DETRIMENTAL EFFECTS OF *Plasmodium cynomolgi* INFECTIONS ON THE SURVIVAL RATE OF *Anopheles dirus* (DIPTERA : CULICIDAE)

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OBJECTIVES :

1. To determine if the longevity of mosquitoes infected with *Plasmodium* spp. is significantly different from that of mosquitoes that are not infected.

2. To determine if the longevity among mosquitoes with different "infection rates" of *Plasmodium* spp. is significantly different.

3. To determine if the longevity of mosquitoes infected with different *Plasmodium* spp. is significantly different among groups.

BACKGROUND : Malaria transmission in nature is the result of physiological and environmental factors which are dependent on the vector host and the human population. The probability of malaria transmission occurring is the direct result of mosquito density, mosquito susceptibility, anthropophilism, frequency and duration of feeding, the extrinsic cycle, infectivity of sporozoites, sporozoite rate, susceptibility and infectivity of the human population, and mosquito longevity (1, 2). Macdonald (3) suggested that under natural conditions, i.e., without the use of pesticides, etc., mosquito mortality assumes the form of a geometric progression, where the death rate does not change with age. He further proposed a mathematical rational for estimating the sporozoite rate based on the fact that the longevity of infected mosquitoes is not different from that of non-infected mosquitoes.

Early investigations on the effects of malarial species on mosquitoes have not been conclusive. A number of workers (4, 5, 6) suggested that the plasmodium parasites have a detrimental effect on the mortality rate of mosquitoes. However, other workers (7, 8) have concluded that there is no detrimental effect on the mortality rate of the vector host. More recently, Hacker (9) demonstrated that the fecundity of *Aedes aegypti* (L) is reduced in populations infected with *P. gallinaceum* Brumpt. Schiefer, et al., (10) also demonstrated that the flight performance of *Anopheles stephensi* Liston infected with *Plasmodium cynomolgi* Mayer is negatively correlated to the severity of the infection. Gad, et al., (11) have provided more conclusive evidence by

demonstrating that there is a significant difference in the longevity between non-infected *An. stephensi* and *stephensi* infected with *P. berghei* Vincke and Lips.

The longevity of infected mosquitoes directly affects the malaria transmission rate, making it increasingly important to understand the parasite-vector relationship. Vector longevity studies, based on infection rate, will further our knowledge and provide a better understanding of the epidemiology of human malaria.

MATERIALS AND METHODS : The B strain of *Plasmodium cynomolgi* (*P.c. bastianellii*), originally isolated from a *Macaca fascicularis* Raffles, in Pahang, Malaya, in 1959 (12), and later provided to Walter Reed Army Institute of Medical Research was used. *Plasmodium cynomolgi* was maintained in a colony of rhesus monkeys (*Macaca mulatta* Zimmerman) by the monkey-mosquito-monkey sporozoite innoculation technique (13). 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 smear was prepared from the monkeys and the parasitemia, gametocyte level and gametocyte sex ratio were determined.

Anopheles (Cellia) dirus Peyton and Harrison, Bangkok colony strain was used as the vector in this study. This colony was established in 1964 by Esah and Scanlon (14), then supplemented with eggs from wild females in 1971 (15). This species was selected for study because it has demonstrated a high degree of susceptibility to *P. c. bastianellii* (16), and is suspected to be a natural vector of Simian malaria in Southeast Asia. *Anopheles dirus* is a known primary vector of human malaria in Thailand (17, 18, 19).

Anopheles dirus was reared under laboratory conditions similar to those described by Esah and Scanlon (14). Newly emerged adults were held in a cage for 3-5 days and maintained on a diet of 5% multivitamin syrup (including 2% sucrose). The study design (Figure 1) and methods used are discussed below.

Female mosquitoes starved 5-6 hours prior to feeding on monkeys, were randomly selected and placed in 2 separate lots. The first lot, consisting of 40 adult females, was allowed to feed on a non-infected rhesus monkey for approximately 30 minutes. Twenty engorged females were randomly selected and placed in a screened cardboard specimen cup (control group) and provided a 5% solution of multi-vitamin syrup throughout the study. The remaining engorged females were held in a cage until day 3, when they were discarded. Mosquitoes observed dead in the control group from day 0-3 were recorded and replaced with females from the first lot. Mosquitoes observed dead from day 3 on, or until all of the control group were dead, were discarded, with the day of mortality recorded for longevity calculations.

The mosquitoes in the second lot, consisting of 100 adult females were allowed to feed on an infected monkey for approximately 30 minutes and during the same time as the first lot above. Sixty engorged females were randomly selected and placed into 3 separate cardboard specimen cups (Test groups 1, 2 and 3), 20 per cup, and provided with a 5% multi-vitamin solution for the remainder of the study. The remaining engorged females were held in a cage

until day 3, when they were discarded. Individuals observed dead in each of the test groups from day 0-3 were replaced with females from the cage and the day of mortality recorded.

