Immune response of *Anopheles gambiae* to the early sporogonic stages of the human malaria parasite *Plasmodium falciparum*

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Deciphering molecular interactions between the malaria parasite and its mosquito vector is an emerging area of research that will be greatly facilitated by the recent sequencing of the genomes of *Anopheles gambiae* mosquito and of various *Plasmodium* species. So far, most such studies have focused on *Plasmodium berghei*, a parasite species that infects rodents and is more amenable to studies. Here, we analysed the expression pattern of nine *An.gambiae* genes involved in immune surveillance during development of the human malaria parasite *P.falciparum* in mosquitoes fed on parasite-containing blood from patients in Cameroon. We found that *P.falciparum* infection triggers a midgut-associated, as well as a systemic, response in the mosquito, with three genes, NOS, defensin and GNB1, being regulated by ingestion of gametocytes, the infectious stage of the parasite. Surprisingly, we found a different pattern of expression of these genes in the *An.gambiae*–*P.berghei* model. Therefore, differences in mosquito reaction against various *Plasmodium* species may exist, which stresses the need to validate the main conclusions suggested by the *P.berghei*–*An.gambiae* model in the *P.falciparum*–*An.gambiae* system.

Keywords: Anopheles gambiae/immune response/interaction/Plasmodium berghei/Plasmodium falciparum

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**Introduction**

Malaria is a devastating disease in many tropical and subtropical regions that results in the death of ~2.7 million people each year. Malaria is essentially due to *Plasmodium falciparum*, one of the four malaria species that specifically infect humans. *Plasmodium falciparum* is exclusively transmitted by *Anopheles* mosquitoes, mainly from members of the *Anopheles gambiae* complex in Africa. This host–parasite combination is one of the most effective to maintain transmission of *P.falciparum* to human beings. Measures to control malaria have been hampered by the spread of malaria parasites resistant to anti-malarial drugs and of insecticide-resistant mosquitoes. To combat malaria, a better understanding of the biology of both parasite and mosquito, as well as of the interactions between *P.falciparum* and *Anopheles* vectors, is needed. The recent sequencing of the genomes of *An.gambiae* mosquito (Holt et al., 2002) and of various *Plasmodium* species will be very helpful towards this aim (Carlton et al., 2002; Gardner et al., 2002).

When ingested by a female mosquito during a blood meal, *Plasmodium* gametocytes differentiate into male and female gametes, which fuse to form zygotes. Each zygote elongates to become a motile ookinete in the blood bolus and crosses the peritrophic matrix and the midgut epithelium to reach the haemocoel gut side. The ookinete becomes attached to the midgut wall and transforms into an oocyst, which undergoes divisions to form sporozoites. Sporozoites are released into the haemolymph and invade salivary glands, where they attain maturity and can be injected into a new host during the next blood meal.

During these crucial sporogonic developmental steps in the mosquito midgut lumen, the midgut epithelium and the haemolymph, parasites face a hostile environment, leading to a considerable reduction in the number of parasites reaching the oocyst stage (Vaughan et al., 1994; Beier, 1998; Gouagna et al., 1998). The exact biological processes implicated in the mosquito response to the presence of *Plasmodium* parasites are still largely unknown. Recent studies using the rodent malaria parasite *P.berghei* have provided evidence that several mosquito genes presumably involved in immune surveillance are activated transcriptionally in infected mosquitoes (Dimopoulos et al., 1997, 1998; Richman et al., 1997; Oduol et al., 2000). In addition, nitric oxide synthase (NOS) was found to play an important role in controlling the rate and intensity of infection by *Plasmodium* parasites of *An.stephensi* (Luckhart et al., 1998).

*Anopheles gambiae* and *An.stephensi* support the development of *P.berghei* in the laboratory, but these host–parasite systems never occur in nature. The laboratory conditions used for *P.berghei* development inside mosquitoes involve large numbers of gametocytes and usually lead to the formation of more than fifty oocysts on the mosquito midgut wall. In contrast, in the field, only a handful of oocysts can be seen in the midgut of *Anopheles* infected by *P.falciparum* (Pringle, 1966; Collins et al., 1984). In this latter case, even if a mosquito ingests a large number of gametocytes, only a few will ultimately develop to the oocyst stage (Sinden and Billingsley, 2001). On the other hand, as few as 10 gametocytes per microlitre of ingested blood can be sufficient for establishing infection in *An.gambiae* (Gouagna et al., 1998; Bonnet et al., 2001). These observations suggest that *P.falciparum* development depends on a fine balance between the ability of the mosquito to build a strong defence response to the
Table I. *Anopheles gambiae* infections by *P. falciparum*

