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Anopheles gambiae pathogen susceptibility: The intersection of genetics, immunity and ecology

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Abstract

Mosquitoes are the major arthropod vectors of human diseases such as malaria and viral encephalitis. However, each mosquito species does not transmit every pathogen, due to reasons that include specific evolutionary histories, mosquito immune system structure, and ecology. Even a competent vector species for a pathogen displays a wide range of variation between individuals for pathogen susceptibility, and therefore efficiency of disease transmission. Understanding the molecular and genetic mechanisms that determine heterogeneities in transmission efficiency within a vector species could help elaborate new vector control strategies. This review discusses mechanisms of host-defense in *Anopheles gambiae*, and sources of genetic and ecological variation in the operation of these protective factors. Comparison is made between functional studies using *Plasmodium* or fungus, and we call attention to the limitations of generalizing gene phenotypes from experiments done in a single genetically simple colony.

INTRODUCTION

Serial blood-feeding behavior allows hematophagous arthropods to access a rich protein resource for egg production, but also exposes them to distinct microbes, thereby making them potential disease vectors. A combination of pre-existing genetic variation, pathogen selective pressure and fitness trade-offs have shaped the ways that vectors respond to their co-evolved pathogens by innate immunity and other mechanisms of host-defense, leading to vector-pathogen specificity. Even a competent vector species displays a wide range of infection phenotypes, including complete blockade of pathogen development in genetically resistant individuals [1]. It is difficult to measure fitness trade-offs in nature, and fitness measures made in the laboratory probably have little relevance for nature, but the transmitted pathogens are also under constraints to mitigate the effects of pathogenesis in order to maximize their own transmission. A pathogen that makes the vector too sick to fly and transmit has a reproductive fitness of zero, and pathogens that impose high fitness cost may also promote the evolution and spread of specific pathogen resistance in the host population. Identifying the molecular components of natural resistance, and understanding

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how they are genetically structured in wild *A. gambiae* populations, could help to improve applied strategies for surveillance and control of malaria transmission.

Genetics of *Plasmodium* resistance

A field-based genetic mapping survey of the wild population revealed that at least 40% of the *A. gambiae* s.s. tested carried a significant locus controlling susceptibility to natural *P. falciparum*, either for parasite numbers (*P. falciparum* intensity loci, *Pfin*) or melanization (*P. falciparum* melanization, *Pfmel*). About half of those loci (~20% of mosquitoes tested) are strong-effect resistance factors that can completely protect mosquitoes from infection [2,3]. This latter genetically resistant fraction of the *A. gambiae* population therefore probably does not transmit malaria, highlighting an important heterogeneity within the malaria transmission system that could potentially be manipulated for purposes of malaria control. A significant cluster of independently mapped resistance loci on chromosome 2L was termed the *Plasmodium* Resistance Island (PRI) in the *A. gambiae* genome [3]. This same genomic region controlling *P. falciparum* development was also mapped in East African *A. gambiae* [4,5], suggesting that the genetic mechanisms of malaria resistance in *A. gambiae* are widespread and shared throughout sub-Saharan Africa, rather than a local geographic adaptation.

The genes and the specific mutations underlying the QTLs mapped in nature have not yet been identified. Efforts are underway to identify nucleotide variation association with resistance to *P. falciparum* infections [6,7], however confirmation of causative mutations associated with the outcome of natural infection will involve large, carefully designed studies that control for population substructure and, as far as possible, identify effects that are clearly independent of genetic background. Nevertheless, candidate gene studies within a region genetically identified by QTL or association can be profitable, even if experimentally demonstrating the phenotype of a gene in the interval is, of course, not evidence that it is the causative agent. For example, in the PRI locus, candidate gene filtering by bioinformatics and function highlighted a paralogous family of genes, *APL1A, APL1B* and *APL1C*, encoding leucine-rich repeat (LRR) proteins that share ≥50% peptide identity with each other [8] and that demonstrate function in the laboratory, discussed further below.

Genetic mapping in wild pedigrees indicated that variants with significant effect on parasite development are collectively frequent in the field population [3], but the allelic spectrum of the variants is not known. The observed resistance loci could be due to either a few individually frequent variants of strong effect, or conversely due to many distinct, individually rare variants with functionally equivalent effects. As a model, the *APL1A* and *TEP1* genes display high genetic diversity in the natural population [9,10]. If polymorphism of these genes has phenotypic consequences for malaria resistance in nature (which is not yet known), there could be many individually rare causative mutations in the genes. This is reminiscent of the case of human hemoglobinopathies such as sickle cell anemia and the thalassemias, which result from a large number of independent and individually rare mutations in different genes, and overall yield a similar phenotype of protection against severe malaria [11,12].

