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Deposition and Development of Eggs of *Angiostrongylus cantonensis in Vitro*

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Studies on the *in vitro* cultivation of *Angiostrongylus cantonensis* have been limited. The first presentation concerning the *in vitro* cultivation of *A. cantonensis* was done by Weinstein *et al.* (1962). They cultured the adults in medium NCTC 109 supplemented with horse, rat or calf serum for 64 to 80 days, and 1 to 6 % of eggs deposited *in vitro* were hatched during further cultivation.

Moreau and Lagraulet (1972) and Blockelman *et al.* (1979) have also cultivated the third or the first stage larvae of *A. cantonensis in vitro* but the development and/or nutritional requirement for the cultivation have not been described in detail.

We have reported on the *in vitro* cultivation of *A. cantonensis* eggs collected from the uterus of adult worms (Uga and Matsumura, 1982), and discussed culture conditions such as optimal serum species and its concentration. In NCTC 109 with 50 % normal rat serum, about 60 % of the eggs embryonated after 5 days of cultivation and the hatching began after 8 days of cultivation. However, there are no studies on the maintenance of adult *A. cantonensis* worms and their egg deposition *in vitro* except the study by Weinstein *et al.* (1962, 1963) as above.

Experiments have been conducted to investigate the egg depositing activity of adult worms and the further development of these eggs deposited *in vitro*.

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Materials and Methods

Recovery of Adult Worms

A. cantonensis was maintained in our laboratory by using aquatic snails, *Biomphalaria glabrata* and albino rats. Rats were infected with 20 to 25 third stage larvae orally and the adult worms were recovered from the rats which had been discharging the first stage larvae in their feces 50 to 70 days postinfection. The rats were killed with ether. Immediately after death, the heart and pulmonary artery and its branches were carefully dissected and examined for worms. The worms thus collected were washed twice in the medium prewarmed at 37°C and used for cultivation.

Medium and Culture Vessels

The medium and culture vessels were the same as described in the previous paper(Uga and Matsumura, 1982) excepting horse serum substituted for rat serum because the horse serum was available in quantity and in the same lot,and preliminary experiments confirmed that the same effects were expectable for both sera. Briefly, NCTC 109 (Difco) was used as a basic medium and supplemented with horse serum at a concentration of 0, 25, 50, 75 or 100 % (unless otherwise indicated, the concentration was 50 %). Antibiotics, penicillin (300 units/ml) and streptomycin (100 μg/ml), were added. In almost all instances, 1 ml of culture medium was used per worm, and each glass tube (16 mm in diameter, 16 mm in height) contained one male or female worm. Cultures were maintained in a CO_2 incubator with 5 % in the atmosphere

at 37°C for 30 days. The culture medium was replaced every three days. All of the culture procedures were carried out under aseptic conditions.

Cultivation and Observation of Adult Worms and Eggs

The condition of the worms was followed daily under a stereomicroscope. The motility of the worms was graded into four classes; intense, moderate, faint and stopped. The eggs deposited during the culture period of an adult worm were cultivated for further 5 days according to Uga and Matsumura (1982) and examined for the appearance rate of embryonated eggs. The whole number of eggs was calculated by counting 0.01 ml out of 1 ml of egg suspension in the culture medium. This was repeated three times and the average was used.

Results

Cultivation of Adult Worms

A male or female worm was cultured in the medium NCTC 109 with 0, 25, 50, 75 or 100% of horse serum to examine the optimum serum concentration for the survival of worms. Consequently, no effect of the serum concentration on the survival of worm was observed. The females survived for longer than 2 weeks and the males for a month in all conditions tested. The females kept the intense motile by day 2nd and, however, their movement became gradually slower, and showed moderate or faint by days 5 to 11 and after 11 th day of cultivation all showed faint movement. On the contrary, the male moved intensely for up to 21 days and was still moderate on day 30 when the cultures were terminated. The addition of pyruvate, glucose or rat red blood cells had no beneficial effect on the survival of adult worms (data not shown).

Soon after the cultivation started, the female deposited eggs and it continued up to about day 5. After that, however, the egg production deteriorated and no other remarkable changes were observed. Although the female survived for more than 2 weeks, it has been thought that the worm did not show normal activity after day 5 judging from the morphology of the uterus and the intestine of the worm.