<table>
<thead>
<tr>
<th>Infection</th>
<th>Number of parasites/μl</th>
<th>Gametocytes</th>
<th>Asexual stages</th>
<th>Ratio of infected mosquitoes</th>
<th>Mean intensity of infectionsª</th>
<th>Mosquito infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>KOU</td>
<td>2325</td>
<td>0</td>
<td>105</td>
<td>10/10 9/10 10/10 30/60 (50%)</td>
<td>7.36</td>
<td>14 h*</td>
</tr>
<tr>
<td>ES64</td>
<td>62</td>
<td>106</td>
<td></td>
<td>10/10 10/10 8/15 22/30 (73%)</td>
<td>12.36</td>
<td>24 h*</td>
</tr>
<tr>
<td>AKU</td>
<td>19</td>
<td>191</td>
<td></td>
<td>10/10 10/10 6/15 24/30 (80%)</td>
<td>7</td>
<td>48 h*</td>
</tr>
</tbody>
</table>

ªNumber of *Pfs25* mRNA positive mosquitoes at 14, 24 and 48 h PBM.
ªNumber and percentage (in parentheses) of infected mosquitoes harboring oocysts at day 7 PBM.
ªMean number of oocysts per positive midgut observed at 7 days.

![Graph](image.png)

**Fig. 1.** Quantitative expression of *An. gambiae* immune-related genes by real-time PCR. mRNA was extracted from a pool of naive or *E. coli*-wounded larvae and adult mosquitoes, reversed transcribed and amplified by real-time PCR. The ribosomal protein S7 mRNA was used to normalize the data expressed as the relative expression level in *E. coli*-wounded mosquitoes (black) to the naive ones (grey). Bars indicate standard deviation from three PCR experiments.

presence of the parasite and that of the parasite to evade the immune response of its host.

Here, we analysed the expression of nine *An. gambiae* genes (*GNBP, Dimopoulos et al., 1997; ICHIT, Dimopoulos et al., 1998; IGALE20, Dimopoulos et al., 1996; ISLP5, Dimopoulos et al., 1997; Sp22D, Gorman et al., 2000; SpiA, Danielli, DDBJ/EMBL/Genebank accession No. AJ271352; AgIMcr14, Odoul et al., 2000; defensin, Richman et al., 1996; and NOS, Dimopoulos et al., 1998) involved in several steps of insect immunity pathways during the sporogonic development of *P. falciparum* in mosquitoes fed on blood of gametocyte carriers. Gene expression was quantified by real-time PCR. We also analysed the expression of several of these genes in *P. berthei*-infected mosquitoes, under the same experimental methodology. Our results indicate that *An. gambiae* immune reactions to *P. falciparum* and *P. berthei* infection are distinct.

**Results**

**Mosquito infection**

To investigate the interaction between *P. falciparum* and *An. gambiae* under natural conditions of transmission, mosquitoes were fed with the blood of three gametocyte carrier volunteers harbouring different parasite loads. *Pfs25*, a well-characterized *Plasmodium* gene that is expressed during the sporogonic development from gametocyte to ookinete stage (Thompson and Sinden, 1994) was used to identify individual midguts containing these stages of *Plasmodium* parasites. The number of *Pfs25*-positive midguts at 14, 24 and 48 h post-blood meal (PBM), the proportion of mosquitoes harbouring oocysts, and the mean intensity of infection observed on day 7 PBM are shown in Table I. RT–PCR amplification of *Pfs25* mRNA revealed that all but one mosquito midguts contained sporogonic stages of *Plasmodium* parasites, presumably zygotes and ookinetes at 14 and 24 h PBM, respectively. At 48 h PBM, the proportion of *Pfs25*-positive midguts varied from 40 to 100%, depending on the initial gametocyte load in the ingested blood. The decrease in the number of mosquitoes with *Pfs25* mRNA between 14 and 48 h PBM in ES64 and AKU infection could be due to the reduction in parasite numbers during sporogonic development (Beier, 1998; Gouagna et al., 1998). Alternatively, it could also be due to the difficulty in detecting *Pfs25* mRNA in late ookinetes or young oocysts by RT–PCR. This was probably the case for the AKU infection, as the proportion of infected mosquitoes on day 7 PBM was higher than that of *Pfs25*-positive mosquitoes at 48 h. On day 7 PBM, 50–80% of mosquitoes had oocysts on their midgut wall. It is noticeable that the high number of gametocytes taken up during KOU infection did not produce high number of oocysts. A similar plateau effect on the oocyst load was also observed with *in vitro*-produced *P. falciparum* gametocytes (Ponnudurai et al., 1987, 1989).

