LABORATORY INFECTION OF MOSQUITO LARVAE BY ENTOMOPATHOGENIC FUNGI WITH PARTICULAR REFERENCE TO *ASPERGILLUS PARASITICUS* AND ITS EFFECTS ON FECUNDITY AND LONGEVITY OF MOSQUITOES EXPOSED TO CONIDIAL INFECTIONS IN LARVAL STAGES

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ABSTRACT

Aspergillus parasiticus was isolated from moribund *Anopheles gambiae* larvae. It was subcultured and identified. It killed 99.8% and 44.4% *Aedes aegypti* first instar and fourth instar respectively. Mortality rates of 83.0% and 45.7% were recorded for *Culex fatigans* first and fourth instar respectively. It killed 100% *Aedes africanus, Aedes simpsoni* and *Anopheles gambiae*. The optimum temperature range was 20–25°C. Mortality rates of 97.0% and 96.7% were obtained for *Aedes aegypti* second instar at 20 and 25°C respectively. The infection progressed very slowly at 15°C. At 30°C the conidia germinated but the majority could not infect. Conidial germination failed at 35°C. *Aspergillus* was found pathogenic to eight species of mosquitoes in four genera (*Anopheles, Culex, Eretmopodites* and *Aedes*).

Infections of *Aspergillus* sp markedly reduced the fecundity and longevity of *Anopheles gambiae, Culex fatigans* and *Aedes aegypti* regardless of the instar that became infected. The reductions were greatest when the 1st and 2nd instars were infected. The tests showed that introduction of *Aspergillus* into natural populations of *Anopheles gambiae, Culex fatigans* and *Aedes aegypti* consisting of mixed larval instars could significantly reduce the number and fertility of eggs resulting in an average reduction of *ca* 34.9% for *Anopheles gambiae, ca* 55.2% for *Culex fatigans* and *ca* 53.6% for *Aedes aegypti* of the F_1 progeny from those females that become infected. Two isolates of *Pythium* pathogenic to mosquito larvae are reported. The importance of these results in biological control is discussed.

INTRODUCTION

ANOPHELES GAMBIAE is the major malaria vector in Uganda. *Culex fatigans* is the main vector of bancroftian filarial infections in Uganda and elsewhere in East Africa. *Aedes aegypti* plays a major role in the urban yellow fever cycle while *A. simpsoni* plays a main role in the sylvatic yellow fever cycles between birds, monkeys and man[1, 2]. Insecticides have been the only practical control measure for these vectors, but recent concern over the continued use of insecticides, insect resistance and particularly, their environmental impact has necessitated the developing of alternative means of biological control.

Among the promising biological control agents are entomopathogenic fungi. *Coelomomyces* is a complex genus of chytridio mycetes-blasto-cladiales which basically infects mosquito larvae[3]. *Coelomomyces* sporangia have been reported in the ovary of female mosquitoes[4, 5]. *Coelomomyces* have been isolated from mosquito larvae in Uganda[5].

Lagenidium giganteum has been isolated around Lake George in Uganda[6]. Studies to date have shown that *L. giganteum* is a promising biological control candidate[7]. *Lagenidium* infections have been reported from eggs of *Anopheles* and *Armigeres dentatus* and *Polyscytalum* infections have been seen in the eggs of *Psorophora howardii*[8]. An ovarian phycomycete has been observed in ovipositing black fly females[9]. However, the oviposition of the fungal spores occurred without great reduction in the number of eggs oviposited[10].

Anopheles larvae were found highly susceptible to *Aspergillus*[11] sp; a high incidence of *Aspergillus* sp was reported in *Culex tritaeniorhynchus summorosus* larvae[12]. *Aspergillus parasiticus* was reported causing high mortality in *Culex gelidus*[13]. A sudden decline in oviposition and high adult mortality of *A. gambiae* which had been exposed to *Aspergillus* sp in larval stages was observed[14]. The pathogenicity of *Aspergillus* species for insects has been discussed[15]. Invasion of the insect is usually through the cuticle, but

penetration through the gut and spiracles also occurs. Insect death may result from any of the several factors, including histolysis, mechanical interference with the function of the insect's tracheoles and spiracles, or the production of potent mycotoxins[16, 17]. Although there is no doubt that *Aspergillus* species can be truly entomopathogenic, many reports of this genus from insects may be due to secondary infections by *Aspergillus* or to its rapid establishment after the insect's death. *Penicillium* and *Aspergillus* conidia would not germinate on the surface of a live CEW larva[18]. *Paecilomyces farionosu* significantly reduces oviposition in female *Leptinolarsa decemlineata*[19]. *Pythium* has been reported as a wound pathogen of mosquito larvae[20].

The purpose of this paper is to report on the pathogenicity of *Pythium* isolates and *Aspergillus* to mosquito larvae and the effect of temperature on the growth and pathogenicity of *Aspergillus* to mosquito larvae. The effects of *Aspergillus* infection on the fecundity and longevity of female mosquito exposed to infection during larval stages are also reported.

MATERIALS AND METHODS

Aedes aegypti Ilobi strain was obtained from Lagos, Nigeria, and has been maintained in the Institute Insectary for over a decade. *Culex* (*fatigans*) *quinquefasciatus* and *A. gambiae* larvae, used in laboratory susceptibility tests were reared on Beemax and dog bisquit and glucose solution at 27°C, relative humidity 100%, in a control condition insectary.

The fungus *A. parasiticus* was isolated from moribund *Anopheles gambiae* larvae at Lubumba ponds. It was cultured and then subcultured using a sterile glass ring technique and single hypha and single conidium techniques on corn meal agar. It was identified and then sent overseas for confirmation.

A. parasiticus was cultured on corn meal agar (20 g) supplemented with 2% glucose in 1000 ml sterilized at 15 lbs pressure for 30 min. Deep petri dishes of 100 × 20 mm were used. Glass ring technique was used to clean the culture.

