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Laboratory Culture and Life Cycle of two species of Mosquito, *Mansonia* (*Mansonioides*) *uniformis* Theobald and *Mansonia* (*Mansonioides*) *annulifera* Theobald from Ceylon.

by

W. A. SAMARAWICKREMA
Medical Research Institute, Colombo 8

Laurence & Smith (1958) and Laurence, Page & Smith (1962) have reported results of successful large scale rearing, in London, of *Mansonia* (*Mansonioides*) *africana* (Theobald) and *M.* (*M.*) *uniformis* from Africa and *M.* (*M.*) *uniformis* from Malaya. Laurence (1960) also studied the biology of these mosquitoes in the laboratory. Ceylon strains of *M. uniformis* and *M. annulifera* were studied concurrently with these species in London during the period 1956-1959 and the colony material was used in studies on the age determination of these mosquitoes (Samarawickrema, 1962). In this paper the results of culture of the two species are discussed first; the culture of the two species in a new medium of liver extract is described next and finally the life cycles of the two species are compared.

MATERIALS AND METHODS

Stock cultures

The two species under consideration were set up in culture from egg batches sent from Ceylon by the author between May and August 1956 prior to his arrival in England. Egg masses of wild-caught females of the two species laid in the laboratory in Colombo were packed in $2\frac{1}{2} \times \frac{1}{2}''$ perspex tubes as described by Smith (1956) and dispatched by air to the Department of Entomology, London School of Hygiene and Tropical Medicine. On arrival in London the material was attended to by Mr. S. A. Smith and Dr. B. R. Laurence. All other materials and methods pertaining to maintenance, feeding and oviposition of adults and rearing of larvae and pupae have been the same as described in detail by Laurence & Smith (1958).

The guinea pig dung medium of Jayawickrema & Niles (1952) modified by Laurence & Smith (1958) was extensively used in stock cultures. A sod of turf placed in the culture trough maintained the beneficial balance. Dog biscuit infusion (Smith, 1956) was tried with little success. Latterly Diet 18 (a proprietary animal diet, E. Dixon & Sons (Ware) Ltd., Great Britain) in water (Laurence, Page & Smith, 1962) was used. Yeast powder was added to the cultures three to four times a week.

Salvinia auriculata was used in the early cultures for larval and pupal attachment. *Pholaris arundinacea*, an aquatic grass, was used during the winter months in the absence

*The insectary in which the mosquitoes were kept maintained a temperature of 26-30° C.

of *Salvinia*. Following the successful experiments of Laurence & Smith (1958) two 14 × 10 cm pieces of wet strength crepe brown papers (manufactured by Reed Paper Group, Kent) were used in each culture bowl.

Liver extract cultures

Experimental work on the nutritional needs of the aquatic stages was done with larval cultures, using various mixtures of the following ingredients: Oxo liver extract (bacteriological), dried yeast from the Distillers Co. Ltd., Edinburgh, Difco yeast extract, sterile Mycostatin powder from Squibb & Son, New York and vitamin mixture (Lea, Dimond & De Long, 1956; modified by Woodall, 1958) with the following composition in milligrammes, biotin 0.05, calcium pantothenate 15, choline chloride 50, glutathione 25, nicotinamide 25, nicotinic acid 5, pteroylglutamic acid 10, pyridoxine hydrochloride 10, riboflavin 10, thiamin hydrochloride 10, dissolved in 50 ml distilled water, B_{12} 0.001, neutralised with 70 mg $NaHCO_3$, riboflavin precipitated and filtered.

