

## SOME FACTORS INFLUENCING INFECTIVITY OF THE HUMAN MALARIA PARASITE *PLASMODIUM VIVAX* (HAEMOSPORIDIA: PLASMODIDAE) TO THE MOSQUITO VECTOR *ANOPHELES TESSELLATUS* (DIPTERA: CULICIDAE)

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**Abstract:** Intake of an infective bloodmeal containing *Plasmodium vivax* gametocytes, by *Anopheles tessellatus* mosquitoes, leads to the detection of zygotes and ookinetes in the midgut 3-8 h after the bloodmeal. An intact peritrophic matrix, first observed approximately 18 h after a bloodmeal, is formed irrespective of the body size and age of the mosquito, but only in a proportion of mosquitoes. Unlike in the case of *P. falciparum* where ookinetes develop at about 24 h, the peritrophic matrix would therefore not be a significant barrier to the infectivity of *P. vivax* due to the relatively rapid formation of *P. vivax* ookinetes. The rate of trypsin production was the same in *An. tessellatus* of different sizes, with the activity peaking about 21 h post bloodmeal. At this time the majority of *P. vivax* ookinetes would have exited the midgut. A tendency for *An. tessellatus* of intermediate size to be more susceptible to infection with *P. vivax* than large or small mosquitoes was observed. However this difference was statistically significant in only one of two experiments. The number of oocysts produced, while dependent on gametocytaemia of the blood meal, was not significantly influenced by mosquito size.

**Key words:** *Anopheles tessellatus*, infectivity, mosquito physiology, peritrophic matrix, *Plasmodium vivax*.

## INTRODUCTION

*Anopheles tessellatus* Theobald (Diptera: Culicidae) mosquitoes are natural vectors of the human malaria parasites *Plasmodium vivax* Grassi and *P. falciparum* Welch (Haemosporidia: Plasmodidae) in Sri Lanka.<sup>1</sup> Development of blood-stage gametocytes of malaria parasites and fertilization occurs in the midgut of an *Anopheles* vector after a blood meal. The resulting zygote loses its spherical shape to become an ellipsoid motile ookinete. Ookinetes reportedly first traverse the peritrophic matrix (PM), a chitinous, lamellar structure that is formed around the blood meal from secretions of the epithelial cells of the midgut.<sup>2,3</sup> Subsequently, the motile ookinetes cross the midgut epithelium, lodge in the basal lamina and develop into oocysts in the posterior region of the midgut. There is evidence to

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suggest that N-acetylglucosamine residues on *An. tessellatus* midgut epithelium/PM glycoproteins, chitin or proteoglycans serve as recognition sites for *P. falciparum* and *P. vivax* ookinetes.<sup>4,5</sup> Sporozoites produced in the oocysts locate to salivary glands, and are inoculated into the host while blood feeding to complete the parasite life cycle.

The intake of a blood meal triggers the synthesis and release of proteolytic enzymes<sup>6,7</sup> and formation of the PM. Light and electron microscopic studies show that in *An. tessellatus*, an intact PM is formed approximately 18 h after a blood meal, beginning in the posterior region of the midgut and generally persisting for up to 48 h after feeding.<sup>8</sup> The time taken from fertilization to the formation of the ookinete, the rate of protease production and the rapidity of blood digestion and PM formation are some of the factors that can influence the susceptibility of *Anopheles* to infection with malaria parasites. Other physiological parameters of the vector mosquito such as body size, age, etc, can also affect the development of the parasite in the vector. Variations in the size of individual mosquitoes are common in nature<sup>9</sup> and body size reportedly influences the susceptibility of *An. gambiae* Giles to infection with *P. falciparum*.<sup>10, 11</sup>

In contrast to *P. falciparum*, little is known about the rate of development of *P. vivax* ookinetes and the influence of mosquito physiology on their infectivity in vector mosquitoes. This information is important for understanding *P. vivax* transmission and developing improved control measures. The kinetics of ookinete generation, PM formation, protease production, and effect of body size of laboratory-reared *An. tessellatus*, on the susceptibility to infection by field isolates of *P. vivax*, are reported in this paper.

