



Anopheles infection responses; laboratory models versus field malaria transmission systems

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Abstract

The molecular biology of disease vectors, particularly mosquitoes, has experienced a remarkable progress in the past two decades. This is mainly attributed to methodological advances and the emerging genome sequences of vector species, which have brought experimental biology to an unprecedented level. It is now possible to determine the entire transcriptome of *Anopheles gambiae* at a variety of conditions, with a low per-gene effort and cost. Proteomic profiles can be generated for as small samples as the hemolymph, and transient reverse genetic and stable germ line based transgenic analyses can be performed to analyze gene function. High throughput screening for receptors and ligands can be used to characterize interactions between vectors and pathogens. At the current breathtaking rates of data production it is essential to question and evaluate the relevance of laboratory infection models to the real disease transmission systems. The majority of scientific discoveries in mosquito molecular biology have been based on highly inbred laboratory strains and rodent malaria parasite infection models, which may differ substantially to their counterparts that transmit human malaria in the field. This review addresses the recent advances in high throughput transcription analyses of *Anopheles* responses to infection, and discusses considerations for the use of laboratory malaria infection models.

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1. Advances in mosquito transcriptomics; analyses of infection responses and refractoriness

The available *Anopheles gambiae* DNA sequence has expanded from about 100 transcripts in the

early 90's to the entire genome in 2002, and there is more to come; the *Aedes aegypti* genome sequence has been completed, and numerous other vector species are currently in the sequencing queue. These genomic advances have conveniently coincided with technological developments that enable simultaneous transcription analyses of thousands of genes. The biological processes underlying vector–parasite interactions and other essential functions for disease transmission, such as blood feeding, are most fre-

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quently reflected by changes at the transcriptome level. Microarray gene expression analyses can therefore provide information on these processes and allow functional predictions of genes.

The first generation *A. gambiae* spotted high-density cDNA microarrays, representing approximately 2300 unique transcripts derived from immune challenged cell lines, have provided an impressive amount of biological information (Dimopoulos et al., 2002b; Christophides et al., 2002; Kumar et al., 2003). In vivo analyses of responses to septic and aseptic injury, and *Plasmodium* infection of the midgut, discriminated injury responsive genes from infection responsive genes, and showed extended overlap between anti-microbial and anti-*Plasmodium* responses at the transcript level (Dimopoulos et al., 2002b).

Gene expression differences between infection responses of susceptible *A. gambiae* mosquitoes and genetically selected refractory mosquitoes, that melanotically encapsulate ookinete stage parasites in their midguts, proposed a link between the mosquito's oxidative status and propensity to kill parasites. This hypothesis was validated with biochemical analyses (Kumar et al., 2003). Assays performed with these first generation microarrays identified members of gene families, as immune responsive, that later were shown to play major roles in anti-*Plasmodium* defense (Dimopoulos et al., 2002b; Blandin et al., 2004; Osta et al., 2004). Another study employing an *A. aegypti* EST-based spotted microarray, analyzed differences in gene expression between two refractory and susceptible *A. aegypti* strains to *P. gallinaceum* (Chen et al., 2004). Several differentially expressed genes were found to be related to blood digestion, and correlated with a previous study that had showed susceptible mosquitoes exhibiting higher enzymatic activity for some digestive proteases (Chen et al., 2004). A more recent study addressed gene transcription of *Plasmodium berghei* and *Anopheles stephensi* during the course of infection, using a spotted cDNA microarray that comprised both mosquito and parasite genes. This analysis defined expression clusters of vector–parasite interaction processes, such as the activity of *Plasmodium*'s invasion machinery during midgut invasion, and the mosquito midgut response to the parasite (Xu et al., 2005). An important advance in *A. gambiae* transcriptomics was the development of the Affymetrix *A. gambiae*, *P. falciparum* gene-chip, and an Agilent Technologies *A. gam-*

biae, *P. berghei* microarray comprising the mosquito's entire predicted transcriptome of approximately 14,000 genes. Analysis at the complete transcriptome level provides a much higher resolution of the biological processes of immune response in the mosquito (Aguilar et al., 2005; Dimopoulos lab, unpublished data).