Freshly sporulated cultures were harvested by removing conidia from (0.5 cm) discs of agar cut at the edges of a fresh culture using 0.03% (V/V) of the nonionic detergent, triton X-100, followed by two washes in sterile distilled water. This procedure effectively wets and disperses the conidia, allowing quantitative manipulations such as titer determinations and plate counting. Conidial suspensions were prepared fresh each time and appropriate dilutions were made immediately after each experiment. To ensure uniformity in conidial dosage, conidial counts were made using a haemocytometer and compound microscope. It was found that each fresh culture 0.5 cm agar disc when harvested yielded 2×10^5 conidia/ml. So, 2×10^5 conidia/ml were added to each experimental plate to which 200 larvae of the required instar were added immediately and the plates kept under observation till the survivors pupated and emerged. Field collected larvae were first washed in 5% sodium hypochlorite and rinsed with sterile distilled water. Controls were run to check whether the controls were pathogen-free. Using a sterile glass syringe, 1 ml conidia was spread over agar plates whose bottoms were just covered with media to enable light to pass through while counting the germinating conidia using a stereomicroscope. Counting of germinating conidia started 12 hr after inoculation and ended 24 hr after inoculation; 40 plates were inoculated.

Test and evaluation procedures: Pupation and emergence records were maintained on each plate to determine variations in rearing efficiency and mortality that might be attributed to the *A. parasiticus* treatment. On the 3rd day after emergence, all mosquitoes were offered blood meals from guinea pigs, and on the following day individual, gravid females were isolated in glass tubes (2.5 × 10 cm) lined inside with a strip of filter paper. To induce oviposition, 3–5 ml of water were added to each tube. Each female mosquito was assigned a number within its respective test group so that records could be maintained on an individual basis. After oviposition, the females were transferred to waxed paper cups covered with nylon netting and supplied with cotton pads saturated with 10% glucose solution. Additional blood meals were offered 2–4 days after each oviposition, and the females were again isolated in tubes. Eggs were counted, and the determined percentage hatch was 72–96 hr after oviposition for *Anopheles* and *Culex*. *A. aegypti* eggs were given two weeks to complete diapause[1] and then hatched for 72–96 hr. Unhatched eggs from a few females in each test group were examined for infections by microscopy at each gonotrophic cycle. Mortality of the females was recorded daily throughout the test period. The status of infection was determined by dissecting each female after death and the presence of mycelium or conidia in the ovary ascertained. No data for uninfected specimens except for the controls were considered in our analysis of results. Also, data were discarded for those females that (1) failed to oviposit, on the 1st gonotrophic cycle, (2) produced sterile eggs

on the 1st gonotrophic cycle or (3) escaped during transfer manipulations.

Pythium sp were cultured on corn meal agar. Fresh culture discs (0.5 cm) were cut at the edges of a young culture and added to test dishes each with 200 larvae in sterile distilled water and infection rates recorded daily.

Temperature experiments were carried out in an incubator using a 100°C calibrated thermometer. Mortality rates were corrected after Abbott's formula (b – k)100/100–k. The following temperatures were employed 15, 20, 25, 28, 30, 35°C and results recorded.

