Molecular mechanisms of *Plasmodium* development in male and female *Anopheles* mosquitoes

Short title: Molecular mechanisms of *Plasmodium* development in mosquitoes

Asako Haraguchi¹, Makoto Takano¹, Jun Hakozaiki¹, Kazuhiko Nakayama¹, Sacré
Nakamura¹, Yasunaga Yoshikawa², Shinya Fukumoto³, Kodai Kusakisako¹, Hiromi Ikadai^{1*}

¹ Laboratory of Veterinary Parasitology, School of Veterinary Medicine, Kitasato University,
Towada, Aomori 034-8628, Japan

² Laboratory of Veterinary Biochemistry, School of Veterinary Medicine, Kitasato
University, Towada, Aomori 034-8628, Japan

³ National Research Center for Protozoan Diseases, Obihiro University of Agriculture and
Veterinary Medicine, Inada, Obihiro 080-8555, Japan

*Corresponding author

E-mail: ikadai@vmas.kitasato-u.ac.jp (HI)

Abstract

Vector competence influences the ability of *Anopheles* mosquitoes to transmit *Plasmodium* parasites. The aim of this study was to determine the competence of male and female *Anopheles* mosquitoes to support the development of *Plasmodium* parasites. Male and female *A. stephensi* (STE2 strain) were infected with *in vitro*-cultured *P. berghei* (ANKA strain) ookinetes. We found that the number of oocysts produced was higher in males than in females. RNA-seq analysis of male and female mosquitoes injected with *P. berghei* ookinetes showed that predominantly genes of unknown function changed in expression levels in response to ookinete infection; however, further studies are required to elucidate their functions. Moreover, male mosquitoes were injected with *in vitro*-cultured *P. falciparum* (3D7 strain) gametocytes or zygotes. The development of *P. falciparum* in males was detected using nested polymerase chain reaction. We found the DNA content was higher on day 15 than on day 0, indicating that *P. falciparum* developed in the mosquito hemocoel. This study revealed promising new mechanisms underlying the interactions between *Plasmodium* and mosquitoes.

Keywords: vector competence; malaria; *Plasmodium berghei*; *Plasmodium falciparum*; *Anopheles stephensi*

Author Summary

Anopheles mosquitoes transmit *Plasmodium* parasites that cause malaria disease. Vector competence is the ability of vectors to support pathogen development. The aim of this study was to determine the competence of male and female mosquitoes for *Plasmodium* parasites. Oocysts were formed in both male and female mosquitoes injected with ookinetes

from *in vitro*-cultured *Plasmodium berghei*, which causes malaria in mice. The number of oocysts was higher in males, indicating that the competence in males was higher than that in females. RNA-seq analysis showed that the expression of genes of unknown function was highly variable in males and females. The genes defining the competence factors need further study, but the results indicate novel genes may be discovered. Furthermore, the development of *Plasmodium falciparum*, which causes the most severe malaria in humans, in male mosquitoes injected with *in vitro*-cultured gametocytes or zygotes was detected. As male mosquitoes do not suck blood, this method may allow us to experiment with *Plasmodium* more safely.

Introduction

Malaria is a serious protozoan disease that infects 200 million and kills 400 000 people per year [1]. It is caused by *Plasmodium* parasites transmitted by *Anopheles* mosquitoes [2–4]. Soon after feeding on an infected vertebrate host, the gametocytes form gametes that, after fertilization, give rise to zygotes that, within approximately 24 h, develop into motile ookinetes. Ookinetes traverse the mosquito midgut epithelial cells and differentiate into early oocysts attached to the basal side of the epithelium, facing the hemocoel. Within 10–15 d, the oocysts mature, releasing thousands of sporozoites into the hemolymph. These sporozoites invade the salivary glands and are transmitted to the next host when mosquitoes feed on another vertebrate host.

Less than one-tenth of the gametocytes ingested by a female mosquito become ookinetes, and less than one-tenth of the ookinetes develop into oocysts [3]. Thus, *Plasmodium* parasites undergo high losses during their development in the mosquito gut. This is a vulnerable period in the life cycle of the parasites and a vulnerable stage for transmission control [3,5,6].