## MATERIALS AND METHODS

*An. tessellatus* production: A self-mating colony of *An. tessellatus* was maintained in the laboratory at  $27 \pm 1^\circ\text{C}$  and 80% relative humidity.<sup>12</sup> The colony was fed on restrained rabbits: the care and use of rabbits were according to WHO guidelines.<sup>13</sup> Adult *An. tessellatus* of different body sizes (i.e. L100: large, L200: medium and L400: small) were raised by varying the larval rearing densities at 100, 200 and 400 larvae in 2 l of water.<sup>14</sup> L100, L200 and L400 derived mosquitoes had wing lengths of  $3.17 \pm 0.02$ ,  $2.95 \pm 0.02$ ,  $2.78 \pm 0.03$  mm and body weights of  $1.10 \pm 0.04$ ,  $0.90 \pm 0.03$  and  $0.60 \pm 0.02$  mg respectively.<sup>14</sup> Mosquitoes produced from L100 larvae were routinely used in experiments unless otherwise stated.

*Trypsin activity*: The activity of trypsin in the midguts of mosquitoes of different sizes was determined at regular intervals between 15 and 27 h after a bloodmeal, exactly as previously described.<sup>7</sup>

**Peritrophic matrix formation:** Groups of 3-4 d old L100, L200 and L400 mosquitoes were blood-fed on a rabbit and held in an insectory with access to sugar. At 18, 24 and 30 h after the intake of the blood meal, the mosquitoes were frozen at  $-20^{\circ}\text{C}$ . The mosquitoes were dissected using a dissecting microscope, and the midgut epithelium then removed gently to expose the food bolus, to establish the presence or absence of a PM around the posterior end of the bolus exactly as described previously.<sup>8</sup> For investigating the influence of age on PM formation, 4, 7 and 9 d old L100 mosquitoes were examined in a similar manner for the presence of a PM after a bloodmeal.

**Studies on the infectivity of *P. vivax*:** The parasitaemia and gametocytaemia of blood used for infection of mosquitoes were determined in Giemsa stained blood films. For determining the kinetics of *P. vivax* ookinete formation, a group of 3-4 d old L100 mosquitoes from the colony were hand fed on a *P. vivax* infected patient, after informed consent was obtained. Midguts of blood fed mosquitoes were dissected at 1-2 h intervals from the time of blood feeding until 18 h afterwards and the epithelium carefully removed. The blood meal was smeared on a glass slide and stained with Giemsa to detect *P. vivax* ookinetes.

For investigating infectivity in mosquitoes of different sizes, parasite infected blood samples were obtained voluntarily, with informed consent, from patients seeking treatment at Government Hospitals at Polgahawela and Rambukkana. These donors had not previously taken drugs for malaria infection. Groups of 3-4 d old L100, L200 and L400 *An. tessellatus* were then infected with the parasite by a membrane feeding technique described previously.<sup>15</sup> Blood from the same donor was used in a given experiment. Fully engorged, blood-fed mosquitoes were separated, kept in cages, and dissected 8 d later for counting oocysts in the midgut. The cumulative mortality of mosquitoes, from the time of the infective blood meal until dissection, was recorded.

