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

5

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

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22 # Abstract

23 Vector competence influences the ability of *Anopheles* mosquitoes to transmit
24 *Plasmodium* parasites. The aim of this study was to determine the competence of male and
25 female *Anopheles* mosquitoes to support the development of *Plasmodium* parasites. Male and
26 female *A. stephensi* (STE2 strain) were infected with *in vitro*-cultured *P. berghei* (ANKA
27 strain) ookinetes. We found that the number of oocysts produced was higher in males than in
28 females. RNA-seq analysis of male and female mosquitoes injected with *P. berghei* ookinetes
29 showed that predominantly genes of unknown function changed in expression levels in
30 response to ookinete infection; however, further studies are required to elucidate their
31 functions. Moreover, male mosquitoes were injected with *in vitro*-cultured *P. falciparum*
32 (3D7 strain) gametocytes or zygotes. The development of *P. falciparum* in males was
33 detected using nested polymerase chain reaction. We found the DNA content was higher on
34 day 15 than on day 0, indicating that *P. falciparum* developed in the mosquito hemocoel. This
35 study revealed promising new mechanisms underlying the interactions between *Plasmodium*
36 and mosquitoes.

37

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

40

41 # Author Summary

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

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

55

56 # Introduction

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

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

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71 Differences in susceptibility to pathogens between male and female hosts have been
72 reported in various animal species. Male mice are more susceptible to *Babesia microti* and *P.*
73 *berghei* than females [7,8]. Vector competence is the ability of vectors to support pathogen
74 development and is one of the factors that define the ability of vectors to transmit pathogens
75 [2,9]. In the dipteran tsetse fly (*Glossina morsitans*), a blood-feeder and pathogen vector like
76 the mosquito, both males and females feed on blood and transmit *Trypanosoma brucei.* Male
77 and female vector competence differ, with male salivary glands being more conducive to *T.*
78 *brucei* development than female salivary glands [10]. Male mosquitoes do not feed on blood,
79 and there are no reports examining male mosquito competence.

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

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

95

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96 # Results

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

99 *P. berghei* oocysts were formed in male and female mosquitoes at 14 d post injection
100 of 2000 ookinetes (Fig 1A). Oocysts were formed in the esophagus, dorsal diverticula, crop,
101 foregut, midgut, Malpighian tubules, hindgut, rectum, and inner abdominal wall of both
102 males and females. At the esophagus, dorsal diverticula, and crop, a median of 42.5 and 9.5
103 oocysts formed in males and females, respectively. At the foregut and midgut, a median of
104 9.5 and 6.0 oocytes was observed in males and females, respectively. In the Malpighian tube,
105 hindgut, and rectum, a median of 18.5 and 5.0 oocytes was detected in males and females,
106 respectively. Last, at the abdominal wall, a median of 60.5 and 30.0 oocytes formed in males
107 and females, respectively (Fig 1B). Oocysts were formed mostly at the esophagus, dorsal
108 diverticula, crop, and abdominal wall in both males and females. In addition, more oocysts
109 were formed in males than in females at every site. At 28 d post injection, *P. berghei*
110 sporozoites were associated with the salivary glands, dorsal diverticula, crop, wings, and legs
111 in males, and with the salivary glands, wings, and legs in females (Fig 1C). Mice infection
112 was confirmed at 4 to 6 d post-tail-vein administration of 2000–10000 sporozoites from the
113 salivary glands and whole body of male- and female-injected mosquitoes (S1 Table). When
114 mice were fed with injected females, infection was confirmed 3 d post-blood feeding,
115 indicating that *P. berghei* sporozoites formed in both males and females were infectious to
116 mice.

117

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

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120 fluorescence microscope at 14 d after injection with 2000 GFP-expressing *P. berghei*
121 ookinetes. DIC, differential interference contrast microscopy. (B) Number of oocysts formed
122 at each site in males (n = 20) and females (n = 20). Mosquitoes were dissected at 14 d post
123 injection. EP: esophagus, dorsal diverticula, and crop; MG: foregut and midgut; HG:
124 Malpighian tube, hindgut, and rectum; AW: abdominal wall. The horizontal bar indicates the
125 median value. Mann–Whitney test was used for comparison (** $p < 0.01$). Data are
126 expressed as the pooled results of replicates. (D) Sporozoites in male and female mosquitoes
127 under a fluorescence microscope at 28 d post injection with 2000 *P. berghei* GFP-expressing
128 ookinetes.
129

