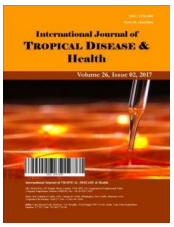

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Vector Competence of Laboratory-reared *Anopheles stephensi* for *Plasmodium vivax*-infected Blood from Pakistani Patients

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Authors' contributions

This work was carried out in collaboration between all authors. Author SW conceived the idea, designed the study, performed the statistical analysis, wrote the protocol and wrote the first and final drafts of the manuscript. Author KU collected the samples and performed the wet labs. Author AF helped in morphological identification of malaria samples and provided clinical inputs. Author SAH managed the insectary and helped in mosquito colony development. All authors read and approved the final manuscript.

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ABSTRACT

Malaria is one of the leading causes of death around the world. *Plasmodium vivax* is supposed to contribute over 80% of total malaria cases in Pakistan. However, data on transmission pattern is inconsistent and scanty in Pakistan.

Aim: The present study was aimed to determine the vector competence based on sporozoite rate rather than oocyst rate.

Study Design: Laboratory reared female *Anopheles stephensi* mosquitoes infected with *Plasmodium vivax* served a study model.

Methodology: *Anopheles stephensi* vectors were reared in the laboratory and fed on *Plasmodium vivax* infected blood obtained from malaria patients. Progression of parasite was determined by

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light or fluorescent microscopic examination of midgut or salivary gland of infected mosquitoes.
Results: Oocyst and sporozoite rates were found to be 64% and 54%, respectively, which confirmed the vector competence of female mosquitoes. Blood feeding contributed the longer survival of the vector irrespective of the presence or absence of *Plasmodium vivax*.
Conclusions: It is concluded that laboratory reared female *Anopheles stephensi* mosquitoes support the development of *Plasmodium vivax*. Blood-fed mosquitoes showed considerable competence for malaria parasite which is dependent on sporozoite rate rather than oocyst rate. The current study exhibited laboratory reared female *Anopheles stephensi* mosquitoes as a potential source of parasite transmission.

Keywords: Malaria; Plasmodium vivax; incrimination; vector competence; infection; Anopheles stephensi.

1. INTRODUCTION

Malaria is one of the major global health burdens which has been the most addressed public health concern worldwide. Malaria is caused by apicomplexa parasite; *Plasmodium* species [1]. Malaria spans over 109 countries and covers about half of population across the globe [2-4]. Eastern Mediterranean region is also endemic for malaria infection, where Pakistan reflects highest malaria burden.

Around 60% population in Pakistan is at risk of malaria infection after its resurgence in 1970. Poor health conditions, natural disasters like floods and earthquakes and mass migration across the borders due to political unrest are major risk factors associated with malaria infections. Among 500,000 annual malaria cases, 50,000 death toll was recorded in Pakistan [5]. Inconsistent data is a major impediment to assess actual trends of malaria infections in Pakistan [6].

Pakistan is a malaria endemic country where parasite transmission is over 90% with highest annual parasite incidence (API) 4.5 cases/1000 people per annum [6,7]. Pakistan is among three countries where *Plasmodium vivax* (*P. vivax*) infection cases exceed 80% of all malaria cases. Pakistan, with 27% confirmed malaria cases, is a second largest country after Sudan (57% confirmed malaria cases) where malaria endemicity is alarming. Over 90% malaria related mortality was recorded in 2013 in these two countries [6]. Nevertheless, scanty epidemiological data is unreliable unless it is reported by evaluating the vector competence.

Therefore, progression of predominant malaria parasite species and subsequent vector competence would warrant further studies to understand the local transmission pattern. Moreover, it would help to understand malaria dynamics in the endemic areas and to design a targeted intervention strategy. Laboratory-reared *Anopheles stephensi* mosquitoes, predominant malaria vector in Pakistan, served as a model to study the vector competence and progression of malaria parasite.

