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DEVELOPMENT OF *BRUGIA PAHANGI* MICROFILARIAE FROM JIRD PERITONEAL CAVITY IN *AEDES AEGYPTI*

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Abstract: A new apparatus for feeding mosquitoes is described. It is constructed without special technique and easy of handling. The usefulness of the apparatus is demonstrated by a report on investigations into the effects of nucleotides on mosquito gorging and the development of the microfilariae from the jird peritoneal cavity and from the dog venous blood in *Aedes aegypti*. As a phagostimulant 10^{-3}M ATP is the most effective of the nucleotides tested. The microfilariae from the jird peritoneal cavity have been proved to reach stage III (i.e. infective stage) as those from the dog, although the rate is lower in the former. It is also proved that the microfilariae develop to infective larvae in the mosquitoes which have not taken blood, serum or protein meals.

INTRODUCTION

Brugia pahangi, whose normal hosts are cats and dogs (Buckley and Edeson, 1956; Laing *et al.*, 1960; Schacher, 1962 a, b), was successfully transmitted to the jird (*Meriones unguiculatus*) with subcutaneous inoculation of infective larvae (Ash and Riley, 1970; Ash, 1973). When the infective larvae were inoculated into the peritoneal cavity of the jird, adults and microfilariae were recovered from the peritoneal cavity (McCall *et al.*, 1973). The adults and microfilariae localized in the peritoneal cavity seem to follow an aberrant mode of development, although the ease of recovery of worms from the small rodents may serve for the studies on the biology, biochemistry, immunology and host-parasite relationships of filariasis. A search in the literature failed to reveal any report on the development of the microfilariae from the peritoneal cavity in mosquitoes.

There have been described several methods of feeding hematophagous arthropods artificially through natural and artificial membranes. Most of the devices have utilized a flat membrane extended in a horizontal plane. The devices with flat membranes have been used for mosquitoes by previous authors (St. John *et al.*, 1930; Woke, 1937; Bishop and Gilchrist, 1946; Eyles, 1952; Kartman, 1953; Bar-Zeev and Smith, 1959; Collins, 1963; Collins *et al.*, 1964; Rutledge *et al.*, 1964; Behin, 1967). In most of such devices it is difficult to maintain the solution offered to the arthropods at a certain temperature suitable for feeding. On the other hand, Pipkin and Connor (1968) have reported a device with a pouch-like membrane of rubber, sheep caecum or pig intestine sheathing a test tube with rounded bottom and also Kitaoka

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and Morii (1970) a feeder with a membrane of chicken crop covering a round bottom tube, which provide sufficient surface areas of the membranes for mass feeding of colonized vectors. In the latter devices the temperature of feeding solution can be maintained with ease, although relatively large quantity of feeding solution has to be filled in the space between the membrane and the tube.

Hosoi (1959) has examined a variety of chemicals dissolved in buffered saline offering to *Culex pipiens pallens* singly confined in a glass tube with a forcible feeding technique and reported that some nucleotides stimulate the mosquito into gorging with a blood meal, which is dispatched only to the stomach. Rutledge *et al.* (1964) found that adenosine triphosphate is a phagostimulant to some mosquitoes.

As the vector mosquitoes for *Brugia pahangi*, *Armigeres subalbatus* and/or *Aedes togoi* were used by previous authors (Ash and Riley, 1970; Ash, 1973; Kan and Ho, 1973; Nakajima *et al.*, 1976). In the present studies Liverpool strain of *Aedes aegypti* has been used because of ease of maintenance and feeding as well as susceptibility to *Brugia pahangi* infection.

This paper describes a new apparatus designed for feeding microfilariae to mosquitoes, the phagostimulant effect of nucleotides on *Aedes aegypti* and the development of microfilariae from the jird peritoneal cavity in the mosquito in comparison with those from the dog venous blood. It also discusses the effect of protein meals for mosquitoes on the development of larvae.

Materials and Methods

I. Feeding apparatus, solutions and mosquitoes

The feeding apparatus consisted of a glass jar about 8 cm in height with a flat bottom about 4 cm in diameter and a rubber stopper inserted with two cannulae. The cannulae were connected to a water pump with two flexible polyvinyl tubes. The inside of the jar was circulated with water from a thermostatically controlled water bath whose temperature was maintained at 38 C.

Mice were sacrificed under ether anesthesia. The fur on the skin was clipped off as closely as possible with a pair of electric clippers. The skin was then removed from the carcass and the subcutaneous and fatty tissues were eliminated. The skin was washed with saline and stored in a refrigerator or a freezer prior to use. When needed, the skin membrane was washed with saline and stretched, the subcutaneous side inward, over the bottom of the jar, to which it was held by a rubber band. A Pasteur pipette was inserted between the membrane and the side of the jar. Commercially available, salted outermost layer of sheep intestine and prophylactic rubber condoms were also tried as feeding membrane. Their use was eventually discontinued, because the former tended to be leaky after freshening and mosquitoes had difficulty in piercing the latter.

The feeding solutions tested were physiological saline, Dulbecco's phosphate-buffered saline without calcium or magnesium (PBS) and bovine serum. The bovine serum was light red in color due to hemolysis. The nucleotides used as phagostimulants were adenosine 5′-monophosphoric acid (from equine muscle,

Type V, Sigma Chemical Co.) (AMP), adenosine 5′-diphosphate (from equine muscle, Sodium salt, Grade I, Sigma Chemical Co.) (ADP) and adenosine 5′-triphosphate (disodium salt, P-L Biochemicals Inc.) (ATP) dissolved in the final concentration of 10^{-4} M or that of 10^{-3} M. The nucleotides were added to the cold solutions just prior to the experiment and placed in an ice bath until feeding.