**Quantification of immune-related gene expression by real-time PCR**

Our first objective was to investigate the insect immune response within the *An. gambiae* midgut during early sporogonic development of *P. falciparum* under seminatural conditions of transmission. For this, we selected a set of genes involved in different pathways of insect innate immunity (Hoffmann et al., 1996, 1999). Three genes (*GNBP, IGALE20 and ICHIT*) belong to the ‘Pattern Recognition Receptor’ (PRR) gene family (Janeway, 1989). Two genes (*ISLP5 and Sp22D*) encode serine proteases presumably involved in activation cascades, although *Sp22D* may also play a role as a PRR. Two other genes (*SpiA and AgIMcr14*) encode serine protease inhibitors, while the two remaining genes encode an
Expression of selected *An.gambiae* immune-related genes during early sporogonic development of *P.falciparum*

We then analysed expression of the nine selected genes after mosquito ingestion of *P.falciparum* gametocytes (Figures 2–4). Gene expression was monitored at 14, 24 and 48 h PBM, corresponding to the transformation of zygotes into ookinetes, to the interaction of ookinetes with the peritrophic matrix and midgut cells, and to the migration and early differentiation of ookinetes into oocysts, respectively. At each time point, gene expression was assessed on RNA pools from Pfs25-positive midguts. For analysis of gene expression in mosquito carcasses, we restricted our analysis to pools of the cognate carcasses from KOU infection. As this infection displayed the highest load of gametocytes and no asexual stages, we anticipated that KOU samples would provide a clear-cut expression profile. In some instances, RNAs pools of carcasses from ES64 and AKU infections were used to confirm gametocyte-specific regulation of gene expression. As controls, similar pools were made from RNAs of 10 midguts and 10 carcasses of mosquitoes fed on non-infected blood or on blood containing asexual stages of *P.falciparum* exclusively, which do not develop in mosquitoes (see Materials and methods).

**PRR gene expression in An.gambiae**

As shown in Figure 2, the expression of the three PRR genes was higher in midguts of mosquitoes fed on blood containing gametocytes (KOU, ES64 and AKU infections) or non-infective asexual stages (P.f1 and P.f2) than in control mosquitoes fed on non-infected blood (C). *ICHIT* and *IGALE20* showed a time-specific expression upon parasite (gametocyte and asexual stage) presence in the blood meal. *ICHIT* was overexpressed at 14 and 24 h PBM, except in AKU infection, with induction rates ranging from 6- to 24-fold. There was a gradual decrease in expression over time after parasite ingestion in midguts, whereas an opposite trend was observed in carcasses: gradual increase of expression from 14 to 48 h PBM. The expression of *IGALE 20* was stimulated strongly 24 h PBM (induction rates 5–62-fold) in both midguts and carcasses after ingestion of gametocytes or asexual parasites. *GNBP* showed the most variable expression pattern in mosquito midguts. Its expression was stimulated in only two infections (AKU and P.f1) at 14 h PBM, and in all infections at 48 h PBM, with induction varying between 3- and 37-fold. At 24 h PBM, *GNBP* expression was either not stimulated (ES64, AKU and P.f2) or slightly stimulated (KOU and P.f1). In contrast, in carcasses, this gene was overexpressed strongly in KOU infection (Figure 2), as well as in ES64 and AKU infections (data not shown), and *GNBP* overexpression in carcasses occurred at 24 h PBM only. This result supports the conclusion that ingestion of *P.falciparum* gametocytes triggers a specific control of *GNBP* expression in *An.gambiae* carcasses.

**Expression patterns of serine protease genes**

The Sp22D gene, which can be classified in both the PRR and protease gene families (Gorman *et al.*, 2000), was overexpressed at 24 h PBM in midguts corresponding to two infections with gametocytes (Figure 3). However,
induction of Sp22D gene expression did not seem to be gametocyte specific, as increased gene expression was also detected in midguts from mosquitoes fed on asexual stages. In carcasses, increased Sp22D gene expression was also noticed at 24 h, but remained moderate compared with midgut expression.

Expression of ISPL5 was only slightly modified in midguts of infected mosquitoes. The most noticeable effect was a 14 h-induced ISPL5 expression in mosquitoes fed on blood that did not contain gametocytes, but high numbers of asexual parasites. In carcasses, ingestion of gametocytes or asexual blood stages induced ISPL5 expression at 24 h PBM.

