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# Studies on *Cardiofilaria nilesi* in Experimental Chickens

by

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SMALL laboratory animals capable of maintaining normal filarial infections have been necessary for investigating host-parasite relationships, for testing filaricidal drugs and for studying various aspects of the pathology of filariasis. The filarial parasite *Cardiofilaria nilesi* is found naturally infecting birds and is transmitted by *Mansonia crassipes* (Niles, Fernando and Dissanaike, 1965; Dissanaike and Fernando, 1965). In the present study attempts were made to maintain a strain of *C. nilesi* in chickens by cyclical transmission through laboratory reared *M. crassipes*. The strain was isolated from wild-caught *M. crassipes* in June,1967 and has since been maintained in our laboratory.

## MATERIALS AND METHODS

The method used for rearing *M. crassipes* in the laboratory was similar to that described by Jayewickreme and Niles (1952) for *Mansonioides* but with certain modifications. The weed, *Alternanthera sessilis*, with which the larvae of *M. crassipes* were found largely associated in nature was used in place of *Pistia* and *Salvinia*. Larvae were reared in cylindrical glass troughs ten inches in diameter and three inches deep. A half inch layer of mud was placed at the bottom of the rearing trough with 6-8 plants and filled with 1000 ml. of five day old guineapig dung infusion diluted with an equal part of tap water. In this volume of medium , corresponding to a depth of two inches, it was possible to rear up to 400 larvae. Egg rafts (each raft consisting of about 200 eggs) were introduced into the medium containing plants to enable the larvae to attach and commence feeding directly on hatching.

Organic matter in suspension was supplied as larval food by the addition of small quantities of dried, powdered and sieved guinea-pig dung at four-day intervals.

Several local species of mosquitoes and an exotic strain of *Aedes* (*Finlaya*) *togoi* were tested out for susceptibility to infection with *C. nilesi*.

Clean laboratory reared *M. crassipes* were used in all transmission experiments but on a few occasions due to poor yields from the laboratory cultures wild *M. crassipes* caught from an infection free area were availed of.

Infective larvae from *M. crassipes* were freshly dissected out of the mosquito in 0.85% saline, counted, and inoculated subcutaneously into clean chickens with the aid of a syringe and a 25-gauge needle. One day old and one week old chickens were used. The course

of the infection in chickens was follwed by sampling the blood for microfilariae. Although it was known that more microfilariae appear towards evening, the sampling was confined to a time between 2.30 and 4.30 p.m. for convenience. The method adopted for examining microfilariae in samples of blood was similar to that described by Anderson (1956) with slight alterations. The sample of blood was collected from the brachial vein into a 0.05 ml. pipette rinsed with a 3.8% sodium citrate solution. This was discharged on to a slide containing a drop of the citrate solution, covered with a No. 1 cover glass (22 × 50 mm.), and examined directly under a magnification of × 100.

A few chickens were transplanted with adult worms recovered from experimentally infected chickens. The donor chickens were killed by severing the carotid artery and jugular vein on one side under light ether anaesthesia to prevent bleeding from the mesenteric veins during subsequent manipulations. The adult worms were carefully collected from the peritoneal cavity after the addition of saline in which they were found to swim actively. They were kept in saline with penicillin and streptomycin till required for implantation.

The recipient chicken which was 2-3 weeks old was starved overnight preparatory to the transplantation. It was anaesthetised with ether and the deplumed abdominal region was swabbed with rectified spirits. An incision of about a centimeter was made posterior to the keel. The adult worms to be transplanted were picked up with a Pasteur pipette of suitable bore which was inserted through the incision and the worms deposited in the lower region of the abdominal cavity. The incision was sutured with cat gut, a piece of sticking plaster being placed over the suture after swabbing with rectified spirits. The chicken was allowed to recover from the anaesthesia in a heated cage.