Controls were the solutions without nucleotides as well as dog serum and

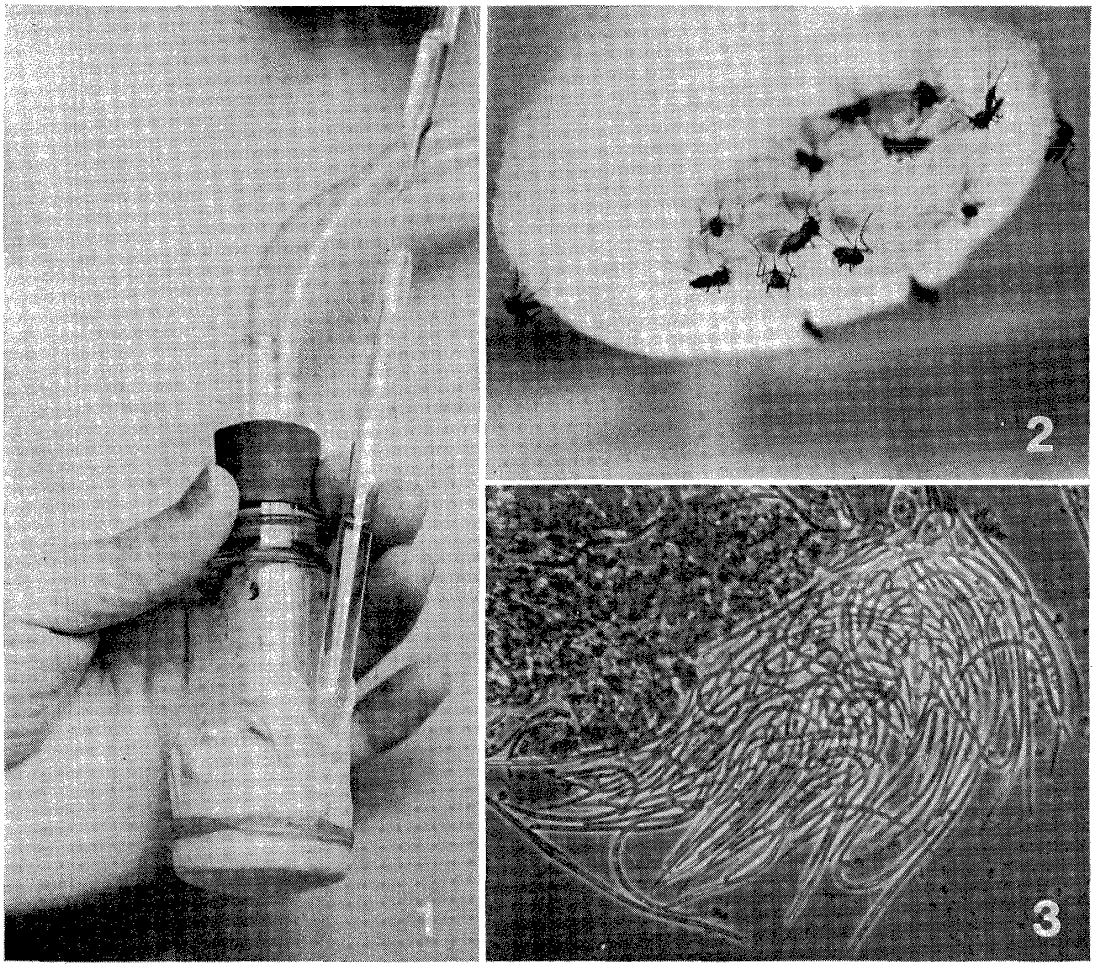

Figure 1 Feeding apparatus for mosquitoes.
A skin membrane is stretched over the flat bottom of the glass jar. Feeding solution is placed into the space between the jar bottom and the membrane through Pasteur pipette. Inside of the jar was circulated with water from a temperature-controlled water bath through a water pump, two flexible tubes and two cannulae.

Figure 2 Mosquitoes piercing the membrane of the apparatus hung in the cage and gorging feeding solution.

Figure 3 Phase-contrast microphotograph of stomach contents of *Aedes aegypti* dissected immediately after artificial feeding of microfilariae.
As shown in the figure, overcrowding of microfilariae in the stomach was occasionally observed in the group of mosquitoes offered the microfilariae from jird peritoneal cavity at the density of 1,000 to 1,500 per 0.03 m*l* of PBS.

defibrinated rabbit blood. In some experiments a drop of Indian ink or a drop of one per cent patent blue solution was added to each 2 to 3 m*l* solution for easy identification of the solutions in the alimentary tract of mosquitoes.

The feeding solution, 2 to 6 m*l*, was placed into the space between the jar bottom and the skin membrane through the Pasteur pipette, which was then removed (Figure 1). The side of the jar was covered by a polystyrene sheet.

The mosquitoes used were *Aedes aegypti* (Liverpool strain) which were maintained in our laboratory. For feeding trials 28 to 100 female mosquitoes were put into a cage about 40 cm long, 30 cm wide and 30 cm high. Age range was 5 to 7 days from the day of emergence. The diet, 2 per cent sucrose solution, was removed 24 hours before the trials.

The jar was hung in the cage through the top hole of the cage (Figure 2). The ambient temperature was 17 to 25.5 C. The cage was covered up with a sheet of black cloth. The test period for each cage was 30 minutes. Immediately after the feeding, the mosquitoes were anesthetized with ether and dissected under an operating microscope.

II. Infection of *Aedes aegypti* with *Brugia pahangi* microfilariae

Microfilariae were recovered from the peritoneal cavity of the infected jirds (*Meriones unguiculatus*) which had been inoculated intraperitoneally with the infective (i.e. stage III) larvae of *B. pahangi*. After the injection of 10 m*l* of PBS into the peritoneal cavity, the syringe was removed and the 18G×1 ½″ (1.2×38 mm) needle was left to recover the injected fluid containing microfilariae. The fluid dripping through the needle was collected, mixed with cold PBS and centrifuged for 5 min at 6,000 rpm, 0 C. The sediment was resuspended in cold PBS or bovine serum to yield 700 to 2,000 microfilariae per 0.03 m*l*. Just prior to the feeding, ATP was added in the final concentration of 10^{-3} M.

Controls were the direct feeding of *Aedes aegypti* (Liverpool strain) on an infected dog and the artificial feeding of the mosquitoes with the microfilariae obtained from the venous blood of the same dog. The microfilaria count of the dog was 495 per 0.03 m*l* of peripheral blood. The mosquitoes were allowed to feed for 15 min on the infected dog under ketamine hydrochloride (Ketalar^{R}) anesthesia. Venous blood was obtained from the vein of the hind limb of the dog with plastic syringes which were prepared by wetting the walls with 1,000 U/m*l* heparin. To each 5 m*l* of venous blood, 45 m*l* of cold one per cent saponin solution was added to attain hemolysis. The mixture was centrifuged for 5 min at 6,000 rpm, 0 C. The sediment was resuspended in cold saline and centrifuged under the same conditions twice. Then the sediment was resuspended in cold bovine serum and PBS to make 100 and 600 microfilariae per 0.03 m*l* respectively, to which 10^{-3} M ATP was added just prior to the feeding.

