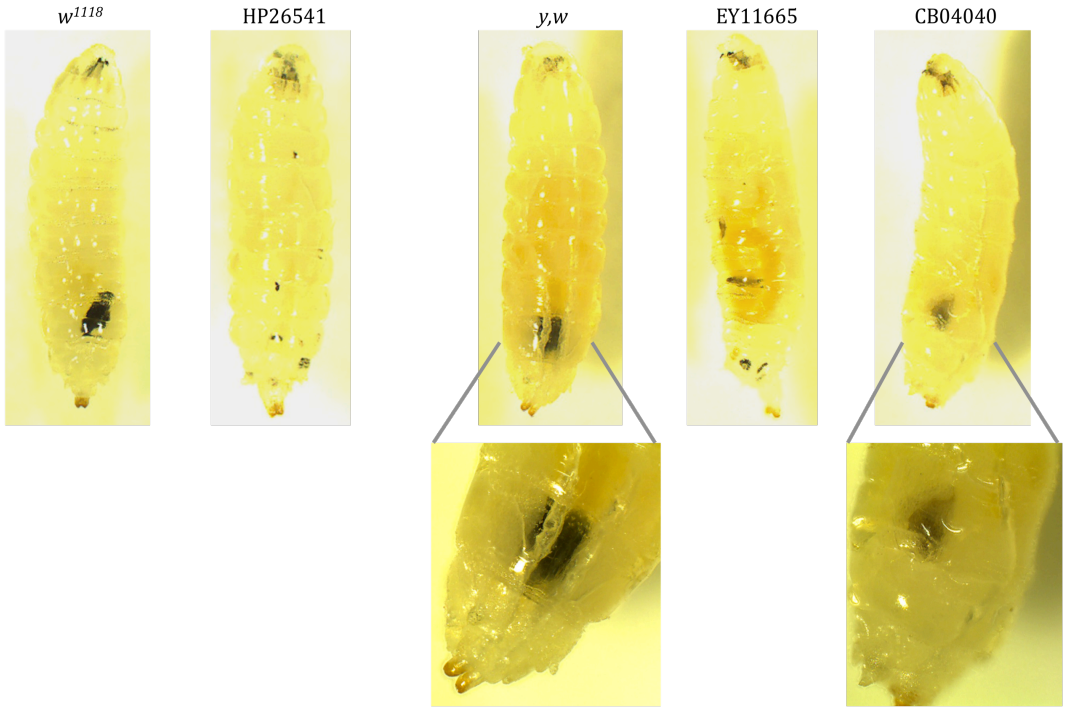


Fig 2. Representative LcNet melanization response of edl insertion strains and background controls. Close-ups of CB04040 and y,w are given to highlight that CB04040 LcNet melanization tended to be light brown, and not black as in control larvae.

Fig 3

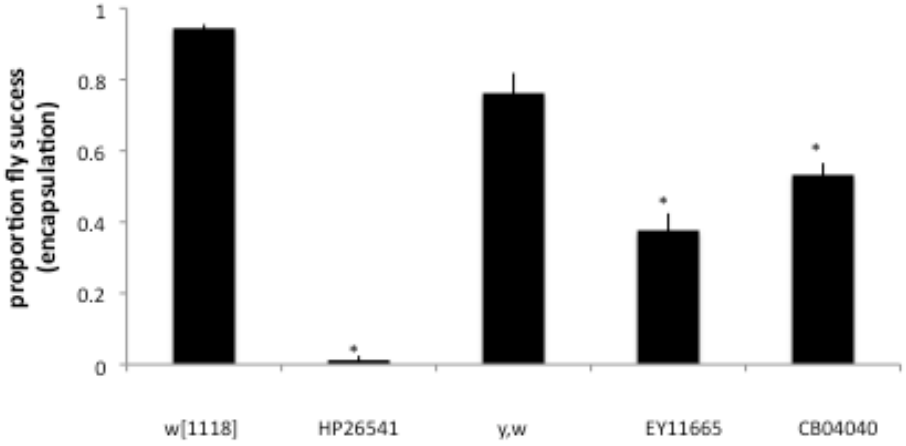


Fig 3. Proportion fly success judged by LcNet encapsulation ability. Average of 3 reps +/- SEM. * $p<0.05$.

Fig 4

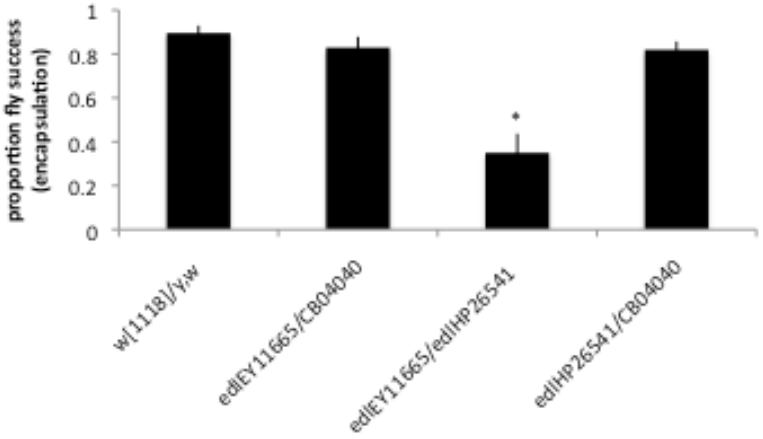
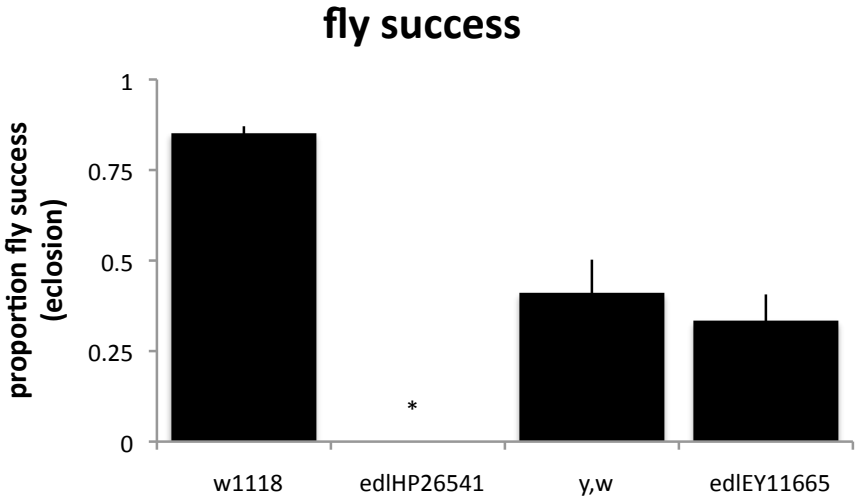