LRR regulation and protective function

In *A. gambiae,* orthologs and homologs of intracellular *Drosophila* factors belonging to the Toll and Imd innate immune pathways have been identified [13]. At the terminus of the *A. gambiae* Toll pathway, the NF-κB transcription factor Rel1 (also known as Gambif1) serves as the functional analogue of *Drosophila* Dif transcription factor, while for the Imd pathway, *Rel2* is orthologous to the *Drosophila* transcription factor Relish [14]. The *A. gambiae* Nf-KB *Rel2* transcript has further been shown to produce, through alternative mRNA splicing, a

full-length (Rel2-F) and a shorter (Rel2-S) protein isoform [15]. Both isoforms are involved in defense against Gram-positive and Gram-negative bacteria.

An important part of anti-*P. falciparum* protection in *A. gambiae* was shown to be mediated by the Imd/Rel2-S pathway, while reduction of *P. berghei* parasites is largely based on Toll/Rel1 signaling [8,15–17]. These are probably not the only protective mechanisms, because after inactivating the relevant pathway for the parasite, the infection distribution shifts towards higher oocyst prevalence and/or intensity, but nevertheless there are still uninfected and/or low-infected mosquitoes, suggesting the operation of other lines of host defense. Functional analysis showed that, of the *APL1* paralogs, only paralog *APL1A* protected *A. gambiae* against infection with *P. falciparum,* whether from wild parasites or *in vitro* gametocyte culture, while only paralog *APL1C* protected against the rodent malaria parasites, *P. berghei* and *P. yoelii* [8,16]. Furthermore, protection against *P. falciparum* correlates with the transcriptional regulation of *APL1A* by the isoform Rel2-S but not Rel-2F [16], whereas protection against *P. berghei* correlates with the transcriptional regulation of *APL1C* by the Toll/Rel1 pathway [8]. The APL1C protein was shown to form a heterodimer with another LRR protein, LRIM1, as part of a complex with the complement-like protein TEP1 [18–20]. *LRIM1* displayed protective activity against the rodent parasites *P. berghei* and *P. yoelii* [21,22] and is also physically located in the PRI locus. Indeed, the PRI locus holds the largest genomic cluster of LRR genes in *A. gambiae* [3].

Because *A. gambiae* is not a natural vector of rodent malaria and there is no shared evolutionary history that could have selected specific resistance [23], the host-defense activity encoded in the PRI locus by at least *APL1C* and *LRIM1* probably also protects mosquitoes against other yet unknown natural pathogens, which would have been the actual selective agents. The criteria of specificity are not yet known for APL1C- and LRIM1-mediated protection against the presumptive natural pathogen targets. Taken together, these data indicate quite fine pathogen specificity, where the host can discriminate between different members of the genus *Plasmodium* via APL1 paralogs A and C, with each protein required for protection against a distinct class of malaria parasite, and without detectable reciprocal effect upon the other class. These observations together suggest the operation of a gene-for-pathogen-class protection system in *A. gambiae,* where the functions of LRR proteins are required for fine orientation of host-defense towards distinct classes of pathogens that include but are probably not limited to *Plasmodium* [16]. The pathogen classes were termed A-class pathogens, protected against by APL1A (currently *P. falciparum*), and C-class pathogens, protected against by APL1C (currently the rodent malaria parasite species *P. berghei* and *P. yoelii*).

Immune specificity

The basis of specificity of such pathogen classes is unknown, nor the identities of other pathogens that might be similarly recognized within a class. The APL1 paralog system distinguishes two kinds of eukaryotic pathogen that otherwise share broadly similar cellular morphology and pathogenic behavior, which would seem to require finer power of discrimination than, for example, the known ability of insects to differentiate between broad groups such as Gram-negative bacteria, Gram-positive bacteria, and fungus [24]. In the rodent malaria model, parasite killing is a collaboration between an LRR dimer that appears to confer target specificity, and a putative effector subunit, TEP1, which in the absence of the LRR dimer binds non-specifically to tissues of self [18]. There are numerous other LRR and TEP genes in the *A. gambiae* genome, some of which interact in protein complexes to yield protective function [25,26]. There are also important protective molecules that are not yet integrated with the LRR-TEP protein complex model, for example AgDscam and fibrinogen-domain proteins [27,28]. Overall, the observations to date suggest the capacity

for considerable combinatorial complexity of immune specificity, and emphasize how little is currently known.

