

Short Communication

Ovipositional Behavior of *Anopheles gambiae* Mosquitoes

Jeffrey Agyapong¹, Joseph Chabi², Aikins Ablorde², Worlasi D. Kartey¹, Joseph H.N. Osei¹, Dzedzom K. de Souza¹, Samuel Dadzie¹, Daniel A. Boakye¹, Nobuo Ohta³, Melinda P. Hadi² and Takashi Suzuki^{1,3*}

Received 31 May, 2014 Accepted 7 September, 2014 Published online 13 September, 2014

Abstract: Mosquito eggs laid within two hours are necessary for transgenic (injection) studies, because mosquito eggs become hard after that period. Thus, in order to have eggs available within this two-hour window, it is important to understand the ovipositional behavior of *Anopheles gambiae* s.s.. In the present study, the ovipositional behavior of *An. gambiae* s.s. (Kisumu) was investigated in several different conditions: age of mosquitoes, time post blood meal to access oviposition substrate, and light conditions. Two groups of mosquitoes, 3–5 days old and 9–11 days old were blood-fed. For those mosquito groups, an oviposition dish was set either at 48 hours or 72 hours after the blood meal either in a light condition or in an artificial dark condition. The number of laid eggs was compared among the different conditions. The 3–5 day-old mosquitoes apparently produced a higher number of eggs than 9–11 day-old mosquitoes, while there was no significant difference between the two groups. The number of laid eggs per one surviving blood-fed mosquito in the dark condition was significantly higher than that in the light condition ($p = 0.03$). Providing an oviposition dish at 72 hours after blood meal resulted in a significantly higher number of laid eggs per one surviving blood-fed mosquito than at 48 hours after blood meal ($p = 0.03$). In conclusion, the optimal condition to have readily available egg supply for transgenic analysis was as follows: 3–5 day-old mosquitoes with an oviposition dish placed at 72 hours after the blood meal in a dark environment.

Key words: *Anopheles gambiae*, ovipositional behavior, transgenic study

INTRODUCTION

Malaria is caused by *Plasmodium*, a protozoan parasite and transmitted by the bite of infected mosquitoes of genus *Anopheles*. Annually 250 million people are infected and close to one million people, mostly children in sub-Saharan Africa were killed by malaria [1]. Insecticide-based control such as massive use of insecticides and insecticide-impregnated bed nets has been one of the most successful methods to control malaria [2]. However, emergence and spread of insecticide-resistant *Anopheles* observed throughout the world including Africa pose a threat to the gains made in malaria control [3, 4]. Novel approaches to the control of vectors are therefore urgently needed to combat malaria.

Advances in insect molecular biology, in particular the development of germ-line transformation, have opened a new avenue for the control of malaria [5]. One of the ma-

ajor goals of gene-modification studies is to generate malaria parasite-resistant (refractory) *An. gambiae* s.s., the most important vector of human malaria in Africa, and to replace the wild-type *An. gambiae* s.s. population that is susceptible to malaria parasites with the genetically modified mosquito population.

The first key step for germ-line transformation is to obtain a requisite number of mosquito eggs for injection. Since mosquito eggshell becomes hard within two hours after being laid [6], it becomes difficult to inject genes into mosquito eggs using a glass needle after that period. Therefore clarification of the conditions in which mosquito eggs laid within two hours can be efficiently obtained is a crucial prerequisite for gene-modification studies. Compared to *Anopheles stephensi*, which is the predominant *Anopheles* vector in the Indian subcontinent, *An. gambiae* s.s. has been used less frequently in embryo transgenic studies and relatively little information is available regard-

¹ Department of Parasitology, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon LG 581, Ghana

² Vestergaard-NMIMR Vector Labs (VNVL), Department of Parasitology, Noguchi Memorial Institute for Medical Research, University of Ghana, Legon LG 581, Ghana

³ Section of Environmental Parasitology, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan

*Corresponding author:

Tel: +81-3-5803-5191

Fax: +81 3 5803 5191

E-mail: 0897vip@tmd.ac.jp

ing its ovipositional behavior [7]. In the present study, we analyzed ovipositional behavior of *An. gambiae* s.s. with reference to the following conditions: age of mosquitoes, time post blood meal to access oviposition substrate, and light conditions at oviposition.