After preliminary trials it was found that in a medium of 1.25 gm of liver extract in a litre of distilled water the host plant, *Salvinia*, did not wither and the larvae grew steadily. This dilution was adopted as the basis of experimental media in the whole series of observations. 500 ml portions of the medium were used in each test culture and supplemented appropriately with the yeast fraction as recorded in the results. The freshly prepared medium was poured into 600 ml beakers without spout and covered with square glass sheets to minimise aerial contamination. When freshly prepared the medium was clear and amber in colour. At the end of twenty four hours, the medium became slightly turbid. Turbidity was at its peak further twenty four hours later. A thick scum had formed on the surface. The medium at this stage was rich with bacteria, flagellates and ciliate protozoa. From this point turbidity decreased gradually, but some micro-organisms were to be found for a period of about ten days. The scum was removed on the day the culture was set up, before the introduction of the larvae. The cultures were set up when, on the fifth day, turbidity had passed and the medium was judged clear and "stable". In practice, the basal medium was prepared and put into culture beakers on the day of oviposition of the mosquitoes. It was ready for use when the eggs hatched five days later. *Salvinia* was used in the early experiments for larval attachment. Crepe paper replaced *Salvinia* in later experiments. Two strips of the paper, each 7 × 10 cm, were put in each culture and renewed once in two days. The yeast component (dried yeast, autoclaved yeast or yeast extract) of the culture was added three times a week, 30 mg per culture each time. Each culture was carefully examined daily and any dead larvae were removed. The pH of the cultures varied from 6.8 to 7.2. As the medium evaporated the level of each culture beaker was made up with distilled water.

The number of larvae pupating daily was noted in each culture and removed with a piece of the paper to 3 × 1″ specimen tubes, containing water and plugged with cotton wool. It was possible, by this method, to determine the duration of the individual pupae. During pupation the crepe paper was replaced in the cultures daily.

RESULTS

Oviposition

The 'ovipot' method of Laurence & Smith (1958) for oviposition using *Salvinia* and paper discs yielded successful results with *M. uniformis*. *M. annulifera*, however, never laid under these conditions. Under more humid conditions supplied by a wet pad, and with *Salvinia*, these mosquitoes laid only at night. The egg masses of the two species were of the same size.

Larval development, pupation and adult emergence in stock cultures.

The results of culture experiments were analysed according to the method adopted by Laurence & Smith (1958) so that a direct comparison could be made with their results. The percentage emergence of adults of first instar larvae reared in different culture media (Table 1) and the percentage emergence in the dilute guinea pig dung infusion medium, but with different attachment hosts for larvae (Table 2), showed, for *M. uniformis*, similar results to those of Laurence & Smith (1958) for the same species. These authors, however, had better yields of adults with *Pholaris*. The average emergence of *M. uniformis* in guinea-pig dung infusion cultures and diet 18 infusion cultures was very close indicating that the faeces medium could be replaced by diet 18 medium (Laurence, Page & Smith, 1962).

TABLE 1

Emergence data for different larval media in rearing first instar larva to adult in the two species of *Mansonioides* (100-250 larvae used in each test).

Type of culture	Species	Average Emergence		Range of Emergence	Percentage complete failures	Number of trials
Guinea pig dung infusion	*uniformis*	27	23.5	0-96	4	170
(Laurence & Smith, 1958)	*annulifera*	20		0-99	30	200
Dog biscuit infusion	*uniformis*	1.6	0.8	0-5	80	5
(Smith, 1956)	*annulifera*	0.0		0	100	5
Dog biscuit infusion — sod	*uniformis*	10	5.3	0-155	33	12
of turf	*annulifera*	0.5		0-57	80	10
Diet 18 infusion (Laurence,	*uniformis*	26		0-90	5	20
Page & Smith, 1962)	*annulifera*	—		—	—	—

While *M. uniformis* adapted well to laboratory culture, much difficulty was experienced with *M. annulifera*. *M. annulifera* gave lesser yields throughout with a significantly high proportion of complete failures. Cultures of *M. annulifera* set up together from the same guinea-pig dung infusion often behaved differently. While in some cultures larvae developed satisfactorily in many others they did not survive. On account of this difficulty *M. annulifera*

cultures were set up mainly in guinea-pig dung infusion medium. Twice the strain of *M. annulifera* died out during the winter months when *Salvinia* was scarce and fresh batches of eggs were flown out from Colombo. Cultures of *M. annulifera* were tried out in smaller troughs of 14 cm diameter with more success. The longest duration of the *M. annulifera* strain was twelve generations extending through twelve months. Finally, comparing these results with those of Laurence & Smith (1958) it was evident that while colony breeding of *M. africana* and *M. uniformis* was successful *M. annulifera* culture was a difficult proposition.