Characterization of Eggs Produced in vitro

The number of eggs produced by female worms were followed every 24 hr (Table 1). No egg deposition was observed in No. 1 worm throughout the culture period. In Nos. 2 to 8 worms the mean number of total eggs was 18, 548±6,043, although there was a tendency to be erratic. About 60% of them were produced within 24 hr of cultivation. The number of eggs produced then decreased gradually and only a small number of eggs were produced after 5 days of cultivation. These eggs deposited at every 24 hr were further cultivated for 5 days in another fresh

Table 1 Number of eggs deposited from adult worms of *A. cantonensis in vitro*

No.	Days of cultivation					Total no. of eggs
	1	2	3	4	5	
1*	0	0	0	0	0	0
2	13,208	3,972	3,398	1,843	513	22,934
3	7,073	1,388	431	1,036	246	10,174
4	13,330	3,932	562	140	1,716	19,680
5	8,424	1,419	1,954	2,480	207	14,484
6	15,064	3,769	990	1,353	648	21,824
7	13,011	3,475	6,921	2,496	1,344	27,247
8	6,857	3,393	1,129	1,168	946	13,493
	10,995±3,418	3,050±1,145	2,198±2,317	1,502±843	803±566	18,548±6,043

* The data of No. 1 worm were omitted from the statistical analysis.

Table 2 Embryonation rate of eggs deposited from adult worms every day after start of cultivation

No.	Days of cultivation				
	1	2	3	4	5
1	79/123*(64.2)	4/96 (4.2)	0/15 (0)	—	—
2	384/640 (60.0)	1/105(1.0)	0/94 (0)	—	—
3	147/273 (53.8)	3/66 (4.5)	0/352(0)	—	—
4	135/306 (44.1)	2/241(0.8)	0/621(0)	—	—
5	75/125 (60.0)	1/78 (1.3)	0/89 (0)	—	—
Mean	820/1,467(55.9)	11/586(1.9)	0/1,171(0)	—	—

* Number of eggs embryonated/Number of eggs examined.
Figures in parentheses are embryonation rate in per cent.

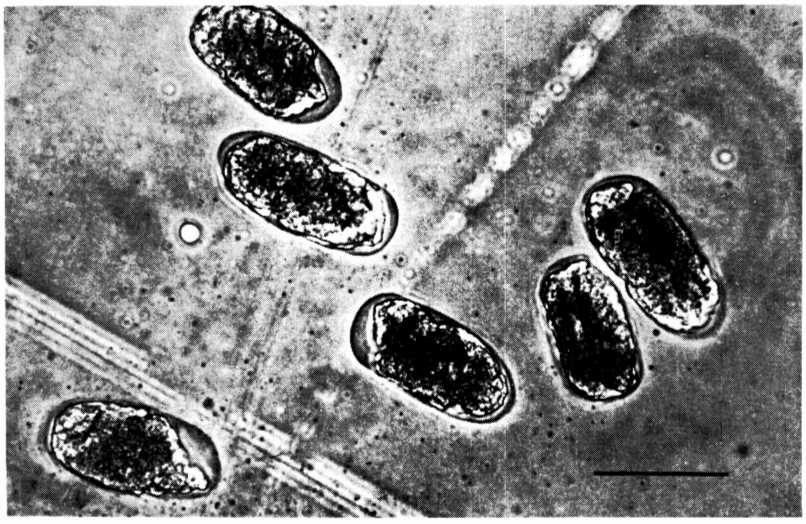

Fig. 1 The oblong-shaped eggs deposited during the first 24 hr. The eggs, having a thin egg-shell, seemed to be mature. Bar indicates 50 μm.

medium, and examined for the appearance rates of embryonated eggs. Embryonation rates of eggs deposited for the first 24 hr were 55.9 %, and 1.9 % for the next 24 hr. After 2 days of cultivation, no eggs were embryonated at all (Table 2). Of the eggs produced within 24 hr of cultivation, 97.6% were of oblong shape (Fig. 1), while only 22.4 % of the eggs after 48 hr were oblong and the remains were sphere shape (Fig. 2). Throughout the culture period, the sphere-shaped eggs did not show any development. Hence, the oblong-shaped eggs deposited within 24 hr after cultivation were examined more in detail and the results are shown in Table 3. Of the eggs deposited within 30 min of cultivation of adult worms, 93.3 % developed to the embryonated egg stage on the 5 th day of cultivation. On the contrary, 18.0 % of the eggs deposited between 4 and 6.5 hr developed to the embryonated egg stage, although no morphological differences from the eggs within 30 min were observed microscopically. Only 4.7 % of oblong-shaped eggs deposited between 6.5 and 24 hr developed.

