** REPORT **

Metarhizium anisopliae (Metsch.) Sorok.: POTENTIAL TO RECYCLE IN MOSQUITO LARVAE

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In the course of a study aimed to assess natural occurrence of entomopathogenic fungi in mosquitoes two isolates of a *Metarhizium anisopliae* strain occurring under laboratory conditions were obtained from the tree hole mosquito *Aedes triseriatus* (Say). mosquitoes.

In May 1992, about 100 *Ae. triseriatusi* larvae were collected from a tree hole in the Stair Park, Vestal-NY (C#05-92) and brought to the laboratory. As a standard procedure the larvae were kept in closed plastic dishes (8.5 cm diameter) with water collected from the breeding site, in groups of 20 to 30 larvae, and with no additional food. One week after sampling, one out of 12 dead late instar larvae (L4) showed a fungus growing from the siphon tip. That larva remained floating after death. The greenish mycelial growth emerging from the siphon strongly suggested *M. anisopliae* as the causative agent (FIG. 1).

That fungus was isolated (IF#1) and cultured in SDAY-combiotic media. Nine days after this first isolation, the fungus identity as *M. anisopliae* was confirmed and an experiment to proceed with Kock's postulates was carried out. The experiments in the present report were carried out under laboratory conditions of 26+-2^{0}C and uncontrolled photoperiod.

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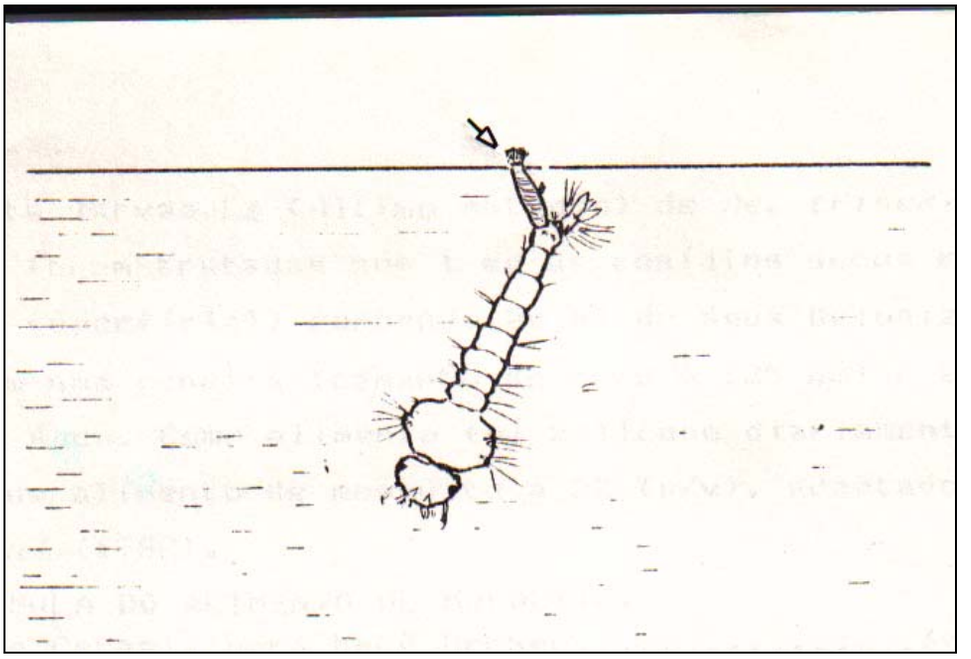

FIG.1 Schematic representation of late instar *Ae. triseriatusi* larvae with *M. anisopliae* sporulated growth in siphon opening.

1. FIRST EXPERIMENT -

***Aedes triseriatus* (Say). C#05-92 and C#06-92**

The assay to Kock's postulates was based on the procedures described by Daoust & Roberts (1982). Field collected mosquito larvae were preferred instead of laboratory reared ones. Three trials of virulence analysis for *Aedes aegypti* larvae from the Cornell Culture (Medical Entomology) showed to be unsuccessful due to low hatching rates. Additionally, the use of laboratory reared *Ae. aegypti* to evaluate *M. anisopliae* virulence led Chang & Liu (1990) to doubtfully indicate either a higher virulence of the fungus or a greater susceptibility of the mosquito strain used, when comparing their results with those obtained by others authors.

Thirty L4 (late instar) field collected *Ae. triseriatusi* larvae were treated with 1 mg of dry conidia in a beaker (16 cm^2 surface area) with 20 ml of deionized water. The fungus was passed through a sieve (pore size 125 um) and spread on the water

surface. As food, 0.1 ml of a 2% (w/v) suspension of mosquito food adapted from Daoust et al. (1982) was applied daily.

Mosquito food formulae: 60g Gerber Mixed Cereal for Baby (oat, corn, wheat and rice flour) + 25 g Brewer's yeast + 10 g crude blood meal + 5 g non-fat dry milk.

Mortality was recorded daily and reached 93.3% after a week (FIG. 2) while only 3.3% mortality occurred in the control group. No mycelial growth among dead larvae was observed in the following days. All the larvae sunk and bacterial growth overcame.

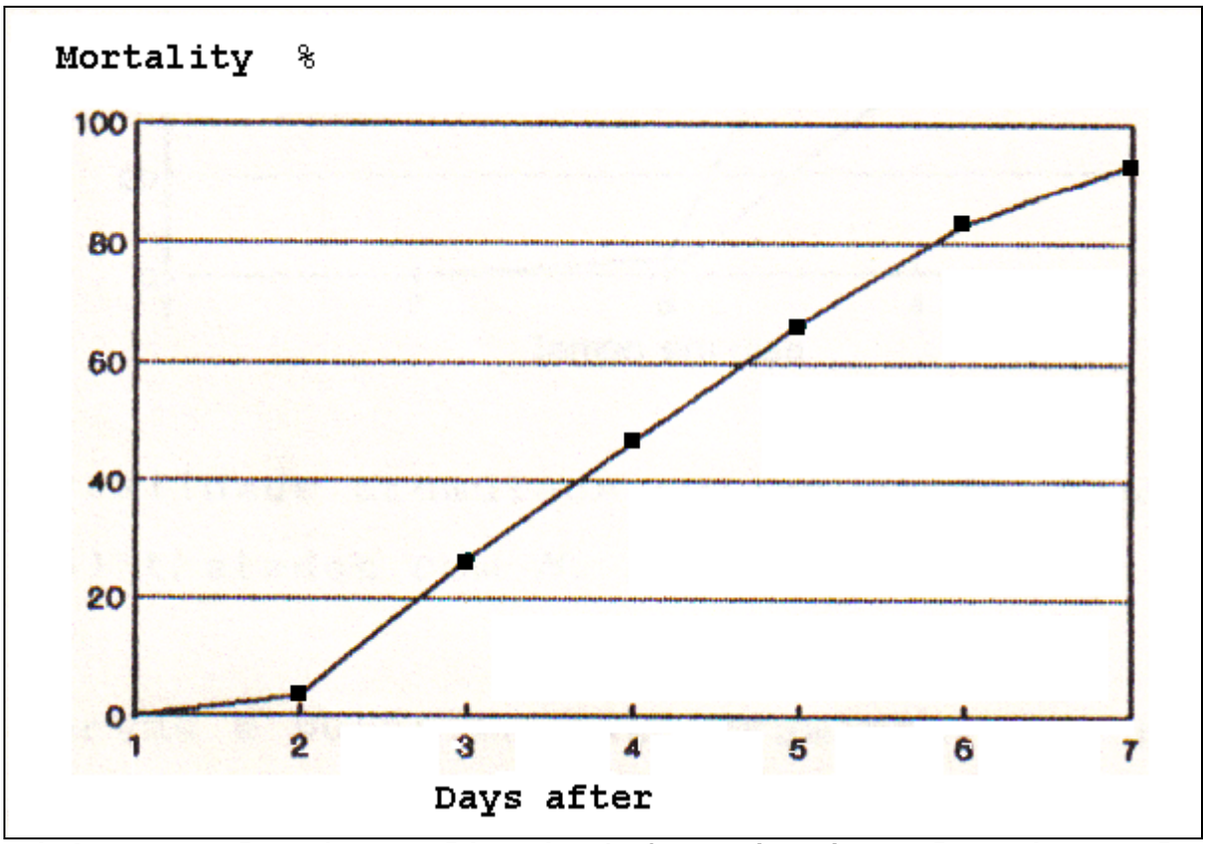

Fig2. Accumulated mortality in *Aedes triseriatus* late instar larvae treated with *Metarhizium anisopliae.*

2. SECOND EXPERIMENT -

***Aedes triseriatus* (Say). C#07-92)**

A second experiment was carried out with *Ae. triseriatus* larvae collected from discarded tires. Thirty L3 and 30 L4 larvae were used per beaker and a higher concentration of 2 mg dry conidia/ 20 ml was spread on the water surface. Total mortality occurred in 4 to 5 days and 40% died as pupae among L4 larvae treated (FIG.3).