130 ## Higher oocyst number in male than in female mosquitoes

131 When 2000 ookinetes were injected into male and female mosquitoes, a median of
132 132 and 51 oocysts formed in males and females, respectively (Fig 2A). When 5000
133 ookinetes were injected, the median number of oocysts was 125 and 57 in males and females,
134 respectively (Fig 2B). In both set-ups, the number of oocysts was significantly higher in
135 males than in females ($p < 0.01$–0.05) and the prevalence was 100% in all mosquito groups.
136

137 **Fig 2. Oocyst numbers in male and female mosquitoes injected with *Plasmodium berghei***
138 **ookinetes.** (A) Oocysts were counted at 14 d post injection of 2000 ookinetes in males (n =
139 28) and females (n = 24). (B) Oocysts were counted at 14 d post injection of 5000 ookinetes
140 in males (n = 34) and females (n = 30). Horizontal bars indicate median values.
141 Mann–Whitney test was used for comparison (* $p < 0.05$, ** $p < 0.01$). Data are expressed as
142 the pooled results of three replicates.
143

144 ## RNA-seq analysis of male and female mosquitoes

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145 To explore the genes that regulate *P. berghei* infection, we performed RNA-seq
146 analysis of male and female mosquitoes at 24 h post injection, when ookinetes had
147 differentiated into oocysts. More genes were differentially regulated in females than in males
148 injected with 5 000 *P. berghei* ookinetes. In total, 140 genes were upregulated and 89 were
149 downregulated in males, whereas 304 were upregulated and 167 were downregulated in
150 females (fold change ≥|2|) (Fig 3A).
151
152 **Fig 3. RNA-seq analysis of mosquitoes injected with *Plasmodium berghei* ookinetes.** (A)
153 Number of upregulated and downregulated genes in mosquitoes at 24 h post injection of 5000
154 *P. berghei* ookinetes (fold change ≥ |2|). Up: upregulated gene, Down: downregulated gene.
155 (B) Percentage of variable genes annotated as biological process, cellular component,
156 molecular function, or unknown function in gene ontology analysis. (C) Detailed functions of
157 genes categorized as biological process.
158
159 Gene Ontology (GO) analysis showed there were 668 genes of known function and
160 193 genes with unknown functions. In males, 29.78% were classified as biological processes,
161 17.15% as cellular components, 25.56% as molecular functions, and 27.51% with unknown
162 functions, whereas in females, the percentages were 30.74%, 21.43%, 24.90%, and 22.93%,
163 respectively (Fig 3B). Among the biological processes, metabolic processes were the most
164 common in both males and females, whereas immune system processes were observed at
165 0.76% in males and 0.48% in females (Fig 3C). The top 20 upregulated and downregulated
166 genes in males and females are shown in Table 1. Most have unknown functions in both
167 sexes.
168

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169 **Table 1. Genes with the most variable expression in male and female mosquitoes**

170 **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

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ASTEI056831	PC	4.908	Unspecified	ASTEI10751	PC	8.144	Unspecified
ASTEI11561	PC	4.720	Unspecified	ASTEI00445	PC	8.110	Unspecified
Downregulated							
ASTEI10516	PC	-6.443	Unspecified	ASTEI08024	PC	-6.875	Unspecified
ASTEI11598	PC	-5.533	Unspecified	ASTEI01867	PC	-6.824	Unspecified
ASTEI07580	PC	-5.187	Osiris 11	ASTEI08168	PC	-5.130	Unspecified
ASTEI08089	PC	-4.601	Unspecified	ASTEI12087	SR	-4.499	U2 spliceosomal RNA
ASTEI04108	PC	-4.359	Unspecified	ASTEI04832	PC	-4.428	Unspecified
ASTEI11712	PC	-3.838	Innexin	ASTEI02029	PC	-3.944	Unspecified
ASTEI07542	PC	-3.786	Unspecified	ASTEI01289	PC	-3.725	Unspecified
ASTEI06186	PC	-3.394	FLYWCH-type domain-containing protein	ASTEI01294	PC	-3.716	Unspecified
ASTEI01075	PC	-3.232	Unspecified	ASTEI12263	SR	-3.484	U2 spliceosomal RNA
ASTEI06944	PC	-3.101	Unspecified	ASTEI10808	PC	-3.300	Histone H2A
ASTEI10499	PC	-3.068	Unspecified	ASTEI10804	PC	-3.253	Histone H2A
ASTEI11858	SR	-2.979	U6 spliceosomal RNA	ASTEI01619	PC	-3.249	Unspecified
ASTEI07844	PC	-2.884	Unspecified	ASTEI00798	PC	-3.207	Unspecified
ASTEI08071	PC	-2.865	Phosphatidylserine decarboxylase proenzyme, mitochondrial	ASTEI02104	PC	-3.144	Leucine rich immune protein (Short)

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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).