## OBSERVATIONS AND RESULTS

*Laboratory rearing of M. crassipes*

Organic matter in suspension is the essential food of the larvae which appear to thrive best in heavy concentrations. The culture medium supported a vigorous growth of the plant and larvae were seen feeding at the bottom of the rearing trough almost buried in the layer of mud while attached to the roots of the plant. Pupae began to appear on the 15th. day after the larvae hatched and were seen on the roots and submerged stems of the plant. The pupae have the meatus of the air-trumpet tracheoid with a line of weakness at the base of the pinna which is broken off when the pupa detaches itself from the plant in order to rise to the surface. In its life-cycle *M. crassipes* is most vulnerable in the pupal stage.

The earliest adults which are male, emerge about the 20th. day after oviposition,and adults keep on emerging upto the 30th. day. Stenogamy is not pronounced as in species of *Mansonioides* and mating of adults was not observed in the laboratory. Adult females fed on chickens almost exclusively at dusk and were reluctant to feed at other times.

The following data regarding the lifecycle of *M. crassipes* were obtained :—

| | | |
|---|---|---|
| Eggs | .. | incubation period, 3 days |
| Mature larvae | .. | first seen 13th.—14th. day after hatching |
| | .. | last seen 16th.—24th. day after hatching |
| Pupae | .. | first seen on roots 15th.—16th. day after hatching |
| | .. | last seen on roots 21st.—26th. day after hatching |
| Adults | .. | earliest emergence 16th.—18th. day after hatching |
| | .. | latest emergence 22nd.—27th. day after hatching |

Under laboratory conditions therefore, the period of development from egg to adult is from 19 to 30 days.

*The efficiency of M. crassipes as a laboratory vector of C. nilesi*

Observations on the efficiency of *M. crassipes* as a laboratory vector of *C. nilesi* are presented in Table 1. *M. crassipes* is an extremely efficient host in which larvae reach maturity as early as the eighth day. All the mosquitoes become infected and almost all the larvae are stage III by the eleventh day. In addition they contain unusually large numbers of larvae, e.g. 11 mosquitoes produced a total of 859 infective larvae when dissected 10.5 days after feeding on an experimentally infected chicken with 10.9 microfilariae per c. mm., an average of 78 infective larvae per mosquito.

TABLE I

Observations on the efficiency of *Mansonia crassipes* as a laboratory-vector of *C. nilesi* (mosquitoes dissected up to 10.5-11.0 days after feeding).

| No. of microfilariae per c. mm. of blood | No. of mosquitoes dissected | Percentage of mosquitoes infective | Total no. of infective larvae | Stage III larvae per infective mosquito |
|---|---|---|---|---|
| 0.075 | 52 | 48 | 53 | 1.1 |
| 0.56 | 28 | 93 | 233 | 8.9 |
| 1.5 | 34 | 91 | 227 | 9.7 |
| 7.8 | 17 | 100 | 792 | 49.5 |
| 10.9 | 11 | 100 | 859 | 78.0 |

*Infection of other mosquitoes with C. nilesi.*

In swamp lands on the outskirts of Colombo where birds infected with *C. nilesi* occur, mosquitoes of the genus *Mansonia* are predominant and *M. crassipes* is the only species found regularly infected with *C. nilesi* (Niles, 1966). However, a wide variety of mosquitoes occur and attempts were made to feed as many as possible to see which were receptive to *C. nilesi.* The following local species of mosquitoes were tested out and found insusceptible to infection. *Culex fatigans, C. gelidus, C. tritaeniorhynchus, C. fuscanus, Aedes aegypti, Aed. albopictus, Armigeres subalbatus, Mansonia uniformis, M. annulifera, Anopheles peditaeniatus, A. nigerrimus and A. aconitus.*