TABLE 2

Emergence data for different attachment hosts using dilute guinea pig dung infusion, yeast and sod turf in rearing first instar larva to adult in the two species of *Mansonioides* (100-250 larvae used in each test)

Air Store	Species	Average Emergence	Range of emergence	Percentage complete failures	Number of trials
Salvinia	*uniformis*	29.5 } 26.3	0-96	2	55
	annulifera	23.0 }	0-74	20	50
Pholaris	*uniformis*	11.7 } 5.8	0-61	33	12
	annulifera	0.0 }	0	100	6
Wet strength crepe paper	*uniformis*	24.5 } 21.0	0-94	4	55
and *Salvinia* at pupation	*annulifera*	17.5 }	0-76	25	26
Wet strength crepe paper only	*uniformis*	28.0 } 24.0	4-90	0	42
	annulifera	20.0 }	0-99	20	51

Average emergence of *M. uniformis* from 100 - 250 first instar larvae was found to be insufficient for experimental use. This was due to the loss of first instar larvae and pupae in the cultures as demonstrated by Laurence & Smith (1958). Thus in an attempt to get higher yields ten to twelve egg masses were introduced to each culture dish. Using guinea-pig dung infusion and diet 18 medium with crêpe paper for larval attachment an average emergence of 300 adults per culture was achieved.

Simultaneous rearing of **M. uniformis** *and* **M. annulifera** *in dilute guinea-pig dung infusion medium in one container.*

Four trials of rearing the two species in one container using the above culture method yielded the following results. A total of 410 first stage larvae of both species, equal number of each, was set up in culture. 60.5% of the first instar larvae of the two species pupated and 48.8% of the first instar larvae emerged as adults. Of these the percentage emergence as adults of first instar larvae of *M. annulifera* was 31. 2 and of *M. uniformis* was 66.3. The pattern of emergence is shown in Figure 1. The lesser response to laboratory stock culture of *M. annulifera* was well marked. These experiments showed clearly the shorter duration of the life cycle of *M. annulifera*.

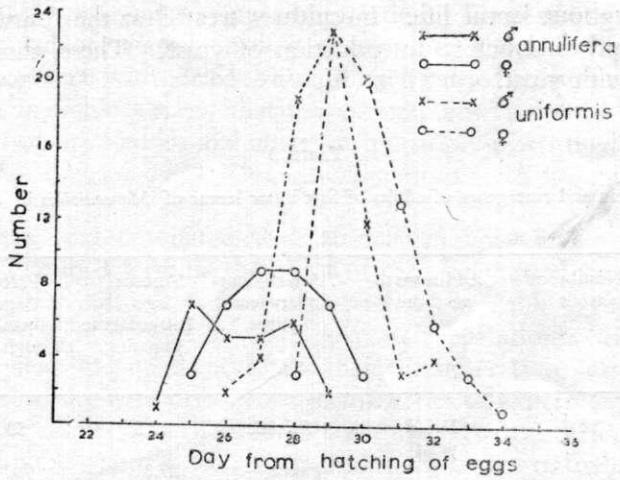

FIG. 1

FIG. 1. Emergence of sexes in *M. uniformis* and *M. annulifera* reared together in one container in guinea-pig dung infusion medium with crepe paper as attachment host for larvae and pupae.

Liver extract cultures

The results of experiments are discussed in chronological series along the steps in which the investigation was conducted. A summary of the results is given in Table 3. The basal medium consisted of 500 ml portions of 1.25 gm of liver extract in a litre of distilled water.

