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MASS SEPARATION OF INFECTIVE STAGE FILARIA LARVAE FROM SUSPECTED MOSQUITO VECTORS PRELIMINARY RESULTS OF TESTS CONDUCTED IN COASTAL TANZANIA[1]

by

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CONTENTS

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1. INTRODUCTION

Wild-caught mosquitos are routinely examined for infective larvae of Wuchereria bancrofti, Brugia malayi and other filarial infections to determine the degree of transmission potential in suspected foci of the diseases. A knowledge of the infectivity rates in available vectors is essential toward understanding the bionomics of transmission and is a necessary pre-requisite to evaluate the success or failure of long-term control programmes. In most cases, this information is obtained by individually dissecting mosquitos in saline and recording the percentage developing filaria larvae. Dissections performed in this manner are normally undertaken at or near the point of collection since mosquitos must be examined fresh. If specimens must be transported any distance to the laboratory, it is common practice to keep the collection at low temperature until dissections can be accomplished.

The results obtained by individual dissections identify those mosquito species participating in transmission, the percentage of infective mosquitos harbouring developing filaria larvae and the actual number of larvae in each infective mosquito. The relative infectivity rates found in different stages of the gonotrophic cycle can be determined for each mosquito species if the collection is divided into unfed, engorged and gravid samples at the time of dissection.

Although data obtained in this manner give valuable information on transmission potential, the mechanics involved in processing each mosquito individually limits the total number of specimens which can be dissected at one time. As a result, bulk data must be gathered over an extended period during which changing ecological conditions and fluctuations in infection rates undoubtedly occur.

It would appear that examination of large numbers of suspected vectors at closely spaced intervals might better serve to clarify the role played by each of the mosquitos involved in transmission and thereby reveal the presence or absence of cyclic transmission due to fluctuations in temperature, rainfall or species composition. Nelson (1958) describes a method for the preservation and staining of mosquitos for the detection of filaria larvae. Although mosquitos are still dissected individually by this method, the technique allows more latitude in sample size since dissections can be accomplished at a later date when time is available.

Using a modified Baermann technique (Baermann, 1917; Cort et al., 1923), a new approach of examining large numbers of mosquitos is being tested at five locations in coastal Tanzania, East Africa. The preliminary results from three of these areas are presented in this paper. Mosquitos are pooled in a screening process similar to the methods employed in virus testing. Results, therefore, yield the total number of infective larvae in the sample rather than the percentage of infected mosquitos examined. It is the thesis of this research that total infective larvae available for transmission can be as valuable an index for epidemiological assessment as individual infection rates.

2. MATERIALS AND METHODS

2.1 Areas under investigation

Three areas in coastal Tanzania have been selected for analysis of the data obtained by mass separation. The areas of Tanga, Muheza and Pandeni were chosen primarily on the basis of contrast, ranging from urban to strictly rural in composition. Tanga, a port on the Indian Ocean, is the second largest city in Tanzania. Mosquito collections were made in the Ngamiani section of the city popularly referred to as Tanga Town. Muheza, located 35 km from the coast, offers an intermediate between urban and rural environments. Mosquito collections were made at several of the small villages immediately surrounding the town proper. Pandeni is a small rural village located 45 km from the coast in the foothills of

the Eastern Usambara Mountains. There is no town of appreciable size in the immediate vicinity.

Microfilaria surveys conducted six months prior to these studies give an indication of the comparative prevalence rates in each of the areas under observation (Crans, unpublished data). Men and women have been combined in the results; children below the age of 15 years have been omitted. In Tanga-Ngamiani, a sample of 231 persons was found to have a microfilaria rate of 31.6% with microfilaria densities of 54.2 per positive case and 17.1 per total. In the Muheza area, 150 adults were examined and a microfilaria rate of 42.0% was found with microfilaria densities of 68.0 per positive case and 28.6 per total. In Pandeni, 77 individuals were found to have a microfilaria rate of 37.6% with microfilaria densities of 86.0 per positive case and 32.3 per total. All samples were taken between the hours of 8.00 p.m. and 10.00 p.m.; microfilaria densities were based on a 50 mm^3 volume of blood. Although isolated cases of Dipetalonema perstans were found, W. bancrofti was the only human filarial infection of consequence detected in these surveys. Full details of the methods and results will be published at a later date.