The eggs developed *in vitro* to the 8- to 16-cell stage by day lst after cultivation, to the 16- to 32-cell stage by day 2, and to the 32 or more-cell stage by day 3. The embryonated stage was attained by day 5, and the hatching

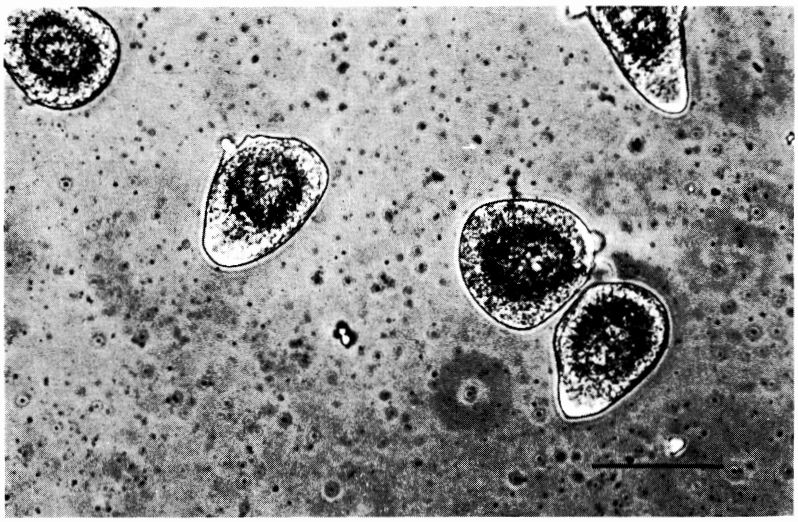

Fig. 2 The eggs deposited after 48 hr, sphere-shaped, had no distinct structure correspond to egg-shall, and seemed to be immature. Bar indicates 50μm.

Table 3 Embryonation rate of eggs deposited from adult worms 0.5, 4, 6.5 and 24 hours after start of cultivation

No.	Hours of cultivation			
	0-0.5	0.5-4	4-6.5	6.5-24
1	58/62 (93.6)	ND*	ND	0/106(0.0)
2	105/109(96.3)	ND	ND	ND
3	201/221(91.0)	17/18 (94.4)	18/47 (38.3)	42/295(14.2)
4	219/231(94.8)	93/100(93.0)	6/26 (23.1)	1/351(0.3)
5	48/54 (88.9)	85/176(48.3)	17/122(13.9)	1/160(0.6)
6	55/58 (94.8)	125/153(81.7)	12/99 (12.1)	5/122(4.1)
Mean	686/735(93.3)	320/447(71.6)	53/294(18.0)	49/1,034(4.7)

* Not done which were omitted from the statistical analysis.

began after 8 days of cultivation (Uga and Matsumura, 1982). Oblong-shaped eggs deposited between 0 and 0.5 hr, 0.5 and 4 hr, 4 and 6.5hr and 6.5 and 24 hr of cultivation of adult worms were examined for developing rates at each stage. The results are shown in Fig. 3. All of the eggs deposited during 0.5 hr of cultivation developed to 8 to 16 cells on the first day, and most of the 8- to 16-cell-stage eggs developed to embryonated eggs on the 5 th day. Of eggs deposited between 0.5 and 4 hr, 97.4 % developed to 8 to 16 cells and the survival rate of these eggs decreased with the increasing culture period, and finally, 81.8 % of the eggs were embryonated on the 5 th day of cultivation. Of eggs deposited between 4 and 6.5 hr, 92.9 % developed to 8 to 16 cells. The number of the eggs which stopped their development between 8-cell and embryonated egg stages were increased, and only 72.7 % developed to embryonated stage. On the contrary, 44.1 % of eggs which deposited between 6.5 and 24 hr did not show any development and only 32.6 % developed to embryonated egg stage.