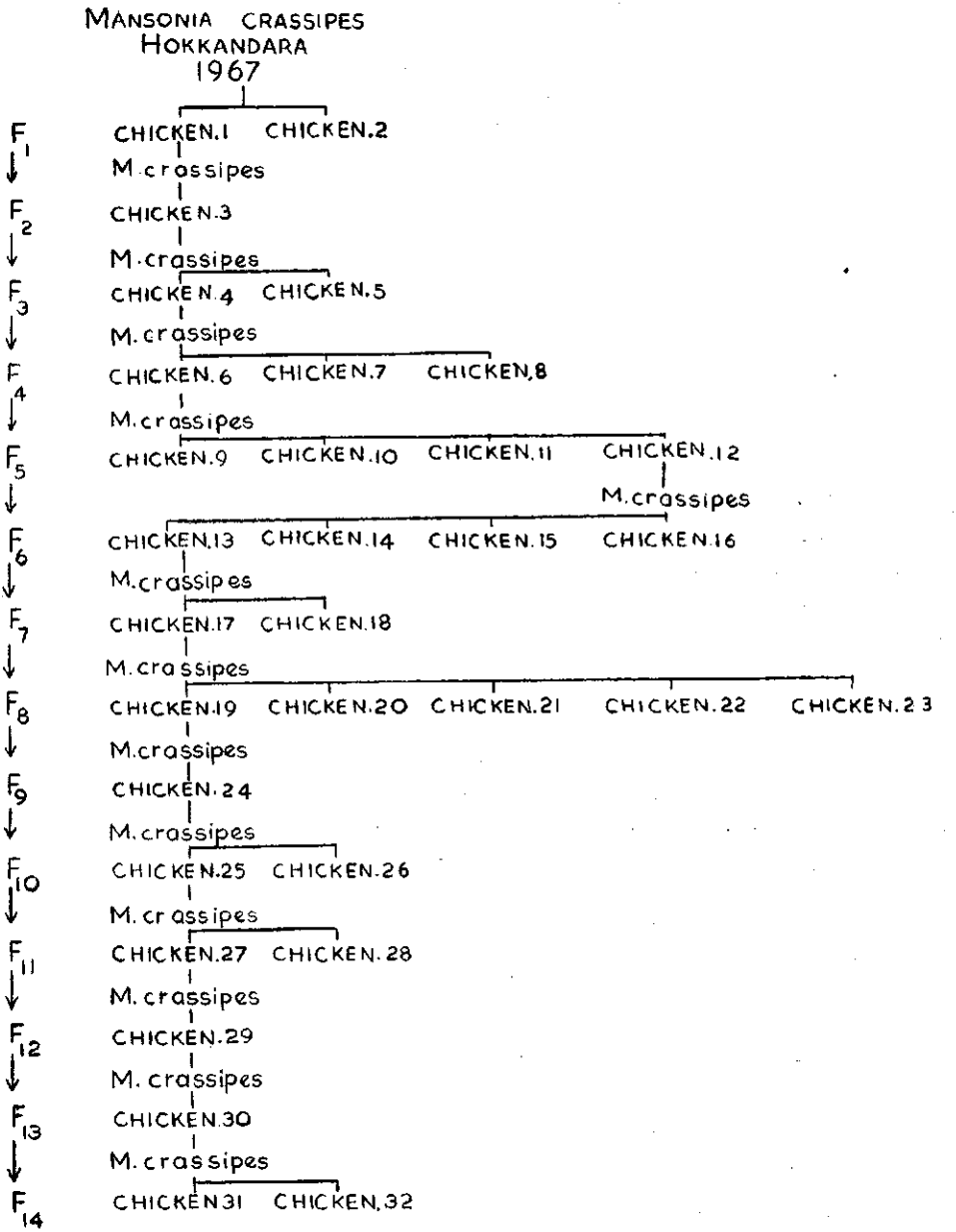


FIG.1. Sets out the transmission of C. nilesi to 32 chickens and shows that the strain has been maintained through 14 generations of microfilariae

Feeding experiments were also carried out with an exotic strain of *A. togoi*. Eggs of this strain were obtained from the London School of Hygiene and Tropical Medicine in September 1968. Seventy five laboratory reared *A. togoi* were fed on an experimentally infected chicken showing a high microfilarial count. The mosquitoes were dissected 12 days after feeding and 17 infective larvae were recovered of which 5 larvae had a dark deposit of melanin over a portion of the body but appeared normal in other respects. All 17 infective larvae were inoculated into a clean chicken. The chicken developed a microfilaraemia 30 days later. Another batch of 30 lab-bred *A. togoi* were fed on this microfilaria positive chicken and the mosquitoes dissected 14 days later. No infective larvae were seen. The vast majority of larvae died in the sausage stage and were found melanised in the thoracic muscles.