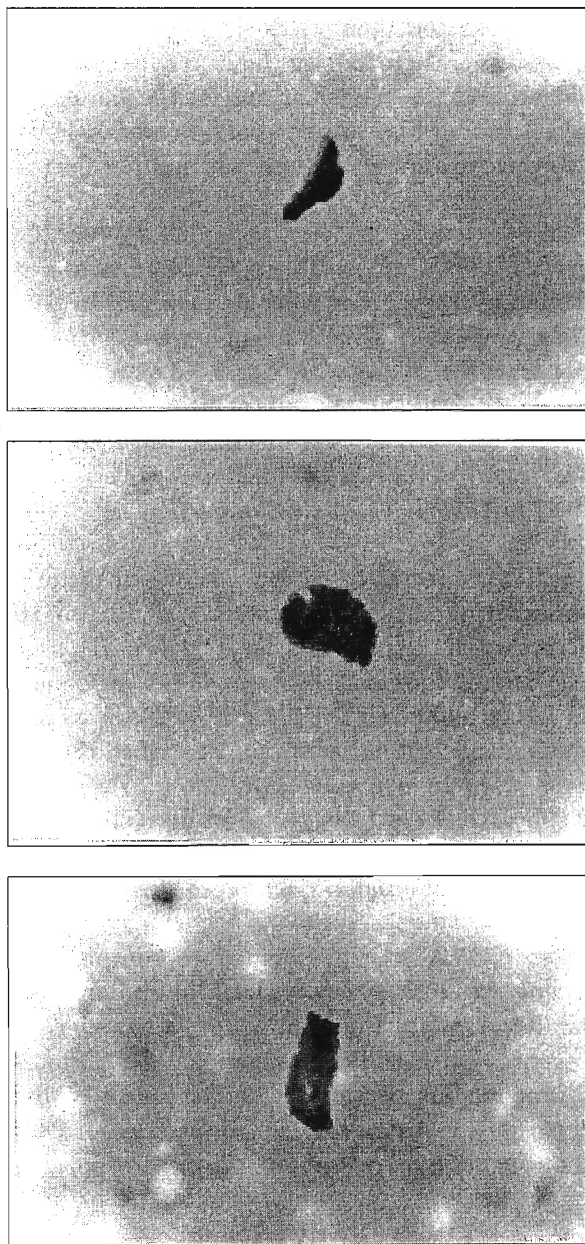
**Statistical analyses:** ANOVA was performed to compare the proportions of mosquitoes of different ages [of a given size, L100] and different sizes [of a given age, 3-4 d old] that developed a PM. Chi-square tests were done to assess the significance of differences between the proportions of mosquitoes infected with *P. vivax* by the membrane feeding technique. The Kruskal-Wallis one-way ANOVA on ranks was used to analyse differences in oocyst numbers between mosquitoes. Tests were carried out using Sigma Stat 2.0 (Jandel Corporation, USA) software.

## RESULTS

### **Kinetics of the development of *P. vivax* ookinetes in *An. tessellatus***

Microscopic examination of stained smears of the contents of *P. vivax* infected midguts of *An. tessellatus* showed the presence of mature zygotes and ookinetes, 3-

8 h after the infective bloodmeal (Figure 1). In contrast, ookinetes were rarely observed in smears made 18 h after the bloodmeal.

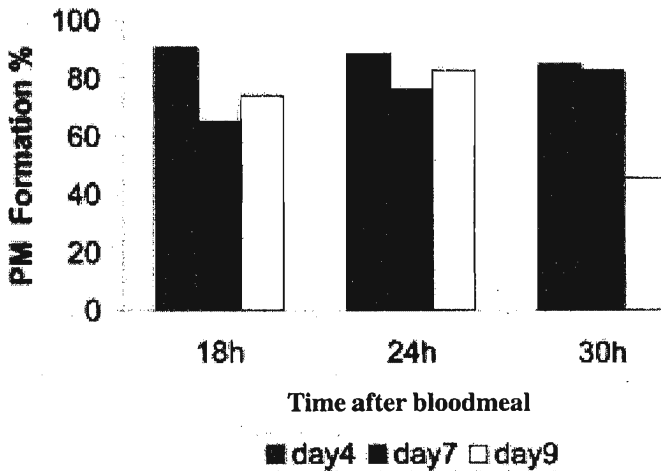


**Figure 1a-c:** *P. vivax* in smears of the midgut contents of *Anopheles tessellatus* after the ingestion of a bloodmeal (observed under x1000 magnification). a- ookinete at 3 h, b- zygote at 5 h and c-ookinete at 8 h, after a bloodmeal.

**Effects of age and size on PM formation in *An. tessellatus***

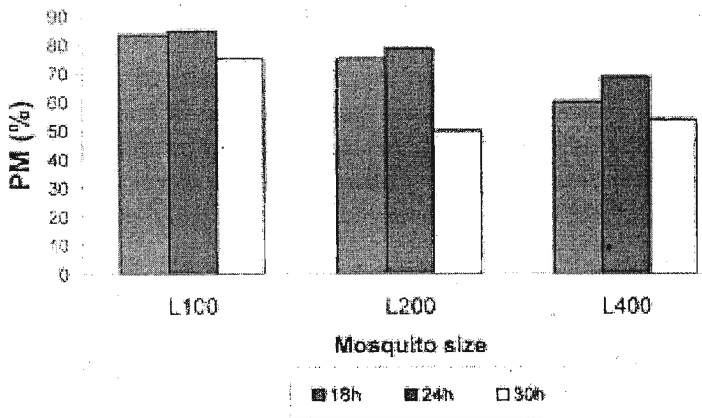
When a PM was present, it was characteristically observed under a microscope as a gelatinous layer extending from the posterior end of the midgut towards the centre of the food bolus.<sup>8</sup> The PM in *An. tessellatus* was present in 60-80% blood fed mosquitoes. The remaining mosquitoes failed to develop a PM. An intact or continuous sleeve of PM was first observed approximately 18h after the intake of a bloodmeal.