2. MATERIALS AND METHODS

2.1 Rearing of *Anopheles stephensi* Mosquitoes

Anopheles stephensi mosquitoes were reared (after collection from the field) in the insectary as described previously [8,9]. Continuous colonies of the mosquitoes were maintained in the laboratory for around one year. Briefly, adult *Anopheles stephensi* mosquitoes were feed on cotton pads soaked in 10% glucose solution. Mosquitoes were allowed to bite on mice for blood intake and facilitated to lay eggs in the water filled beaker and subsequently hatching onto the water in egg pans. After hatching, 200-250 larvae were distributed in the egg pans. The larvae were fed on powdered chicken liver. After 8-10 days the larvae developed into pupae which were collected in a beaker and the lid was closed to avoid flying after they develop into adult mosquitoes. Temperature and humidity was maintained at 26±4°C and 80±5%, respectively.

All the protocols used in project were approved by the Bio-Ethical Committee of Quaid-i-Azam University (BEC-FBS-QAU-10).

2.2 Inclusion/Exclusion Criteria

The patients with apparent symptoms of malaria including high grade fever, vomiting, shivering, profuse sweating, chill and headache were subjected to blood sampling after informed consent. Blood samples were examined microscopically to confirm the presence of malarial parasite (gametocyte stage). Mixed

infections were also ruled out. The patients having blood group O^+, irrespective of the age and gender, were selected and 2-3 mL of their blood was taken in vacutainers (BD, Belgium) and stored at ambient temperature. Total iron binding capacity (TIBC) and serum protein contents of the donors' blood samples were determined (data not shown here) before feeding to minimize the donors' effect on the mosquito survival rates.

2.3 Artificial Blood Feeding

The mosquitoes were fed on *Plasmodium vivax* gametocyte-infected blood as described previously [10-13]. Briefly, after providing free feeding and mating chances, female *Anopheles stephensi* mosquitoes (20-30 mosquitoes/cage) were selected and separated in small cages. The female mosquitoes were kept starved for 12 h before artificial blood feeding through membrane feeder.

The feeder was connected to a water jacket, thermostatically controlled, to maintain a specific temperature (37 ± 02°C). Around 2 mL infected blood was supplied through feeder along with water at 37°C to prevent exflagellation of gametocytes. Starved female *Anopheles stephensi* mosquitoes were feed for 30-35 min. After 24-36 h, unfed or partially fed female mosquitoes were removed and fully fed female mosquitoes were left in the cage. A control group was fed on sugar solution.

2.4 Dissection

Blood-fed female mosquitoes were provided with 10% glucose solution for water and energy. Female mosquitoes were dissected 5-7 or 10-14 days post blood feeding to confirm the presence of oocysts (in midgut) and sporozoites (in salivary glands), respectively, as described previously (11). Midgut and salivary glands were recovered under dissecting microscope in phosphate buffer saline (PBS) (Biochrom, UK) and examined under compound microscope (IRMECO, Germany) after staining with Giemsa (Merck, Germany) or unstained [9].

2.5 Immunofluorescent Assay (IFA)

Salivary glands were disrupted in PBS solution to release sporozoites as described previously [14, 15]. Sporozoites were stained with anti circumsporozoite protein (CSP) antibody conjugated with fluorescence as per manufacturer's instructions (Innova Biosciences, UK). The stained sporozoite were mounted on slide and examined under fluorescent microscope (Olympus, Japan). Sporozoite rate was determined using the following equation (1).

Sporozoite rate =

$$\frac{Number\ of\ female\ mosquitoes\ with\ sporozoites}{Number\ of\ female\ mosquitoes\ examined} \times 100 \quad (1)$$

2.6 Survival Curve

Female *Anopheles stephensi* mosquitoes were divided into three groups. Each group had equal number of mosquitoes (150 mosquitoes/group) and subjected to same conditions (temperature and humidity). Group one was kept starved, group two and three were fed on un-infected and infected blood meals, respectively. The number of mosquitoes died or remained alive each day were recorded up to 10 days post blood feeding. A survival curve was drawn using the number of dead and alive mosquitoes.