Mosquitoes remaining alive in test group 1 were dissected for midguts on day 7. The midguts were examined for oocysts and, if positive, the number of oocysts counted. However, midguts observed with more than 100 or 200 oocysts were estimated as > 100 or > 200, respectively. In addition, 5 oocysts were measured and the mean diameter calculated. The largest and smallest oocysts were measured to provided an estimate of the size range. The mean size of oocysts for all mosquitoes measured were determined to be within the range of expected growth and development. Mosquitoes observed dead prior to day 7 were dissected and the number of oocysts determined.

Mosquitoes in test group 2 remaining alive on day 14 were dissected for both midguts and salivary glands. The midguts were examined for oocysts and the remnants of burst oocysts. Salivary glands were examined and the sporozoite rate determined, i.e., +1 (1-10 sporozoites), +2 (11-100 sporozoites), +3 (101-1,000 sporozoites), and +4 (> 1,000 sporozoites). The general condition of the salivary glands and midgut was also noted, i.e., ruptured, discolored, etc. Mosquitoes dying prior to day 14 were also dissected as described above.

Mosquitoes in test group 3 were observed at least twice daily for signs of morbidity and mortality. Those observed dead prior to day 3 were recorded and replaced with a live adult from the same feeding lot. Specimens demonstrating signs of morbidity, i.e., unable to fly or right themselves, for experimental purposes were considered dead. Mosquitoes observed dead or considered dead from day 3 on, to the end of the experiment, were immediately dissected or preserved as described by Ward (20), and held for later dissection. Midguts were dissected from mosquitoes dead on days 3-8 and an estimate of the oocyst frequency and stage of oocyst development determined. Both midguts and salivary glands were dissected from mosquitoes observed dead on day 9 and thereafter until all were dead. An estimate of the oocyst frequency, sporozoite rate and condition of the salivary glands and midgut were recorded. The daily mortality was recorded throughout the study and the range and mean longevity were calculated for each replicate. An accurate count of the number of oocysts for all mosquitoes in test group 3 was not possible because many mosquitoes either had ruptured oocysts or midguts that were partially or totally decomposed. For this reason, the mean number of oocysts in test group 1 was used as the estimate of the mean number of oocysts in test group 3. Based on specimens in test group 3 in which mature and ruptured oocysts were countable, there was no significant difference in the mean number of oocysts between test groups 1 and 3.

The temperature throughout the study ranged from 22.2°-32.2°C for both the infected and control groups. The mean temperature for infected groups ranged from 25.5° to 26.6°C with a mean of 26.0°C, while the control groups ranged from 25.8°-26.6°C with a mean of 26.1°C.

The humidity ranged from 52-88 RH for both the infected and control groups. The mean humidity for the infected groups ranged from a low of 64.9 and high of 72.2 RH, average 66.49 RH, while the control groups ranged from a low of 65.1 and high of 70.4 RH, average 66.43 RH.

RESULTS : Thirty six replicates were performed from 9 August 1979 through 25 March 1980, with 2,160 adult females dissected. The mean number of oocysts per mosquito for different replicates (calculated from test group 1) ranged from 0.05 (1 oocyst per 20 mosquitoes) to 169.8 with a mean of 48.35 for all replicates. The percentage of infected mosquitoes for all test groups ranged from 5.0 to 100%. The mean percentage of infected mosquitoes was 78.8, 76.3 and 72.3 for test groups 1, 2 and 3, respectively[1]. There was no significant difference in the percentage of infected mosquitoes among different test groups, $F_{2,83} = .3064$.

The number of oocysts per mosquito was also determined for nine sets of triplicates, where each triplicate was obtained from mosquitoes feeding on an infected monkey at the same time. This gives an idea of the reliability of using the number of oocysts in test group 1 to estimate the number of oocysts for mosquitoes in test group 3 that died (and hence often could not have a reliable count of the number of oocysts). The within replicate variance was 146.78 (18 df) and the among replicate variance was 2,077.56[2] (8 df); the intraclass correlation can be estimated by $r_I = (s_a^2 - s_w^2)/(s_a^2 - 2s_w^2)$ (Snedecor and Cochran, 1967) and equals .814, which is reasonably high. The within replicate variance from this experiment was also used to find unbiased estimates of regressions on the number of oocysts.

The mean longevity of the control group ranged from 22.0 to 54.8 days, mean 40.2 days, for all replicates. The mean longevity of test group 3 ranged from 15.7 to 48.6 days, with a mean of 32.5 days for all replicates. The differences in the mean longevity of the control groups and the test groups ranged from -6.5 to 34.9 days, mean +7.75 days, for all replicates. The regression of the difference of the mean longevity of the control groups and test group on the mean number of oocysts per mosquito is positive and highly significantly different from 0, $t_{34} = 7.89$ (Figure 2, Table 1), and the correlation coefficient is +.80. The regression on the mean longevity of test group three on the mean number of oocysts per mosquito has a negative slope that is significantly[1] different from 0, $t_{34} = -6.25$, and this correlation coefficient is -.73 (Figure 3, Table 1)

The differences in the mean longevity of the test groups with > 0-10, > 10-40, > 40-70 and > 70 mean number of oocysts and the control groups are shown in Table 2. There was no significantly difference in the mean longevity of the control groups and that of the test groups with 0-10 oocysts. However, the mean longevities for test groups with > 10-40[3], > 40-70[4] and > 70[4] oocysts per mosquito were significantly different from the mean longevity of the control groups.