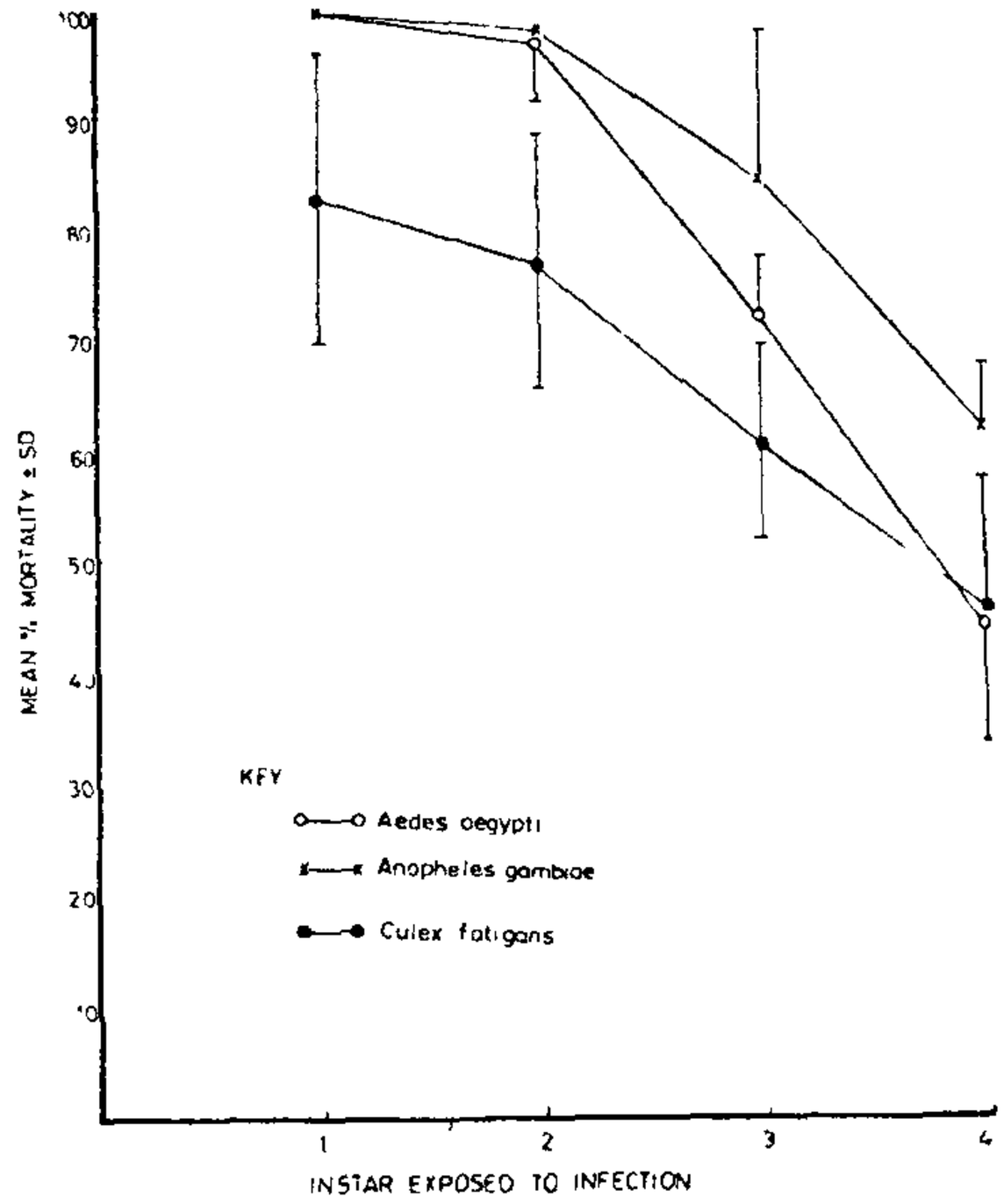

Figure 1a. The susceptibility of mosquito larval instars to *A. parasiticus* conidial infection.

RESULTS AND OBSERVATIONS

The susceptibility of mosquito larvae to entomopathogenic fungi *A. parasiticus* and two isolates of *Pythium* sp are presented in figures 1a and 1b. First instars were most susceptible while fourth instars were least susceptible.

Figure 2a represents data on the effect of temperature on the susceptibility of *A. aegypti* larvae exposed to *A. parasiticus* infection. Temperatures above 25°C reduced susceptibility of mosquito larvae to infection. There was no infection at 35°C. Figure 2b represents data on the effect of temperature on the germination and growth of *A. parasiticus*. There was low germination rates at 15°C and the growth rate was low. The optimum temperature was 25–28°C. At 30°C the conidia germinated but the colony growth rate was very slow and the colony was very poor. There was no germination at 35°C. Probably the temperature killed the embryo.

The number of gonotrophic cycles, the number of eggs and the percentage hatch for all the replicates

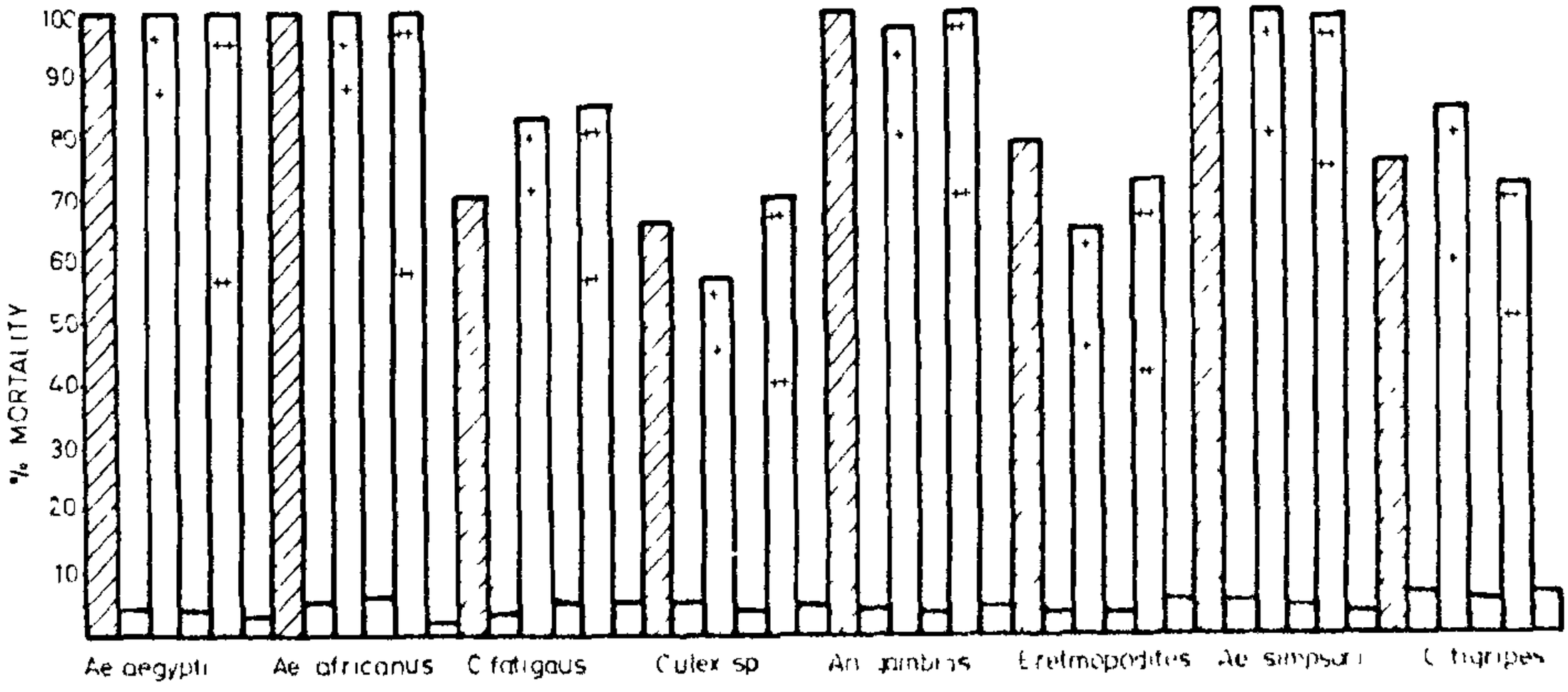

Figure 1b. The comparison of mortality rates of eight species of mosquito larvae exposed to entomopathogenic fungi. ☐ Control ⊡ Pythium with filamentous sporangia – No oogonia ⊡ Pythium with sphaerical sporangia. No oogonia ▨ Aspergillus Test.

