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Evolutionary Dynamics of Immune-Related Genes and Pathways in Disease-Vector Mosquitoes

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Mosquitoes are vectors of parasitic and viral diseases of immense importance for public health. The acquisition of the genome sequence of the yellow fever and Dengue vector, *Aedes aegypti* (*Aa*), has enabled a comparative phylogenomic analysis of the insect immune repertoire: in *Aa*, the malaria vector *Anopheles gambiae* (*Ag*), and the fruit fly *Drosophila melanogaster* (*Dm*). Analysis of immune signaling pathways and response modules reveals both conservative and rapidly evolving features associated with different functional gene categories and particular aspects of immune reactions. These dynamics reflect in part continuous readjustment between accommodation and rejection of pathogens and suggest how innate immunity may have evolved.

Repeatedly during evolution, mosquitoes and other insects have adopted hematophagy to sustain abundant progeny production. In turn, blood feeding provided a new point of entry for pathogens. To counter assaults, innate immunity has evolved to recognize and respond to numerous pathogens, in a dynamic playoff where either host or pathogen may win. Although fundamental concepts mostly derive from *Dm*, *Ag* is now an important model for studies of innate immunity. A previous comparative analysis of *Ag* and *Dm* immune-related gene families (*1*) highlighted their diversification and pointed toward an expanded conceptual framework of insect innate immunity. The sequencing of the *Aa* genome (*2*) permitted deeper understanding of insect immune systems, as displayed by two quite different mosquito species that diverged ~150 million years ago (Ma) and *Dm*, which separated from them ~250 Ma. This three-way comparison is considerably more powerful than the previous *Dm-Ag* study, because it allows measuring true genetic distances rather than unrooted sequence similarities. Taking advantage of the added value from multiple species comparisons, we explore the evolutionary dynamics of innate immunity in insects and how they can address both common and species-specific immune challenges.

Multiple large-scale bioinformatic methods, manual curation, and phylogenetic analyses (*3*) identified 285 *Dm*, 338 *Ag*, and 353 *Aa* genes from 31 gene families and functional groups implicated in classical innate immunity or defense functions such as apoptosis and response to oxidative stress (table S1). Additional limited analysis of nine sequenced genomes from four holometabolous insect orders, spanning 350 million years of evolution, further defined conserved family features and assisted manual gene model curation by gene family experts. The detailed core analysis (*Aa*/*Ag*/*Dm*) is presented in the supporting online material (SOM) text and in figs. S1 to S22, and the total data set is organized into a web-accessible resource (http://cegg.unige.ch/Insecta/immunodb/), offering a comparative perspective across higher insects. All but 24 previously named *Aa* genes, as well as 79 previously unnamed *Ag* genes, were named in accordance with the nomenclature scheme devised for the *Ag* genome (*1*) with the use of additional guidelines as described in the SOM; this information will be incorporated in the forthcoming manual annotations of the VectorBase resource (www.vectorbase.org).

Our conservative bioinformatic analysis of the complete genomes identified 4951 orthologous trios (1:1:1 orthologs in the three species) and 886 mosquito-specific orthologous pairs (absent from both *Dm* and the honeybee, *Apis mellifera*). Combined bioinformatic analysis and manual curation of the immune repertoire identified 91 trios and 57 pairs, plus a combined total of 589 paralogous genes in the three species. Paralogs derive from family expansions and gene losses, or cases of exceptionally high sequence divergence obscuring phylogenetic relationships. Orthologs most likely serve corresponding functions in respective organisms, whereas paralogs may have acquired different functions.

By definition, orthologous trios represent a numerically conserved subset of genes. Nevertheless, a plot of *Dm-Aa* and *Dm-Ag* phylogenetic distances, measured in terms of amino acid substitutions, revealed that, on average, immunity trio orthologs are significantly more divergent (~20%) than the totality of trios in the genomes

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(Fig. 1A). Indeed, the immune repertoire is one of the most divergent functional groups as defined by Gene Ontology classifications (fig. S1A). Furthermore, with *Dm* as reference, several *Ag* immunity genes are considerably more divergent than their *Aa* orthologs. A similar trend among all 1:1:1 orthologs was detected, implying greater accumulation of amino acid substitutions in *Anopheles*. One hypothesis that merits detailed testing is whether this reflects a higher speciation rate and diverse habitat colonization by *Anopheles* as opposed to the more cosmopolitan *Aedes*.