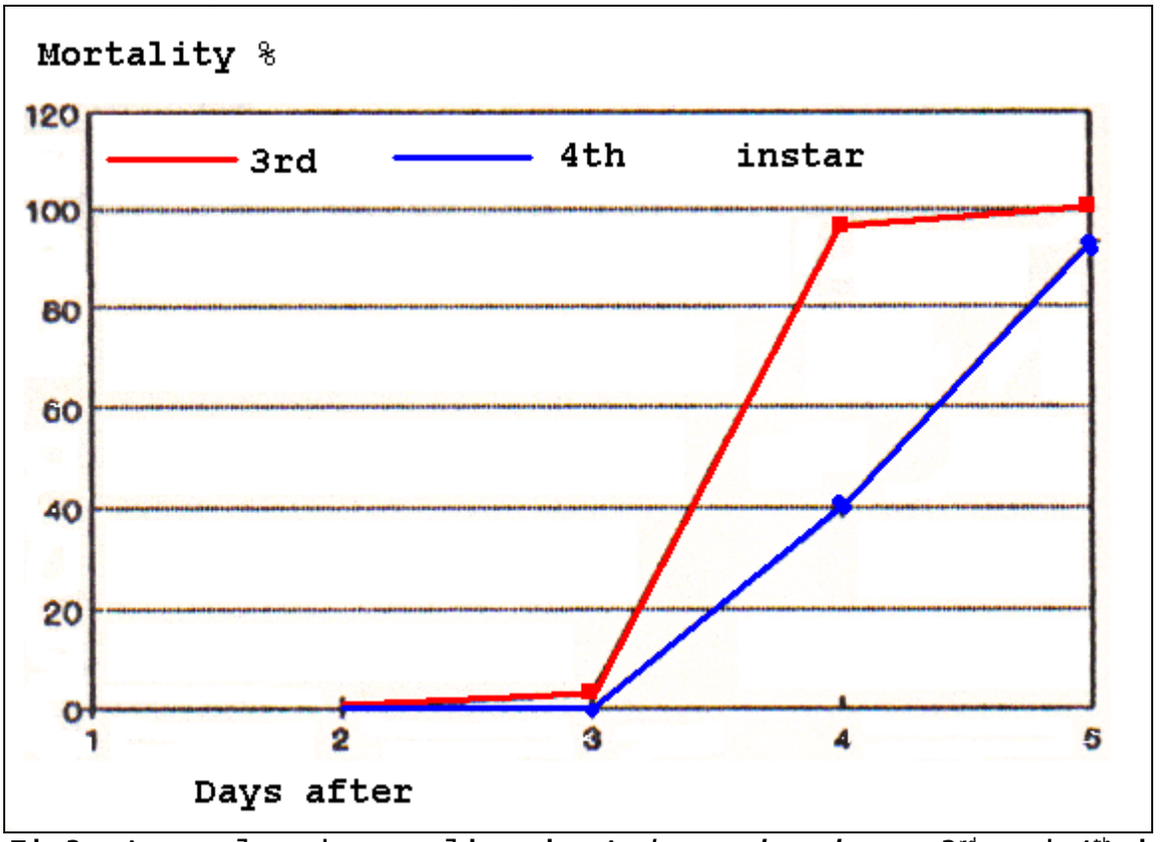

Fig3. Accumulated mortality in *Aedes triseriatus* 3rd and 4th instar larvae treated with *Metarhizium anisopliae.*

Dead larvae and pupae were maintained for 4 days in the beakers for aerial mycelial growth. As before no recycling fungus was observed. Attempts to isolate the fungus from dead larvae in SDAY-Combiotic media were unsuccessful. Meanwhile, light microscopic examination of stained smears and whole larvae showed germinated spores and hyphae in the tracheal trunks as well as and abundance of virus

inclusion bodies in the adipose tissue. The most probable cause of death can be attributed to the combined action of the viruses and the fungus, since only 5% mortality occurred in the same period among 20 L4 larvae maintained as control.

During the experiments described above, a second late instar *Ae. triseriatus* larva (C#05-92) was found with a brownish-green mycelial growth upon its body. This larva had not been selected (L3) for the first experiment and had been left in an uncovered petri dish with just a thin layer of water. The semi submerse condition of that larva enabled the fungus to grow on the thorax as well as on some abdominal segments (FIG. 4). A second isolate (IF#2) was then obtained and confirmed as *M. anisopliae*.

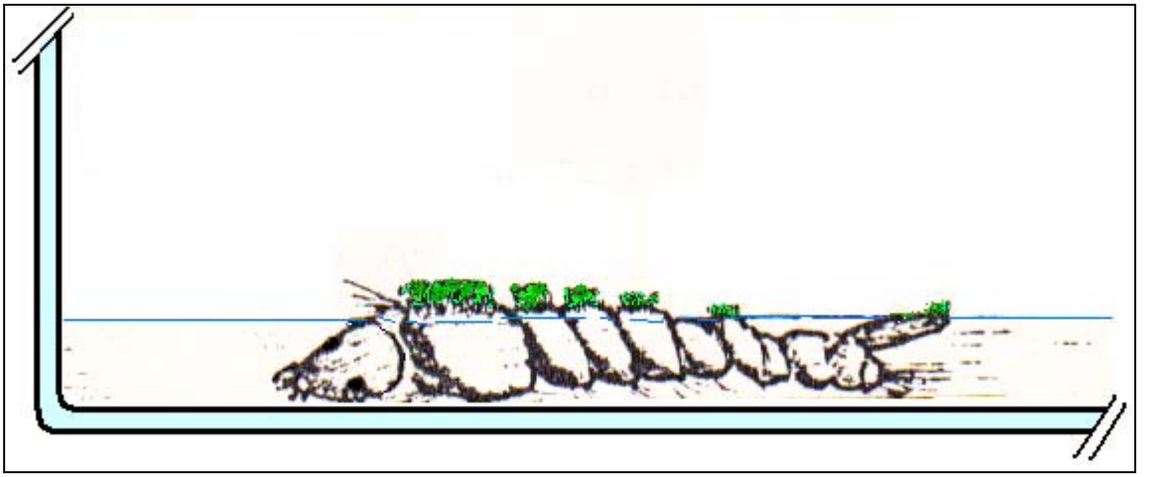

Figure 4. Schematic representations of late instar *Ae. triseriatusi*larvae with *M. anisopliae* sporulated growth upon the body.

Although it might be possible that the two *Ae. triseriatusi* larvae acquired *M. anisopliae* infection under field conditions it should be considered improbable. No mortality was observed by the time of larvae collection. A sample of the tree hole water along with some soil from the bottom was collected some weeks later. Two out of 15 field collected Cx. restuans larvae died when exposed to the tree hole

water and soil, but no symptoms of infection disease occurred. On the other hand, the laboratories in the Insect Pathology Resource Center (at Boyce Thompson Institute) have been receiving strains of entomopathogenic fungi from all over the world. Hypothesis for these isolates origin remains uncertain, since most likely the larvae acquired infection from laboratory air born spores. It should be noted that no work with sporulated cultures of *M. anisopliae* was being conducted in the same laboratory simultaneously or close before the first isolation. These premisses indicate that two high virulent *M. anisopliae* isolates with a particular high ability to infect and recycle in mosquito larvae were obtained.

3. THIRD EXPERIMENT. COMPARING IF#1 and IF#2 ISOLATES FOR VIRULENCE. *Culex restuans* Theobald. C#08-92)

The two *M. anisopliae* isolates were assayed against L2 *Cx. restuans* larvae. An egg raft was obtained from the field and incubated under laboratory conditions. Four repetitions with 15 larvae each (in 16 cm^2 beakers) were used for each isolate and 1 mg of dry conidia was applied as described previously. Total mortality was obtained in 68 hr among treated larvae and no mortality occurred among the 15 larvae maintained as control (FIG. 5). Some treated larvae died during ecdysis to third instar and remained floating. They failed to achieve siphonal ecdysis and stayed attached to the exuviae (FIG. 6). Microscopic examination showed mycelial growth in the siphon and respiratory trunks but no sporulation occurred in the larvae that remained floating.

