Transmission Blocking Immunity in the Malaria Non-Vector Mosquito Anopheles quadriannulatus Species A

Tibebu Habtewold, Michael Povelones, Andrew M. Blagborough, George K. Christophides*

Immunology and Infection, Division of Cell and Molecular Biology, Faculty of Natural Sciences, Imperial College London, London, United Kingdom

Abstract

Despite being phylogenetically very close to Anopheles gambiae, the major mosquito vector of human malaria in Africa, Anopheles quadriannulatus is thought to be a non-vector. Understanding the difference between vector and non-vector mosquitoes can facilitate development of novel malaria control strategies. We demonstrate that An. quadriannulatus is largely resistant to infections by the human parasite Plasmodium falciparum, as well as by the rodent parasite Plasmodium berghei. By using genetics and reverse genetics, we show that resistance is controlled by quantitative heritable traits and manifested by lysis or melanization of ookinetes in the mosquito midgut, as well as by killing of parasites at subsequent stages of their development in the mosquito. Genes encoding two leucine-rich repeat proteins, LRM1 and LRM2, and the thioester-containing protein, TEP1, are identified as essential in these immune reactions. Their silencing completely abolishes P. berghei melanization and dramatically increases the number of oocysts, thus transforming An. quadriannulatus into a highly permissible parasite host. We hypothesize that the mosquito immune system is an important cause of natural refractoriness to malaria and that utilization of this innate capacity of mosquitoes could lead to new methods to control transmission of the disease.


Editor: David S. Schneider, Stanford University, United States of America

Received November 27, 2007; Accepted April 14, 2008; Published May 23, 2008

Copyright: © 2008 Habtewold et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by a Wellcome Trust Programme grant (GR077229/Z/05/Z) and a NIAID-NIH Programme Project grant (2PO1AI044220-06A1). Funders had no role in the design and conduct of the study, in the collection, analysis, and interpretation of the data, or in the preparation, review, or approval of the manuscript. Authors and funders have no financial interest interest on the results presented herein.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: g.christophides@imperial.ac.uk

Introduction

The Anopheles gambiae Giles complex comprises seven mosquito species and several incipient species [1–3]. Sibling species are closely related to each other, are morphologically indistinguishable, and can crossbreed in captivity; however, they vary greatly in their capacity to transmit human malaria [3–5]. An. gambiae sensu stricto (henceforth An. gambiae) and Anopheles arabiensis are highly efficient vectors in sub-Saharan Africa and surrounding islands. Other species of this complex are only locally important vectors: Anopheles melas in western Africa, Anopheles nerois in eastern Africa, and Anopheles bicambui in Uganda. Finally, An. quadriannulatus Theobald species A and B, found in southern Africa and Ethiopia, respectively, are exceptional in that they are considered medically unimportant; human malaria parasites have never been detected in wild caught An. quadriannulatus females [4]. Both species display characteristics that are believed to have existed in ancestral forms of the complex, i.e. standard chromosomal arrangements, disjointed distribution and adaptation to temperate climates [1]. Furthermore, they have been considered strictly zoophilic although recent laboratory and field studies report equal feeding preference for human and cattle [6–8]. Although laboratory reared An. quadriannulatus species A can be infected with cultured P. falciparum, the infection prevalence is significantly lower than in An. gambiae and Anopheles stephensi [9].