Fig 4. Proportion fly success (encapsulation) of complementation crosses between *edl* insertion strains compared to control cross ($w^{1118}/y,w$). Average of 3 reps +/- SEM. * $p<0.05$.

Fig 5a



b

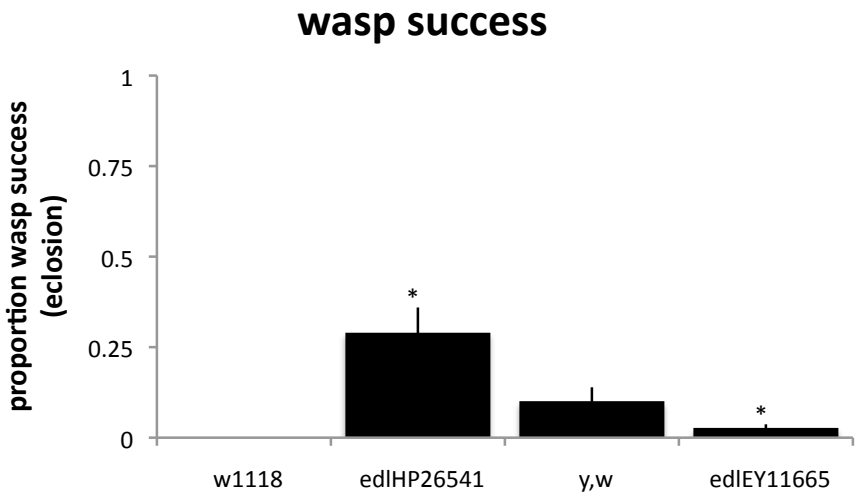


Fig 5. Proportion (a) fly success and (b) wasp success judged by LcNet eclosion ability. Average of 5 reps +/- SEM. * $p<0.05$.

Supplemental Table 2

Baseline strain encapsulation success against LcNet

w1118 won significantly more often than edlHP26541 ($p < 0.0001$)

significant overall difference between y,w, edlEY11665 , and CB04040 ($p < 0.0001$)

y,w won significantly more often than edlEY11665 ($p < 0.001$)

y,w won significantly more often than CB04040 ($p = 0.00669$)

significance groups:

a	y,w
b	edlEY11665
b	edlCB04040

Complementation cross encapsulation success against LcNet

significant overall difference between crosses ($p < 0.0001$)

w1118 x y,w won significantly more often than edlHP26541 x edlEY11665 ($p < 0.001$)

edlEY11665 x CB04040 won significantly more often than edlHP26541 x edlEY11665 ($p < 0.001$)

CB04040 x edlHP26541 won significantly more often than edlHP26541 x edlEY11665 ($p < 0.001$)

no other significant pairwise differences (all $p > 0.44$)

significance groups:

a: w1118 x y,w	b: edlHP26541 x edlEY11665
edlEY11665 x CB04040	
CB04040 x edlHP26541	
HeGal4 x YanAct	

Overexpression cross encapsulation success against LbG486

no significant difference between w1118 x HeGal4 and edlHP26541 x HeGal4 (p=0.0942)

w1118 x C833 never encapsulated, and the binomial logit link function requires taking log(p / (1 - p)), where p = probability of success, in this case encapsulation of all wasp eggs. Therefore, the model based on the raw data may be unreliable because log(0) is undefined, so analysis was performed after adding 1 to the data.

modified: no significant difference between w1118 x C833 and edlHP26541 x C833 (p = 0.0936)(binomial errors)

Eclosion success against LcNet

fly success

w1118 won significantly more often than edlHP26541 ($p < 0.0001$)

no significant difference between y,w and edlEY11665 ($p = 0.505$)

wasp success

wasps eclosed significantly more often from edlHP26541 than w1118 ($p < 0.001$)

wasps eclosed significantly more often from y,w than edlEY11665 ($p = 0.0413$)

Supplemental Table 2: Statistics for encapsulation and eclosion studies.

