PART IV

Pathogens and their Hosts

CHAPTER 20

Rapid evolution of innate immune response genes

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20.1 The evolution of immunity

The immune system is a central mediator of inherently antagonistic interactions between hosts and pathogens. Genes in the immune system often evolve more rapidly than genes in other physiological systems (e.g. Murphy 1991; Schlenke and Begun 2003), presumably as a consequence of this antagonism. The mode of immune system evolution, however, can depend on a multitude of factors, including whether the pathogens are generalists or specialists, the prevalence and diversity of infectious agents in hosts' natural environments, and pleiotropic functions of immune genes. Even within the immune system, there is every reason to expect that selective pressures will vary across functionally distinct components.

Host immune systems are generally defined in terms of the physiological process of recognizing and eliminating potentially pathogenic infection. In order to be effective, any immune system must therefore possess mechanisms for surveillance, for signal transduction and stimulation of appropriate antipathogen activity, and for sequestration and killing of the pathogen. For the pathogen, surviving the immune response is essential. Pathogens, therefore, may experience strong selective pressure to evade recognition, subvert or suppress signal transduction, and/or resist host killing mechanisms. Pathogen success on any of these fronts, however, imposes renewed selective pressure on the host to evolve re-established immunity. Thus, hosts and pathogens may reciprocally adapt to each other, serially evolving under positive Darwinian selection but without achieving any substantial change in the relationship status quo. This particular coevolutionary model is sometimes termed a 'coevolutionary arms race' or 'Red Queen dynamics,' the former referring to serial escalation that maintains parity between antagonists and the latter referring to the Lewis Carroll character's assertion to Alice that in Wonderland 'it takes all the running you can do, to keep in the same place' (van Valen 1973; Dawkins and Krebs 1978).

This chapter provides an overview of the evolutionary dynamics of insect antimicrobial and antiviral immune systems, emphasizing the fruit fly, *Drosophila*. Insects have no analog to the charismatic antibody-mediated acquired immunity that allows vertebrates to generate hyperdiversity and memory of previous infection through somatic recombination and clonal expansion (Murphy et al. 2007). Instead, insects rely solely on 'innate' immunity. Innate immune systems, which are also central components of vertebrate defense, are hardwired into the genome and therefore might be more sensitive to host–pathogen coevolutionary dynamics. Innate immune responses to microbes include defensive phagocytosis and the production of broad-spectrum antimicrobial peptides (reviewed in Lemaitre and Hoffmann 2007). Innate immunity to RNA viruses and transposable elements is mediated by RNA interference (RNAi), a cellular mechanism for recognizing and degrading double-stranded RNA (dsRNA), and subsequently single-stranded RNA homologous to the activating dsRNA (van Mierlo et al. 2011). Components of both the antimicrobial immune system and antiviral RNAi have been shown to evolve rapidly and adaptively in *Drosophila* and other insects.

20.2 Orthology and gene family evolution in antimicrobial immunity

Insect immune responses to microbes can include both defensive phagocytosis and production of secreted antimicrobial peptides (AMPs). The mechanistic basis for the systemic production of AMPs has been well studied and appears from comparative genomic analyses to be highly conserved across invertebrates (reviewed in Lazzaro 2008). There are two primary signaling pathways used to activate AMP production, named the Toll pathway and the Imd pathway after key constituent genes. The Imd pathway has homology to the mammalian tumor necrosis factor pathway, and mammalian Toll-like signaling pathways are named for their homology to their insect counterpart. Nearly all the core signaling proteins in both the Toll and Imd pathways are conserved as strict orthologs across available sequenced invertebrate genomes.

The Imd and Toll pathways can each be stimulated by host recognition of microbial cell wall components. This recognition is achieved by peptidoglycan recognition proteins (PGRPs) and the Gram-negative binding proteins (GNBPs; misleadingly named because their recognition spectrum is not restricted to Gram-negative bacteria). PGRPs and GNBPs each exist as multigene families of roughly four to 15 members in most insects and mammals. These gene families remain evolutionarily stable over short time periods, but family members undergo considerable duplication and deletion over longer evolutionary timescales (e.g. Evans et al. 2006; Sackton et al. 2007; Waterhouse et al. 2007; Zhou et al. 2007). Fig. 20.1 shows the distribution of turnover rates of genes involved in recognition, signaling, and effector classes, showing that the signaling class has the lowest rate. Genes encoding antimicrobial peptides show extremely high rates of gene family expansion and contraction. While genes encoding some peptides, such as cecropins and defensins, are nearly ubiquitous in insects, most peptide gene families are much more taxonomically restricted (e.g. Evans et al. 2006; Sackton et al. 2007; Waterhouse et al. 2007; Zhou et al. 2007). Peptides in the Defensin class are the most taxonomically widespread, being found in insects, mammals, and plants. The most distantly related Defensins may, however, be the product of convergent evolution (Broekaert et al. 1995) to a similar tertiary structure.