METHODS

Mosquitoes

An. gambiae s.s. (Kisumu strain) mosquito colony was maintained by the following procedures. The mosquitoes were reared in a climate controlled insectary at a temperature of $28 \pm 1^\circ\text{C}$, 12L:12D photoperiod and relative humidity of $70 \pm 10\%$. The adults were kept in holding cages ($30 \times 30 \times 30$ cm) with access to 10% sucrose (WAKO) solution. 4–6 day-old females were blood-fed on rabbits and provided with oviposition dishes, covered with moist filter paper. The eggs laid on the filter paper were transferred to plastic trays. Hatched larvae were fed on fish meal (Tetramin®). Pupae were collected in small cups and transferred to holding cages.

Oviposition

Two groups of *An. gambiae* s.s. mosquitoes were used: 3–5 day-old mosquitoes and 9–11 day-old mosquitoes. For each group, approximately 100 female mosquitoes were kept in one cage (4 cages per group). The

mosquitoes in each group were maintained in a photoperiod of 12L:12D and blood-fed at halfway of the light period, after being starved for at least 3 hours. An oviposition dish was then set in each cage either at 48 hours or at 72 hours after the blood meal either in a light condition or in an artificial dark condition. The number of eggs laid on filter papers was counted every 30 minutes up to 2 hours for each cage. All experiments were done at a temperature of $28 \pm 1^\circ\text{C}$ in triplicate.

Statistical analysis

The number of laid eggs was adjusted by the number of surviving blood-fed female mosquitoes. The obtained values (numbers of eggs per one surviving blood-fed mosquito) were compared separately for each condition: age of mosquitoes, time post blood meal to access oviposition substrate, and light conditions. Significant differences were analyzed by Student's t-test (significance at $p < 0.05$).

RESULTS AND DISCUSSION

In order to understand the ovipositional behavior of *An. gambiae* s.s., we compared the effect of mosquito age on egg-laying behavior. The 3–5 day-old mosquitoes apparently produced a higher number of eggs than the 9–11 day-old mosquitoes (Table 1), while there was no signifi-

Table 1. Numbers of eggs produced by female *An. gambiae* mosquitoes in different conditions.

		Light				Dark			
		48 hours		72 hours		48 hours		72 hours	
		3–5 day	9–11 day	3–5 day	9–11 day	3–5 day	9–11 day	3–5 day	9–11 day
Number of eggs laid at	0–30 min	0 ± 0	1.0 ± 1.0	13.3 ± 13.3	0.3 ± 0.3	63.7 ± 63.7	0.3 ± 0.3	301.0 ± 112.2	140.0 ± 67.6
	30–60 min	0 ± 0	0 ± 0	3.7 ± 3.7	1.7 ± 1.7	27.0 ± 27.0	25.0 ± 25.0	206.0 ± 91.0	50.0 ± 42.7
	60–90 min	0 ± 0	0 ± 0	0 ± 0	1.0 ± 1.0	0 ± 0	1.0 ± 1.0	14.7 ± 10.4	0.3 ± 0.3
	90–120 min	0 ± 0	0.3 ± 0.3	16.7 ± 8.4	47.3 ± 29.3	0 ± 0	17.0 ± 17.0	0 ± 0	46.0 ± 46
Number of eggs (total)		0 ± 0	1.3 ± 1.3	33.7 ± 25.4	50.3 ± 32.3	90.7 ± 90.7	43.3 ± 43.3	521.7 ± 213.2	236.3 ± 156.6
Number of female (total) mosquitoes		107.0 ± 3.0	88.0 ± 10.4	108 ± 6.5	85.3 ± 13.9	109.3 ± 4.1	88.7 ± 12.3	112.0 ± 3.8	84.3 ± 11.5
Number of blood-fed mosquitoes		78.0 ± 3.5	61.3 ± 7.6	83 ± 4.2	55.3 ± 9.6	88.3 ± 3.7	61.0 ± 8.5	73.0 ± 13.0	59.0 ± 5.5
Dead (blood-fed)		2.3 ± 1.9	7.3 ± 1.5	5 ± 2.9	10.3 ± 4.3	1.3 ± 0.9	11.0 ± 4.2	3.3 ± 1.5	11.7 ± 3.8
Dead (blood-unfed)		1.0 ± 0	3.7 ± 1.5	2.7 ± 0.9	3.7 ± 0.3	0.7 ± 0.3	5.3 ± 2.6	1.0 ± 0.6	6.7 ± 2.2
Feeding Rates		72.9%	69.7%	76.9%	64.8%	80.8%	68.8%	65.2%	70.0%
Mortality Rates		3.1%	12.5%	7.1%	16.4%	1.8%	18.4%	3.9%	21.7%
Number of laid eggs (total) / number of blood-fed alive mosquitoes		0 ± 0	0 ± 0	0.4 ± 0.3	1.2 ± 1.2	1.1 ± 1.0	1.1 ± 1.3	7.6 ± 3.9	5.8 ± 2.7