From these results, it is suggested that the oblong-shaped eggs deposited *in vitro* consisted of the following three groups: (1) The

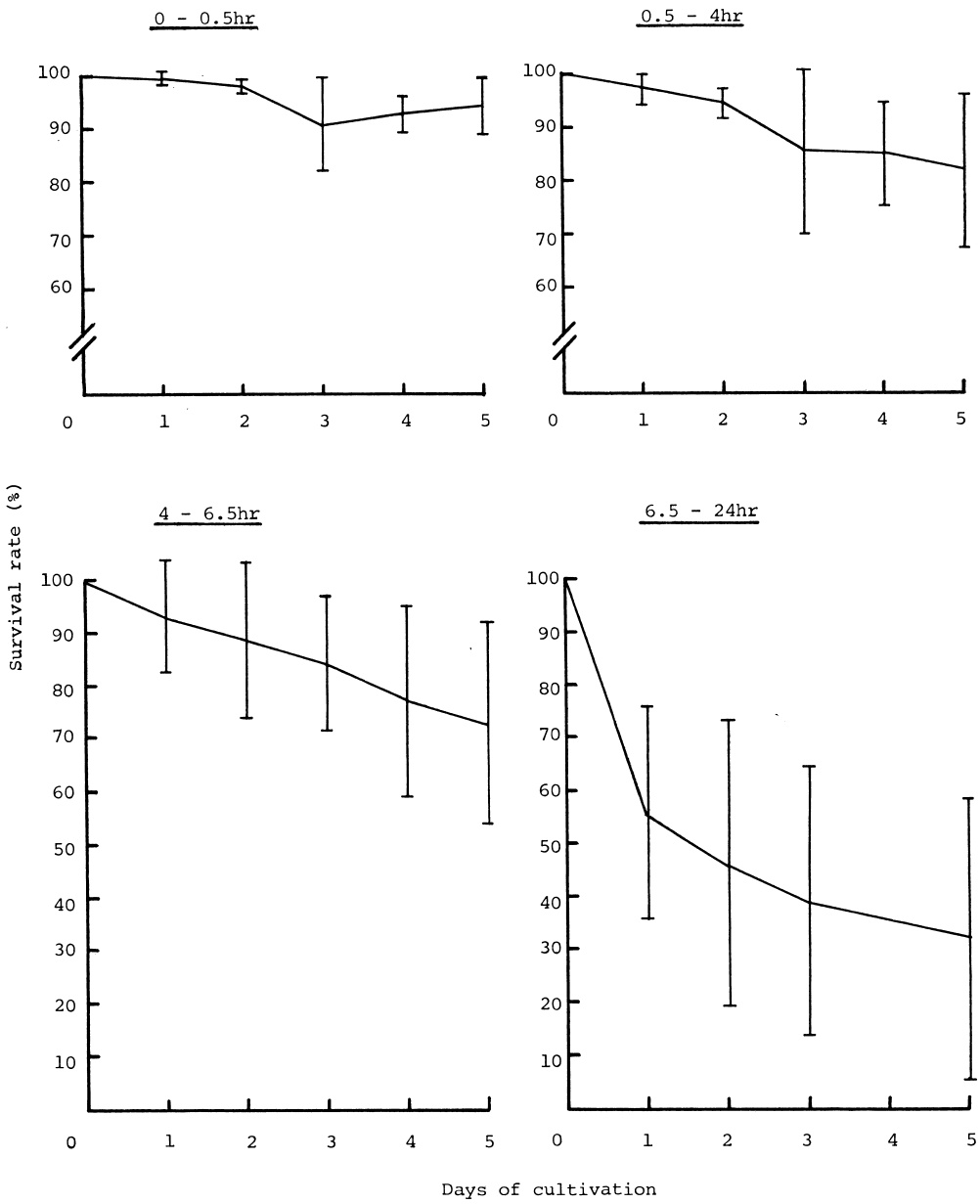

Fig. 3 Development of eggs deposited at each period of cultivation of adult females. The 0, 1, 2, 3, 4 and 5 days of cultivation are equivalent to 1 cell, 8-16 cell, 16-32 cell, 32 or more cell, pre-embryonated and embryonated egg stages, respectively.

eggs almost all of which developed to embryonated-egg stage; they are deposited within 30 min after the cultivation and the time course of the development is shown in Fig. 3 as a horizontal line. (2) The eggs which stopped their development between the stages of 8 cells and embryonated egg; this percentage is induced by subtracting the percentage of the embryonated eggs from that of 8- to 16-cell stage eggs. The occurrence rates of

these eggs are 15.6 % at 0.5 to 4 hr, 20.2 % at 4 to 6.5 hr and 23.3 % at 6.5 to 24 hr. They are shown in Fig. 3 as a declining line between 8 to 16 cells and the embryonated egg. (3) The eggs which did not show any development; the number of this kind of egg was increased with the increasing culture period of adult worm. The ratios of this egg were 2.6 % at 0.5 to 4 hr, 7.1 % at 4 to 6.5 hr and 44.1 % at 6.5 to 24 hr. Afterwards a remarkable increase in the ratio was observed; 92.2 % at 24 to 48 hr and 95.0 % at 48 to 72 hr. It is shown in Fig. 3 as a line between 1-cell and 8- to 16-cell stages.

