EMBO
reports

scientific report

Anopheles and *Plasmodium*: from laboratory models to natural systems in the field

Anna Cohuet[1+], Mike A. Osta[2], Isabelle Morlais[3], Parfait H. Awono-Ambene[3], Kristin Michel[2], Frederic Simard[3], George K. Christophides[2], Didier Fontenille[1] & Fotis C. Kafatos[2++]

[1]Institut de Recherche pour le Développement—Laboratoire de Lutte contre les Insectes Nuisibles, UR 016, Montpellier, France, [2]Division of Cell and Molecular Biology, Imperial College London, South Kensington Campus, London, UK, and [3]Organisation de Coordination de la lutte contre les Endémies en Afrique Centrale, Laboratoire de Recherche sur le Paludisme, Yaoundé, Cameroon

Parasites that cause malaria must complete a complex life cycle in *Anopheles* vector mosquitoes in order to be transmitted from human to human. Previous gene-silencing studies have shown the influence of mosquito immunity in controlling the development of *Plasmodium*. Thus, parasite survival to the oocyst stage increased when the parasite antagonist gene *LRIM1* (leucine-rich repeat immune protein 1) of the mosquito was silenced, but decreased when the C-type lectin agonist gene *CTL4* or *CTLMA2* (*CTL mannose binding 2*) was silenced. However, such effects were shown for infections of the human mosquito vector *Anopheles gambiae* with the rodent parasite *Plasmodium berghei*. Here, we report the first results of *A. gambiae* gene silencing on infection by sympatric field isolates of the principal human pathogen *P. falciparum*. In contrast with the results obtained with the rodent parasite, silencing of the same three genes had no effect on human parasite development. These results highlight the importance of following up discoveries in laboratory model systems with studies on natural parasite–mosquito interactions.

Keywords: *Anopheles*; immunity; model; natural conditions; *Plasmodium*

EMBO reports (2006) **7**, 1285–1289. doi:10.1038/sj.embor.7400831

INTRODUCTION

Despite efforts in control and prevention, malaria remains one of the most devastating infectious diseases. The situation continues to worsen because of deteriorating socio-economic conditions and the increased resistance of parasites and vectors against the available antimalarial drugs and insecticides, respectively (Greenwood & Mutabingwa, 2002; Castro & Millet, 2005). The global malaria burden is mainly due to infections by *Plasmodium falciparum*—the most important and often deadly human malerial parasite. In much of Africa, where the malaria epidemic is most serious, most infections are transmitted by *Anopheles gambiae*. New approaches to control malaria include interrupting the transmission cycle in the mosquito; however, they require a better understanding of the specific interactions between human *Plasmodium* parasites and their natural vectors.

In life science research, model organisms greatly facilitate the identification of fundamental mechanisms that can be tested later in less tractable systems. For example, the key developmental mechanisms that were discovered in *Drosophila melanogaster* were later shown to be pertinent, even if not invariant, to most metazoa. The mouse model has been instrumental in biomedical research, even though its distinct physiology requires that discoveries in the mouse be validated in primates or humans before potential application to human medicine. Similarly, for complex diseases such as malaria in which three organisms—the human host, the *Plasmodium* parasite and the *Anopheles* vector—are implicated, model organisms greatly facilitate discovery. In recent years, the development of genomic and functional genomic approaches to malarial research has favoured the use of the principal human vector *A. gambiae*, although in combination with tractable non-human hosts and parasites. Such studies have explained important mechanisms of parasite killing in vectors. Testing their relevance to the human malarial system is now the primary goal.

Plasmodium parasites need to overcome several bottlenecks in the vector that result in most wild mosquitoes bearing few, if any, oocysts—that is, showing low infection prevalence and low parasite load (Vaughan *et al*, 1992; Gouagna *et al*, 1998; Sinden, 1999). Studies on the immune system of *A. gambiae* have shown that a fine balance exists between mosquito factors that affect ookinete survival to the oocyst stage, positively (agonists) or negatively (antagonists; Blandin *et al*, 2004; Osta *et al*, 2004).