Each value (mean ± SE) represents the mean of three independent experiments. Feeding rate was calculated as follows: number of blood-fed mosquitoes divided by number of female (total) mosquitoes. Mortality rate was calculated as follows: number of both blood-fed and -unfed dead mosquitoes divided by number of female (total) mosquitoes.

cant difference ($p = 0.86$) between the number of eggs produced by one female mosquito in the two groups. Mortality of *Anopheles* mosquitoes was reported to be age-dependent [8], and indeed the average mortality of 9–11 day-old mosquitoes in our study was significantly higher than that of 3–5 day-old mosquitoes (Table 1). The difference in the numbers of laid eggs between the two groups might therefore be due to the difference in the actual numbers of surviving mosquitoes.

Secondly, light condition was compared. The number of laid eggs per one surviving blood-fed mosquito was significantly higher in the dark conditions than that in the light conditions ($p = 0.03$) (Table 1, Fig. 1), which was consistent with the previous report [9]. Since wild-type *Anopheles* mosquitoes were reported to lay eggs preferably at night [10], artificial dark environment seemed to promote the egg-laying behavior of *An. gambiae s.s.* mosquitoes.

Finally, oviposition rates were compared between mosquito groups for which oviposition dishes were set at 48 hours and at 72 hours after the blood meal. Providing an oviposition dish at 72 hours after blood meal resulted in a significantly higher number of laid eggs per one surviving blood-fed mosquito compared to that at 48 hours after blood meal ($p = 0.03$) (Fig. 1). No significant difference was observed in hatching rate or viability of laid eggs between the two mosquito groups (data not shown). Since female mosquitoes require blood nutrients to produce eggs, the longer period after the blood meal may have allowed

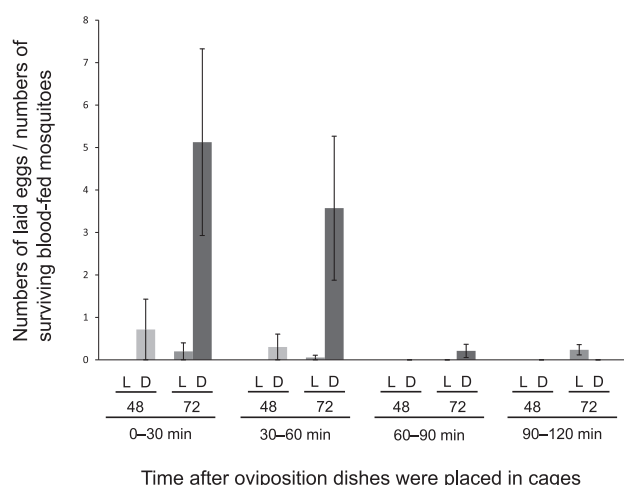


Fig. 1. Comparison of numbers of eggs per one surviving blood-fed mosquito at 0–30 min, 30–60 min, 60–90 min and 90–120 min in different conditions. 3–5 day-old mosquitoes. L denotes light condition. D denotes artificial dark condition. “48” and “72” denote that the oviposition dish was placed at 48 hours and 72 hours after blood feeding, respectively.

mosquitoes to digest the blood and consequently to utilize more nutrients for egg production. Alternatively, the longer period of holding matured eggs may have induced the more active egg laying of the mosquitoes when the oviposition dish was made available in the cage. Also possibly, it may have taken more than 48 hours for mosquitoes to fully recover from the effect of starvation before the blood meal. However, it seems unlikely that a holding period longer than 72 hours would result in higher numbers of eggs in total because mosquito mortality rates would increase [8].

From these results, the best condition for optimal oviposition rates of *An. gambiae s.s.* mosquitoes was determined as follows: 3–5 day-old mosquitoes for which an oviposition dish was placed at 72 hours after the blood meal in a dark environment. It would be also noteworthy that in this condition, most of the eggs were laid within 60 min after the placement of oviposition dishes. To our knowledge, this is the first report on the time-course ovipositional behavior of *An. gambiae* mosquitoes. While *An. gambiae s.s.* strain-specific difference may exist, this egg laying system could provide valuable information for transgenic studies of *An. gambiae s.s.* mosquitoes. Moreover, the above information may be useful for practical malaria vector control.

ACKNOWLEDGMENTS

The study was supported in part by a grant-in aid from the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.

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