DETRIMENTAL EFFECTS OF *PLASMODIUM CYNOMOLGI* INFECTIONS ON THE LONGEVITY OF *ANOPHELES DIRUS*[1]

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ABSTRACT. The effects of *Plasmodium cynomolgi* on the longevity of *Anopheles dirus* were examined. The mortality rates of infected and non-infected groups surviving to the beginning of post-feeding periods 0–3, 4–8 and 31–40 days were not significantly different. However, there was a significant difference in the mortality rates between the 2 groups for periods 9–13, 14–20 and 21–30 days. A comparison of the cumulative mortality rates further illustrates that the mortality rates of the 2 groups were nearly the same until about day 10, when the mortality rate of the infected group was increased greatly. Furthermore, dissection of infected mosquitoes revealed excessive bacteria and deterioration of the midgut and salivary glands, especially for days 9–20. The mortality rates for both the infected and non-infected groups were higher for days 0–3 than for days 4–8, a possible post-blood feeding response.

INTRODUCTION

Malaria transmission in nature is dependent on the vector host, the human population and associated physiological and environmental factors. The probability of malaria transmission occurring is the direct result of a number of factors (Macdonald 1952b, 1953), including the mosquito survival rate. The logarithmic graph of the cumulative weekly survival of *Anopheles quadrimaculatus* Say (Keener 1945) formed almost a straight line, demonstrating that mortality was in the form of a geometric progression unaltered by age (Macdonald 1952a). Based on this and other observations, Macdonald (1952a) suggested that under natural conditions, i.e., without the use of pesticides, etc., mosquito mortality does not change with age. Furthermore, he formulated a mathematical rationale for estimating the sporozoite rate based on the assumption that a malaria infection has no influence on the viability of the anopheline host (Macdonald 1957), suggesting the survival rate of infected mosquitoes is not different from that of non-infected mosquitoes. This assumption generally was accepted and currently is a basic premise for the calculation of the daily survival rate of a vector, a primary component in the mathematical equations of such epidemiological indices as the "vectorial capacity" (Garrett-Jones 1964, Molineaux 1978) and the "entomological inoculation rate" (Onori and Grab 1980).

Actually, investigations of the pathogenicity of malarial parasites in mosquitoes have not been conclusive. A number of early workers (Buxton 1935, Sinton and Shute 1938, DeBuck and Swellengrebel 1935) suggested that *Plasmodium* parasites have a detrimental effect on the longevity of mosquitoes. However, other workers (Boyd 1940, Ragab 1958) concluded that there is no detrimental ef-

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fect. More recently, Hacker (1971) demonstrated that the fecundity of *Aedes aegypti* (Linnaeus) was reduced in populations infected with *Plasmodium gallinaceum* Brumpt. However, Wilkinson et al. (1972) indicated that the development of *Plasmodium falciparum* (Welch) oocysts in laboratory infected *An. dirus* Peyton and Harrison (as *balabacensis* Baisas) and *An. minimus* Theobald, did not influence the mortality rates of the mosquitoes through day 8 to 10 when they were dissected. Recently, Gad et al. (1979), have provided evidence that there is a significant difference in the mortality rate of non-infected *An. stephensi* Liston and those infected with *Plasmodium berghei* Vincke and Lips. These last authors also noted that during the normal egg development and oviposition period (1–3 days) subsequent to a blood meal, blood-fed non-infected *An. stephensi* had a significantly higher mortality rate than non-infected *An. stephensi* fed on a sugar solution.

Accordingly, previous assumptions that the longevity of the vector is neither affected by age nor by infection with *Plasmodium* parasites, and the associated implications that *Plasmodium* parasites have no detrimental effects on mosquitoes are questionable. Further, mathematical models based on these assumptions could lead to serious errors in the estimates of actual "inoculation rates." This study was undertaken to determine if *Plasmodium* parasites affect the survival rate of infected mosquitoes, and if so, to point out the need for re-evaluation of mathematical models based on the above assumptions.