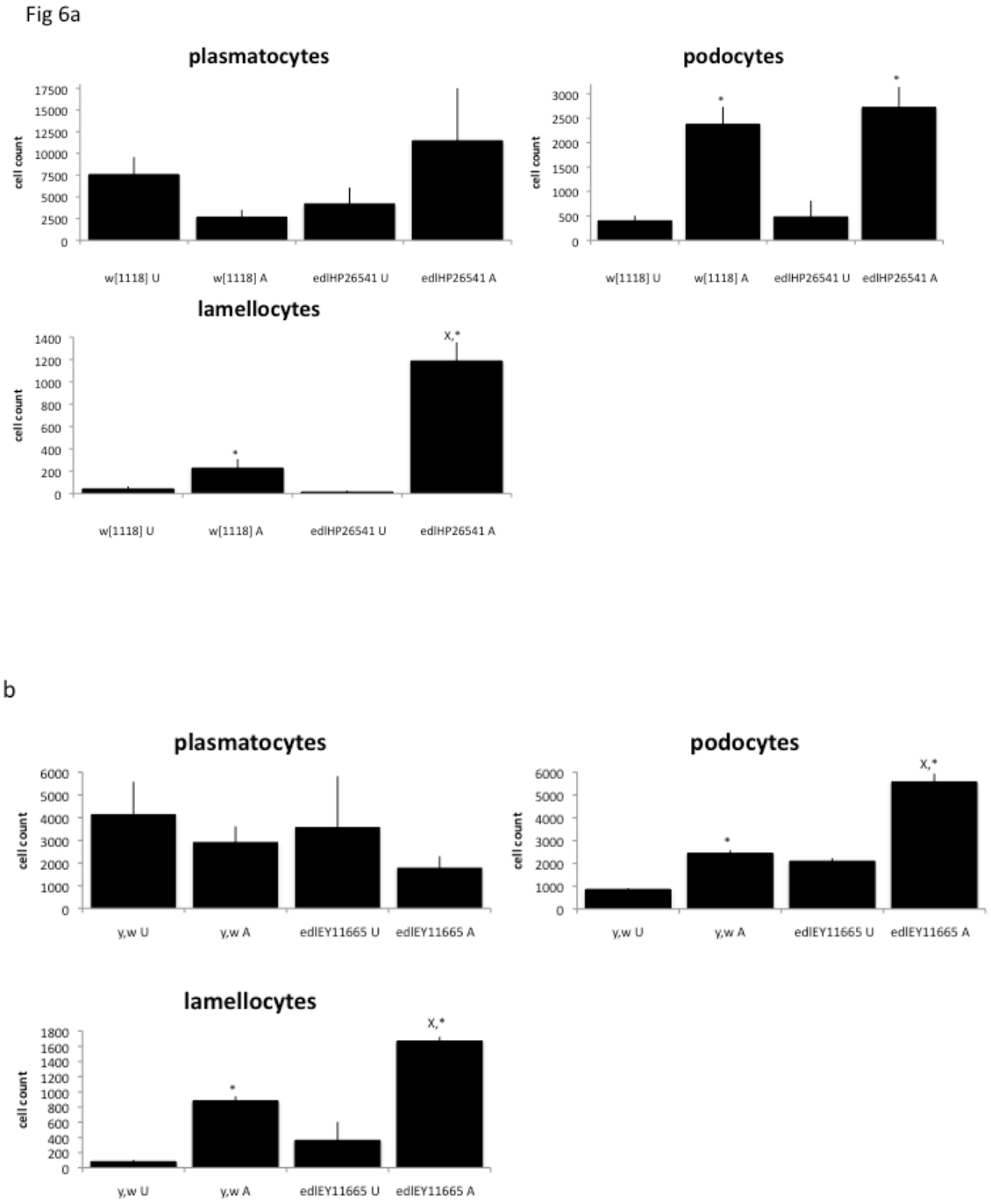


Fig 6. Hemocyte counts in unattacked (U) and attacked (A) (a) w^{1118} and edlHP26541 larvae, and in (b) *y,w*, edlEY11665 , and edlCB04040 larvae. * $p<0.05$ relative to unattacked larvae, X $p<0.05$ relative to control strain.

Supplemental Table 3

strain	treatment	cell type	cell count +/- SEM
w1118	pierced	plasmatocyte	8133.333 +/- 1422.549
edlHP26541	pierced	plasmatocyte	8093.333 +/- 2830.3435
y,w	pierced	plasmatocyte	4586.667 +/- 716.411
edlEY11665	pierced	plasmatocyte	3160.000 +/- 266.333
w1118	pierced	podocyte	3613.333 +/- 558.729
edlHP26541	pierced	podocyte	1386.667 +/- 315.242
y,w	pierced	podocyte	1786.667 +/- 608.532
edlEY11665	pierced	podocyte	3266.667 +/- 996.349
w1118	pierced	lamellocyte	480.000 +/- 161.658
edlHP26541	pierced	lamellocyte	360.000 +/- 83.267
y,w	pierced	lamellocyte	306.667 +/- 70.553
edlEY11665	pierced	lamellocyte	466.667 +/- 13.333
w1118	pierced	crystal cell	506.667 +/- 93.333
edlHP26541	pierced	crystal cell	253.333 +/- 66.667
y,w	pierced	crystal cell	386.667 +/- 188.090
edlEY11665	pierced	crystal cell	440.000 +/- 80.000

Supplemental Table 3: Cell counts.

Supplemental Table 4

Hemocyte counts

w1118 and edlHP26541

Plasmatocytes

attacked counts

no significant interaction between strain and attack ($p = 0.0611$)

no significant effect of strain ($p = 0.442$)

no significant effect of attack ($p = 0.747$)

poked counts

no significant effect of strain ($p = 0.991$)

Podocytes

attacked counts

no significant interaction between strain and attack ($p = 0.950$)

no significant effect of strain ($p = 0.564$)

attacked had significantly more podocytes ($p < 0.0001$)

poked counts

w1118 had significantly more podocytes ($p = 0.0227$)

Lamellocytes

attacked counts

no significant interaction between strain and attack ($p = 0.0703$)

21948 had significantly more lamellocytes ($p = 0.00253$)

attacked had significantly more lamellocytes ($p < 0.001$)

poked counts

no significant effect of strain ($p = 0.532$)

Crystal cells

attacked counts

no significant interaction between strain and attack ($p = 0.875$)

no significant effect of strain ($p = 0.419$)

attacked had significantly more crystal cells ($p = 0.00125$)

poked counts

no significant effect of strain ($p = 0.0900$)

y,w and edlEY11665

Plasmatocytes

attacked counts

no significant interaction between strain and attack ($p = 0.708$)

no significant effect of strain (p = 0.550)

no significant effect of attack (p = 0.233)

poked counts

no significant effect of strain (p = 0.118)

Podocytes

attacked counts

no significant interaction between strain and attack (p = 0.663)

23094 had significantly more podocytes (p < 0.0001)

attacked had significantly more podocytes (p < 0.0001)

poked counts

no significant effect of strain (p = 0.260)

Lamellocytes

attacked counts

no significant interaction between strain and attack (p = 0.320)

23094 had significantly more lamellocytes (p = 0.0228)

attacked had significantly more lamellocytes (p < 0.001)

poked counts

no significant effect of strain (p = 0.116)

Crystal cells

attacked counts

significant interaction between strain and attack (p = 0.0308)

attack led to increased crystal cell counts for 23094

no clear effect of attack on crystal cell counts for y,w; slight decrease

poked counts

no significant effect of strain (p = 0.811)

Supplemental Table 4: Statistics for hemocyte studies.