3. RESULTS

3.1 Progression of *Plasmodium vivax* in Female *Anopheles stephensi* Mosquito

Blood samples were confirmed by the presence of male and female gametocytes of *Plasmodium vivax* in thin blood smears as shown in Fig. 1.

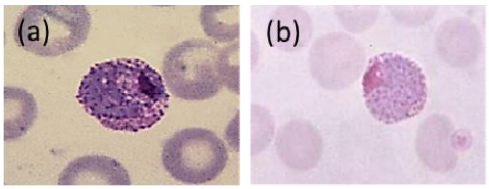

Fig. 1. Gametocytes of *Plasmodium vivax*. A representative Giemsa stained blood sample of a malaria patient representing a) male and b) female gametocytes. Magnification=1000X

Progression of malaria parasite was monitored by inspecting various parts of the mosquito vector. Blood-fed female mosquito (Fig. 2a) has shown half gravid midgut (Fig. 2b). Abdominal part depicts the presence of oocysts (Fig. 2c) which represents the progression of parasite in the mid gut wall after ingestion of infected blood. Salivary glands were dissected from the thorax region. Light microscope image represents three lobes of salivary gland (Fig. 2d). Finally, the parasites (sporozoites) were detected in the salivary glands by staining them with anti-CSP antibodies conjugated with fluorescence (Fig. 2e).

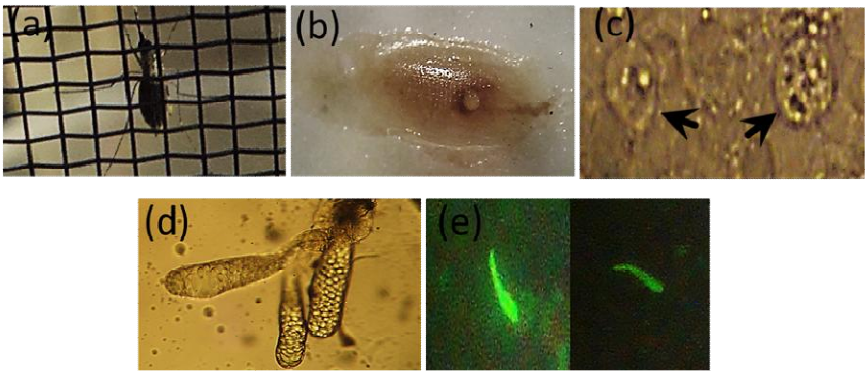

Fig. 2. Progression of malaria parasite in a female *Anopheles stephensi* mosquito. It shows a) a blood-fed female mosquito, b) midgut of a half gravid mosquito, c) the oocysts (black arrows), d) salivary glands and e) sporozoites stained with anti-CSP antibody conjugated with fluorescence (a composite figure). Magnification=1000X

3.2 Oocyst and Sporozoite Rate

Among 320 blood-fed *Anopheles stephensi* mosquitoes, 208 mosquitoes were found positive for oocysts. The mean oocyst rate was found to be 65% (Fig. 3, Table 1). Another set of experiment included 320 female mosquitoes (total 16 experiments) which were infected with *P. vivax*. Among these 320 blood-fed *Anopheles stephensi* mosquitoes, 173 mosquitoes were found positive for sporozoites with an average infectivity or sporozoites rate of 54% (Fig. 3, Table 1). However the sporozoite rate among different experiments varied from 35% to 80% (Table 1).