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188 **Table 2. Detection of *Plasmodium falciparum* in male mosquitoes injected with**
189 **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

190 Male mosquitoes were injected with the indicated number of parasites and at the indicated
191 days, some mosquitoes were homogenized and assayed by PCR for the presence of *P.*
192 *falciparum* 18S rRNA genes. Number of positive mosquitoes out of number analyzed is
193 presented in parentheses. ND: No data
194

195 # Discussion

196 In this study, *P. berghei* oocysts formed throughout the body in male and female
197 mosquitoes injected with ookinetes, particularly in the esophagus, dorsal diverticula, crop,
198 and inner abdominal wall. In blood-feeding female mosquitoes, *P. berghei* ookinetes
199 transform into oocysts by migrating through midgut epithelial cells to face the hemocoel [2–
200 4]. It has been reported that *P. berghei* ookinetes injected into the female *A. gambiae*
201 hemocoel can develop into oocysts anywhere in the hemocoel [11,12], consistent with the
202 observations of this study. However, this study demonstrated that ookinetes can develop into

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203 oocysts in both male and female *A. stephensi* after injection. Many parasites migrate to
204 specific tissues [16]; ookinetes injected into the hemocoel did not exhibit tissue specificity,
205 suggesting that they migrated and adhered randomly to an available surface.
206 Furthermore, sporozoites were formed throughout the body at 28 d post injection and
207 infected mice after administration into their tail vein or via mosquito blood feeding.
208 Mosquitoes have an open circulatory system, and hemolymph from the dorsal vessels
209 promotes circulation throughout the hemocoel [17,18]. Sporozoites move passively in the
210 hemocoel following the hemolymph flow, attach to the salivary glands, and migrate into the
211 salivary cavity [19]. They can be found attached to various tissues when mosquitoes are
212 infected by blood feeding [5]. Thus, it is likely that sporozoites were transported throughout
213 the mosquito body following the hemolymph flow and invaded the salivary glands.
214 Male mosquitoes exhibited more *P. berghei* oocysts than females at every site. The
215 number of total oocysts in males was significantly higher than that in females ($p < 0.01$–
216 0.05), indicating that males exhibit greater competence than females. Moreover, injection of
217 increased numbers of ookinetes did not increase the number of oocysts in both male and
218 female mosquitoes. This suggests that a mosquito has a limited capacity to form an oocyst, or
219 parasites can increase only up to a certain number; these results require further analysis. We
220 attempted to investigate competence factors at the early oocyst stage using RNA-seq analysis
221 at 24 h post injection. There were more gene expression changes in female than in male
222 mosquitoes, those annotated to metabolic processes being the most common. Possibly, these
223 genes participate in blocking early oocyst formation. Most up and downregulated genes in
224 males and females have unknown functions, and differences in competence between males
225 and females may be owing to these unknown-function genes. Vector competence in
226 mosquitoes has been reported to be associated with many biological and environmental
227 factors, such as physiological function, nutritional status, gut microbiota, and immune

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

238 This study showed that *P. falciparum* infects male mosquitoes injected with
239 gametocytes and zygotes, indicating that the development of gametocytes to ookinetes, which
240 normally occurs in the midgut, is midgut-independent and occurs in the hemocoel. In blood-
241 feeding mosquitoes, gametocytes differentiate into mature oocysts with sporozoites in 10–15
242 d [2–4]. The injection of *P. falciparum* gametocytes and zygotes showed infection at 15- and
243 11-d post injection, respectively. In addition, the quantity of *P. falciparum* DNA was greater
244 on day 15 than on day 0 of gametocyte injection. There were differences among individuals,
245 suggesting that mature oocysts with sporozoites formed in male mosquitoes. To confirm the
246 development of a *P. falciparum* gametocyte, it is necessary to observe ookinetes, oocysts, and
247 sporozoites in the injected mosquitoes. Currently, sporozoite vaccines to prevent the spread
248 of *Plasmodium* parasites are being developed; however only one type of vaccine is currently
249 in use, and more research on oocysts, which produce sporozoites internally, and sporozoites,
250 which are infectious to humans and develop inside mosquitoes, is required to enable effective
251 vaccine development [24,25]. A method for the *in vitro* culture of *P. falciparum* oocysts and
252 sporozoites has been reported [26,27]; however, it is complicated and cumbersome, and the