**Expression patterns of protease inhibitor genes**

No major difference was observed in the expression of Spi1A in mosquito midguts in various infection experiments (Figure 3). Nonetheless, a slight induction was observed in two gametocyte-containing infections at 14 h PBM and in the two asexual stage-containing infections at 24 h PBM. In contrast, the expression of this gene was upregulated at 24 and 48 h PBM in carcasses of mosquitoes fed on a large number of gametocytes or asexual parasites (KOU and P.f1 infections, respectively). This result illustrates that the presence of P.falciparum parasites in the blood meal does not lead to midgut overexpression of all immune-related genes.

AgIMcr14 showed an interesting pattern of expression. In midguts of mosquitoes fed on gametocyte- or asexual stage-containing blood, a very high level of induction was observed at 14 h PBM. In addition, although AgIMcr14 expression decreased over time, the level of expression was still 100-fold higher than in control mosquitoes at 24 or 48 h PBM. In carcasses, although AgIMcr14 expression was higher in the two analysed infections than in the control, the expression level was considerably lower than in midguts. The strong expression of AgIMcr14 in response to ingested parasites, in both midguts and carcasses, supports the conclusion that this molecule possibly plays a critical role in mosquito immunity.

**Defensin and NOS expression**

Midgut expression of defensin displayed a 26-fold induction at 14 h PBM in KOU infection, which contained a high load of gametocytes, whereas its expression was not induced in the two other gametocyte-containing infections, nor in the two asexual stage-containing infections (P.f1 and P.f2) (Figure 4). Interestingly, induction of defensin was observed in carcasses of mosquitoes from KOU, Es64 and AKU infections (5- to 15-fold induction, Figure 4; data not shown) at 24 h PBM, whereas asexual stages triggered a 2-fold induction only. This result strongly suggests that expression of defensin in *An.gambiae* carcasses is specifically regulated by the presence of gametocytes in the ingested blood.

The level of NOS midgut expression was equivalent to the control or slightly lower in all infections, except in one gametocyte infection (ES64) at 24 h PBM and in P.f1 infection at 14 h PBM (Figure 4). In this latter infection, a 50-fold induction of NOS was observed. However, in carcasses, NOS expression was increased at 24 h PBM, 15 or 75-fold in KOU and P.f1 infections, respectively. Absence, or low level, of NOS expression in midguts of mosquitoes fed on gametocyte-containing blood may reveal that early sporogonic stages of *P.falciparum* are able to repress NOS expression.

**Specificity of the immune response**

As mentioned earlier, *An.gambiae* is a natural host for *P.falciparum*, but not for rodent malaria parasites such as *P.berghei*. It can be anticipated that host–parasite interaction can vary depending on the species involved (Yoeli, 1973; Templeton et al., 1998; Lecuit et al., 2001; Bonas...
and Lahaye, 2002). We thus tested whether the mosquito immune surveillance system was stimulated differently by *P. berghei* and *P. falciparum*, using real-time PCR to measure gene expression. It should be emphasized that in humans, gametocyte carriers harbour a moderate level of, and sometimes no detectable, asexual stages in peripheral blood circulation and that individuals having symptomatic malaria outburst usually carry a high density of asexual stages without gametocytes, whereas rodents infected with *P. berghei* harbour gametocytes and a high number of asexual stages. In addition, *P. falciparum* infections under field conditions of transmission lead to the formation of few oocysts per mosquito midgut, whereas *P. berghei* laboratory infections usually lead to the formation of at least 50 oocysts per midgut. To limit the number of parameters that vary between the two parasite systems as much as possible, we analysed *P. berghei* infections that led to a mean intensity of midgut infection similar to the *P. falciparum* situation that we analysed above. Furthermore, to distinguish the effects associated with *P. berghei* gametocytes from those associated with *P. berghei* asexual stages, we analysed the expression pattern of some of the previously studied genes in mosquitoes fed on mice infected with a *P. berghei* gametocyte-producing strain or a non-gametocyte-producing one. We restricted our analysis to genes (*NOS*, *defensin*, *GNBP*, *IGALE20* and *AgIMcr14*) and a time point (24 h PBM) that showed the most significant regulation in *P. falciparum*-infected mosquitoes.