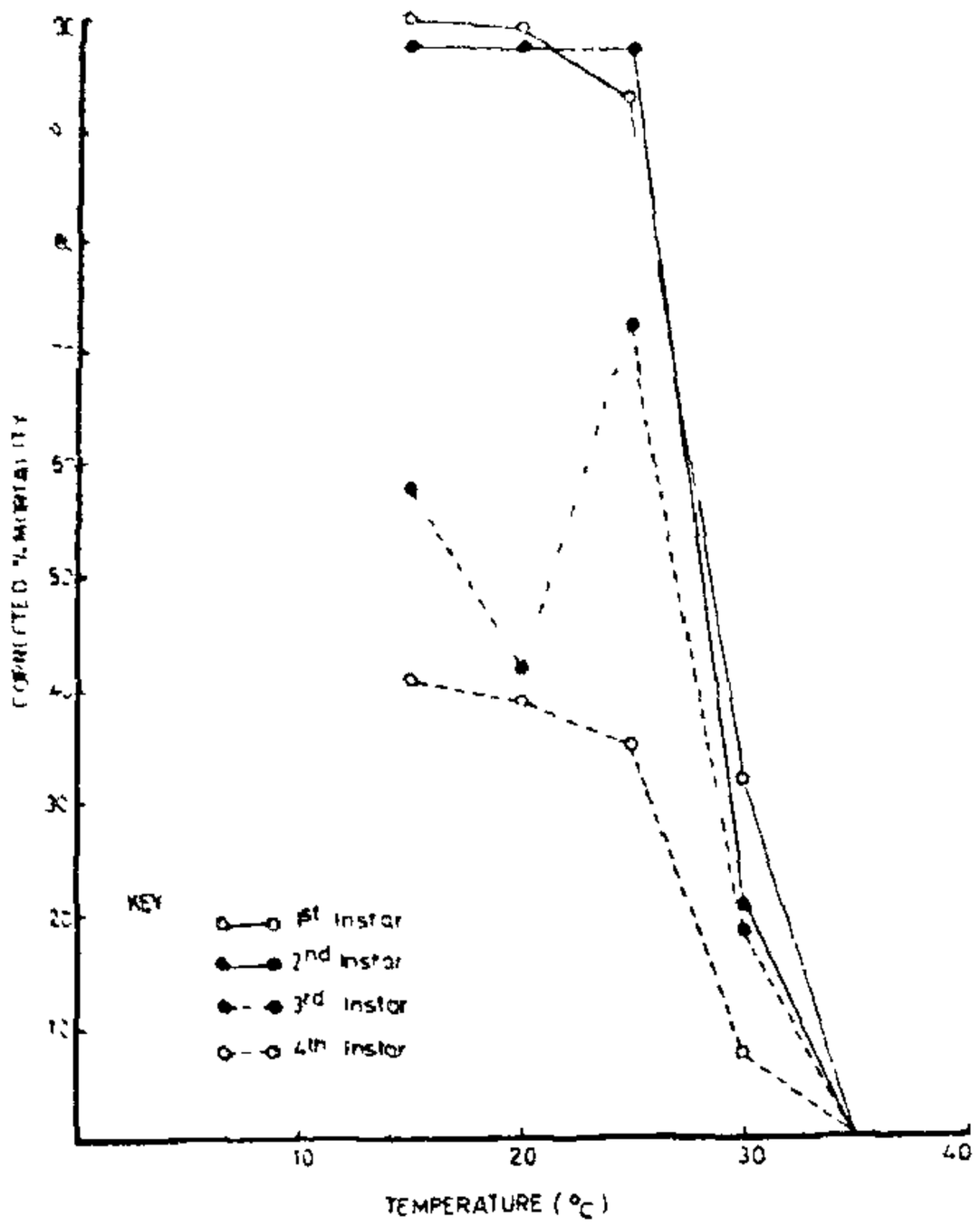

Figure 2a. The effect of temperature on the susceptibility of *A. aegypti* larvae to *A. parasiticus* infection.