Differences in susceptibility to pathogens between male and female hosts have been reported in various animal species. Male mice are more susceptible to *Babesia microti* and *P. berghei* than females [7,8]. Vector competence is the ability of vectors to support pathogen development and is one of the factors that define the ability of vectors to transmit pathogens [2,9]. In the dipteran tsetse fly (*Glossina morsitans*), a blood-feeder and pathogen vector like the mosquito, both males and females feed on blood and transmit *Trypanosoma brucei*. Male and female vector competence differ, with male salivary glands being more conducive to *T. brucei* development than female salivary glands [10]. Male mosquitoes do not feed on blood, and there are no reports examining male mosquito competence.

It has been reported that *in vitro*-cultured *P. berghei* ookinetes microinjected into the body cavity (hemocoel) of female *Anopheline* mosquitoes develop into oocysts and form sporozoites [11,12]. Microinjection of *P. gallinaceum* ookinetes into the dipteran fruit fly (*Drosophila melanogaster*) also yields oocysts and sporozoites [13]. Fruit flies are not natural hosts, and oocysts are not formed via blood feeding or oral ingestion of ookinetes; however, oocysts and sporozoites are formed when microinjected into the hemocoel, which indicates that *P. gallinaceum* cannot traverse the fruit fly midgut wall [13]. Moreover, that macrophages in the fruit fly act as a *P. gallinaceum* exclusion mechanism was clarified by studying fruit flies microinjected with *P. gallinaceum* in detail [13]. Thus, microinjection can be used to study the *Plasmodium* exclusion system or vector competence. Recently, RNA-seq analysis has emerged as a simple and powerful tool for investigating the *Plasmodium* vector competence of mosquitoes [14,15].

The aim of this study was to compare the competence of male and female *Anopheles* mosquitoes for *Plasmodium* by determining oocyst numbers and conducting RNA-seq analysis to investigate underlying molecular mechanisms.

Results

Development of *P. berghei* in male mosquitoes injected with ookinetes

P. berghei oocysts were formed in male and female mosquitoes at 14 d post injection of 2000 ookinetes (Fig 1A). Oocysts were formed in the esophagus, dorsal diverticula, crop, foregut, midgut, Malpighian tubules, hindgut, rectum, and inner abdominal wall of both males and females. At the esophagus, dorsal diverticula, and crop, a median of 42.5 and 9.5 oocysts formed in males and females, respectively. At the foregut and midgut, a median of 9.5 and 6.0 oocytes was observed in males and females, respectively. In the Malpighian tube, hindgut, and rectum, a median of 18.5 and 5.0 oocytes was detected in males and females, respectively. Last, at the abdominal wall, a median of 60.5 and 30.0 oocytes formed in males and females, respectively (Fig 1B). Oocysts were formed mostly at the esophagus, dorsal diverticula, crop, and abdominal wall in both males and females. In addition, more oocysts were formed in males than in females at every site. At 28 d post injection, *P. berghei* sporozoites were associated with the salivary glands, dorsal diverticula, crop, wings, and legs in males, and with the salivary glands, wings, and legs in females (Fig 1C). Mice infection was confirmed at 4 to 6 d post-tail-vein administration of 2000–10000 sporozoites from the salivary glands and whole body of male- and female-injected mosquitoes (S1 Table). When mice were fed with injected females, infection was confirmed 3 d post-blood feeding, indicating that *P. berghei* sporozoites formed in both males and females were infectious to mice.

Fig 1. Oocyst and sporozoite formation in male and female mosquitoes injected with *Plasmodium berghei* ookinetes. (A) Oocysts in male and female mosquitoes viewed with a

fluorescence microscope at 14 d after injection with 2000 GFP-expressing *P. berghei* ookinetes. DIC, differential interference contrast microscopy. (B) Number of oocysts formed at each site in males (n = 20) and females (n = 20). Mosquitoes were dissected at 14 d post injection. EP: esophagus, dorsal diverticula, and crop; MG: foregut and midgut; HG: Malpighian tube, hindgut, and rectum; AW: abdominal wall. The horizontal bar indicates the median value. Mann–Whitney test was used for comparison (** $p < 0.01$). Data are expressed as the pooled results of replicates. (D) Sporozoites in male and female mosquitoes under a fluorescence microscope at 28 d post injection with 2000 *P. berghei* GFP-expressing ookinetes.