The early experiments were designed to determine the effect, on larval growth and pupation, of the addition of dried yeast. The results given in Table 3 series A1 and A2 are very convincing. No pupation took place at all in the absence of yeast. Cultures not treated with yeast remained clear throughout and, qualitatively, few micro-organisms were to be found in them at the end of the tests. In most tests the larvae died out very early. In some they reached the third instar. Occasionally, in *Salvinia* cultures, a few larvae survived to moult into the fourth instar. The yeast treated cultures became somewhat cloudy, then cleared, and contained debris of dead yeast cells at the bottom, together with living yeast cells and other micro-organisms in suspension at the end of the test. The yield of pupae and adults obtained was poor. In cultures with initially a hundred first instar larvae, the mortality was heavy and steady throughout and, in some, the larvae did not survive until pupation. Better results were obtained by rearing fifty and twenty five larvae per culture experiment. As the investigation progressed all tests were carried out with twenty five larvae.

Next step was to determine whether yeast was essential throughout larval life. Separate cultures were treated with dried yeast as follows: (*a*) up to the third larval instar; (*b*) from the fourth instar onwards, and (*c*) throughout larval life. Reference to series A3 of Table 3

shows that pupation and emergence of adults took place most successfully when yeast was available throughout larval life. In cultures treated at the fourth instar most of the larvae, if not all, and died before introduction of yeast. These, therefore, behaved like cultures untreated with yeast.

TABLE 3

Summary of pupation and emergence of adults of first instar larvae of *Mansonioides* in different liver extract-yeast media.

Series	Species	Number of trials	Additions to medium	Attachment for larvae and pupae	Percentage 1 stage larvae pupating	Percentage pupae coming through to adult	Percentage 1 stage larvae coming through to adult
A1	*uniformis*	18	Dried yeast	*Salvinia*	12.5	53.4	6.6
		7		Paper	27.3	70.7	19.3
	annulifera	6		*Salvinia*	16.0	35.7	5.7
A2	*uniformis*	10	Nil	*Salvinia*	0.0	0.0	0.0
		4		Paper	0.0	0.0	0.0
	annulifera	4	Nil	*Salvinia*	0.0	0.0	0.0
		4		Paper	0.0	0.0	0.0
A3	*uniformis*		Dried yeast				
		4	(a) from IV instar	*Salvinia*	0.0	0.0	0.0
		4	(b) up to IV instar	*Salvinia*	23.3	48.6	11.3
		4	(c) throughout	*Salvinia*	33.5	64.2	29.8
A4	*uniformis*	4	(a) Vitamin mixture	Paper	0.0	0.0	0.0
		4	(b) Vitamin mixture Dried yeast	Paper	34.5	55.1	19.0
		2	(c) Dried yeast only	Paper	35.0	65.7	23.0
A5	*uniformis*	7	(a) Dried yeast	Paper	52.7	77.2	40.7
		7	(b) Killed yeast	Paper	43.6	59.2	25.8
		6	(c) Yeast extract	Paper	50.2	62.8	31.6
	annulifera	6	(a) Dried yeast	Paper	50.5	89.1	45.0
		6	(b) Killed yeast	Paper	33.5	79.1	26.5
		5	(c) Yeast extract	Paper	33.1	50.0	16.6
A6	*uniformis*	5	(a) Dried yeast + Mycostatin	Paper	64.8	70.0	56.8
		3	(b) Killed yeast + Mycostatin	Paper	56.0	87.7	50.7
		4	(c) Yeast extract + Mycostation	Paper	33.0	47.2	21.0
	annulifera	6	(a) Dried yeast + Mycostatin	Paper	78.0	83.8	65.3
		5	(b) Killed yeast + Mycostatin	Paper	37.6	89.4	33.6
		5	(c) Yeast extract + Mycostatin	Paper	33.6	78.6	26.4

A vitamin mixture used by Lea, Dimond & De Long (1956), modified by Woodall 1958), was next tested with yeast to improve pupation. Three sets of cultures were treated as follows: (*a*) 0. 1 ml of vitamin mixture three times a week; (*b*) an equal quantity of vitamin mixture together with dried yeast, (*c*) with dried yeast only as control. The results in series A4 in Table 3 show that the vitamin mixture alone did not bring about pupation. It did not have additional effect on pupation when used together with dried yeast.