2.2 Mosquito collection, pooling and processing

All mosquitos processed in these investigations were collected from houses by standard spray-catch methods. Each area was visited once per week during the months of January, February and March, 1971. Collections were placed on a moist filter paper pad in a covered petri dish and transported to a nearby facility for species identification and pooling. Each species was then further subdivided according to the stage of the gonotrophic cycle, i.e.: unfed, engorged or gravid and grouped in pools of 25 each. The entire pool of 25 mosquitos was lightly crushed between two microscope slides and flushed into 25 cm^3 of normal saline. The saline mixture containing the crushed mosquitos was placed in the separation chamber described below where extraction of infective larvae took place by gravitation over a two-hour period.

2.3 Description of mass separation technique

The system uses a modification of the Baermann method of extraction first described by Baermann (1917) for isolating hookworm larvae from soil and redescribed by Cort et al. (1923). Living infective larvae emerge from the ruptured mosquito exoskeleton and into solution when the system is used for filarial extraction. The moving larvae slowly settle to the bottom of the solution passing through a relatively coarse screen which separates them from the floating mosquito parts. Removal of the screen after extraction omits the coarse mosquito parts from the sample leaving the infective larvae together with mosquito eggs and small debris particles which have settled in the sample.

The separation chamber used in this study was a plastic petri dish measuring 50 mm in diameter and 28 mm in depth. The screen divider was a cylinder cut from a disposable plastic cup which was covered at one end with 30 mesh gauze. When the basket-like cylinder was fitted in the petri dish, the chamber effectively divided into an upper and lower section. (Figure 1.)

2.4 Preservation and examination of samples

If the extract in the petri dish was examined immediately under stereoscopic magnification, living infective larvae could be detected in the sample but it was found that preservation and later examination was more convenient. The extract was therefore transferred to a 25 cm^3 screw cap vial and allowed to resettle. Approximately 18 cm^3 of excess saline was then drawn from the top of the extract and replaced with 95% ethyl alcohol. This method was found to preserve the infective larvae without distortion and allow for later mounting and species identification.

For examination, excess alcohol was removed from the top of the vial and the sample was transferred to the lid of a plastic petri dish, lightly scored to facilitate scanning. Infective larvae were readily discernable among the debris particles when the sample was scanned at 20x under a stereoscopic microscope with intense light reflected from below. By this method, each pool of 25 mosquitos could be examined for infective larvae in from two to three minutes' time. All infective larvae were mounted by hanging drop according to the method described by Heisch et al. (1959) and identified to species by the characters set forth by Nelson (1959).

2.5 Presentation of results

The mass separation technique described herein reveals the total number of infective stage larvae recovered from a given sample of the suspected vector. Since large numbers of each vector species can be processed simultaneously, the data can be compared with relative accuracy. Data obtained by this method have been expressed in the following manner. The number of mosquitos in the sample has been divided by the number of infective larvae recovered. The figure obtained has been expressed as a ratio representing the number of mosquitos processed for each infective larva recovered. This figure has been interpreted as an indication of the transmission potential in the population during the time of the collection.

A ratio of 1:50, for example, indicates that one infective larva was recovered for every 50 mosquitos in the sample. Because this figure was derived from hundreds of mosquitos, it can be assumed that one infective larva was present for every 50 mosquitos in the population at the time the collection was made. A total of 200 mosquito bites during this interval would theoretically yield the potential transmission of four infective larvae. These larvae could come from four different mosquitos or all could be transmitted by one bite; the data are incapable of distinguishing which might be the case. These data can be expressed for each individual mosquito species under investigation for comparative infectivity rates or can be combined to obtain an overall index for the area. Biting rates for each of the species at this time would reveal the extent of transmission.

Data presented in tabular form have been accumulated monthly and have been expressed in the method explained above. For the purpose of plotting this information at weekly intervals, the data have been converted to the number of infective larvae recovered from each 100 mosquitos.