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1 Observations of test group 3 were not included when 3 or more individuals could not be dissected.

2 Probability less than 0.5.

3 Time, in days, required for 50% mortality.

4 Time, in days, required for 90% mortality.

The daily mortality rates of the control group and test groups 3 are shown in Figures 4 and 5, respectively. In general, the daily mortality rate of the control group gradually increases from about day 10 until it peaks on day 46. It decreases abruptly from day 46 to day 53, and then gradually decreases until all mosquitoes were dead by day 72. The infected group (test group 3) differs considerably from the control group in that there were 2 peaks rather than one. Mosquito mortality of the infected group abruptly increases on day 9, peaks on day 14 and abruptly decreases until day 20. The general trend of mosquito mortality of the infected group roughly corresponds to the control group after day 20 with a gradual increase, a late peak on about day 45, and an abrupt decrease in the mortality. Although the period from day 20 through day 50 has approximately the same mortality rate for the control and infected groups, the ratio of total dead to total live mosquitoes is much higher in the infected groups.

The general appearance of the salivary glands and midguts was examined for mosquitoes in each test group. These data show that mosquitoes with heavy infections, i.e., mosquitoes taken from test groups with > 70 mean number of oocysts, were more often observed with evidence of bacterial decomposition when dissected on day 14 or after death. Salivary glands in these mosquitoes burst very easily and were often discolored, nearly black or a grey to green color, and often partially decomposed.

A comparison of the daily cumulative mortality rates at different levels of infectivity, i.e., > 0-40, > 40-70 and > 70 mean number of oocysts per test group, was made (Figure 6). The LT_{50}[1] and LT_{90}[2] for the control group and infected groups with > 0-40 and > 40-170 mean number of oocysts per test group were 42, 52; 40, 50; and 25, 45 respectively. The cumulative mortality rate from day 0-9 for both samples of the test groups and the control group was not observed to differ significantly. The total cumulative mortality rate of the infected sample group with 40 or fewer mean number of oocysts is quite similar to the control group cumulative mortality rate (Figure 6). However, the total cumulative mortality rate of the infected sample group with > 40 mean number of oocysts is quite different from the control group, with the highest percentage of mortality on days 9-18.

The difference between the mean longevity of the control groups and infected groups at days 0-3, 4-8, 9-14 and 15-20 are shown in Table 3. In general, there is no significant difference in the mortality rate of the control groups and infected groups for the periods, day 0-3 and 3-8. However, there is a highly significant difference in the mortality rate of the control groups and test groups with > 40 mean number of oocysts for the periods, days 9-14 and 15-20. There was no significant difference in the mortality rate of the control groups and test group with > 0-40 mean number of oocysts for the periods, days 9-14 and 15-20.

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1 Time, in days, required for 50% mortality.

2 Time, in days, required for 90% mortality.

The gametocyte number and gametocyte sex ratio, at the time the mosquitoes fed, were both regressed on the mean number of oocysts. The computations revealed that neither the gametocyte number nor the gametocyte sex ratio had a direct relationship to the mean number of oocysts produced in the mosquitoes (Table 1).

Table 1. Sample Sizes and Statistics for Regression Lines of Figs. 2 and 3 and Gametocyte Number and Ratio.

Mean Number of Oocysts VS	Number of Paired Feeds	Y Intercept	Slope	Coefficient of Correlation	F Value
Survival rate of Infected Groups	36	+40.45	-0.16 -0.18[2]	-0.74	41.45[1]
Mean Diff in Survival rate of non-infected and infected groups	36	-11.39	+0.18 +0.21[2]	+0.80	60.21[1]
Gametocyte Number for Mean Number of oocysts	36	-	-	+ .009	0.0
Gametocyte Ratio for Mean Number of oocysts	36	-	-	- .06	0.14

[1] Probability less than 0.01

[2] Corrected for error when X' is an estimate of X.

Table 2. Effect of the Mean Number of Oocysts on the Mean Survival of Mosquitoes

Group	Mean Number of Oocysts	Number of Observations	Survival Mean	Survival Range	HØ : μ infected group - μ non-infected group - Ø	
Non-infected	-	7	40.5	35.1-49.3	t	.487
Infected	> 0-10		39.5	33.4-48.6		
Non-infected	-	9	42.0	35.7-51.3	t	2.810*
Infected	> 10-40		38.1	30.6-45.8		
Non-infected	-	12	37.7	22.0-49.9	t	4.883**
Infected	> 40-70		29.8	25.3-37.0		
Non-infected	-	8	41.8	29.5-54.8	t	5.696**
Infected	> 70		24.0	15.7-40.2		

* Probability less than 0.05

** Probability less than 0.01

Table 3. Differences in the mortality rate of the non-infected and infected mosquitoes