Discussion

A number of papers have been reported on the *in vitro* cultivation of parasitic helminths (Taylor and Baker, 1968, 1978; Trager, 1978), but reports concerning the *in vitro* cultivation of adult *A. cantonensis* were only made by Weinstein *et al.* (1962, 1963). They stated that in chemically defined medium alone (NCTC 109) females survived for approximately a month and males for 19 days. Survival was considerably enhanced by the addition of rat or horse serum. The maximum survival obtained was 80 days for females and 64 days for males. In our experiments, however, no definite effects of serum addition were observed on survival rate of both male and female adult worms. Further, males survived longer than females in our experiments, the reason for this difference has been yet unknown. The males still maintained the motility even after 1 month when the cultures were terminated. Even the female adult worms with the uterus physically injured when recovered from the rat lung and with eggs filled in the body cavity survived for 7 to 10 days. Although these females did not deposite at all, the eggs filled in the body cavity or uterus were developed despite a little delay compared with eggs deposited normally.

Weinstein *et al.* (1963) noted a phenomenon of egg deposition when they cultured female adults of *A. cantonensis in vitro*. The deposition counts per day were 43 to 15,000, the same as the results by the authors.

The females cultured *in vitro* produced morphologically different two types of eggs, the oblong-shaped and sphere-shaped eggs. The former was deposited chiefly within 24 hr after cultivation of the females, whereas the latter after 48 hr. The sphere-shaped eggs did not grow *in vitro* at all.

The oblong-shaped eggs deposited within 30 min after cultivation of adult worms reached the embryonated egg stage at 93.3 % in the following five-day culture (Table 3). This result suggests the two facts as follows: (1) Our culture system and culture medium are an enough statisfactory method; if the eggs are maturated and fertilized, most of them can be grown up to the embryonated stage on day 5. (2) Most of the eggs deposited within 30 min of cultivation are maturated and fertilized, and they will be able to reach the embryonated stage *in vitro*. In view of the above point, Fig. 3 suggests that the oblong-shaped eggs further include two kinds of eggs; the eggs which cannot grow up to the embryonated stage but can grow halfway and the other eggs which do not grow at all. The rates of these two kinds of eggs in the total deposition count increased with increasing culture period of adult worms. These eggs that can grow halfway may be either those lacking something required for growth up to the embryonated stage despite fertilizing or those causing parthenogenesis bacause of physical stimulation when collected. The eggs showing no growth, though morphologically maturated, may be infertile or physiologically immature enough to start the growth. In any case, these eggs leave a number of unknown points, which are at present under study.

Uga and Matsumura (1982) noted that there are extremely undifferentiated triangular eggs in the upper part of the uterus, sphere-shaped immature eggs in the midpart and oblong-shaped mature eggs in the posterior part of adult female of *A. cantonensis*. Besides this, triangular eggs were observed by Yamamoto *et al.* (1983) and sphere-shaped eggs by Weinstein *et al.* (1963). In the present experiment,

no triangular eggs were deposited at all.

The above results together with the results that the authors had previously reported disclosed the presence of morphologically different three kinds of eggs of *A. cantonensis* and also the presence of three kinds of oblong-shaped eggs showing different growth *in vitro*.

Summary

Male and female worms of *Angiostrongylus cantonensis* recovered from the lung of the rat were cultured in a medium NCTC 109 containing 50% horse serum. The female began to deposit eggs immediately after cultivation and it continued for five days. The overall deposition count per female was 18,548±6,043 on the average, approximately 60% of which were deposited within 24 hr after cultivation. The forms of the eggs deposited were of oblong shape and sphere shape. The eggs in the latter shape did not grow *in vitro*. Although, after all, the females survived for over 2 weeks and the males for over one month, assessment of movement of the worm and intestinal contents suggested no maintenance of normal activity in the latter half of the culture. The oblong-shaped eggs deposited within 24 hr after cultivation of the adult worms were taken as the subjects.