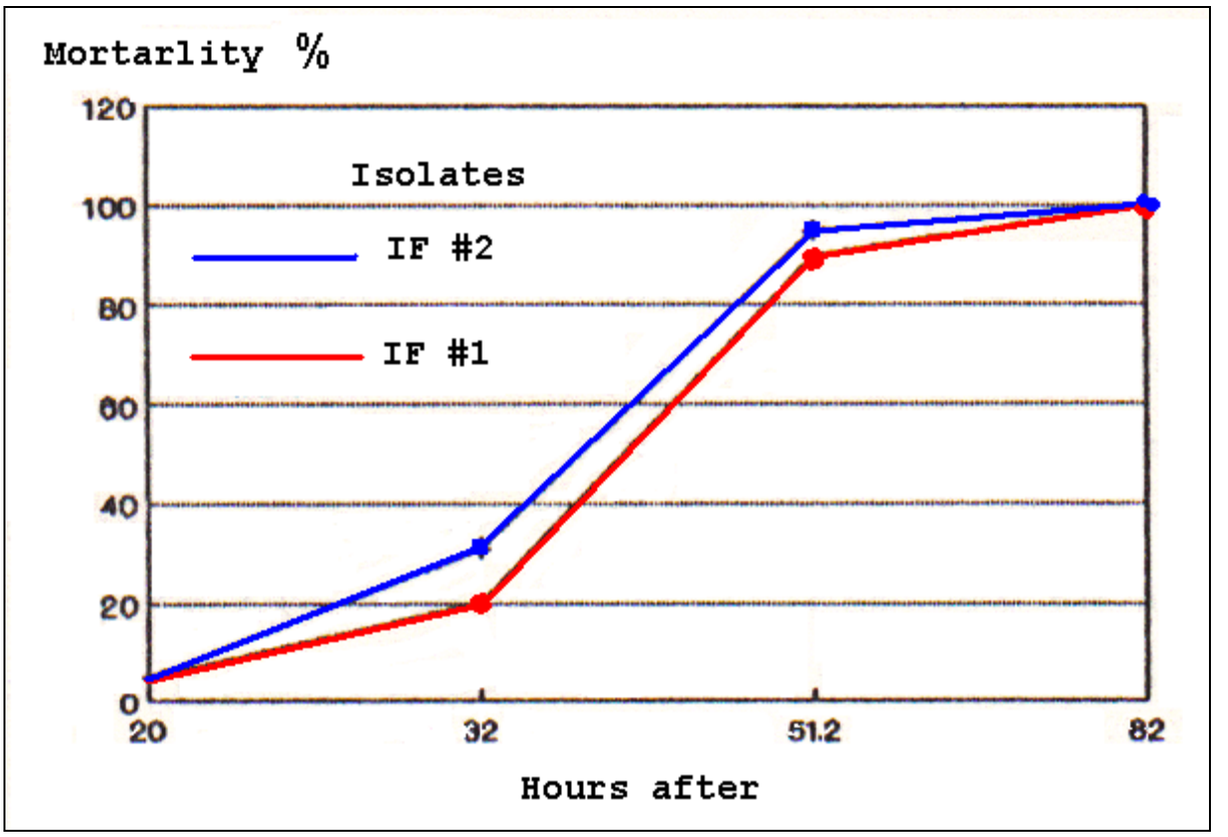

Fig 5. Accumulated mortality in 2nd instar *Culex restuans* larvae treated with two *Metarhizium anisopliae* isolates.

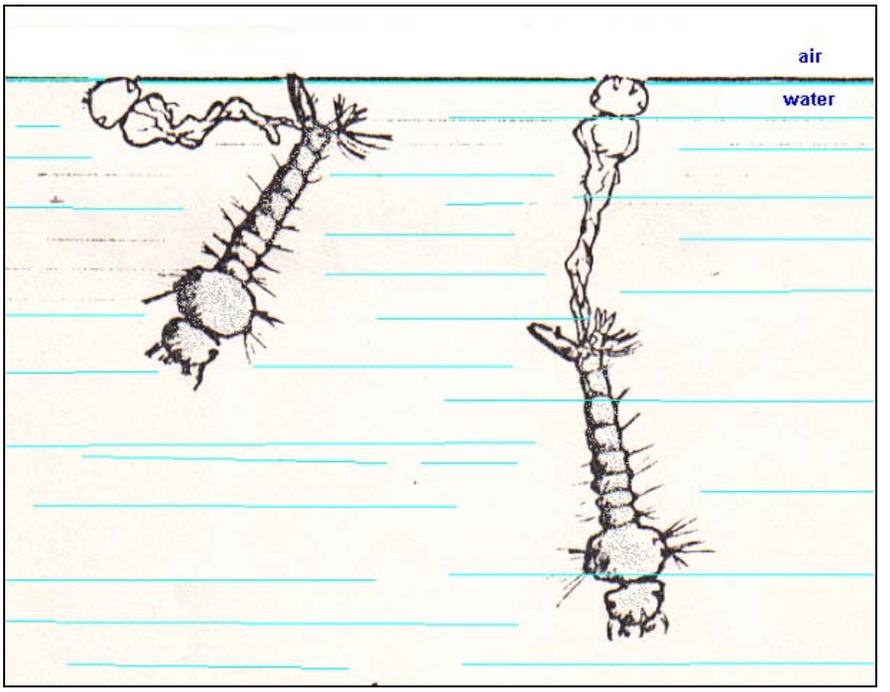

Figure 6. Schematic representation of *Cx. restuans* larvae died during ecdysis

A similar observation was reported by Clark et

al.(1968) when late instar *Cx. pipiens* larvae were exposed to floating spores of *Beauveria bassiana*. They reported that in many cases mortality occurred in pupae, that remained floating with larval siphon held to pupal tail by mycelial growth. No aerial mycelia were observed by those authors.

Although not completely comparable, the present results could also be discussed considering the results obtained by Daoust & Roberts (1982). Forty-seven isolates of *M. anisopliae* var *anisopliae* were screened for virulence against *Cx. pipiens pipiens* larvae by these authors. The most virulent isolates were found to be from Austria, Australia and Brazil, providing LTs_{50} of 1.08, 1.38 and 1.41 days respectively (for the same concentration) and 100% mortality 5 days after treatment. In the present study, *Cx. restuans* larvae showed 50% mortality after a period around 1.6 days (40 hr). In addition, total mortality was obtained against this species after a period of 2.8 days for both isolates IF#1 and IF#2.

Due to the similar virulence among both IF#1 and IF#2 isolates, they were considered as only one strain and deposited in ARSEF collection (Plant Protection Research Unit. US Plant, Soil and Nutrition Lab. ARS, USDA, Ithaca-NY) under Assess Number AESEF-3826, which will be referred to from now on.

4. FOURTH AND FIFTH EXPERIMENTS. RECYCLING *M. anisopliae* IN MOSQUITO LARVAE. *Anopheles walkeri* Theobald. C#08-92)

Two experiments were conducted at the same time with field collected An. walkeri larvae, maintained in beakers (16 cm^2 surface area) with 20 ml of water from the breeding site. In the fourth experiment, 15 L2 larvae were treated with 0.5 mg of *M. anisopliae* (equivalent to 0.03 mg/cm^2) receiving no additional food. In the fifth

experiment, a mixture of 25 L3 and L4 larvae were treated with 0.06 mg/cm^2 of spores as described anteriorly, receiving one hour later 2 mg of the mosquito food spread on the water surface. Due to the typical habit of surface filtering showed by the anopheline larvae, all visible floating spores clusters had been ingested in some minutes in both experiments. As a result only one individual in the fifth experiment survived, by molting to pupae in the next day after treatment, and then to adult. The remainder larvae died in 1 to 4 days in both experiments (FIG. 7). Only one dead L4 larva stayed floating and about one week later mycelial growth was observed emerging from its thorax and abdominal segments. Eight days later the aspect of *M. anisopliae* growth was evident (FIG. 8). For the first time during this study *M. anisopliae* growing and sporulating (recycling) was observed in a controlled application.