[1]Institut de Recherche pour le Développement—Laboratoire de Lutte contre les Insectes Nuisibles, UR 016, BP 64501, 911 Avenue Agropolis, 34394 Montpellier cedex 5, France
[2]Division of Cell and Molecular Biology, Imperial College London, Sir Alexander Fleming Building, South Kensington Campus, London SW7 2AZ, UK
[3]Organisation de Coordination de la lutte contre les Endémies en Afrique Centrale, Laboratoire de Recherche sur le Paludisme, Yaounde BP 288, Cameroon
[+]Corresponding author. Tel: +33 4 67 41 61 55; Fax: +33 67 54 20 44; E-mail: cohuet@mpl.ird.fr
[++]Corresponding author. Tel: +44 20 7594 1267; Fax: +44 20 7594 2056; E-mail: f.kafatos@imperial.ac.uk

Received 13 April 2006; revised 4 September 2006; accepted 4 September 2006; published online 10 November 2006

©2006 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Such factors, and the molecular mechanisms they serve, might prove to be favourable targets for novel interventions towards blocking malaria transmission.

Putative components of the *A. gambiae* immune system were identified by using comparative genomic analysis (Christophides *et al*, 2002; Zdobnov *et al*, 2002) combined with transcriptomic studies of the *A. gambiae* response to *Plasmodium berghei* infections (Oduol *et al*, 2000; Christophides *et al*, 2002; Dimopoulos *et al*, 2002; Vlachou *et al*, 2005). Functional analysis by RNA interference-mediated gene silencing showed that certain genes, which are upregulated in the mosquito during ookinete invasion of the midgut, participate in humoral mechanisms of *P. berghei* killing in the vector. Thus, silencing of the antagonist *LRIM1* (leucine-rich repeat immune protein 1) gene markedly increases the number of developing oocysts (Osta *et al*, 2004), as does the silencing of the complement-like gene thioester-containing protein (gene) 1 (*TEP1*; Blandin *et al*, 2004). Both *LRIM1* and *TEP1* encode circulating haemolymph (blood) proteins that favour the killing of ookinetes by lysis and melanization after midgut invasion. By contrast, two circulating C-type lectins—C-type lectin 4 (*CTL4*) and CTL mannose binding 2 (*CTLMA2*)—inhibit the melanization response and protect the development of ookinetes to the oocyst stage (Osta *et al*, 2004). More recent studies have established that antagonistic immune reactions are controlled, at least in part, by immune signalling pathways such as the Relish/Imd pathway (Meister *et al*, 2005). The number of *P. berghei* oocysts in *A. gambiae* is also regulated by local midgut epithelial responses that engage diverse—both negative and positive—factors (Vlachou & Kafatos, 2005; Vlachou *et al*, 2005).

The interplay of several humoral and cellular regulators of ookinete survival offers potential targets for novel malaria control strategies, aiming to support interactions that are either fatal for the parasite or to disrupt others that are protective. However, the interactions that have been identified in the laboratory combination *A. gambiae–P. berghei* might not fully represent those in the natural *A. gambiae–P. falciparum* systems (Boëte, 2005). Recently, a laboratory study has shown that transcriptional *A. gambiae* immune responses to *P. falciparum* and *P. berghei* are not identical, and might have different effects depending on the parasite species (Dong *et al*, 2006). Therefore, studies of interactions between *A. gambiae* and *P. falciparum* carried out under natural conditions are essential for understanding the specific molecular mechanisms that are pertinent to successful development of the principal human parasite, and to identify proper targets for interrupting human malaria transmission. Here, we have used gene silencing to investigate the role of *CTL4*, *CTLMA2* and *LRIM1* in the development of *P. falciparum* in *A. gambiae*, and the transcriptional responses of these genes to infection by this parasite species. These experiments were carried out under nearly natural conditions near Yaoundé in Cameroon, by membrane-feeding mosquitoes representative of the local vector population with field isolates of *P. falciparum*.

RESULTS AND DISCUSSION

Gene silencing mediated by double-stranded RNA (dsRNA) injection was carried out in female mosquitoes of the Yaoundé strain and was confirmed by reverse transcription–PCR (RT–PCR). The knockdown efficiency (% kd) was determined by comparing, for each gene of interest, the transcript levels in mosquitoes injected 4 days earlier with either dsRNA for that gene (experimental mosquitoes) or with dsRNA for green fluorescent protein (GFP) (controls). The percentage knockdown values were reproducibly high and averaged 81.5% for *CTL4*, 96.9% for *CTLMA2* and 96.8% for *LRIM1*. In previous experiments with *P. berghei*, such kd values resulted in strong effects on parasite prevalence and parasite load (Osta *et al*, 2004). Control and experimental mosquito groups were infected in parallel by feeding on four samples of human blood carrying field isolates of *P. falciparum*, including 20, 16, 16 or 32 gametocytes per microlitre. The respective oocyst prevalence in control midguts was 48.6%, 50.0%, 56.3% and 90.9%. The four replicates showed comparable kd values and were pooled (Table 1).