Supplemental Table 5

strain	proportion fly success +/- SEM
w1118XHe-Gal4	0.076 +/- 0.042
edlHP26541 XHe-Gal4	0.072 +/- 0.018
w1118XC833	0.000 +/- 0.000
edlHP26541 XC833	0.055 +/- 0.037

Supplemental Table 5: Proportion fly success (encapsulation) +/- SEM when *edl* is overexpressed in hemocytes (*Hemes-Gal4*) or in the fat body (*C833*), compared to w^{1118} control cross.

Supplemental Table 6

strain	proportion fly success +/- SEM
HeGal4XYanAct	0.922 +/- 0.019

Supplemental Table 6: Proportion fly success (encapsulation) +/- SEM when Yan^{ACT} is expressed in hemocytes (via the Hemese-Gal4 driver). There is no control strain for Yan^{ACT}, but for statistical analysis, this cross was compared to w^{1118}Xy,w.

Supplemental Table 7

a

	Theta-W	Hd	Hd p-value	Tajima's D	Tajima's D p-value
California *D. melanogaster*	0.009	1.000	1.000	0.023	0.528
Africa *D. melanogaster*	0.009	1.000	1.000	-0.261	0.412
California *D. simulans*	0.009	0.786	0.069	-0.206	0.432
Africa *D. simulans*	0.009	0.889	0.340	0.312	0.671

b

	Synonymous	Nonsynonymous	p-value
All *D. melanogaster* polymorphisms	12	1	0.544
All *D. melanogaster* fixations	7	2	
All *D. simulans* polymorphisms	10	2	0.603
All *D. simulans* fixations	5	2	

Supplemental Table 7: polymorphism (a) and divergence (b) analyses of *edl*. (a) Theta-W is Watterson's theta, a measure of heterozygosity (Watterson 1975). Hd (haplotype diversity) and Tajima's (Tajima 1989) p-values determined from coalescent simulations. (b) McDonald-Kreitman analyses for *edl* in *D. melanogaster* and *D. simulans* populations using *D. yakuba* as an outgroup. p-values from two-tailed Fisher's exact test.

Supplemental Table 8

strain	treatment	crystal cell count +/- SEM
w1118	unattacked	93.333 +/- 35.277
w1118	attacked	506.667 +/- 109.138
edlHP26541	unattacked	80.000 +/- 80.000
edlHP26541	attacked	373.333 +/- 48.074
y,w	unattacked	746.667 +/- 235.891
y,w	attacked	533.333 +/- 70.553
edlEY11665	unattacked	333.333 +/- 150.259
edlEY11665	attacked	1013.333 +/- 176.383

Supplemental Table 8: Crystal cell counts.

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Chapter 5: *pgant35A's* role in the Drosophila anti-wasp immune response.

Host immune responses play an essential role in the defense against invading pathogens and parasites. Immune responses are widely conserved and have been extensively studied in genetic model organisms including the fruit fly *Drosophila melanogaster* [1,2]. Fly immune responses can be broadly classified as either antimicrobial, involving the release of antimicrobial peptides and the phagocytosis of microbes, or antiparasitic, in which the cellular encapsulation response is targeted against macroparasites such as parasitoid wasp eggs [3-5]. Although the study of *D. melanogaster* immunity has largely been focused on antimicrobial responses, recent studies of the mechanisms underlying the cellular encapsulation of parasitoid eggs reveal significant conservation with human cellular immunity [6-8].

The encapsulation response begins with the recognition of the wasp egg and the calcium-dependent activation of fly immune cells following wasp infection [5,8]. These activated hemocytes then migrate towards, and form a layer around, the wasp egg [7,9,10]. Recognition of the wasp egg also induces the differentiation of lamellocytes, a specialized hemocyte subtype that functions in encapsulation, from both circulating hemocytes and hemocyte precursors in the lymph gland [11,12]. These lamellocytes form a multilayered consolidated capsule around the wasp egg, which is subsequently melanized, leading to death of the developing parasitoid [5,7,9]. Despite these advances, there is still much to be learned about the molecular mechanisms underlying cellular encapsulation. Here we show that mucin-type protein O-glycosylation is required for capsule formation.

Protein glycosylation is an evolutionarily ancient post-translational modification and plays roles in protein stability, activity and trafficking [13]. There are two major forms of protein glycosylation, N-linked glycosylation in which preformed carbohydrate groups are covalently attached to asparagine residues, and O-linked glycosylation in which saccharides are covalently attached to serine and threonine residues [14,15]. There are several types of O-linked glycosylation, and mucin-type protein O-glycosylation is among the most common forms in both mammals and flies [16-18]. Proteins modified with mucin-type O-glycans are broadly and dynamically expressed throughout fly development [19-23], and play demonstrated roles in cell adhesion, protein secretion, hematopoesis, and development of the tracheal system [24-28].

