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Effects of Gamma Irradiation on Mating Competitiveness of Male *Culex quinquefasciatus* (Diptera:Culicidae) in Laboratory

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ABSTRACT

Culex quinquefasciatus is the main vector of lymphatic filariasis in Pekalongan City. Sterile Insect Technique (SIT) can be employed as complementary vector control for filariasis. The key success of this technique depends on the ability of laboratory-reared sterile males to mate with the wild-type females. This research aimed to was to determine the mating competitiveness, fecundity and fertility of sterile males of *Cx. quinquefasciatus*. The pupae of *Cx. quinquefasciatus* were gamma irradiated at doses of 60, 70, and 80 Gy, whereas unirradiated pupae were prepared as control. The mosquitoes emerging from the irradiated pupae were found to be able to mate with normal females in the cages. Observation was done for the mean female laying eggs, the fecundity, the fertility and the mating competitiveness. The observation data were analyzed by one-way ANOVA. The results show that the irradiated *Cx. quinquefasciatus* at the test doses does not affect the fecundity and the mating competitiveness, but the fertility is disturbed (sterile). A dose of 70 Gy was found to be the optimum dose, which gave a fertility rate of 1.8 % (98.2 % sterile) with a value of competitiveness (C index) of 0.568. Based on these results, irradiated *Cx. quinquefasciatus* can be recommended for semifield application.

INTRODUCTION

Lymphatic filariasis is a communicable disease caused by filarial worms, which can be transmitted through the bite of various kinds of mosquitoes. There are three species of worms causing lymphatic filariasis in Indonesia, namely *Wuchereria bancrofti*, *Brugia malayi* and *Brugia timori* [1]. These three species exist in Indonesia, but more than 70 percent of cases of filariasis in Indonesia are caused by *Brugia malayi* [2].

Pekalongan City is one of the endemic regions of lymphatic filariasis caused by *Wuchereria bancrofti* with *Culex quinquefasciatus* being the vector [3]. The reports by Pekalongan City Health Office mention that there were 12 cases of chronic lymphatic filariasis in 2007. There are 7 villages in 2 sub-districts with a microfilaria rate of > 1 %. That is why Pekalongan City is considered as a filariasis endemic area. Well-preserved places for *Culex quinquefasciatus* breeding, with small coverage of Mass Drugs Administration (60.3 percent), and people's lack of awareness of environmental sanitation, the resistance of filarial larvae in mosquitoes and vector to insecticides is found to be the factor that keeps filarial transmission going in Pekalongan City [4-6].

This also proves that the mass drug administration in the last five years has not effectively cut the chain of filarial transmission. An important effort that should be made is conducting

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surveilance of vector of filariasis as the basis for vector control in cutting the transmission chain. Furthermore, vector control of filariasis in Indonesia has not been done in a dedicated way due to extreme complex issues. The application of Sterile Insect Technique (SIT) is one of vector control efforts that can be made for this purpose. SIT is a highly specific biological vector control technique which only affects target species. This technique only reduces the population size in the field, not kills off the population, and graduated release can reduce the mosquito population [7]. The SIT principle is that sterile male insects (*Cx. quinquefasciatus*) are released in a considerable number to a target area. The released sterile males of *Cx. quinquefasciatus* will compete with the wild type males to mate wild type females. The mating between sterile *Cx. quinquefasciatus* males and wild type females is expected to produce no offspring, which eventually reduce the population size of *Cx. quinquefasciatus* in that area [8].

SIT is an environment-friendly, effective and potential vector control technique. This technique is also called species-specific control, by which vector is killed by the vector itself (autocidal technique) [9]. The technical procedure of this technique is relatively easy. Male mosquitoes are irradiated in a laboratory and then released to their habitat periodically. Irradiation can cause male mosquitoes to be sterile as it causes damages to the spermatogenesis and stops the production of sperms (aspermia). Sperm inactivation also causes sterilization as the sperms will become unable to move to fertilize the egg cell. The inability to mate is yet another sterilization factor because irradiation causes damages to somatic cells of the internal genital organs and prevents egg cell fertilization [8] .