Plasmodium undergoes a complex developmental lifecycle in the mosquito. As shown for the rodent malaria parasite P. berghei, a standard laboratory model system, the parasite suffers substantial losses during its passage through the mosquito. The greatest reduction in parasite numbers occurs at the ookinete-to-oocyst transition stage [10]. Ookinetes, an invasive parasitic form, are often eliminated by lysis (and clearance) or melanization in the mosquito midgut epithelium, which are controlled by reactions of the mosquito innate immune system [11]. However, the few parasites that survive to reach the oocyst stage, a sessile parasitic form developing on the basal side of the midgut epithelium, multiply and produce thousands of sporozoites. When the oocysts burst, sporozoites are released to the haemolymph, invade the salivary glands and, upon subsequent mosquito bites, infect human hosts. Here, we investigate the mechanisms of refractoriness to Plasmodium in the malaria non-vector mosquito An. quadriannulatus. We show that refractoriness is controlled by partially dominant genetic traits and is manifest by clearance and melanization of ookinetes in the mosquito midgut as well as by killing of other parasitic stages developing later in the mosquito. The mosquito immune system appears to play a fundamental role in these reactions: inactivation of genes known to contribute to parasite killing in the malaria vector An. gambiae renders An. quadriannulatus a highly efficient vector of the rodent parasite P. berghei. We speculate that the same resistance traits may be present in wild vector populations at lower frequencies, since genetic selection for refractoriness apparently generates An. gambiae lines with phenotypes that are similar to that of An. quadriannulatus [12,13]; these phenotypes can be reversed after silencing specific immunity genes [14]. Our data suggest that resistance to malaria may be an ancestral state of mosquitoes and prompt us to hypothesize that...
Malaria is a mosquito-borne infectious disease that threatens almost half of the human population and kills 1 to 3 million people every year. In sub-Saharan Africa, where the vast majority of deaths occur, the capacity of mosquitoes to transmit malaria varies greatly even between closely related species. We compared the ability of malaria parasites to develop in two very closely related mosquitoes, one vector and one non-vector, and found that non-vector mosquitoes kill parasites at various stages, predominantly when they invade the mosquito midgut. This is achieved by parasite clearance, possibly by lysis in the midgut cells and by melanization, both of which are reactions of the mosquito immune system. This phenotype depends on heritable and dominant traits that can be passed on to vector/non-vector mosquito hybrids. We examined whether specific components of the mosquito immune system affect the resistance of these mosquitoes to infection. By silencing the activity of three immunity genes, we transformed mosquitoes of the resistant species into highly susceptible. Our results suggest that the mosquito immune system may affect refractoriness to malaria in non-vector mosquitoes. This innate capacity of mosquitoes to kill malaria parasites could be utilized in future integrated efforts to control and ultimately eradicate the disease.

Results/Discussion

Plasmodium killing in An. quadriannulatus

We tested the ability of An. quadriannulatus species A, strain SKUQA (henceforth An. quadriannulatus), to support development of P. falciparum. An. gambiae mosquitoes of the Yaoundé strain [15] were used as a reference. Three to four-day-old female mosquitoes were fed via a membrane with cultured P. falciparum gametocytes, and 10 days later their midguts were dissected and examined for oocysts. The results from two independent feeding experiments showed 0 of 18 (0/18) and 6/39 An. quadriannulatus midguts infected (0% and 15.4% infection prevalence, respectively); the corresponding median oocyst densities were 0.0 in both experiments (Table 1). In the paired feedings of An. gambiae, a known host for P. falciparum, 13/38 and 13/30 midguts had at least one viable oocyst (34.2% and 43.3% infection prevalence, respectively) with corresponding median oocyst densities of 5.0 and 12.0. Two subsequent An. quadriannulatus infections showed no live oocysts, but melanized ookinete were occasionally observed in the mosquito midguts (Figure 1A); however, control An. gambiae mosquitoes were not used in these experiments, and thus comparisons cannot be made. Clearance of pre-oocyst parasitic stages and melanization of ookinete are established important immune reactions of mosquitoes against Plasmodium. Thus, these data suggested that mosquito immunity could contribute to the reduced susceptibility of An. quadriannulatus.

To investigate further this possibility, we utilized the convenient laboratory parasite, P. berghei, against which an extensive repertoire of mosquito immune responses has been previously documented. In these experiments, An. quadriannulatus and control An. gambiae mosquitoes were infected with a transgenic P. berghei parasite line that constitutively expresses green fluorescent protein (GFP) throughout its lifecycle [16]. Data from four independent infection experiments showed that An. quadriannulatus mosquitoes are highly refractory to P. berghei, in terms of oocyst prevalence, parasite density and ookinete melanization. A representative picture of an infected An. quadriannulatus midgut, with melanized P. berghei ookinetes, is shown in Figure 1B. The four experiments were analyzed by the Residual Maximum Likelihood (REML) variance components analysis, which revealed that the outcomes of these experiments were homogeneous and unlikely to be the result of random effects; thus justifying pooling of the data. Compared to An. gambiae (n = 118, where n is the number of midguts in the pooled data), An. quadriannulatus (n = 167) exhibited both reduced oocyst prevalence (67% vs. 100%; P<0.001) and increased ookinete melanization prevalence (93% vs. 13%; P<0.001) in their midguts 7–10 days post infection. As shown in Figure 1C, the density of melanized parasites per midgut of An. quadriannulatus was markedly greater than in An. gambiae (P<0.001) where melanization was only sporadically observed. In contrast, the live oocyst density was much lower (P<0.001) in An. quadriannulatus than in An. gambiae. The distributions of oocyst densities varied significantly (P<0.01) between the two mosquito species, as one-third of An. quadriannulatus had no oocysts whereas almost every An. gambiae midgut had one or more (Figure S1).