MATERIALS AND METHODS

The B strain of *Plasmodium cynomolgi* Mayer (*P. c. bastianellii*), originally isolated from *Macaca fascicularis* Raffles, in Pahang, Malaya, in 1959 (Garnham 1959) and later provided to Walter Reed Army Institute of Research, was used. *Plasmodium cynomolgi* was maintained in rhesus monkeys (*Macaca mulatta* Zim-

merman) by the mosquito-sporozoite-monkey inoculation technique (Schmidt et al. 1963). Mosquitoes were fed on monkeys at the second peak of parasitemia and on successive days thereafter when both male and female gametocytes were present. Prior to the mosquito feed a thin blood film was prepared from the monkeys, and the parasitemia, gametocyte level and gametocyte sex ratio were determined.

Anopheles dirus, Bangkok colony strain, was used as the vector in this study. This colony was established in 1964 by Esah and Scanlon (1966), then supplemented with eggs from wild females in 1971 (Wilkinson et al. 1972). This species was selected for study because it is highly susceptible to *P. cynomolgi bastianellii* in the laboratory (Rutledge et al. 1970), is suspected to be a natural vector of simian malaria parasites in Southeast Asia (Eyles et al. 1964), and is a known primary vector of human malaria parasites in Thailand (Gould et al. 1966, Peyton and Harrison 1979, Scanlon and Sandhinand 1965).

Anopheles dirus was reared under laboratory conditions similar to those described by Esah and Scanlon (1966). Specimens used during the study came from eggs oviposited by numerous females in screened cages, and were reared and maintained in the same room under essentially identical environmental conditions.

Adults were maintained on a 5% solution of commercially produced multivitamin syrup (2% sucrose) throughout the entire study. Three to 5 day old female mosquitoes were separated into 2 lots (control and experimental) and starved for 5–6 hours prior to feeding on monkeys. The control lot (40 females) and experimental lot (100 females) were fed simultaneously on a non-infected and infected rhesus monkey, respectively, for approximately 30 minutes. Twenty engorged female mosquitoes were selected from each of the 2 lots above and placed as separate groups (non-infected and infected) in screened cardboard specimen

cups for the remainder of the study. Excess engorged females from the original 2 lots (non-infected and infected) were held in separate reserve cages until day 3. These served as replacements for the corresponding dead females from the non-infected and infected groups that might die due to blood feeding (Gad et al. 1979). During the first 3 days, dead mosquitoes from each of the groups were recorded and replaced with females from the corresponding reserve cage. Following day 3, however, mosquitoes that died from each of the groups were recorded (by day) for longevity calculations and discarded without replacement. The substitution of females up until day 4 served to eliminate mortality directly due to blood engorgement (Gad et al. 1979). After day 3, dead and moribund mosquitoes from the infected group were immediately dissected or preserved as described by Ward (1962) and held for later dissection. Dissections for midguts were performed from days 4–8, while both midguts and salivary glands were examined on day 9 and thereafter, until there were no surviving mosquitoes. The daily mortality and an estimate of the percentage of infected mosquitoes per group were determined.

The environmental conditions experienced during the study were more variable than anticipated, possibly due to the insectary facilities being in a large room (96 m³) on the 7th floor. However, all replicates of non-infected and infected lots were the same age and were reared and maintained simultaneously under nearly identical environmental conditions. Temperatures and humidities throughout the study were 22.2°–32.2°C and 52%–88% RH. The mean temperatures and humidities for the infected groups were 25.5°–26.6°C and 64.9%–72.2% RH, while the mean temperatures and humidities of the non-infected groups were 25.8°–26.6°C and 65.1%–70.4% RH. The slight differences recorded for both temperature and relative humidity probably reflect differences in the calibration and/or sensitivity of the 2 hygrothermographs used.