Mucin-type O-glycosylation begins with the addition of N-acetylgalactosamine (GalNAc) to serine and threonine residues in nascent proteins in the golgi apparatus [16,29]. This addition of GalNAc is catalyzed by the family of polypeptide α-N-acetylgalactosaminyltransferases (known as GALNTs in mammals and PGANTs in flies). The human genome encodes 20 GALNT family members, and between 12 and 14 PGANTs are found in the *D. melanogaster* genome [30,31]. It has been demonstrated that GALNT/PGANT family members have distinct peptide substrates in both mammals [16,30,32] and flies [22,26,33], although there is some overlap in substrate specificity. Additionally these enzymes show differential expression patterns in mammals [16,30,32] and flies [23], leading to tissue specific patterns of mucin-type protein O-glycosylation [32]. This O-GalNAc residue can be further modified by β1-3-galactosyltransferase

enzymes which catalyze the addition of a β1-3 linked galactose [29]. This structure can also be further modified to produce increasingly elaborate glycans in mammals [16,29], although these highly elaborated forms are rare in flies [17].

Functional assays have demonstrated the transferase activity of eight *D. melanogaster* PGANT family members [22,34]. These PGANTs are broadly expressed throughout fly development [22], although with increasingly tissue specific patterns [23]. One of these PGANTs, *pgant35A*, is an essential gene in fly development [34], and tissue specific knockdown demonstrates that it is required in the mesoderm, trachea (fly respiratory system) and digestive system [35]. The development of the embryonic tracheal system is disrupted in *pgant35A* mutant flies. The main tracheal branches take on an unusual convoluted morphology and lose paracellular barrier function due to defects in the trafficking of secreted and transmembrane proteins in tracheal cells. This leads to the mislocalization of septate junction proteins, and consequently a failure to establish or maintain septate junctions between tracheal cells [27]. Here we demonstrate a novel role for *pgant35A* in the fly cellular immune response following wasp infection. *pgant35A* mutant larvae have decreased resistance to wasp infection, and we show that this is due to defective capsule formation in these mutants.

Results

Larval hemocyte surface proteins are modified by O-glycosylation.

D. melanogaster embryonic hemocytes are stained by the mucin-type O-glycan specific lectin Peanut Agglutinin (PNA) [28,36] suggesting that fly hemocyte surface proteins are modified by mucin-type O-glycosylation. To test whether larval hemocytes also carry O-glycosylated surface proteins, we stained hemocytes from third instar larvae with Jacalin, a lectin that binds O-glycans [37]. We observed that circulating hemocytes from fly larvae were positive for Jacalin staining in both uninfected controls (Fig 1a) and wasp infected larvae (Fig 1b). Furthermore we also observed that the lamellocytes produced in response to wasp infection were also positive for Jacalin staining, though at lower levels than plasmatocytes (Fig 1b). This suggests that mucin-type protein O-glycosylation, and therefore PGANT family members, may play a role in wasp egg encapsulation.

***pgant35A* is required for wasp egg encapsulation.**

We hypothesize that PGANT enzymes are involved in the encapsulation response, and to test this hypothesis we analyzed the data from a genome-wide association study recently completed in our lab (KH and TAS, in preparation) to look for associations between genetic variation in PGANT encoding genes and wasp resistance. We found significant associations for 6 of the 14 putative PGANT genes (Table 1), suggesting that mucin-type O-glycosylation may play a role in the cellular immune response to wasp infection. Most of these PGANTs are essential for fly viability [34,35], so we were only able to obtain viable alleles for 2 of the 6 PGANT genes, *pgant35A* and *CG30463*.

To assay the putative roles of *pgant35A* and *CG30463* in the encapsulation response, we measured egg encapsulation rates in mutant larvae infected by the avirulent wasp *Leptopilina clavipes*, eggs of which are consistently encapsulated in wild type control larvae (Fig 2)[7]. We first tested $pgant35A^{B335}$, a viable allele caused by the insertion of a transposon into the 3' untranslated region of the *pgant35A* transcript. We found that $pgant35A^{B335}$ failed to complement the embryonic lethality of $pgant35A^{3}$ demonstrating that it is an allele of *pgant35A* [34,38]. $pgant35A^{B335}$ mutant larvae had profound defects in their ability to encapsulate *L. clavipes* eggs following infection (Fig 2). We next tested the $CG30463^{MB07284}$ transposon insertion allele, and found that mutant larvae were able to encapsulate *L. clavipes* eggs at a rate comparable to controls (Fig 2). These findings demonstrate that a specific subset of O-glycosylated proteins play an essential role in the encapsulation response, and that our association study is capable of identifying wasp resistance loci.

To test the specificity of the role of *pgant35A*, we assayed the encapsulation ability of viable alleles of two additional PGANT genes, *pgant3* and *GalNAc-T2* (also known as *pgant7*), neither of which were identified in the association study as being associated with wasp resistance (Table 1). We found that both $pgant3^{EY05266}$ and $GalNAc\text{-}T2^{EY12918}$ mutant larvae were able to encapsulate *L. clavipes* eggs (Fig 2) suggesting that, at least among testable PGANT genes, the encapsulation deficient phenotype is specific to *pgant35A*.

Loss of *pgant35A* does not affect larval hematopoeisis.

It has been previously demonstrated that mucin-type protein O-glycosylation plays a role in fly hematopoeisis [28]. Specifically, a mutation in *C1GalTA*, a *D. melanogaster* β1-3-galactosyltransferase homolog, results in a significant decrease in the number of circulating hemocytes in third instar larvae [28]. Because hemocyte number has been linked to antiwasp immunity in Drosophila species [39-41], we hypothesized that the decreased encapsulation rate seen in *pgant35A* mutants may be due to similar defects in hematopoeisis. To test this hypothesis we counted the total number of circulating hemocytes in control and $pgant35A^{B335}$ third instar larvae, and found that there was no significant difference between the genotypes (Fig 3a,b). However, it is possible that $pgant35A^{B335}$ larvae have defects in hematopoeisis following wasp infection, and to test this idea, we used a sterile wounding assay to produce a cellular immune response [42]. We found that sterile wounding provoked the differentiation of lamellocytes in both genotypes (Fig 3d), and that again there were no differences between control and $pgant35A^{B335}$ larvae in either total hemocyte count or number of circulating lamellocytes (Fig 3c,d). These findings demonstrate that *pgant35A* is not required for fly hematopoeisis in either control or immune induced larvae, but instead suggests that *pgant35A* is required for hemocyte function during the encapsulation process.