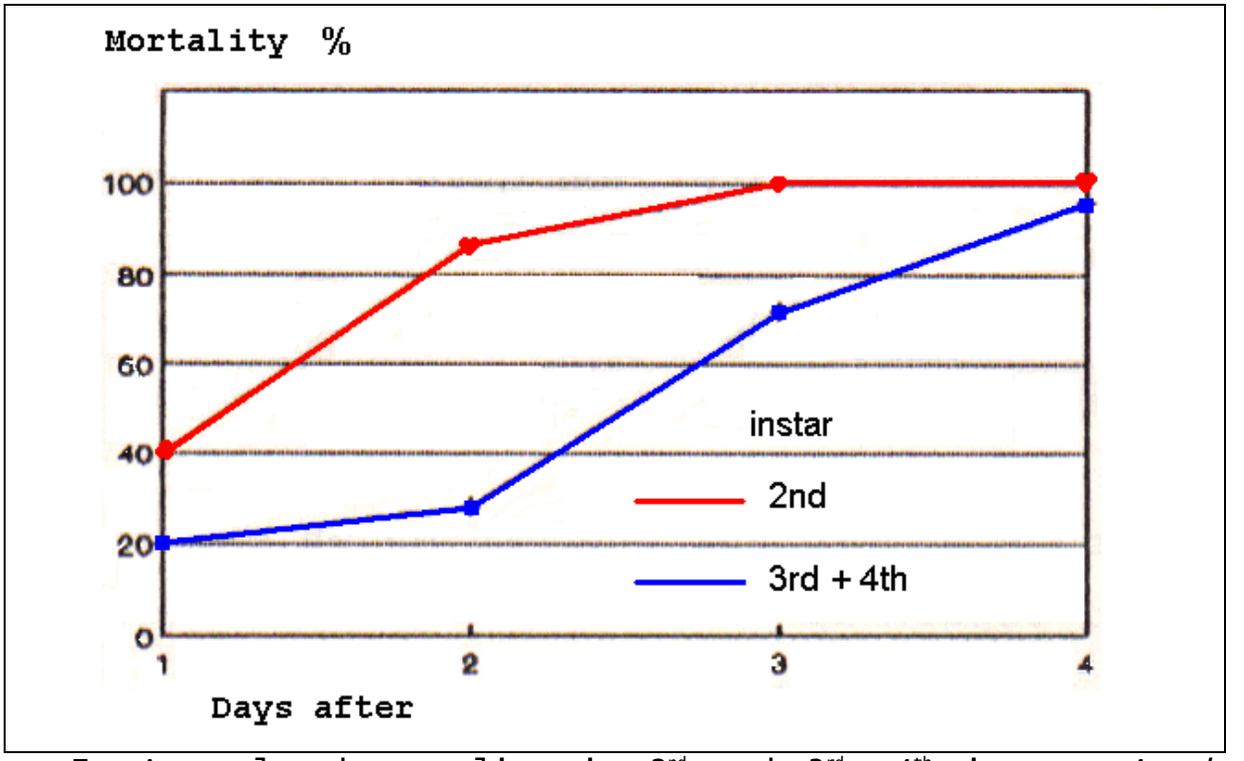

Fig. 7. Accumulated mortality in 2nd and 3rd+ 4th instars *Anopheles walkeri* larvae treated with different concentrations of *M. anisopliae*.

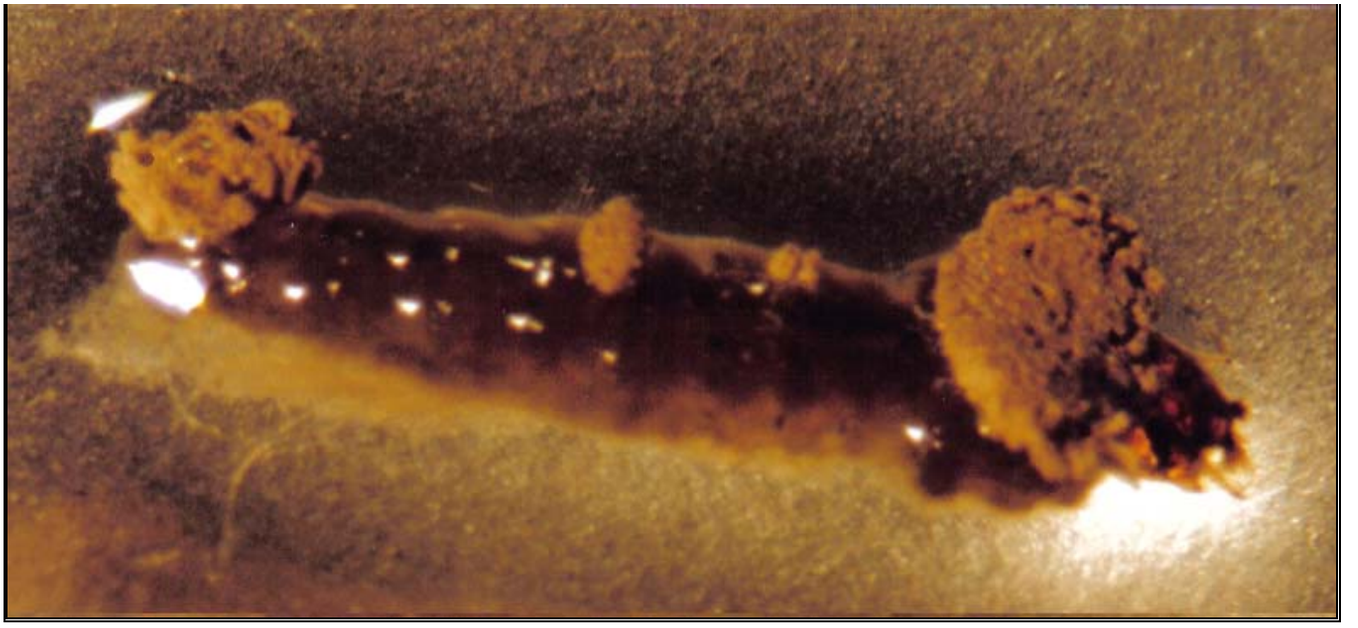

Fig. 8. Late instar *Anopheles walkeri* larvae infected with *M. anisopliae*.

5. MODE OF ACTION

As pointed out by several authors, the mode of action of *M. anisopliae* in mosquitoes depends upon many factors and can be either by:

1. Ingestion of a high inoculum of spores followed by toxin release and rapid toxaemia causing death in 6 to 24 hr (Roberts, 1967; Crisan, 1971, Lacey et al., 1988).

2. Ingestion of a small inoculum followed by germination in hind gut (*Aedes* larvae) or mid gut (*Culex* larvae) with death occurring due to infection in several days (Roberts, 1974; Ravallec et al., 1989).

3. Spore germination and hyphae penetration through spiracular valves, which causes suffocation by occluding the breathing openings. Death will also occur in some days (Roberts, 1970; Al-Aidroos & Roberts, 1978; Lacey et al., 1988).

According to Lacey et al. (1988) only one of either the first or third mode of action can occur (in *Cx. quinquefasciatus* larvae) depending on the treatment method used, submersed or floating spores respectively.

In relation to the present results on *An. walkeri* it can be supposed that any mode of action could be involved. This is because the treated larvae were observed filtering virtually all visible spores from the water surface in few minutes and that mortality occurred after 1 to 4 days. In any case, due to the occurrence of one larvae recycling the fungus, one out of the second or the third mode of action might be involved in that case. Indeed, microscopic examination revealed germinated spores in the respiratory openings and tracheal trunks in many dead *An. walkeri* larvae that actually didn't recycle the fungus. Although no mycelial growth was observed in the gut this rout of infection remains possible. Dead larvae were examined at least one week after dying permitting no accurate diagnostic in many tissues due to natural decomposition.

6. SIXTH AND SEVENTH EXPERIMENT: EVALUATING *M. anisopliae* HORIZONTAL TRANSMISSION IN *Anopheles walkeri*

The present experiments were conducted in plastic dishes (57 cm^2 surface area) with 80 ml of water from the breeding site. In an attempt to use the previously obtained infected *An. walkeri* larva as a source of inoculum, it was placed along with field-collected larvae (C#10c-92) in two consecutive assays. The infected larvae remained floating during all the time in both experiments. The hydrophobic properties of the sporulated mycelial growth avoided the larva to sink even when touched with a brush.

In the sixth experiment 22 cospecific *An. walkeri* larvae in different instar (2-L4, 10-L3 and 10-L2) and 5 (L3) Cx. *restuans* larvae were assayed. No additional fungal inoculum or food source was applied. The two L-4 larvae pupated in the first day after treatment and molted to adult some days later. Total mortality overcame to the remaining

larvae in 8 days after (FIG. 9a) but no mycelial growth occurred. All the *Cx. restuans* larvae died by the sixth day after treatment. *M. anisopliae* germinated spores and hyphae as well as viral inclusion bodies could be observed in microscopic examination.

It was observed that the treated anopheline larvae frequently scraped the body surface of that one used as inoculum. Also, two *Cx. restuans* larvae were totally eaten and some *An. walkeri* larvae were partially cannibalized.

In the seventh experiment 31 conspecific larvae (28-L2 and 3-L3) were used. In the same way, no additional food or inoculum other than the previously obtained infected larvae was applied. High mortality occurred in the first day after treatment, with 22.5% of the larvae being totally cannibalized. Due to this, some dead larvae were put apart in small petri dishes with distilled water as soon as they were detected. No mycelial growth occurred among the 14 individualized larvae and total mortality overcame 5 days after treatment (FIG. 9b).