Large variation exists in different immune families in their proportions of orthologous trios, mosquito pairs, and species-specific genes (Fig. 1B). Some families display exclusively species-specific genes, some mostly trios, and others intermediate variation. At one extreme are apoptosis inhibitors (IAPs), oxidative defense enzymes [superoxide dismutases (SODs), glutathione peroxidases (GPXs), thioredoxin peroxidases (TPXs), and heme-containing peroxidases (HPXs)], and class A and B scavenger receptors (SCRs), all of which show predominantly trio orthologs. At the opposite extreme are highly diverse immune effector gene families, including three shared antimicrobial peptide (AMP) families that collectively exhibit no orthologous trio and only one confident mosquito orthologous pair. The C-type lectins (CTLs), which have been implicated in immunity as opsonins and modulators of melanization (see below), are intermediate, exhibiting large expansions while retaining nine trios and one pair. The present study reaffirms the family diversity observed in our previous *Dm-Ag* comparison and further reveals substantial diversity between the two mosquito species, at just over half the evolutionary distance.

A fascinating picture emerged when we disarticulated the immune responses into sequential phases (Figs. 2 and 3). Immune responses begin with molecular recognition of microbial patterns, producing immune signals. Some signals are modulated and/or transduced before activating effector mechanisms. We observed that each of the phases is characterized by different evolutionary dynamics, which may collectively account for the flexibility of the innate immune system that enables adaptation to new challenges.

The immune recognition phase seems to achieve flexibility through divergent evolution: Gene duplications result in species- or lineage-specific expansions and generation of novel genes, whereas domain duplications lead to new gene architectures. Consequently, fruit fly and mosquito recognition proteins mostly form distinct clades within each family (see SOM). Nevertheless, sequence divergence between reduplicated recognition genes or domains remains limited, possibly reflecting the relatively limited diversity of microbial molecular patterns that are known to trigger immune responses. The peptidoglycan recognition proteins (PGRPs) and the Gram-negative binding proteins (GNBPs) are recognition receptor families that trigger signaling through Toll or Imd pathways as indicated in Fig. 2 (*4*). The Gram-negative recognition protein *Dm* PGRP-LC, which functions in the Imd pathway, and its *Anopheles* ortholog each have three functional PGRP domains; however, these are more similar within species than between species, indicating phylogenetically separate domain reduplications. A sequence gap obscures the full structure of the *Aedes PGRP-LC* ortholog, which apparently derives from the same domain reduplication events that created *Ag PGRP-LC*. Separate reduplication of two adjacent *PGRP-LC* domains in *Drosophila* generated a novel gene, *PGRP-LF*, which is absent from mosquitoes.

The function of PGRP-LC in *Dm* is antagonized by catalytic PGRPs that cleave and inactivate peptidoglycan (*5*, *6*). Mosquitoes also possess catalytic PGRPs, but most have emerged as species-specific paralogs (*Ag* PGRPS2/3 and *Aa* PGRPS4/5). The fruit fly recognizes Gram-positive bacteria activating Toll using the species-specific *Dm* PGRP-SD, as well as *Dm* PGRP-SA, which belongs to a trio and functions in conjunction with GNBP1, a recognition protein that processes polymeric peptidoglycan (*7*). The two additional *Dm* GNBPs are also fruit fly–specific; one of them, GNBP3, recognizes fungi, possibly through binding β1,3-glucans (*8*). A large expansion has generated five mosquito-specific B-type