Table 1 | *Plasmodium falciparum* oocyst distribution in the midguts of knockdown *Anopheles gambiae* of the Yaoundé strain

	Gene knockdown					
	CTL4	GFP	CTLMA2	GFP	LRIM1	GFP
Midguts*	92	92	85	85	65	65
Prevalence	50	59.8	58.8	62.3	58.5	61.5
Range	0–10	0–17	0–28	0–17	0–16	0–16
Mean oocyst numbers	2.0^{NS}	3.1	3.0^{NS}	3.3	2.2^{NS}	3.3

Mean oocyst numbers include midguts from fed mosquitoes with 0 oocysts.
The results are pooled from four experimental replicates. Oocyst distributions for each silenced gene and the control GFP knockdown were tested by using the Kolmogorov–Smirnov test.
GFP, green fluorescent protein; NS, nonsignificant difference between experimental and control (GFP knockdown) samples.
*Different numbers of mosquitoes were available for each gene-silencing pool, owing to the death of individuals after injection or between blood meal and dissection 7 days later, and to the removal of unfed and partially fed mosquitoes. For each replicate, equal numbers of control and experimental mosquitoes were used to avoid bias owing to different levels of infection in the replicates.

The mean number of oocysts per midgut in all control group mosquitoes (even when lacking oocysts) was 3.02 (s.e. = 0.37); the oocyst load (mean oocyst number among mosquitoes showing at least one oocyst) was 4.98 (s.e. = 0.47). These values are consistent with previous data on natural infections with *P. falciparum* in human blood (Tahar *et al*, 2002; Gouagna *et al*, 2004; Boudin *et al*, 2005; Lambrechts *et al*, 2005). By contrast, infections of the same vector with *P. berghei* frequently show mean oocyst numbers higher than 50 (Blandin *et al*, 2004; Osta *et al*, 2004; Abraham *et al*, 2005). This difference, between the laboratory model and the human malaria system in the field, might be inherent to the parasite itself or the host–parasite interactions.

Kolmogorov–Smirnov tests showed that at day 7 after infection, the distributions of developing *P. falciparum* oocysts in control and *CTL4*, *CTLMA2* or *LRIM1* knockdown mosquitoes are statistically similar (Table 1). Additionally, melanized ookinetes were not detected in the *CTL* knockdowns. These results are in contrast with our previous results from gene-silencing laboratory experiments on *P. berghei* infections (Osta *et al*, 2004). These previous experiments were carried out in the G3 strain, which originated from a mix of various populations of M and S molecular forms of *A. gambiae* and is polymorphic for chromosomal inversions. The G3 strain differs considerably from the Yaoundé strain used here, which is the offspring of a pure, standard

©2006 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

Table 2 | *Plasmodium berghei* oocyst distribution and melanized ookinetes in the midguts of knockdown *Anopheles gambiae* of the Yaoundé strain

	Gene knockout			
	GFP	CTL4	CTLMA2	LRIM1
Midguts	17	14	23	13
Prevalence	100	100	100	100
Mean parasite numbers	122.4	186.1	129.7	582.8
Percentage of melanized parasites	0	93.4	66.1	0
Mean oocyst numbers	122.4	12.2**	44.0*	582.8*

Mean parasite numbers include melanized ookinetes and live oocysts.
The oocyst distribution for the tested gene and the control GFP knockdown was analysed using the Kolmogorov–Smirnov test. *$P<0.05$, **$P<0.001$.
GFP, green fluorescent protein.

chromosomal and molecular M form population from Cameroon. As an appropriate control, we used *P. berghei* to infect Yaoundé mosquitoes and confirmed both their high susceptibility to *P. berghei* and pronounced effects of gene silencing (Table 2); the results were comparable with those reported previously for infections of the G3 strain (Osta *et al*, 2004). Therefore, the effects of gene silencing on *P. berghei* and *P. falciparum* infections differ because of the parasite species and not the mosquito strain.