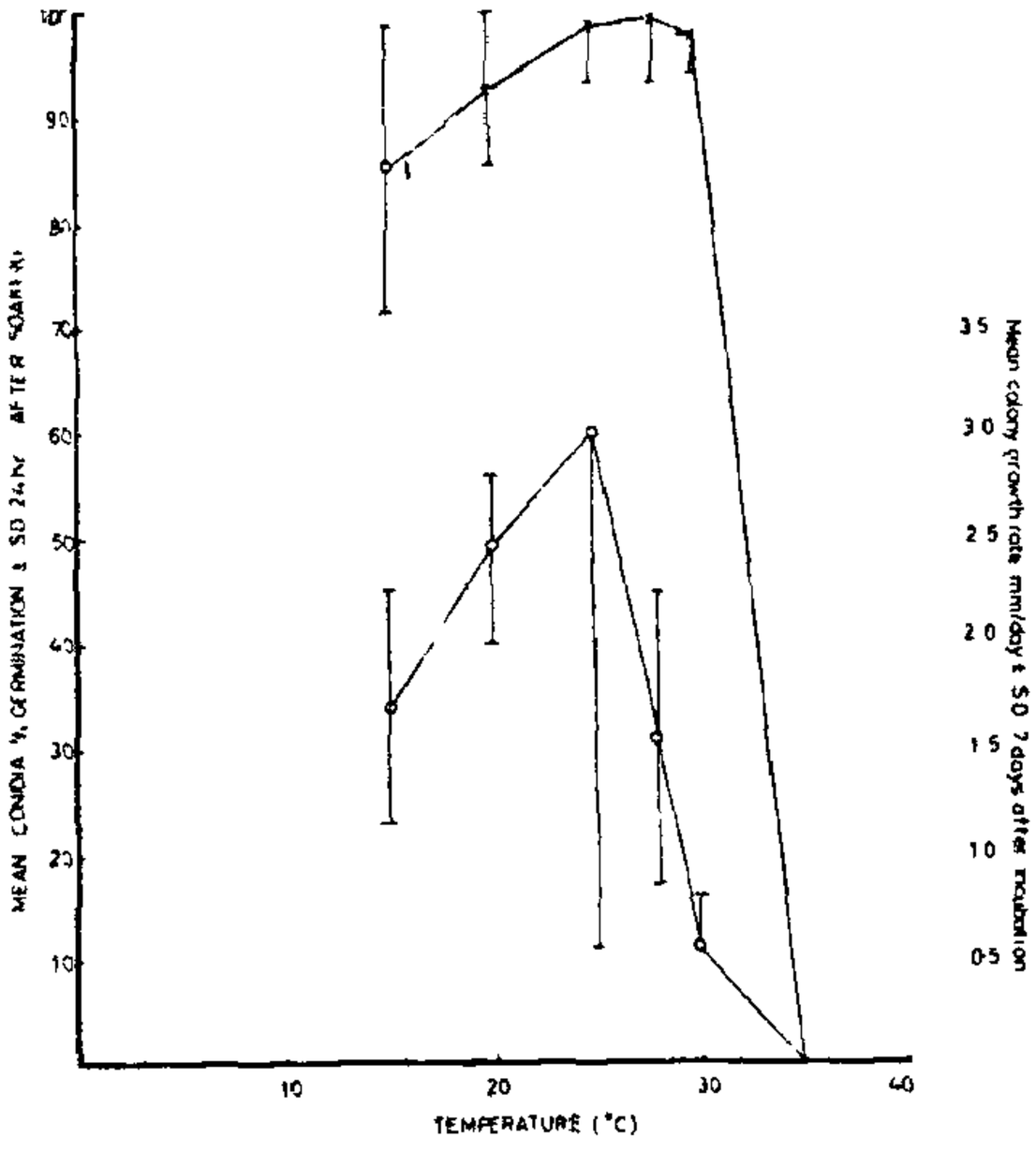

Figure 2b. The effect of temperature on the germination of conidia and the growth rate of *A. parasiticus* colonies on corn meal agar supplemented with 2% dextrose 7 days after inoculation.

were subjected to multiple regression analyses. Comparisons of these analyses showed no statistically significant differences among the replicates; therefore, the data reported here are average values based on the total number of female mosquitoes used in the experiments. The daily mortality data were subjected to probit analysis to compute LT-50 and LT-90 values.

Tables 1a, 1b and 1c represent data on the effects of *A. parasiticus* on the fecundity of *A. gambiae*, *Culex fatigans* and *A. aegypti* respectively. Figure 3a, 3b and 3c show the cumulative percentage mortality of *A. gambiae*, *C. fatigans* and *A. aegypti* respectively. Tables 2a, 2b and 2c show the effect of *A. parasiticus* on the longevity of *A. gambia*, *C. fatigans* and *A. aegypti*. The data showed that the infection reduced fecundity and longevity of all mosquitoes that became infected.

The average infection rates for adults in *A. gambiae* 1st and 2nd instars exposed were 92%, 3rd instars 81%, 4th instars exposed 91% and control 0%.

The average infection rates for adults in *C. fatigans* 1st and 2nd instars exposed were 88%, 3rd instars exposed 85%, 4th instars exposed 77.5% and controls 0%. The average infection rates for adults in *A. aegypti* 1st and 2nd instars exposed were 92%, 3rd instars 81%, 4th instars exposed 91% and control 0%.

DISCUSSIONS

Aspergillus is an ubiquitous and easily recognized genus, which is readily isolated from nearly any organic substrate; it is hardly surprising that many of its species belonging to several sections of the genus should affect insects. The pathogenicity of *Aspergillus* species for insects was discussed[15]. Invasion of insects is usually through the cuticle, but penetration through the gut and spiracles also occurs. Insect larval death may result from any of the several factors including histolysis, mechanical interference with the function of the insects' tracheoles and spiracles or the production of potent mycotoxins[16]. Culture filtrates of *A. flavus* isolated from moribund mosquito larva contained two chloroform extractable compounds toxic to *Culex* larvae[17].

A. parasiticus isolated from moribund *A. gambiae* larvae was purified using a sterile glass ring technique and subcultured using single hypha and single conidium techniques. It was possible to extract a toxin from culture filtrates that was highly toxic to *A. gambiae*, *A. aegypti* and *C. fatigans*. Conidia concentration of (2×10^5) at 25°C killed 97.4% 2nd instar *A. aegypti* and

Table 1a *The effects of A. parasiticus on the fecundity of A. gambiae when infection were induced in different larval instars.*

Parameters Evaluated average per female	Instars in infected with *Aspergillus*				Controls
	1st	2nd	3rd	4th	
Total females tested	85	85	85	85	85
Gonotrophic cycles	1.8	1.8	2.2	2.2	3.6
Eggs laid	247.6	226.4	309.4	298.7	398.6
Eggs hatched	193.5	198.5	294.6	261.3	366.8
Reduction in eggs hatched % compared to controls	41.2	46.9	24.3	27.2	

Table 1b *The effects of A. parasiticus on the fecundity of C. fatigans when infections were induced in different larval instars.*

Parameters Evaluated average per female	Instars infected with *Aspergillus*				Controls
	1st	2nd	3rd	4th	
Total females tested	85	85	85	85	85
Gonotrophic cycles	1.7	1.8	1.9	1.8	3.6
Eggs laid	195.6	246.8	253.7	257.9	498 2
Eggs hatched	156.2	203.0	198.4	204.5	468.5
Reduction in eggs hatched % compared to controls	64.5	53.7	51.3	51.3	

Table 1c *The effects of A. parasiticus on the fecundity of A. aegypti when infections were induced in different larval instars.*

Parameters Evaluated average per female	Instars infected with *Aspergillus*				Controls
	1st	2nd	3rd	4th	
Total females tested	85	85	85	85	85
Gonotrophic cycles	2.4	2.4	1.9	1.9	3.8
Eggs laid	254.4	233.6	245.7	258.5	499.6
Eggs hatched	199.5	202.8	209.4	210.3	469.2
Reduction in eggs hatched % compared to controls	52.2	56.7	54.1	51.4	