The application of sterile insect technique in Indonesia has been tested on *Aedes aegypty* in vector control of high fever, and this technique has successfully reduced the size of mosquito population by up to 95.23 percent. This condition lasts for 3-6 months until high fever cases resurface. In the research on *Ae. aegypti* conducted by Batan, a Gamma irradiation dose of 70 Gy can sterilize mosquitoes by up to 100 percent with a mating competitiveness value of 0.31, and a dose of 65 Gy can sterilize by 98.53 percent with a mating competitiveness value of 0.45 [10]. On *Anopheles maculatus*, a dose of 110 Gy can sterilize by 97 percent with a mating competitiveness value of 0.65, and a dose of 120 Gy dose can sterilize by 99.99 percent [11]. Setiyaningsih *et al*. have reported that irradiation doses of 40 Gy, 50 Gy, 60 Gy and 70 Gy applied to *Cx. quinquefasciatus* result in sterilization of 20.92 percent, 48.99 percent, 89.48 percent and 100 percent, respectively [12].

SIT application for *Cx. quinquefasciatus* control has never been done in Indonesia, thus a preliminary test should be conducted for supporting the success of SIT application in nature. Some preliminary tests are needed to support SIT application in the field, one of which is mating competitiveness of the sterilized male *Cx. quinquefasciatus*. This research was intended to evaluate the effect of gamma irradiation on fecundity, fertility and mating competitiveness of sterile male *Cx. quinquefasciatus* at laboratory scale. It is expected that the research results support the feasibility of SIT application in lymphatic filariasis vector control.

EXPERIMENTAL METHODS

This research is part of a doctoral dissertation at the Bogor Agricultural Institute (2018, Bogor, Indonesia). The study was carried out from June 2016 to June 2017. The rearing of mosquitoes were carried out as joint research with Banjarnegara Vector Control Research Unit, National Institute of Health Research and Development

Sample mosquitoes

The research sample consisting of *Culex quinquefasciatus* pupae of second to third generation from a field isolate in Pabean Subdistrict, Pekalongan City, were kept at the Laboratory of Entomology of the Center for Research and Development of Animal-Borne Disease Control of Banjarnegara. The colony was from the *Culex spp* larvae in Pabean Subdistrict, Pekalongan City, and was then kept at the laboratory, and the larvae were fed with uncrushed dog feed (Pedigree®). The administration of larva feed was adjusted in accordance to the age. After instar 3 they were moved to cultivation tray sized 27 cm x 35 cm x 5 cm with a density of 400-600 mosquitoes/tray. The larvae were raised until they grew into pupae. The pupae were taken and put into a mosquito cage. The emerging mosquitoes in the cage were identified and separated from *Cx. quinquefasciatus* and given 10 % sugar solution and marmot blood to enable them to lay eggs. The eggs produced were incubated and raised until they turned into pupae. The male and female pupae derived from the colonization at the laboratory were separated (male pupae are smaller than female pupae) with a sieving (90-95 percent small pupae are males) according to the method of Nurhayati *et al*. (2010) [13]. The identified male pupae were inserted into a bottle, kept in a cool box and carried to PAIR BATAN Jakarta to be irradiated. Female pupae were inserted in a 40 cm x 40 cm x 40 cm sized mosquito cage and then kept at the Laboratory of Entomology of the Center for Research and Development of Animal-Borne Disease Control of Banjarnegara before test.

Gamma irradiation

Irradiation was conducted at the Center for Isotopes and Radiation Application BATAN, Jakarta. Irradiation was carried out when *Cx. qinquefasciatus* pupae were ≥ 15 hours old using Gamma Cell-220 irradiator with adjustment of the position of radioactive sources around the material, so more uniform absorption doses can be obtained. The sample was placed in plastic pots with a lower diameter of 4.5 cm, upper diameter of 6.5 cm and height of 6.5 cm, filled with 20 ml of water. Every plastic pot was filled with 200 pupae to which irradiation doses of 60, 70 and 80 Gy were administered separately and alternately. The doses were determined based on the results of dose test in the previous research. The control group consisted of non-irradiated male *Cx. quinquefasciatus* prepared at the Laboratory of Entomology of the Center for Research and Development of Animal-Borne Disease Control of Banjarnegara at the same age as that of the irradiated pupae. After the gamma irradiation process, male *Cx. quinquefasciatus* pupae were raised until they became mature, and observation was conducted on their fertility, fecundity and mating competitiveness.