Results presented in Figure 5A and B indicate that *IGALE20*, *AgIMcr14* and *defensin* are overexpressed in the midgut of mosquitoes infected with the gametocyte-producing clone as compared with control mosquitoes and mosquitoes infected with the non-gametocyte-producing clone. In contrast, midgut *GNBP* expression remained at similar levels in mosquitoes infected with either parasite clones or in controls. Finally, *NOS* expression was increased in all infections, but overexpression did not appear to be gametocyte-specific (Figure 5C). In mosquito carcasses (Figure 5D), even if *NOS* was overexpressed in *P. berghei*-fed mosquitoes as compared with control mosquitoes, the level of expression was considerably lower than in cognate midguts. Carcass expression of *defensin* and *IGALE20* was also higher in *P. berghei*-fed mosquitoes than in control mosquitoes; this induction was not dependent on the presence of gametocytes in the blood meal. Expression of *GNBP* and *AgIMcr14* remained almost unchanged.

Most of these results are in agreement with published results obtained with semi-quantitative analysis of the *P. berghei–An. gambiae* system (Richman et al., 1997; Dimopoulos et al., 1998). However, we did not observe the previously reported gametocyte-specific expression of *GNBP* (Richman et al., 1997). Another discrepancy concerns the level of *NOS* expression in infected midguts, which was reported lower than expression in carcasses (Dimopoulos et al., 1998). By using the two *P. berghei* strains, our data provided two novel observations: non-gametocyte-specific expression of *NOS* and gametocyte-specific induction of *IGALE20* in midguts of *P. berghei*-infected *An. gambiae*. When comparing these results with those obtained with *P. falciparum*-infected mosquitoes, all the studied genes, with the exception of midgut expression of *GNBP*, were regulated differently in the two systems.

**Discussion**

During development in mosquitoes, *P. falciparum* parasites suffer major population losses at two developmental stages: when gametocytes transform into migrating ookinetes, and when sporozoites invade salivary glands. Presumably, mosquito innate immunity contributes to these losses. Evidence has been provided that the *An. gambiae* immune response is indeed stimulated at different stages of *P. berghei* development, particularly after ingestion of gametocytes (Dimopoulos et al., 2001; and references herein). However, *P. berghei* is not naturally transmitted by *An. gambiae*, and the number of developing *P. berghei* ookinetes in experimental infections exceeds the number of *P. falciparum* ookinetes under natural transmission conditions by far. Therefore, we asked here whether a similar immune response is triggered in *An. gambiae* upon infection with *P. falciparum* or *P. berghei*, under the same experimental design. The results described in this report support several important conclusions.

The first conclusion is that most genes whose expression is regulated by the presence of the *P. falciparum* parasite in the blood meal are not specifically regulated by the presence of gametocytes. The presence of asexual stages in two of the studied gametocyte infections may be the source of this confounding effect for some genes (i.e. *GNBP* midgut expression). Alternatively, parasite molecules shared by gametocytes and asexual stages, rendered accessible to the immune surveillance system as a consequence of the digestion process stimulated by blood feeding, could trigger these responses. In a few instances, however, gene expression was modified specifically after gametocyte ingestion: *NOS* was repressed in midguts, and *GNBP* and *defensin* were induced in carcasses (which contain the fat body cells that behave...
as an important source of immune response in insects; Boman, 1995). Results obtained from the KOU infection, which harboured gametocytes only, clearly suggest that *P. falciparum* gametocytes specifically upregulate GNB1 and defensin expression in mosquito carcasses, but not in midgut midguts, at 24 h PBM. The timing of expression corresponds to the invasion of the mosquito midgut by ookinetes. As discussed by Richman and collaborators (Richman et al., 1997), it is not known whether such an effect (in the carcasses) results from receptor-mediated immune recognition of developing parasites or from midgut injury (and possibly induced by bacteria contained in the blood bolus) associated with ookinete invasion. In *Glossina* flies, establishment of *Trypanosoma* parasites triggers a sustained defensin expression in the fat body over several days (Hao, 2001). The fact that in our system, defensin and GNB1 expression was tightly upregulated at 24 h PBM and not expressed later on suggests that, once established in the mosquito midgut epithelium, *P. falciparum* ookinetes might be able to repress defensin, and possibly GNB1, expression. Alternatively, this tight regulation might be solely due to bacterial or ooinkete components accessible to the immune system in a narrow window. It is intriguing that defensin was overexpressed in midguts of mosquitoes fed on KOU infection early in the infection process (14 h PBM). As expression of this gene was similar to the control in all other infections, it may be hypothesized that this overexpression is controlled by the large number of ingested gametocytes, which leads to the loss in parasites reaching the oocyst stage (Table I). It is also worth noting that not all genes were regulated by parasite ingestion in our system. Parasite presence had almost no effect on Spi1A midgut expression, and ingestion of gametocytes did not induce or even repress midgut expression of NOS. Spi1A was reported not to be regulated by bacterial infection in *An. gambiae* cell culture (Dimopoulos et al., 2000), whereas NOS was found to be upregulated in *P. berghei*-infected *Anopheles* mosquitoes (Dimopoulos et al., 1998; Luckhart et al., 1998).