We compared the expression levels of *CTL4*, *CTLMA2* and *LRIM1* 24 h after feeding Yaoundé mosquitoes with human blood, which was either non-infected or infected with *P. falciparum*. At this crucial time, parasites emerge at the basal surface of the midgut, come into contact with haemolymph components and either begin the transformation into oocysts or die. The data from two independent experiments showed an average of 2.4-, 1.7- and 1.5-fold upregulation of these genes in the carcasses of infected mosquitoes (Fig 1). A comparable level of upregulation in carcasses—where these genes are predominantly expressed—was detected in previous infections with *P. berghei* (Osta *et al*, 2004). Carcasses are enriched in haemocytes (mosquito blood cells) that predominantly expresses these genes (M.A.O., unpublished data). The midguts showed low expression levels of these genes and no significant upregulation on infection. It should be noted that, at least with respect to *P. berghei*, the upregulated genes include both a parasite antagonist (*LRIM1*) and two agonists (*CTL4*, *CTLMA2*). Thus, transcriptional upregulation does not correlate directly with the outcome of infections, although further studies are warranted to test this conclusion more rigorously.

In conclusion, we have observed an important difference in the impact of silencing immunity genes on infection levels between the laboratory model (rodent) parasite and the sympatric natural (human) parasite isolates. Three *A. gambiae* genes encoding systemically circulating proteins, which strongly influence *P. berghei* development (positively or negatively), did not show comparable effects on *P. falciparum* infections in the same mosquito species and strain. It could be argued that the latter result might be due to the low infection levels of that parasite in *A. gambiae*. Future experiments to test this hypothesis definitively, by manipulating infection levels for both parasites, are conceivable but difficult—especially in the field—and are required to address this hypothesis. At least in terms of transcription,

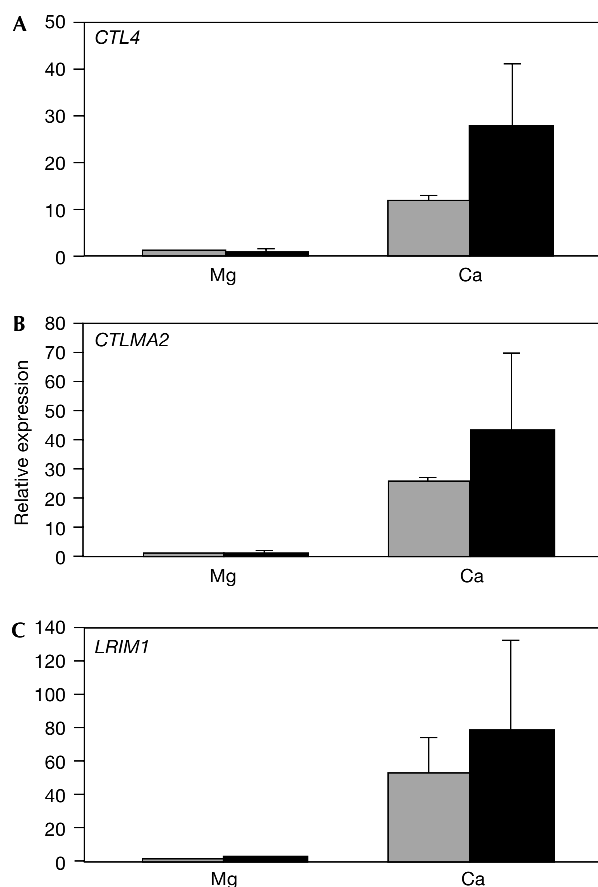

Fig 1 | *Plasmodium falciparum* infection activates *Anopheles gambiae* immune response. Transcript levels of *CTL4* (**A**), *CTLMA2* (**B**) and *LRIM1* (**C**) genes in midguts (Mg) and carcasses (Ca) of mosquitoes 24 h after feeding on blood from gametocyte carriers (black bars) or uninfected persons (grey bars). The ordinate represents relative expression values. Error bars represent s.d. in the pooled data set of two independent biological experiments.