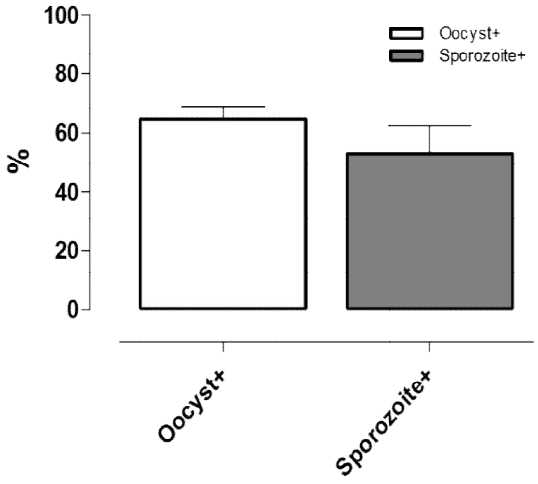

Fig. 3. Infection rate. The percentage of oocyst or sporozoite positive blood-fed *Anopheles stephensi* mosquitoes. Data was measured as mean±SE

3.3 Survival Rate in Correlation with Blood Meal

The total number of mosquitoes died were 64, 36 and 40 from the unfed (D1), un-infected blood fed (D2) and infected blood fed (D3) mosquito groups, respectively, after 10 days (Table 2).

The number of mosquitoes remained alive after 10 days were 86, 114 and 110 of the unfed, un-infected blood fed and infected blood fed mosquito groups, respectively (Fig. 4). The mean survival rate was 0.95 ± 0.04, 0.97 ± 0.02 and 0.97 ± 0.02 for unfed, un-infected blood fed and infected blood fed mosquitoes, respectively.

3.4 Statistical Tool

All statistical analyses including mean, average and *p* values where applicable, were performed on GraphPad Prism (Ver. 0.5)

4. DISCUSSION

The data suggest that laboratory reared *Anopheles stephensi* is a suitable vector for *P. vivax* because oocysts and sporozoites develop only when both the parasite and vector are compatible with each other [16].

The data revealed that mean oocyst rate of laboratory reared *Anopheles stephensi* was 65%. It indicates *Anopheles stephensi* as a suitable vector for *P. vivax*. The highest mean oocyst rate reported previously was 77% for *Anopheles stephensi* [11]. However, 51-64% oocyst rate of different laboratory reared *Anopheles stephensi* populations was reported for *Plasmodium berghei* [17].

Table 1. Sporozoites rate of blood-fed *Anopheles stephensi* mosquitoes

Experiment number	No of dissected mosquitoes	No of sporozoites positive mosquitoes	Sporozoite rate (average %)
1	20	10	50
2	20	7	35
3	20	8	40
4	20	14	70
5	20	15	75
6	20	11	55
7	20	9	45
8	20	10	50
9	20	14	70
10	20	9	45
11	20	10	50
12	20	7	35
13	20	12	60
14	20	16	80
15	20	13	65
16	20	8	40
Total	**320**	**173**	**(Average = 54)**

Table 2. The number of mosquitoes died per day out of 150 mosquitoes per group

Days	Unfed mosquitoes dead (D1)	Un-infected blood fed mosquitoes dead (D2)	Infected blood fed mosquitoes dead (D3)
1st	3	2	1
2nd	1	1	3
3rd	5	4	2
4th	7	5	6
5th	2	2	1
6th	9	1	3
7th	5	4	5
8th	8	2	4
9th	11	8	9
10th	13	7	6
Total	**64**	**36**	**40**