We assessed mosquito salivary gland infection by sporozoites to determine whether the losses of midgut parasitic stages in An. quadriannulatus can ultimately affect the transmission capacity of these mosquitoes. Importantly, we observed that parasite losses continue at later stages of the parasite lifecycle. In pooled data from three infection experiments (different from the above), the prevalence of P. berghei salivary gland sporozoites at day 21–22 post infection was much lower (20%; n = 115) compared to the prevalence of oocysts (57%; n = 104) at day 10 in the corresponding infection (P<0.001; Figure 1D and Table S1). No significant difference in prevalence between midgut (n = 105) oocysts and salivary gland (n = 85) sporozoites was detected in the paired infections of An. gambiae (76% vs. 79%, respectively). Moreover, salivary gland sporozoites in An. quadriannulatus appeared to be less infective compared to those in An. gambiae. From four bite-back experiments, using equal numbers of An. quadriannulatus or An. gambiae females (ranging from 10 to 15), which were infected 21–22 days earlier with P. berghei and then allowed to feed on naive TO mice (one per experiment per mosquito species), only one resulted in mouse infection. In contrast, all four An. gambiae control bite-back experiments were infectious.

Resistance to Plasmodium is heritable and dominant

A great variability in the degree of refractoriness to P. berghei was observed between An. quadriannulatus individuals, indicating genetic polymorphism within the mosquito population. The majority of mosquitoes in the four infection experiments described above displayed an intermediate phenotype with high numbers of both live oocysts and melanized parasites; others were fully resistant exhibiting strong ookinete melanization and no live oocysts, and yet others were highly susceptible, displaying many oocysts and few or no melanized ookinete. No correlation (R2 = 0.001) was detected between oocyst and melanized ookinete counts (Figure S2). This variability within the population suggested that these phenotypes are determined by quantitative genetic traits. Two independent crossing experiments were carried out to determine whether the refractory traits of An. quadriannulatus are heritable. In these experiments, F1 females were generated by mass mating of An. quadriannulatus males and An. gambiae females (the reciprocal cross is uninformative because it predominantly yields males [17]). The resulting F1 females were then backcrossed to An. quadriannulatus males to obtain backcrossed F2 females. First
generation F1 and F2 progenies, and the parental *An. quadriannulatus* and *An. gambiae* populations were compared for their ability to support parasite development (see Materials and Methods). The data resulting from the two crossing and infection experiments were pooled and analyzed with Kruskal-Wallis non-parametric ANOVA.

In terms of infection, the F1 hybrids were phenotypically similar to the *An. quadriannulatus* but different from the *An. gambiae* parental populations in both prevalence of infection (P<0.001) and oocyst density (P=0.03; Figure 2). They displayed 80% oocyst prevalence with a median density of 5.5 per midgut (n = 66) compared to 71% and 5.0 in *An. quadriannulatus* (n = 49) and 98% and 14.0 in *An. gambiae* (n = 53), respectively. F1 and parental *An. quadriannulatus* mosquitoes exhibited marked similarity in their pattern of ookinete melanization. The prevalence of melanization was 77% in F1 hybrids and 80% in parental *An. quadriannulatus*.

Table 1. *P. falciparum* infection of *An. quadriannulatus* and *An. gambiae*.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Species</th>
<th>n</th>
<th>Oocyst prevalence P</th>
<th>Oocyst density</th>
<th>Range</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>An. quadriannulatus</em></td>
<td>18</td>
<td>0.0</td>
<td>ns</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td><em>An. gambiae</em></td>
<td>38</td>
<td>34.2</td>
<td>5.0</td>
<td>1–43</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td><em>An. quadriannulatus</em></td>
<td>39</td>
<td>15.4</td>
<td>&lt;0.001</td>
<td>0.0</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td><em>An. gambiae</em></td>
<td>30</td>
<td>43.3</td>
<td>12.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Mosquito midguts were examined for *P. falciparum* live oocysts 10 days post-infection. Two independent experiments were performed. Oocyst prevalence is the percentage of mosquitoes displaying at least one live oocyst, and oocyst density is the median number of oocysts in infected mosquitoes. The range of oocyst numbers is also presented. In both experiments, the oocyst prevalence and density were substantially less in *An. quadriannulatus* compared to *An. gambiae*. Infection of *An. quadriannulatus* was not observed in experiment 1. The oocyst prevalence was analyzed with the Chi-square Fishers exact test with Yates correction, and the oocyst density with the Kruskal-Wallis non-parametric ANOVA. n, number of mosquitoes; ns not significant.