P. falciparum indeed affects the expression of some immune genes (Fig 1). Furthermore, an extensive series of studies on *CTL4* failed to show a correlation between the degree of *P. berghei* ookinete melanization and infection levels (M.A.O., unpublished data). At present, we favour the hypothesis that the observed contrasting phenotypes might reflect co-evolution of the human parasite and its natural vector. Indeed, *P. falciparum* is naturally transmitted, but *P. berghei* might encounter *A. gambiae* only accidentally. This rodent parasite is naturally transmitted by *A. dureni* in Central Africa (reviewed by Gillies & De Meillon, 1968), and is unlikely to encounter the highly anthropophilic *A. gambiae*. The opposite is true for the human parasite *P. falciparum*, which shares an evolutionary history with the strongly anthropophilic *A. gambiae*, potentially resulting in unique co-adaptations. This supposition is consistent with the tantalizing observation that the *A. gambiae* refractory strain L3-5 is able to melanize numerous species of *Plasmodium*, including non-African *P. falciparum* isolates, but not—or only poorly—African *P. falciparum* isolates that are sympatric with *A. gambiae*

(Collins *et al*, 1986). Co-adaptation would have favoured low infection levels if, as proposed by Boëte (2005), both parasite and host benefit by limiting the parasite numbers; infection is costly for the vector (Anderson *et al*, 2000; Ferguson & Read, 2002) and reducing the burden might favour transmission to the vertebrate. The bottleneck that natural parasites suffer at the ookinete-to-oocyst transition (Gouagna *et al*, 1998) might at first be interpreted as evidence for selection of host responses that reduce the parasite load. However, such selection is unlikely to be unconstrained: an excessive immune response could be detrimental not only for the parasites but also for the host itself, as immune system overactivation might have costly side effects (Moret & Schmid-Hempel, 2000; Schmid-Hempel, 2003). Thus, co-adaptation might have benefited both parties by favouring a robust equilibrium on the basis of limited, but sufficient to keep the parasite load low, responses of several *A. gambiae* genes (not just the ones we tested) to *P. falciparum*. Our data suggest that, despite the lower infection levels compared with *P. berghei*, *P. falciparum* induces an immune response of *A. gambiae* genes including, but probably not limited to, upregulation of *LRIM1*, *CTL4* and *CTLMA2* 24 h after blood feeding. In fact, a recent genome-wide comparison of *A. gambiae* expression profiles after infection with *P. berghei* and *P. falciparum* parasites (Dong *et al*, 2006) showed that the former induces a wide and profound impact on the transcriptome, whereas the latter elicits a more specific immune response, possibly suggesting co-adaptation of the immune system to *P. falciparum* infection.

It remains unclear whether natural parasite–mosquito interactions are sufficiently distinct to preclude the possibility of extrapolating results obtained in *P. berghei–A. gambiae* to the natural transmission system. Future work will need to test whether other potent circulating immune-active proteins of the mosquito, such as TEP1 (Blandin *et al*, 2004) or RFABG (Vlachou *et al*, 2005), also show disparate effects on the rodent model compared with field isolates of the human parasite. In addition, proteins that are synthesized and locally active within the invaded epithelium, such as cytoskeleton-modifying components (Vlachou *et al*, 2005), also need to be tested. Genes in quantitative trait loci, shown by investigations in field conditions, such as Anopheles Plasmodium-responsive leucine-rich repeat 1 (*APL1*; Riehle *et al*, 2006) are also excellent candidates for affecting *P. falciparum* in natural transmission systems.

Although the possibility of parasite–vector genotypic co-adaptation is largely conjectural, a recent study has reported local effects of parasite genotype on the vector competence of specific mosquito genotypes (Lambrechts *et al*, 2005). To evaluate the possibility of parasite–vector co-adaptation and mosquito immune response in detail, this intriguing initial evidence needs to be followed up by studies on the infection and transcriptional regulation in diverse populations of *A. gambiae* by various *Plasmodium* species and *P. falciparum* genotypes. Ultimately, such studies will need to be combined with the current analysis of the role of human genotypes in malaria prevalence (Ruwende *et al*, 1995; Modiano *et al*, 2001; Fowkes *et al*, 2006). Although the complexity of genetic interactions in the host–parasite–vector trio might seem daunting, the availability of genomic and functional tools in all three organisms will make such studies feasible. In turn, such studies might lead to a deeper understanding of the patterns of malaria transmission and potentially provide new approaches to controlling this devastating global disease.