The second observation concerns the spatio-temporal regulation of the studied immune-related genes following ingestion of *P. falciparum*. Some genes were expressed at the same time in both midguts and carcasses (IGALE20, Sp22D and AgIMcr14), others were expressed later in carcasses rather than in midguts (ICHIT and defensin). In addition, NOS, whose midgut expression was virtually unchanged after gametocyte ingestion, was clearly induced in carcasses by both asexual stages and gametocytes. A similar observation can be made for Spi1A after ingestion of gametocytes and asexual stages. Since tissue components of the carcasses were never directly in contact with parasites at any time point studied, these results would indicate that signals arising from the parasite midgut interaction reached these tissues, which might then behave as a second line of defence. As mentioned earlier, the nature of the signals could be molecules common to both gametocytes and asexual stages, released from the parasites through the action of the mosquito digestive enzymes or ookinete-specific molecules during midgut penetration. It might also come from a midgut injury response possibly involving midgut bacteria moving along with the migrating ookinetes. Of particular interest are the differential expression patterns of AgIMcr14 and ICHIT. Midgut expression of these two genes gradually decreased from 14 to 48 h PBM. Moreover, carcass expression of ICHIT gradually increased during that same time period. Such a regulation involving an early induction in the midgut and transferred (transposed) and sustained response in the carcasses suggests that this pathway may contribute efficiently to pathogen clearance. ICHIT encodes a protein with chitin-binding and mucin domains that are found also in several molecules involved in defence mechanisms (Dimopoulos et al., 1998), and could be involved in opsonization of microorganisms. Such a function may similarly be associated with AgIMcr14. This gene has been recently characterized as the first insect member of the mammalian α2-macroglobulin and C3 complement family and was shown to be specifically activated by *P. berghei* infection rather than by bacterial infection in whole mosquitoes (Oduol et al., 2000). We show here its expression pattern in both midguts and carcasses. In midguts of *P. falciparum*-infected mosquitoes, AgIMcr14 was strongly upregulated after ingestion of both gametocytes and asexual stages. Our data indicate that this immune pathway, which has recently been documented in an established *An. gambiae* cell line and in haemocytes (Levashina et al., 2001), is also present in the mosquito midgut, clearly confirming the status of the midgut as an immune organ.

A third observation was made by comparing the regulation of immune-related genes in the natural *An. gambiae*–*P. falciparum* system and in the model *P. berghei*–*An. gambiae* system. Regulation of gene expression was compared at 24 h PBM, a time that corresponds to the initial interaction of ookinetes with the mosquito midgut. A global analysis of our results obtained with the two host–parasite systems showed that the *An. gambiae* immune surveillance behaves differently in the presence of *P. falciparum* or *P. berghei*. First, whereas no gene was specifically regulated by gametocyte in midguts of *P. falciparum*-infected mosquitoes, three genes (IGALE20, AgIMcr14 and defensin) were upregulated upon ingestion of *P. berghei* gametocytes. Second, expression of defensin and GNB1 was increased upon ingestion of gametocytes in carcasses of *P. falciparum*-infected mosquitoes, but not in carcasses of *P. berghei*-infected mosquitoes. Third, *P. berghei* gametocytes and asexual stages triggered NOS expression midguts, whereas *P. falciparum* gametocytes and asexual stages did not. On the contrary, both gametocytes and asexual stages of *P. falciparum* upregulated NOS expression in mosquito carcasses, while *P. berghei* gametocytes and asexual stages did not. Lastly, the level of AgIMcr14 expression differed considerably between the two systems.

As we used infection conditions that were as similar as possible in the two parasite systems (see Results and Materials and methods), it is likely that the above differences are mainly due to the two different species involved. For example, the difference in the infection pathway by ookinetes of the two species may account in part for the differences in mosquito immune reaction. Indeed, the mode of ookinete migration across the midgut epithelium appears to be different in the two species, either via an intracellular (*P. berghei*) or an intercellular (*P. falciparum*) mode (Meis et al., 1989). Previous work
confirmed the intracellular route followed by *P. bergheri* ookinetes (Han et al., 2000).