For feeding, 3 m*l* of solution was placed in the apparatus to a cage of 89 to 140 female mosquitoes. The mosquitoes were allowed to feed for 15 min at the ambient temperature of 25±1 C. To count the number of microfilariae ingested, the mosquitoes were anesthetized and dissected under an operating microscope immediately after feeding. To observe the exsheathing of microfilariae, 5 to 10 mosquitoes of

each cage were anesthetized and dissected every hour from one to 8 hours after feeding except 7 hours. The content of the stomach was smeared on a glass slide (Figure 3), dried rapidly, fixed with methylalcohol and stained with Giemsa solution. Also 5 to 10 mosquitoes per cage were dissected every day from one to 13 days after feeding to observe the development of larvae. The larvae were classified into three developing groups according to the morphological characteristics described by Schacher (1962 a). Measurement of body length was done on the infective larvae recovered from the mosquitoes dissected 11 days after feeding.

Results

I. Response of *Aedes aegypti* to nucleotide solutions

Physiological saline without nucleotide was not detected in the stomach nor in the diverticula of *Aedes aegypti* (Table 1). Saline containing 10^{-4} M AMP was

Table 1 Response of *Aedes aegypti* to nucleotides in saline with localization and degree of intake of solutions†

Nucleotide	No. of mosquitoes			
	dissected	fed	in stomach (degree of intake)††	in diverticula (degree of intake)††
None	30	0	0	0
AMP 10^{-4}M*	29	5	5 (p, 3: s, 2)	1 (s, 1)
ADP 10^{-4}M*	29	14	14 (p, 10: s, 4)	0
ATP 10^{-4}M*	30	18	18 (f, 4: p, 13: s, 1)	0
AMP 10^{-3}M**	94	81	80 (f, 27: p, 53)	1 (s, 1)
ADP 10^{-3}M**	83	80	80 (f, 27: p, 53)	0
ATP 10^{-3}M**	86	73	73 (f, 20: p, 53)	0

† Artificial feeding at ambient temperature of 25–25.5 C.

†† f; fully distended: p; partially distended: s; slightly distended.

* A drop of 1% patent blue solution was added to each 2 m*l*.

** A drop of Indian ink was added to each 3 m*l*.

found in 5 of 29 mosquitoes, in 3 of which the stomach was partially distended, in two slightly, and in one the diverticula also contained small quantity of the solution. Saline containing 10^{-4} M ADP was found in 14 of 29 mosquitoes; in 10 of them the stomach was partially distended and in four slightly. The phagostimulant effect of 10^{-4} M ADP was more marked than that of 10^{-4} M AMP: The difference in the stomach engorgement of the solution was significant ($p<0.0254$) in the chi square test. Saline containing 10^{-4} M ATP was found in 18 of 30 mosquitoes; in four of them the stomach was fully distended, in 13 partially and in one slightly. ATP was more effective than AMP: The difference in the stomach engorgement between 10^{-4} M AMP and 10^{-4} M ATP was statistically significant ($p<0.0019$). When 10^{-3} M nucleotides were added to saline, more mosquitoes gorged and the

rates of full stomach distension were higher than those in 10^{-4} M concentrations (Table 1): Using the chi square test the differences in the stomach engorgement were significant in AMP ($p<0.0001$), ADP ($p<0.0001$) and ATP ($p<0.0096$).

PBS without nucleotides was detected in 4 of 30 mosquitoes, in one of which the stomach was fully distended, in one partially and in two slightly (Table 2). When 10^{-4} M AMP or 10^{-4} M ADP was added to PBS, the rate of stomach intake seemed to be a little higher than the solution without nucleotides, but the computed values of chi square were not statistically significant. PBS containing 10^{-4} M ATP was engorged by 23 of 29 mosquitoes, in 22 of which the stomach was fully distended. As a phagostimulant, ATP was significantly more effective than AMP ($p<0.0015$) or ADP ($p<0.0003$). On the dissection, sucrose solution was found to remain in the diverticula of the mosquitoes offered 10^{-3} M nucleotides in PBS. It might probably have inhibited the feeding of nucleotide solutions to a certain extent. Only 46 of 90 mosquitoes took 10^{-3} M AMP solution (Table 2). Although the rate of engorgement was significantly higher than the control solution ($p<0.0015$), the difference between 10^{-4} M AMP and 10^{-3} M AMP was not statistically significant. On the other hand, the difference between 10^{-4} M ADP and 10^{-3} M ADP was highly significant ($p<0.0002$). The rate of engorgement of 10^{-3} M ADP in PBS was higher than that of 10^{-3} M AMP in PBS: The difference was statistically significant ($p<0.032$). As a phagostimulant in PBS, 10^{-3} M ATP was more effective than 10^{-3} M ADP: The difference was also significant ($p<0.020$).

Table 2 Response to nucleotides in PBS with localization and degree of intake of solutions†

Nucleotide	No. of mosquitoes			
	dissected	fed	in stomach (degree of intake)††	in diverticula (degree of intake)††
None	30	4	4 (f, 1: p, 1: s, 2)	0
AMP 10^{-4}M*	30	9	9 (f, 1: p, 1: s, 7)	0
ADP 10^{-4}M*	30	7	7 (f, 7)	3 (s, 3)
ATP 10^{-4}M*	29	23	23 (f, 22: p, 1)	3 (p, 3)
AMP 10^{-3}M**	90***	46	46 (f, 19: p, 27)	0
ADP 10^{-3}M**	93***	63	63 (f, 34: p, 29)	0
ATP 10^{-3}M**	96***	80	79 (f, 34: p, 45)	1 (p, 1)

† Artificial feeding at ambient temperature of 22–24 C.

†† f; fully distended: p; partially distended: s; slightly distended.

* A drop of 1% patent blue solution was added to each 2 m*l*.