The proportion of mosquitoes of the L100 size category developing a PM was higher among 4 d old mosquitoes that took a bloodmeal, than in 7 or 9 d old mosquitoes (Figure 2). Also an intact PM was observed consistently until 30h in a larger number of 4 d old mosquitoes compared to 7 and 9 d old ones (Figure 2). However, differences in the proportions of L100 mosquitoes developing a PM were not statistically significant by ANOVA ( $p>0.05$ ) in mosquitoes of different ages.



**Figure 2: PM formation at different times after a bloodmeal in large sized (L100) *An. tessellatus* of different ages.**

PM formation was also explored in 3-4 d old *An. tessellatus* of the three experimental sizes. Accordingly, 81 % (n=37) of L100 mosquitoes developed a PM after a bloodmeal compared to 68% (n=38) and 61 % (n=44) of L200 and L400 mosquitoes respectively. More L100 mosquitoes were observed to have a continuous sleeve of PM at 30h after the blood meal compared to L200 and L400 mosquitoes (Figure 3). However, the differences in the proportions of 3-4 d old mosquitoes of varying sizes developing a PM were not statistically significant by ANOVA ( $p>0.05$ ).



**Figure 3: PM formation at different times after a bloodmeal in 3-4 d old *An. tessellatus* of different sizes.**

#### **Kinetics of trypsin production in *An. tessellatus* of different sizes**

The development of trypsin activity in the midguts of large (L100), medium (L200) and small (L400)- sized mosquitoes was similar, with maximal enzyme activity occurring about 21 h after the blood meal in mosquitoes of all three size groups. Detailed data are not shown because they are the same as the kinetics of trypsin production reported previously by us with large (L100) *An. tessellatus*.<sup>7</sup>

#### **Infectivity of wild *P. vivax* isolates to *An. tessellatus* of different sizes**

Three to four day old mosquitoes of all three size groups were readily infected with *P. vivax* (Table 1). In experiment 1, the highest incidence of infection was observed in L200 mosquitoes followed by L400 and L100 mosquitoes. However, in this experiment, the proportions of infected mosquitoes were not significantly related to body size ( $\chi^2 = 5.66$ ,  $df=2$ ,  $p>0.05$ ). In Experiment 2, the proportion of infected mosquitoes was again higher among L200 mosquitoes, than L400 or L100 mosquitoes. In this experiment the proportions of infected mosquitoes were significantly different between the three size categories ( $\chi^2 = 9.58$ ,  $df=2$ ,  $p<0.05$ ).

Oocysts were present mainly in the posterior of the midgut and the numbers of oocysts developed by mosquitoes was dependent on the gametocytaemia of the infective bloodmeal. In Experiment 1, where the gametocytaemia was high, the numbers of oocysts in L100, L200 and L400 mosquitoes analysed by Kruskal-Wallis ANOVA on ranks was not significantly different ( $H = 1.55$ ,  $df=2$ ,  $p>0.05$ ). This was also the case in Experiment 2 with lower gametocytaemia ( $H=0.52$ ,  $df=2$ ,  $p>0.05$ ). Therefore, the number of *P. vivax* oocysts formed in the mosquito midgut, were not significantly related to mosquito body size.

**Table 1: Effect of body size of *An. tessellatus* on its susceptibility to infection with *P. vivax*.**

Mosquito size	% Infection* (n)	Oocyst range [median]**	% Cumulative mortality (n)***
Experiment 1 gametocytaemia 0.3%			
L100	18 (56)	4-226 [23] <sup>a</sup>	12.5 (64)
L200	39 (38)	3-300 [30] <sup>a</sup>	20.8 (48)
L400	32 (50)	12-160 [65] <sup>a</sup>	26.5 (68)
Experiment 2 gametocytaemia 0.06%			
L100	25 (24)	01-58 [4] <sup>b</sup>	69.6 (79)
L200	73 (15)	02-72 [3] <sup>b</sup>	79.7 (74)
L400	33 (21)	01-25 [5] <sup>b</sup>	69.5 (69)

For logistical reasons, (i.e. the quantity of infected blood withdrawn from a patient) the number of mosquitoes used could not be increased. (n)= number of mosquitoes

\* In experiment 1 the proportions of infected mosquitoes are not different among the three body sizes (p>0.05). In experiment 2 the proportions of infected mosquitoes are significantly different among the three body sizes (p<0.05).