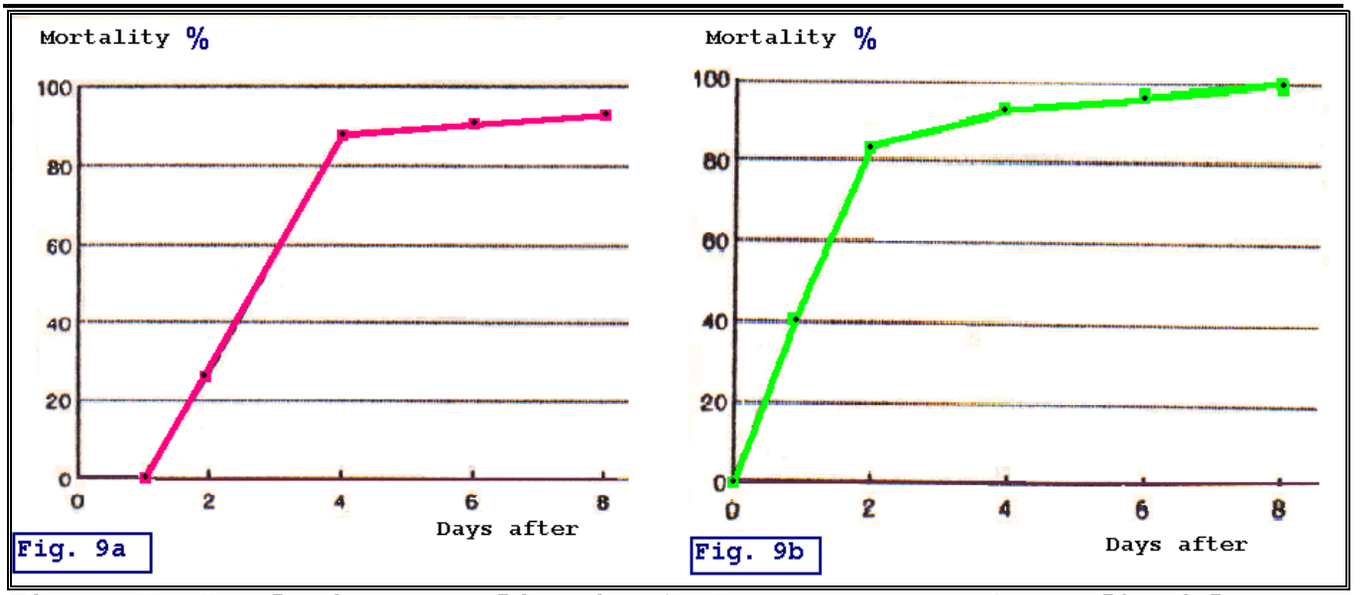

Figure 9. Cumulative mortality in a) *Cx. restuans* + *An. walkeri* larvae, and b) *An. walkeri* larvae, inoculated with one infected *An. walkeri* larvae.

From these experiments, the first ones carried-out with such

a small inoculum of *M. anisopliae* (only one dead larvae with fungal growth), it became obvious that food plays an important role in the process under study. The proportion of cannibalized larvae at the end of the seventh experiment would probably be greater than the final score of missing larvae (55%) if some of them had not been taken out from the recipients. This was the first time cannibalism was observed in the present work.

A series of trials showed that once healthy larvae were killed or seriously injured by warm water, they were promptly cannibalized when offered to other healthy *An. walkeri* larvae, despite the presence of floating mosquito food. While filtering, the larvae are frequently touching each other and apparently scraping (or trying to do so) the body surface of its neighbors. Such a behavior sometimes seems to anger the scraped healthy larva that may show a fast flight movement. Otherwise, if this reaction does not occur, cannibalism proceeds. Such observations indicate that the larvae that first acquire infection or become intoxicated by the ingestion of spores could be more easily cannibalized by others while moribund. This would decrease the chances for producing larvae with sporulated fungal growth upon the body thus reducing the recycling potential of *M. anisopliae*. It should be noted that the infected larva used as inoculum was scraped by the browsing buccal movements of the treated larvae in both experiments, but wasn't consumed. By the end of the experiments the infected larva remained apparently as it was before indicating that less reduction, if so, occurred in its potential as inoculum.

7. EIGHTH EXPERIMENT: EFFECT OF FOOD CONSUMPTION IN *An. walkeri*

The following test was done in order to estimate the role of food in the expression of the mortality by *M. anisopliae* in *An. walkeri*

larvae. Forty field collected late instar larvae (C#11-92) were treated in plastic dishes (57 cm^2) containing 80 ml of deionized water. One mg of spores of the *M. anisopliae* (ARSEF 3826) was spread in the water surface as previously described. The larval behavior was observed during one hour after the application of the spores. By the end of that period, no evident spore clusters could be noted on the water surface, meaning total consumption. Two groups of 20 larvae each were then separated at random and transferred to others dishes in non-treated water. The first group received 100 mg of the described mosquito food applied as powder on the water surface. The second group received no food. Mortality and cannibalism were recorded daily and showed to be quite different when both groups were compared (FIG. 10).

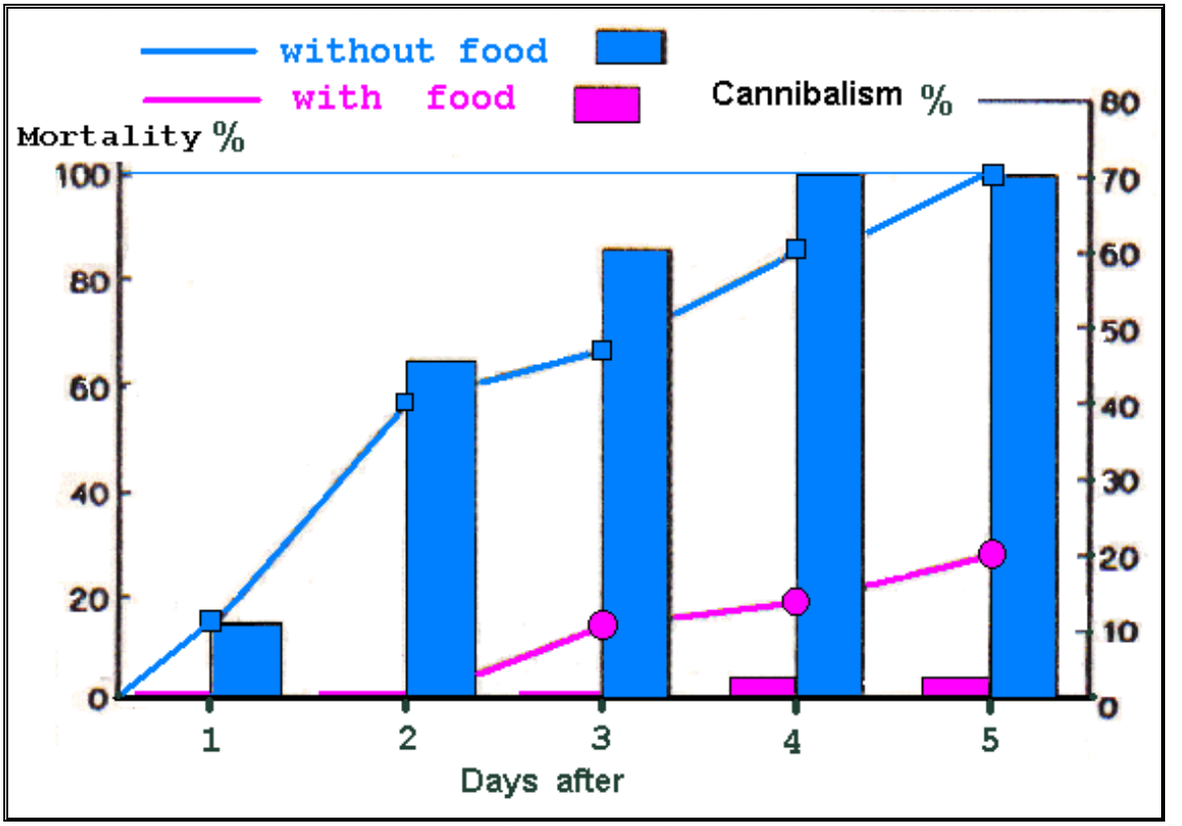

FIG. 10. Accumulated mortality (--) and Cannibalism (bars) for *Anopheles walkeri* larvae treated with *M. anisopliae* WITH or WITHOUT food supply.

Only one larva was cannibalized in the group that received food. Final mortality scored 30% and the surviving larvae normally

molted to pupae and adult. In the other group, without food, mortality reached 100% occurring in larvae, pre-pupae and pupae. Due to the high cannibalism observed in this treatment (70%), few dead individuals remained in the dishes.

No *M. anisopliae* was observed sporulating among the dead larvae in either treatment. It was meanwhile observed one submersed dead pupa with mycelial growth upon the body in the group deprived of food, by the second day after treatment (FIG. 11). A trial to cultivate part of the mycelia in SDAY media showed to be unsuccessful. Even though difficult to associate that mycelial growth to *M. anisopliae*, it should be emphasized that the pupa was completely eaten in the subsequent days. This observation can be reinforced by others observations done during the present study. Submersed or floating dead larvae were noted with typical saprolegniales fungi growing all over the body being partially or totally eaten, specially if a small amount or no food at all was supplied.

Figure 11. *An. walkeri* pupa found with mycelial growth upon the body when larvae were treated with *M. anisopliae*.

8. A PREMISE

The previously reported observations led to the following premise about the recycling potential of *M. anisopliae*:

The present obtained strain of *M. anisopliae* could recycle in *Anopheles* larvae if: a) applied as a small inoculum not enough to kill the larvae in a short period by toxaemia; AND b) If by some way the feeding process is inhibited in order to permit that the larvae that first become infected were not cannibalized by the others.