Information was also obtained on the numbers of infective larvae present in mosquitos of differing stages of gonotrophic development with particular reference to the comparative numbers of larvae in engorged and gravid mosquitos. Since engorged mosquitos are examined immediately after obtaining a blood-meal, they would be expected to yield the fewest numbers of infective stage larvae, having already transmitted a proportion of the larvae which were fully developed. The comparative numbers of infective larvae found in engorged and gravid mosquitos may be indicative of the transmission efficiency in nature. For this purpose an E/G factor (engorged/gravid) has been calculated for each mosquito species. The figure indicates the factor by which results from engorged mosquitos must be multiplied to equate with gravid specimens. An E/G factor of 2.0 implies that twice as many infective larvae were found in gravid mosquitos as in engorged specimens. The contrast in E/G factors for the three mosquito vectors has been presented.

3. RESULTS AND DISCUSSION

During the first three months of 1971, more than 20 000 mosquitos were processed by the technique of mass separation and approximately 260 infective stage larvae were recovered and tentatively identified. The small number of Setaria spp. and Dirofilaria spp. that were found have been removed from the data, therefore all results are believed to apply to W. bancrofti. A total of 12 871 mosquitos were collected in the three areas included in this paper and are the basis of all results.

3.1 Infectivity rates

Tanga-Ngamiani

Culex p. fatigans was the only vector of human filariasis collected in numbers from houses in the Ngamiani section of Tanga. Although small numbers of Anopheles gambiae were present, no reliable information on the species could be ascertained. Table I presents results from 4081 C.p. fatigans examined in the first three months of 1971. During the three-month interval, 46 infective larvae of W. bancrofti were recovered or one larva for every 89 C.p. fatigans that were processed. The data in Table I indicate that the transmission potential was extremely low in January, increased substantially in February and decreased again during March. Figure 2 plots the same data over weekly intervals for this period. Throughout January and the first week of February, transmission potential appeared to be negligible with a maximum of 0.4 infective larvae for each 100 mosquitos in the sample. Infectivity rates apparently reached a peak during the remainder of February, increasing in the third week by more than 10-fold, and slowly diminished in intensity through March.

Muheza Area

Two known vectors of human filariasis, Anopheles gambiae and Anopheles funestus, were collected in Muheza area. Although Culex p. fatigans is also known to occur, specimens were not taken in sufficient numbers for testing during the collection period. Table II presents the results from 3888 mosquitos collected in January, February and March, 1971. Throughout this interval, 89 infective larvae of W. bancrofti were recovered or one larva for every 44 mosquitos that were processed. Infectivity rates were highest in January, decreased substantially during February and were minimal in the month of March. Figure 3 plots the same data at weekly intervals with both species combined.

It is evident from Table II that the two vector species in Muheza area demonstrated a distinct contrast in transmission potential throughout the three-month interval. In January, when A. gambiae populations were highest, one infective larva was recovered for every 15 A. gambiae that were processed. The ratio remained nearly constant in February although numbers of the species dropped considerably. In March, the A. gambiae population was too low to render the data reliable. Considerably fewer infective larvae were recovered from A. funestus that were examined simultaneously. In January, one infective larva was found for every 61 mosquitos that were processed. This figure decreased to 1:95 in February, when the population apparently increased, and reached a low of 1:315 in March. Figure 4 plots these data for each species at weekly intervals throughout the period. In all cases, A. gambiae appeared to be carrying a proportionally greater number of infective larvae and was therefore the species of greatest risk but biting counts at the time would have undoubtedly shown that the greater numbers of A. funestus in February and March provided nearly equal transmission from both species.

Pandeni

A totally different pattern was operative at Pandeni where all three vector species were collected. Table III presents the results from 4862 mosquitos processed in the first three months of 1971. A total of 90 W. bancrofti infective larvae were recovered or one larva for every 54 mosquitos that were processed. Infectivity rates appeared to be relatively constant throughout the period with only a slight rise from January through March. Figure 5, plotted at weekly intervals, shows that transmission potential did not fluctuate appreciably through January or February but dropped sharply in the first week of March, then rose rapidly for the remainder of the month.