Group	Day	Number of Observations (> 0-40 oocysts)	% Mean Mortality Rate	t value	Number of Observations (> 40 oocysts)	% Mean Mortality Rate	t value	Total Number of Observations	Total % Mean Mortality	t value
Non-infected	0-3	16	2.03	1.758	20	1.46	-.780	36	1.72	0.341
Infected			0.60			2.20			1.49	
Non-infected	4-8	16	0.31	-1.370	20	1.00	-.024	36	0.69	-0.906
Infected			1.24			1.02			1.11	
Non-infected	9-13	16	0.31	-1.379	20	1.53	-3.323**	36	0.98	-3.249**
Infected			1.25			11.07			6.70	
Non-infected	14-20	16	3.54	0.544	20	8.11	-4.171**	36	6.08	-3.399**
Infected			2.86			29.97			17.93	
Non-infected	21-30	16	10.14	-2.258	20	15.10	-3.801**	36	12.89	-4.353**
Infected			18.38			31.33			25.57	
Non-infected	31-40	16	34.31	-0.256	20	31.96	-1.705	36	33.01	-1.617
Infected			35.82			35.68			41.30	

** Probability less than 0.01

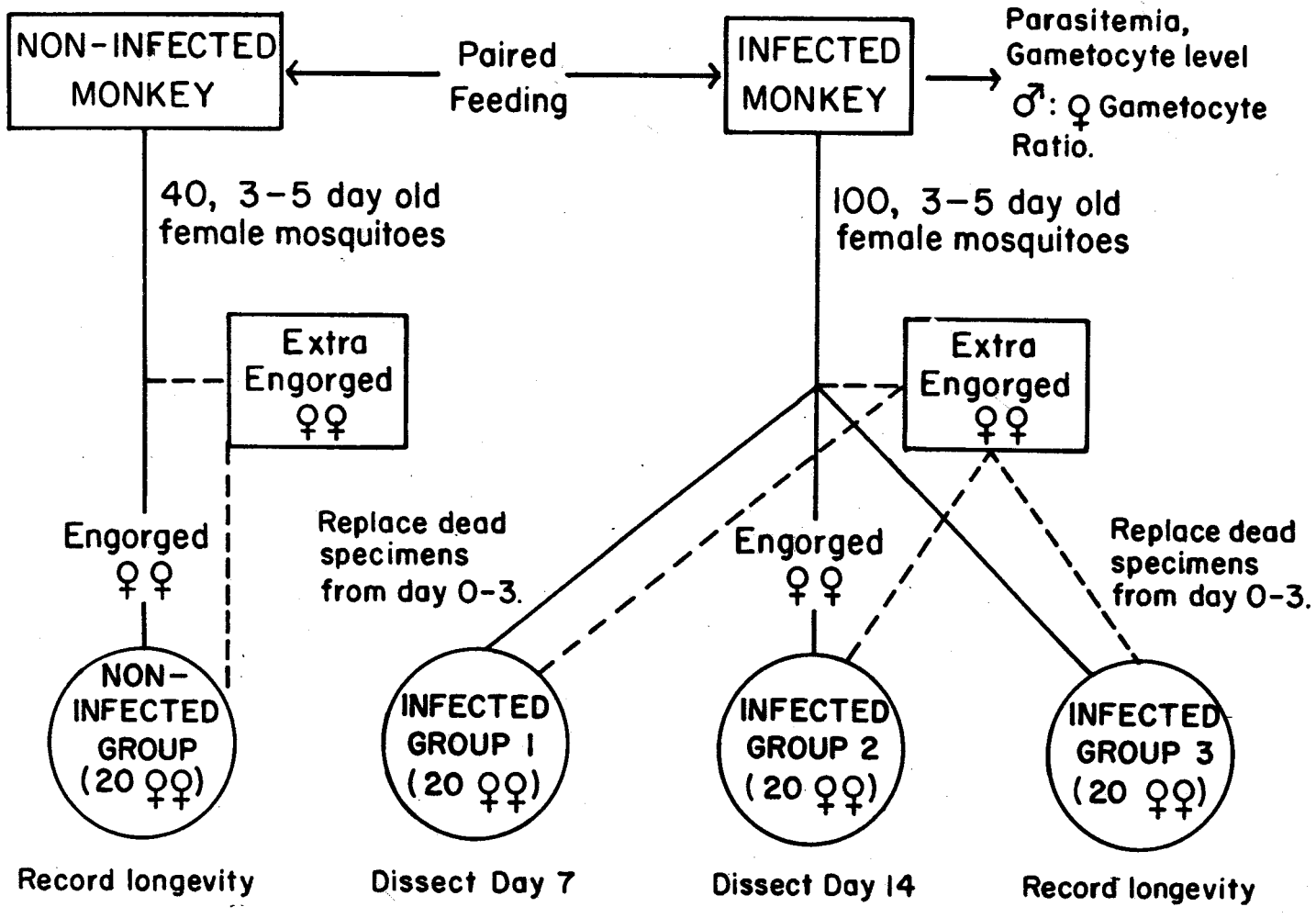

Figure 1. Diagram illustrating the sequence of events for each replicate

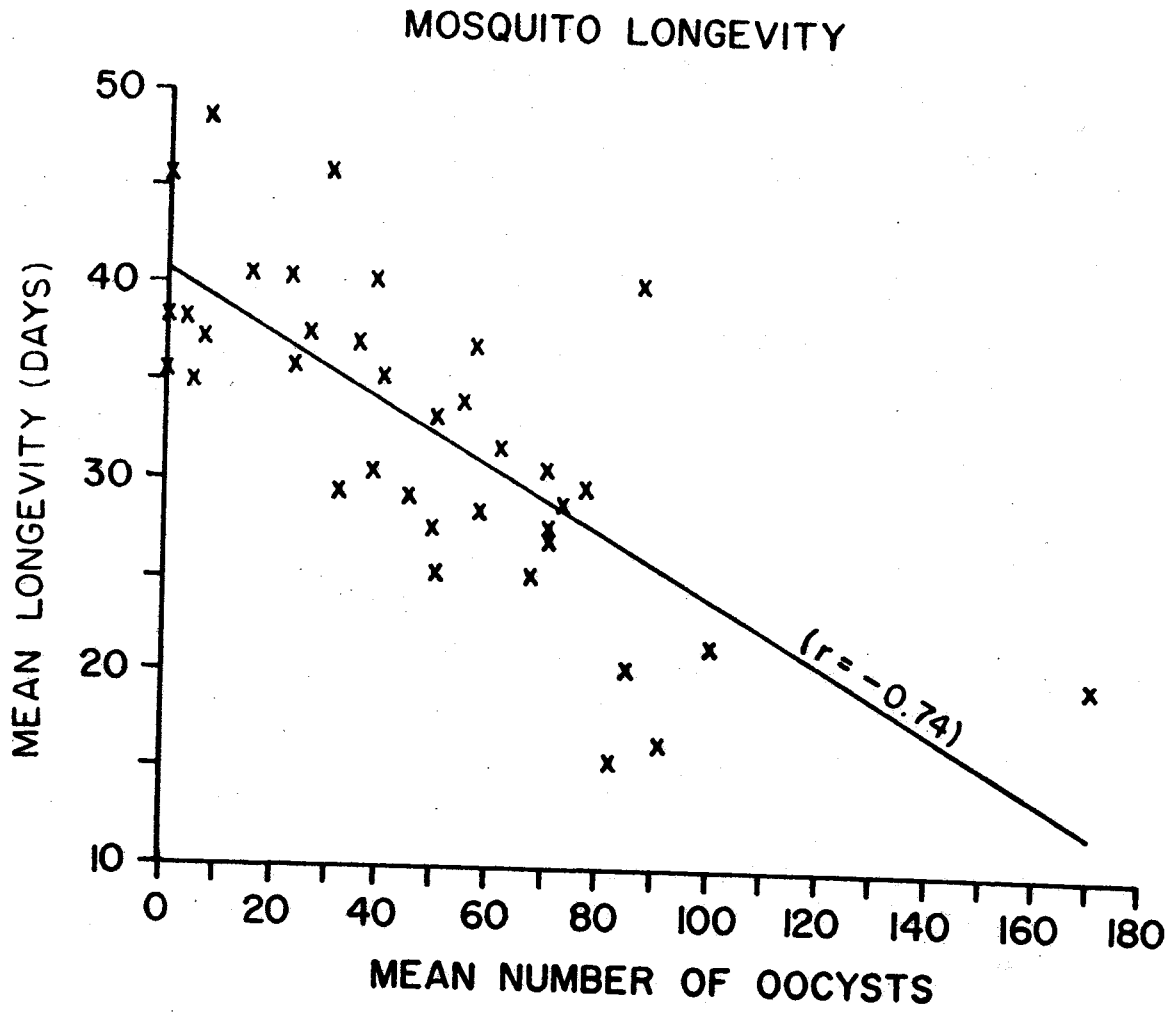

Figure 2. Regression of the mean longevity of infected group 3 on the mean number of oocysts per mosquito (Infected group 1).