doi:10.1371/journal.ppat.1000070.t001

Figure 1. *Plasmodium* parasite killing in *An. quadriannulatus*. (A, B) Melanized ookinetes (arrows) of *P. falciparum* (A) and *P. berghei* (B) while crossing the *An. quadriannulatus* midgut. (C) Melanized ookinete and live oocyst density in the midguts of *An. quadriannulatus* and *An. gambiae* females infected with *P. berghei*. Four independent paired experiments were performed and their results were analysed by REML variance components analysis by fitting the mixed effect model. The geometric means±SD of the pooled data from the four independent experiments are shown. The melanized parasite densities (black bars) were 12.4±3.1 for *An. quadriannulatus* (n = 167) and 0.2±0.5 for *An. gambiae* (n = 118; P<0.001), and the oocyst densities were 3.6±3.3 and 12.8±2.6, respectively (P<0.001). n, number of midguts. (D) Prevalence (% of mosquitoes with at least one live parasite) of midguts at day 10 and salivary glands in corresponding infections at day 21–22 showing live *P. berghei* oocysts and sporozoites, respectively. The results of three independent experiments (see Table S1) were pooled and analyzed using the Chi-square goodness-of-fit test. A significant decrease in prevalence is detected in *An. quadriannulatus* (P<0.001), but not in *An. gambiae*. Bars represent standard errors.

doi:10.1371/journal.ppat.1000070.g001
both of these were very different from the prevalence of melanization in An. gambiae (23%; P<0.001). A more striking similarity of refractoriness with the parental An. quadriannulatus was detected in the F2 backcrossed mosquitoes (n=86; 69% oocyst prevalence and 4.0 oocyst density). Melanized ookinetes were detected in 75 of the 86 F2 females (87% prevalence) with median density 12.0 per midgut. All these measurements were significantly different from those reported above for An. gambiae (all at P<0.001 but the oocyst density which was at P=0.01). Together these data suggested that the refractoriness exhibited by An. quadriannulatus is heritable and that both traits contributing to this phenotype, reduction in the number of oocysts and increase in the number of melanized ookinetes, are dominant or partially dominant.

### Innate immunity controls resistance to Plasmodium

Given that the refractory mechanisms of An. quadriannulatus are under genetic control, we sought to determine if these are due to reactions of the mosquito innate immune system. Several An. gambiae genes have been implicated in lysis, clearance and melanization of P. berghei ookinetes in the mosquito midgut. In this initial study, we examined three genes, LRIM1, LRIM2 (previously called APL1 [13]; synonym is suggested here for systematization) and TEP1, all of which exhibit potent antagonistic effects against P. berghei. LRIM1 and LRIM2 encode leucine-rich repeat proteins, and silencing of either gene by RNA interference (RNAi) remarkably increases live oocyst densities in An. gambiae [13,16]. LRIM1 also mediates melanization of ookinetes in mosquitoes that are deficient for the melanization inhibitor C-type lectin 4, CTL4. TEP1 is the founder member of a thioester-containing protein family; it binds ookinetes promoting their lysis or melanization [14].