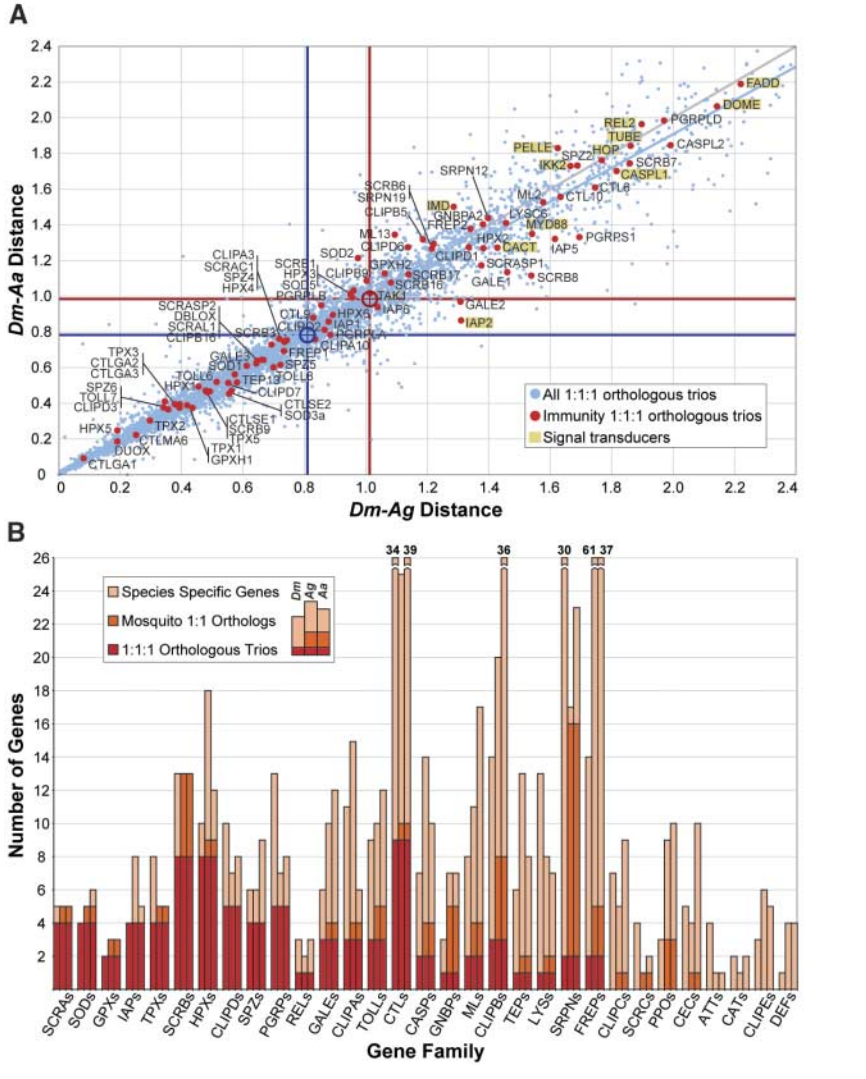
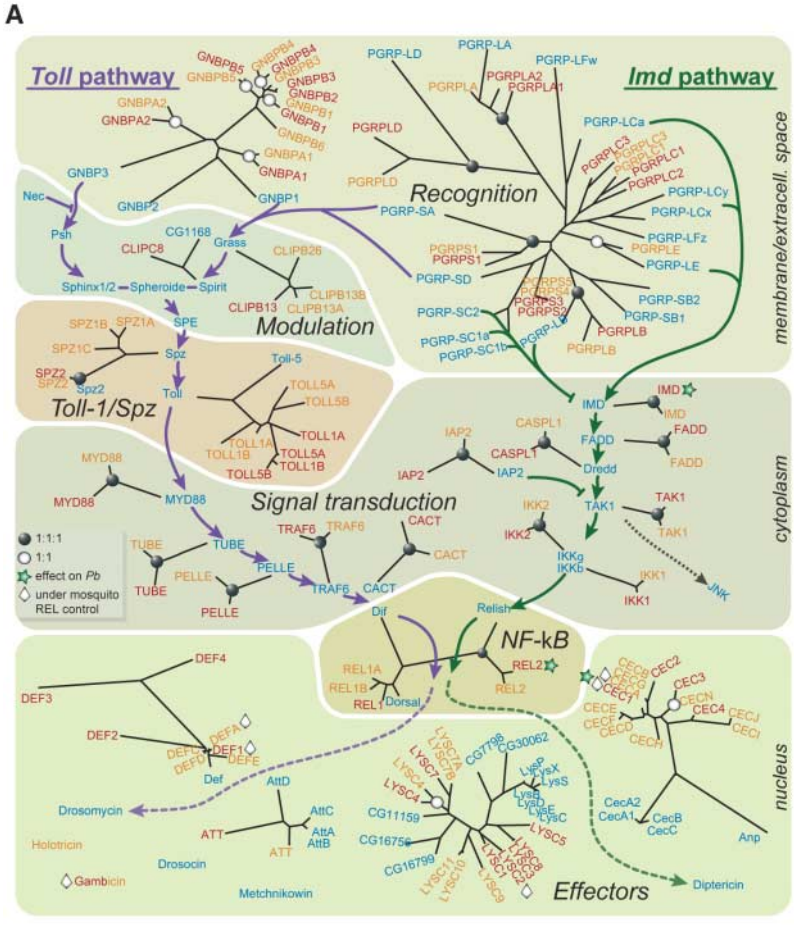


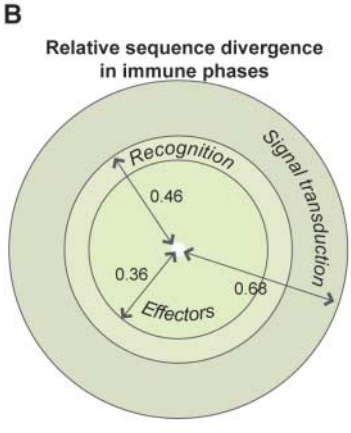
Fig. 1. (**A**) Divergence of orthologous trios. Immunity single-copy trios are compared with all single-copy trios in terms of genetic distances of each mosquito species (*Ag* or *Aa*) protein to the corresponding *Dm* ortholog (*3*) (fig. S1B). Signal transducers are highlighted. Red and blue lines indicate distance means for immunity (red dots) and all trios (blue dots), respectively. (**B**) The repertoire of putative immune-related gene families. The numbers of 1:1:1 orthologous trios (red), mosquito-specific 1:1 orthologs (orange), and species-specific genes (light brown) are summed to give the total number of genes identified in *Dm* (first bar), *Ag* (second bar), and *Aa* (third bar) for each gene (sub)family. Families are arranged from left to right, according to the decreasing proportion of 1:1:1 orthologous trios within the family. Family acronyms that are not defined in the text include: CASPs, caspases; CATs, catalases; FREPs, fibrinogen-related proteins; GALEs, galectins; MLs, MD2-like receptors.