77.4% *C. fatigans* 2nd instar larvae. *A. gambiae* 2nd instars suffered 98.2% mortality within 72 hr. The fungus killed 100% *A. simpsoni* and *A. africanus*. Larvae of *A. aegypti* exposed to infection at 15°C took 7 days to score 97.3% mortality, but larvae of the same age (2nd instar) exposed to infection at 25°C yielded to infection only in 72 hr (96.7%). The infection varied with age and was temperature-dependent. Younger larvae suffered higher mortality rates while 4th instar larvae suffered least infection. Fresh pupae when exposed to infection did not die, probably because it takes only three days for the pupae to emerge and pupal cuticle is much harder than that of 1st instar larvae. A few pupae resulting from infected 4th instar were found infected and died of obvious mycosis. The others that acquired the infection did not die but carried the infection into adult female mosquitoes. Examination showed that dead larvae turned black

when the hyphae invaded the larval haemocoel. Hyphae were encapsulated by haemocytes and melanin formation occurred. This would account for the blackening of the dead larvae. Melanization was taken to be an evidence that foreign bodies continued to live following encapsulation. Since the hyphal invasion of the haemocoel was not extensive at death, it was concluded that the toxin largely accounted for the

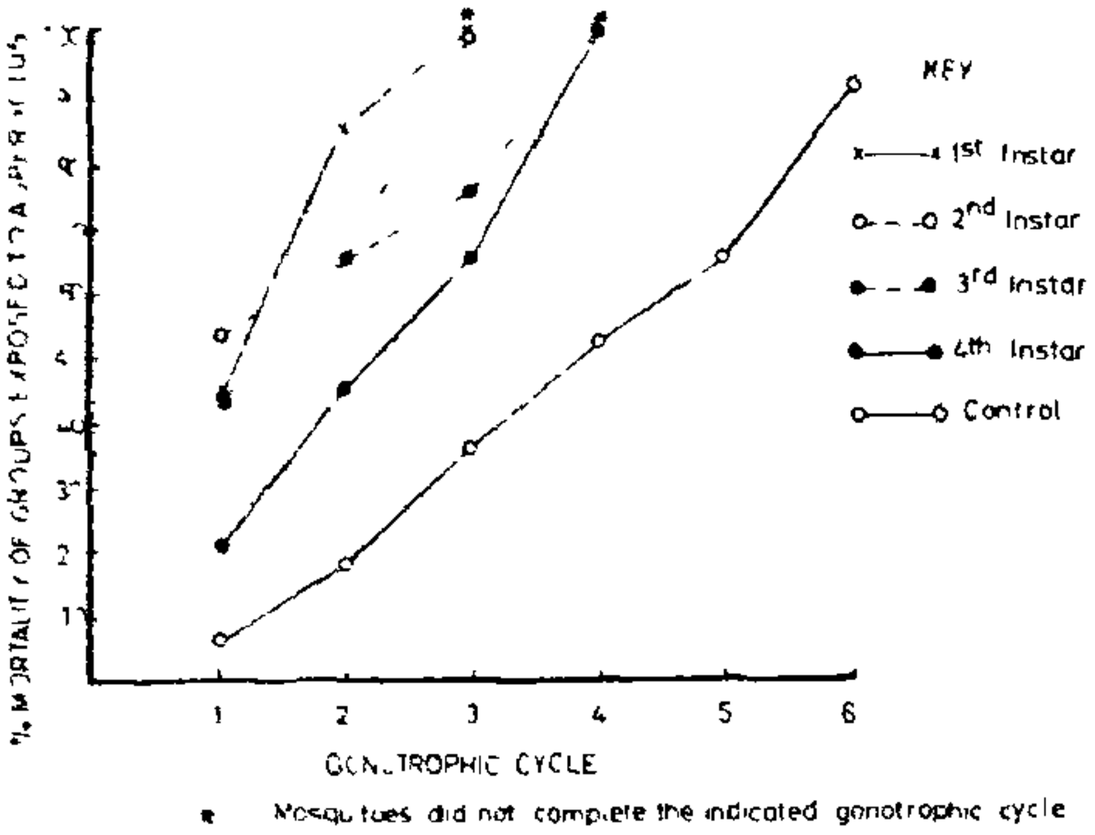

Figure 3a. Cumulative percentage mortality of *A. gambiae* females after each gonotrophic cycle when infections of *A. parasiticus* were induced in different larval instars.