Dried yeast was next compared with autoclaved yeast and yeast extract. Several trials were carried out to determine the effect of each yeast product. Larval growth and pupation took place in all cultures, as shown in series A 5 in Table 3, suggesting that growth and pupation factors were present in all three yeast preparations. However, dried yeast gave rather better yields of pupae and adults than the other two yeast components. The yields of pupae and adults with dried yeast in this series of experiments showed a marked improvement on the results of earlier cultures with dried yeast. This is mainly due to the experience gained by the writer in techniques during the course of the investigation. However, in all these combinations of media some cultures always failed to give good yields of pupae and adults.

If the paper was allowed to stand for more than two days fungal growths appeared at the bottom of the culture, on the paper within the medium and at the surface. These moulds often enmeshed the larvae attached to the paper, preventing further development. They appeared on paper before the latter became waterlogged and due for change. The three principal genera of fungi isolated from the cultures were *Chaetomium* sp. with black perithecia attached to paper, *Cephalosporium* sp. also on paper and *Absidia* sp. from the bottom of the culture. *Chaetomium* was the commonest of the three.

Control of these fungal infections was attempted although this implied, in theory, destruction or interference with the yeast culture and constituents. An antifungal substance, mycostatin, derived from *Streptomyces noursei* and used in various laboratory techniques especially in bacterial tissue culture, was tried. In a preliminary experiment different quantities of the dry powder were suspended in the basal medium and vigorously stirred and the cultures set up with larvae. No trace of the troublesome fungi was found in the cultures throughout the experiments. However, larvae survived best in cultures treated with 125,000 units (5 mg) and less. Thus, in the final series of experiments with the three products of yeast and mycostatin, pupation and emergence took place, the yields from dried yeast cultures being better than those of the other two (A6 , Table 3).

With mycostatin a somewhat numerically higher yield of pupae was achieved although not consistently. so. The effect of mycostatin on larval life was, except for yeast extract cultures with *M. uniformis*, favourable to rather better yields of pupae and adults. This numerical result is in agreement with the observed freedom from mechanical entanglement if the fungicide is present. One might, however, doubt the significance of the differences shown by the presence or absence of mycostatin on these numerical yields of pupae and adults, because the yields were generally much greater in both these types of experiments

than in other comparable series (A1, A3, A4). It is, therefore, concluded on these results, that, while mycostatin eliminated the mechanical entanglement of larvae, it did not help to increase the yield to any appreciable degree.

The duration of stages of the life cycle in liver extract and yeast media.

Length of larval life.

The duration of the larval instars was determined from a a set of cultures of liver extract media treated with dried yeast. Each culture was emptied into a basin daily and the stage of individual larvae were determined daily under a stereoscopic microscope. The daily percentage of larval instars of the survivors until the appearance of pupae is shown in figure 2. Moults from one instar to the next took place over a long period in both species. This feature was more marked in *M. uniformis*. The early moulting in *M. annulifera* was repeated in all stages as judged by the peak for each instar. The mean duration of larval life and range in all liver extract yeast media for the two species is given in Table 4.

TABLE 4

Mean length and range of larval and pupal life (days) of the two species of *Mansonioides* from all liver extract - yeast media.

Species	Length of Larval life in all media		Length of Pupal life in all media			
	Mean	Range	Mean		Range	
			♂	♀	♂	♀
uniformis	27.8	26.2-30.0	3.41	3.58	3.11-3.62	3.44-3.72
annulifera	22.8	20.8-24.3	3.37	3.31	3.00-3.71	3.12-3.40

Length of pupation and pupal stage.

The onset of pupation in different conditions of nutrition showed a variation. But apart from nutritional factors involved pupation is delayed by the death of some mature larvae. The mean duration of the total pupal period for *M. uniformis* was 24 days and for *M. annulifera* 18 days. But, as seen in figure 3, pupation in the two species was nearly complete in 16 and 13 days respectively. The mean length of pupal life for both sexes in the two species, given in Table 4, was more or less the same.