RESULTS

Thirty-six paired replicate lots of mosquitoes were fed on non-infected rhesus monkeys and rhesus monkeys infected with *Plasmodium cynomolgi*. The percentage of infected mosquitoes, based on specimens that could be dissected for all infected groups, ranged from 7.1% to 100%, mean 66.1%. The mean longevity of infected groups with low mean oocyst values was much higher than that of infected groups with high mean oocyst values (unpublished data). The mean longevity of the non-infected groups ranged from 22.0 to 54.8 days with a mean of 40.3 days for all replicates, while the infected groups ranged from 15.7 to 48.6 days, with a mean of 32.6 days for all replicates. A t test shows that the differences between the mean longevities (days) of the non-infected and infected groups were significant $t_{35} = 5.576$ (p=<0.01). The differences between the mean longevities of the non-infected minus the infected group ranged from −6.5 to +34.9 days with an average of +7.8 days for all replicates.

The daily mortality rates of the non-infected and infected groups are shown in Figs. 1 and 2, respectively. In general, the daily mortality rate of the non-infected group gradually increased from about day 15 until it peaked on day 46.
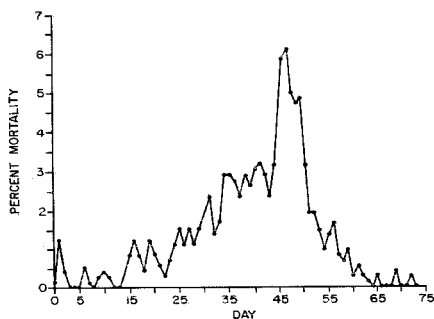

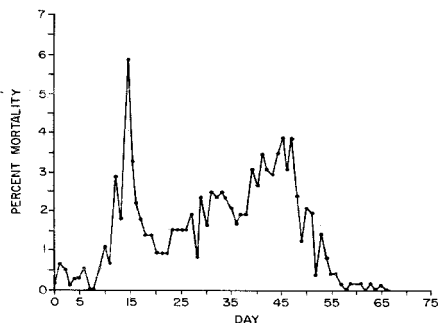Fig. 1. Percent daily mortality of non-infected mosquitoes.

Fig. 2. Percent daily mortality of infected mosquitoes.

Following day 46 there was an abrupt decrease in the mortality rate to about day 55 whereby it gradually decreased until all mosquitoes were dead by day 72. The mortality rate of the infected group differed considerably from the non-infected group in that there are 2 peaks rather than one. The first peak, not observed in the non-infected group, occurred on about day 14 following a sharp increase in the mortality rate on day 10. The mortality rate rapidly declined from day 14 until about day 20. Following day 20, the general trend of mosquito mortality of the infected group roughly corresponded to the non-infected group, i.e., gradually increasing until it peaked on about day 45, sharply decreasing until about day 55, and then gradually decreasing until all mosquitoes were dead by day 66.

The general appearance of the salivary glands and midguts of infected mosquitoes were compared with those of non-infected mosquitoes. Salivary glands in "heavily infected" mosquitoes (midguts with more than 100 oocysts) appeared to burst easily and were often discolored, nearly black-gray to green, and partially decomposed. The midgut and other abdominal organs also were partially decomposed and infiltrated by bacteria in a number of these individuals. Glands and midguts of "lightly infected" mosquitoes (midguts with less than 20 oocysts) were

similar in appearance to glands and midguts dissected from non-infected mosquitoes. An analysis of the effects of *Plasmodium cynomolgi* on *An. dirus* as measured by oocyst densities will be described in another publication.

The differences between the percent mean mortality rates in the non-infected and infected groups surviving to the beginning of post-feeding periods 0–3, 4–8, 9–13, 14–20, 21–30 and 31–40 days are shown in Table 1. There was no significant difference in the mortality rates of the non-infected and infected groups for the periods 0–3, 4–8 and 31–40 days. However, there was a highly significant difference in the mortality rates of these groups for the periods 9–13, 14–20 and 21–30 days. Mortality for the period 0–3 days for both groups was higher than the subsequent period, 4–8 days.