GNBPs, distinct from the two A-type orthologous pairs that resemble fruit fly GNBPs.

Recent studies in *Ag* identified two types of putative malaria parasite recognition receptors belonging to distinct structural classes: thioester-containing proteins (TEPs) and leucine-rich repeat (LRR) proteins. Members of each class have been associated with the killing and disposal of parasites by lysis or melanization. The TEP family is related to the vertebrate complement factors C3/C4/C5 and pan-protease inhibitors α2-macroglobulins. *Ag* TEP1 binds to the surface of *Plasmodium berghei* and mediates parasite killing (*9*); it also binds to bacteria and promotes phagocytosis (*10*, *11*). TEPs exhibit only one orthologous trio and otherwise form two groups: one with both *Dm* and mosquito TEPs and another with only mosquito species-specific clades (the latter group includes *Ag* TEP1) (Fig. 3). The second class of putative receptors include LRR immune gene 1, the pioneer *P. berghei* LRR antagonist (*12*); others of similar function are *Anopheles Plasmodium*-responsive LRR 1 and LRR domain 7, which have been additionally implicated in resistance to *P. falciparum*, the human malaria parasite (*13*, *14*). Like TEP1, none of the three has identifiable orthologs in *Aa* or *Dm*.

Immune modulation is an important process that regulates both the immediate aftermath of recognition and subsequent effector functions and evolves in a "mix and match" mode. Examples are modulation of Toll pathway activation and the melanization reaction, respectively. In both contexts, modulation uses a vast reservoir of serine proteases and their inhibitors [serpins or serine protease inhibitors (SRPNs)] or other regulators, from which particular components are picked to constitute species-specific regulatory modules.





Immune phases	species specific	○ 1:1	● 1:1:1
Recognition diversity by gene and domain duplication	21	12	12
Modulation distinct modules from large, highly divergent families	13	0	0
Toll-1/Spz distinct interacting pairs	14	0	3
Signal transduction strict orthology, coherent sequence diversification	0	0	36
NF-kB function transfer across paralogs	5	0	3
Effectors diversity by family invention & gene duplication	65	9	3

Fig. 2. Evolution of immune signaling phases in insects. (**A**) Genes and gene families implicated in two immune signaling pathways, Toll and Imd (green and purple, respectively). The well-recognized phases of signaling, from recognition to effector production, are outlined. Genes known to be part of these pathways in *Dm* are indicated in blue, with their closest phylogenetic relatives in *Ag* in red and *Aa* in yellow (based on the analysis presented in the SOM). Single-copy orthologs (1:1:1) in all three genomes are indicated with solid circles at the branching node and mosquito 1:1 orthologs are indicated with open circles, respectively. *Ag* genes affecting survival of the malaria parasite *P. berghei* are marked with stars, and mosquito genes transcriptionally regulated by NF-κB–like mosquito REL factors are marked with diamonds; *Aa CECA* and *Aa DEFA* effectors are controlled by both REL1A and REL2 (*33*, *39*); similarly, *Ag* REL2 controls expression of immune effectors, including *CEC1/3* and *GAM* (*40*). *Dm* LYSs show little response to bacterial infection, but several are up-regulated after infection by microsporidia (*41*). The mosquito *Ag LYSC1/2* and *Aa LYSC11* (*LysA*) genes are up-regulated after bacterial challenge (*42*, *43*), and *Ag LYSC2* is controlled by REL1. We constructed radial trees using similarity distances of the conserved sequence cores computed by maximum likelihood. Branch-length scaling is preserved within, but not between, trees. (**B**) Gene families implicated in the three major immune phases (recognition, signal transduction, and effector production) are clearly different in relative sequence divergence (left panel; sum of branch lengths divided by number of members). Quantitative analysis of evolutionary divergence modes in all six phases defined in (A) is based on gene numbers: trios, mosquito pairs, and genes found in only one species (right panel). All signal transduction genes form trios but are maximally divergent in sequence. In contrast, effector families diversify not by sequence divergence but by gene duplication and creation of new families (e.g., Gambicin in mosquitoes and Diptericin, Drosocin, and others in *Dm*). This mode results in numerous species-specific effectors but very few trios, contrasting with the pattern seen in signal transduction. The species-specific modulators are selected separately in each species, from very large, divergent families such as SRPNs and CLIPs. Although the Toll and SPZ families are rich in trios, the mosquito genes most closely related to the *Dm* Toll-1/Spz interaction module are largely species-specific. Finally, the recognition phase shows an intermediate level of diversification, with species-specific genes approximately equal in number to the gene sum of trios and mosquito pairs; in this case, diversification arises by duplication of both genes and domains within genes [see (A)].