We used An. gambiae-specific oligonucleotide primers to amplify exon sequences of these genes from a cDNA pool constructed from An. quadriannulatus adult females. Sequencing the amplified fragments revealed a high degree of sequence similarity between An. gambiae and An. quadriannulatus for all the three genes: 98.9% for LRIM1, 96.2% for LRIM2 and 98.7% for TEP1 (Figure S3). This was not surprising as the two species are very closely related in the evolutionary scale and genetic introgression has likely taken place for some time after their separation. Using these gene fragments as templates, we produced double stranded RNA (dsRNA) sequences for each of these genes, which were microinjected separately in the body cavity of freshly emerged An. quadriannulatus females, as described for An. gambiae [19]. Mosquitoes injected with dsRNA of the LacZ gene were used as a control. Quantitative RT-PCR (qRT-PCR) revealed robust and specific silencing of cognate gene expression 4 days later, which ranged from 98% for LRIM1 to 89% for LRIM2. As shown in Figure 3, silencing of LRIM1, LRIM2 or TEP1 in An. quadriannulatus resulted in a striking increase in P. berghei oocyst density (P<0.001) and complete inhibition of ookinete melanization compared to the control (P<0.001). While the oocyst density was 2.0 per midgut in control mosquitoes, this number increased to 102.5 in LRIM1 and 141.0 in LRIM2 knockdown (kd) mosquitoes. A similar increase was observed by silencing TEP1 compared to the control: 116.5 vs. 1.1, respectively. These results suggest an effect for these genes in both ookinete melanization and clearance of parasites (possibly by lysis), as the number of oocysts in kd mosquitoes is much higher than the sum of oocysts and melanized ookinetes in the controls.

The very high sequence similarity between An. gambiae and An. quadriannulatus, which is expected for most genes in the two genomes, pointed to an intriguing possibility: that An. gambiae-specific dsRNA can be directly used to silence genes in An.
Indeed, An. gambiae-specific dsRNA for LRIM1 fully rescued the susceptibility phenotype when injected into An. quadriannulatus females, causing an approximately 4-fold increase in the oocyst density, 71% increase in oocyst prevalence (from 29% to 100%) and complete inhibition of ookinete melanization (data not shown). This result would constitute an important breakthrough if it pertains to additional genes: this non-vector species could then be utilized in conjunction with the An. gambiae vector as a model system, to further understand differences contributing to its reduced vectorial capacity.

Our results clearly indicate that a mosquito innate immune response accounts for most of the resistance of An. quadriannulatus to P. berghei. LRIM1, LRIM2 and TEP1 are essential elements of this response and likely to operate in the same pathway, since their effects on the parasite are very similar. We examined with qRT-PCR whether the transcriptional profile of any of these three genes is different between the two mosquito species. In two independent experiments, mosquitoes were allowed to feed either on naive mice or on mice infected with P. berghei, and RNA samples from whole mosquitoes were prepared 24 hrs later. Sugar-fed mosquitoes of the same generation and age were also included. The results showed that the expression levels of LRIM1 and TEP1 were similar between the two species at all three conditions, with minor variations (Figure S4). However, the levels of LRIM2 in sugar-fed and naive blood-fed mosquitoes were consistently elevated in An. quadriannulatus compared to An. gambiae. It remains to be explored whether this difference in LRIM2 expression is directly related to the refractoriness phenotype of An. quadriannulatus. Furthermore, although only few nucleotide differences were identified in the sequenced gene fragments between An. gambiae and An. quadriannulatus, some of these differences in LRIM1 and LRIM2 lead to non-synonymous amino acid substitutions. Future research will aim to determine if any of these changes (or others in the non-sequenced gene segments) can enhance or otherwise alter the function of these genes, thus contributing to the refractoriness phenotype.

The An. gambiae LRIM2 gene is located in a genomic region that was recently identified to control the density of mosquito infection.

Figure 3. Silencing LRIM1, LRIM2 and TEP1 transforms An. quadriannulatus into a vector species. (A) Representative microscopy images of midguts dissected from P. berghei-infected mosquitoes which were injected with either LacZ dsRNA (control) or dsRNAs for each of the examined genes. GFP-fluorescent oocysts are shown in the right panels whereas arrows in the bright field images indicate melanized ookinetes. No melanized ookinetes and higher oocyst densities are observed in kd mosquitoes. (B) Quantitative effects of gene kds on melanized ookinete density (black bars) and oocyst density (green bars) in mosquito midguts compared to LacZ dsRNA-treated controls. The pooled results from two independent experiments were analyzed using the REML variance component analysis by fitting the mixed effects model. Geometric means and standard deviations are shown. Experiments with LRIM1 and LRIM2 kds were performed separately from those with TEP1 kd; thus they are presented in separate graphs.