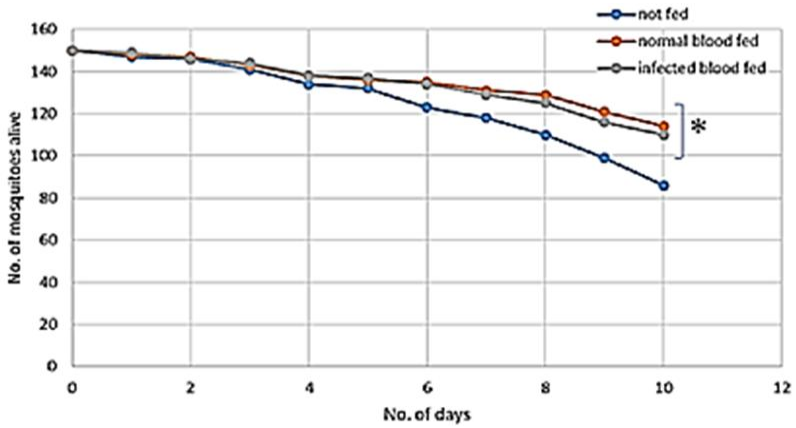

Fig. 4. Survival curve of female *Anopheline mosquitoes.* Survival rate curve of unfed, un-infected blood fed and infected blood fed female *Anopheles stephensi mosquitoes.* *represents significance (p<0.01)

Sporozoites rate (54%) indicated the successful progression of malaria parasite in *Anopheles stephensi*. However sporozoite rate reported here is in line with previously published data in India [11]. Sporozoite rate of *Anopheles darlingi* and *Anopheles culicifacies* for *P. falciparum* and *P. vivax* were reported to be 41% and 56%, respectively [11,18], which corroborate our findings. Around half of the mosquito population was negative for sporozoite in its salivary gland. Another explanation is that the mosquitoes which did not show any progression of malaria parasite might have retained their anti-parasite immunity even after infected blood meal. Mosquito specific nitrous oxide (NO) shows anti-parasitic activity and blocks the parasite development [19]. Variation of sporozoite rates among mosquitoes might be due to the variations in access to feeding chances or intrinsic receptors for nutrients.

Generally, gametocytes inside gut wall of female mosquitoes were able to develop into oocysts. However not all oocysts were developed into sporozoites which might be due to anti-parasitic mechanisms of mosquitoes. It was reflected in our findings where oocyst rate was higher than sporozoites rate. Thousands of sporozoites are released from an oocyst however around 20% are able to localize at salivary gland. CSP and (thrombospondin related anonymous protein) TRAP act as ligands to bind with cognate receptors on salivary gland. CSP is the major antigen expressed on the surface of sporozoite. It is also involved in the progression of malaria parasite from midgut to salivary gland through its adhesive properties [20]. Rest of sporozoites (80%) are neutralized in the midgut by anti-parasite mechanisms of mosquito.

The mean survival rate per day (0.97 ± 0.02) of human blood fed female *Anopheles stephensi* mosquitoes is in line with other vectors in the region [21]. The higher survival probability per day exhibits better vector competence and makes the *Anopheles stephensi* a suitable vector for transmission of malaria parasite. The survival index calculated for other *Anophelines* species like *Anopheles dirus*, *Anopheles maculatus*, *Anopheles minimus*, and *Anopheles jayporiensis* in Laos, Iran and India corroborates our findings [22].

Data showed a positive correlation between survival rate and blood meal (uninfected or infected). Female mosquitoes which were not fed on blood died earlier than blood fed female mosquitoes. It is not surprising because blood fed female mosquitoes receive necessary proteins, iron and energy required for egg development while un-fed female mosquitoes remain starved. Presence of *P. vivax* in the gut of female mosquito has no role to play with its longer survival rate.

Longevity of mosquito survival is in favour of malaria parasite as it needs to complete its life cycle in the female mosquito [23]. Previous studies showed inconsistent and contrary data regarding survival probabilities of blood-fed mosquitoes. Some studies revealed no effect on survival probability [24-29] while others revealed reduction in survival probability [30-34]. Our data suggested that infected *Anopheles stephensi* mosquitoes survive longer than the mosquitoes who did not get a blood meal, but survive slightly lesser than the uninfected mosquitoes that got normal blood meal.

5. CONCLUSIONS

It is concluded that laboratory reared female *Anopheles stephensi* mosquitoes support the development of *P. vivax*. Blood-fed mosquitoes showed considerable competence for malaria parasite which is dependent both on sporozoite and oocyst rates. Vector competence exhibited female *Anopheles stephensi* mosquitoes as a potential source of parasite transmission. Data warrant further studies regarding malaria vectorial capacity of field isolates (female *Anopheles stephensi* mosquitoes). It would help to manage the major public health concern in the region.

CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the authors.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Hay SI, Okiro EA, Gething PW, Patil AP, Tatem AJ, Guerra CA, et al. Estimating the global clinical burden of *Plasmodium falciparum* malaria in 2007. PLoS Med. 2010;7(6):1000290.
2. Thongsahuan S, Baimai V, Junkum A, Saeung A, Min GS, Joshi D, et al. Susceptibility of *Anopheles campestris*-like and Anopheles barbirostris species complexes to *Plasmodium falciparum* and *Plasmodium vivax* in Thailand. Mem Inst Oswaldo Cruz. 2011;106(1):105-12.
3. Organization WH. World malaria report 2016. Geneva: WHO Embargoed until. 2016;13.
4. Gholizadeh S, Zakeri S, Djadid ND. Genotyping *Plasmodium vivax* isolates infecting Anopheles stephensi, an Asian main malaria vector. Experimental Parasitology. 2013;134(1):48-51.
5. Khattak AA, Venkatesan M, Nadeem MF, Satti HS, Yaqoob A, Strauss K, et al. Prevalence and distribution of human Plasmodium infection in Pakistan. Malar J. 2013;12(1):297.
6. Who. World malaria report 2013: World Health Organization; 2014.
7. Kakar Q, Khan M, Bile K. Malaria control in Pakistan: new tools at hand but challenging epidemiological realities. 2010.
8. Organization WH. Malaria Entomology and Vector Control. 2013.
9. Williams J, Pinto J. Training Manual on Malaria Entomology for Entomology and Vector Control Technicians (Basic Level) Roll Back Malaria WHO PAHO: RTI International; 2012. Available:pdf.usaid.gov/pdf_docs/PA00J1Z9.pdf
10. Rutledge L, Ward R, Gould D. Studies on the feeding response of mosquitoes to nutritive solutions in a new membrane feeder. Mosq News. 1964;24(4):407-9.
11. Adak T, Kaur S, Singh O. Comparative susceptibility of different members of the *Anopheles culicifacies* complex to *Plasmodium vivax*. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1999;93(6):573-7.
12. Bonnet S, Gouagna C, Safeukui I, Meunier J-Y, Boudin C. Comparison of artificial membrane feeding with direct skin feeding to estimate infectiousness of *Plasmodium falciparum* gametocyte carriers to mosquitoes. Transactions of the Royal Society of tropical Medicine and Hygiene. 2000;94(1):103-6.
13. Sattabongkot J, Maneechai N, Phunkitchar V, Eikarat N, Khuntirat B, Sirichaisinthop J, et al. Comparison of artificial membrane feeding with direct skin feeding to estimate the infectiousness of *Plasmodium vivax* gametocyte carriers to mosquitoes. The American Journal of Tropical Medicine and Hygiene. 2003;69(5):529-35.
14. Touray MG, Warburg A, Laughinghouse A, Krettli AU, Miller LH. Developmentally regulated infectivity of malaria sporozoites for mosquito salivary glands and the vertebrate host. The Journal of Experimental Medicine. 1992;175(6):1607-12.
15. Russell H, Sampson JS, Schmid GP, Wilkinson HW, Plikaytis B. Enzyme-linked immunosorbent assay and indirect immunofluorescence assay for Lyme disease. Journal of Infectious Diseases. 1984;149(3):465-70.
16. Alavi Y, Arai M, Mendoza J, Tufet-Bayona M, Sinha R, Fowler K, et al. The dynamics of interactions between Plasmodium and the mosquito: A study of the infectivity of *Plasmodium berghei* and *Plasmodium gallinaceum* and their transmission by *Anopheles stephensi, Anopheles gambiae* and *Aedes aegypti*. International Journal for Parasitology. 2003;33(9):933-43.
17. Basseri HR, Hajipirloo HM, Bavani MM, Whitten MM. Comparative Susceptibility of different biological forms of *Anopheles stephensi* to *Plasmodium berghei* ANKA Strain. PloS one. 2013;8(9).
18. Grieco JP, Achee NL, Roberts DR, Andre RG. Comparative susceptibility of three species of Anopheles from Belize, Central America, to *Plasmodium falciparum* (NF-54). Journal of the American Mosquito Control Association. 2005;21(3):279-90.
19. Luckhart S, Vodovotz Y, Cui L, Rosenberg R. The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. Proceedings of the National Academy of Sciences. 1998;95(10):5700-5.
20. Aly AS, Vaughan AM, Kappe SH. Malaria parasite development in the mosquito and infection of the mammalian host. Annual Review of Microbiology. 2009;63:195.
21. Edalat H, Moosa-Kazemi SH, Abolghasemi E, Khairandish S. Vectorial capacity and Age determination of *Anopheles stephens* Liston (Diptera: Culicidae), during the