** A drop of Indian ink was added to each 3 m*l*.

*** Sucrose solution remained in diverticula.

Bovine serum was light red in color due to hemolysis. At the ambient temperature of 17 C, bovine serum without nucleotides was taken by 19 of 30 mosquitoes, in 18 of which serum was detected in the stomach and in three in the diverticula (Table 3). Only 8 of 30 mosquitoes took dog serum without nucleotides, which had been obtained without apparent hemolysis. The difference in the stomach engorgement

between bovine serum and dog serum was statistically significant ($p<0.0191$). The addition of 10^{-4} M AMP or 10^{-4} M ADP to bovine serum did not enhance the rate of feeding: The computed values of chi square were not statistically significant. On the other hand, the addition of 10^{-4} M ATP stimulated a little the stomach engorgement: The difference was statistically significant ($p<0.0487$). At the ambient temperature of 25 C, 39 of 100 mosquitoes gorged bovine serum without nucleotides; only in eight of them the stomach was fully distended and in 31 partially (Table 4). The addition of 10^{-3} M nucleotides to serum enhanced the rates of feeding and full stomach distension. The differences in the stomach engorgement were highly significant ($p<0.0001$) in the three nucleotides. However, the addition of 10^{-3} M AMP to bovine serum was not so effective as that of 10^{-3} M ADP or of 10^{-3} M ATP. When ADP or ATP was added, the rate of feeding was equal to that in rabbit defibrinated blood. The difference between 10^{-3} M AMP and rabbit defibrinated blood was highly significant ($p<0.0012$), while the computed values of

Table 3 Response to 10^{-4}M nucleotides in bovine serum† and to dog serum††

Nucleotide	No. of mosquitoes			
	dissected	fed	in stomach (degree of intake)**	in diverticula (degree of intake)**
None, bovine serum*	30	19	18 (f, 4: p, 2: s, 12)	3 (s, 3)
AMP 10^{-4}M*	29	18	18 (f, 4: p, 5: s, 9)	3 (p, 1: s, 2)
ADP 10^{-4}M*	28	22	22 (f, 6: p, 4: s, 12)	3 (p, 1: s, 2)
ATP 10^{-4}M*	29	25	25 (f, 20: s, 5)	2 (s, 2)
None, dog serum	30	8	8 (f, 1: p, 1: s, 6)	0

† Bovine serum was a little hemolysed.

†† Artificial feeding at ambient temperature of 17 C.

* A drop of 1% patent blue solution was added to each 2 m*l*.

** f; fully distended: p; partially distended: s; slightly distended.

Table 4 Response to 10^{-3}M nucleotides in bovine serum† and to rabbit defibrinated blood††

Nucleotide	No. of mosquitoes			
	dissected	fed	in stomach (degree of intake)**	in diverticula
None, bovine serum*	100	39	39 (f, 8: p, 31)	0
AMP 10^{-3}M*	95	74	74 (f, 40: p, 34)	0
ADP 10^{-3}M*	94	90	90 (f, 77: p, 13)	0
ATP 10^{-3}M*	100	96	96 (f, 75: p, 21)	0
None, rabbit defibrinated blood*	89	85	85 (f, 68: p, 17)	0

† Bovine serum was a little hemolysed.

†† Artificial feeding at ambient temperature of 25 C.

* A drop of Indian ink was added to each 3 m*l*.

** f; fully distended: p; partially distended: s; slightly distended.

chi square were not statistically significant in ADP and ATP. The rate of full stomach distension was also lower in AMP than in ADP, ATP or defibrinated blood (Table 4).

These results indicated that ATP was the most effective phagostimulant to *Aedes aegypti*, that ADP was next to ATP and that AMP was the least of three nucleotides. It was also clear that 10^{-3} M concentration was generally more effective than 10^{-4} M concentration.

II. Artificial feeding and development of larvae

The mosquitoes which fed directly on the dog with microfilaremia of 495 per 0.03 m*l* of peripheral blood became infected at a rate of 88.5 per cent (Table 5). At the dissection immediately after feeding, the average number of microfilariae in the stomach of the infected mosquito was 18.0 and the range was two to 75. The mosquitoes fed on PBS containing the microfilariae from the dog venous blood at the density of 600 per 0.03 m*l* became infected at a rate of 92.3 per cent. The number of microfilariae in the stomach of the infected mosquito was 21.5 on an average, varying from four to 47. The mosquitoes taken PBS containing the microfilariae from the jird peritoneal cavity at the density of 700 per 0.03 m*l* became infected at a rate of 33.3 per cent. The average number of microfilariae per the infected was 38.0 and the range was five to 204. When the density was raised to 1,000 in PBS, the infection rate was 77.7 per cent, and the average number was 204.0 with the range of four to 653. The microfilarial density of 1,500 in PBS produced the infection rate of 84.2 per cent and the average number of 336.6 with the range of one to 1,213. Thus, the infection rate seemed to be lower in the artificial feeding with PBS than in the direct feeding, if the microfilarial counts were similar in both groups.

Table 5 Infection rates in mosquitoes and numbers of microfilariae in stomach on dissection immediately after feeding

Source of mf.	Feeding medium*	Mf. count per 0.03 m*l*	No. of mosquitoes		Mean no. of mf. per infected (range)
			dissected	infected (%)	
Dog peripheral blood	(direct)	495	26	23 (88.5)	18.0 (2–75)
Dog venous blood	PBS	600	13	12 (92.3)	21.5 (4–47)
	bovine serum	100	12	5 (41.7)	5.2 (2–9)
Jird peritoneal cavity	PBS	700	27	9 (33.3)	38.0 (5–204)
		1,000	27	21 (77.7)	204.0 (4–653)
		1,500	19	16 (84.2)	336.6 (1–1,213)
	bovine serum	1,000	20	15 (75.0)	19.0 (1–79)
		1,500	20	20 (100)	180.2 (3–505)
		2,000	20	20 (100)	137.8 (1–1,002)

* As phagostimulant 10^{-3}M ATP was contained.

The average number of microfilariae per infected mosquito was greater and the individual count per mosquito varied more widely in the artificial feeding with PBS than in the direct feeding. The infection rates with bovine serum were similar to those with PBS (Table 5). The computed value of chi square was not statistically significant at the density of 1,000. In bovine serum, however, the average number of microfilariae per the infected seemed to be less and the individual count per mosquito varied less widely than in PBS.

The microfilariae from the dog venous blood exsheathed in the stomach of *Aedes aegypti* after the artificial feeding. Exsheathing reached the high percentage of 38.9 at one hour and that of 54.8 at 2 hours after the feeding with bovine serum (Table 6). Thereafter, the percentage of the exsheathed microfilariae among the ones remaining in the stomach ranged from 26.8 to 58.4. Exsheathing of the microfilariae from the dog venous blood occurred at lower rates with PBS than with bovine serum: The percentages were only 6.4 to 18.4 with PBS.