These experiments show that *A. togoi* (London strain) is capable of acting as a vector of *C. nilesi* and that infective larvae will develop into adult worms in experimentally infected chickens.

*Experimental transmission of C. nilesi to chickens.*

Thirty two chickens were exposed to infective larvae by subcutaneous inoculation of which the first ten chickens in the series were 1 day old and the rest 1 week old. Figure 1 sets out the transmissions to all the chickens and shows that the strain of *C. nilesi* isolated from wild-caught *M. crassipes* has been transmitted through 14 successive generations.

The chickens received from 11 to 150 infective larvae, the number given depending on the number of larvae available at the time of exposure. It was not found necessary to give large numbers of larvae to obtain an infection. Two chickens (one day old) which received 100 infective larvae and three chickens (one week old) which received 200 infective larvae died shortly after inoculation. Two of the chickens (Nos. 20 and 25) which had developed a heavy microfilaraemia also died during the ninth week of the patent period. All other chickens lived until the worms matured and microfilariae were detected in the blood. The results obtained from the subcutaneous inoculation of infective larvae in thirty two chickens are summarized in Table 2.

TABLE 2

Results of exposure of chickens to subcutaneous inoculation of the infective larvae of *C. nilesi*.

| Chicken No. | Date Inoc. | No. larvae | Date positive | Pre-patent period in days | Date autopsied | Worms recovered | Percent recovered |
|---|---|---|---|---|---|---|---|
| 1 | 1 June 67 | (24) | 23 June 67 | 22 | 20 Sept. 67 | 2 Males<br>2 Females | 16.7 |
| 2 | 1 June 67 | (35) | 25 June 67 | 24 | 19 July 67 | 11 Males<br>7 Females | 51.4 |
| 3 | 16 Aug. 67 | 31 | 12 Sept. 67 | 27 | * | | |
| 4 | 4 Sept. 67 | (11) | 5 Dec. 67 | 31 | 10 Feb. 68 | 2 Males<br>2 Females | 36.4 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 5 | 27 Dec. 67 | (26) | 24 Jan. 68 | 28 | 29 Feb. 68 | 3 Males<br>2 Females | 19.2 |
| 6 | 5 Jan. 68 | (35) | 31 Jan. 68 | 26 | 6 March 68 | 4 Males<br>7 Females | 31.4 |
| 7 | 18 Jan. 68 | 27 | 10 Feb. 68 | 23 | * | | |
| 8 | 23 Jan. 68 | (35) | 19 Feb. 68 | 27 | 14 March 68 | 9 Males<br>16 Females | 71.4 |
| 9 | 5 Feb. 68 | (22) | 29 Feb. 68 | 24 | 30 March 68 | 7 Males<br>4 Females | 50.0 |
| 10 | 9 Feb. 68 | 35 | 3 March 68 | 23 | * | | |
| 11 | 21 Feb. 68 | 80 | 20 March 68 | 28 | * | | |
| 12 | 7 March 68 | 70 | 7 April 68 | 30 | * | | |
| 13 | 16 April 68 | (20) | 14 May 68 | 28 | 18 May 68 | 13 Males<br>3 Females<br>(1M in pleural-cavity) | 80.0 |
| 14 | 23 May 68 | 7 | — | — | * | | |
| 15 | 29 Aug. 68 | 31 | 23 Sept. 68 | 25 | * | | |
| 16 | 12 Oct. 68 | 27 | 5 Dec. 68 | 24 | * | | |
| 17 | 16 Jan. 69 | 35 | 14 Feb. 69 | 29 | * | | |
| 18 | 7 Feb. 69 | 40 | 28 Feb. 69 | 21 | * | | |
| 19 | 28 May 69 | 112 | 28 June 69 | 31 | * | | |
| 20 | 12 June 69 | 150 | 18 July 69 | 36 | Died<br>(11 Sept. 69) | | |
| 21 | 19 June 69 | 112 | | | * | | |
| 22 | 28 June 69 | 95 | | | * | | |
| 23 | 1 July 69 | 93 | | | * | | |
| 24 | 8 Aug. 69 | 70 | | | * | | |
| 25 | 30 Sept. 69 | 102 | 30 Oct. 69 | 30 | Died<br>(12 Jan. 70) | | |
| 26 | 2 Nov. 69 | 114 | 3 Dec. 69 | 31 | * | | |
| 27 | 17 Nov. 69 | (113) | 16 Dec. 69 | 29 | 10 April 70 | 12 Males<br>46 Females<br>(1F in pleural cavity) | 51.3 |
| 28 | 29 Nov. 69 | 75 | 28 Dec. 69 | 29 | * | | |
| 29 | 3 Feb. 70 | 80 | 3 March 70 | 27 | * | | |
| 30 | 11 March 70 | 40 | | | | | |
| 31 | 20 June 70 | 50 | | | | | |
| 32 | 20 June 70 | 50 | | | | | |
| Total larvae in autopsied chickens | | (321) | | | | 152 | 47.4 |