Fecundity and fertility

The mature *Cx. quinquefasciatus* males (n=25) on which gamma irradiation was administered were mated with normal female mosquitoes (n=25) in a cubic cage sized 30 cm x 30 cm x 30 cm. The control group consisted of normal *Cx. quinquefasciatus* males (n=25) that were mated with normal female mosquitoes (n=25). After three days of mating period, the male mosquitoes were released from the cage using aspirator. Female mosquitoes were fed with blood (marmot) until they were full, and then individually moved to a transparent plastic vial tube for oviposition process [14]. This treatment was repeated four times. The observation of the number of eggs, eggs that hatched, and eggs that did not hatch was conducted manually using stereo microscope with a 4x magnification. Eggs with open operculum were eggs that hatched, while eggs that did not hatch were marked with closed operculum [15].

Mating competitiveness

The observation of mating competitiveness was conducted at the Entomology Laboratory of the Center for Research and Development of Animal-Borne Disease Control of Banjarnegara at a temperature of 22-25 ^{o}C and humidity of 70-79 percent. After the irradiation process, each mosquito was inserted into the cage by taking into account the ratio between irradiated males, normal males and normal females. As many as 25 *Cx. quinquefasciatus* males and 25 *Cx. quinquefasciatus* females were used in every mating combination with four repetitions. The mating process took place naturally, where males and females were placed in a 30 cm x 30 cm x 30 cm sized cubic cage for 2-3 days for maximizing the mating. The irradiated male mosquitoes were marked with code R, while the normal female and male mosquitoes were marked with code N. Then, all mosquitoes were mated following the combinations in the method of Bellini *et al*. [16] as shown in Table 1.

Table 1. Mating combinations of *Cx. quinquefasciatus* for the observation of mating competitiveness.

Doses (Gy)	Replication	S/N	Mating Combinations
			composition of each dose:
60	4	3	Ha = 25 ♂N X 25 ♀N
70	4	3	Hs = 25 ♂R X 25 ♀N
80	4	3	E = 75 ♂R X 25 ♂N X 25 ♀N

Ha is the percentage of hatching eggs from normal male-normal female mating combination. Hs is the percentage of hatching eggs from irradiated male-normal female mating combination with a ratio of 1:1. E is the percentage of hatching eggs from irradiated male, normal male and normal female mating combination with a ratio of 3:1:1. S/N is the ratio of irradiated males to normal males in mating combination E.

The mosquitoes in the mating cage were given 10 % sugar solution. The mosquitoes were given marmot blood for one hour each day for five days starting from the fourth day. Gravid female mosquitoes were taken individually and inserted into 150 ml sized paper glass filled with 50 ml of 10 % straw water as a place to lay eggs. The observation of the number of eggs and hatching eggs was conducted every day for five days. The observation was conducted for one gonotrophic cycle.

Data analysis

The parameters measured in this research were the number of gravid female mosquitoes, number of eggs produced by female mosquitoes (fecundity), number of hatching eggs (fertility) and the value of mating competitiveness obtained from Fried's Competitiveness Index (C index). Fertility rate was calculated by comparing the number of hatching eggs and the number of all eggs. The mating competitiveness value or C index was obtained from the equation according to the method of Bellini *et al.* (2013) [16].

The data were analyzed using SPSS version 16, and one-way Analysis of Variance (ANOVA) was used for evaluating the effect of gamma irradiation doses on fecundity, fertility and mating competitiveness with p value of < 0.05. Duncan's post hoc test was used for further analysis of the comparison between means.

RESULTS AND DISCUSSION

Fecundity

The data of fecundity and number of normal female *Cx. quinquefasciatis* mosquitoes successfully laying eggs by mating with irradiated males and normal males (non-irradiated) as control group are presented in Table 2.

Table 2. Fecundity of female *Culex quinquefasciatus* in mating with irradiated male mosquitoes with different dosages.

Doses (Gy)	Average % of female mosquitoes laying eggs	Number of eggs	Average number of eggs per mosquito ± SD (95 % CI)
Control	40.00[a]	4528[a]	111.8±10.2 (95.5-128.1)[a]
60	39.00[a]	4264[a]	109.3±26.5 (67.5-151.4)[a]
70	43.00[a]	5160[a]	120.7±11.1 (103.1-38.4)[a]
80	41.00[a]	3939[a]	96.4±27.5 (52.7-140.2)[a]

[a] $p > 0.05$

The percentage of female mosquitoes successfully laying eggs is 40 percent in the control group and 39-43 percent in the treatment group, and there is no statistical difference (F = 2.201; $p = 0.099$). The ability of mosquitoes to lay eggs is related to the structures and functions of the reproductive organs. Mosquitoes with healthy condition of reproductive organs and the ability to function normally tend to have more successful oviposition than otherwise. Mosquitoes may be unable to lay eggs because of their ovaries' inability to produce eggs or to have oviposition. The two factors are related to the biological aspect, especially the factor that is related to abnormalities of the organ function due to genetic factors [17].