The vector potential of the two Anopheline species did not show the marked contrast that was evident in the Muheza area. Both A. gambiae and A. funestus seemed to possess equal transmission potential throughout the three-month period. C.p. fatigans was not

numerous in this area but did show some evidence of infectivity during the brief periods when sufficient numbers were collected.

Figure 6 plots the infection rates in each of these three vector species at weekly intervals. Unlike the data obtained at Muheza, it is clear that distinct differences in transmission potential were not operative in this area. A. gambiae and A. funestus showed parallel infectivity rates and it is interesting to note that at no time during the three-month period was there total absence of infective larvae available for transmission. When C.p. fatigans populations appeared and when infection levels increased in March all three vector species could have been simultaneously transmitting W. bancrofti in this village. Biting counts at this time would have revealed considerable information on the actual transmission of bancroftian filariasis.

3.2 Examination of engorged and gravid mosquitos

Throughout these investigations, all unfed, engorged and gravid mosquitos were tested separately in an attempt to ascertain whether or not the stage of gonotrophic development appreciably affected results. It was assumed that gravid mosquitos would carry a greater number of infective larvae in as much as engorged mosquitos had recently fed and would have transmitted a proportion of the fully developed larvae that they were carrying. Dissections performed with engorged specimens would give lower results than dissections performed on the same mosquitos prior to taking the blood-meal. Since the majority of mosquitos collected by spray-catch are fully engorged, a correction factor might be necessary to equate results.

The findings of these investigations were surprising since considerable variation was found for species taken in different study areas. Table IV presents the results from the three areas under investigation. Unfed mosquitos were taken in very small numbers and have been omitted from these data.

In Tanga, where C.p. fatigans was the only vector of consequence, infective larvae were found at a ratio of one to every 97 engorged C.p. fatigans and one to every 65 gravid specimens. Thus 1.5 times as many larvae were recovered from proportional numbers of gravid mosquitos. This figure has been termed the E/G factor (engorged/gravid) and is a convenient reference to express the degree of difference found for each species. Likewise, if the 24 infective larvae recovered from the engorged specimens at Tanga were multiplied by the factor 1.5, the calculated ratio in the engorged group would equate identically with that found in the gravid specimens.

In Muheza area, the ratios obtained from gravid and engorged A. gambiae were nearly identical with a calculated E/G factor of 1.2. A. funestus showed a wider range with an E/G factor of 2.3. In the case of A. gambiae, dissections performed on either engorged or gravid specimens would have given similar results; with A. funestus, however, the greatest proportion of infective larvae would have been missed if only engorged mosquitos were dissected.

At Pandeni, a totally different pattern was found in that 4.2 times as many infective larvae were found in the gravid samples of both A. gambiae and A. funestus. In this area, dissections performed only on engorged specimens would have missed more than two-thirds of the larvae available for transmission.

The significance of these variations and their application to future dissections with these species is debatable considering that these data were only accumulated over a three-month period. The variation in the numbers of infective larvae found in gravid and engorged mosquitos could well be an indication of the degree to which infective larvae are actually being voided from the mosquitos when they feed. If such were the case, a high E/G factor would indicate a high degree of transmission. It is also possible that these

data merely reflect an upward or downward trend in the degree of transmission potential in the area over the interval that the collections were made.

Until greater numbers of engorged and gravid mosquitos are tested over a longer time period, it is not possible to draw firm conclusions on the significance of gonotrophic development and its impact on dissection results. It would also be unwise to devise a correction factor for each species at this time. The state of gonotrophic development might have a pronounced effect on dissection results but its extent and reasons must as yet be speculative.

4. CONCLUSIONS

The technique of mass separation of infective larvae from suspected mosquito vectors appears to show promise for future investigations of human filariasis and its vectors. In an area where the vectors of the disease are unknown, large numbers of many mosquito species could be processed in a short period of time. The presence of infective larvae in any of the samples would incriminate certain mosquito species and detailed studies could be undertaken without an extended pre-investigation period.

The system may also have value in the evaluation of long-term filariasis control programmes. If mosquitos were screened prior to the application of control measures and then re-examined at intervals once the project was terminated, the mean density of infective stage larvae in the mosquito population could be used as an index of suppression.