Higher oocyst number in male than in female mosquitoes

When 2000 ookinetes were injected into male and female mosquitoes, a median of 132 and 51 oocysts formed in males and females, respectively (Fig 2A). When 5000 ookinetes were injected, the median number of oocysts was 125 and 57 in males and females, respectively (Fig 2B). In both set-ups, the number of oocysts was significantly higher in males than in females ($p < 0.01$ – 0.05) and the prevalence was 100% in all mosquito groups.

Fig 2. Oocyst numbers in male and female mosquitoes injected with *Plasmodium berghei* ookinetes. (A) Oocysts were counted at 14 d post injection of 2000 ookinetes in males (n = 28) and females (n = 24). (B) Oocysts were counted at 14 d post injection of 5000 ookinetes in males (n = 34) and females (n = 30). Horizontal bars indicate median values. Mann–Whitney test was used for comparison (* $p < 0.05$, ** $p < 0.01$). Data are expressed as the pooled results of three replicates.

RNA-seq analysis of male and female mosquitoes

To explore the genes that regulate *P. berghei* infection, we performed RNA-seq analysis of male and female mosquitoes at 24 h post injection, when ookinetes had differentiated into oocysts. More genes were differentially regulated in females than in males injected with 5 000 *P. berghei* ookinetes. In total, 140 genes were upregulated and 89 were downregulated in males, whereas 304 were upregulated and 167 were downregulated in females (fold change $\geq |2|$) (Fig 3A).

Fig 3. RNA-seq analysis of mosquitoes injected with *Plasmodium berghei* ookinetes. (A) Number of upregulated and downregulated genes in mosquitoes at 24 h post injection of 5000 *P. berghei* ookinetes (fold change $\geq |2|$). Up: upregulated gene, Down: downregulated gene. (B) Percentage of variable genes annotated as biological process, cellular component, molecular function, or unknown function in gene ontology analysis. (C) Detailed functions of genes categorized as biological process.

Gene Ontology (GO) analysis showed there were 668 genes of known function and 193 genes with unknown functions. In males, 29.78% were classified as biological processes, 17.15% as cellular components, 25.56% as molecular functions, and 27.51% with unknown functions, whereas in females, the percentages were 30.74%, 21.43%, 24.90%, and 22.93%, respectively (Fig 3B). Among the biological processes, metabolic processes were the most common in both males and females, whereas immune system processes were observed at 0.76% in males and 0.48% in females (Fig 3C). The top 20 upregulated and downregulated genes in males and females are shown in Table 1. Most have unknown functions in both sexes.

Table 1. Genes with the most variable expression in male and female mosquitoes injected with *Plasmodium berghei* ookinetes.