Table 2 shows the number of eggs produced by *Cx. quinquefasciatus* females mating with the irradiated males. The highest number is produced at an irradiation dose of 70 Gy (5,160 eggs), and the lowest number at 80 Gy (3,939 eggs). The results of the statistical analysis show that there is no significant difference between the number of eggs produced by female *Cx. quinquefasciatus* at various irradiation doses and the control group (F = 1,544; $p = 0.254$; $p > 0.05$).

The largest number of eggs produced by female *Cx. quinquefasciatus* per oviposition is at the irradiation dose of 70 Gy (120.7 ± 11.1) and the lowest is at the dose of 80 Gy (96.4 ± 27.5). The results of the observation show that there is no difference between the average number of eggs per female *Cx. quinquefasciatus* at different irradiation doses and the control group (F = 0.907; $p = 0.498$; $p > 0.05$) (Table 2). These results are in line with the results of the research by Sasmita and Ernawan [18] on *Ae. aegypti* and *An. maculatus* [19].

The number of eggs produced by female mosquitoes is affected by the amount of blood sucked, biological factor and ecological factor [17]. According to Clements, to produce 85.5 eggs, on average, a mosquito needs 3-5 mg of blood. Eggs will not be produced if the amount of blood sucked is less than 0.5 mg [20]. The size of the mosquito's body is a biological factor, which affects the size of its abdomen and ovary. Larger ovary, which functions as a place in which eggs are produced, has bigger capacity and productivity. Large mosquitoes tend to have larger abdomens. In addition, the greater the amount of blood is sucked, the bigger the number of the eggs is produced [21].

Fertility

Fertility rate is the percentage of the number of hatching eggs out of the number of eggs produced by female mosquitoes mating with sterile or normal male mosquitoes. The fertility rates of female mosquitoes mating with irradiated and control male mosquitoes are shown in Table 3.

Table 3. Fertility rate of female *Culex quinquefasciatus* mating with irradiated male mosquitoes at different doses.

Doses (Gy	Average number of eggs	Average number of hatching eggs	Average % of hatching eggs ± SD (95 % CI)
Control	1132.00	1076.25	94.1±5.4 (85.6-102.7)
60	1066.00	51.17	4.8±1.5 (2.4-7.3)[a]
70	1290.00	23.22	1.8±0.6 (1.0-2.6)[a]
80	984.75	12.80	1.3+0.6 (0.4-2.4)[a]

[a] $p < 0.05$

Normal female mosquitoes mating with normal males have the highest fertility at 94.1 percent, while those mating with irradiated males have fertility of around 1.3-4.8 percent. The higher the irradiation dose, the lower the fertility rate will be resulted. The results of the statistical test show a significant difference in the fertility rate of treatment group and control group (F = 896.614; p = 0.00).

The eggs produced by female *Cx. quinquefasciatus* show that the insemination process may progress, but the embryo formed can not survive due to the dominant lethal mutation properties carried by irradiated male mosquito sperm cells. Dominant lethal mutation does not inhibit the process of male nor female gametogenesis, and there is no inhibition in the zygote formation either, but the embryo will experience death [9]. The dominant lethal mutation can be seen from the decreasing fertility rate with the increase in gamma irradiation doses [22].

The control group has a high fertility rate because the sperm cells transferred by male mosquitoes to female spermatheca in the mating process are normal. The meeting of normal sperm cell and egg cell of a female mosquito will produce a fertile egg [20].

Setiyaningsih *et al*. have reported that SIT application affects the decrease in the fertility of the eggs of *Ae. aegypti*, both in and out of home [20]. The decrease in the percentage of fertility is due to the mating between the sterilized *Cx. quinquefasciatus* males and normal females in the nature. Sterile male mosquitoes will transfer sterile sperm cells to the spermatheca of female mosquitoes and produce sterile eggs. Sterile eggs may also be formed when sterile male mosquitoes fail to mate with female mosquitoes due to morphological changes in the male mosquitoes' sex organs because of the irradiation process. The morphological changes in the sex organs of male mosquitoes may hinder the transfer of sperm cells to female mosquitoes and prevent egg cell fertilization [20,23].