The data obtained from the three areas in coastal Tanzania tend to emphasize several important points. Transmission of W. bancrofti is apparently governed by local factors. The three areas of investigation were located within a 20 km radius yet simultaneously each showed distinctly different patterns of transmission potential. It would appear most unwise to take results obtained from a single study area and interpret them as being applicable to a broader geographic situation.

The practice of making a quick survey in an area might not lead to accurate results. Spot checks at each area would not have been indicative of the infectivity levels in the vector populations. Dissections performed in Tanga-Ngamiani in January or March would have detected little or no transmission potential for C.p. fatigans. Dissections performed in the same area in February would have suggested the opposite. Likewise, studies performed in either Muheza or Pandeni at any one point in time would have indicated little since both areas were apparently experiencing a gradual increase or decrease in infectivity levels. A limited number of dissections performed at intermittent intervals may also have failed to reveal accurate information since data would have been pooled over the period and a single figure for infectivity would have been obtained.

The data presented in this paper on each of the areas under investigation cannot be construed as meaningful for anything but the time interval that collections were made. These preliminary results have been presented primarily to demonstrate the application of the technique. All of the data were obtained during the dry season and it appears obvious that cyclic phenomena occur to a greater or lesser extent at each site. Until similar investigations are continued for a full 12 month period, very little can be ascertained on the overall importance of each of the mosquito vectors involved in transmission. Data must be compiled for a span of one year and correlated with biting densities at each site before meaningful information on the transmission of bancroftian filariasis to man can be established.

SUMMARY

1. A technique for the separation of infective stage larvae from large numbers of suspected mosquito vectors is described. Results yield the total number of infective larvae in a given sample of mosquitos; individual infection rates cannot be determined by the method.

2. The method was field tested for three months in three areas in coastal Tanzania, East Africa, where bancroftian filariasis was known to exist. The sites included an urban area where Culex p. fatigans was the only vector of consequence, an area surrounding a town where Anopheles gambiae and Anopheles funestus were found and a small village where all three vector species were collected.

3. A total of 12 871 mosquitos were collected and processed over the three-month interval and 226 Wuchereria bancrofti infective larvae were recovered and identified.

4. Differing degrees of transmission potential were found at each site although areas were located within close proximity of one another. Results suggested that transmission was governed largely by local factors.

5. Mosquitos tested in different stages of gonotrophic development showed differences in the numbers of infective larvae that were carried but results varied widely from one area to another.

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TABLE I. THE RESULTS OF MASS SEPARATION OF INFECTIVE STAGE WUCHERERIA BANCROFTI FROM MOSQUITO VECTORS AT TANGA-NGAMIANI DURING JANUARY, FEBRUARY AND MARCH, 1971

Month	Anopheles gambiae			Anopheles funestus			Culex p. fatigans			All species		
	No. mosquitos examined	No. infective larvae	Ratio[1]	No. mosquitos examined	No. infective larvae	Ratio	No. mosquitos examined	No. infective larvae	Ratio	No. mosquitos examined	No. infective larvae	Ratio
January	28	1	1:28	0	-	-	819	2	1:409	847	3	1:282
February	4	0	-	0	-	-	1 481	32	1:46	1 485	32	1:46
March	8	0	-	0	-	-	1 781	12	1:149	1 789	12	1:149
Total	40	1	1:40	0	-	-	4 081	46	1:89	4 121	47	1:88

[1] The number of mosquitos processed for each infective larva that was recovered.

TABLE II. THE RESULTS OF MASS SEPARATION OF INFECTIVE STAGE WUCHERERIA BANCROFTI FROM MOSQUITO VECTORS IN MUHEZA AREA DURING JANUARY, FEBRUARY AND MARCH, 1971

Month	Anopheles gambiae			Anopheles funestus			Culex p. fatigans			All species		
	No. mosquitos examined	No. infective larvae	Ratio[1]	No. mosquitos examined	No. infective larvae	Ratio	No. mosquitos examined	No. infective larvae	Ratio	No. mosquitos examined	No. infective larvae	Ratio
January	803	52	1:15	666	11	1:61	0	-	-	1 469	63	1:23
February	154	8	1:19	1 237	13	1:95	0	-	-	1 391	21	1:66
March	83	2	1:42	945	3	1:315	0	-	-	1 028	5	1:206
Total	1 040	62	1:17	2 848	27	1:105	0	-	-	3 888	89	1:44

[1] The number of mosquitos processed for each infective larva that was recovered.