Table 6 Exsheathing of microfilariae in stomach after artificial feeding

Source of mf.	Feeding medium*	No. of dissected mosquitoes	No. of exsheathed mf./total no. of mf. (%) on hourly dissection after feeding**							
			1	2	3	4	5	6	8	total
Dog venous blood	PBS	50	39/610 (6.4)	19/156 (12.2)	51/440 (11.6)	33/399 (8.3)	20/140 (14.3)	N.D.	16/87 (18.4)	178/1,832 (9.7)
	bovine serum	52	21/54 (38.9)	51/93 (54.8)	15/56 (26.8)	34/81 (42.0)	31/70 (44.3)	66/113 (58.4)	21/53 (39.6)	239/520 (46.0)
Jird peritoneal cavity	PBS	31	2/740 (0.3)	0/111 (0)	5/257 (1.9)	16/433 (3.7)	16/693 (2.3)	9/312 (2.9)	1/988 (0.1)	49/3,534 (1.4)
	bovine serum	57	0/452 (0)	4/427 (0.9)	3/109 (2.8)	14/112 (12.5)	12/130 (9.2)	35/137 (25.5)	32/174 (18.6)	100/1,541 (6.5)

* As phagostimulant 10^{-3}M ATP was contained.

** Dissection was not done 7 hours after feeding.

The microfilariae from the jird peritoneal cavity exsheathed also in the stomach of mosquito, although less than one per cent of the microfilariae exsheathed at one hour and 2 hours after the feeding both with PBS and bovine serum. After 4 hours of feeding the percentage of the exsheathed microfilariae varied from 9.2 to 25.5 with bovine serum, while less than 4 per cent of microfilariae exsheathed with PBS. Thus, the microfilariae from the jird peritoneal cavity exsheathed at much lower rates than those from the dog venous blood with both media. The differences in the overall exsheathing between the microfilariae from the jird peritoneal cavity and those from the dog venous blood were highly significant ($p<0.0001$) in both media.

As shown in Table 7, the death rates of mosquitoes were lower in the groups taken the microfilariae from the jird peritoneal cavity than in the groups received the microfilariae from the dog venous blood, although the density of microfilariae in the feeding medium and the average number of microfilariae taken up by a mosquito were higher in the former groups. The difference in the death rate between the

Table 7 Numbers of deaths in mosquitoes 1 to 11 days after artificial feeding

Source of mf.	Feeding medium*	Mf. count per 0.03 m*l*	Total no. of mosquitoes	Mean no. of mf. per infected (range)**	No. of mosquitoes died on days after feeding											total
					1	2	3	4	5	6	7	8	9	10	11	
Dog venous blood	PBS	600	89	21.5 (4–47)	1	3	24	7	1	0	2	0	0	0	0	38 (42.7)
	bovine serum	100	114	5.2 (2–9)	0	8	8	22	3	0	4	0	0	0	0	45 (39.5)
Jird peritoneal cavity	PBS	700	132	38.0 (5–204)	0	0	1	3	7	6	8	3	5	1	0	34 (25.8)
		1,000	122	204.0 (4–653)	0	3	4	0	2	2	0	0	0	0	0	11 (9.0)
		1,500	105	336.6 (1–1,213)	1	9	3	0	0	0	1	2	1	0	0	17 (16.2)
	bovine serum	1,000	140	19.0 (1–79)	1	0	1	0	5	5	4	10	3	1	1	31 (22.1)
		1,500	97	180.2 (3–505)	4	1	1	0	0	0	0	1	1	1	0	9 (9.3)
		2,000	100	137.8 (1–1,002)	5	2	0	0	0	0	0	0	0	1	0	8 (8.0)

* As phagostimulant 10^{-3}M ATP was contained.

** Numbers immediately after feeding.

group received the microfilariae from the dog at the density of 600 per 0.03 m*l* in PBS and the group taken those from the jird at the density of 700 in PBS was statistically significant ($p<0.013$). Also the difference was significant ($p<0.0044$) between the group received the microfilariae from the dog at the density of 100 in bovine serum and that taken the microfilariae from the jird at the density of 1,000 in bovine serum. The mosquitoes received the microfilariae from the dog most frequently died 3 or 4 days after feeding. In the groups taken those from the jird at the density of 700 in PBS and at that of 1,000 in bovine serum, most deaths occurred 5 to 7 days after feeding. When the density was higher, most deaths occurred much earlier, within one to 3 days of infection.

On and after one day of infection, the infection rate of mosquitoes taken the microfilariae from the dog venous blood at the density of 600 in PBS was as high as that in the group which fed directly on the dog (Table 8). In the group offered the microfilariae from the dog at the low density of 100 in bovine serum, the infection rate was as low as 12.9 per cent 10 to 13 days after feeding. In the groups given the microfilariae from the jird in PBS, the infection rates were low; although the density of those from the jird was raised to 1,000 or 1,500, the rates at the period between 10 to 13 days of infection seemed to be lower than that of the microfilariae from the dog offered at the density of 600. Also the rates were low in the groups received the microfilariae from the jird in bovine serum. The infection rate was 30 per cent in the mosquitoes given the microfilariae from the jird at the density of 1,000 in bovine serum 10 to 13 days after feeding, seemingly higher than that in the mosquitoes taken the microfilariae at the density of 100. But the difference was not statistically significant. When the density of those from the jird was raised to 1,500 or 2,000 in bovine serum, the rate at the same period became significantly higher ($p<0.039$ at 1,500; $p<0.0017$ at 2,000) than that of the microfilariae from the dog given at the density of 100.

Table 8 Infection rates in mosquitoes one to 13 days after direct or artificial feeding

Source of mf.	Feeding medium*	Mf. count per 0.03 m*l*	No. of mosquitoes Infected/dissected (%) for periods		
			1–5 days	6–9 days	10–13 days
Dog peripheral blood	(direct)	495	18/25 (72.0)	16/20 (80.0)	9/10 (90.0)
Dog venous blood	PBS	600	13/15 (86.7)	12/13 (92.3)	9/10 (90.0)
	bovine serum	100	5/14 (35.7)	2/12 (16.7)	4/31 (12.9)
Jird peritoneal cavity	PBS	700	25/61 (41.0)	10/42 (23.8)	25/76 (32.9)
		1,000	42/63 (66.7)	29/42 (69.0)	51/75 (68.0)
		1,500	20/27 (74.1)	17/22 (77.3)	11/20 (55.0)
	bovine serum	1,000	14/28 (50.0)	2/21 (9.5)	12/40 (30.0)
		1,500	22/27 (81.5)	11/21 (52.4)	10/20 (50.0)
		2,000	21/27 (77.8)	13/20 (65.0)	14/25 (56.0)

* As phagostimulant 10^{-3}M ATP was contained.