doi:10.1371/journal.ppat.1000070.g003
Mosquito Resistance to Malaria

with *P. falciparum* in a natural malaria transmission system in Mali, West Africa [13]. The same locus was responsible for almost 90% of parasite-free mosquitoes and 100% of mosquitoes with melanized parasites. These responses are highly similar to those we report here for *An. quadriannulatus*, making LRIM2 a strong candidate for regulating natural mosquito refactoriness to the human malaria parasite. On the other hand, LRIM1 was recently shown to have undergone strong positive selection in the other major African vector, *An. arabiensis*; this “arabiensis-like” allele has been introduced in *An. gambiae* populations at lower frequencies through multiple introgression events, but it is not present in other, less competent species of the complex including *An. quadriannulatus* [20]. These data in conjunction with the failure to demonstrate an apparent effect of *An. gambiae* LRIM1 on sym pathetic field isolates of *P. falciparum* in a laboratory transmission setting in Cameroon [21], could suggest that LRIM1 is subject to evolutionary adaptation to the human parasite; however, the effect of LRIM1 on allatopatic isolates or laboratory strains of *P. falciparum* is yet to be examined. Finally, *An. gambiae* TEP1 was shown to have a strong antagonistic effect against a laboratory *P. falciparum* line [22]. Furthermore, a refractory allele of this gene is found in a genetically selected mosquito strain which kills and melanizes all *Plasmodium* species or strains that have been tested, except sympatric isolates of *P. falciparum* [12]. The phenotype of this refractory *An. gambiae* strain is identical to that of *An. quadriannulatus*. Therefore, it is tempting to speculate that persistent interaction of *An. gambiae* (and other major vectors) with *P. falciparum* might have led to an evolutionary co-adaptation between the mosquito immune responses and this parasite, whereas the resistance phenotype of the mostly zoophilic *An. quadriannulatus* could represent the ancestral function of the mosquito immune system against the parasite.

**Perspective**

Malaria kills up to three million people every year and threatens the lives of almost half of the global population. Of the several hundreds of mosquito species only some anophelines can transmit human malaria. Even within the *An. gambiae* species complex, which includes some of the most important malaria vectors in Africa, the two *An. quadriannulatus* species are considered non-vectors. Researchers have proposed that understanding the differences between vector and non-vector mosquitoes could provide a new means for malaria control. Our research establishes for the first time a model laboratory system to study these differences at a genetic and molecular level. It demonstrates that mosquito immunity, which regulates the density of infection by the model rodent parasite, *P. berghei*, in the most competent vector of human malaria, *An. gambiae*, is the main cause of refractoriness to *P. berghei* in its non-vector sibling *An. quadriannulatus* species A. It remains to be revealed whether these findings also apply to infections with the human parasite *P. falciparum*.

**Materials and Methods**

**Mosquito colonies, infections and dissections**

The *An. quadriannulatus* SKUQUA strain was established from wild mosquitoes collected from an area near Skukuza, Kruger National Park, South Africa, in December 1995. The *An. gambiae* Yaoundé strain was colonized from wild mosquitoes collected from the Yaoundé area in 1988 [13]. Both mosquito colonies were raised at 28°C, 65–70% relative humidity, under a 12 hr light/dark cycle; adult mosquitoes were maintained on a 10% (w/v) sucrose solution. Infections with *P. berghei* were performed using the PbGFP_{co} parasite line [16], cultured using standard methods [23]. For infection, 50–70 female mosquitoes were randomly separated in paper caps, fed on anaesthetized mice infected with *P. berghei* (parasitaemia >5%) and kept at 20–21°C until the day of dissection. Midguts of mosquitoes were dissected 7–10 days post infectious blood meal, fixed in 4% paraformaldehyde and mounted on microscopy slides using VectaShield (Vector Laboratories Inc) before visualized with a light/fluorescence microscope. Killed parasite that appear as melanized oocinates in the midguts and living oocyst that fluoresce green were separately quantified; melanized oocinates were detected in bright field and oocysts were visualized with the fluorescein isothiocyanate filter.

For *P. falciparum* infections, erythrocytic stages of the 3D7 clone of the NF54 isolate were cultured as described [24], followed by induction of gametocytogenesis [25]. Cultures were then added to RBCs with HI AB serum at packed cell volume (ca. 40%) and introduced into membrane feeders. Mosquitoes were exposed to the membrane feeders for 25–30 min, and thereafter kept at standard insectary conditions until dissection. Mosquito midguts were dissected 7–9 days post infection, stained with 0.5% mercuricchrome and examined for live oocysts and melanized oocinates using a light microscope.