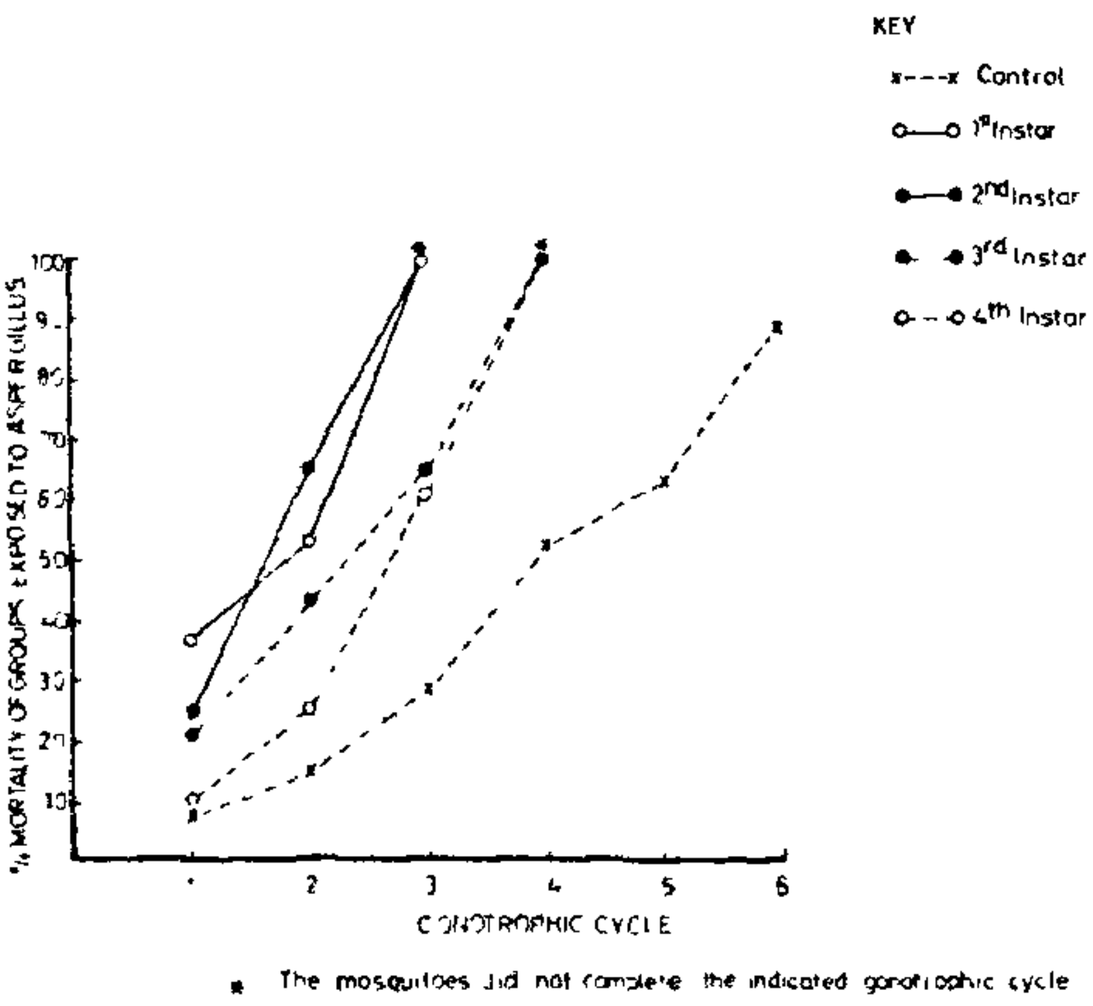

Figure 3b. Cumulative percentage mortality of *C. fatigans* females after each gonotrophic cycle when infections of *A. parasiticus* were induced in different larval instars.

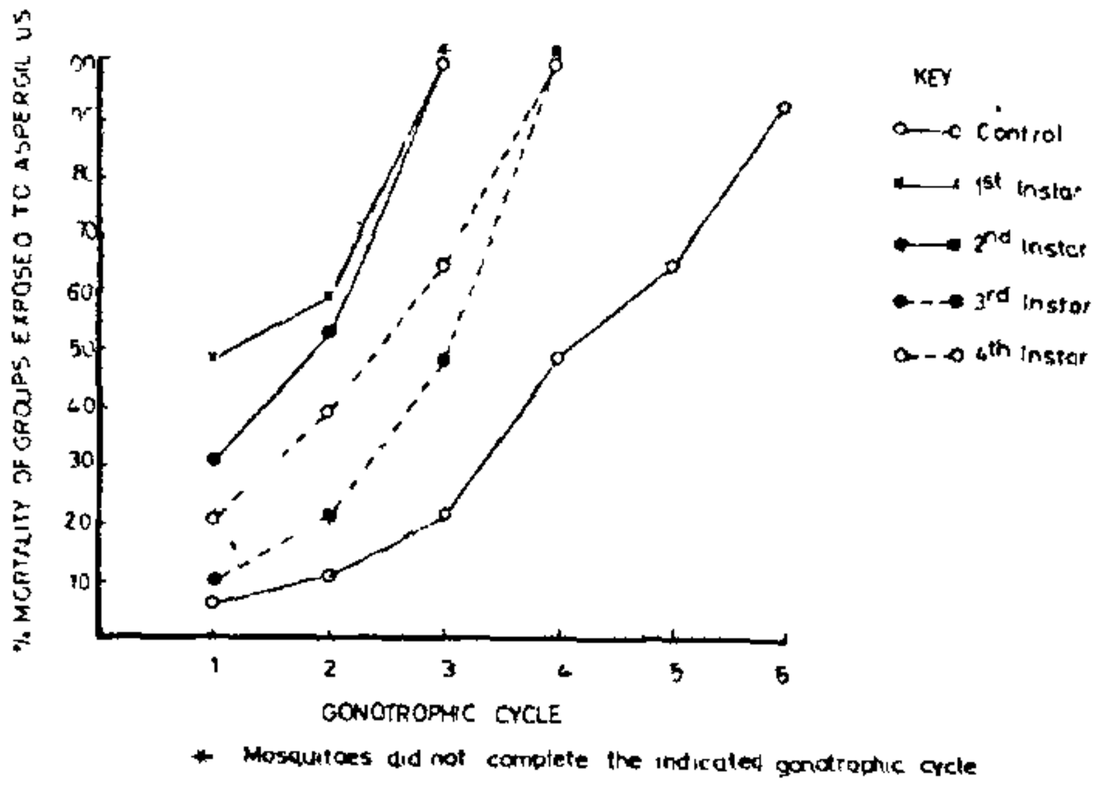

Figure 3c. Cumulative percentage mortality of *A. aegypti* females after each gonotrophic cycle when infections of *A. parasiticus* were induced in different larval instars.

death of the larvae. The toxin was temperature-resistant.

The optimum temperatures for the germination and growth in culture of Ugandan isolate of *A. parasiticus* were between 20 and 25°C. At 30°C the fungus germinated but did not grow. The most favourable temperature for growth and germination of some other insect pathogenic fungi has been found to lie between 20 and 30°C[21]. Larvae in enamel plates of water incubated with conidia were not infected when incubated at 30°C, though the conidia adhered and germinated on the cuticle.