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253 number of oocysts and sporozoites obtained is low. Injection of gametocytes into male
254 mosquitoes may be a simpler way to develop oocysts and sporozoites, and as male
255 mosquitoes are used, there is no risk of infection by blood feeding. This is expected to
256 advance the study of the mosquito stages of these parasites.
257

258 Conclusion

259 This study reveals that *P. berghei* ookinete injections into male and female
260 mosquitoes, result in males having a higher competence than females. The differentially
261 regulated genes in these males and females provide insight into the underlying molecular
262 mechanisms involved in vector competence. In addition, *P. falciparum* infection succeeded in
263 males when injected with gametocytes and zygotes. Hence, this study helps elucidate the
264 factors involved in vector competence and access the mosquito stages of parasites more
265 safely.
266

267 Materials and Methods

268 Mosquito rearing and microinjection of parasites

269 STE2 strain *A. stephensi* mosquitoes were reared in an insectary at 19 °C under a
270 14:10 h light:dark cycle with 10% (w/v) sucrose solution. A total of 69 nL of purified
271 ookinete, gametocyte, and zygote suspensions was injected into the hemocoel of <10-day-old
272 male and female mosquitoes using a Nanoject II automatic nanoliter injector (Drummond
273 Scientific Company, Broomall, PA, USA) [11].
274

275 *P. berghei* and ookinete culture

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276 *P. berghei* (ANKA strain) that constitutively expresses green fluorescent protein
277 (GFP) was maintained via serial passage in ICR mice (Charles River Laboratories Japan Inc.,
278 Yokohama, Japan) from frozen stock. Ookinete culture of *P. berghei* was performed as
279 previously described [28,29]. Blood from infected mice was collected via cardiac puncture
280 and placed in 10 volumes of ookinete medium containing RPMI medium 1640 (Thermo
281 Fisher Scientific Inc., Waltham, MA, USA), 25 mM HEPES (Dojindo Laboratories,
282 Kumamoto, Japan), 0.4 mM hypoxanthine (Sigma-Aldrich, St. Louis, MO, USA), 24 mM
283 $NaHCO_3$ (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), and 12.5 mg L^{-1}
284 gentamicin reagent solution (Gibco) before incubation for 24 h at 19 °C.
285

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

287 *In vitro* culture of *P. falciparum* (3D7 strain) was performed according to the standard
288 protocol [30] and maintained at 5% hematocrit in A^+ human red blood cells (RBCs) on a
289 complete medium at 37 °C under 5% CO_2 and 5% O_2. The complete medium comprised
290 RPMI medium 1640, 25 mM HEPES, 0.1 mM hypoxanthine, 24–26 mM $NaHCO_3$, 11 mM
291 D(+)-glucose (Fujifilm Wako Pure Chemical Corporation), 12.5 mg L^{-1} gentamicin reagent
292 solution, and 10% (v/v) heat-inactivated type A^+ human plasma (Japan Red Cross Society,
293 Tokyo, Japan). Gametocyte cultures were initiated from asexual parasites at 0.5–3.9%
294 parasitemia and 6–10% hematocrit. The cultures were maintained for 14–27 d with daily
295 medium changes without adding fresh RBCs. From day 1 to 5, the culture medium was
296 supplemented with N-acetyl-D-glucosamine (Sigma-Aldrich) at a final concentration of 50
297 nM. Mature gametocytes were resuspended at 20% hematocrit in an exflagellation-inducing
298 medium containing 10 mM Tris (pH 7.6, Fujifilm Wako Pure Chemical Corporation), 170
299 mM NaCl (Fujifilm Wako Pure Chemical Corporation), 25 mM $NaHCO_3$, 10 mM D(+)-

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300 glucose, and 50–100 µM xanthurenic acid (Sigma-Aldrich) at 19 °C for 30 min for gamete
301 maturation and zygote fertilization.
302