The meaning of the term specificity also needs to be interrogated in the context of *Plasmodium* protection. The bacterial elicitors that are recognized as pathogen-associated molecular patterns (PAMPs, [29]), and corresponding host pattern-recognition receptors, are reasonably well known, less so for fungi [30], but are largely unknown for protozoa [31]. Indeed, there is no current direct evidence that host-defense against *Plasmodium* is PAMP-based. It was recently reported that rodent malaria ookinetes, during their passage through the midgut epithelium in a storm of reactive oxygen and cell damage, are surface-labeled by protein nitration, and that the nitration is an important determinant of subsequent binding of at least TEP1 and then ookinete death [32]. It was proposed that a proportion of ookinetes stochastically escape surface nitration, and that this unlabeled fraction therefore evades TEP1 binding and can develop unhindered. The work was done with rodent malaria, and validity for *P. falciparum* was not reported. However, the findings raise the interesting possibility that nitration could serve as a danger- or damage-associated molecular pattern (DAMP, [33]), thus potentially simplifying the immune challenge of recognizing various parasite PAMPs to, instead, a simpler requirement to merely sense surface nitration. However, this hypothesis might predict that the host requires only minimal complexity of host immune receptors to sense the density of nitrates on the ookinete surface, which may not be consistent with the observed complexity of host molecules that protect against malaria midgut infection [16,25–28]. Similarly, host defense against extracellular hemocoel sporozoites prior to salivary gland invasion [34] probably requires an explanation other than intracellular nitration.

Probing vector immunity with entomopathogenic fungi

In addition to adult female mosquitoes, APL1 paralogs are also expressed by adult males and during larval stages [25], which are never in contact with malaria parasites, indicating a more general protective role for these factors. Both male and female *A. gambiae* feed on plant nectar [35], which is a source of exposure to a shared pathogen repertoire such as bacteria and fungi. In addition, the female mosquito contacts aquatic and soil pathogens during oviposition. Entomopathogenic fungi such as *Metarhizium anisopliae* and *Beauveria bassiana* are environmentally ubiquitous, found in soil, air, and plants that mosquitoes feed on for sugars [36–38].

Fungi are being examined in the lab and field as a potential new vector control tool [39–43]. Reducing vector longevity is an effective approach to reduce transmission of malaria parasites, and is the basis of transmission control by indoor residual insecticide spraying. The oocyst stage in the vector has a long period of latency, producing infectious sporozoites in the salivary glands only just before the demographic end of the mosquito lifespan [44]. Thus, killing the vector at any point in that extended period is equally effective in reducing the proportion of infected mosquitoes that become infectious. One argument in favor of fungal control of vectors is that, as a delayed killing agent, the cost in reproductive fitness to the mosquito is lowered and pressure for the development of resistance mechanisms is lessened [39].

Understanding the genetics and functional biology of resistance to fungal pathogens may reveal new features of *A. gambiae* immunity. While these fungi are evolutionarily distant from *Plasmodium*, they are also eukaryotes, and aspects of mosquito immunity to both pathogens may not be completely independent. As a practical question, it should be determined whether widespread use of fungal entomopathogens to control malaria, including the eventual selection of genetic resistance to fungus, could have collateral consequences, either beneficial or detrimental, for mosquito susceptibility to and transmission of

Plasmodium. Interestingly, fungus infection can preferentially kill mosquitoes infected with rodent malaria, although the observation needs to be confirmed for *P. falciparum* [43]. If so, it might be possible to use a conditionally lethal fungus, which preferentially kills malaria-infected mosquitoes, to drive natural malaria-resistance alleles to higher levels in the population, by imposing selection against infected and therefore genetically susceptible mosquitoes in the population [3,45].

Immunity of mosquito larvae

Larval stages of anophelines are aquatic, and the water in breeding sites harbors a repertoire of microbes, including bacteria, fungi, and protozoans [46,47]. A subset of these microbes may be incorporated into a stable gut microbiome in larvae and subsequent adult mosquitoes [48]. Larval-derived exposure to bacteria [49] or protozoa [50] can influence adult mosquito susceptibility to malaria parasites. Of the few genes tested to date, mosquito immune genes regulated transcriptionally in adults were also regulated in larvae [51,52], although larval immunity has not been systematically studied. In adult mosquitoes, functional genomic studies are performed by injection of double stranded RNA (dsRNA). In mosquito larvae this method is more difficult, at least because of the need to perform injections out of the water, with consequent higher mortality, small size, and the tough and elastic larval cuticle. A non-invasive RNAi method was reported for *A. gambiae* using self-assembled dsRNA nanoparticles to mediate gene silencing through larval feeding [53]. Entomopathogenic fungi have been proposed for larval mosquito control because larval exposure with the right formulation of spores causes high larval mortality [54], and thus fungi can also be used for functional studies with larval stages of *A. gambiae*.