Capsule formation is defective in *pgant35A* mutant larvae.

Following wasp infection, the fly cellular immune response results in the formation of a melanized capsule. To test the hypothesis that $pgant35A^{B335}$ mutant larvae are defective in capsule formation, we imaged wasp eggs following infection of control and mutant

larvae by the avirulent wasp *L. clavipes*. We found that in control larvae, within the first 48 hours following infection, the wasp egg becomes surrounded by a multilayered capsule of hemocytes (Fig 4a). However, in *pgant35A*^{B335} mutant larvae this multilayered capsule fails to form, and instead, only small regions of the wasp egg become covered in hemocytes (Fig 4b). We further found that capsule consolidation was complete in control larvae 24 hours later. By contrast, *L. clavipes* eggs had hatched in *pgant35A*^{B335} mutants and live wasp larvae were observed in the hemocoel at this time, supporting the idea that hemocytes in *pgant35A*^{B335} mutant larvae are deficient in capsule formation.

***pgant35A* plays a role in wasp resistance.**

Finally we wanted to test whether this role for *pgant35A* in wasp egg encapsulation reflects a requirement in wasp resistance. To assay resistance we reared *L. clavipes* infected control and *pgant35A*^{B335} larvae to adulthood and measured the fly and wasp eclosion success rate. We found that relative to controls, *pgant35A*^{B335} mutant flies showed a significant decrease in fly success (Fig 5a), and an increase in wasp success (Fig 5b) following wasp infection. These data demonstrate that the failure of *pgant35A*^{B335} larvae to encapsulate wasp eggs is correlated with decreased fitness following wasp infection.

Discussion

The results of our genome-wide association study suggest that multiple PGANT family members may be important for *D. melanogaster* cellular immunity. Because many PGANT genes are essential [35], we were only able to test two PGANT genes for roles in the encapsulation response, but we found that one of them, *pgant35A*, is required for wasp egg encapsulation. This suggests that the association study is able to identify important anti wasp immunity factors, and that analysis of the complete results (KH and TAS, in preparation) will likely identify additional genes required for encapsulation.

Our data further supports the idea that additional PGANT family members may also be important for the encapsulation response. We found that larval hemocytes and immune induced lamellocytes are positively stained by the mucin-type specific lectin Jacalin. However, this staining is not completely abolished in *pgant35A* mutants (unpublished data), suggesting that additional PGANT enzymes are active in these cells.

Furthermore, although *C1GalTA* mutants are defective in hematopoeisis, *pgant35A* mutants do not have hematopoeitic deficits. β1-3-galactosyltransferases such as *C1GalTA* modify glycans formed by the activity of PGANT enzymes, and the lack of hematopoeitic defects in *pgant35A* mutants also suggests that a second PGANT is active in hemocytes and required for hematopoeisis. Since total hemocyte numbers are correlated with cellular immunity [39-41], it is likely that the *C1GalTA* (and addtional PGANT) dependent hematopoeisis is also important for the encapsulation response. Indeed this O-glycosylation dependent hematopoeisis may require one of the other (essential) PGANTs identified in our association study. This requirement for multiple

PGANTs is likely based on the specificity of PGANT function [22,26,33], and potentially reflects a role for several mucin-type O-glycosylated proteins in fly cellular immunity.

In developing fly embryos, *pgant35A* is required for septate junction formation in the tracheal (respiratory) system [27]. Interestingly, septate junctions are also important for capsule formation in the antiwasp immune response [9,10], perhaps providing mechanistic insight into the *pgant35A* encapsulation phenotype. We attempted to image interhemocyte septate junctions within the capsule as previously described [10]. The pieces of capsule that form on wasp eggs in *pgant35A* mutants have apparently normal septate junction staining (unpublished data), but perhaps the cells that fail to participate in capsule formation do so because of defects in septate junction protein localization.

Together these data demonstrate a role for mucin-type protein O-glycosylation in the fly cellular encapsulation response to parasitoid infection. They further suggest the existence of two subsets of mucin-type O-glycosylated proteins that may play complementary roles in encapsulation; *pgant35A* independent (but *C1GalTA* dependent) O-glycosylation that plays a role in hematopoeisis, and *pgant35A* dependent O-glycosylation that is important for capsule formation. While these activities are also distinct from the previously described role for protein N-glycosylation in capsule consolidation [7], they collectively demonstrate the importance of protein post-translational modifications in *D. melanogaster* cellular immunity.

Materials and methods

Insect strains

Fly strains used were CG30463^{MB07284} (w^{1118} background, Bloomington), and pgant3^{EY05266}, GalNAc-T2^{EY12918}, and pgant35A^{B335} (y,w background, Bloomington). Wasp strain used was *Leptopilina clavipes* strain LcNet.

Jacalin stains

Late 2nd instar larvae were unexposed or exposed to 4 LcNet females for 24 hours and bled onto diagnostic slides (Tekdon, Inc.) immediately and 24 hours later. Unattacked images were taken immediately, and attacked hemocyte images were captured 24 hours post infection period. Hemocytes were allowed to adhere to slides for 5 minutes and then stained with 20 μg/ml Fluorescein Jacalin (Vector Laboratories) for 3 minutes and washed three times with Drosophila Ringer's solution. Hemocytes were then visualized using the Olympus BX51 microscope with a FITC filter and Olympus DP2-BSW software.