303 Purification of parasites

304 *P. berghei* ookinetes and *P. falciparum* gametocytes and zygotes were purified using
305 a MidiMACS separator system (LS Columns, Miltenyi Biotec, Bergisch Gladbach,
306 Germany), as previously described [28,29]. Ookinetes, gametocytes, and zygotes were
307 recovered by passing ookinete medium, incomplete medium comprising complete medium
308 without 10% (v/v) heat-inactivated type A^{+} human plasma, and exflagellation-inducing
309 medium, respectively.
310

311 Examination of mosquitoes injected with *P. berghei*

312 Male and female mosquitoes were dissected at 14 d post injection, and oocyst
313 localization, number, and prevalence were examined using an Eclipse E600 (Nikon, Tokyo,
314 Japan) fluorescence microscope. At 28 d post injection, sporozoite localization was observed
315 using a fluorescence microscope. Sporozoites from whole bodies or salivary glands of the
316 injected males and females were administered into the tail vein of mice. Seven injected
317 females were blood-fed on mice, and the infection in the mice was checked every day using a
318 blood smear test.
319

320 DNA extraction

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

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324 extracted using a ZymoBIOMICS Miniprep (Zymo Research Corporation, Irvine, CA, USA)
325 according to the manufacturer's protocol or using the phenol-chloroform isoamyl alcohol
326 method. Briefly, a 500 µL genomic lysis buffer (Zymo Research Corporation) and 500 µL of
327 phenol:chloroform:isoamyl alcohol (25:24:1) (Nippon Gene Co., Ltd., Tokyo, Japan) were
328 added to extract the DNA, and then the DNA was precipitated using ethanol. The extracted
329 DNA was dissolved with 10–50 µL of TE buffer and stored at -20 °C.
330

331 Detection of *P. falciparum* using nested PCR

332 Prime STAR® HS (Premix) (Takara Bio Inc., Shiga, Japan) was used for PCR
333 amplification, according to the manufacturer's protocol, and the extracted DNA samples were
334 used as template DNA. For *A. stephensi* S7 gene (loading control) amplification, PCR was
335 conducted using primers AsRpS7-F (5′-CCTGGATAAGAACCAGCAGA-3′) and AsRpS7-R
336 (5′-GGCCAGTCAGCTTCTTGTA-3′). The PCR conditions were as follows: 1 cycle of 95
337 °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
338 cycle of 72 °C for 6 min. Distilled water as a negative control. Amplification of the *P.*
339 *falciparum* 18S rRNA gene for detecting *P. falciparum* was conducted using nested PCR.
340 The primers for the first PCR reaction were 18S rRNA-1F (5′-
341 TTAATTTGACTCAACACGGG-3′) and 18S rRNA-1R (5′-
342 TATTGATAAAGATTACCTA-3′) [13]. The PCR conditions were as follows: 1 cycle of 95
343 °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
344 1 cycle of 72 °C for 6 min. The primers for the second reaction were 18S rRNA-2F (5′-
345 TAATAGCTCTTTCTTGAT-3′) and 18S rRNA-1R. The PCR conditions were as follows: 1
346 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
347 for 30 s, and then 1 cycle of 72 °C for 6 min. Genomic DNA from *in vitro*-cultured *P.*

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348 *falciparum* was used as a positive control and the 7–11-d-old male mosquitoes as a negative
349 control. Eight PCR-positive samples were sequenced using 18S rRNA-2F and 18S rRNA-1R
350 primers (Fasmac Co., Ltd., Kanagawa, Japan).
351

352 Real-time PCR

353 Among the PCR-positive mosquitoes injected with 20 stage-V gametocytes, DNA
354 was quantified in three mosquitoes at 0 and 15 d post injection using KOD SYBR qPCR Mix
355 (Toyobo Co., Ltd., Osaka, Japan), and 18S rRNA-3F (5′-GGATGGTGATGCATGGCCG-3′)
356 and 18S rRNA-2R (5′-CAGTGTAGCACGCGTGCAG-3′) primers. The analysis was
357 conducted using Applied Biosystems StepOnePlus (Thermo Fisher Scientific) with the
358 following cycling conditions: 1 cycle at 98 °C for 3 min, followed by 40 cycles of 98 °C for
359 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
360 °C for 15 s. *P. falciparum* DNA was quantified using a calibration curve, and each value was
361 corrected by dividing by the quantity of template DNA used in the PCR reaction.
362

363 RNA-seq analysis

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

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372 read quality, and statistical analysis was performed using fold change per comparison pair of
373 mosquitoes injected with *P. berghei* ookinetes and with ookinete medium. GO analysis was
374 performed using VectorBase (https://www.vectorbase.org/).
375