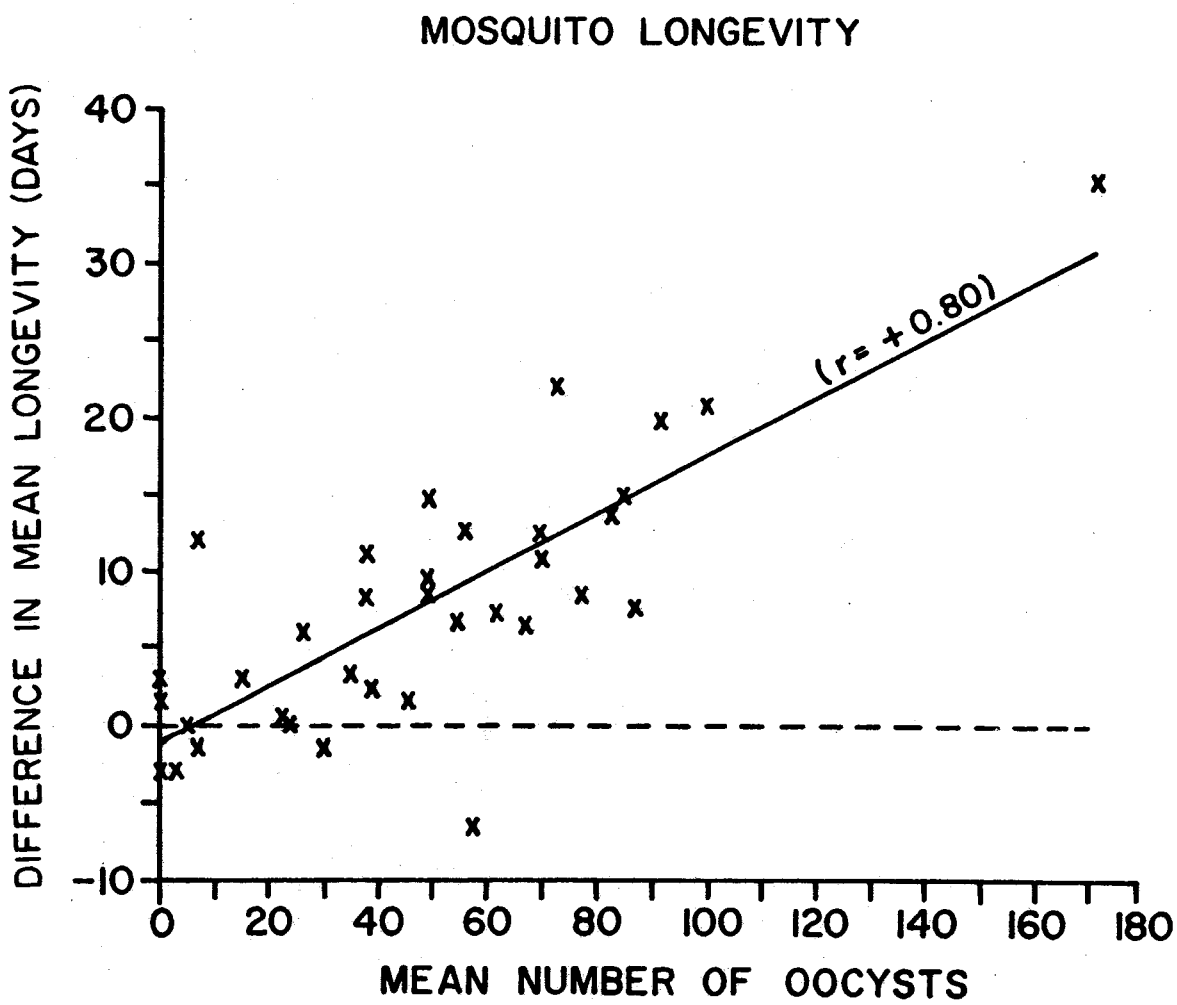

Figure 3. Regression of the difference in the mean longevity of the non-infected groups and infected groups 3 on the mean number of oocysts per mosquito (infected-group 1).