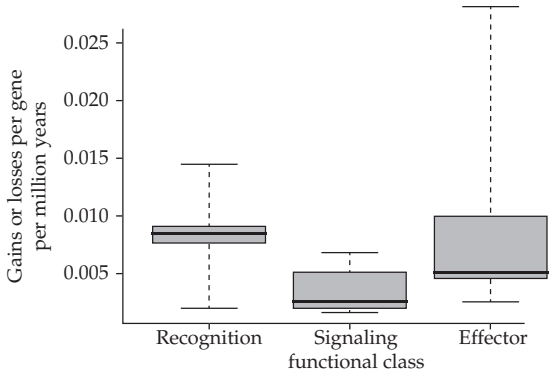

Figure 20.1 Rates of turnover of copy number of different classes of innate immune genes, as inferred from the 12 *Drosophila* genome sequences. On the *Drosophila* phylogeny, the gene copy number of each class was determined in the 12 species, and a maximum likelihood procedure was used to estimate the rate of change in copy number along the branches of the phylogeny. The clear conservativeness of copy number of the signaling genes stands in contrast to both the recognition and effector (antimicrobial) peptides. Redrawn from Sackton et al. (2007).

There are exceptions to the pattern of strong conservation of the Toll and Imd pathways and diversification of PGRPs, GNBPs, and AMPs. For example, the pea aphid genome sequence indicates that aphids have lost key genes in the Imd pathway and are completely without PGRPs (Gerardo et al. 2010). Even though the honeybee has intact Toll and Imd pathways, the bee exhibits reduced copy number in most multigene families, resulting in a nearly two-thirds reduction in the complement of identifiable immune system genes (Evans et al. 2006). It is unclear whether these insects are actually immunocompromised. The gene losses may be offset by indirect protection from infection through hygienic hive behavior in the case of the honeybee or protection by secondary symbionts in the case of the aphid. Alternatively, the immunological functions lost with the deletions of these genes may be regained through other, yet-unidentified genetic mechanisms. These questions cannot be addressed by comparative genomic analysis, but can only be answered with careful functional study.

The evolutionary examination of genes involved in defensive phagocytosis has been less thorough.

Nonetheless, there are clear indications that, like PGRPs and GNBPs, phagocytic receptors duplicate and delete at rates that are significantly higher than the genome average (e.g. Sackton et al. 2007). For example, receptors in the eater/nimrod/hemese class are highly diversified in *Drosophila* (Sackton et al. 2007; Zou et al. 2007; Somogyi et al. 2008). Scavenger receptors are also diversified across distinct insect taxa. *Class C scavenger receptors* have expanded from one progenitor to four genes in the melanogaster group of *Drosophila* (Lazzaro 2005; Sackton et al. 2007), and the *Class B scavenger receptor* family is greatly expanded in *Tribolium castaneum* (Zou et al. 2007). The *Tep* gene family encodes protease-activated opsonins that tag microbes and other pathogens for phagocytosis and immunological elimination. *Tep* genes are highly diversified in mosquitoes (Waterhouse et al. 2007) and experience rapid gene family evolution across insect taxa (Evans et al. 2006; Zou et al. 2007; Gerardo et al. 2010).

In summary, the rate of gene copy number evolution varies greatly across different functional components of the immune system, but is relatively consistent across insect taxa. Core signaling genes in the Imd and Toll pathways tend to be maintained as strict orthologs across insect taxa. In contrast, PGRP and GNBP recognition proteins that activate these pathways and the AMPs that are among their downstream targets are highly diversified across insects. This diversification in recognition and effector proteins may arise as a consequence of different species' ecological exposure to distinct suites of microbes. Alternatively, these genes may be subject to 'threshold' evolution, where gene copies can duplicate and delete nearly neutrally provided some minimum capacity for microbial recognition and clearance is retained. Whichever model is more correct, rates of evolution for these gene families are considerably higher than for most genes in the genome.