376 Statistical analysis

377 All statistical analyses were performed using GraphPad Prism version 8.4.3 software
378 (GraphPad Software Inc., San Diego, CA, USA). We determined the significance of oocyst
379 intensity in injected mosquitoes using the Mann–Whitney test ($p < 0.01–0.05$) and the
380 significance of DNA content for real-time PCR using the unpaired t-test ($p < 0.05$).
381

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386 Japan. The *Anopheles stephensi* (STE2 strain, MRA-128) was provided by BEI Resources.
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484

485 # Supporting Information

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

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489 post injection was 7.3. Unpaired t-test was used for comparison ($p < 0.05$). Data are from
490 three biological replicates.
491
492 **S1 Table. Days confirming infection by administration of sporozoite of male and female-**
493 **injected mosquito**. At 28 d post injection, 2000–10000 *P. berghei* sporozoites from whole
494 bodies or salivary glands of the injected males and females, respectively, were administered
495 into the tail vein of mice. Seven injected females were blood-fed on mice, and the infection in
496 the mice was checked every day using a blood smear test. I.V.: administration into the tail
497 vein.

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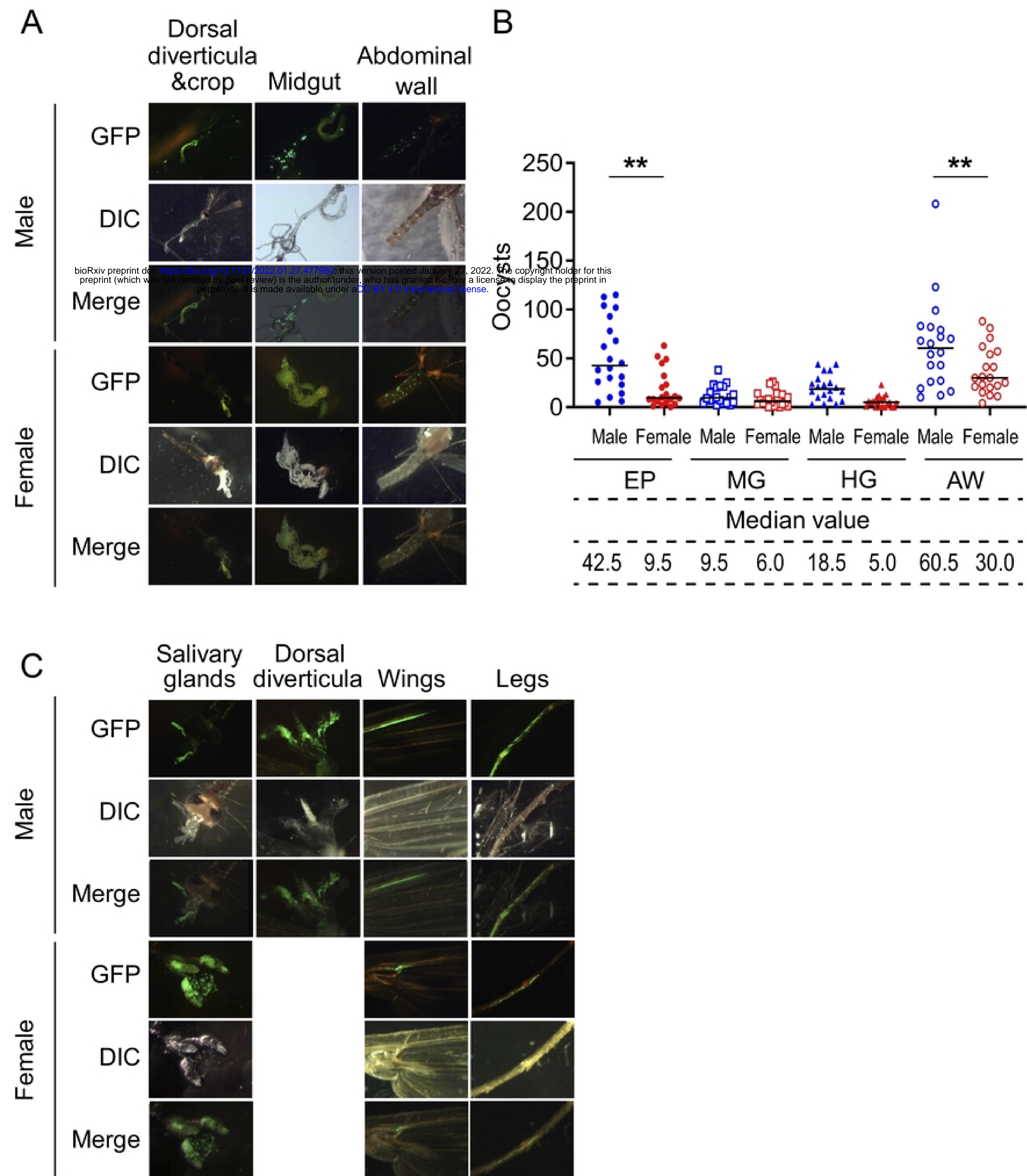