To evaluate these possibilities, the next experiments were carried-out. Due to some scarcity of *An. walkeri* larvae in the sites commonly sampled another field collected species was utilized. Consistently, cannibalism was found to be also common in this species.

9. NINTH AND TENTH EXPERIMENTS: *Anopheles punctipennis* (Say).

As many as 1000 *An. punctipennis* larvae mainly as L3 and L4 were obtained from a sample in a pond located at Homer C. Thompson Vegetable Research Farm (NY State College of Agriculture and Life Science Cornell University, Freeville-NY) (C#15-92). Some larvae of *Cx. territans* Walker were also collected together with the anopheline larvae.

Once in the laboratory the larvae were separated into two groups and placed in plastic pots (1700 cm^2) with 2 l of water from the breeding site. In order to approach the natural breeding conditions, some aquatic plants as well as small floating twigs and dead leaves were also collected and placed with the larvae. Microfauna composed of free swimming nematodes and crustaceans were also present.

Ninth experiment.

This experiment was carried out to evaluate a possible synergism resulting from a treatment with a sub-lethal concentration of Bacillus thuringiensis israelensis (B.t.i.) followed by a treatment with a small concentration of *M. anisopliae*. The primary site of action of B.t.i. toxins is in the larval midgut epithelia and typical gut paralysis occurs as a result. By this means the B.t.i. treatment would interfere with conidia ingestion and cannibalism due to food intake inhibition. Additionally, part of the *M. anisopliae* conidia would remain floating for a period longer enough to improve attachment to spiracular valves and to initiate infection.

About 420 *An. punctipennis* larvae and 25 Cx. territans larvae were treated with 2.12 mg of Bactimos-WP (equivalent to 0.125 Kg/ha). The B.t.i. product was applied as powder on the water surface, passed through a sieve (pore size 125 um). Although formulated as wetable powder, most of the product remained on the water surface for some minutes, being visibly filtered by the anopheline larvae.

No mortality or cannibalism were recorded among the treated larvae up to one hour after B.t.i. application. At this time, all the 25 Cx. territans (L3 and L4) larvae and 16 randomly collected *An. punctipennis* (L4) larvae were removed from the pot and were separately transferred to two small dishes (57 cm^2 surface area) with 80 ml of the treated water. For *An. punctipennis* the relation amount of larvae/water surface/water volume in this dishes continued to be approximately the same as in the pot. These two groups were maintained as indicative of susceptibility to only B.t.i. treatment. The remained *An. punctipennis* larvae in that original pot received then 7.5 mg of *M. anisopliae* applied as sieved dry conidia on the water surface (equivalent to 0.44 Kg/ha or 0.0044 mg/cm^2). The second pot with 384 *An. punctipennis* L3 and L4 larvae received no treatment at this time and was observed as control for a twelve hours period. After this time, 7.5 mg of *M.*

anisopliae dry conidia was applied and this group was scored as only *M. anisopliae* treatment, twelve hours later.

Test for the fungus viability was done by counting germinated conidia in SDAY-combiotic media, incubated for 24 hr at 26 C (Daoust & Roberts, 1982). For two counts of 500 conidia each, average germination rate was 80.5%.

Mortality and cannibalism were recorded 12 hr after applications and discriminated among L3 and L4 larvae when subjected to both pathogens (FIG. 11). Larvae that pupated during that period was not considered for calculations.

TABLE 1. Percent mortality (MORT.) and cannibalism (CANN.) in *An. punctipennis* and *Cx. territans* larvae treated with B.t.i. or *M. anisopliae* (M.a.) or both, after 12 hours.

	Cx. territans	*An.punctipennis*				
TREAT.	B.t.i.	B.t.i.	M.a.	B.t.i.+M.a.		CONTROL
n;Ln*	25;L3/L4	16;L4	384;L3/L4	133;L3	156;L4	384;L3/L4
	%					
MORT.	0.0	37.5	0.0	72.9	62.5	0.0
CANN.	-	0.0	0.0	48.1	43.7	0.0

*number of treated larvae; instar.

The present results indicate an apparent high susceptibility of *An. punctipennis* to B.t.i., suffering 37.5% mortality in only 12 hr. The used concentration was equivalent to the lowest concentration recommended by the producer to mosquitoes in clear water. It was also equivalent to half of the LT_{50}-20 hr after treatment to *An. triannulatus* (Andrade, 1992). Contrarily, some mortality was expected among *Cx. territans* larvae considering that species in the genus *Culex* are usually more susceptible to B.t.i. than that in the genus *Anopheles*. The application of B.t.i. as powder on the water surface, certainly improved its intake by the anopheline larvae and consequently reduced the amount to which *Cx. territans* was exposed, since this is a bottom

feeding species. As noted by Lacey & Lacey (1990) the use of granular floating formulations of B.t.i. in rice lands permits an improved control of anopheline with less active ingredient per unit area. The non-occurrence of cannibalism among the B.t.i. control larvae, suggests that possible alimentary inhibition occurred.

The non-occurrence of mortality in the group which received only *M. anisopliae* treatment indicated that a low concentration was really used and possibly no larvae became intoxicated enough to be cannibalized. Actually, the concentration used (44 mg/m^2) corresponds to approximately 1/10 of the mid-point of the dose range recommended to Anopheles control (300 to 600 mg/m^2)(WHO, Data sheet, 1980).

The scored mortality in *An. punctipennis* larvae that received B.t.i. and a subsequent application of *M. anisopliae* was considerably higher than that in the group receiving only B.t.i. or only the fungus (non-lethal at this time). The present results indicate Potentiating Synergism (as proposed by Benz, 1971) at this point.

It should be noted that for both L3 and L4 larvae, the percent cannibalism contributed significantly to the high mortality observed. It was also noted that in many cases, the cannibalized larvae weren't totally eaten and remained in the bottom of the recipient. As the mortality in this experiment was considered as early as 12 hr after treatment, it can be assumed that it was due to the combined toxic action of the two pathogens and to the cannibalism upon intoxicated larvae. Mycelial growth was not expected to overcome among the dead larvae in this case since toxin release in the gut after ingestion of spores occurs with no apparent tissue invasion (Lacey et al., 1988).

Tenth experiment.

This experiment was aimed to assess the proportion of *An. punctipennis* larvae recycling *M. anisopliae* in both, *M. anisopliae* alone and B.t.i.+ *M. anisopliae* treatments. Also, to clarify whether cannibalism could play an important role in the expression of the recycling.

The tenth experiment consisted only in dividing the surviving larvae from the ninth experiment in 2 new treatments. In one treatment larvae were just individualized in small plastic dishes (8 cm^2 surface area) with 6 ml of water from the original treatment. In the other treatment, groups of 12 larvae each were just sorted and placed in plastic dishes bigger than that (57 cm^2 surface area) with 80 ml of the same previously treated water. The original relation larva/area for this grouped larvae became 0.21/cm^2, slightly inferior than 0.23/cm^2 and 0.24/cm^2 for the *M. anisopliae* and the B.t.i. + *M. anisopliae* treatments respectively. The dishes for grouped larvae were covered with plastic lids and the small recipients for individual larvae were placed in enamelled pans covered with acrylic plates.

Mortality, cannibalism (in the grouped larvae) and sporulated growth of *M. anisopliae* were scored until the adult stage, twenty days later (TABLE 2.). A pinch of the described mosquito food of about 4 mg or 40 mg were respectively spread on
the water surface for the individualized or grouped larvae daily.

TABLE 2. Percent final mortality (MORT.), cannibalism (CANN.) and

sporulated mycelial growth (RECY.) in grouped or individualized *An. punctipennis* larvae treated with B.t.i., *M. anisopliae* (M.a.) or both. Values between [ ] corrected by Abbott's formula.

INSTAR	L3			L4			
TREAT.	M.a.	B.t.i.+M.a.	CTR	B.t.i.	M.a.	B.t.i.+M.a.	CTR
Grouped larvae (n)	(96)	(12)	(24)	(16)	(96)	(48)	(24)
% MORT.	60.4 [40.6]	99.0 [98.5]	33.3	56.2 [4.4]	56.2 [4.4]	91.8 [82.1]	54.2
% CANN.	57.5 [43.3]	59.6 [46.1]	25.0	0.0 [-]	33.3 [5.8]	62.5 [47.0]	29.2
% RECY.	1.0	0.0	0.0	-	5.2	0.5	0.0
Individualizes larvae (n)	(96)	(24)	(28)	-	(96)	(48)	(28)
% MORT.	56.2 [35.5]	95.0 [92.6]	32.1	- -	88.5 [78.5]	93.3 [87.5]	46.4
% RECY.	2.1	0.8	0.0	-	17.7	1.9	0.0

The present data show that the combined action of B.t.i. and *M. anisopliae* resulted in Supplemental Synergism (Benz, 1971) for both L3 and L4 larvae regardless the grouped or individualized treatment. In any case more than 90% total mortality was scored. Although only permitting comparison among the three different treatments (B.t.i., M.a. and B.t.i.+M.a.) against grouped L4 larvae, the corrected mortality also confirms the synergism.