20.3 Molecular evolution of the antimicrobial immune system

Despite their strict maintenance of orthology across very distantly related taxa, signaling genes in the Toll and Imd pathways evolve surprisingly rapidly at the amino acid level. Genes in these pathways are among the most divergent in the immune system in comparisons between *D. melanogaster* and the mosquitoes *Anopheles gambiae* and *Aedes aegypti* (Waterhouse et al. 2007), and several individual signaling genes exhibit significant evidence of adaptive evolution within *Drosophila* (Begun and Whitley 2000; Schlenke and Begun 2003; Jiggins and Kim 2007; Sackton et al. 2007). This observation has been interpreted in light of the capacity of some pathogens to subvert or block host immune signaling (Begun and Whitley 2000; Schmid-Hempel 2008). The essential requirement of these pathways for antimicrobial immunity and their highly conserved orthology may be the very features that expose them to pathogen manipulation. Whereas recognition proteins and AMPs are comprised of diverse and varied gene families, the two signaling pathways are a 'bottleneck' at which pathogens can choke off the immune response. The ubiquitous orthology of these pathways may further serve to make them attractive targets for interference by generalist pathogens. The adaptive evolutionary signature in these pathways may be amplified by the correlated amino acid substitutions within and among proteins that maintain pathway function while escaping pathogen manipulation (DePristo et al. 2005).

Compared to other functional classes of genes in the innate immune system, genes encoding receptors display the strongest signature of positive selection (Fig. 20.2). Genes encoding opsonins and receptors for phagocytosis tend to evolve under positive selection at the amino acid level. In particular, *Tep* genes have been shown to evolve adaptively in *Drosophila* (Jiggins and Kim 2006; Sackton et al. 2007), *Anopheles* (Little and Cobbe 2005), and the cladoceran crustacean *Daphnia* (Little et al. 2004), with selected sites predominantly found in and around the domain that is proteolytically cleaved for TEP activation. The expanded class C scavenger receptor family in the *melanogaster* species group also evolves unusually quickly at the amino acid level (Lazzaro 2005), as do several other scavenger receptors and bacteria-binding phagocytosis receptors in the nimrod class (Sackton et al. 2007). In contrast, there is little indication of adaptive amino-acid level evolution in PGRP and GNBP

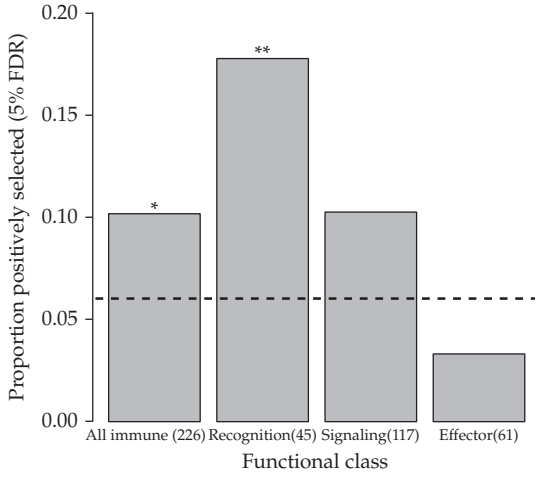

Figure 20.2 Rates of amino-acid substitution are accelerated in recognition and signaling proteins (as estimated by the maximum likelihood fits to the codon substitution model as implemented in PAML), resulting in a greater proportion of genes in these classes showing evidence for positive selection ($K_A/K_S > 1$). The dotted line represents the genome-wide average proportion of positively selected genes. Redrawn from Sackton et al. (2007).

recognition proteins that activate Toll and Imd signaling (Schlenke and Begun 2003; Jiggins and Kim 2006; Sackton et al. 2007). The observation of adaptive evolution in signaling genes but not in the recognition factors that activate signaling seems to be generalizable across invertebrates (e.g. Little et al. 2004; Little and Cobbe 2005; Bulmer and Crozier 2006), although individual genes may vary in the degree to which they are selected in different taxa (e.g. Levine and Begun 2007; Sackton et al. 2007). The distinct evolutionary trajectories of phagocytosis receptors versus PGRPs and GNBPs may stem from differences in binding affinity. Opsonins and phagocytic receptors bind to a diversity of pathogen molecules, some of which may be evolutionarily very labile. In contrast, GNBPs and PGRPs that activate the immune system are targeted to highly conserved microbial cell wall compounds like peptidoglycan and β-glucans.