Male				Female			
Gene ID	Type	Fold change	Function	Gene ID	Type	Fold change	Function
Upregulated							
ASTEI08011	PC	840.051	Unspecified	ASTEI06560	PC	56.734	Unspecified
ASTEI05475	PC	15.863	Unspecified	ASTEI01256	PC	41.215	Unspecified
ASTEI02008	PC	12.868	Unspecified	ASTEI11350	PC	40.660	Unspecified
ASTEI07456	PC	8.058	Unspecified	ASTEI05300	PC	33.923	Unspecified
ASTEI04711	PC	7.873	Unspecified	ASTEI02744	PC	28.089	Unspecified
ASTEI10579	PC	7.053	Unspecified	ASTEI01747	PC	24.236	Unspecified
ASTEI05300	PC	6.884	Unspecified	ASTEI02745	PC	20.332	Unspecified
ASTEI11350	PC	6.265	Unspecified	ASTEI02028	PC	20.188	Unspecified
ASTEI00942	PC	6.252	Insulin-like peptide 5	ASTEI05302	PC	17.607	Unspecified
ASTEI06560	PC	6.009	Unspecified	ASTEI10660	PC	17.323	Unspecified
ASTEI05299	PC	5.973	Unspecified	ASTEI07614	PC	13.962	Unspecified
ASTEI00668	PC	5.743	Unspecified	ASTEI01070	PC	13.674	Unspecified
ASTEI10541	PC	5.615	Unspecified	ASTEI10165	PC	12.857	Unspecified
ASTEI10164	PC	5.194	Unspecified	ASTEI05298	PC	10.623	Unspecified
ASTEI06287	PC	5.152	Transferrin	ASTEI05299	PC	10.281	Unspecified
ASTEI08428	PC	5.083	Unspecified	ASTEI04630	PC	8.567	Unspecified
ASTEI06585	PC	5.079	Unspecified	ASTEI04764	PC	8.551	Unspecified
ASTEI02745	PC	4.912	Unspecified	ASTEI02027	PC	8.522	Unspecified

ASTEI05683						
1	PC	4.908	Unspecified	ASTEI10751PC	8.144	Unspecified
ASTEI11561	PC	4.720	Unspecified	ASTEI00445PC	8.110	Unspecified
Downregulated						
ASTEI10516	PC	-6.443	Unspecified	ASTEI08024PC	-6.875	Unspecified
ASTEI11598	PC	-5.533	Unspecified	ASTEI01867PC	-6.824	Unspecified
ASTEI07580	PC	-5.187	Osiris 11	ASTEI08168PC	-5.130	Unspecified
ASTEI08089	PC	-4.601	Unspecified	ASTEI12087SR	-4.499	U2 spliceosomal RNA
ASTEI04108	PC	-4.359	Unspecified	ASTEI04832PC	-4.428	Unspecified
ASTEI11712	PC	-3.838	Innexin	ASTEI02029PC	-3.944	Unspecified
ASTEI07542	PC	-3.786	Unspecified	ASTEI01289PC	-3.725	Unspecified
			FLYWCH-type			
ASTEI06186	PC	-3.394	domain-containing protein	ASTEI01294PC	-3.716	Unspecified
ASTEI01075	PC	-3.232	Unspecified	ASTEI12263SR	-3.484	U2 spliceosomal RNA
ASTEI06944	PC	-3.101	Unspecified	ASTEI10808PC	-3.300	Histone H2A
ASTEI10499	PC	-3.068	Unspecified	ASTEI10804PC	-3.253	Histone H2A
ASTEI11858	SR	-2.979	U6 spliceosomal RNA	ASTEI01619PC	-3.249	Unspecified
ASTEI07844	PC	-2.884	Unspecified	ASTEI00798PC	-3.207	Unspecified
			Phosphatidylserine			
ASTEI08071	PC	-2.865	decarboxylase proenzyme, mitochondrial	ASTEI02104PC	-3.144	Leucine rich immune protein (Short)

ASTEI11218	PC	-2.855	Unspecified	ASTEI02414	PC	-3.074	Unspecified
ASTEI02041	PC	-2.808	Unspecified	ASTEI03548	PC	-3.071	Unspecified
ASTEI08776	PC	-2.775	Unspecified	ASTEI11477	PC	-2.989	Unspecified
ASTEI08651	PC	-2.752	Unspecified	ASTEI02978	PC	-2.977	Unspecified
ASTEI09420	PC	-2.743	Unspecified	ASTEI04415	PC	-2.911	Unspecified
ASTEI00613	PC	-2.629	Unspecified	ASTEI02025	PC	-2.902	Angiotensin- converting enzyme

The top 20 upregulated and downregulated genes in males and females are listed in order of

the magnitude of change. Genes with unknown function are marked as unspecified.