Encapsulation and eclosion assays

Flies were allowed to lay eggs onto molasses medium supplemented with yeast paste. 72 hours after the egg lay period, 40 second instar larvae were transferred to 35 mm Petri dishes containing standard Drosophila medium and three female LcNet were placed into

the Petri dish and allowed to attack fly larvae for approximately 72 hours. For encapsulation assays, approximately 30 larvae were dissected to measure proportion fly success. For eclosion assays, all larvae were transferred to food vials containing standard Drosophila medium and allowed to eclose as either flies or wasps to measure proportion fly success, and proportion wasp success. Eclosed flies were dissected to ensure that they were attacked, and all unattacked flies (those without wound or wasp egg melanization) were discarded from analyses.

Hemocyte counts

Flies were allowed to lay eggs for 24 hours on molasses medium with yeast paste. Early 3rd instar larvae were cleaned in Ringer's solution, dried, and placed on double sided tape to prevent movement during which time their cuticle was pierced with a 0.125 mm tungsten carbide needle (Fine Science Tools). Following piercing, larvae were transferred to 35 mm diameter Petri dishes containing standard Drosophila medium. Unpierced controls were treated in the same manner except for needle injury. 24 hours later, 5 size matched larvae were bled into 20ul of 1X PBS 0.01% phenylthiourea (PTU). Solution containing bled hemolymph was pippetted into Incyto C-Chip DHC-N01 hemocytometer chips and hemocytes were counted from sixteen 0.25X0.25X0.1 mm squares and normalized to per larva values. Each experiment was replicated three times.

Fig 1

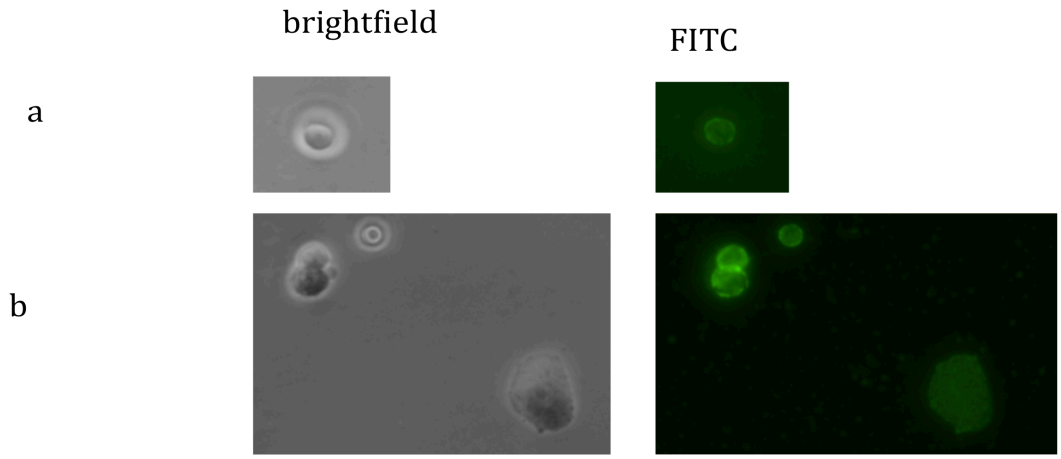

Fig 1: Images of Jacalin-stained hemocytes from (a) unexposed and (b) wasp exposed *y,w* larvae.

Fig 2

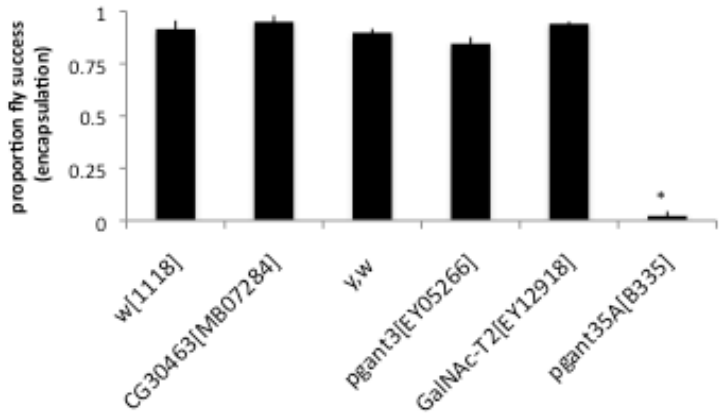

Fig 2: Proportion fly encapsulation success of various wild-type and mutant fly strains. * $p<0.05$.