TABLE III. THE RESULTS OF MASS SEPARATION OF INFECTIVE STAGE WUCHERERIA BANCROFTI FROM MOSQUITO VECTORS AT PANDENI DURING JANUARY, FEBRUARY AND MARCH, 1971

Month	Anopheles gambiae			Anopheles funestus			Culex p. fatigans			All species		
	No. mosquitos examined	No. infective larvae	Ratio[1]	No. mosquitos examined	No. infective larvae	Ratio	No. mosquitos examined	No. infective larvae	Ratio	No. mosquitos examined	No. infective larvae	Ratio
January	658	9	1:73	1 365	21	1:65	6	0	-	2 029	30	1:68
February	429	7	1:61	596	14	1:43	211	1	1:211	1 236	22	1:56
March	657	15	1:44	619	22	1:28	321	1	1:321	1 597	38	1:42
Total	1 744	31	1:56	2 580	57	1:45	538	2	1:269	4 862	90	1:54

[1] The number of mosquitos processed for each infective larva that was recovered.

TABLE IV. A COMPARISON OF THE NUMBERS OF WUCHERERIA BANCROFTI INFECTIVE LARVAE RECOVERED FROM ENGORGED AND GRAVID MOSQUITOS

	Tanga						Muheza area						Pandeni					
	Anopheles gambiae		Anopheles funestus		Culex p. fatigans		Anopheles gambiae		Anopheles funestus		Culex p. fatigans		Anopheles gambiae		Anopheles funestus		Culex p. fatigans	
	E	G	E	G	E	G	E	G	E	G	E	G	E	G	E	G	E	G
No. mosquitos tested	-	-	-	-	2387	1438	669	364	1664	1089	-	-	1255	483	1467	1026	292	244
No. infective larvae	-	-	-	-	24	22	37	24	10	15	-	-	12	19	14	41	0	2
Ratio[1]	-	-	-	-	1:97	1:65	1:18	1:15	1:166	1:73	-	-	1:105	1:25	1:105	1:25	0:292	1:122
E/G factor[2]	-		-		1.5		1.2		2.3		-		4.2		4.2		2.4+	

[1] The number of mosquitos processed for each infective larva that was recovered.

[2] Engorged/Gravid ratio obtained by the following formula:

$$\frac{\frac{\text{No. Engorged mosquitos processed}}{\text{No. Infective larvae recovered}}}{\frac{\text{No. Gravid mosquitos processed}}{\text{No. Infective larvae recovered}}}$$