2. Considerations for the choice of infection model in the study of mosquito–parasite interactions; differences and similarities

Plasmodium has to go through a complex journey in the mosquito, involving specific interactions with several different organs and cell types. So far, most studies addressing the molecular interactions between *Anopheles* and *Plasmodium* have employed the rodent malaria parasite model, *P. berghei*, which is more amenable than *P. falciparum* (Sinden, 1978; Dimopoulos et al., 2002a). The genome organization is largely conserved between the rodent and the human parasites, and about 3900 rodent malaria parasite genes have orthologs in *P. falciparum*, representing the universal *Plasmodium* gene set (van Lin et al., 2000; Carlton et al., 2002; Hall et al., 2005). However, despite the overall similarity between the biology of human and rodent malaria parasites, differences exist that are directly related to the interaction of the parasites with their vectors. A major question is how closely the biology of the natural malaria transmission system resembles the laboratory models with rodent malaria parasites and highly inbred mosquito strains.

2.1. Vector permissiveness to different parasite species

During *Plasmodium*'s development through the mosquito it suffers large losses at three major stages: in the blood meal during several developmental transitions from gametes to ookinetes, in the midgut epithelium and in the haemocoel when sporozoites migrate to the salivary glands (Ghosh et al., 2001; Shahabuddin and Costero, 2001; Sinden et al., 2004). These losses vary between different parasite–mosquito species combinations. A certain mosquito species always shows different permissiveness to different *Plasmodium* species. For instance, *P. berghei* will develop at least 50 oocysts in *A. gambiae*, while *P.*

falciparum rarely exceeds three to four oocysts per midgut in the field and even in laboratory infections (Pringle, 1966; Collins et al., 1984). Different mosquito species will also frequently display different permissiveness to the same *Plasmodium* species. For instance, *P. falciparum* achieves different infection levels in six *Anopheles* species, and comparative susceptibilities in order are: *A. freeborni* \geq *A. gambiae*, *A. arabiensis*, *A. dirus* $>$ *A. stephensi*, *A. albimanus* (Vaughan et al., 1994). *P. falciparum* in *A. freeborni* appeared to have an enhanced production of ookinetes, passing through the midgut with greater efficiency than in other species. The population dynamics of sporogony of wild parasites, which have co-adapted to their local vector species, is probably different from the population dynamics of laboratory-adapted strains (Githeko et al., 1992; Beier et al., 1992). These variations of infection phenotypes, for different *Anopheles*–*Plasmodium* species and strain combinations, must be considered when interpreting and comparing experimental data, and in particular when assaying the mosquito's response to infection, which may be directly dependent on infection levels, and the molecular and temporal details of interaction.

2.2. Ookinete migration pattern across the midgut epithelium

The midgut epithelium is the major site of mosquito anti-*Plasmodium* immune responses and therefore an essential site to address potential differences in interactions with the human and model malaria parasites. An early study suggested that *P. falciparum* migrates through *A. stephensi* midgut epithelium via an intercellular mode and did not cause the type of cell damage that resulted from *P. berghei* invasion, while a more recent analysis supported an intracellular route (Meis et al., 1989; Han et al., 2000; Vlachou et al., 2004; Baton and Ranford-cartwright, 2004). The discrepancy between the two studies could be due to a different sampling procedure, or biological differences between the *P. falciparum* strains NF54 and 3D7A. In the latter and more detailed study, *P. falciparum* ookinetes were not found to prefer a particular midgut cell type, and invasion resulted in dramatic changes that were indicative of host cell apoptosis and expulsion from the epithelium into the lumen, similarly to what had been described for *P. berghei* in *A. stephensi* (Han et al.,

2000; Vlachou et al., 2004). The *A. aegypti* mosquito midgut epithelium is also invaded by *P. gallinaceum* through an intracellular route, and suffers damage, which is repaired by a unique actin cone zipper mechanism, rather than the actin ring mechanism that has been described in *A. gambiae* (Gupta et al., 2005). In summary, recent analyses suggest a similar route of invasion for the human and rodent parasites, and a similar cellular response of the *A. gambiae* midgut epithelium to the two parasite species.

2.3. Mosquito immune responses to *P. berghei* and *P. falciparum* infections

Transcription analyses have mostly been used to assess *Anopheles* immune responses to *Plasmodium* infection, and the transcriptome will eventually reflect even the smallest and most subtle differences in interaction and infection intensity for different Plasmodia in *A. gambiae*. Analyses of *A. gambiae* transcriptional immune responses to ingested *P. berghei* and *P. falciparum* infected blood, has indeed revealed substantial qualitative and quantitative differences; these comparative analyses were first performed with a limited set of selected immune genes, and more recently at the global transcriptome level (Tahar et al., 2002; Dimopoulos lab, unpublished). The observed differences may have resulted from a variety of factors, ranging from variations in infection intensities, interactions with mosquito epithelium, secretion of parasite metabolites and proteins and recognition of parasite antigens by the mosquito's immune surveillance system. Differences in assayed response patterns may also have been attributed to differences in temperature (*P. berghei* infections are done at 21 °C while *P. falciparum* develop at 27 °C) and developmental rates for the two parasite species (Dimopoulos et al., 2002a).