A comparison of the cumulative survival of the non-infected and infected groups is shown in Fig. 3. The LT_{50} (time in days 50% of mosquitoes dead) for the non-infected and infected groups was 41.5 and 33.5, respectively, while the LT_{90} was 52.0 and 47.5, respectively. The cumulative survival for days 0–9 for both groups was not different. On day 10 and

Table 1. Differences in the mortality rates of non-infected and infected mosquitoes alive at the beginning of each post-feeding period.

Group*	Period (days)	Percent mean mortality	t-value
Non-infected	0–3	1.71	0.341
Infected		1.49	
Non-infected	4–8	0.69	−0.906
Infected		1.11	
Non-infected	9–13	0.98	−3.249**
Infected		6.70	
Non-infected	14–20	6.07	−3.399**
Infected		17.92	
Non-infected	21–30	12.89	−4.353**
Infected		25.57	
Non-infected	31–40	33.01	−1.617
Infected		41.30	

* 36 observations in each group.
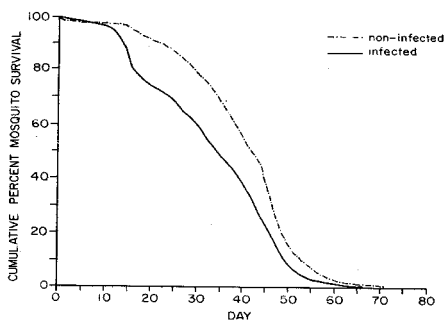** Probability less than .01.

Fig. 3. Cumulative percentage of survival in non-infected and infected groups of *Anopheles dirus*.

thereafter, however, the cumulative survival of the infected group decreased more rapidly than the non-infected group, with the highest percentage of mortality occurring on days 10–20. On about day 60 the percentage of living mosquitoes in both non-infected groups and infected groups was nearly the same.

DISCUSSION

Our data show that the mean longevities of non-infected groups and groups infected with *Plasmodium cynomolgi* were significantly different. The infection rate of mosquitoes within the infected groups ranged from 7.1 to 100% with an average of only 66.1%. Even with only two-thirds of the mosquitoes infected, these data still show that the mean longevities of non-infected and infected groups were significantly different ($p=<0.01$), i.e., the non-infected mosquitoes in general lived longer than infected mosquitoes.

Figures 1 and 2 illustrate differences in the mortality rates of the non-infected and infected groups. The infected group has 2 peak mortality periods while the non-infected group only has one. The early peak on about day 14 in the infected group can be attributed partially to the rupturing of oocysts, penetration of saliv-

ary gland cells by sporozoites and the subsequent rupturing of the salivary glands. Following day 14, when most oocysts have burst, there is an abrupt decrease in the mortality rate of the infected group until it approaches the mortality rate of the non-infected group on about day 20. Thereafter, the mortality rate of both groups gradually increases until about day 46, abruptly decreases until about day 55, and then gradually decreases until all mosquitoes are dead. The data in Figs. 1 and 2 suggest that if the infected mosquitoes survive through day 20, then the mortality rate of the infected and non-infected groups approach each other. However, as shown by Table 1, this is not the case. A comparison of the percentage of observed mortality of the remaining mosquitoes between the 2 groups clearly shows that the daily mortality rate of the infected group is greater than the non-infected group.

Initial high mortality rates attributed to the acquisition of a blood meal have been observed in experiments using non-infected *An. stephensi* (Gad et al. 1979). Therefore, mosquitoes in this study (both non-infected and infected groups) that died during the first 3 days after blood feeding, were replaced with mosquitoes from reserve cages from the corresponding feeding lots. This eliminated a possible bias (mortality) due to a post-blood feeding response. In our study an initial mortality peak was observed for both non-infected and infected groups on day 1 after the blood meal, however, mortality then declined until about day 3 (Figs. 1 and 2). In addition the early high mortality rates observed in *An. stephensi* (Gad et al. 1979) were not observed in *An. dirus* during this study. There was no significant difference in the percentage of mortality between the non-infected and infected groups for the period 0–3 days (Table 1). There was also no significant difference between the 2 groups for the period 4–8 days, prior to the rupturing of the oocysts. After the initial blood meal, the non-infected and infected lots were not offered an additional blood meal.