As expected when utilizing field collected larvae mortality in control groups showed to be high and no diagnostic could be achieved. The dead larvae were maintained in the dishes to assess the accidental occurrence of *M. anisopliae* and bacterial growth overcame by the end of the experiments.

Cannibalism over the grouped larvae used as control was high occurring at 25.0% and 29.2% for L3 and L4 larvae respectively. As final mortalities within "instar" category has scored close values when

comparing grouped versus individualized (cannibalism free) categories, it should be concluded that cannibalism occurred mainly over larvae that actually would die.

The premise that B.t.i. could inhibit cannibalism was partially confirmed, since no cannibalism occurred in this treatment. Contrarily, cannibalism was enhanced when the larvae were exposed to a subsequent application of *M. anisopliae*, scoring 46.1 % and 47 % for L3 and L4 grouped larvae respectively. When comparing such corrected final proportions with those scored 12 hours after treatment (cf. TABLE 1: 48.1 % and 43.7 % for L3 and L4 respectively) it becomes clear that cannibalism occurred mainly during the first 12 hours after application, as mentioned before, upon intoxicated larvae.

Cannibalism in the group receiving only *M. anisopliae* initiated 3 days after treatment and may have occurred otherwise mainly upon infected larvae. Canibalism was significantly greater among L3 larvae (43.3 %) than upon L4 larvae (5.8%). Consistently, the greatest occurrence of grouped larvae recycling the fungus (5.2 %) was among the L4 larvae treated with *M. anisopliae*, that showed the lowest cannibalism rate.

With one exception, the cannibalism-free individualized larvae showed essentially the same final mortalities as those mortalities scored for grouped larvae in both *M. anisopliae* and B.t.i.+*M. anisopliae* treatments. Again, confirming that the incidence of cannibalism may be primarily upon moribund diseased larvae rather than healthy ones. The exception was the L4 larvae treated only with M.a.. Subjected to this treatment, the late instar larvae showed 78.5 % corrected mortality followed by the highest recycling occurrence (17.7 %). Lower values of 4.4 % mortality and 5.2 % recycling were recorded among grouped larvae subjected to this treatment.

The survivors adults in these experiments were kept in the

original dishes aftyer death and no mycelial growth developed over its body.

The potential to *M. anisopliae* recycling upon *An. punctipennis* larvae was finally evaluated by counting the amount of spores produced in some infected L3 (n=2) and L4 larvae (n=12). Each whole larva was suspended in 1 ml of 0.5 % Tween 80 with 500 mg of small glass beads. The tubes were then shaken vigorously and two samples of spore suspension were evaluated in hemacytometer for each larva.

The mean values (S.D.) found for spores counting/larva were $1.3x10^6$ (1.0) and $2.2x10^6$ (0.01) for L3 and L4 respectively. The relation appraised by Daoust et al. (1982) indicate that 1 mg of *M. anisopliae* conidia contains about $3x10^7$ conidia. Considering that figure, each L3 or L4 larvae recycling *M. anisopliae* produced a mean value of 43.3 mg or 73.3 mg of conidia.

The following conclusions can be obtained from the present results against *An. punctipennis*.

1.- B.t.i. can operate synergically with *M. anisopliae* both in a short-term (improving toxaemia) or in a medium-term (improving infection).

2.- A low concentration (44 mg/m^2) of the *M. anisopliae* isolate alone can result in small to medium control related to the larval instar (L3 or L4).

3.-A relative small proportion (1 % to 5.2 %) of larvae subjected to that low concentration becomes infected recycling the fungus and some long-term control may be expected.

4.-Cannibalism occurs mainly upon moribund larvae and do interfere with the incidence of infected larvae recycling the fungus.

A comparative view of the present results with some records on Anopheles susceptibility to *M. anisopliae* is presented in Table 3.

TABLE 3.

Anopheles SPECIES / INSTAR/SOURCE	*M. anisopliae* STRAIN / VIAB.(%) (isolated from)	WATER TYPE	FOOD SUPPLY	CONCENT. mg/cm²	% MORTALITY AFTER (days) 1	2	3	4	5	6	8	Final	REFERENCE
An. stephensi													Roberts (1970)
L1-L2 / Lab.	np / np	deion.	Y	0.022	0		10	10	20			65	
				0.044	0		5	5	5			82	
				0.087	0		5	10	15			100	
				CTR.	0		0	0	0			97	
L2-L3 / Lab.	np / np	deion.	Y	0.022	0		5	15				65	
				0.044	0		5	25	30			77	
				0.087	0		35	55	80			100	
				CTR.				0	15			100	
L3 / Lab	E9CS1 / 65% (Homoptera. Enhanced in *Cx. pipiens*)	deion.	Y	0.033					31			np	Daoust *et al.* (1982)
				0.067					72			np	
				CTR. (used for correction by Abbott's formula)									
L3 / Lab	Various/83 to 99% (Non-mosquito hosts)	deion.	Y	0.062					4-100			np	Daoust & Roberts (1982)
				CTR.					=< 6				
L1 / np	IP-F3-142-78/ np (sick dead *Cx. fatigans* larvae surface sterilized)	dist.	np	0.033	100		-	-	-	-		100*	Balaranan *et al.*(1979)
L2 / np				0.033	18		51		10	10		89*	
L3 / np				0.033	18		50		3	0		71*	
L4 / np				0.033	22		50		1	0		73*	
				CTR. (used for correction by Abbott's formula)									
An. gambiae													Roberts (1975)
np / Field	F84-1-1/>90% (Coleoptera: Elateridae)	site	Y	0.02	20-76		93-100	100	-	-	-	100	
				0.042	53-76		96-100	100	-	-	-	100	(data for two assay)
				CTR.	7-16		7-40	20-40				20-40	
An. albimanus													Ramoska *et al.* (1981)
L2 / Lab.	E9 / np (Homoptera: Cercopidae)	demin. +dechl.	Y	0.05				97				np	
				0.15				100	-	-	-	100	
				0.5	63		100	-	-	-	-	100	
				CTR.	(np)								
An. walkeri	**PRESENT RESULTS**												RECYCLING
L2 / Field	ARSEF..../>80% (esporulated growth in *Ae. triseriatusi*)	site	N	0.031	40		100	-	-	-	-	100	
L3-L4/Field			Y	0.062	20		72	96	96	96	96	96	4.0 %
L4 / Field			Y	0.017	0		15	20	30	30	30	30	
			N	0.017	15		70	90	100	-	-	100	
			Y	CTR.			0	0	0	4	5	8	
An. punctipennis													
L3 / Field (grouped)		Site	Y	0.0044	0	4	31	23	29	33	37	40	1.0 %
L4 / Field (grouped)			Y	0.0044	2	5	9	0	0	0	4	3	5.2 %
L3 / Field (individualized)			Y	0.0044	0	9	6	0	8	0	0	18	2.1 %
L4 / Field (individualized)			Y	0.0044	1	3	0	0	25	25	42	78	17.7 %
				CTR. (used for correction by Abbott's formula)									

np= not provided. * Cited as "Total mortality", probably not corrected.

Although not completly comparable due to differences in methodology some few contrasts can be noted from the results presented in TABLE 3:

1. Almost the same mortality was obtained by Roberts (1970) in L2-L3 *An. stephensi* larvae 4 and 5 days after treatment (25% and 30%) when comparing to the present results against L3 *An. punctipennis* grouped larvae (23% and 29%) subjected to one tenth of *M. anisopliae* inoculum, viz 0.044 mg/cm^2 (Roberts, 1970) and 0.0044 mg/cm^2 (present results).

2. When comparing to Daoust et al. (1982) results (0.033 mg/cm^2 5 days after treatment) close mortality percent against L3 larvae was also obtained in the present work with a relative inoculum of 1/7.5 (31% and 29% respectively).

3. Higher mortalities were obtained to lower concentrations when comparing the present results with those obtained by Balaranan et al. (1979) against *An. stephensi*.

Additional comparison upon toxicity could be merely speculative and since the present results differs mainly as regards the recycling competence, the ARSEF .... strain may be considered in further studies as a good candidate for mosquitoes control.