*Not autopsied.

*Prepatent period*

The time required for development between the inoculation of infective larvae and the appearance of microfilariae in the blood was usually about 24 days. The prepatent period was shortest, 21 days* in chicken 18, which received 40 infective larvae and longest, 36 days in chicken 20, which received 150 larvae.

*Patent period*

At intervals during the patent period of the infection the number of microfilariae was counted in the 0.05 ml sample of blood. The largest counts of microfilariae occurred about the second month of the infection and were followed by diminishing densities untill few or no microfilariae were seen about six months or so after transmission. The patent period seems to last for at least five months. In general the developmental pattern of the microfilariaemia was similar in all chickens except in chicken 27. In this chicken the infection was much heavier than the others. It had a microfilaria count of 742 in 0.05 ml at 3.00 p.m., 4 weeks after the microfilariae were first detected in the blood. The numbers gradually declined for the next six weeks and four weeks later had risen to 1112 at 3.00 p.m. The decline in the number of microfilariae followed by an increase was similar to a phenomenon observed by Anderson (1956) in ducklings infected with *Ornithofilaria fallisensis*.

TABLE 3

Showing the results obtained in periodicity studies in 3 chickens with transmitted infections of *C. nilesi* (figures in heavy type indicate peak counts).

| No. of larvae inoculated | Microfilaria count per 20 c.mm. of blood on the 24-hour clock | | | | | | |
|---|---|---|---|---|---|---|---|
| | 10.00 hrs. | 14.00 hrs. | 18.00 hrs. | 22.00 hrs. | 02.00 hrs | 06.00 hrs. | 10.00 hrs. |
| 33 | 2 | 13 | **17** | 13 | 12 | 5 | 1 |
| 44 | 3 | 11 | 16 | **23** | 17 | 8 | 1 |
| 50 | 4 | 23 | **31** | 20 | 15 | 11 | 3 |

Periodicity studies done earlier on three other experimentally infected chickens are also reported here. The studies were based on the examination of 0.02 ml. blood films at 4-hourly intervals for 24 hours. Blood films were taken two months after the appearance of microfilariae. The details are given in Table 3. Microfilariae were present in the peripheral blood at all times but there was a marked fluctuation with an increase in numbers between 4. 00 p.m. and 8. 00 p.m. This tendency to a marked evening periodicity is of interest since observations made by us both in the field and in the laboratory indicate that *M. crassipes* is essentially crepuscular in its feeding habits.

* Since the above was written, a chicken inoculated with infective larvae has shown microfilariae in the blood 18 days subsequent to inoculation.

*Distribution of adult worms.*

More than 45% of the infective larvae (321) given to chickens were recovered as adult worms (152). We have not found adult worms in any part of the host other than the peritoneal cavity except for two instances, in which one of the thirteen male worms (chicken 13) and one of the forty six female worms (chicken 27) were in the pleural cavity, suggestive of migratory activity in search of a mate. Chicken 13 had a preponderance of male worms (12) over female worms (3) in the peritoneal cavity and chicken 27 had a preponderance of female worms (45) over male worms (12) in the peritoneal cavity.