malaria transmission in Southern Iran. 2015.
22. Vythilingam I, Phetsouvanh R, Keokenchanh K, Yengmala V, Vanisaveth V, Phompida S, et al. The prevalence of Anopheles (Diptera: Culicidae) mosquitoes in Sekong Province, Lao PDR in relation to malaria transmission. Tropical Medicine & International Health. 2003;8(6):525-35.
23. Schwartz A, Koella J. Trade-offs, conflicts of interest and manipulation in Plasmodium-mosquito interactions. . Trends in Parasitology. 2001;17:189-19.
24. Boyd M. On the correlation between the incidence of stomach and gland infection in Anopheles quadrimaculatus infected with *Plasmodium vivax*. American Jounal of Trophical Medicine and Hygiene. 1940; 20:129-31.
25. Chege G, Beier J. Effect of *Plasmodium falciparum* on the survival of naturally infected Afrotropical Anopheles (Diptera: Culicidae). Journal of Medical Entomology. 1990;27:454-8.
26. De Buck A. Some results of six years' mosquito infection work. American Journal of Hygiene. 1936;24:1-18.
27. Gamage-Mendis A, Rajakaruna J, Weerasinghe S, Mendis C, Carter R, Mendis K. Infectivity of *Plasmodium vivax* and *P. falciparum* to *Anopheles tessellatus*; relationship between oocyst and sporozoite development. Transactions of the Royal Society of Tropical Medicine and Hygiene. 1993;87:3-6.
28. Hogg J, Hurd H. The effects of natural *Plasmodium falciparum* infection on the fecundity and mortality of Anopheles gambiae s.l. in north east Tanzania. Parasitology. 1997;114:325-31.
29. Robert V, Verhave J, Carnevale P. *Plasmodium falciparum* infection does not increase the precocious mortality rate of *Anopheles gambiae*. Trans R Soc Trop Med Hyg. 1990;84:346-7.
30. Ferguson H, Read A. Genetic and environmental determinants of malaria parasite virulence in mosquitoes. Proceedings of the Royal Society of London. 2002;269:1217-24.
31. Hogg J, Hurd H. Plasmodium yoelli nigeriensis: The effect of high and low intensity upon the egg production and bloodmeal size of Anopheles stephensi during three gonotrophic cycles. Parasitology. 1995;111:555-62.
32. Klein T, Harrison B, Andre R, Whitmire R, Inlao I. Detrimental effects of *Plasmodium cynomolgi* infections on the longevity of Anopheles dirus. Mosquito News. 1982; 42:265-71.
33. Klein T, Harrison B, Grove J, Dixon S, Andre R. Correlation of survival rates of Anopheles dirus A (Diptera: Culicidae) with different infection densities of Plasmodium cynomolgi. Bulletin of the World Health Organization. 1986;64:901-7.
34. Gad A, Maier W, Piekarski G. Pathology of Anopheles stephensi after infection with *Plasmodium berghei* Journal of Parasites Client-Research Parasitology. 1979; 60:249-61.

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