Thus, any mortality that might be caused by additional blood meals was avoided.

For the periods 9–13, 14–20 and 21–30 days, the mortality rates for the infected and non-infected groups were significantly different. The periods 9–13 and 14–20 days correspond to the period of time when the oocysts rupture and the sporozoites migrate and penetrate the salivary glands as well as other areas and parts of the body. By day 20 most of the oocysts have already burst. Thereafter, other factors (reduced vitality of the mosquitoes, etc.) may contribute to increasing susceptibility to bacterial or fungal infections, thereby increasing the mortality rate among the infected groups. Apparently, infected specimens surviving to approximately day 30 have overcome most of the detrimental effects of the infection or, perhaps all females with a heavy malaria infection have died by that day, and the survivors had either low or no infections.

A comparison of the cumulative survival rates for the non-infected and infected groups (Fig. 3) further illustrates that there is no difference in the mortality rate of the 2 groups for periods 0–3 and 4–8 days. However, following day 10 the cumulative survival rates for the 2 groups diverge rapidly with more mosquitoes surviving in the non-infected groups. By day 20, however, the daily percentage of mortality among infected groups decreased. The LT_{50} for the non-infected and infected groups was 41.5 and 33.5 days, respectively, a difference of +8.0 days, while the LT_{90} for the non-infected and infected groups was 52 and 47.5 respectively, a difference of only +4.5 days. One possible explanation for the decreased difference in the LT_{90} of the non-infected and infected groups is that within the infected groups approximately 34% were negative for parasites and these assumed the same general longevity pattern as the "non-infected" groups. The calculation of the LT_{50} and LT_{90} is useful in estimating that proportion of the population which will survive to a particular day. Since the longevity of heavily infected mosquitoes is reduced, the number of potential ovarian cycles for those mosquitoes also is reduced. This further decreases the number of blood meals, i.e., the number of human exposures to infected vectors, and therefore reduces the "vectorial capacity" of the vector.

Salivary gland and midgut dissections of dead mosquitoes further support the hypothesis that infected mosquitoes have a decreased longevity. From days 9–20, dead mosquitoes were often "heavily infected" while dead or dying mosquitoes dissected after day 20 were usually either "non-infected" or only "lightly infected." "Heavily infected" mosquitoes often had abnormal salivary glands, which burst easily, were discolored, black-gray to green and were partially decomposed, with numerous bacteria. In several cases, all that remained of the glands were the salivary ducts.

In conclusion, these data show that the longevity of certain groups of mosquitoes infected with *Plasmodium cynomolgi* is reduced when compared with groups of non-infected mosquitoes under identical laboratory conditions. In nature, where more environmental stress is placed upon the individuals, these differences may be more pronounced than were observed under laboratory conditions. A reduced vector longevity is very significant epidemiologically, and implies a reduction in vectorial capacity by decreasing the number of blood meals taken by infected females. These observations indicate that investigations on the effects of human malarias on primary mosquito vectors, e.g., *An. dirus*, should be initiated to determine if human malaria parasites affect them in the same manner as does *P. cynomolgi*.

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References Cited

Boyd, M. F. 1940. On the correlation between the incidence of stomach and gland infection in *Anopheles quadrimaculatus* infected with *Plasmodium vivax*. Am. J. Trop. Med. 20:129–131.

Buxton, P. A. 1935. The effect of *Proteosoma* upon the survival of *Culex*. Parasitology 27:547–550.

De Buck, A. and N. H. Swellengrebel. 1935. On the seasonal longevity of *Anopheles maculipennis* in Holland with references to their ability to act as malaria vectors. Proc. R. Acad. Sci., Amsterdam 38:335–343.

Esah, S. and J. E. Scanlon. 1966. Notes on a laboratory colony of *Anopheles balabacensis* Baisas, 1936. Mosq. News 26:509–511.