*Transplantation of adult worms.*

The location of *C. nilesi* in its host suggested that the adults might be transplanted from one chicken to another. Studies on the transplantation of *C. nilesi* to clean hosts commenced in 1967 and since then nine experiments have been carried out. Table 4 indicates the results in a summarized form. The difficulty of obtaining a surplus of lab-bred *M. crassipes* for experimental use prevented a more detailed study. The density and duration of the microfilaraemia in recipient chickens appeared to be dependent on the number of female worms implanted. The longest duration was 177 days in chicken TF/2A into which 10 female worms were implanted. This chicken also showed a high count of 109 microfilariae at 3.30 p.m. on the 21st. day after the implantation; a marked increase in numbers compared to the 24 microfilariae observed on the 15th. day at 3.30 p.m. and 22 microfilariae observed on the next sampling day, i.e. on the 35th. day at the same time. The adult worms were 51 days old when implanted into this chicken. The microfilarial density in most of the recipient chickens was low. The largest number of adult worms was implanted into chicken TF/6A but unfortunately the chicken was older (8 weeks) than the rest of the recipients and hence no detailed microfilarial counts were made on it.

TABLE 4

Transplantation experiments with *C. nilesi*.

| Recipient chicken No. | No. and sex of worms implanted | Age of worms implanted (day) | Day first examined for microfilariae | Day microfilariae first observed | No. of mf. at first observations per 0.05 ml. blood | Duration of microfilaraemia (days) |
|---|---|---|---|---|---|---|
| TCN/1 | 2F + 1M | 98 | 11th. | 11th. | 1 | 38 |
| TF/1 | 2F | 64 | 3rd. | 8th. | 7 | 33 (died) |
| TF/2A | 10F | 51 | 4th. | 4th. | 33 | 177 |
| TF/2B | 3F + 6M | 51 | 4th. | 4th. | 6 | 11 (died) |
| TF/3A | 5F | 92 | 2nd. | 2nd. | 2 | 97 |
| TF/3B | 2F + 4M | 92 | 2nd. | 2nd. | 1 | 2 |
| TF/4A | 3F | 48 | 1st. | 1st. | 2 | 67 (died) |
| TF/5A | 2F | 32 | 2nd. | 4th. | 1 | 76 |
| TF/6A | 55 unsexed | 144 | 111th. | 111th. | 7 | 150 |

The developmental pattern of the microfilaraemia in all recipient chickens was more or less similar and there was no sign that the number of microfilariae had begun to decline in the blood of those birds which had been implanted with older worms.

## DISCUSSION

The essentials for continuous rearing of *Mansonia* (*Mansonioides*) mosquitoes the in laboratory were given by Jayewickreme and Niles (1952) and over the last few years there have been improvements in *Mansonioides* breeding techniques (Wharton, 1957; Laurence, Page and Smith, 1962; Samarawickrema, 1968). Unlike *Mansonioides*, *M. crassipes* which belongs to the subgenus *Coquillettidia*, is restricted in its choice of plants and does not readily utilize *Pistia*, *Salvinia* or *Eichhornia* for breeding purposes. Wharton (1962) reports that in Malaya this species prefers aquatic grasses (*Panicum amplexicaule*) and Bonne-Wepster (1939) states that in a lake in Borneo the larvae were associated with the common vegetable *Ipomoea* and not with the water-hyacinth (*Eichhornia crassipes*) which usually covered the lake. In swampy grass fields situated on the outskirts of Colombo, *M. crassipes* has been found breeding largely in association with the weed *Alternanthera sessilis* and not with *Pistia* and *Salvinia*, the common host plants of the indigenous *Mansonioides*. The weed, commonly known as 'Mukunuvenna', is extensively cultivated in Ceylon for its edible leaves and is readily available. It survived well in the larval culture medium for the duration of an experiment Using this plant for rearing the larvae adequate numbers of adults were obtained for transmission purposes but more work needs to be done before a continuous culture of *M. crassipes* can be established in the laboratory.