PC: Protein coding, SR: SnRNA

Detection of *P. falciparum* in male mosquitoes injected with gametocytes or zygotes

Male mosquitoes were injected with *P. falciparum* gametocytes or zygotes, and parasitic DNA in mosquitoes was measured on different days after injection. *P. falciparum* DNA was detected by nested polymerase chain reaction (PCR) up to 15 d post injection (Table 2). For 20 stage-V gametocytes injected into males, the detection rate of *P. falciparum* was 100% on day 0 and 30% on day 15. As for the 2, 1, and 1 stage-V gametocytes, the detection rates were 100.0%, 50.0%, and 81.3%, respectively, on day 0, and 30%, 0%, and 0%, respectively, on day 15. For 50 zygotes injected, the detection rate was 100% on days 0 to 11. Eight PCR-positive samples were sequenced and confirmed to be the *P. falciparum* 18S rRNA gene. In addition, the DNA content of *P. falciparum* in mosquitoes injected with 20 stage-V gametocytes was higher on day 15 than on day 0 (S1 Fig).

Table 2. Detection of *Plasmodium falciparum* in male mosquitoes injected with gametocytes or zygotes using nested polymerase chain reaction (PCR).

Injected parasites /mosquito	Positive rate of PCR [%]				
	(PCR-positive mosquitoes/total mosquitoes)				
	Days post injection				
	0	1	11	12	15
20 gametocytes	100 (12/12)	100 (2/2)	ND	ND	30 (3/10)
2 gametocytes	100 (6/6)	0 (0/2)	ND	ND	30 (3/10)
1 gametocyte	50 (1/2)	71 (5/7)	ND	ND	0 (0/2)
1 gametocyte	81 (13/16)	40 (4/10)	0 (0/1)	10 (1/10)	0 (0/17)
50 zygotes	100 (13/13)	100 (6/6)	100 (1/1)	ND	ND

Male mosquitoes were injected with the indicated number of parasites and at the indicated days, some mosquitoes were homogenized and assayed by PCR for the presence of *P. falciparum* 18S rRNA genes. Number of positive mosquitoes out of number analyzed is presented in parentheses. ND: No data

Discussion

In this study, *P. berghei* oocysts formed throughout the body in male and female mosquitoes injected with ookinetes, particularly in the esophagus, dorsal diverticula, crop, and inner abdominal wall. In blood-feeding female mosquitoes, *P. berghei* ookinetes transform into oocysts by migrating through midgut epithelial cells to face the hemocoel [2–4]. It has been reported that *P. berghei* ookinetes injected into the female *A. gambiae* hemocoel can develop into oocysts anywhere in the hemocoel [11,12], consistent with the observations of this study. However, this study demonstrated that ookinetes can develop into

oocysts in both male and female *A. stephensi* after injection. Many parasites migrate to specific tissues [16]; ookinetes injected into the hemocoel did not exhibit tissue specificity, suggesting that they migrated and adhered randomly to an available surface.

Furthermore, sporozoites were formed throughout the body at 28 d post injection and infected mice after administration into their tail vein or via mosquito blood feeding. Mosquitoes have an open circulatory system, and hemolymph from the dorsal vessels promotes circulation throughout the hemocoel [17,18]. Sporozoites move passively in the hemocoel following the hemolymph flow, attach to the salivary glands, and migrate into the salivary cavity [19]. They can be found attached to various tissues when mosquitoes are infected by blood feeding [5]. Thus, it is likely that sporozoites were transported throughout the mosquito body following the hemolymph flow and invaded the salivary glands.

Male mosquitoes exhibited more *P. berghei* oocysts than females at every site. The number of total oocysts in males was significantly higher than that in females ($p < 0.01$ – 0.05), indicating that males exhibit greater competence than females. Moreover, injection of increased numbers of ookinetes did not increase the number of oocysts in both male and female mosquitoes. This suggests that a mosquito has a limited capacity to form an oocyst, or parasites can increase only up to a certain number; these results require further analysis. We attempted to investigate competence factors at the early oocyst stage using RNA-seq analysis at 24 h post injection. There were more gene expression changes in female than in male mosquitoes, those annotated to metabolic processes being the most common. Possibly, these genes participate in blocking early oocyst formation. Most up and downregulated genes in males and females have unknown functions, and differences in competence between males and females may be owing to these unknown-function genes. Vector competence in mosquitoes has been reported to be associated with many biological and environmental factors, such as physiological function, nutritional status, gut microbiota, and immune