3. Laboratory reared colonies versus field mosquito populations

Our current knowledge on mosquito responses to infection is mainly based on studies performed with highly inbred laboratory strains of *A. gambiae* that have been maintained under laboratory conditions for the past 15–30 years. The environmental and microbial exposure of these mosquitoes is significantly milder and less diverse than that of field mosquitoes.

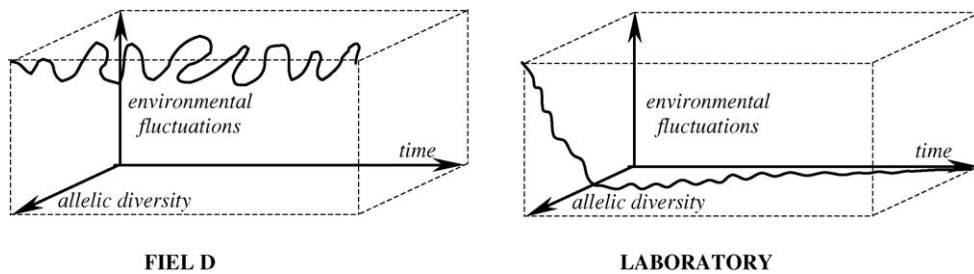


Fig. 1. Divergence between laboratory and field mosquitoes over time is determined by differences in environmental conditions (climate, nutrition, microbial exposure), and population size. Laboratory colonies will rapidly suffer a dramatic loss of allelic diversity and fitness, while field populations may change over time, while responding and adapting to varying conditions.

Reduction in selective pressure, taken together with increased inbreeding depression, due to the initial small parental population size and subsequent population bottlenecks, will result in loss of genetic variability and fitness (Wright, 1977; Charleston and Charleston, 1987; Falconer and Mackay, 1996; Whitlock and Fowler, 1999; Reed and Frankham, 2003). Indeed, studies with *Drosophila* showed that inbred populations had lower fitness and were less adaptable than their outbred parental population (Reed et al., 2003; Frankham et al., 2000; Woodworth et al., 2002; Hoffmann et al., 2001). *A. gambiae* microsatellite DNA polymorphisms are dramatically reduced in laboratory colonies compared with field populations, due to the small parental strain size used to initiate laboratory colonies (Norris et al., 2001).

The microbial exposure represents a strong evolutionary force for immune gene divergence between laboratory and field mosquitoes and between field mosquito populations. In addition to the necessity of maintaining a pattern recognition repertoire that is compatible to the microbial species-specific exposure of each ecological niche, selective pressure is also exerted by pathogens' ability to interfere with various host mechanisms (Spriggs, 1996; Hueck, 1998). Sequence diversity of immune genes can be predicted to be higher in field mosquitoes, to cope with a broader diversity of microbes (Hughes, 1997; Wang et al., 2003). Strong directional selection has indeed been documented for pathogen recognition receptor genes in *Drosophila* and *Anopheles* (Christophides et al., 2002; Schlenke and Begun, 2003; Morlais et al., 2004). *A. gambiae* field mosquito immune genes exhibit high levels of nucleotide polymorphism, and pathogen recognition receptors show a higher degree of replacement

substitutions (Morlais et al., 2004). Sequence polymorphisms in immune genes have also been correlated with the prevalence of natural *P. falciparum* infections in *A. gambiae* field populations (Luckhart et al., 2003). Even in a seemingly homogeneous mosquito strain, a variety of alleles appear to exist that can affect susceptibility to the parasite (Medley et al., 1993; Niare et al., 2002). For these reasons, various physiological systems, including the immune system, of laboratory and field mosquitoes will inevitably diverge. Divergence is also expected to occur between different field mosquito populations that inhabit different ecological niches, as well as between different laboratory strain mosquitoes that may have different rearing conditions and have originated from different parental strains (Fig. 1). Most studies have addressed divergence between species and populations at the DNA and protein sequence level, while the degree and significance of transcriptomic (gene-expression) divergence has not been thoroughly studied. A recent study documented positive coupling between gene expression polymorphisms and divergence with protein sequence divergence in *Drosophila*, suggesting similar evolutionary dynamics for gene-expression and protein sequences (Lemos et al., 2005). Microarray analyses in *A. gambiae* indicated broad differences between microbial challenge response profiles of the susceptible *A. gambiae* G3 and pink eye (4A r/r) strains (Kumar et al., 2003). The GNBPB1 (gram-negative bacteria-binding protein B1) gene has shown interesting variations in infection responsive transcriptional regulation between different *A. gambiae* laboratory strains. Richman et al. (1997) and Dimopoulos et al. (1998) documented transcriptional up-regulation of GNBPB1 in the midgut of the refractory *A. gambiae* L3–5 strain mosquitoes at 24 h after ingestion of *P. berghei* infected blood