Despite their rapid gene family turnover, AMP genes in *Drosophila* show little indication of rapid evolution at the amino acid level (e.g. Lazzaro and Clark 2003; Jiggins and Kim 2005; Sackton et al. 2007). This contrasts with the observation that AMP gene duplication is frequently associated with adaptive amino acid diversification in vertebrates (Tennessen et al. 2005). AMP gene duplication has also been coupled with amino acid divergence in termites and mosquitoes (Bulmer and Crozier 2004; Dassanayake et al. 2007), so the data from *Drosophila* may represent a departure from the norm. Amino acid diversification may result in altered antimicrobial activity (Tennessen 2005; Yang et al. 2011), and both gene family expansion and amino acid diversification may be driven by adaptation to commonly encountered microbes. Drosophila species may associate less with specific coevolving microbes, obviating the need for amino-acid level adaptation in AMP genes. The limited survey work that has been conducted suggests that most microbes associated with *D. melanogaster* in the field are generalist opportunists (Corby-Harris et al. 2007; P. Juneja and B.P. Lazzaro unpublished data), and the *Drosophila* antimicrobial immune system may be adapted to management of these more persistent but less threatening challenges (Hultmark 2003).

20.4 The evolution of defense against viruses and transposable elements

Genes responsible for defense against viruses and transposable elements (TEs) can exhibit exceptionally fast evolutionary rates. Double-stranded RNA (dsRNA) associated with RNA viruses and active transposons are targeted for silencing and degradation by RNAi machinery in plant, insect, and mammalian cells. Three *Drosophila* genes that are required for processing and silencing of transposon- and virus-derived dsRNA (*Dicer-2*, *R2D2*, and *Argonaut-2*) are among the fastest evolving 3% of genes in the *D. melanogaster* genome (Fig. 20.3). These genes exhibit highly elevated Ka/Ks ratios and McDonald–Kreitman test statistics that indicate strong positive selection across the melanogaster subgroup species (Obbard et al. 2006). *Dicer-2* and *Argonaut-2* in particular appear to have been recent targets of selective sweeps, resulting in significantly reduced genetic diversity at these loci in *D. melanogaster* and related species (Obbard et al. 2006, 2011). Modeling of the selective process suggests multiple recurrent, recent, and independent

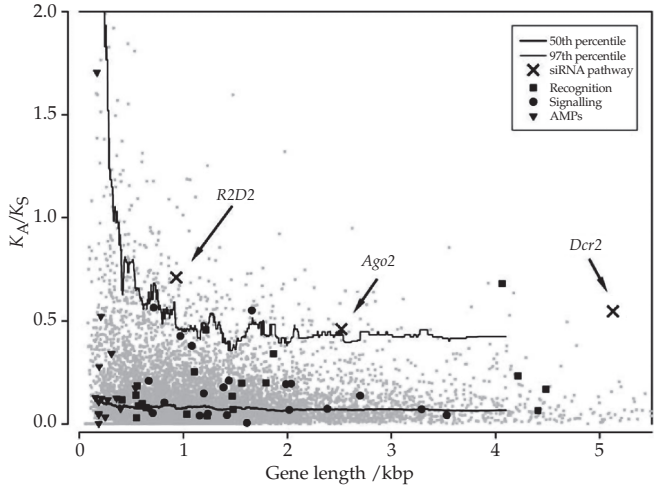

Figure 20.3 Rates of adaptive evolution of genes involved in immune response, expressed as the ratio of nonsynonymous (K_A) to synonymous (K_S) rates of nucleotide substitution. Three genes involved in antiviral response, *R2D2*, *Ago2*, and *Dcr2* are among the top 3% most rapidly evolving genes in *Drosophila*. From Obbard et al. (2006).

sweeps at *Argonaut-2* in *D. melanogaster*, *D. simulans*, and *D. yakuba* (Obbard et al. 2011).

RNAi is an effective defense against RNA viruses, and several viruses have mechanisms for suppressing or subverting the host defensive RNAi of plants, mammals, insects, and worms (reviewed in Li and Ding 2006). Viral suppression of RNAi (VSR) can occur through a variety of mechanisms which may spur molecular arms races between hosts and viruses. These could include competitive binding and sequestration of processed siRNAs (which would dampen the host RNAi response), competitive binding of full-length dsRNA (which would prevent access by endogenous RNAi machinery), and direct inhibition of host RNAi proteins (Li and Ding 2006; Obbard et al. 2009). The sites that are putatively evolving adaptively in host RNAi genes tend not to be restricted to known functional domains, but are distributed throughout the proteins (Obbard et al. 2006; Kolaczkowski et al. 2011; Obbard et al. 2011). Putatively adaptive substitutions occur in a domain critical for RNA-binding by *D. melanogaster* Dicer-2 (Kolaczkowski et al. 2011), and are particularly prevalent on molecular surfaces of other genes (Obbard et al. 2006, 2011; Kolaczkowski et al. 2011), which could perhaps indicate coevolution with viral genes that physically interact with host RNAi machinery or correlated compensatory coevolution among physically correlated amino acid residues or interacting RNAi proteins (as in DePristo et al. 2005; Callahan et al. 2011). An arms race between hosts and viruses implies not only rapid evolution in the antiviral machinery, but also rapid evolutionary turnover in viral VSRs. This condition is satisfied by the rapid molecular evolutionary origin and elimination of VSRs across viral taxa. VSRs are often encoded by overlapping genes that differ in reading frame, which arise when an existing gene sequence becomes translated in an alternative reading frame, a process known as overprinting (Li and Ding 2006). This results in gene sets that vary in age, with structurally novel proteins arising instantaneously in viral lineages and resulting in remarkable VSR functional diversity. As would be expected under an arms race model, VSRs show elevated rates of protein divergence relative to other viral genes (Obbard et al. 2009).