Adult emergence.

The duration time of adult emergence is governed by the duration of the total pupal period. The pattern of adult emergence from all cultures followed the pattern of pupation as shown in figures for the two species. The earliest emergence of adults after hatching of eggs was in 21 days for *M. uniformis* and in 18 days for *M. annulifera*. The early emergence of males followed by females, in both species, is also shown in figure 4. These findings confirm in detail the shorter duration of the life history in *M. annulifera* observed during the simultaneous stock cultures of the two species.

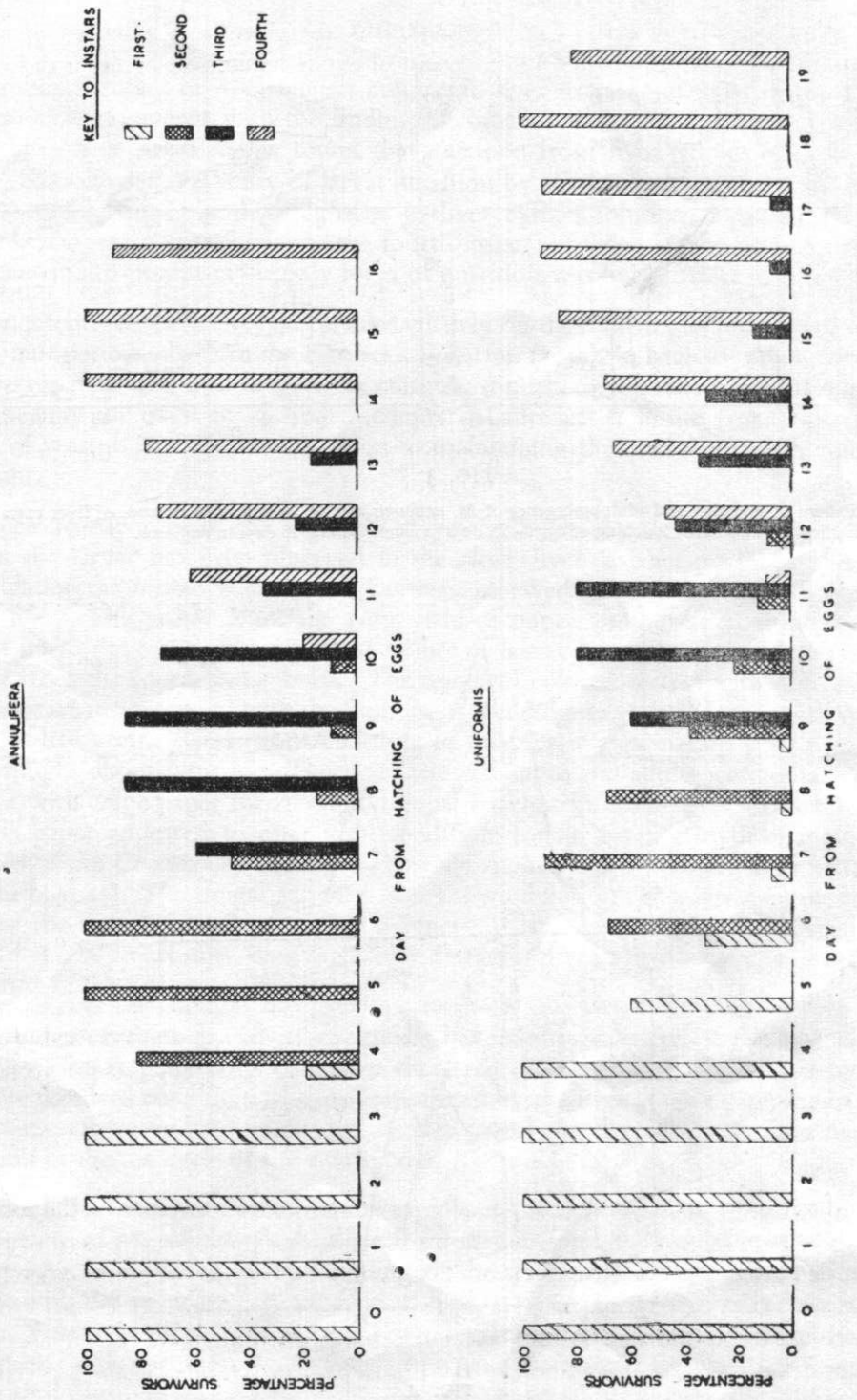

FIG. 2

FIG. 2. Daily larval moulting of *M. uniformis* and *M. annulifera* reared in a medium of liver extract and yeast.