Successful triggering of the *Dm* Toll pathway after fungal and Gram-positive recognition engages a dedicated proteolytic activation cascade of serine proteases and SRPNs, of which several have been identified recently (*15*). None of these proteins exhibit mosquito orthologs, and only Spirit and Grass have recognizable paralogs (Fig. 2). The cascade culminates in cleavage of Spaetzle by the Spaetzle proteolytic enzyme (SPE), releasing a cytokine that binds to Toll. Mosquitoes have several genes encoding Spaetzle-like proteins (SPZs), but their SPE has not been recognized. Suggestively, the short and very specific SPE cleavage site (*16*) recurs in *Ag* CLIP-domain serine protease B5 (*Ag* CLIPB5) and *Aa* CLIPB38, which are otherwise phylogenetically unrelated.

Similarly, activation of prophenoloxidases (PPOs) to phenoloxidases (POs), the executors of melanization, is induced by a protease cascade (mostly CLIPBs). The cascade is positively and negatively regulated by a network of inactive protease homologs (CLIPAs), CTLs, and SRPNs (Fig. 3). This melanization module is tightly controlled, because it generates toxic byproducts including reactive oxygen species. Reverse genetic analyses have identified a large set of *Ag* regulators for melanization of *P. berghei* (*17*–*19*) or Sephadex beads (*20*, *21*): one SRPN, two CTLs, eight CLIPBs, and three CLIPAs (Fig. 3). Notably, all are members of mosquito-specific expansions, none has a definitive 1:1:1 ortholog, and only SRPN2 has a clear *Aa* ortholog. The reservoir of *Aa* proteases shows an underrepresentation of CLIPAs and massive expansions of CLIPBs as compared with both *Ag* and *Dm*. Finally, the melanization module may encompass additional regulators, because the genetic background determines which components are important in specific *Ag* strains (*19*).

The observed diversity of modulation components suggests that related but distinct regulatory modules may evolve in different species and even in subspecific taxa. Recruitment of individual members from very large multigene families may be followed by modulatory fine-tuning through selection imposed by particular microbes. For example, several of the genes that negatively control *P. berghei* melanization in *Ag* [*CTL4*, *CTL mannose-binding 2* (*CTLMA2*), and *SRPN2*] do not affect *P. falciparum* (*22*, *23*). Because *Ag* is a natural vector of *P. falciparum* but not of *P. berghei*, it is appealing to speculate that the sets of regulators of the melanization module evolve with and are manipulated by parasites. This modular mix and match evolution hinders detailed knowledge transfer between vector species but reinforces its importance in shaping the immune response. Future experimental studies of the melanization module in *Aa*, which can melanize bacteria and filarial worms, as well as sporozoites of the avian parasite *P. gallinaceum* (*24*, *25*), will be fruitful in further exploring this fascinating mode of immune evolution.

Although Toll-like receptors (TLRs) are found throughout the animal kingdom, phylogenetic and functional studies have suggested that insect Tolls and mammalian TLRs evolved independently (*26*). Most *Dm* Tolls serve developmental functions, and the recruitment of the Toll (Toll-1) receptor to immune signaling has been ascribed to convergent evolution. Even within insects, our analysis detects diversity: species-specific Toll expansions and only three trios. *Dm* Toll-1 has no clear orthologs; reduplications have created a clade of four *Ag* and four *Aa* genes, all related to both *Dm* Toll-1 and *Dm* Toll-5 (Fig. 2). In addition to its role in antifungal and antibacterial responses, *Dm* Toll-1 has been implicated in cellular antiviral responses (*27*). Thus, the possibility that the expanded Toll-1/Toll-5 clade in mosquitoes is related to their interactions with viruses merits detailed functional investigation. An unexpected evolutionary pattern was also observed for Spaetzle, the cytokine partner of *Dm* Toll-1, which shows three *Aa* paralogs and no identifiable *Ag* ortholog. *Aa* SPZ1C acts together with *Aa* TOLL5A to activate antifungal responses (*28*); however, the absence of an *Ag* Spaetzle ortholog raises questions about the evolution of this pair of molecules as an immune module, especially because the cytokine-Toll interaction is not required for mammalian TLR signaling. The only insect Tolls that cluster with TLRs are *Dm* Toll-9, *Ag* TOLL9, and *Aa* TOLL9A/9B. Because *Dm* Toll-9 is the only other Toll linked to *Drosophila* immunity (*29*), it is possible that this clade represents the most ancient immune-related insect Tolls. Whether these receptors can directly recognize microbial or viral immune inducers remains to be seen; it is worth noting that they are