In conclusion, our work provides the first description of the immune response of *An. gambiae* against the human malaria parasite *P. falciparum*, during the interaction of ookinetes with the mosquito midgut barrier. The study of the immune response at different time points during this infection process emphasizes that mosquitoes are able to mount a finely tuned response against the parasite. Furthermore, our analysis shows that *An. gambiae* immune response to the presence of *P. falciparum* is different to that of *P. bergheri*. Although *P. bergheri* is more amenable to manipulation than *P. falciparum*, *P. falciparum* is the major human malaria pathogen. The differences reported here emphasize the need to validate the main conclusions suggested by the *P. bergheri–An. gambiae* model in the *P. falciparum–An. gambiae* system, especially if one wants to exploit the mosquito immune system to produce mosquitoes refractory to the development of the human malaria parasite.

**Materials and methods**

**Gametocyte carriers**
Asymptomatic Cameroonian schoolchildren aged <10 years old were mass-screened to detect parasite carriers in four villages located ~100 km east of Yaoundé, the capital city of Cameroon, during the rainy season in May–June 2000. Thick blood smears from finger-pricked samples were stained with 10% Giemsa, and the number of *P. falciparum* gametocytes was determined by microscopy observation. Children with at least 10 gametocytes/μl of blood who had not taken any anti-malarial treatment within the previous two weeks were enrolled in our study. Children with lower gametocytaemia or mixed infections with *P. ovale* and/or *P. malariae* were excluded. Children with >1000 parasites/μl and symptoms associated with malaria were treated with sulfadoxine–pyrimethamine. All participants were volunteers and their parents’ consent was obtained. The study was approved by the Cameroonian National Ethics Committee.

**Mosquitoes and *P. falciparum* infection**
*Anopheles gambiae* (Yaoundé strain, M cytotype) was reared under standard conditions, in the insectarium set up at OCEAC, Yaoundé. This strain originated from mosquitoes collected in Yaoundé city and was maintained under laboratory conditions for several generations (Tchuinkam et al., 1993). Venous blood (10 ml) from gametocyte carrier volunteers was collected in a heparin-coated tube and immediately used for infecting mosquitoes. For each experiment, batches of 50 nulliparous females (5 days old) starved of sugar 24 h prior to blood feeding were fed on patient’s blood for 20 min, using the artificial membrane feeding technique (Tchuinkam et al., 1993). Fully engorged females were maintained in the insectarium until dissection. Three independent infections, referred to as AKU, ES64 and KOU, were performed by feeding mosquitoes on blood containing 19, 62 and 2325 gametocytes/μl, respectively. As gametocyte carriers usually harbour asexual parasites also, a set of experiments was performed with blood containing asexual parasites, without gametocytes, with parasitaemia of 20 000 parasites/μl or 200 parasites/μl. The latter sample was obtained by diluting the former in non-infected blood so that the asexual parasite density corresponds to that found in gametocyte carriers. A series of mosquitoes were also fed on an infected blood. Mosquitoes were dissected in cold phosphate-buffered saline (PBS pH 7.2), at 14, 24 and 48 h PBVM. Thus, 180 midguts and corresponding carcasses (remaining tissues) were individually isolated and conserved in RNA Later® (Ambion) for further RNA isolation. For each feeding on infected blood, oocyst detection was performed on day 7 PBVM on the remaining mosquitoes (n > 30).

**Plasmodium bergheri infection**
The same *An. gambiae* Yaoundé strain was reared at the Pasteur Institute and fed on *P. bergheri* infected mice (Swiss). Two infections were performed with the ANKA gametocyte-producing strain 2.34 (parasitemia: 7.25 and 5.9%) and two with the ANKA gametocyte-defective strain 2.33 (parasitemia: 4.1 and 5%). A series of mosquitoes was fed on an uninfected mouse. Mosquito midguts and carcasses were isolated at 24 h PBVM as described above, and their RNA content isolated for real-time RT–PCR analysis. Mosquito infection was scored on day 11 PBVM. Prevalence of infection and mean number of oocysts per positive midgut were 51.7% and 10.5, and 68.7% and 7.3, respectively, for *P. bergheri* 2.34. No oocyst was detected on midguts from mosquitoes fed on *P. bergheri* 2.33, as expected.

**Bacterial infection**
Larvae and adult females (*An. gambiae*, G3 strain) were wounded with a needle dipped into a concentrated *E.coli* suspension. Larvae and adults surviving after 24 h were used for RNA extraction.

