appear to either have been lost or forced to diversify. Interestingly, the largest number of orthologues and smallest diversification between the two species is found in gene families that are involved in the highly conserved signal transduction pathways of the innate immune system and developmental processes. Mosquito orthologues have for instance been identified for all Toll pathway components. In contrast, the largest family expansions and diversifications have occurred within gene families implicated in pathogen recognition, serine protease cascades and effector systems. Diversification of the protein repertoire for some gene families is also increased through alternative splicing of transcripts, as in the case of the PGRP genes (discussed above). The TEP families (discussed above) are represented by six and 15 members in *D. melanogaster* and *A. gambiae*, respectively. Only one 1:1 orthologous pair has been identified and most of the other members have resulted from species-specific expansions which may reflect adaptations to different microbial exposure (Christophides *et al*., 2002). One of the most remarkable examples of a massive mosquito-specific gene family expansion is represented by the FREP family (described in earlier sections). Whereas only 13 FREP homologues have been identified in the *D. melanogaster* genome, the *A. gambiae* has as many as 58 different members (Zdobnov *et al*., 2002). The reason for this massive expansion of the *A. gambiae* family is probably linked to haematophagy and exposure to *Plasmodium*. The bacteria and blood cell binding nature of FREPs may be important in controlling the midgut bacteria flora, which is highly boosted upon blood feeding, or prevent blood coagulation through competitive inhibition (Demaio *et al*., 1996; Gokudan *et al*., 1999). The malaria infection responsive nature of some mosquito members suggest them being a part of the antimalarial defence system (Christophides *et al*., 2002; Dimopoulos *et al*., 2000; 2002; unpublished data). The *A. gambiae* antimicrobial peptide, Gambicin, has also been identified in the Aedes aegypti mosquito but does not exist in the *D. melanogaster* genome (Dimopoulos *et al*., 2000; Vizioli *et al*., 2001; Christophides *et al*., 2002). Gambicin may have evolved specifically to cope with the mosquitos' microbial flora or the malaria parasite.

The mosquitos' innate immune responses and *Plasmodium* killing

The lifecycle of *Plasmodium* in the mosquito is complex and involve several developmental transformations and spatial translocations through and between epithelial tissues (Fig. 1). The parasite suffers large losses during its sporogonic development. A small proportion of ingested gametocytes will develop into ookinetes and of these only a fraction will reach the oocysts stage. At the later stages of infection, more than 80% of the haemocoel sporozoites will fail to relocate into the salivary glands and are instead rapidly cleared from the haemolymph through unknown mechanisms. The magnitude of these losses can differ greatly between infections with different parasite and mosquito species combinations and their molecular basis is unknown (Beier, 1998; Ghosh *et al*., 2000; Dimopolous *et al*., 2002b). Parasite elimination in mosquitoes have been linked to the innate immune system and may be crucial for the successful transmission of malaria (discussed below; Luckhart *et al*., 1998; Lowenberger *et al*., 1999). High infection levels may seriously affect the mosquitos' fitness whereas a too low infection level may prevent transmission of the parasite. The lowest possible infection level that can permit transmission appears to be most favourable for both the parasite and the vector. In fact, several studies have indicated increased mosquito mortality upon high malaria infection levels that are more likely to occur in infections between unnatural mosquito–parasite species as opposed to low infection levels that are characteristic for natural mosquito–parasite combinations (Ferguson and Read, 2002). A finely tuned equilibrium between the mosquitos' immune system and the parasites immune evasive capability is likely to contribute towards the establishment of a low but transmissive infection level. Incompatibility of the biochemical environment in the mosquito, the receptor–ligand interactions involved in epithelial invasion and the temporal kinetics of parasite development and mosquito processes, such as blood digestion, are also likely to constitute important determinants of mosquito permissiveness to malaria infection and thus important regulators of transmission.

Mosquito refractoriness to Plasmodium *infection*

Several *Anopheles–Plasmodium* combinations are incompatible because of highly efficient parasite killing by mosquito refractory mechanisms. The best characterized refractory mosquitoes belong to the genetically selected L3-5 *A gambiae* strain which melanotically encapsulates late ookinetes and early oocysts as they reach the basal side of the midgut epithelium between 20 and 40 h after infection (Fig. 1). This humoral encapsulation mechanism has a certain degree of specificity and key components of the melanization reaction appear to originate from the haemolymph (Collins *et al*., 1986; Gorman *et al*., 1998; Paskewitz *et al*., 1998; Dimopoulos *et al*., 2001). Melanized Plasmodia are rarely seen in field collected *A. gambiae* mosquitoes (Schwartz and Koella, 2002). Three quantitative trait loci (QTL) are controlling the refractory trait (Zheng *et al*., 1997) and a 528 kb section of the major QTL, *Pen1,* chromosomal region has been sequenced and revealed 48 genes that are currently being analysed for potential implication in the refractory mech-