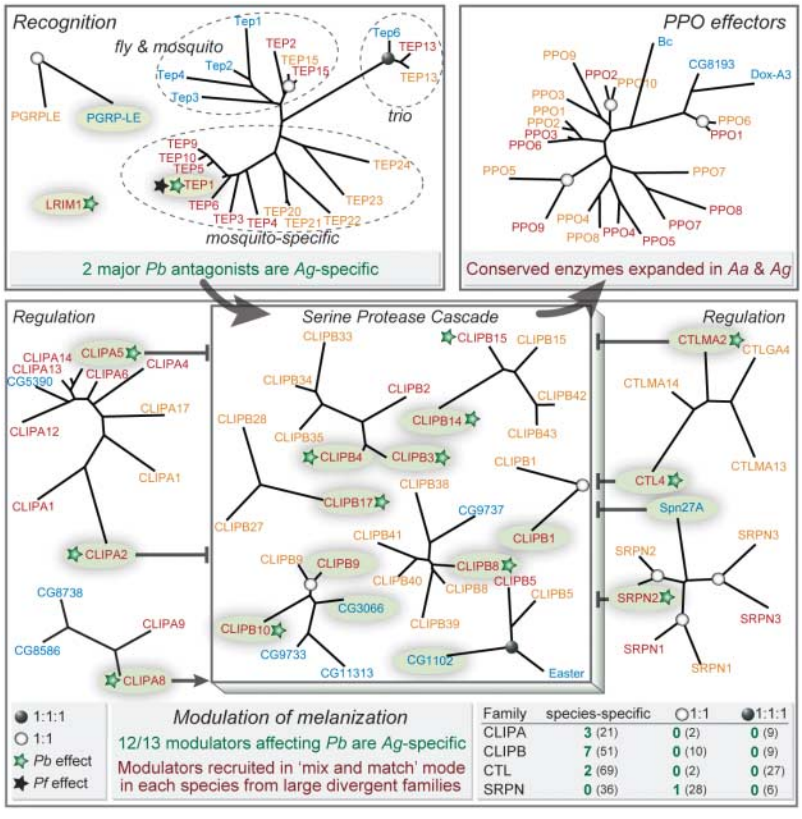


Fig. 3. The melanization immune response evolves by convergence and is based on pathogen-related, species-specific regulatory modules. Components are highlighted and shown in relation to their closest phylogenetic relatives in *Dm* (blue), *Ag* (red), and *Aa* (yellow). They are grouped in three phases: recognition, signal modulation, and effectors. TEPs exhibit only one orthologous trio and otherwise form two groups: one with both *Dm* and mosquito genes and another with species-specific mosquito clades. Recognition genes affecting *P. berghei* (*Pb*) melanization (green stars) are *Ag*-specific. Similarly, among modulators, those affecting *Pb* melanization (numbers in green in the bottom right box) are almost exclusively specific for *Ag* and are recruited from large divergent families (numbers in parentheses). In the modulation phase, CLIPB cascades are regulated positively and/or negatively by serine protease homologs (CLIPAs), CTLs, and SRPNs. Among those, CLIPB1, 4, 8, 9, and 10 are involved in melanization of Sephadex beads. The PPO effectors remain conserved in sequence to preserve their enzymatic function, but the family is expanded in mosquitoes. *Ag* genes marked with black stars affect survival of *P. falciparum* (*Pf*). Single-copy orthologs (1:1:1) in all three genomes are indicated with solid circles, and mosquito 1:1 orthologs are indicated with open circles on respective nodes. We constructed radial trees using similarity distances of the conserved sequence cores computed by maximum likelihood, with branch-length scaling preserved within but not between trees.

more similar to lipid-binding TLRs rather than to nucleic acid–binding TLRs.

Signal transduction components exhibit an unexpected mode of evolution. Rather than duplicating to create novel cascades responding to distinct challenges, or picking up members of multiprotein families to promote adaptive interactions, these components show robustness, maintaining their distinctive identity and functionality in the face of sequence evolution. The cytoplasmic signal transduction of the Toll pathway includes a chain of interacting partners, almost invariably encoded by orthologous trios: myeloid differentiation factor 88 (MYD88), TUBE, PELLE, tumor necrosis factor receptor–associated factor 6 (TRAF6), and CACT (Fig. 2). The same is true for the components of the IMD pathway: IMD, Fas-associated death domain protein (FADD), Dredd (CASPL1), IAP2, transforming growth factor β–activated kinase (TAK1), and inhibitor of nuclear factor κB kinase subunits γ and β (IKKγ and IKKβ). Despite persistent orthology, these components show marked divergence in sequence (Fig. 1A). A similar pattern is observed in the signal transducers Dome and Hop of the immune signaling Janus kinase–signal transducers and activators of transcription (JAK-STAT) pathway, which is activated in *Dm* by virus infections (*30*). We hypothesize that the requirement for these factors to interact productively with others in the same chain causes escalating sequence divergence: A mutation in one may enhance the acceptability of certain mutations in its interacting partner, maintaining pathway function through coherent evolution rather than stasis. Consistent with this interpretation, evidence has been reported for an association between natural sequence variation of core signaling pathway components and immune competence in *Drosophila* (*31*). Similar evolutionary patterns are detected among members of the RNA interference antiviral pathway, Dicer-2 and Ago-2 (*32*), which also form highly divergent trios.