Figure 1

A

*

1000
800
600
400
200
0

Oocysts/mosquito

Male Female

B

**

1000
800
600
400
200
0

Oocysts/mosquito

Male Female

Figure 2

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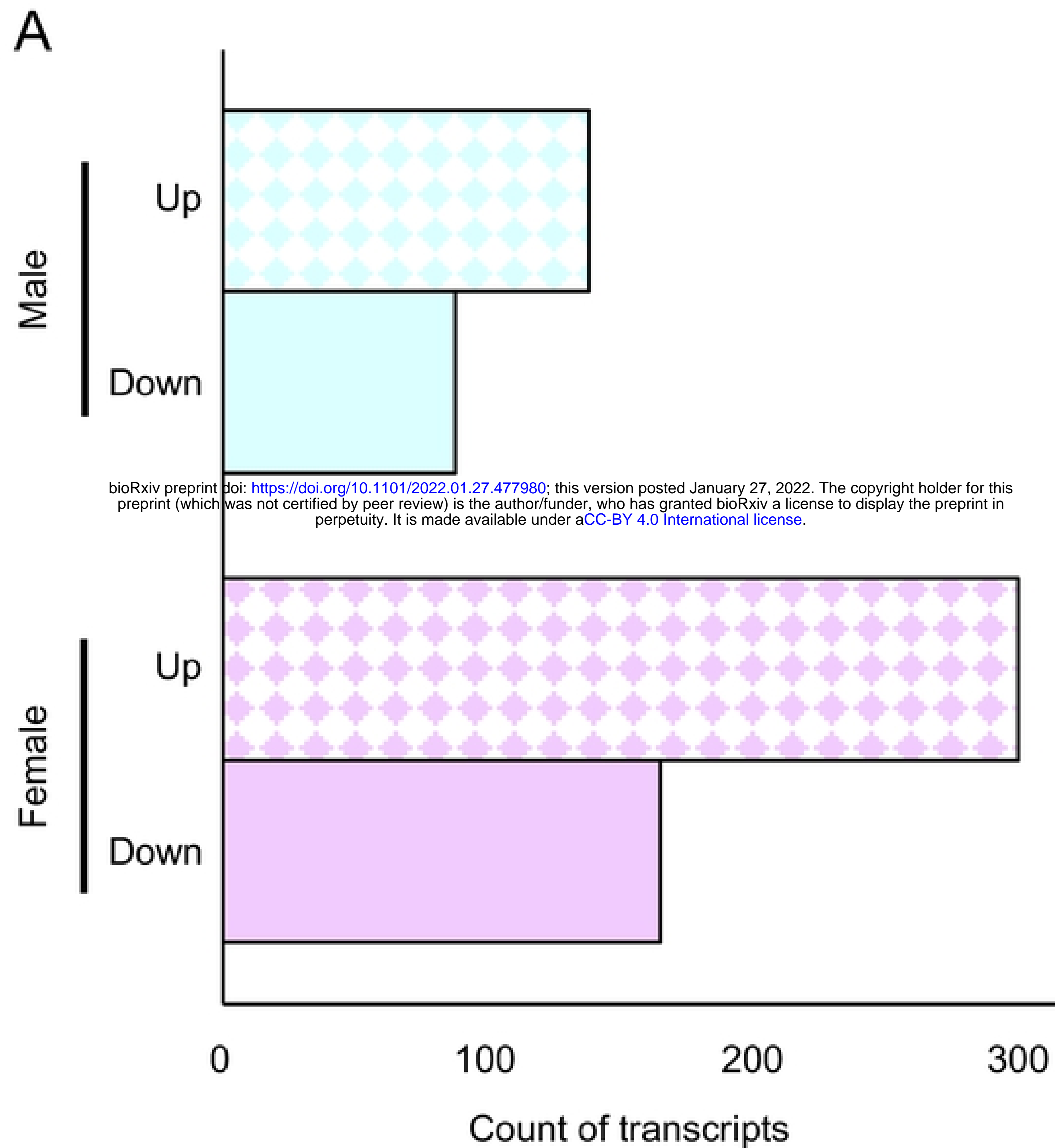