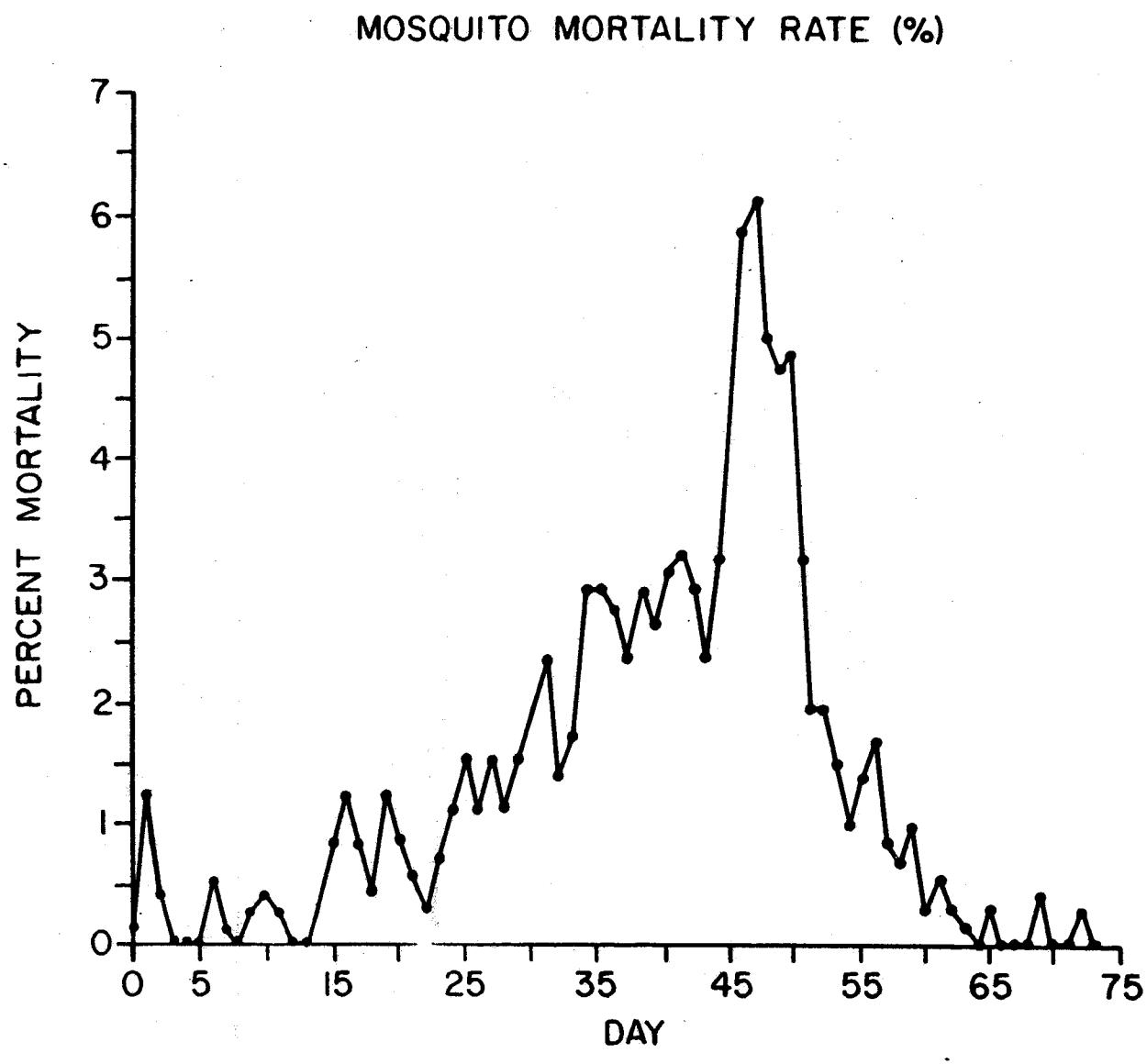

Figure 4. Percent daily mortality of non-infected mosquitoes.