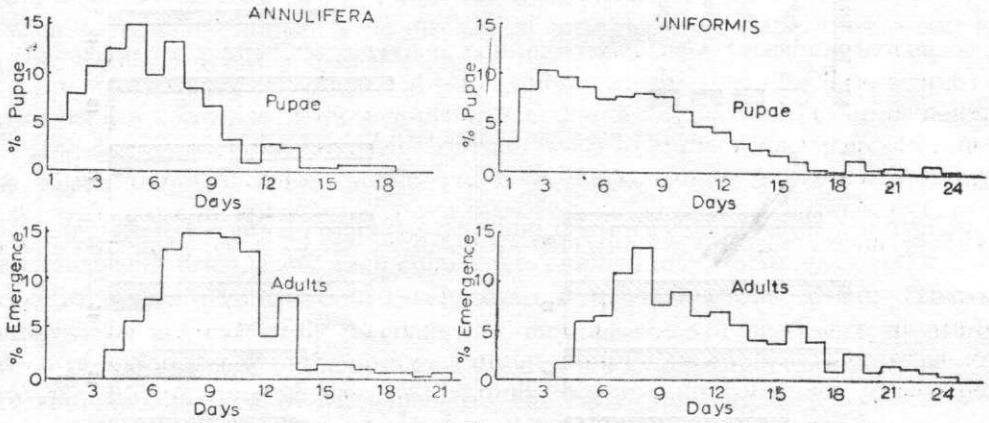

FIG. 3

FIG. 3. Pattern of pupation and adult emergence of *M. uniformis* and *M. annulifera* in cultures of liver extract and yeast Earliest pupation after hatching of eggs - 15 days in *annulifera* and 18 days in *uniformis*.

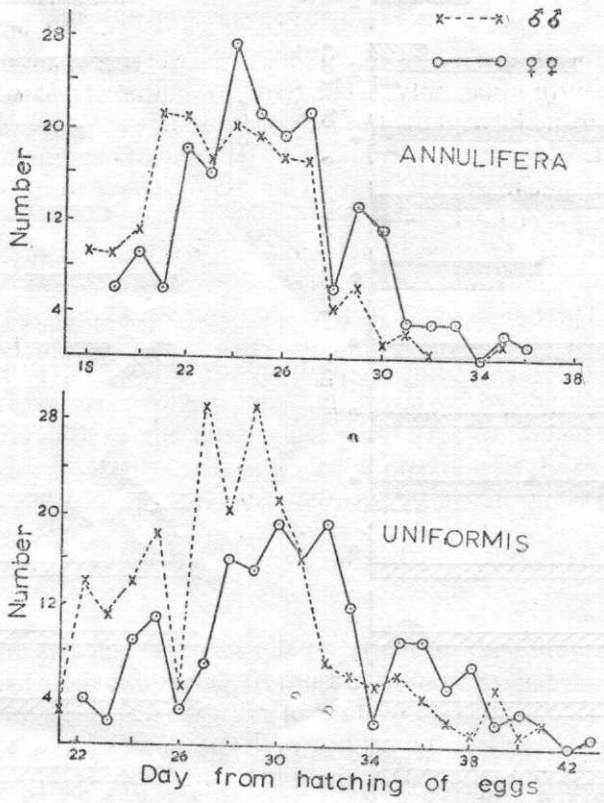

FIG. 4

FIG. 4. The order of emergence of sexes in the two species in liver extract and yeast media.