METHODS

Mosquito strain and gene silencing. The sympatric mosquitoes used in this study were *A. gambiae* of the Yaoundé strain. The Yaoundé mosquito strain was established in 1988 from field specimens caught in the suburbs of Yaoundé city, in the south of Cameroon (Tchuinkam *et al*, 1993). Mosquitoes that fed through a parafilm membrane were used and then maintained under standard insectary conditions. The colony belongs to the M molecular and Forest chromosomal forms (standard chromosomal arrangements). The Yaoundé strain is representative of the local natural transmission system in the south of Cameroon, where malaria transmission is mainly due to *A. gambiae* (M and S molecular forms, Forest chromosomal form), although *A. funestus*, *A. nili* and *A. moucheti* can also be locally important (Antonio-Nkondjio *et al*, 2002).

Gene silencing was achieved by injecting 207 ng of dsRNA into the thorax of 1-day-old females, as described previously (Blandin *et al*, 2002). In each of five replicates carried out, 60–100 females were injected with dsRNA for each of the genes tested. DsRNA for *GFP* was used as reference. The efficiency of gene silencing was determined as described by Osta *et al* (2004).

Plasmodium falciparum **gametocyte carriers-recruitment and experimental infection.** *P. falciparum* gametocyte carriers were recruited among 5- to 12-year-old children at schools in Mfou, a town located 30 km from Yaoundé city in an area endemic for malaria. Blood samples were collected by finger-prick from each volunteer and thick blood smears were stained with Giemsa and examined by microscopy for the presence of *P. falciparum*. Sexual and asexual stages were counted in observed fields that cumulatively contained at least 500 leukocytes; an estimate of parasite density was obtained by assuming a standard number of 8,000 leukocytes/μl of blood. Children with asexual parasitaemia exceeding 1,000 parasites/μl were immediately treated with the amodiaquine and artesunate drug combination according to national guidelines. Asymptomatic gametocyte-positive children were enrolled as volunteers. The recruitment procedures were approved by the Cameroonian and WHO ethical review committees.

For each experimental replicate, mosquitoes were injected with dsRNA and 4 days later were fed on blood from different *P. falciparum* gametocyte carriers. Five millilitres of venous blood was collected in heparinized vacutainers. To limit the effect of human factors, such as transmission-blocking immunity (Boudin *et al*, 2005), the blood was centrifuged for 3 min at 2,000 r.p.m. and the serum was replaced with the same volume of AB serum from a French donor with no exposure to malaria. Mosquitoes were starved for 16 h and allowed to feed for 15 min on this mixture using standard membrane-feeding (Tchuinkam *et al*, 1993). Unfed and partially fed mosquitoes were discarded. After 7 days, mosquitoes were dissected and midguts were stained with 0.4% mercurochrome in distilled water to count oocyst numbers by light microscopy. Experiments were considered successful when the infection prevalence of control mosquitoes (*GFP* dsRNA) was at least 30%. Mosquito infections with the *P. berghei* GFP-CON strain (Franke-Fayard *et al*, 2004) were carried out as described by Osta *et al* (2004).

Transcript level analysis in infected mosquitoes compared with non-infected mosquitoes. Females of the Yaoundé *A. gambiae* strain were fed on either gametocyte-positive blood or non-infected human blood. Transcript level analysis of *CTL4*, *CTLMA2* and *LRIM1* was carried out by using QRT–PCR. Briefly, midguts

©2006 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION

and carcasses of Yaoundé mosquitoes, fed on uninfected human blood or *P. falciparum*-infected human blood, were dissected 24 h after blood feeding, and were kept separately in RNAlater (Ambion, Austin, TX, USA) before RNA isolation. A batch of 30 control mosquitoes was dissected 7 days after blood feeding to determine the level of infection. Total RNA was isolated by using the TRIzol Reagent (Invitrogen, Paisley, UK) according to the supplier's instructions, and contaminant genomic DNA was removed by DNase I (Invitrogen) treatment. Complementary DNA was synthesized from total RNA (2–3 μg) by using the SuperScript II RNase H^- Reverse Transcriptase and oligo$(dT)_{12–18}$, as described by the supplier (Life Technologies Inc., Paisley, UK). QRT–PCR was carried out with the ABI Prism 7700 Sequence Detection System, by using the SYBR Green PCR Master Mix kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Primer sequences are described by Osta *et al* (2004). Relative gene expression values were calculated by using the comparative C_T method after checking for efficiency of target amplification as described in the User Bulletin #2. The S7 ribosomal protein gene was used as an internal reference.