when ookinete invasion occurs. In contrast, Tahar et al. (2002) showed that GNBPB1 transcription ranged between two-fold repression and no regulation in two replica infection assays with the same parasite strain in the Yaoundé *A. gambiae* strain. A third study, by Christophides et al. (2002), did not detect induction of GNBPB1 in the midgut tissue of the pink eye (4A r/r) *A. gambiae* strain, at similar conditions to the previous studies. Such variations are not surprising, considering the different adaptations and inherent different genetic background of these strains. GNBPB1 is a pattern recognition receptor that may diverge rapidly at the gene regulatory level while it adapts to different microbial exposures. Studies have been initiated to address transcriptomic differences between the field *A. gambiae* incipient M and S species (M and S molecular forms) and an inbred laboratory strain. Preliminary data suggest a stronger and potentially more rapid response of field mosquitoes to the challenge with bacteria; the number of significantly regulated genes at a very early time point after microbial challenge is three times larger in the field than in laboratory strain mosquitoes (Dimopoulos lab, unpublished).

4. Concluding remarks

Interactions between *Anopheles* and *Plasmodium* are complex and exhibit variations, even within the same vector–parasite species combination. These variations will inevitably affect gene expression and hence the outcome of transcriptomic analyses that aim at the characterization of mosquito responses to infection. Most of our knowledge on mosquito infection responses has derived from studies performed with *A. gambiae* laboratory strains and the rodent parasite *P. berghei*, and it would simply not have been possible to reach the current level of knowledge through exclusive use of field mosquitoes and *P. falciparum* (Richman et al., 1997; Dimopoulos et al., 1998, 2001, 2002a, 2002b; Han et al., 2000; Christophides et al., 2002; Tahar et al., 2002; Kumar et al., 2003; Blandin et al., 2004; Osta et al., 2004; Sinden et al., 2004; Vlachou et al., 2004; Xu et al., 2005).

Laboratory models provide certain advantages, in both technical and biological context, against the natural field organisms. Performing infections with *P. falciparum* in *A. gambiae* is plagued by difficulties

in establishing an infectious parasite culture and achieving adequate infection levels in the mosquitoes. The use of *P. falciparum* gametocyte carrier blood sources is for most laboratories not possible. Infection assays with *P. falciparum* will furthermore require a high safety insectary. The unnaturally high infection levels of *P. berghei* in *A. gambiae*, renders it possible to assess effects of mosquito gene silencing on *Plasmodium* development with a greater precision. The low infection levels of *P. falciparum* may not enable detection of infection induced transcription for certain mosquito genes that may be specific for invaded midgut cells (Ghosh et al., 2001).

It is complicated to establish laboratory colonies from field caught mosquitoes, and in many cases impossible. Such colonies are frequently unstable and may undergo dramatic changes in allele frequencies over time, which in turn can affect the biology of interaction and response to *Plasmodium*. Laboratory strains are inbred and thereby more standardized, and will therefore produce more consistent data over time (Charleston and Charleston, 1987; Whitlock and Fowler, 1999; Norris et al., 2001; Hoffmann et al., 2001; Woodworth et al., 2002).

The choice of biological system for any given study should eventually be based on the various tradeoffs, if options for choice exist at all. A small compromise in biological validity may in many cases be preferred over the time and resource consuming technical obstacles that would have to be solved for the use of a natural system. Initial screens and assays can be performed with laboratory models, and interesting findings must subsequently be validated with natural mosquito–parasite combinations. The nature of *Anopheles* anti-*Plasmodium* responses is not expected to differ fundamentally for the different parasite species and preliminary analyses indicate similar anti-*P. falciparum* and anti-*P. berghei* activities for many tested immune genes (Baton and Ranford-cartwright, 2004; Vlachou et al., 2004; Gupta et al., 2005; Han et al., 2000; Dimopoulos lab, unpublished data).

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