The individual counts of larvae recovered from the mosquitoes fed directly on the dog did not vary widely throughout the development of larvae (Table 9). In this group, the average number of larvae per mosquito was 5.7 to 14.0 during the period between one and 3 days of infection and did not fluctuate widely in the subsequent period of infection. In the mosquitoes given the microfilariae from the dog venous blood at the density of 600 per 0.03 m*l* in PBS, the average number of larvae per mosquito was equal to or somewhat higher than that of direct feeding group, although the individual counts of larvae varied more widely. In the mosquitoes fed on the microfilariae from the dog venous blood at the low density of 100 in bovine serum, only a few larvae were recovered even in the early period of infection.

The average number of larvae per mosquito was much lower in the group offered the microfilariae from the jird peritoneal cavity at the density of 700 per 0.03 m*l* in PBS than in the group taken those from the dog venous blood at the density of 600 (Table 9): Only 2 of 179 mosquitoes dissected during 13 days of infection harbored 10 larvae or more. When the microfilarial density in PBS was raised to 1,000, the average number approached to that of the direct feeding group. In the group fed on PBS containing the microfilariae at the density of 1,500, the average number was higher than that of the direct feeding group during the period between one and 6 days of infection and equal to that of the latter subsequently. In the groups given the microfilariae from the jird peritoneal cavity with bovine serum, the average number of larvae per mosquito was far below that of the direct feeding group. In the artificial feeding groups, especially those given the microfilariae with PBS, the individual counts of larvae recovered from the mosquitoes varied widely, more frequently in the early period of infection than in the late period.

Table 9 Numbers of larvae developed in mosquitoes after direct or artificial feeding

Source of mf.	Feeding medium*	Mf. count per 0.03 m*l*	Mean no. of larvae per infected mosquito (range) on days after feeding					
			1	2	3	4	5	6
Dog peripheral blood	(direct)	495	14.0 (4–26)	5.7 (1–13)	9.3 (2–16)	4.7 (1–8)	8.3 (4–12)	3.0 (1–6)
Dog venous blood	PBS	600	9.3 (8–12)	19.3 (3–35)	27.5 (4–51)	5.0 (2–7)	9.5 (3–16)	30.0 (2–57)
	bovine serum	100	4.0 (4–4)	1.0 (1–1)	6.0 (6–6)	4.0 (4–4)	1.0 (1–1)	ND
Jird peritoneal cavity	PBS	700	3.0 (1–4)	4.0 (1–8)	1.3 (1–2)	1.6 (1–3)	1.0 (1–1)	1.0 (1–1)
		1,000	5.0 (1–9)	18.6 (1–72)	18.0 (2–48)	9.8 (2–25)	5.4 (1–19)	10.7 (1–23)
		1,500	50.5 (16–115)	4.0 (2–8)	21.3 (1–61)	15.0 (3–30)	16.3 (13–20)	20.3 (3–57)
	bovine serum	1,000	2.3 (1–4)	1.0 (1–1)	2.0 (1–3)	2.5 (2–3)	2.0 (1–3)	ND
		1,500	27.0 (3–61)	4.3 (3–7)	4.5 (1–12)	6.3 (1–16)	4.0 (3–6)	5.0 (1–11)
		2,000	3.8 (2–8)	6.3 (2–12)	3.8 (1–7)	3.8 (1–8)	5.3 (1–13)	2.5 (1–4)

Source of mf.	Mean no. of larvae per infected mosquito (range) on days after feeding						
	7	8	9	10	11	12	13
Dog peripheral blood	8.3 (7–9)	4.8 (2–10)	6.8 (4–12)	9.6 (3–16)	10.8 (5–20)	ND	ND
Dog venous blood	17.3 (2–41)	10.0 (4–16)	6.7 (2–12)	14.7 (3–21)	11.8 (4–21)	ND	ND
	1.0 (1–1)	1.0 (1–1)	ND	1.0 (1–1)	1.0 (1–1)	ND	ND
Jird peritoneal cavity	6.0 (3–9)	2.7 (1–5)	8.5 (2–15)	1.0 (1–1)	1.1 (1–2)	1.8 (1–3)	2.7 (1–10)
	1.9 (1–3)	8.2 (1–17)	8.7 (1–21)	9.2 (1–25)	10.8 (1–44)	5.8 (1–18)	1.8 (1–4)
	8.3 (1–24)	15.0 (3–45)	6.4 (1–14)	6.3 (3–15)	10.6 (5–15)	ND	ND
	ND	1.0 (1–1)	ND	1.0 (1–1)	1.6 (1–3)	ND	ND
	3.0 (1–7)	2.6 (2–3)	1.5 (1–2)	3.7 (2–5)	4.6 (2–10)	ND	ND
	6.0 (4–8)	4.5 (1–7)	3.0 (1–6)	2.2 (1–4)	7.0 (1–16)	ND	ND

* As phagostimulant 10^{-3}M ATP was contained.

As shown in Figure 4, stage I larvae were detected in the thoracic muscle of the mosquitoes fed directly on the dog and dissected one to 5 days after feeding. Only stage II larvae were found in the direct feeding mosquitoes dissected 6 to 9 days after feeding. In the mosquitoes dissected on 10 days of infection, 72.9 per cent of larvae were still in stage II and localized in the thoracic muscle, while 8.9 per cent of larvae had reached stage III and were detected in the head, and 18.8 per cent had also reached stage III and were found in the abdomen. The mosquitoes dissected on 11 days harbored stage II larvae in the thoracic muscle (11.6%), and stage III larvae in the head (74.4%), in the thorax (4.7%) and in the abdomen (9.3%). The larvae in the mosquitoes received the microfilariae from the dog venous blood followed the gradual development of those in the direct feeding mosquitoes without much delay. Only stage I larvae were found in the thoracic muscle of the mosquitoes which took up the microfilariae from the dog venous blood in PBS and were dissected one to 5 days after feeding. In this group of mosquitoes dissected on 6 days of infection, 3.3 per cent of larvae were still in stage I and 96.7 per cent had reached stage II in the thoracic muscle. All larvae found in the mosquitoes dissected 7 to 9 days after feeding were in stage II and localized in the thoracic muscle. On 10 days of infection 47.7 per cent of larvae were still in stage II localized in the thoracic muscle, while 18.2 per cent had attained stage III in the head, 29.5 per cent had attained the same stage in the thorax and 4.6 per cent in the abdomen. The mosquitoes dissected on 11 days had stage III larvae in the head (69%), thorax (11.3%) and abdomen

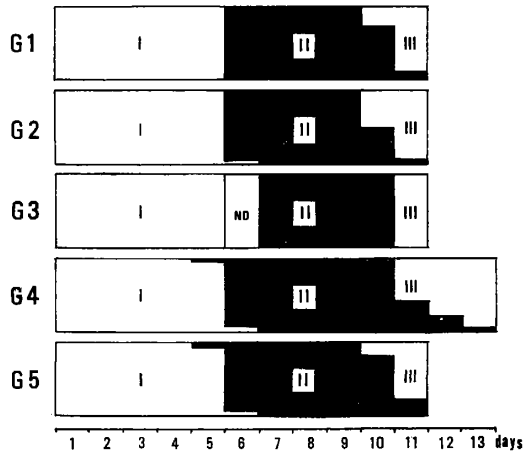

Figure 4 Development of *Brugia pahangi* larvae in *Aedes aegypti* after direct and artificial feeding.