Although there is no doubt that *Aspergillus* species can truly be entomopathogenic, many reports of this genus from insects may be due to secondary infections by *Aspergillus* or its rapid establishment after the insects' death. But it has been proved beyond doubt that our isolate of *A. parasiticus* was truly toxic to mosquito larvae.

Infection by *A. parasiticus* markedly reduced the reproductive capacity of *A. gambiae*, *C. fatigans* and *A. aegypti* regardless of the larval instars that become infected (tables 1a, 1b and 1c). Although the greatest effects were seen in those exposed as 1st and 2nd instars, the fecundity of all females exposed as larvae was significantly (0.01% level) reduced when compared to the controls. There were no significant differences between those exposed as 1st and 2nd or between those exposed as 3rd and 4th instar larvae in regard to number of gonotrophic cycles, eggs laid or percentage hatch; however, the differences between those exposed as 2nd and 3rd instars were highly significant. This was explained at least in part, by the

Table 2a *Effects of A. parasiticus on the longevity of A. gambiae when infections were induced in different larval instars.*

Instars Infected	LT-50 (days)	% reduction in longevity compared to controls	LT-90 (days)	% reduction in longevity compared to controls
1st	9.8	50.5	15.7	50.2
2nd	11.5	41.9	16.4	47.9
3rd	12.6	36.4	24.5	22.2
4th	14.2	28.3	24 8	21.3
Uninfected controls	19.8		31.5	

Table 2b *Effects of A. parasiticus on the longevity of C. fatigans when infections were induced in different larval instars.*

Instars Infected	LT-50 (days)	% reduction in longevity compared to controls	LT-90 (days)	% reduction in longevity compared to controls
1st	11.8	43.8	17.5	52.4
2nd	9.5	54.8	18.6	49.4
3rd	13.5	35.7	24.7	32.8
4th	14.6	30.5	25.2	31.5
Uninfected controls	21.0		36.8	

Table 2c *Effects of A. parasiticus on the longevity of A. aegypti when infections were induced in different larval instars.*

Instars Infected	LT-50 (days)	% reduction in longevity compared to controls	LT-90 (days)	% reduction in longevity compared to controls
1st	9.8	56.3	15.9	58.7
2nd	11.4	49.1	19.4	49.6
3rd	14.3	36.2	25.6	33.5
4th	14.6	34.8	25.9	32.7
Uninfected controls	22.4		38.5	

timing of exposures to conidia to coincide with the larval molt. Conidia were administered to the group as follows: First instar day 1 immediately after set up; 2nd instar—late day 2; 3rd instar—day 5 and 4th instar—late day 6 and early day 7. Thus, nearly 3 full days elapsed between exposures of the 2nd and 3rd instar groups. If a given dosage has a specific pathogenic effect on fecundity or longevity when the infection reaches a certain level, then the attainment of this level is primarily a function of time and not of the instar infected. The fecundity data (tables 1a, 1b and 1c) and the longevity data (figures 3a, 3b and 3c) and (tables 2a, 2b and 2c) support this proposition that the dosage of conidia used in these tests and infection of *A. parasiticus* developed at a similar rate regardless of the instar that became infected.

Figures 3a, 3b and 3c give the cumulative mortality of each group after each gonotrophic cycle for *A. gambiae*, *C. fatigans* and *A. aegypti* respectively. Among the replicates, high rates of mortality were evident among the females exposed as 2nd instar larvae within 24 hr after the mosquitoes were offered the 2nd blood meal. There was no explanation for this early mortality since there was no evidence of significant change in the laboratory environment, and similar mortalities did not occur in other groups that were given blood meals from guinea pigs on the same day. However, examination of the organs of these dead

mosquitoes showed that all were heavily infected with *A. parasiticus* conidia-like bodies and mycelium in the ovary. Throughout the studies it was observed that many of the infected mosquitoes died soon after consuming blood or soon after being placed in the oviposition vials (2.5 × 10 cm glass tubes). Also, many died on the water, apparently attempting to oviposit. The control mosquitoes died at a relatively constant rate and no unusual mortality was observed.

The LT-50's and LT-90's computed from the daily mortality data for *A. gambiae*, *C. fatigans* and *A. aegypti* are presented in tables 2a, 2b and 2c respectively. The 50.2%, 52.4% and 58.7% reduction in longevity of *A. gambiae*, C. *fatigans* and *A. aegypti* females (respectively) exposed as 1st instars larvae was observed. The reduction of 22.2 and 21.3% for *A. gambiae* females exposed as a 3rd and 4th instar larvae and the calculated reduction of 24.3% and 27.2% in F_1 progeny (based on the percentage of eggs hatched compared with the percentage of eggs hatched in the control) showed that *A. parasiticus* had a marked effect on the longevity and fecundity of *A. gambiae* even when infections were induced in the late instars.

The reductions of 32.8% and 31.3% for C. *fatigans* 3rd and 4th were recorded. This, coupled with the calculated reductions of 51.3% for 3rd and 4th instars respectively in F_1 progeny, showed that *A. parasiticus* has a marked effect on the longevity and fecundity of *C. fatigans* even when the infections were induced in the late instars.

The reduction of 33.5% and 32.7% for *A. aegypti* 3rd and 4th instar were recorded. This, coupled with the calculated reduction of 54.1% and 51.4% in F_1 progeny, showed that *A. parasiticus* had a marked effect on the longevity and fecundity of *A. aegypti* even when infections were induced in the late instars.

The eggs that failed to hatch were not found infected with the fungus. The study showed that the effects of chronic diseases are significant in understanding the totality of the pathogen beyond immediate larval or adult mortality. It is concluded that successful introductions of *A. parasiticus* into natural breeding areas of *A. gambiae*, *C. fatigans* and *A. aegypti* could reduce their vectorial capacity and substantially reduce malaria transmission rates and transmission rates of arboviruses.

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