Ecological differences between larval pools may also be an aspect of niche differentiation by *A. gambiae* population subgroups, such as the reproductively isolated natural strains referred to as M and S molecular forms [46,55]. Preferential larval site selection by ovipositing females probably exposes their progeny to particular microbial communities and perhaps differential immune pressure, although little is known. It was recently reported that wild M-form mosquitoes display significantly reduced genetic diversity at two unlinked immune gene loci, *TEP1* and *APL1*, as compared to S-form mosquitoes [9,56]. The most likely explanation may be that specific and costly pathogen exposure of M-form larvae, based on larval site ecology, has selected for only a few alleles of the protective immune genes, but the hypothesis requires empirical evidence.

Immune phenotypes and study systems

Wild *A. gambiae* populations display high genetic diversity [57], which is partitioned between reproductively isolated population subgroups, and between diverged long allelic sequence blocks locked within polymorphic chromosome inversions [58–61]. Laboratory-reared *A. gambiae* colonies have been necessarily used for almost all reported functional studies of mosquito-pathogen interactions, because such experiments require controlled conditions (mosquito numbers, age, etc.) that are difficult to produce in nature. However, all colonies display low genetic diversity, and therefore segregate only a subset of alleles [62,63]. Thus, the cost of the common practice of working with just one colony in a given laboratory is that resulting genetic claims, including determination of the phenotype of the gene, are based on an experiment that queried only a small number of alleles in a particular genetic background. It is possible that some reported mosquito gene phenotypes would give a different result in a different colony.

The best way to collect high quality phenotypic data that is comparable between laboratories might be to make phenotypic determinations across a panel of independent colonies initiated from nature, modeled on the Genetic Reference Panel of Drosophila [64]. Functional tests of

a mosquito gene would be replicated in a panel of reference strains, compensating for genetic simplicity in any one strain by the summation of the same observation repeated across strains. The result would define the gene phenotype not as a single point, but instead as many points that differ according to target gene allelic variation and genetic background of each test strain, and overall would better approximate the true range of the phenotype in the natural genetic system. In fact, otherwise protective genes may even enhance pathogen development in some genetic contexts [22]. One challenge with this approach is that *Anopheles* culture is more difficult than Drosophila, and there is no method of strain storage other than continuous culture, which requires insectary space and resources. A panel of reference strains initiated from nature could also serve as a useful genetic resource for mapping, because of their individually simplified allelic spectra and extended linkage.

The other important aspect of malaria vector study systems is the parasite model. The rodent malaria laboratory model (*P. berghei*, *P. yoelii*, etc.) has been extraordinarily productive, and its robustness and easy availability is largely responsible for the emergence of *A. gambiae* as a model organism for host-pathogen interactions. However, the accuracy of rodent malaria interactions in *A. gambiae* as a model for vector response to the naturally transmitted parasite, *P. falciparum*, has not been systematically examined or validated. When comparisons have been made, the theme is often more about differences than similarities. For example, functional tests show different signaling pathways, and different outcomes for many protective genes [16,17,21,65]. The mapped *A. gambiae* QTLs that control resistance, even though the overall number is relatively small, are also different between rodent parasites in the laboratory and human malaria in the field [3]. Mosquito transcriptional profiling shows a combination of both overlap and distinct responses between the two parasite classes [21,65]. It was suggested that rodent malaria infection in the mosquito *A. stephensi*, rather than *A. gambiae*, may be more representative of *P. falciparum* interaction with *A. gambiae* [21]. Overall, these differences encourage caution in generalizing about mechanisms of “protection against Plasmodium,” in particular against *P. falciparum*, from results obtained only in the rodent malaria model, and mitigate in favor of studies that include direct comparison using challenge with *P. falciparum* gametocyte culture whenever possible.

CONCLUSION

Mosquitoes are the most important arthropod vectors of diseases. Understanding the molecular and genetic components of vector competence for transmission of a given pathogen could help generate new vector control tools. In addition, the sheer quantity of mosquito-malaria research coupled with genomic technologies has now made *A. gambiae* a model system for the study of host-pathogen interactions. The *A. gambiae* – *P. falciparum* system results from an old and specific evolutionary history due to the obligatory nature of human malaria transmission by Anopheles. This is unlike other model organisms such as Drosophila or *Arabidopsis*, which are infected by facultative combinations of more generalist pathogens [66]. Thus, the mosquito-malaria study system may be especially advantageous for detecting a clear genetic signal resulting from an evolutionary interplay between pathogen virulence and host defense.