Eyles, D. E., R. H. Wharton, W. H. Cheong and M. Warren. 1964. Studies on malaria and *Anopheles balabacensis* in Cambodia. Bull. W.H.O. 30:7–21.

Gad, A. M., W. A. Maier and G. Piekarski. 1979. Pathology of *Anopheles stephensi* after infection with *Plasmodium berghei berghei*. I. Mortality Rate. Z. Parasitenkd. 60:249–261.

Garnham, P. C. C. 1959. A new subspecies of *Plasmodium cynomolgi*. Riv. Parasitol. 20:273–278.

Garrett-Jones, C. 1964. The human blood index of malaria vectors in relation to epidemiological assessment. Bull. W.H.O. 30:241–261.

Gould, D. J., J. E. Scanlon and R. A. Ward. 1966. *Anopheles* vectors of malaria in Southeast Asia. Army Sci. Conf. Proc. 1:361–371.

Hacker, C. S. 1971. The differential effect of *Plasmodium gallinaceum* on the fecundity of several strains of *Aedes aegypti*. J. Invertebr. Pathol. 18:373–377.

Keener, G. G., Jr. 1945. Detailed observations on the life history of *Anopheles quad-*rimaculatus. J. Natl. Malaria Soc. 4:263–270.

Macdonald, G. 1952a. The analysis of the sporozoite rate. Trop. Dis. Bull. 49:569–586.

Macdonald, G. 1952b. The analysis of equilibrium in malaria. Trop. Dis. Bull. 49:813–829.

Macdonald, G. 1953. The analysis of malaria epidemics. Trop. Dis. Bull. 50:871–889.

Macdonald, G. 1957. The epidemiology and control of malaria. Oxford Univ. Press, London, Appendix 1.

Molineaux, L. 1978. Entomological parameters in the epidemiology and control of vector-borne diseases. pp. 100–105, in Med. Entomol. Centenary Symp., Proc. R. Soc. Trop. Med. Hyg.

Onori, E. and B. Grab. 1980. Indicators for the forecasting of malaria epidemics. Bull. W.H.O. 58:91–98.

Peyton, E. L. and B. A. Harrison. 1979. *Anopheles (Cellia) dirus*, a new species of the Leucosphyrus Group from Thailand (Diptera: Culicidae). Mosq. Syst. 11:40–52.

Ragab, H. A. A. 1958. Effect of *Plasmodium* on the transmitting mosquito host from the point of view of the longevity of the infected mosquito. J. Egypt Med. Assoc. 41:447–454.

Rutledge, L. C., D. E. Hayes and R. A. Ward. 1970. *Plasmodium cynomolgi:* Sources of variation in susceptibility of *Anopheles quadrimaculatus, A. balabacensis* and *A. stephensi.* Exp. Parasitol. 27:53–59.

Scanlon, J. E. and U. Sandhinand. 1965. The distribution and biology of *Anopheles balabacensis* in Thailand (Diptera: Culicidae). J. Med. Entomol. 2:61–69.

Schmidt, L. H., R. N. Rossan and K. F. Fisher. 1963. The activity of a repository form of 4, 6-diamino-1-(p-chlorophenyl)-1, 2-dihydro-2, 2-dimethyl-s-triazine against infections with *Plasmodium cynomolgi*. Am. J. Trop. Med. Hyg. 12:494–503.

Sinton, J. A. and P. G. Shute. 1938. A report on the longevity of mosquitoes in relation to the transmission of malaria in nature. Rep. Public Health Med. Subjects, Ministry of Health, London, No. 85:1–45.

Ward, R. A. 1962. Preservation of mosquitoes for malarial oocyst and sporozoite dissections. Mosq. News 22:306–307.

Wilkinson, R. N., D. J. Gould and P. Boonyakanist. 1972. Comparative susceptibility of *Anopheles balabacensis* and *Anopheles minimus* to naturally occurring *Plasmodium falciparum* in central Thailand. Proc. Helminthol. Soc. Wash. 39(Special issue):423–427.