10. CANNIBALISM AND EPIZOOTIOLOGY

Cannibalism is a well recorded relationship involving many genera of container-breeding predator mosquitoes such as Toxorhinchytes, Megarhinus, Lutzia, Armigeres, Eretmapodites, and Psorophora (Bay, 1974). Besides the fact that preying a conspecific results in food intake, the ecological reasons why such behavior evolved is explainable by the consideration of selective pressures towards

intraespecific competition reduction. To corroborate this statement, studies devoted to evaluate the potential of *Toxorhinchytes* spp in the biological control of vectors have shown that cannibalism may occur even upon eggs (Kazana et al., 1983; Linley, 1988; Linley & Duzak, 1989). Also, larvae in any instar may kill, but not necessarily eat, a conspecific (Rubio & Ayesta, 1984; Annis & Rusmiarto, 1988), resulting in small or no nutritional gain. A possible defensive behavior has also been argued for *Tx. brevipalpis* which kills and eventually eats conspecifics during the few days immediately before pupation (Corbet, 1985). The availability of food (preys) or it's renewal in small containers may be critical for a mosquito predator and beyond cannibalism, *Toxorhynchites* late instar larvae are able to survive without feeding for up to six months, or sometimes longer (Corbet & Griffiths, 1963; Trpis, 1970).

Cannibalism has also been recorded among larvae of nonpredacious mosquitoes that breed in small containers, such as *Ae. aegypti* (Mc Gregor, 1915; Misch et al., 1992) and *Ae. triseriatusi* (Livdahl & Koenekoop, 1985). But not only cannibalism seems to regulate competition. In this latter species egg hatching is regulated by larval density, with a significant decrease in hatching rate occurring as larval density increases (Livdahl & Edgerly, 1987). In *Ae. albopictus*, another container-breeding species, developmental rate and size at pupation have been showed to be density-dependent, and diminution of adult size has important reproductive consequences (Mori, 1979). Indeed, studies in Japan have indicated the probable presence of density-dependent effects also on larval mortality (Mori & Wada, 1978; Toma et al., 1982).

CANNIBALISM IN *ANOPHELES*.

Considering the limited environment of the trapped water as in tires, tree holes, bamboo stumps and stone basins may represent, inter- and intraspecific competition really can not be misconsidered. Unfortunately few ecological studies has been done on predation and cannibalism in filter-feeding mosquitoes. In a demonstrative study, Petersen et al. (1969) make reference to at least three early reports only mentioning the apparent predacious nature of larvae of *An. barberi* Coquillett, a tree-hole mosquito. In their experimental design, Petersen et al.(1969) placed 10 first instar larvae of Cx. quinquefasciatus (as prey) along with only one late instar *An. barberi* larvae in each repetition. Due to this design cannibalism was not possible to be observed but may be likely to occur in *An. barberi.*

Cannibalism has being early recorded also among Anopheles species that breed in relatively open habitats such as ponds and lakes (Roy, 1931). More recently, experimental evidence indicates for at least three *Anopheles* species a density- or food-dependent nature of cannibalism, viz *An. stephensi* Liston (Reisen, 1975; Reisen & Emory, 1976), *An. pharoensis* Theo. (Shoukry, 1980) and *An. messeae* Fall. (Gordeev & Troshkov, 1991). In this late species cannibalism is genetically determined and is argued to be responsible of differentiation in overpopulated biotopes as a result of intraspecific competition. According to Gordeev & Troshkov (1991) adaptation occurred in two directions during K-selection in *An. messeae*. The original population in the southwestern part of the range (mosquitoes with XL0 inversion dominant) evolved adaptations (not discussed by the authors) to more effective consumption of resources by young instar larvae. With the further advance of the

species northeastward in new water bodies, inversion XL1 dominant determining cannibalism among old instar larvae becomes common. In their experiment cannibalism was also observed upon already immobilized larvae and permits to suppose that as in the present study, occurred upon moribund larvae as well.

It is my personal hypothesis that cannibalism and even the observed scraping behavior that normally precede cannibalism may have evolved as an adaptive trait independent of competitive interactions. A mutual scraping could permit removal and ingestion of ectoparasites. No references were found regarding this subject but some inferences are possible. In the present study many moribund or dead larvae were observed with the body surface infested by colonies of *Vorticella* and related ciliates. Accordingly, Jupp & Smith (1986) reported that high mortality may occur due to *Vorticella* infestation in mosquitoes cultures. Also, self removing and ingesting larval mites attached to body surface by *An. cruscians* larvae is pointed as adaptive: mites not dislodged or eaten can parasitize the emerging adult mosquitoes (Lanciani, 1988). Although not mentioned by this author it remains possible that one larva could remove mites from another.

Infection through the gut wall occurs commonly with many host-coevolved viruses. Vertical and horizontal transmission of the Iridovirus (IV-1) of *Tipula paludosa* occur simply by larval cannibalism alone. When the mosquito iridescent virus Chloriridovirus (IV-3) is acquired by late instar *Ae. taeniorhynchus* larvae through cannibalism of infected cadavers there may be occult passage through the adult and the disease will be expressed in the following generation (Evans & Entwistle, 1987). In infections caused by some fungi the same is not true.

Cannibalism upon moribund individuals or cadavers that not yet produced spores or other infective stage may otherwise reduce secondary inoculum. In the present study the higher occurrence of larvae recycling *M. anisopliae* was in the treatment were lowest cannibalism occurred. Individualized larvae receiving the same treatment showed indeed higher rate of individuals recycling the fungus.

11. CYCLING RECORDS OF *M. anisopliae* IN MOSQUITOES AND CONCLUSION

In general, *M. anisopliae* has not been observed naturally growing and sporulating over mosquito larvae, although germination in siphon or gut and some growth in the hemocoel and tracheal trunks can occur. Due to this fact, the isolation of the fungus from mosquito larvae has been achieved only by culturing either excised siphons (from laboratory treated larvae - Daoust & Roberts, 1982) or surface sterilized larvae (from field collected larvae -Balaraman et al., 1979). Even so, recycling seems to be by some way possible under field conditions. Roberts & Panter (1985) reported a personal communication from P. K. Rajagopalan giving account of *M. anisopliae* naturally recycling in mosquito habitats in India along a three-week period. Also, Ramoska (1982) refers to his personal unpublished data about *M. anisopliae* suppressing mosquito populations in artificial container breeding sites for nearly one month. No details about the recycling mechanism were mentioned, however, by these authors.

Ramoska et al. (1981) showed two pictures to compare *Cx. quinquefasciatus* siphon openings after death by two different methods; submerged or floating *M. anisopliae* spores. In the pictures, typical

mycelial growth can be noted emerging from the siphon opening in larva killed by the latter method. No details were offered about sporulation, or whether the larvae died floating or sunk after death being then placed under aerial conditions to permit mycelial growth.

Wilson et al. (1990) reported that between 45.3% and 100% of adult *Ae. aegypti*, subjected to *M. anisopliae* treatment as larvae became infected and produced mycelia under bioassay conditions, regardless of the quantity of spores added to the medium. Following adult ecdysis, all but 0.5 ml of the treated water was drained from the holding vials. The mosquitoes were retained without food until death and then placed on moist paper. They concluded that with the resulting adults becoming infected, dissemination to new sites and perpetuation in treated sites are thereby promoted. Although maintaining dead larvae in the treatment beakers, no mycelial growth was recorded for this developmental stage.

As indicated early in this report, the present study was been hosted by Dr. D. W. Roberts, who confess this being the first case he has ever observed of a *M. anisopliae* strain with such ability to grow and sporulate in mosquito larvae.

Although not recorded as naturally sporulating in mosquito larvae, *M. anisopliae* was recently found naturally infecting adult Ae. crinifer (Theobald) females in Argentina (Lopez-Lastra, 1989). The absolute scarcity of such records leads to the conclusion that *M. anisopliae* infecting mosquitoes possibly represents a feebly coevolved (if so) or relatively new pathogen-host relationship. Humber (1984) discussed associations patterns of members in Entomophthorales with their respective insect hosts, suggesting that pathogenic species in that taxa might have evolved from a rapid killing of the hosts to slower parasitism. Regarding

to *M. anisopliae* having mosquito larvae as host some analogy is possible. In early contacts, highly toxic strains could rapidly kill the larvae by ingestion of its spores but the maintenance of the disease in such population would ever depend upon alternative hosts developing the mycosis. By the other side strains with lower toxicity but high infectivity and competence to recycle could well be expected as resulting from a coevolved process.

M. anisopliae is judged a non-colonizing agent in mosquitoes and has being considered as a mycoinsecticide due to the short-term nature of its effect upon mosquito populations (Roberts & Panter, 1985). In such terms most of the known *M. anisopliae* strains could be compared to B.t.i., and the search for mutants or strains with increased virulence accordingly has been accomplished (Al-Aidroos & Roberts, 1978; Daoust & Roberts, 1982). Lopez-Lastra (1989) findings that *M. anisopliae* can naturally occurs upon adult mosquitoes, as well as the present results on the occurrence in mosquito larvae, recycling and again causing death, may permit otherwise new considerations under the epizootiological point of view. In confirming under field conditions the ability of *M. anisopliae* strains to recycle upon mosquitoes it may be better compared to *B. sphaericus*, meaning new weapons in mosquito's management programs.

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