*M. crassipes* is an extremely efficient host for *C. nilesi* but requires a long period to develop from egg to adult. The use of a more easily reared alternative mosquito host with a shorter developmental period from egg to adult would be advantageous for the laboratory transmission of *C. nilesi*. *A. togoi* seems to hold promise, since, as already noted, infective larvae derived from this mosquito when inoculated into chickens develop into adult worms and produce a microfilaraemia. It might be possible to increase the infectivity of *C. nilesi* to *A. togoi* by selection of the microfilariae as shown by Laurence and Pester (1967) in the adaptation of *Brugia patei* to this mosquito species. Gooneratne (1969), fed *A. togoi* on experimentally infected chickens obtained from our present series and found that 8.2% of those fed contained one infective larva (rarely two) per mosquito.

A successful peritoneal transplant of 5 adult worms found in one of four chickens obtained from the present series is reported by Gooneratne (1968) According to him, microfilariae appeared in the peripheral blood of the recipient chicken on the sixth day after the transplant and microfilaraemia lasted 63 days. Our findings show that microfilariae appear very much earlier, in one case (chicken TF/4A), on the very next day after the transplant. They also remain in the peripheral circulation for as long as 177 days (chicken TF/2A). The fact that a smaller sample (0.02ml) of blood was examined may account for the results obtained by Gooneratne. Wenk and Höfler (1967) found that an inoculation of $3.5 \times 10^6$ microfilariae of *Litomosoides carinii* into the peritoneal cavity of each of two cotton rats produced peripheral microfilaraemia on the third and sixth day respectively and that it continued till the 56th day showing a longevity of at least 56 days. Bagai and Subrahmanyam (1968) reported similar numbers of *L. carinii* inoculated into the pleural cavity of albino rats produced peripheral microfilaraemia on the following day similar to what we observed in chickens implanted with *C. nilesi*. The durations of the patent periods in the recipient chickens com pared favourably with those in the primarily infected chickens and appeared to be dependent on the number of female worms implanted.

Gooneratne (1969*a*) believed that the absence of microfilariae in the peripheral blood of a chicken which harboured fecund female worms showing mature microfilariae in the uteri, was due to an " immune response " and that trapping of the microfilariae in the lungs was an early manifestation of this " response ". Bagai and Subrahmanyam (1968) have shown conclusively that at least in the case of infections with *L. carinii* the absence of microfilariae in the circulating blood was due to a local immunity at the site of worm development, independent of any humoral antibodies, preventing the microfilariae from reaching the general circulation. There is no evidence to show that this is contradicted in the case of chickens infected with *C. nilesi*. The microfilariae observed by Gooneratne (1969*a*) in the lung smears could still be circulating microfilariae. The sample of peripheral blood examined was infinitesimal compared to the total volume in which the microfilariae could hide. Furthermore, it is well known, that when microfilariae decrease or disappear from the peripheral blood during the negative phase of the periodic cycle they concentrate in the lung to reappear in the peripheral circulation during the positive phase. This may well be the explanation for the presence of microfilariae in the lung smears at a time when they were not demonstrable in the peripheral blood. Low fluctuant counts lasting several weeks, with the blood negative on occasions, can be expected in experimentally infected chickens with small worm loads. Our findings do not indicate that there could be a total absence of microfilariae from the peripheral blood stream due to an immunological interference as suggested by Gooneratne (1969*a*). The manifestation of immunity to *C. nilesi* in chickens judged by the output of microfilariae needs much further elucidation.

Considerable attention has been given during recent years to filarial species in animals convenient for laboratory maintenance. *C. nilesi*, as evidenced in the present findings, has a particular advantage over other known filarial worms of animals in being the only filarial worm which becomes an adult in less than 21 days. Moreover, the adult worms are easily accessible. The maintenance of a strain of *C. nilesi* in the chicken might prove useful for quantitative experiments on host-parasite relationships and for drug testing.

## SUMMARY

Methods are described for the breeding of *Mansonia crassipes* and the transmission of *Cardiofilaria nilesi* to chickens. A strain of *C. nilesi* has been maintained in the laboratory for over a period of 3 years and a general description of the experience obtained with this parasite is given. It is hoped that the maintenance of this filarial infection in the chicken will prove useful in the laboratory as an experimental model not only for many basic studies on host-parasite relationships but also for the evaluation of filaricidal drugs.

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