**Bite-back experiments**

Detection of the infectivity of salivary gland sporozoites was carried out by mosquito bite-back experiments as described [26], with minor modifications. Female *An. quadriannulatus* and *An. gambiae* mosquitoes were infected with PbGFP_{co} *P. berghei* after feeding on an infected TO mouse. Non-blood-fed mosquitoes were removed. The presence of oocysts on the mosquito midguts was confirmed at day 8–10 post infection. At day 21–22 post infection, 10–15 of these mosquitoes were allowed to feed on naïve 8–10 week-old TO mice. The mice were then screened for blood staged parasites on day 5 after the mosquito bite, and the screening was continued every other day until day 15. The bite-back was considered non-infective if no blood-staged parasites were detected by day 15. Four independent replicate experiments were performed.

**Crossing experiments**

In each crossing experiment, male *An. quadriannulatus* (300–400) and female *An. gambiae* (100–150) adult mosquitoes were allowed to mass mate to produce the F1 progeny. 100–150 females from this F1 progeny were then backcrossed to 300–400 *An. quadriannulatus* males to obtain the F2 progeny. In parallel to this backcrossing, the initial crossing of the parental populations was repeated in order to obtain first generation F1 progeny which were of the same age as the F2 progeny. Females of the parental populations and the F1 and F2 progenies were allowed to feed on *P. berghei*-infected mice as described above. Because there were four groups of females for each infection experiment, we used two mice of similar parasitaemia, each of which was randomly allocated to two of these groups; after 10 min in feeding, mice were swapped between group pairs. The entire crossing and infection experiment was repeated twice.

**DsRNA production and qRT-PCR**

DsRNA production was performed as previously described, using gene specific oligonucleotide primers tailed with the short T7 promoter sequence TAAATCAGACTACTATAGGG [27]. The sequences of these primers are: LRIM1 F, AATATC-TATCTCAGCGACAGATAA; LRIM1 R, TGGACAGGTA-CATCTTGC; LRIM2 F, GCTTACGGCGACACTATTCA; LRIM2 R, GCTATTGGGATGCTTGCTA; TEPI F, TTTGCGGCCCTTAAAGCCTGT; TEPI R, ACCAGG-TAACCGGCTCGGATGA; LACZ F, AGAATCCGACGCGTTGT-
TACT; LACR, R, CACCAAGCTCATCGATAATT. Injection of dsRNA in adult female mosquitoes was performed as described [19]. The aforementioned primers were also used to PCR amplify and determine the sequence of the An. quadriannulatus gene. Two additional primers were used for sequencing another fragment of the LRIM1 gene in An. quadriannulatus, which was not part of the dsRNA-targeted sequence: LRIM1 988 F, ATCGGCAGGAGCGCAAG; LRIM1 1530 R, TTATCCACGTGGCTGCTAAATTCTG.

qRT-PCR was performed as described previously [27], with the following modifications. Total RNA was extracted from approximately 10 adult mosquitoes with 1 ml of TRIzol reagent (Invitrogen) and treated with Turbo DNAfree (Ambion) according to the manufacturer’s directions. 1 μg of total RNA was used for reverse transcriptions using Superscript II (Invitrogen). Transcript abundance was measured with an Applied Biosystems 7700 Real-Time PCR system using the ribosomal S7 gene as an internal control. Reactions of 25 μl consisted of 1× SYBR green mix (Applied Biosystems) and cDNA, corresponding to 2.5 ng of total RNA. The primer sequences and concentrations in the final reaction are: LRIM1 1914 F (0.9 μM), CATCCCGGATTGG-GATATGTG; LRIM1 1983 R (0.9 μM), CTTCTTTGACCCGTGT-CATTTC; LRIM2 825 F (0.9 μM), GGCAAGAAGTGACAAAGCGG; LRIM2 884 R (0.3 μM), CGCTGTCAGGGCAATGTA; TEP1 2676 F (0.9 μM), AAGGCTTGTGACCACGG; TEP1 2750 R (0.3 μM), TTCTCCACACACACCAAC; S7 R (0.3 μM), GTCGCCAGTTGGAGAAGA; S7 R (0.3 μM): ATCGGTTTGGG-CAAGCCGTAT. 