\*\* Medians followed by the same superscripts are not significantly (p<0.05) different by Kruskal-Wallis one way analysis of variance on ranks.

\*\*\* In experiments 1 and 2 the proportions of dead mosquitoes are not significantly different among the three body sizes (p>0.05)

The cumulative mortality among infected mosquitoes varied from 12.5-26.5% in Experiment 1 and from 69.5-79.7% in Experiment 2. However, mortality was not significantly correlated to body size of the mosquito in either Experiment 1 ( $\chi^2 = 4.05$ , df=2, p>0.05) or in Experiment 2 ( $\chi^2 = 2.57$ , df=2, p>0.05).

Furthermore, the cumulative mortality up to dissection at 8 d, in Experiment 1 where there was higher gametocytaemia in the bloodmeal and consequently higher oocyst numbers on dissection, was lower than in Experiment 2, with lower gametocytaemia and oocyst numbers. This suggests that the intensity of infection with *P. vivax*, as measured by oocyst numbers, influenced mortality less than other aspects of batch to batch variation among mosquitoes.

**DISCUSSION**

The kinetics of ookinete formation and penetration in *P. vivax* is different from that of *P. falciparum*, where ookinete development has been observed 24 h after the vector takes a blood meal.<sup>16</sup> The maximal ookinete penetration activity occurs at around 22 h following a bloodmeal in *An. atroparvus* van Theil infected

with *P. berghei*<sup>17</sup> and between 15–18 h in *An. omori* infected with *P. yeolli nigeriensis*.<sup>18</sup> In *Aedes aegypti* (L) infected with *P. gallinaceum*, the maximal penetration of the midgut by ookinetes takes place between 30–35 h after a bloodmeal<sup>19</sup> and in *An. stephensi* infected with *P. falciparum*, maximal ookinete penetration occurs between 32–36 h after the bloodmeal.<sup>20</sup> The rate of development of *P. vivax* in the vector midgut, like *P. yeolli* and *P. berghei*, is faster than *P. falciparum* or *P. gallinaceum*. In *An. tessellatus*, mature oocysts of *P. vivax* are detected 6–7 d after the infective bloodmeal, while those of *P. falciparum* are seen 8–10 d after the infective blood meal.<sup>5</sup> This may be partly attributed to the ookinetes of *P. vivax* establishing themselves earlier than those of *P. falciparum* in the basal lamina. Like in other malaria parasites, the oocysts of *P. vivax* establish themselves in the posterior end of the *An. tessellatus* midgut probably due to the presence of VATPase—over expressing cells in this region.<sup>21</sup>

The PM is formed at varying times in different mosquitoes and it has been suggested that this is one cause for the differential infectivity of *Plasmodium* for mosquitoes.<sup>22</sup> In *An. stephensi* Liston the PM is first seen around 24 h<sup>20</sup> and the presence or absence of a PM in *An. stephensi* did not influence *P. berghei* Vincke infectivity,<sup>23</sup> although the PM is reportedly a barrier to penetration of *P. gallinaceum* inside the midgut of *Ae. aegypti*.<sup>24</sup> We observe here that ookinetes of *P. vivax* are formed 3–8 h after the ingestion of the infective blood meal. The intact PM is formed at about 18 h, irrespective of the age and size of the *An. tessellatus*, and this may allow the majority of *P. vivax* ookinetes sufficient time to cross the midgut epithelium and form oocysts. There was no relationship observed between the formation of PM in mosquitoes of different sizes and the relative infectivity of *P. vivax* in the present experiments. Although chitinase synthesised by *P. falciparum* ookinetes helps them penetrate the PM,<sup>25</sup> there is no evidence for the production of chitinase by the ookinetes of *P. vivax*. These findings are consistent with the proposal that most ookinetes of *P. vivax* cross the midgut before a PM barrier is fully established and do not have to cross the PM before developing into oocyst. Because anti-midgut antibodies concomitantly reduce *P. vivax* infection to *An. tessellatus*<sup>15</sup> and inhibit PM formation in the posterior midgut,<sup>8</sup> it was previously postulated<sup>26</sup> that the PM is not a physical barrier to the establishment of this parasite. Our present findings further support the assertion that the PM is not a significant barrier to the establishment of *P. vivax* infections in *An. tessellatus*.