response [2,9]. Previous reports have compared strains or species of *Anopheles* mosquitoes with varying competence for *Plasmodium* parasites, indicating the involvement of the immune system and other factors [20–23]. However, in these studies, it was difficult to equalize genetic backgrounds and growth environments of mosquitoes, exclude these factors, and compare the competence factors. Therefore, the detailed molecular mechanisms that define vector competence remain unclear. As male and female mosquitoes of the same species and strain (e.g., *A. stephensi* [STE2 strain, MRA-128]), have the same genetic background and growth environment, a detailed examination of the genes revealed by the comparison of males and females in this study could unveil new facts concerning the molecular mechanisms of competence.

This study showed that *P. falciparum* infects male mosquitoes injected with gametocytes and zygotes, indicating that the development of gametocytes to ookinetes, which normally occurs in the midgut, is midgut-independent and occurs in the hemocoel. In blood-feeding mosquitoes, gametocytes differentiate into mature oocysts with sporozoites in 10–15 d [2–4]. The injection of *P. falciparum* gametocytes and zygotes showed infection at 15- and 11-d post injection, respectively. In addition, the quantity of *P. falciparum* DNA was greater on day 15 than on day 0 of gametocyte injection. There were differences among individuals, suggesting that mature oocysts with sporozoites formed in male mosquitoes. To confirm the development of a *P. falciparum* gametocyte, it is necessary to observe ookinetes, oocysts, and sporozoites in the injected mosquitoes. Currently, sporozoite vaccines to prevent the spread of *Plasmodium* parasites are being developed; however only one type of vaccine is currently in use, and more research on oocysts, which produce sporozoites internally, and sporozoites, which are infectious to humans and develop inside mosquitoes, is required to enable effective vaccine development [24,25]. A method for the *in vitro* culture of *P. falciparum* oocysts and sporozoites has been reported [26,27]; however, it is complicated and cumbersome, and the

number of oocysts and sporozoites obtained is low. Injection of gametocytes into male mosquitoes may be a simpler way to develop oocysts and sporozoites, and as male mosquitoes are used, there is no risk of infection by blood feeding. This is expected to advance the study of the mosquito stages of these parasites.

Conclusion

This study reveals that *P. berghei* ookinete injections into male and female mosquitoes, result in males having a higher competence than females. The differentially regulated genes in these males and females provide insight into the underlying molecular mechanisms involved in vector competence. In addition, *P. falciparum* infection succeeded in males when injected with gametocytes and zygotes. Hence, this study helps elucidate the factors involved in vector competence and access the mosquito stages of parasites more safely.

Materials and Methods

Mosquito rearing and microinjection of parasites

STE2 strain *A. stephensi* mosquitoes were reared in an insectary at 19 °C under a 14:10 h light:dark cycle with 10% (w/v) sucrose solution. A total of 69 nL of purified ookinete, gametocyte, and zygote suspensions was injected into the hemocoel of <10-day-old male and female mosquitoes using a Nanoject II automatic nanoliter injector (Drummond Scientific Company, Broomall, PA, USA) [11].

P. berghei and ookinete culture

P. berghei (ANKA strain) that constitutively expresses green fluorescent protein (GFP) was maintained via serial passage in ICR mice (Charles River Laboratories Japan Inc., Yokohama, Japan) from frozen stock. Ookinete culture of *P. berghei* was performed as previously described [28,29]. Blood from infected mice was collected via cardiac puncture and placed in 10 volumes of ookinete medium containing RPMI medium 1640 (Thermo Fisher Scientific Inc., Waltham, MA, USA), 25 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), 0.4 mM hypoxanthine (Sigma-Aldrich, St. Louis, MO, USA), 24 mM NaHCO₃ (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), and 12.5 mg L⁻¹ gentamicin reagent solution (Gibco) before incubation for 24 h at 19 °C.