Fig. I. SEPARATION CHAMBER USED TO EXTRACT INFECTIVE FILARIAL LARVAE FROM MOSQUITOES

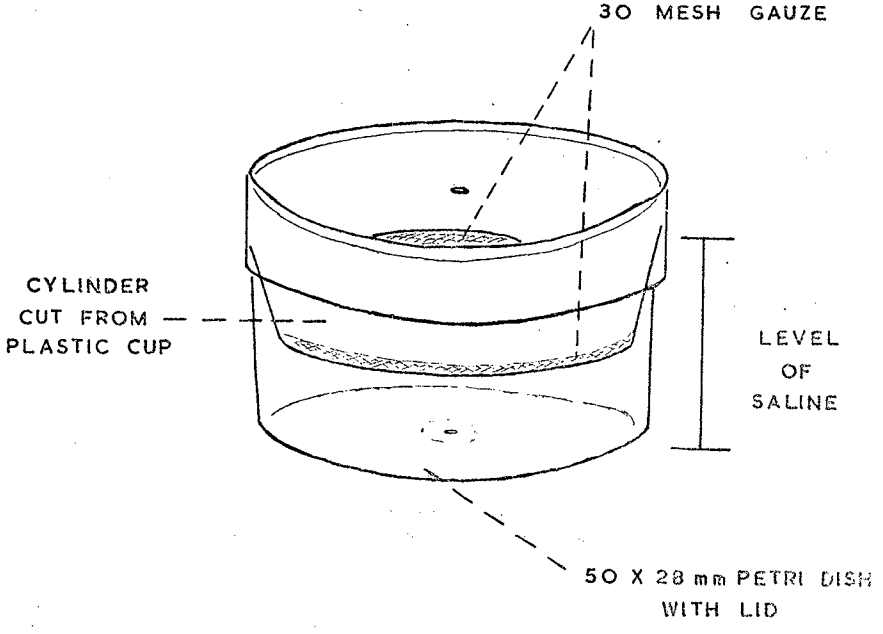

Fig. 2. THE NUMBER OF INFECTIVE LARVAE OF WUCHERERIA BANCROFTI RECOVERED FROM EACH 100 MOSQUITOES PROCESSED AT TANGA - NGAMIANI DURING JANUARY, FEBRUARY AND MARCH, 1971

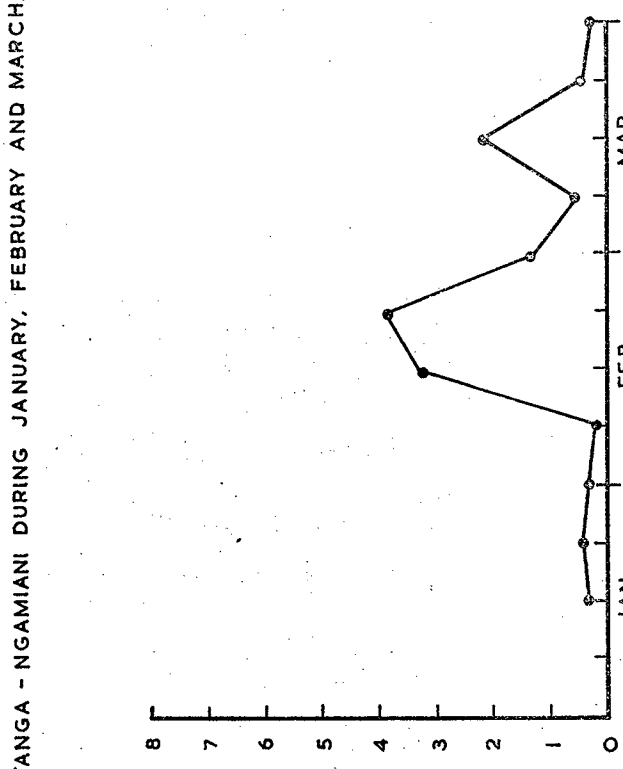

Fig. 3. THE NUMBER OF INFECTIVE LARVAE OF WUCHERERIA BANCROFTI RECOVERED FROM EACH 100 MOSQUITOES PROCESSED AT MUHEZA AREA DURING JANUARY, FEBRUARY AND MARCH, 1971