Pupal stadium is a developmental stadium where young organs transform or develop into mature organs [24]. In this stadium, spermatogenesis normally takes place. A small dose of radiation (65-70 Gy) may have caused sterilization already. In the permatogenesis process, the sperm cells split rapidly. If the irradiation interacts with the sperm cells, some changes will occur and abnormal sperm cells are produced. Abnormal sperm cells have small heads, short tails and low mobility, while normal sperm cells are bigger and have higher mobility [23].

The percentage of egg hatching of female mosquitoes used in this research is fairly good. It reaches 94.1 percent. This is different from the research by Sasmita and Ernawan on *Aedes aegypti* [18], where the percentage of egg hatching was 50.6 percent. The variation in gamma irradiation doses affects the fertility and sterilization of *Cx. quiquefasciatus*, which agrees with Setiyaningsih *et al*. [12]. Shetty *et al*. have reported the effect of gamma irradiation dosage of 0-50 Gy on fertility, egg hatching, occurrence, and age of *Ae. Aegypti* [27].

Some eggs do not hatch because there is no fertilization between sterile sperm cells and egg cells because the *Cx. quiquefasciatus* males are unable to perfectly copulate with normal females as their genital organs change, preventing the sperm cells to be delivered perfectly [23]. Decreased egg fertility also shows the ability of the sterile male *Cx. quiquefasciatus* to compete with normal male *Cx. quiquefasciatus* in the nature in finding mates for mating.

Mating competitiveness

The mating competitiveness values of the sterilized *Cx. quinquefasciatus* at various irradiation doses are presented in Table 4. The highest C index value was obtained at the irradiation dose of 70 Gy (C index = 0.53), while the lowest was at the irradiation dose of 80 Gy (C index = 0.36). The statistical test results show no significant difference in the mating competitiveness of the sterile male *Cx. quinquefasciatus* at different irradiation doses (F = 0.526; $p > 0.05$).

Table 4. The Mating Competitiveness Values of sterile *Cx. quinquefasciatus* at different irradiation doses with mating combinations at the laboratory scale.

Doses (Gy)	Percentage of the hatching of mating combination ♂R: ♂N: ♀N			S/N	C index
	0 : 25 : 25	25 : 0 : 25	75 : 25 : 25		
60	91.52	3.15	43.65	3	0.44[a]
70	91.54	2.27	38.32	3	0.53[a]
80	91.51	1.05	45.13	3	0.36[a]

[a] $p > 0.05$

High mating competitiveness shows that the gamma irradiation dose of Co-60 administered in the pupal stadium does not have any effect on the mating competitiveness. Every species needs a certain optimum gamma irradiation dose for the sterilization of eggs without affecting its mating competitiveness [22,26].

In this research the irradiation dose does not have any effect on the mating competitiveness statistically as it is possibly caused by the relatively small range of the doses tested. Some researches, however, have proven that higher doses will cause negative effect on mating competitiveness [26] and this is also found in *Ae. Aegypti* [15] and *An arabiensis* [23]. Higher irradiation doses (> 120 Gy) applied in the sterilization of males may also cause the males to be unable to transfer sperm cells into female mosquitoes [13].

Sterile males' success in mating may be caused by their ability to compete with normal males in getting mates. The main factor of the decrease in mating competitiveness in SIT is the gamma irradiation sterilization process. The ionizing capability of gamma radiation may cause damages to somatic cells, which may also deteriorate male *Ae. aegypti*'s form [16,25]. Low physical form may also worsen males' ability to mate with females. The results of some previous researches show that the mating competitiveness of male mosquitoes has a negative correlation with the increase in gamma irradiation dose [16,18].

A dose of 70 Gy is an optimum dose to be used at a fertility rate of 1.8 percent, with a competitiveness value of 0.53. This indicates that the irradiation dose of 70 Gy can decrease the egg hatching rate by 98.2 percent. Mating competitiveness value or C Index is useful for determining the number of sterile male mosquitoes spread in the nature following the method of Sasmita and Ernawan [18]. In this research the mating competitiveness of *Cx. quinquefasciatus* was 0.53. This means that the number of sterile males released should be at least twice of the population of males in nature to increase mating possibility with normal females.