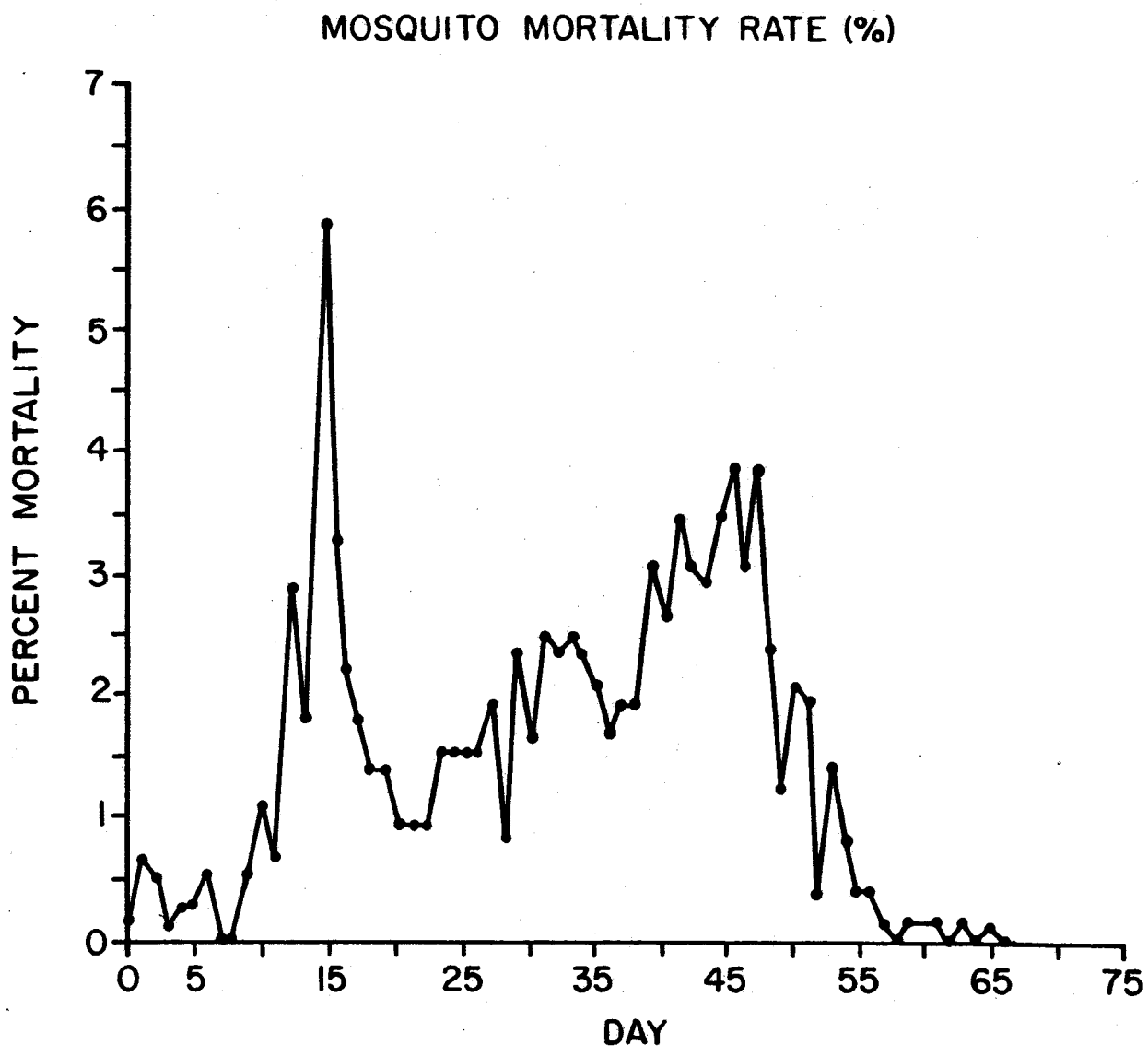

Figure 5. Percent daily mortality of infected mosquitoes.

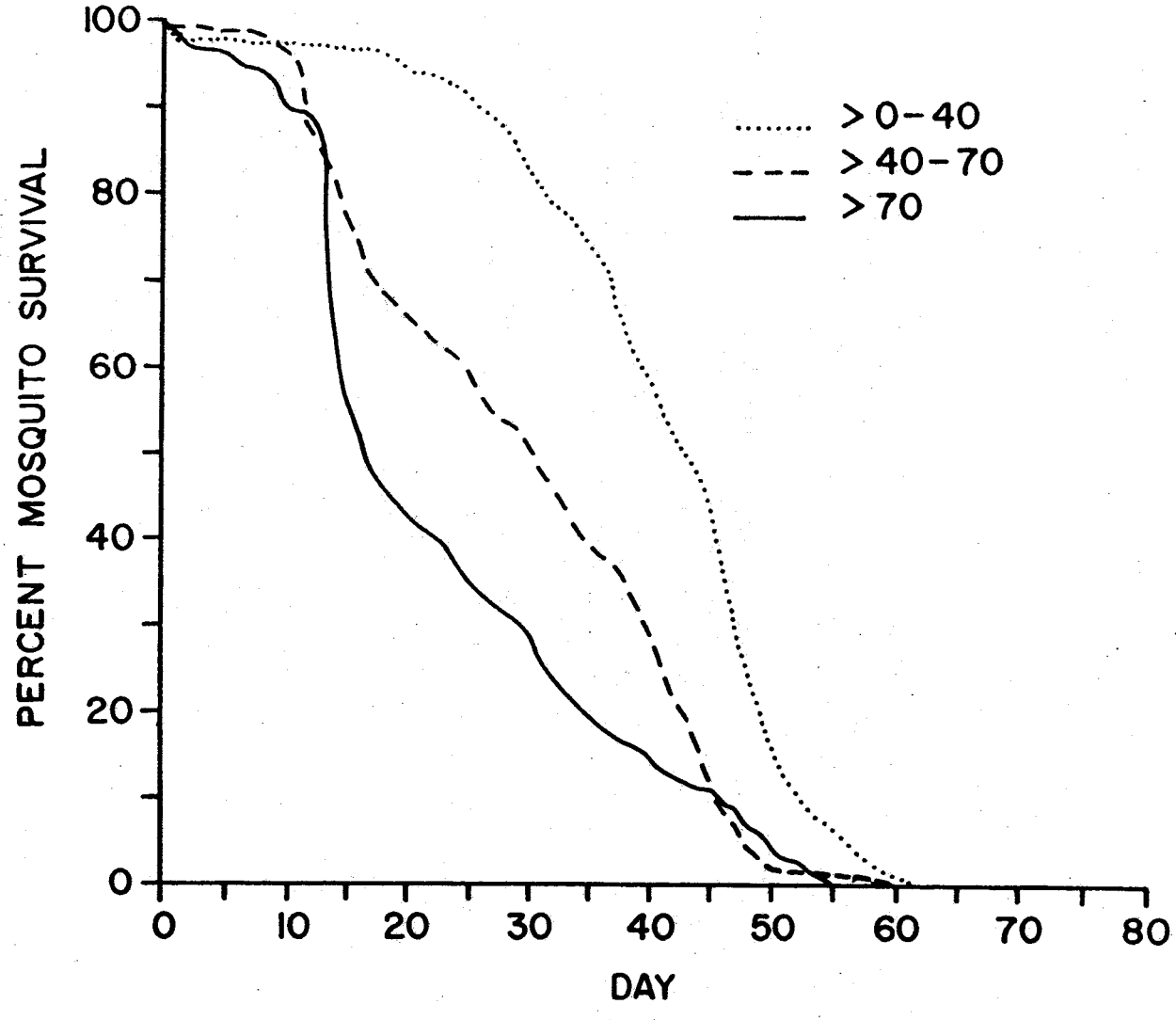

Figure 6. Cumulative daily survival rate of infected groups with >0–40, > 40–70 and >70 mean number of oocysts.

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