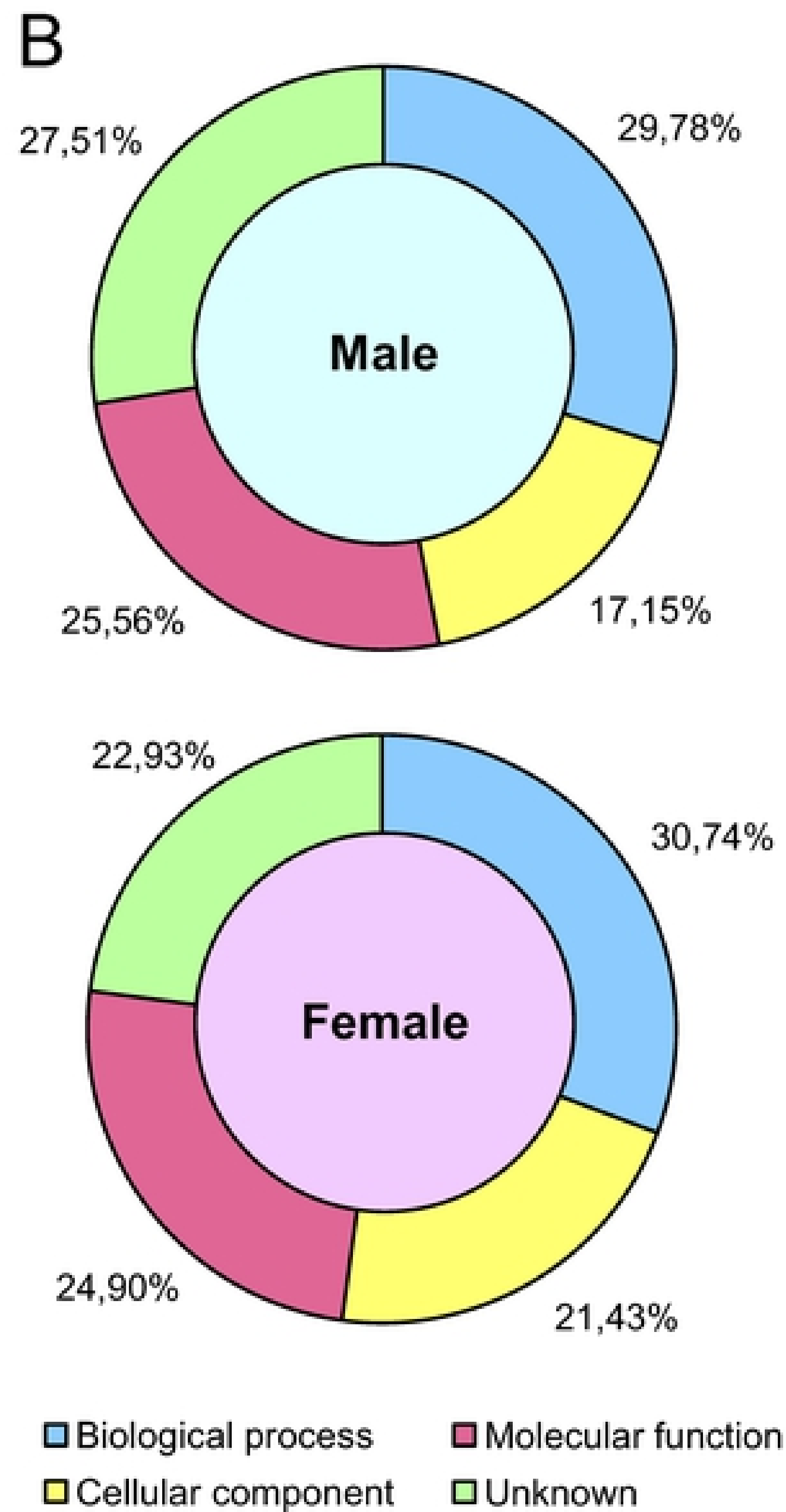

Figure 3