Another factor that needs to be considered is whether a bloodmeal containing malaria parasites will influence formation of the PM as a consequence of infection. This possibility was not clarified in the present work but requires further investigation.

The observation that an intact PM is not formed in a proportion of the *An. tessellatus* after a bloodmeal warrants further examination. It is argued that the Type I PM, characteristic of fluid-feeding insects, and observed in adult female



mosquitoes, has evolved primarily to protect against bacteria contaminating the feed.<sup>2</sup> A PM is absent in some Dipterans that feed on relatively sterile fluids like blood and nectar,<sup>2</sup> and it may be that it is therefore largely vestigial in adult female mosquitoes. This will be the case if their reproductive ability is not significantly affected by infection with blood-borne parasites. A proportion of blood fed female mosquitoes may therefore not develop a PM, due perhaps to insufficient stimulus, and not show significant ill effects.

When proteolytic enzymes affect the susceptibility of the vector to the malaria parasite, the effects should be evident during the time of zygote to ookinete transition. In *An. tessellatus*, at 5-6 h after the blood meal, the activity of trypsin in the midgut is low<sup>8</sup> and therefore, trypsin is unlikely to be deleterious to early *P. vivax* ookinetes. However as the trypsin levels increase in the midgut, later ookinete formation may be significantly affected. Midgut proteases reduce the susceptibility of *Anopheles* spp. to infection with *P. falciparum*, but the enzymes alone do not affect zygote formation.<sup>27</sup> In this context, we have previously reported<sup>7</sup> that the presence of trypsin inhibitors in a bloodmeal delayed the onset of peak trypsin activity by 6-12 h, depending on the inhibitor and its concentration. The trypsin inhibitors increased the infectivity of *P. vivax* but decreased the infectivity of *P. falciparum* to *An. tessellatus*.<sup>7</sup> The opposite effects were attributed to delayed trypsin activity reducing damage to zygotes and immature ookinetes of *P. vivax*, while increasing damage to the more slowly developing *P. falciparum* zygote/ookinete or inhibition of the activation of a prochitinase that is required by *P. falciparum* ookinetes.<sup>7</sup>

In laboratory-reared *An. dirus*, larger females tend to be more frequently infected than smaller females and developed more oocysts when infected with cultured *P. falciparum* although these differences were not statistically significant.<sup>28</sup> In field caught *An. gambiae*, larger adults are reported to have fewer sporozoite infections than medium sized mosquitoes.<sup>10</sup> The proportion of mosquitoes infected was independent of size, but the number of oocysts harboured increased with size of the mosquito. These authors hypothesized that heavier parasite loads in larger mosquitoes would result in greater mosquito mortality in the field. The pattern is interpreted as a combination of high survival rate of large, uninfected mosquitoes and of low survival rate of mosquitoes infected with many oocysts. However, in *An. maculatus* Theobald naturally infected with *P. falciparum*, *P. vivax* and *P. malariae*, there was no observed correlation between body size and parasite infectivity.<sup>30</sup> But the same authors reported that females infected with oocysts were significantly larger than those with sporozoites.<sup>29</sup> Experimental larval rearing densities influenced the adult body size of *An. tessellatus* and an inverse relationship between wing lengths ranging from 2.78 – 3.17 mm and larval rearing densities were observed although the mosquitoes were similar metabolically.<sup>14</sup> In *An. tessellatus* infected with *P. vivax* in the laboratory, irrespective of gametocytaemia in the blood meal, there was no consistent relationship between the numbers of oocysts

formed and body size. The proportion of infected mosquitoes however tended to be greatest with mosquitoes of intermediate size. The cause of this is presently unclear.

Our results demonstrate that mortality of *P. vivax*-infected laboratory-bred *An. tessellatus* was independent of the body size and, under the experimental conditions, also not affected by the numbers of oocysts. That field caught *An. tessellatus* did not show increased mortality following infection with the malaria parasite<sup>30</sup> is consistent with the latter observation. Furthermore, although mortality among field caught *An. gambiae* was high, the presence of maturing oocysts reportedly did not affect mortality.<sup>12</sup>

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