RNAi genes more conventionally associated with germline silencing of transposable elements (TEs) also show evidence of recent and recurrent adaptation (Obbard et al. 2009; Kolaczkowski et al. 2011). TE silencing in the germline is executed by the PIWI-interacting, or piRNA, pathway. Active TEs can be severely deleterious to host lineages and are strongly selected against (reviewed in Lee and Langley 2010). Theory predicts, and empiricism bears out, that piRNAs which silence transposons should be adaptive through reducing the deleterious effects of TE mobilization (Lu and Clark 2010).

piRNA pathway genes show strong evidence of adaptive evolution and are among the 5% most rapidly evolving genes in *D. simulans* (Obbard et al. 2009). Several piRNA genes also show evidence of recent selective sweeps in *D. melanogaster* (Kolaczkowski et al. 2011).

Because there is mechanistic overlap between antiviral and anti-transposon RNAi functions, it is difficult to definitively declare that rapid evolution of RNAi genes is due to coevolution specifically with viruses or with TEs at the exclusion of the other. Several piRNA components appear to have additional antiviral functions and some VSRs may affect piRNA pathway genes (reviewed in Obbard et al. 2009, 2011; Kolaczkowski et al. 2011), which could result in the rapid evolution of piRNA genes without invoking transposon-driven selection. At the same time, the antiviral RNAi genes *Dicer-2* and *Argonaut-2* are recruited for anti-TE function in an RNAi mechanism so far thought to be unique to *Drosophila* (Obbard et al. 2009). Unlike transposons, however, viruses have a known mechanism for suppressing host RNAi, and have themselves the capacity to rapidly evolve in response to evolutionary change in the host. These factors suggest that antagonistic host-virus coevolution may be a more probable driver of rapid evolution in RNAi genes than is host–TE coevolution.

20.5 Concluding remarks

Immune systems tend to evolve rapidly and adaptively, and the innate immune system of insects and invertebrates is no exception. The precise nature of evolution in immune system genes unsurprisingly depends on gene function, but not all immune genes evolve in a manner that is necessarily intuitive. For example, pathogen recognition proteins that activate antimicrobial immune signaling show little evidence of adaptive amino acid evolution, suggesting that these genes tend not to coevolve. At the same time, the gene families encoding these recognition proteins recurrently show taxon-specific expansion and deletion, potentially indicating adaptation to the spectrum of microbes encountered by distinct species. Cell-surface and secreted proteins that bind microbes for phagocytosis, on the other hand, show both rapid gene family diversification and pervasive adaptive amino acid evolution. The difference in evolutionary profiles between the two classes of pathogen-recognition proteins is likely a function of differences in the ligands which they recognize. Perhaps surprisingly, intracellular signaling proteins that activate systemic antimicrobial immunity tend to evolve rapidly at the amino acid level, even though these are not expected to have obvious contact with pathogens and show virtually no diversification at the gene family level across very distantly related taxa. This has been interpreted to result from coevolution with pathogens that interfere with these highly conserved signaling processes. Although antimicrobial peptides are ubiquitous components of innate immune systems, specific peptide families are typically highly taxonomically restricted and vary across taxa in the rate at which they evolve at the amino acid level. Finally, RNAi genes that defend against viruses and transposable elements evolve extraordinarily quickly, probably in reflection of tight coevolution with highly specific and quickly evolving viruses that have high mutation rates and short duplication times.

Also surprisingly, there is little indication that genes in the innate immune system maintain polymorphism through balancing natural selection. Instead, where there is evidence of adaptive evolution, the data reveal rapid directional selection more consistent with coevolutionary arms races. This may partially stem from the fact that serial directional selection is experimentally easier to detect than balanced polymorphism, but also is indicative of the nature of invertebrate immune system evolution.

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