Observations of their growth conditions disclosed the presence of eggs having three different forms: (1) Most of the eggs deposited within 30 min after cultivation of the adult worms, grow up to the embryonated egg stage by the next 5 days. (2) The eggs deposited between 4 and 6.5 hr, could not grow up to the embryonated egg stage, but could grow halfway. (3) The eggs deposited between 6.5 and 24 hr showed no growth.

The above results together with the results that the authors had previously reported disclosed the presence of morphologically different three forms of eggs of *A. cantonensis* and also the presence of three forms of oblong-shaped eggs showing different growth *in vitro*.

References

1) Brockelman, C. R., Chavalsilp, C. and Sitthitavorn, P. (1979): *Angiostrongylus cantonensis*: Infectivity of first stage larvae in the presence of rat fecal materials as tested by the *in vitro* cultivation technique. Southeast Asian J. Trop. Med. Pub. Hlth., 10, 164–165.
2) Moreau, J. P. and Lagraulet, J. (1972): Survie *in vitro* des larves de troisième stade d' *Angiostrongylus cantonensis*. Annales de Parasitologie (Paris), 47, 525–529.
3) Taylor, A. E. R. and Baker, J. R. (1968): The cultivation of parasites *in vitro*. Blackwell Scientific Publications, Oxford.
4) Taylor, A. E. R. and Baker, J. R. (1978): Methods of cultivating parasites *in vitro*. Academic Press, London, pp. 151–285.
5) Trager, W. (1978): Cultivation of parasites *in vitro*. Am. J. Trop. Med. Hyg., 27, 216–222.
6) Uga, S. and Matsumura, T. (1982): *In vitro* cultivation of *Angiostrongylus cantonensis* eggs. Jpn. J. Parasitol, 31, 59–66.
7) Weinstein, P. P., Rosen, L., Laqueur, G. L. and Sawyer, T. K. (1962): *Angiostrongylus cantonensis* infection in rats and rhesus monkeys, and survival of the parasite *in vitro*. J. Parasitol. (Suppl.), 48, 51–52.
8) Weinstein, P. P., Rosen, L., Laqueur, G. L. and Sawyer, T. K. (1963): *Angiostrongylus cantonensis* infection on rats and and rhesus monkeys, and observations on the survival of the parasite *in vitro*. Am. J. Trop. Med. Hyg., 12, 358–377.
9) Yamamoto, H., Ogura, N., Kobayashi, M., Tsuruoka, H., Chigusa, Y. and Mishima, S. (1983): Balanced salt solution for helminthic cells (1). Jpn. J. Parasitol (Suppl.), 32, 30.

In vitro における広東住血線虫の産卵と虫卵の発育について

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ラット肺より回収した広東住血線虫の雌雄成虫を用い，NCTC 109 に馬血清を50%の割合に含む培養液で培養した．雌は培養開始直後から産卵を開始し，それらはほぼ5日間持続した．この間における雌成虫1隻当りの総産卵数の平均は18,548±6,043であり，その約60%が成虫の培養開始後24時間以内に産卵された．産卵された虫卵の形態は長円形 (oblong-shape) と円形 (sphere-shape) であり，後者は *in vitro* では全く発育しなかつた．最終的に雌は2週間以上，雄は1カ月以上 *in vitro* で生存しつづけたが，培養の後半では虫体の動き，腸管内容物等から判断して正常な activity を保つているとは考えられなかつた．成虫の培養開始後24時間以内に産卵された長円形卵を対象とし，その *in vitro* における発育状況を観察したところ，3種の異つた態度を示す虫卵が存在することが明らかとなつた．それらは①成虫培養開始後30分以内に産卵される卵；ほとんどの虫卵がその後の5日間の培養で幼虫包蔵卵にまで発育する．②成虫培養開始後4～6.5時間の間に産卵される卵；幼虫包蔵卵にまでは発育出来ないが途中まで発育する．③成虫培養開始後6.5～24時間の間に産卵される卵；*in vitro* では全く発育を示さない．であつた．

以上の結果より，著者等がすでに報告した結果と合せ，広東住血線虫卵には形態的に異る3種の卵と，*in vitro* で異つた発育を示す3種の長円形卵が存在することが明らかとなつた．