DISCUSSION

The present studies of *Mansonioides* cultures in liver extract and yeast media showed some features of agreement with the findings of other workers. Trager (1935, 1947), in his sterile culture of *Aedes aegypti* found that nutrition from liver extract utilised in true solution. No such detailed study of larval nutrition by sterile culture was attempted with *Mansonioides*. However, in these cultures in liver extract solution, but untreated with yeast, the larvae could grow as far as the fourth instar but failed to pupate. Apart from the incidental micro-organism the only form of nutrition was liver extract in true solution.

Jayewickreme & Niles (1952) depended entirely on the centrifuged portions of micro-organisms put periodically into the cultures. Wharton (1957), in Malaya, relied on similar diet for larvae. In dog biscuit infusion cultures, (Smith, 1956) micro-organism content was renewed by the addition of fresh medium. Laurence & Smith (1958) appreciated the value of yeast as larval food in addition to maintaining the micro-organism content in the medium.

In the present experiments the ingestion of yeast particles by the movement of mouth brushes in the larvae has been observed in the clear liver extract medium. Since the yeast population diminishes with time, it has to be renewed at regular intervals throughout the larval life. This accounts for the poor yield of pupae and adults in cultures treated with yeast up to the fourth instar and the failure of larvae to pupate in cultures provided with yeast from the fourth instar only. The results of cultures treated with different fractions of yeast are in agreement with the findings of Buddington (1941) regarding the greater efficiency of live yeast. Under the conditions of the present experiments yeast wholly, or in part, brought about pupation. The best yields of pupae and adults were obtained with dried yeast with crepe paper for larval and pupal attachment. The liver extract medium with crepe paper appeared to offer ideal conditions for the rapid growth of moulds like *Chaetomium*. Both *Chaetomium* and *Cephalosporium* are associated with cellulose matter and paper (Smith, 1954). The fungal growths have shown indication of control by mycostatin, judging by the fact that mechanical entanglement of larvae has not been observed in the presence of it. Within limits, therefore, liver extract and yeast media have been successful in the culture of the two species of *Mansonioides*.

The studies on these special culture media have clearly shown that both *M. uniformis* and *M. annulifera* responded to culture to the same degree. This could have been due to the more standard conditions of nutrition offered by the liver extract yeast media. The equal response shown by both species has directly resulted in elucidating the facts regarding the duration of the life cycle of the two species.

Although *M. annulifera* develops more rapidly than *M. uniformis* in mixed cultures the poorer response of *M. annulifera* to stock culture suggests that this species does not have an advantage over *M. uniformis*. The breeding of the two species in the same culture is in agreement with the occurrence in the field of both species side by side in certain localities in Ceylon. Wherever the two species are found breeding together *M. uniformis* outnumbers *M. annulifera*. Also whereas *M. uniformis* is widely distributed in swamps *M. annulifera* has a restricted distribution.

SUMMARY

Laboratory studies are reported on the culture and life cycle of *Mansonia* (*Mansonioides*) *uniformis* (Theobald) and *M.* (*M.*) *annulifera* (Theobald) from Ceylon.

Larvae of the two species were successfully reared in a medium of guinea-pig dung infusion in tap water with a sod of turf; dried yeast was added three times a week as larval food in addition to micro-organisms provided by infusion. *Salvinia*, at first, and later 37 lb strength crepe paper were used as attachment hosts for larvae and pupae. Later Diet 18 infusion gave similar successful results as the guinea-pig dung infusion. While *M. uniformis* was successfully reared throughout the period, *M. annulifera* did not last more than twelve generations.

Both species were cultured in a small scale with equal success in a more standard nutritive medium of 1.25 gm of oxo liver extract per litre of distilled water and different yeast products — crepe paper, mainly, being the attachment host. Yeast was essential for pupation. Better yields of adults and pupae were obtained from dried yeast cultures than from autoclaved yeast and yeast extract media. Fungal growths appearing in crepe paper causing mechanical entanglement of larvae and pupae were eliminated by mycostatin.

The life cycle in *M. annulifera* was shorter than in *M. uniformis*.

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