ACKNOWLEDGEMENTS
We thank Dr M. Engelbert, C. Efemba and E. Bozewan of the Mfou hospital for assistance; A.M. Mendes and Dr D. Vlachou for sharing materials; and A.C. Koutsos and Dr C. Boëte for stimulating discussions. We are grateful to the inhabitants of Mfou for their cooperation. M.A.O. was supported by a Marie Curie Intra-European fellowship. This investigation received financial support from the UNICEF/UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases (TDR), grant A50241.

REFERENCES

Abraham EG, Pinto SB, Ghosh A, Vanlandingham DL, Budd A, Higgs S, Kafatos FC, Jacobs-Lorena M, Michel K (2005) An immune-responsive serpin, SRPN6, mediates mosquito defense against malaria parasites. *Proc Natl Acad Sci USA* **102:** 16327–16332

Anderson RA, Knols BG, Koella JC (2000) *Plasmodium falciparum* sporozoites increase feeding-associated mortality of their mosquito hosts *Anopheles gambiae* s.l.. *Parasitology* **120:** 329–333

Antonio-Nkondjio C, Awono-Ambene P, Toto JC, Meunier JY, Zebaze-Kemleu S, Nyambam R, Wondji CS, Tchuinkam T, Fontenille D (2002) High malaria transmission intensity in a village close to Yaounde, the capital city of Cameroon. *J Med Entomol* **39:** 350–355

Blandin S, Moita LF, Kocher T, Wilm M, Kafatos FC, Levashina EA (2002) Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the *Defensin* gene. *EMBO Rep* **3:** 852–856

Blandin S, Shiao SH, Moita LF, Janse CJ, Waters AP, Kafatos FC, Levashina EA (2004) Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell* **116:** 661–670

Boëte C (2005) Malaria parasites in mosquitoes: laboratory models, evolutionary temptation and the real world. *Trends Parasitol* **21:** 445–447

Boudin C, Diop A, Gaye A, Gadiaga L, Gouagna C, Safeukui I, Bonnet S (2005) *Plasmodium falciparum* transmission blocking immunity in three areas with perennial or seasonal endemicity and different levels of transmission. *Am J Trop Med Hyg* **73:** 1090–1095

Castro J, Millet D (2005) Structural Adjustment Programs and malaria: any link? In *Genetically Modified Mosquitoes for Malaria Control*, C Boëte (ed), pp 16–23. Georgetown, TX, USA: Landes Biosciences

Christophides GK *et al* (2002) Immunity-related genes and gene families in *Anopheles gambiae*. *Science* **298:** 159–165

Collins FH, Sakai RK, Vernick KD, Paskewitz S, Seeley DC, Miller LH, Collins WE, Campbell CC, Gwadz RW (1986) Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. *Science* **234:** 607–610

Dimopoulos G, Christophides GK, Meister S, Schultz J, White KP, Barillas-Mury C, Kafatos FC (2002) Genome expression analysis of *Anopheles gambiae*: responses to injury, bacterial challenge, and malaria infection. *Proc Natl Acad Sci USA* **99:** 8814–8819

Dong Y, Aguilar R, Xi Z, Warr E, Mongin E, Dimopoulos G (2006) *Anopheles gambiae* immune responses to human and rodent *Plasmodium* parasite species. *PLoS Pathog* **2:** 513–525

Ferguson H, Read A (2002) Why is the effect of malaria parasites on mosquito survival still unresolved? *Trends Parasitol* **18:** 256–261

Fowkes FJ, Imrie H, Migot-Nabias F, Michon P, Justice A, Deloron P (2006) Association of haptoglobin levels with age, parasite density, and haptoglobin genotype in a malaria-endemic area of Gabon. *Am J Trop Med Hyg* **74:** 26–30

Franke-Fayard B, Trueman H, Ramesar J, Mendoza J, van der Keur M, van der Linden R, Sinden RE, Waters AP, Janse CJ (2004) A *Plasmodium berghei* reference line that constitutively expresses GFP at a high level throughout the complete life cycle. *Mol Biochem Parasitol* **137:** 23–33