Signal transduction culminates in the next phase: nuclear translocation of transcription factors. The cytoplasmic nuclear factor κB (NF-κB) transcription factors remain inactive until a processed immune signal frees them from inhibitors, permitting their entry into the nucleus and transcription of effector genes. The evolutionary pattern in this phase combines aspects observed in other phases. The NF-κBs of the Imd pathway [Relish in *Dm* and Rel-like NF-κB protein 2 (REL2) in mosquitoes] form an orthologous trio that displays high sequence divergence, as in signal transducer trios (Figs. 1A and 2). A recent duplication in *Aa* has resulted in an orthologous quartet (*Ag* REL1, *Dm* Dorsal, *Aa* REL1A, and *Aa* REL1B). In contrast, Dif is absent from both mosquito species, although the intronless *Aa REL1B* gene may have originated by retrotransposition. Transgenic analysis has shown that REL1A controls *Aedes* antifungal responses, as does Dif in *Dm* (*33*); this represents an interesting case of functional transfer between paralogs. STAT, the transcription factor of the JAK-STAT pathway, shows high sequence divergence like REL2 and has been duplicated in *Ag*.

Immune effectors are required to target and neutralize the microbial source of the immune signal. We observed varied evolutionary dynamics for different categories of effectors, reflecting their modes of action. Those acting directly on microbes diversify rapidly or are species-specific, whereas effector enzymes that produce chemical cues to attack invaders remain conserved but independently expand in each species.

The production of AMPs, which act on bacterial membranes causing lysis, is a classic immune-inducible effector response (Fig. 2). Seven AMP families exist in *Dm*, but only three of them were detected in mosquitoes: Defensins (DEFs), cecropins (CECs), and attacins (ATTs) are highly diverse, together displaying no orthologous trio and only one confident 1:1 orthologous pair. Conversely, gambicins are only encountered in mosquitoes. The apparent paucity of mosquito AMPs in contrast to *Dm* may be attributable to different prevalence of bacteria in their respective environments.

As diverse as AMPs, the large family of antibacterial peptidoglycan-hydrolyzing lysozymes (LYSs) shows only one identifiable trio and one mosquito pair among 28 members (Fig. 2). A marked expansion in *Dm* is ascribable to the use of LYSs for digestion of bacteria as a food resource: These peptides are atypically acidic and are expressed in the midgut but not in other immune tissues (*34*). Apart from these digestive *Dm* LYSs, the family forms two groups: one with both *Dm* and mosquito LYSs and the other with only species-specific clades of mosquito LYSs—a very similar pattern to that observed for TEPs, which are also thought to function both as recognition receptors and as complement effectors.

The family of PPO melanization effectors has expanded greatly in mosquitoes as compared with *Dm* and larger model insects. *Ag* PPO1/*Aa* PPO6 is the only orthologous pair that clusters with *Dm* PPOs; the remaining 17 mosquito PPOs form a distinct clade, created by reduplication events both before and since *Ag-Aa* diverged (Fig. 3). The invariable catalytic activity of POs (conversion of tyrosine to melanin) is likely to restrict their functional diversification, suggesting that observed expansions may reflect diversification to accommodate differential developmental, topological, or temporal activation. Indeed, several *Aa* and *Ag* PPOs show developmental or physiological specificity (*35*, *36*).

In *Ag*, increased systemic levels of hydrogen peroxide (H_2O_2) have been associated with *Plasmodium* melanization (*37*). H_2O_2 is used as an electron acceptor by HPXs that catalyze various oxidative reactions. This effector family shows a small expansion in *Aa* and a large one in *Ag*, while retaining a set of eight orthologous trios including *DUOX* (dual HPX and NADPH-oxidase, where NADPH is the reduced form of nicotinamide adenine dinucleotide phosphate). The latter is associated with peroxidase-mediated nitration during the apoptotic response of midgut cells to *Plasmodium* invasion (*38*). Numerous trio orthologs of HPXs and other enzyme families implicated in oxidative defense show low sequence divergence, suggestive of constraints to preserve ubiquitous catalytic activities.

The availability of the genome sequences of distantly related insects has allowed us to apply comparative genomic methods to analyze the evolutionary dynamics of the insect innate immune repertoires. Notably, we identified distinct and seemingly contrasting evolutionary modes characterizing different immune modules, which together serve to provide a flexible system capable of adapting to new challenges. The repertoire of recognition receptors of microbial groups such as bacteria and fungi, which are encountered by all species, is achieved through expansion and fine-tuning of model genes. New functions (e.g., recognition of malaria parasites) are acquired from genes bearing powerful and ancient recognition domains such as LRRs. Protein networks modulating immune signals are assembled independently in each species, in the mix and match mode of evolution described as "bricolage" by François Jacob; they therefore coevolve with pathogens and may be subject to evasion. Pathways of signal transduction, on the other hand, remain highly conserved, and their constituent genes seem to evolve always in concert. Finally, effector mechanisms follow evolutionary patterns that depend on their mode of action; most are highly divergent or even species-specific, in contrast to the ancient, conserved oxidative defense mechanisms.