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Highlights

- Mosquito vectors of disease vary between individuals for pathogen infection.
- Pathogen susceptibility is controlled by immunogenetics and ecological factors.
- Studies of pathogen susceptibility in a single mosquito colony are oversimplified.

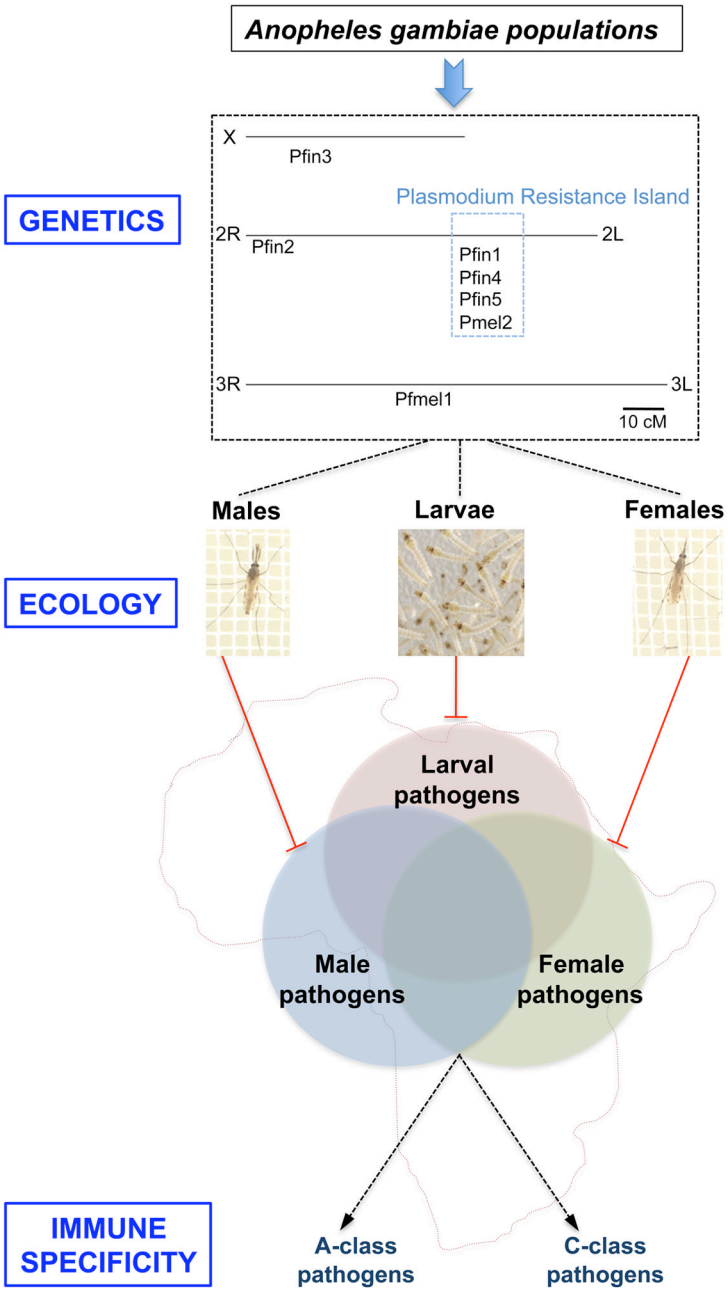

Figure.

Synthetic model depicting the intersection of genetics, ecology and immunity on Anopheles gambiae pathogen susceptibility. Upper panel (***Genetics***): The genetic location of loci that control *P. falciparum* infection in the natural *A. gambiae* population are indicated [3][4,5]. *Pfin* loci control numbers of normal oocysts following an infected blood meal, *Pfmel* loci control ookinete melanization, and the Plasmodium Resistance Island comprises a cluster of protective loci on chromosome 2L. Middle panel (***Ecology***): Genetic and functional mechanisms of *Plasmodium* resistance can only be tested in female mosquitoes, but some of the same host-defense mechanisms or close variants may be deployed against other pathogens in adult male mosquitoes and larvae of both sexes. Males and larvae are subject to

distinct microbial repertoires and immune selective pressure, but microbial repertoires common to all forms may shape shared components of immunity. Lower panel (***Immune Specificity***): Classes of pathogens that are distinguished by mosquito immunity can be hypothesized, based on differential modes of fine specificity mediated by either APL1A (A-class pathogens, including *P. falciparum* but not rodent malaria), or APL1C (C-class pathogens, including rodent malaria parasite species but not *P. falciparum*). Functional tests of other pathogens and immune genes will be necessary to fully define the protective spectra of these and other immune genes, and the network of their interactions [Mosquito pictures: CEPIA, Institut Pasteur].