Our results show that wild *Cx. quinquefasciatus* males irradiated at the optimum dose (70 Gy) can compete successfully with the unirradiated male for wild females in the laboratory.

CONCLUSION

Irradiation of *Culex quinquefasciatus* pupae at a dose of 60-80 Gy affects fertility, but does not affect the fecundity and mating competitiveness. A dose of 70 Gy is found to be the optimum dose, which yields a fertility rate of 1.8 percent (98.2 percent sterile) and a mating competitiveness value of 0.53 (i.e., the number of sterile male mosquitoes released should be about twice the number of male mosquitoes in the population).

The results of this research can be used as a baseline for further testing at semi-field and limited-field scale and for assessing the feasibility of SIT for vector control of lymphatic filariasis in Pekalongan City.

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REFERENCES

1. Anonymous, Situasi Filariasis di Indonesia Tahun 2015 (2016). (in Indonesian)
2. Anonymous, Laporan Bulanan dan Tahunan Subdit Filariasis (2009). (in Indonesian)
3. T. Ramadhani, Suyoko and S. Sumarni, Journal of Health Ecology **9** (2010) 1303. (in Indonesian)
4. Windiastuti, Suhartono and Nurjazuli, Kesehatan Lingkungan Indonesia **12** (2013) 51.
5. T. Ramadhani and B.F. Wahyudi, Journal of Disease Vector **9** (2015) 1. (in Indonesian)
6. B.F. Wahyudi and N. Pramestuti, Balaba **12** (2016) 55. (in Indonesian)
7. V.A. Dyck, J. Hendrichs and A.S. Robinson, Sterile Insect Technique Principles and Practice in Area-Wide Integrated Pest Management, The Netherlands: Springer (2005) 412.
8. E.F. Knipling, J. Econ. Entomol. **48** (2005) 459.
9. S. Sutrisno, J. Ilmiah Aplikasi Isotop dan Radiasi **2** (2006) 35. (in Indonesian)
10. S. Nurhayati, B. Santoso and A. Rahayu, Proceedings of the National Seminar on Safety and the Environment **VI** (2010) 163.
11. Widiarti, Report of research (2010) (unpublish).
12. R. Setiyaningsih, Widiarti and H.J. Heriyanto, Vektora **7** (2015) 71.
13. S. Nurhayati, B. Santoso, A. Rahayu *et. al.*, Proceedings of the National Seminar on Safety and the Environment V (2009) 78. (in Indonesian)
14. D.J. Zhang, R.S. Lees and Z.Xi, PloS ONE **10** (2016) 1.
15. M.L. Zheng, D.J. Zhang and D.D Damiens, Parasites & Vectors **10** (2015) 1.

16. R. Bellini, F. Balestrino and A. Medici, J. Med. Entomol. **50** (2013) 94.
17. Iswanto, S.J. Mardihusodo and T. Baskoro, J. Sain Kesehatan **17** (2004) 89. (in Indonesian)
18. H.I. Sasmita and B. Ernawan, J. Ilmiah Aplikasi Isotop dan Radiasi **10** (2014) 149. (in Indonesian)
19. A.N. Clements, The Physiology of Mosquitoes, Pergamon Press, New York (1963) 393.
20. R. Setiyaningsih, M. Agustini and A. Rahayu, Vektora **7** (2015) 71.
21. B.J. Herms, Medical Entomology 4th ed., Mc Millan Co, New York (1950) 643.
22. S. Nurhayati, B. Yunianto and T. Ramadhani, J. Sains dan Teknologi Nuklir Indonesia **14** (2013) 1. (in Indonesian)
23. M.E.H. Helinski, A.G. Parker and B.G.J. Knols, Acta Tropica **109** (2008) 64.
24. G.H.S. Hoper, J. Econ. Entomol. **64** (1976) 464.
25. C.O. Calkins and A.G. Parker, The Netherlands, Springer (2005) 269.
26. M.E.H. Helinski and B.G.J. Knols. J. Med. Entomol. **45** (2008) 698.
27. V. Shetty, N.J. Shetty and B.P. Harini *et. al*., Parasite Epidemiology and Control **1** (2016) 26.