anism (Thomasova *et al*., 2002). Factors such as pattern recognition receptor molecules or components of serine protease cascades represent promising candidates for the regulation of melanotic encapsulation. Microarray gene expression studies on the refractory L 3–5 mosquitoes have indicated major differences from the susceptible mosquitoes in expression signatures involving immunity and oxidoreductive genes. The potential implication of the innate immune system and/or the mosquitos' oxidative status in the determinants of the refractory phenotype is currently investigated (C. Barillas-Mury, R. Cantera, G. Christophides, G. Dimopoulos, F. C. Kafatos, unpublished data).

Other refractory mosquito species and strains that kill Plasmodia with different mechanisms exist. A genetically selected strain of the oriental vector *Anopheles dirus* is almost totally refractory to *Plasmodium yoelii nigeriensis* infection whereas it will permit normal development of *P. falciparum* and *Plasmodium vivax*. In this strain, *Plasmodium yoelii* oocyst development is arrested within 12 h and then followed by melanization of dead early oocysts. The melanization reaction does not seem mediate the killing itself but occurs as a subsequent event (Somboon *et al*., 1999). In a genetically selected *A. gambiae* strain, all *Plasmodium gallinaceum* ookinetes die in the midgut epithelium through a lytic mechanism. The dying ookinetes are first vacuolated and will then appear as degraded and broken within the midgut cell cytoplasm. Genetic crossing experiments suggest that this lytic killing mechanism is controlled by a single dominant locus (Vernick *et al*., 1995). The Mexican malaria vector *Anopheles albimanus* is highly refractory to the *P. vivax* CS VK247 variant. The development of this parasite is compromised at three different stages in the midgut: the first portion of parasites is believed to be destroyed by mosquito digestive enzymes in the ectoperitrophic space close to the internal midgut surface; a second portion disintegrates within the midgut epithelium and; a third portion is arrested during early oocyst development on the basal side of the midgut epithelium. (Gonzalez-Ceron *et al*., 2001). Although the implication of the mosquitos' immune system in these refractory mechanisms is strongly suggested, their molecular basis remains unknown. The same mechanisms that are responsible for total refractoriness are also most likely operating at a lower level in susceptible mosquitoes where they reduce parasites at the crucial transition stages within and between epithelia. For instance, melanized Plasmodia have been documented in natural mosquito populations (Schwartz and Koella, 2002). Other killing mechanisms, such as lysis, are more difficult to document because of the lack of a visible phenotype. Hence, genetically selected refractory strains are important for the study of mechanisms mediating *Plasmodium* killing in natural susceptible mosquitoes.

Anopheles gambiae *immune responses*

Upon malaria infection, the mosquito is mounting robust local and systemic immune responses that correlate temporally and spatially with the parasite's development. During midgut invasion by the ookinete, several immune genes have been shown to be upregulated in both the midgut epithelium and in the fat body. The activation of immune responses in the fat body at this stage, when the parasites are still located within the midgut epithelium, strongly suggest the existence of immune signalling cascades between the different tissues. At the later stages of infection, when sporozoites translocate from the oocysts to the salivary glands, immune responses have been documented in the salivary gland and in the fat body. Genes implicated in these immune responses encode putative pattern recognition receptors, serine proteases, signalling pathway components, antimicrobial peptides and nitricoxide synthase (Dimopoulos *et al*., 1997; 1998; 2002; Richman *et al*., 1997; Luckhart *et al*., 1998; Oduol *et al*., 2000). A significant degree of overlap between responses to bacteria and malaria challenge has been demonstrated by gene expression analysis of 2300 genes on a microarray. The spectrum of genes that are regulated by malaria infection is significantly smaller than that of the bacteria infection induced genes and do not overlap with sterile injury induced genes. In this assay, the malaria infection responsive immune genes included a GNBP, a PGRP, a FREP, a TEP, a serine protease, a phagocytic component and a leucin rich repeat protein gene that share homology with Toll receptors (Dimopoulos *et al*., 2002). The documented immune responses to malaria infection may partly result from the injury that is caused by parasite invasion of epithelial tissues as well as microbial components of the midgut that may be present at the invasion site. However, the lack of overlap between sterile injury responsive and malaria infection responsive gene expression signatures, and the strong activation of immune genes in antibiotic-treated malaria-infected mosquitoes strongly suggest the existence of a *Plasmodium* recognition-specific mechanism of immune induction (Richman *et al*., 1997; Dimopoulos *et al*., 2002). A comprehensive gene expression study using a whole genome microarray, representing the entire *A. gambiae* transcriptome, will provide a much more detailed view on the regulation of malaria infection responses and the implicated components. The significance of the documented immune responses in the elimination of parasites is strongly supported by the lower prevalence of malaria infection in mosquitoes that have been preimmune challenged with bacteria, the implication of the immune responsive nitricoxide synthase in *Plasmodium* killing and the correlation of immune