For analysis of the data, the prevalence of infection and parasite density were treated as two independent infection variables, although they are likely to be partly connected. The prevalence data were analysed using the chi-square goodness-of-fit test, except for comparing the prevalence of *P. falciparum* infection between *An. quadriannulatus* and *An. gambiae* where Fisher exact test with Yates correction was used. For the analysis of density of oocysts and melanised ookinetes, mosquitoes showing no parasites (neither live oocysts nor melanised ookinetes) in their midguts were excluded, and the data were subjected to normality and homogeneity tests. As counts of both live and dead parasites (x) displayed right-skewed distributions, the geometric means were computed after data normalization by log10(x + 1) transformation. The log-transformed data from all replicates within a study (dataset) were analyzed by REML (Residual Maximum Likelihood) variance components analysis by fitting the mixed effect model. In this model, we treated mosquito species or the control kd status as a random component and mosquitoes fed 24 hrs earlier either on naïve or *P. berghei*-infected mice. Transcripts of the S7 ribosomal protein gene were used as internal normalization control. All data points for each gene were calibrated to the transcript levels in sugar-fed *An. gambiae*, which were set at 100%. The standard error of two independent experiments is shown.

Data analysis

For comparison of the prevalence of infection and parasite density, the data were treated as two independent infection variables, although they are likely to be partly connected. The prevalence data were analysed using the chi-square goodness-of-fit test, except for comparing the prevalence of *P. falciparum* infection between *An. quadriannulatus* and *An. gambiae* where Fisher exact test with Yates correction was used. For the analysis of density of oocysts and melanised ookinetes, mosquitoes showing no parasites (neither live oocysts nor melanised ookinetes) in their midguts were excluded, and the data were subjected to normality and homogeneity tests. As counts of both live and dead parasites (x) displayed right-skewed distributions, the geometric means were computed after data normalization by log10(x + 1) transformation. The log-transformed data from all replicates within a study (dataset) were analyzed by REML (Residual Maximum Likelihood) variance components analysis by fitting the mixed effect model. In this model, we treated mosquito species or the control kd status as a random component and mosquitoes fed 24 hrs earlier either on naïve or *P. berghei*-infected mice. Transcripts of the S7 ribosomal protein gene were used as internal normalization control. All data points for each gene were calibrated to the transcript levels in sugar-fed *An. gambiae*, which were set at 100%. The standard error of two independent experiments is shown.

For analysis of the data, the prevalence of infection and parasite density were treated as two independent infection variables, although they are likely to be partly connected. The prevalence data were analysed using the chi-square goodness-of-fit test, except for comparing the prevalence of *P. falciparum* infection between *An. quadriannulatus* and *An. gambiae* where Fisher exact test with Yates correction was used. For the analysis of density of oocysts and melanised ookinetes, mosquitoes showing no parasites (neither live oocysts nor melanised ookinetes) in their midguts were excluded, and the data were subjected to normality and homogeneity tests. As counts of both live and dead parasites (x) displayed right-skewed distributions, the geometric means were computed after data normalization by log10(x + 1) transformation. The log-transformed data from all replicates within a study (dataset) were analyzed by REML (Residual Maximum Likelihood) variance components analysis by fitting the mixed effect model. In this model, we treated mosquito species or the control kd status as a random component and mosquitoes fed 24 hrs earlier either on naïve or *P. berghei*-infected mice. Transcripts of the S7 ribosomal protein gene were used as internal normalization control. All data points for each gene were calibrated to the transcript levels in sugar-fed *An. gambiae*, which were set at 100%. The standard error of two independent experiments is shown.

Table S1 Prevalence of *An. quadriannulatus* and *An. gambiae* infection with *P. berghei*. Mosquito midguts were dissected 10 days post-infection and salivary glands were dissected 21–22 days post-infection to determine the prevalence of live oocysts and score the presence of sporozoites, respectively. Three independent experiments were performed. Prevalence values show the percentage of mosquitoes displaying *P. berghei* oocysts or salivary gland sporozoites, respectively; these values within each species were compared using the Chi-square goodness-of-fit test, n, number of midguts and salivary glands (SG); ns, not significant. 

*Acknowledgments*

We thank Maureen Coetzee and Willem Takken for providing us with the *An. quadriannulatus* colonies. We are grateful to Fotis C. Kafatos for fruitful discussions of the results, and to Robert E. Sinden for advice with the *P. falciparum* infections and critical reading of the manuscript.

*Author Contributions*

Conceived and designed the experiments: TH GC. Performed the experiments: TH MP AB. Analyzed the data: TH MP GC. Wrote the paper: GC.
References