**RNA extraction and reverse transcription**
Total RNAs from individual midguts and carcasses was extracted with Tri-Reagent™ following the manufacturer’s instructions. After DNase I treatment (Amibo DNA-free), the pellet was suspended in a final volume of 20 μl of Tris–EDTA buffer. RNA (2 μl) was transcribed using a random hexamer mixture and MMLV reverse transcriptase (Gibco-BRL), in a final volume of 40 μl. The absence of contaminating genomic DNA was checked in each RNA sample by specific amplification of the actin gene (Salazar et al., 1994).

**Detection of Pf25 transcripts in infected mosquitoes**
In order to identify infected mosquitoes harbouring sporogenic stages of *P. falciparum*, the presence of Pf25 transcripts in RNA sample from each mosquito midgut was assessed by RT–PCR. After reverse transcription, 5 μl of cDNAs were subjected to a series of nested PCR using the following primers: PCR1, Pf25–1U (5’-ATGCGGAATTTACGCTGGAT-3’) and Pf25–1L (5’-CAAGCCTATGAAGACGGATT-3’); and PCR2. Pf25–2U (5’-ATAATGCCAAGTTACCCG-3’) and Pf25–2L (5’-CACGGTTTACTTTTTTCTT-3’). cDNAs of Pf25-positive midguts corresponds to one infection and to one time point after infection were pooled. The cDNAs of the cognate carcasses were pooled as well.

**Quantitative real-time PCR analysis**
Real-time PCR was performed using the dsDNA dye SyberGreen (MasterMix Perkin Elmer) and the iCycler from Bio-Rad. Primers were designed using the Oligo 5 software (NIBI) in such a way that the amplification products fall in the 100 bp size range and that primers do not form unwanted dimers. PCR was performed in triplicate in a 25 μl final volume containing 900 nM of each forward and reverse primers, and 5 μl of a 1/5 dilution of the RT products. Signals were normalized to the ribosomal protein S7 mRNA. Normalized data were used to quantitate relative levels of a given mRNA in uninfected and infected samples according to the ΔΔCt analysis (Hoopers et al., 2001). Before proceeding to the relative quantification using this method, similarity in amplification efficiency of target and reference was verified (User Bulletin 2, ABI). The following primers were used for amplification of the 37mRNA and quantification of the expression of nine immune-related genes: S7-U, 5’-CACCGCCTTGATACGACATCCA-3’, S7-L, 5’-ATGTTGCTGTCGGTTTCT-3’, GNB-U, 5’-CAGGACCAAGGTTACCCGAAAG-3’, GNB-L, 5’-ATCCTGCCGTTGATGCTTA-3’, IGLE20-U, 5’-TTAACCGGACGATTGATGTG-3’, IGLE20-L, 5’-GGACGACACCGGCGCACAC-3’, ICHT-U, 5’-GGACGTTGGCACTTATTTT-3’, ICHT-L, 5’-CCCTACGTGCCATTCGTAAGAC-3’, ISPL-U, 5’-GGCTACCTCGGCTTGGTTCT-3’, ISPL-L, 5’-GGTTGCTTGTTCGTACTCTGC-3’, Spi1A-U, 5’-CGTTGAACTGCGGGCAAAAC-3’, Spi1A-L, 5’-GCTGCGCCCGACCTTC-3’, AgMc14-U, 5’-ATATTTGCTGCTGCTGAC-3’, AgMc14-L, 5’-TACCCGATTCGACAGGT-3’, Sp22D-U, 5’-TGTGATCCACGCTTCTTCTAC-3’, Sp22D-L, 5’-GGCTTGGCCGATGGTTCTTCT-3’, IGLE15-U, 5’-TGTGATCCACGCTTCTTCT-3’, IGLE15-L, 5’-GGCCGATTCGACTTCATCT-3’, IGLE20-U, 5’-TTACCGGACGATTGATGTG-3’, IGLE20-L, 5’-GGACGACACCGGCGCACAC-3’, ICHT-U, 5’-GGACGTTGGCACTTATTTT-3’, ICHT-L, 5’-CCCTACGTGCCATTCGTAAGAC-3’, ISPL-U, 5’-GGCTACCTCGGCTTGGTTCT-3’, ISPL-L, 5’-GGTTGCTTGTTCGTACTCTGC-3’, Spi1A-U, 5’-CGTTGAACTGCGGGCAAAAC-3’, Spi1A-L, 5’-GCTGCGCCCGACCTTC-3’, AgMc14-U, 5’-ATATTTGCTGCTGCTGAC-3’, AgMc14-L, 5’-TACCCGATTCGACAGGT-3’, Sp22D-U, 5’-TGTGATCCACGCTTCTTCTAC-3’, Sp22D-L, 5’-GGCTTGGCCGATGGTTCTTCT-3’.

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