G 1, direct feeding on an infected dog; G 2, microfilariae from dog venous blood fed artificially with PBS; G 3, microfilariae from dog venous blood fed artificially with bovine serum; G 4, microfilariae from jird peritoneal cavity fed artificially with PBS (three experiments with different microfilarial densities combined); G 5, microfilariae from jird peritoneal cavity fed artificially with bovine serum (three experiments with different microfilarial densities combined); I, stage I larvae; II, stage II larvae; III, stage III larvae.

(2.7%), besides stage II larvae in the thoracic muscle (7%). The number of infected mosquitoes were small, only 11, in the group which were offered the microfilariae from the dog venous blood in bovine serum and dissected one to 11 days after feeding. In this group all larvae found at one to 5 days' dissection were in stage I. Only stage II larvae were detected 7 to 10 days after feeding. All larvae reached stage III on 11 days.

In the group of mosquitoes received the microfilariae from the jird peritoneal cavity in PBS at the density of 700, only stage I larvae were found on 5 days of infection, and only stage II larvae were detected on 6 days. On the other hand, stage II larvae were found on 5 days at a rate of 3.7 per cent in the group offered the microfilariae from the jird in PBS at the density of 1,000 and at a rate of 2.5 per cent in the group offered at 1,500. On 6 days of infection, 10.3 per cent of larvae were still in stage I in the group taken the microfilariae at 1,000 in PBS, and all larvae reached stage II in the group taken at 1,500. Stage II larvae were found at a rate of 12.5 per cent in the group received the microfilariae from the jird in bovine serum at the density of 1,500 on 5 days, while only stage I larvae were detected in the group received at 1,000 and that at 2,000. On 6 days 6.7 per cent were still in stage I in the group received the microfilariae at 1,500, and all larvae reached stage II in that at 2,000. Thus, some of the microfilariae from the jird peritoneal cavity reached stage II one day earlier than the microfilariae from the dog. On the other hand, stage I larvae were still found on 6 days in the groups taken the microfilariae from the jird peritoneal cavity as in the group received the microfilariae from the dog venous blood. The development of the microfilariae from the jird to stage II seemed to be not influenced by the density of microfilariae in the medium, nor by the nature of the medium (i.e. PBS or bovine serum).

In the groups received the microfilariae from the jird in PBS at the density of 700 and 1,000, all larvae were still in stage II on 10 days of infection, and stage II larvae were 25.0 per cent and 43.4 per cent respectively on 11 days. Stage II larvae were still found on 12 days at a rate of 11.1 per cent and 20.0 per cent respectively. In the former group 7.4 per cent of larvae remained at stage II even on 13 days of infection. In the group taken the microfilariae from the jird in PBS at the density of 1,500, stage II larvae were 96.0 per cent on 10 days and 17.6 per cent on 11 days. In the mosquitoes received the microfilariae from the jird in bovine serum, the rate of stage II larvae on 10 days was 33.3 per cent at the density of 1,000, 81.8 per cent at 1,500 and 72.2 per cent at 2,000, and that on 11 days was 28.6 per cent, 25.0 per cent and 17.0 per cent respectively. Thus, the development of the microfilariae from the jird peritoneal cavity into the final form in the mosquito seemed to be slower than that of the microfilariae in the direct feeding on the dog and that of the microfilariae from the dog venous blood (Figure 4). Also the rate of stage III larvae situated in the head was 26.7 to 41.9 per cent on 11 days in the groups taken the microfilariae from the jird, much lower than that in the groups fed on the microfilariae from the dog.

On the dissection 10 to 11 days after feeding, 9 of 10 mosquitoes which fed directly on the dog harbored the infective (i.e. stage III) larvae (Table 10). The number of infective larvae per infected mosquito ranged from one to 17 with an

Table 10 Numbers of stage III larvae in mosquitoes 10 to 11 days after feeding and lengths of larvae

Source of mf.	Feeding medium*	Mf. count per 0.03 m*l*	No. of mosquitoes dissected	No. of mosquitoes with stage III larvae (%)	Mean no. of stage III larvae per infected (range)	Range of lengths (mean)**
Dog peripheral blood	(direct)	495	10	9 (90.0)	5.7 (1–17)	1,422–1,676 (1,464.3)
Dog venous blood	PBS	600	10	9 (90.0)	9.9 (1–21)	1,294–1,627 (1,431.6)
	bovine serum	100	31	3 (9.7)	1.0 (1–1)	ND
Jird peritoneal cavity	PBS	700	76	18 (23.7)	2.2 (1–9)	1,451–1,789 (1,584.2)
		1,000	75	43 (57.3)	5.8 (1–21)	
		1,500	20	8 (40.0)	7.8 (1–11)	
	bovine serum	1,000	40	10 (25.0)	1.2 (1–2)	1,235–1,643 (1,440.0)
		1,500	20	8 (40.0)	3.3 (1–7)	
		2,000	25	11 (44.0)	5.0 (1–13)	

* As phagostimulant 10^{-3}M ATP was contained.

** Lengths in micron on 11 days.

average of 5.7. The length of infective larva on 11 days varied between 1,422 μ and 1,676 μ with an average of 1,464.3 μ. In the group taken the microfilariae from the dog venous blood in PBS at the density of 600, the percentage of mosquitoes harboring the infective larvae was 90 per cent, and the average number of infective larvae was 9.9, somewhat higher than the direct feeding group. In the mosquitoes taken the microfilariae at the density of 100, the infection rate was low and the number of larvae was small. In the groups received the microfilariae from the jird, the percentage of mosquitoes harboring the infective larvae was 23.7 to 57.3 per cent, lower than that in the mosquitoes fed directly on the dog and in those offered the microfilariae from the dog in PBS. The average number of infective larvae was also smaller in the groups received the microfilariae from the jird than in the group taken the microfilariae from the dog at the density of 600. The lengths of infective larvae in the groups received the microfilariae from the jird in bovine serum were similar to those in the group fed on the microfilariae from the dog in PBS. The length of infective larva in the mosquitoes offered the microfilariae from the jird in PBS varied between 1,451 μ and 1,789 μ with an average of 1,584.2 μ

Discussion

A new apparatus for feeding mosquitoes is small and easy of handling without a sophisticated self-contained heater and thermistor. Its construction is economical and involves no special techniques, using a plain laboratory glassware, mouse skin

etc. It can be used with any mosquito cage that has a top hole. The capacity for feeding medium is approximately 10 m*l*. When the conservation of medium is important, 2 m*l* or less can be used. Thus, the usefulness of the new apparatus is demonstrated in the present studies.