Gillies M, De Meillon B (1968) *The Anophelinae of Africa South of the Sahara (Ethiopian Zoogeographical Region)*. Johannesburg, South Africa: The South African Institute for Medical Research

Gouagna LC, Mulder B, Noubissi E, Tchuinkam T, Verhave JP, Boudin C (1998) The early sporogonic cycle of *Plasmodium falciparum* in laboratory-infected *Anopheles gambiae*: an estimation of parasite efficacy. *Trop Med Int Health* **3:** 21–28

Gouagna LC, Bonnet S, Gounoue R, Verhave JP, Eling W, Sauerwein R, Boudin C (2004) Stage-specific effects of host plasma factors on the early sporogony of autologous *Plasmodium falciparum* isolates within *Anopheles gambiae*. *Trop Med Int Health* **9:** 937–948

Greenwood B, Mutabingwa T (2002) Malaria in 2002. *Nature* **415:** 670–672

Lambrechts L, Halbert J, Durand P, Gouagna LC, Koella JC (2005) Host genotype by parasite genotype interactions underlying the resistance of anopheline mosquitoes to *Plasmodium falciparum*. *Malar J* **4:** 3

Meister S *et al* (2005) Immune signaling pathways regulating bacterial and malaria parasite infection of the mosquito *Anopheles gambiae*. *Proc Natl Acad Sci USA* **102:** 11420–11425

Modiano D, Luoni G, Sirima BS, Lanfrancotti A, Petrarca V, Cruciani F (2001) The lower susceptibility to *Plasmodium falciparum* malaria of Fulani of Burkina Faso (west Africa) is associated with low frequencies of classic malaria-resistance genes. *Trans R Soc Trop Med Hyg* **95:** 149–152

Moret Y, Schmid-Hempel P (2000) Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290:** 1166–1168

Oduol F, Xu J, Niare O, Natarajan R, Vernick KD (2000) Genes identified by an expression screen of the vector mosquito *Anopheles gambiae* display differential molecular immune response to malaria parasites and bacteria. *Proc Natl Acad Sci USA* **97:** 11397–11402

Osta MA, Christophides GK, Kafatos FC (2004) Effects of mosquito genes on *Plasmodium* development. *Science* **303:** 2030–2032

Riehle MM *et al* (2006) Natural malaria infection in *Anopheles gambiae* is regulated by a single genomic control region. *Science* **312:** 577–579

Ruwende C, Khoo SC, Snow RW, Yates SN, Kwiatkowski D, Gupta S (1995) Natural selection of hemi- and heterozygotes for G6PD deficiency in Africa by resistance to severe malaria. *Nature* **376:** 246–249

Schmid-Hempel P (2003) Variation in immune defence as a question of evolutionary ecology. *Proc Biol Sci* **270:** 357–366

Sinden RE (1999) *Plasmodium* differentiation in the mosquito. *Parassitologia* **41:** 139–148

Tahar R, Boudin C, Thiery I, Bourgouin C (2002) Immune response of *Anopheles gambiae* to the early sporogonic stages of the human malaria parasite *Plasmodium falciparum*. *EMBO J* **21:** 6673–6680

Tchuinkam T, Mulder B, Dechering K, Stoffels H, Verhave JP, Cot M, Carnevale P, Meuwissen JH, Robert V (1993) Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. *Trop Med Parasitol* **44:** 271–276

Vaughan JA, Noden BH, Beier JC (1992) Population dynamics of *Plasmodium falciparum* sporogony in laboratory-infected *Anopheles gambiae*. *J Parasitol* **78:** 716–724

Vlachou D, Kafatos FC (2005) The complex interplay between mosquito positive and negative regulators of *Plasmodium* development. *Curr Opin Microbiol* **8:** 415–421

Vlachou D, Schlegelmilch T, Christophides GK, Kafatos FC (2005) Functional genomic analysis of midgut epithelial responses in *Anopheles* during *Plasmodium* invasion. *Curr Biol* **15:** 1185–1195

Zdobnov EM *et al* (2002) Comparative genome and proteome analysis of *Anopheles gambiae* and *Drosophila melanogaster*. *Science* **298:** 149–159

©2006 EUROPEAN MOLECULAR BIOLOGY ORGANIZATION