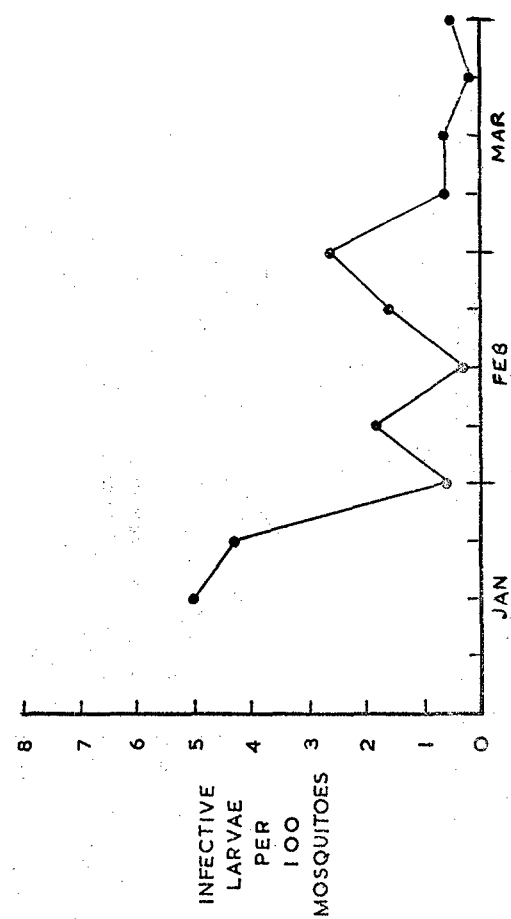

Fig. 4. A COMPARISON OF THE INFECTION RATES IN EACH OF THE MOSQUITO VECTORS OF WUCHERERIA BANCROFTI AT MUHEZA AREA DURING JANUARY, FEBRUARY AND MARCH, 1971

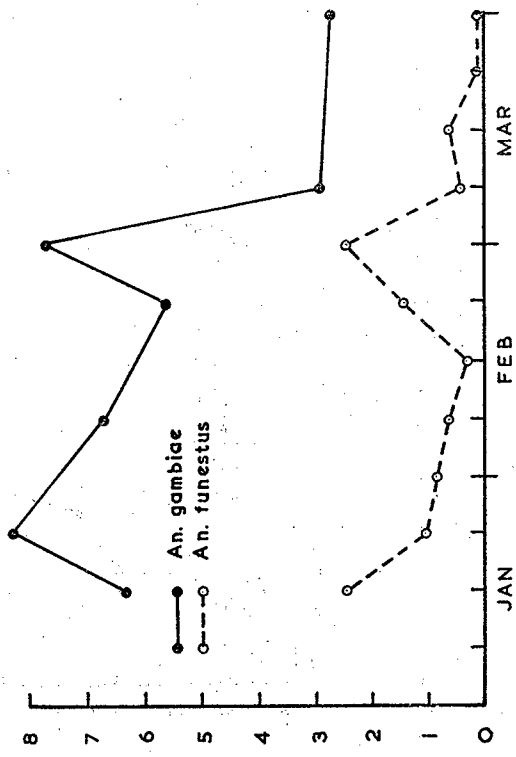

Fig. 5. THE NUMBER OF INFECTIVE LARVAE OF WUCHERERIA BANCROFTI RECOVERED FROM EACH 100 MOSQUITOES PROCESSED AT PANDENI DURING JANUARY, FEBRUARY AND MARCH, 1971

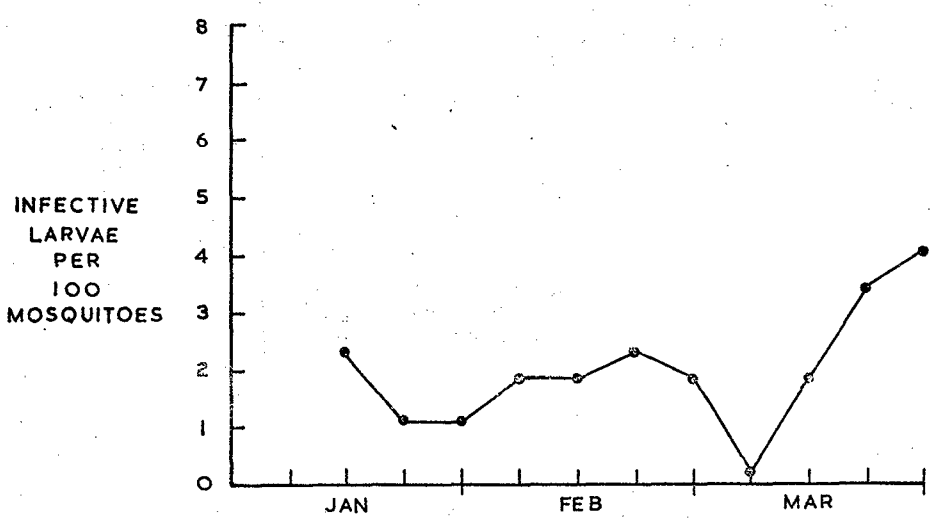

Fig. 6. A COMPARISON OF THE INFECTION RATES IN EACH OF THE MOSQUITO VECTORS OF WUCHERERIA BANCROFTI AT PANDENI DURING JANUARY, FEBRUARY AND MARCH, 1971