Fig 3

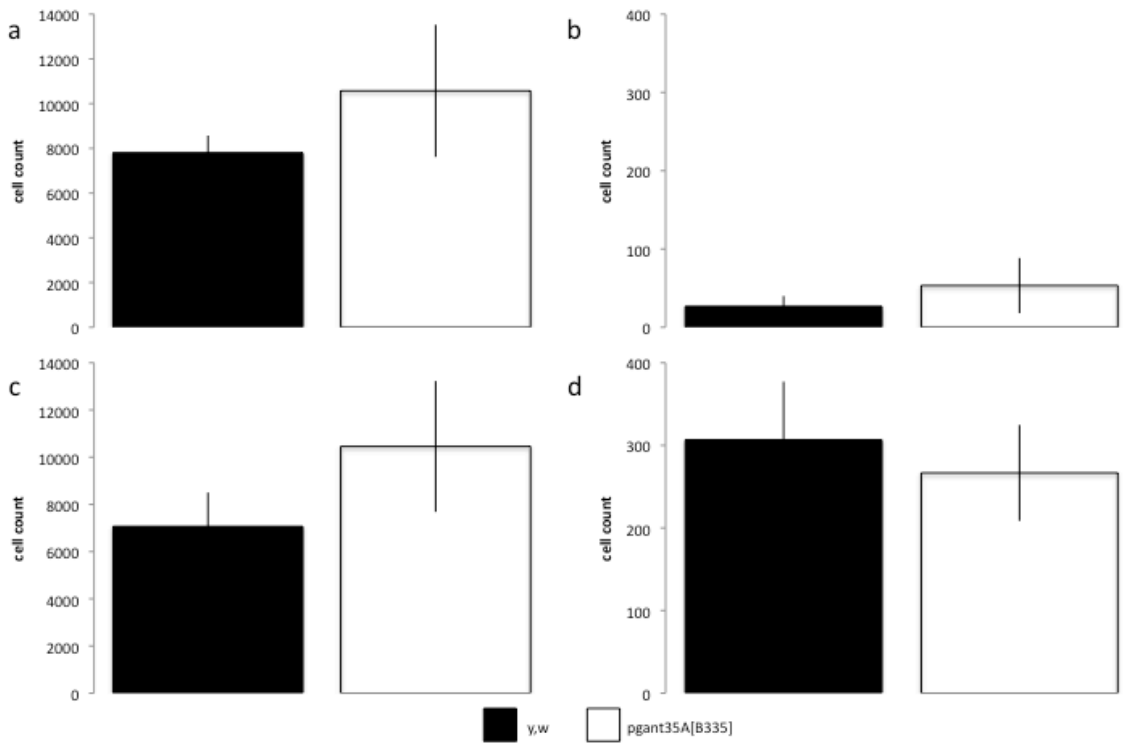

Fig 3: (a) Total hemocyte and (b) lamellocyte counts from unpierced larvae. (c) Total hemocyte and (d) lamellocyte counts from pierced larvae.

Fig 4

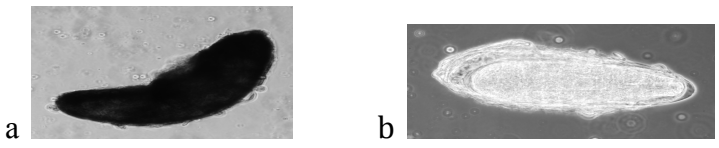

Fig 4: (a) image of *y,w* LcNet encapsulation and (b) *pgant35A*^{B33} LcNet encapsulation.

Fig 5

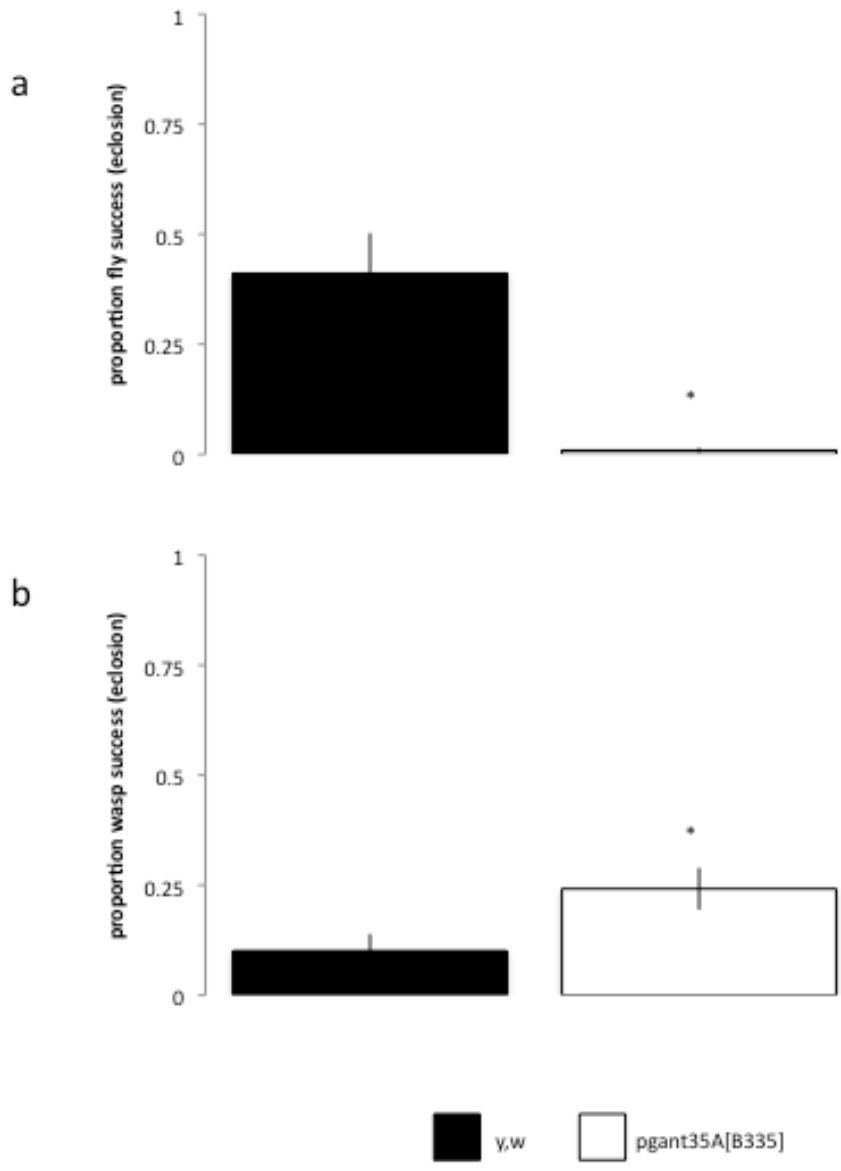

Fig 5: Proportion (a) fly success and (b) wasp success post LcNet attack. *$p<0.05$ compared to control.

Table 1

Gene	# of SNPs	p value (of best SNP)
pgant2	8	8.57 x 10-7
pgant4	1	1.87 x 10-6
pgant5	13	4.55 x 10-9
pgant35A	1	2.84 x 10-9
CG30463	7	9.82 x 10-11
CG31776	1	7.64 x 10-7
GalNAc-T2	-	-
pgant3	-	-

Table 1: Table of 98 DGRP strain wasp-resistance association results (KH and TAS in prep).

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