In the present studies ATP is the most effective phagostimulant of the nucleotides dissolved in saline, PBS or bovine serum. Hosoi (1959) reported that AMP and ADP were more effective than ATP to *Culex pipiens pallens* with a kind of forcible feeding. The difference in the species of mosquitoes and/or feeding techniques may have caused disagreement between the results. The fact that nucleotides in 10^{-3} M concentration are generally more effective than those in 10^{-4} M concentration is in accord with observations by Rutledge *et al.* (1964) that 0.005 M was near the optimum of ATP to Bangkok strain of *Aedes aegypti.* Thus, 10^{-3} M ATP is an adequate phagostimulant for the artificial feeding of Liverpool strain of *Aedes aegypti.*

Some microfilariae from the dog venous blood become inactive within 30 min in 5 per cent glucose solution or saline both containing 10^{-3} M nucleotides at 37 C, while inactive microfilariae are detected after 90 min in PBS containing 10^{-3} M nucleotides and after 20 hours in bovine serum containing them (unpublished data). So, PBS and bovine serum are suitable for the media for microfilarial feeding.

It is suggested that microfilariae are maldistributed in PBS, twining round debris, by the findings that the infection rates in mosquitoes are lower for the artificial feeding with PBS than for the direct feeding and that the individual count of microfilariae per mosquito varies widely in that with PBS, when the microfilarial densities are similar in both groups. In the artificial feeding with bovine serum, the infection rates are low as in that with PBS, although the microfilarial densities are higher than in the direct feeding group. However, the average numbers of microfilariae per infected mosquito are lower and the individual count per mosquito varies less widely with bovine serum than with PBS. It seems likely that the maldistribution of microfilariae is less in bovine serum than in PBS, probably due to the viscosity of serum.

The exsheathing of microfilariae from both the dog venous blood and the jird peritoneal cavity occurs higher in the artificial feeding with bovine serum than in that with PBS. The exsheathing may be disturbed in the mosquitoes taken up a number of microfilariae by the overcrowding in the stomach: it is more frequent with PBS than with bovine serum as indicated by the individual count per mosquito. It is also possible that the viscosity of serum facilitates the exsheathing in the stomach. Aoki (1971 a, b) and Aoki *et al.* (1976) reported that microfilariae exsheathed well on agar pads. The findings support the view that the exsheathing is switched on by the change of milieu toward solidification.

The death rates of mosquitoes are lower in the groups taken the microfilariae from the jird peritoneal cavity than in the groups received those from the dog venous blood. It is of interest to speculate whether the developing larvae inflict damage on mosquitoes (Rosen, 1955; Wharton, 1957; Townson, 1970; Hockmeyer *et al.*, 1975; Christensen, 1978), and whether some microfilariae from the jird are inadequate to the development, which results in the low death rate of mosquitoes. It may be also that most deaths occurring one to 3 days after feeding in the group taken high density of microfilariae are brought by a number of microfilariae penetrating the stomach

wall and developing in the thoracic muscle as stage I larvae.

On and after one day of infection, the rates of infected mosquitoes are low in the group taken the microfilariae from the dog venous blood at the density of 100 per 0.03 *ml* in bovine serum: it seems likely that the low rates of infection are brought by the low density of microfilariae offered. In the artificial feeding groups, especially those with PBS, individual counts of larvae vary widely one to 3 days of infection. It probably reflects the fact that the number of microfilariae taken varies greatly among the mosquitoes in the same groups.

The microfilariae from the jird peritoneal cavity exsheathe in both PBS and bovine serum at lower rates than those from the dog venous blood. The rate of infected mosquito is low in the groups received the jird microfilariae: 10 to 13 days after feeding, the rate in the group offered the jird microfilariae at the density of 1,000 is not significantly different from that in the group taken the dog microfilariae at the density of 100. The average number of larvae per infected tends to be low in the group taken the jird microfilariae. These findings suggest that the microfilariae from the jird peritoneal cavity includes the ones inadequate to the development in the mosquitoes, such as the newborn and the aged, at a higher rate than those from the dog venous blood.

Some microfilariae from the jird peritoneal cavity reach stage II one day earlier than the microfilariae from the dog. However, the development of the microfilariae from the jird to stage III is generally slower than that of the microfilariae from the dog. It probably causes the low rate in the mosquitoes harboring the stage III larvae and the low average number of stage III larvae per infected in the group received the microfilariae from the jird and dissected 10 to 11 days after feeding. It is clear from the present findings that the microfilariae develop to the stage III larvae in the mosquitoes which have not taken blood, serum or protein meals, and that the infective larvae sometimes become even longer with PBS.

Brugia pahangi inoculated, localized and producing microfilariae intraperitoneally and the microfilariae thus produced and localized in the peritoneal cavity seem to follow an aberrant mode of development. The present studies, however, have revealed that the microfilariae from the jird peritoneal cavity can reach stage III in *Aedes aegypti*, although at a lower rate than the microfilariae produced by the lymphatic adults in a natural host.

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スナネズミ腹腔より得た *Brugia pahangi* ミクロフィラリアの *Aedes aegypti* における発育

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蚊に対する人工摂取装置を新たに考案した。製作に特殊な技術を要せず，使用に便利である。その装置を用いて，*Aedes aegypti* の胃内摂取に対するヌクレオチドの影響と，スナネズミ腹腔ならびにイヌ静脈血より得たミクロフィラリアの，*Aedes aegypti* における発育の比較を行った。摂食刺戟物としては，用いたヌクレオチドの中で，10^{-3} M ATP が最も有効である。イヌ静脈血より得たミクロフィラリアと同じく，スナネズミ腹腔より得たものは，第 III 期幼虫（感染幼虫）に成長することが証明されたが，その率はイヌ静脈血より得たものより低い。また，血液，血清，蛋白を食物として摂取していない蚊においても，ミクロフィラリアが感染幼虫になることが証明された。

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