***P. falciparum* and culture of gametocytes and zygotes**

In vitro culture of *P. falciparum* (3D7 strain) was performed according to the standard protocol [30] and maintained at 5% hematocrit in A⁺ human red blood cells (RBCs) on a complete medium at 37 °C under 5% CO₂ and 5% O₂. The complete medium comprised RPMI medium 1640, 25 mM HEPES, 0.1 mM hypoxanthine, 24–26 mM NaHCO₃, 11 mM D(+)-glucose (Fujifilm Wako Pure Chemical Corporation), 12.5 mg L⁻¹ gentamicin reagent solution, and 10% (v/v) heat-inactivated type A⁺ human plasma (Japan Red Cross Society, Tokyo, Japan). Gametocyte cultures were initiated from asexual parasites at 0.5–3.9% parasitemia and 6–10% hematocrit. The cultures were maintained for 14–27 d with daily medium changes without adding fresh RBCs. From day 1 to 5, the culture medium was supplemented with N-acetyl-D-glucosamine (Sigma-Aldrich) at a final concentration of 50 nM. Mature gametocytes were resuspended at 20% hematocrit in an exflagellation-inducing medium containing 10 mM Tris (pH 7.6, Fujifilm Wako Pure Chemical Corporation), 170 mM NaCl (Fujifilm Wako Pure Chemical Corporation), 25 mM NaHCO₃, 10 mM D(+)-

glucose, and 50–100 μ M xanthurenic acid (Sigma-Aldrich) at 19 °C for 30 min for gamete maturation and zygote fertilization.

Purification of parasites

P. berghei ookinetes and *P. falciparum* gametocytes and zygotes were purified using a MidiMACS separator system (LS Columns, Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described [28,29]. Ookinetes, gametocytes, and zygotes were recovered by passing ookinete medium, incomplete medium comprising complete medium without 10% (v/v) heat-inactivated type A⁺ human plasma, and exflagellation-inducing medium, respectively.

Examination of mosquitoes injected with *P. berghei*

Male and female mosquitoes were dissected at 14 d post injection, and oocyst localization, number, and prevalence were examined using an Eclipse E600 (Nikon, Tokyo, Japan) fluorescence microscope. At 28 d post injection, sporozoite localization was observed using a fluorescence microscope. Sporozoites from whole bodies or salivary glands of the injected males and females were administered into the tail vein of mice. Seven injected females were blood-fed on mice, and the infection in the mice was checked every day using a blood smear test.

DNA extraction

Mosquitoes with *P. falciparum* gametocytes or zygotes from 0 to 15 d post injection were collected and stored at –80 °C. Whole mosquitoes were homogenized using a Micro SmashTM MS-100R (Tomy Seiko Co., Ltd., Tokyo, Japan) at 2500 rpm for 30 s. DNA was

extracted using a ZymoBIOMICS Miniprep (Zymo Research Corporation, Irvine, CA, USA) according to the manufacturer's protocol or using the phenol-chloroform isoamyl alcohol method. Briefly, a 500 µL genomic lysis buffer (Zymo Research Corporation) and 500 µL of phenol:chloroform:isoamyl alcohol (25:24:1) (Nippon Gene Co., Ltd., Tokyo, Japan) were added to extract the DNA, and then the DNA was precipitated using ethanol. The extracted DNA was dissolved with 10–50 µL of TE buffer and stored at -20 °C.