Recognition of the role of Toll in *Drosophila* immunity led directly to the identification of TLRs as a fundamental aspect of mammalian innate immunity. Similarly, the diverse evolutionary modes of insect immunity that we detected in the present study can guide future studies on the evolution of innate immune mechanisms in vertebrates and other animals. They can also facilitate targeted studies of immunity in the two mosquito species, which together transmit some of the most devastating infectious diseases of humankind.

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Supporting Online Material

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Culling Prey Promotes Predator Recovery—Alternative States in a Whole-Lake Experiment

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Many top-predator fish stocks in both freshwater and marine systems have collapsed as a result of overharvesting. Consequently, some of these communities have shifted into seemingly irreversible new states. We showed, for predators feeding on prey that exhibit food-dependent growth, that culling of fish prey may promote predator recovery. We removed old stunted individuals of a prey-fish species in a large, low-productive lake, which caused an increase in the availability of small-sized prey and allowed the predator to recover. The shift in community state has been sustained for more than 15 years after the cull ended and represents an experimental demonstration of an alternative stable state in a large-scale field system. Because most animals exhibit food-dependent growth, shifts into alternative stable states resulting from overcompensating prey growth may be common in nature and may require counterintuitive management strategies.

Rapid changes observed in many ecological systems, such as the collapse of major fish stocks (*1*–*4*), have prompted an increased interest in alternative stable states during recent years (*5*, *6*). Theoretical studies (*7*, *8*) suggest that size-selective predation (*9*–*11*) may be a major mechanism behind shifts between alternative stable states if reduced competition for resources among remaining prey (*6*, *12*) accelerates prey growth. Predation on small individuals in this case leads to an overcompensating response because surviving prey mature more rapidly and achieve higher population reproductive outputs. Counterintuitively, densities of small prey hence increase and not decrease when predators forage on such small prey individuals (*7*, *13*). Thus, size-selective predators shape the biotic environment to their own advantage. These predator-prey systems are, however, prone to irreversible collapse of the predator if overharvested. A drop in predator density causes prey to grow and reproduce more slowly and consequently produce lower abundances of vulnerable, small-sized prey. This change in prey-size distribution subsequently prohibits recovery of the predator, making the collapse seemingly irreversible (*7*, *8*). Next to the predator-prey state the community thus possesses an alternative stable state with only prey (*7*). Size-selective harvesting of prey may offer a route to predator recovery, because it should stimulate rates of prey growth and reproduction and thereby shift prey-size distribution toward smaller individuals. Once the prey-size constraint for recovery is lifted, the recovered predator population should itself be able to sustain the system in the new state.

In the early 1900s, the top-predator brown trout (*Salmo trutta*) was the only species in the low-productive Lake Takvatn, in northern Norway (*14*–*16*). Overharvesting reduced trout to low levels, and Arctic charr (*Salvelinus alpinus*)—prey for, but also a potential competitor of, small brown trout for invertebrates (*14*, *16*–*19*)—was introduced in about 1930. The charr soon dominated the fish community, and by 1980 trout were almost absent (Figs. 1 and 2, A and D). To improve lake fisheries, 666,000 charr (31.3 metric tons) were removed during 1984 to 1989 (*14*, *15*). By 1991 charr density had decreased by 80%, subsequently rebounded to less than half its 1984 density, and ultimately exhibited a decelerating decrease toward a new steady state (1992 to 2006, regression $F_{1,14} = 14.7$, $P = 0.002$) (Fig. 1). Trout density increased from 1989 to 1992, remaining steady afterward (1992 to 2006, regression $F_{1,14} = 0$, $P = 0.99$). On average (1992 to 2006), the trout density was 12% of the charr density, similar to the value of 15% observed in a control lake (*16*). Although charr may compete with small trout for food (*14*, *17*, *18*), we found no evidence for a negative density–dependent effect of either total charr density or density of charr <150 mm in size on the body condition of 100-mm trout (regressions, $F_{1,16} = 0.10$ to 2.27, $P > 0.1$).

We generated seven testable expectations of this prey-culling management strategy with an existing resource-prey-predator, food-chain model (*7*, *13*), in which prey exhibit food-dependent growth and reproduction and predators forage on small prey only. At low densities, invading predators cannot increase in density in the stable prey-resource state (Fig. 3). Culling prey induces oscillations in prey density and strong pulses in prey recruitment, which allow predators to increase in numbers and reach high densities. Predators can subsequently control the prey, driving the system toward an alternative, stable resource-prey-predator equilibrium (Fig. 3) characterized by (1) lower prey density (smallest-size

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