responses and parasite losses (Dimopoulos *et al*., 1997; 1998, 2000; 2002; Richman *et al*., 1997; Luckhart *et al*., 1998; Lowenberger *et al*., 1999). The immune responsive mosquito-specific antimicrobial peptide Gambicin has been shown to possess lethal activity against ookinete stage Plasmodia (Vizioli *et al*., 2001). Antiplasmodial activity against the oocyst and sporozoite stages of *Plasmodium* has also been shown *in vitro* for defensins from other insects (Shahabuddin *et al*., 1998).

Other insect–parasite models

Whereas most of our knowledge on the insect innate immune system and its interactions with parasites has derived from studies in *D. melanogaster* and *A. gambiae*, respectively, studies of other insect–pathogen models are revealing novel and complementary aspects on insects' immune system and resistance to human parasites.

For example, ookinete stage Plasmodia that have been injected into the *D. melanogaster* hemocoel will develop oocysts and produce infectious sporozoites that are rapidly cleared from the haemolymph. Parasite development was not compromised in mutants with constitutively active Toll receptors, nor was transcription of antimicrobial peptide genes induced in infected flies. Killing of sporozoites in the haemolymph appeared to involve haemocytes and other unknown components (Schneider and Shahabuddin, 2000). Drosophila immune responses and killing mechanisms of Plasmodia are likely to differ significantly from those taking place in the co-adapted mosquito vector. However, the fly with its powerful genetic and transgenic tools may provide a useful model to identify, study and engineer anti-Plasmodial proteins.

Studies in tsetse (*Glossina* spp.) vectors of African sleeping sickness and nagana have shown upregulation of several antimicrobial peptides upon infection with *Trypanosoma*. Interestingly, the tsetses' immune surveillance system appears to be capable of discriminating between parasites and bacteria, and between different parasite life stages. The implication of tsetse immune responses in parasite killing is suggested by the significantly lower *Trypanosoma* infection levels in flies that have been preimmune challenged with bacteria (Hao *et al*., 2001; Boulanger *et al*., 2002). A recently implemented gene discovery project have identified over 60 tsetse fly genes with potential implication in its immune system and will permit a more detailed dissection of tsetse responses to *Trypanosoma* infection (M. J. Lehane, personal communication).

Conclusions and discussion

The past decade has experienced a revolution in our knowledge on the insect innate immune system and novel aspects are continuously added. For instance, the apparent connections between immune cascades and the apoptotic machinery may prove to play a key role in the mosquitos' interactions with the malaria parasite (Christophides *et al*., 2002; Hoffmann and Reichhart, 2002). *Plasmodium* infection has been suggested to induce apoptosis of both invaded mosquito midgut cells and follicular epithelial cells (Han *et al*., 2000; Hopwood *et al*., 2001).

The available *A. gambiae* genome sequence in combination with high throughput gene expression analysis and transgenic technologies will allow a more comprehensive dissection of the mosquitos' responses to infection. These studies can be expanded and include different refractory mosquito strains, that are easily selected in the laboratory (H. Hurd, personal communication), and mosquitoes from the field that may have diverged significantly from the currently studied lab strains.

The accumulated knowledge on vector–parasite interactions will ultimately allow the development of disease control strategies based on transgenic refractory insects and other transmission blocking approaches where host antibodies can block the parasites' lifecycle in the mosquito (Collins, 1994; Stowers and Carter, 2001; Ito *et al*., 2002). The mosquitos' innate immune system could be utilized in various ways for the development of malaria control strategies based on genetically engineered mosquitoes or the spread of resistance genes in mosquito populations with mobile elements. In one scenario the mosquito could have a boosted anti-Plasmodial immune-surveillance system that would recognize the parasite as non-self more efficiently and/or stronger activate *Plasmodium* killing mechanisms. In another scenario, the engineered mosquito could express a blood meal inducible immune protein that would kill ookinetes in the midgut epithelium. The implementation of such control strategies will, in addition to a comprehensive dissection of the mosquitos' immune system, also require consideration of the transgenic mosquitos' fitness, the possible selection of resistant parasites to the killing mechanism and a detailed knowledge of the field mosquito populations that frequently are composed of several sympatric, but reproductively isolated, sibling species (Coetzee *et al*., 2000; Alphey *et al*., 2002; Enserink, 2002).

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