Detection of *P. falciparum* using nested PCR

Prime STAR® HS (Premix) (Takara Bio Inc., Shiga, Japan) was used for PCR amplification, according to the manufacturer's protocol, and the extracted DNA samples were used as template DNA. For *A. stephensi* S7 gene (loading control) amplification, PCR was conducted using primers AsRpS7-F (5'-CCTGGATAAGAACCAGCAGA-3') and AsRpS7-R (5'-GGCCAGTCAGCTTCTTGTA-3'). The PCR conditions were as follows: 1 cycle of 95 °C for 3 min, followed by 35 cycles of 98 °C for 10 s, 55 °C for 5 s, and 72 °C for 18 s, and 1 cycle of 72 °C for 6 min. Distilled water as a negative control. Amplification of the *P. falciparum* 18S rRNA gene for detecting *P. falciparum* was conducted using nested PCR. The primers for the first PCR reaction were 18S rRNA-1F (5'-TTAATTTGACTCAACACGGGG-3') and 18S rRNA-1R (5'-TATTGATAAAGATTACCTA-3') [13]. The PCR conditions were as follows: 1 cycle of 95 °C for 3 min, followed by 40 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 33 s, and 1 cycle of 72 °C for 6 min. The primers for the second reaction were 18S rRNA-2F (5'-TAATAGCTCTTTCTTGAT-3') and 18S rRNA-1R. The PCR conditions were as follows: 1 cycle of 95 °C for 3 min, followed by 35 cycles of 98 °C for 10 s, 55 °C for 15 s, and 72 °C for 30 s, and then 1 cycle of 72 °C for 6 min. Genomic DNA from *in vitro*-cultured *P.*

falciparum was used as a positive control and the 7–11-d-old male mosquitoes as a negative control. Eight PCR-positive samples were sequenced using 18S rRNA-2F and 18S rRNA-1R primers (Fasmac Co., Ltd., Kanagawa, Japan).

Real-time PCR

Among the PCR-positive mosquitoes injected with 20 stage-V gametocytes, DNA was quantified in three mosquitoes at 0 and 15 d post injection using KOD SYBR qPCR Mix (Toyobo Co., Ltd., Osaka, Japan), and 18S rRNA-3F (5'-GGATGGTGATGCATGGCCG-3') and 18S rRNA-2R (5'-CAGTGTAGCACGCGTGCAG-3') primers. The analysis was conducted using Applied Biosystems StepOnePlus (Thermo Fisher Scientific) with the following cycling conditions: 1 cycle at 98 °C for 3 min, followed by 40 cycles of 98 °C for 10 s, 60 °C for 10 s, and 68 °C for 30 s, and 1 cycle of 95 °C for 15 s, 60 °C for 1 min, and 99 °C for 15 s. *P. falciparum* DNA was quantified using a calibration curve, and each value was corrected by dividing by the quantity of template DNA used in the PCR reaction.

RNA-seq analysis

Male and female mosquitoes at 24 h post injection with *P. berghei* ookinetes or with ookinete medium (negative control) were collected for RNA-seq analysis. Three mosquitoes were collected as one sample and stored at -80 °C. Total RNA was extracted using Nucleo Spin RNA Blood (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol, delivered to MacroGen Japan Corp. (Tokyo, Japan) for library construction using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, CA, USA), and sequenced using the NovaSeq 6000 system (Illumina). FastQCv0.11.7 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>) was used to analyze the raw

read quality, and statistical analysis was performed using fold change per comparison pair of mosquitoes injected with *P. berghei* ookinetes and with ookinete medium. GO analysis was performed using VectorBase (<https://www.vectorbase.org/>).

Statistical analysis

All statistical analyses were performed using GraphPad Prism version 8.4.3 software (GraphPad Software Inc., San Diego, CA, USA). We determined the significance of oocyst intensity in injected mosquitoes using the Mann–Whitney test ($p < 0.01$ – 0.05) and the significance of DNA content for real-time PCR using the unpaired t-test ($p < 0.05$).

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Supporting Information

S1 Fig. *Plasmodium falciparum* DNA content in male mosquitoes injected with gametocytes. One mosquito injected with 20 stage-V gametocytes was used in this experiment. The mean DNA content on day 0 post injection was set as 1.0, and that on day 15

post injection was 7.3. Unpaired t-test was used for comparison ($p < 0.05$). Data are from three biological replicates.

S1 Table. Days confirming infection by administration of sporozoite of male and female-injected mosquito. At 28 d post injection, 2000–10000 *P. berghei* sporozoites from whole bodies or salivary glands of the injected males and females, respectively, were administered into the tail vein of mice. Seven injected females were blood-fed on mice, and the infection in the mice was checked every day using a blood smear test. I.V.: administration into the tail vein.

Figure 1

Figure 2

A

B

C

Figure 3