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Studies on the Free-Living Generations of *Strongyloides planiceps* Rogers, 1943. I. Effects of Quantity of Food and Population Density on the Developmental Types

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Parasites belonging to the genus *Strongyloides* have a complicated life cycle. The eggs produced by the parasitic females which are thought to be parthenogenetic, develop into the infective larvae (so called direct development) or the free-living males and females (indirect development) outside the host. The mechanism of the differentiation into the two courses of development, direct and indirect, has been the subject of much controversy. Some authors stressed that environmental factors influenced the selection of the courses of development (Nishigori, 1928; Beach, 1936; Premvati, 1958; Little, 1962; Varju, 1966; Hansen *et al.*, 1969), while others believed that the inherent factors played a major role (Sandground, 1926; Kreis, 1932; Faust, 1933; Tanabe, 1938 a, b; Abe *et al.*, 1966). Such divergent results may come from the incomplete culture systems or from the natures specific to the species of *Strongyloides*. In the latter case the stage of worms transferred to cultures should be considered. Premvati (1958) observed the development of *Strongyloides fülleborni*, and stated that the differentiation was influenced by the environmental conditions before the first molt. Hence in the cases of *S. stercoralis* in which the larvae have hatched out from the eggs when they are passed from the host, and of *S. ratti* which passes both eggs and rhabditoid larvae in the feces, the course of development of the worms might be already determined when they were transferred to the culture systems.

The present study deals with the effect of quantity of food (feces) and egg population density upon the development of free-living generations of *Strongyloides planiceps*, which passes only eggs in the host feces.

Materials and Methods

Strongyloides planiceps used in this study was originally recovered from a dog in Kyoto in 1973 and has been maintained by serial passage in puppies.

Eggs were collected from the feces of an infected puppy by the floatation method using saturated NaCl solution, and immediately washed about 10 times with distilled water by centrifugation. For estimation of the number of eggs for cultures, the number of embryonated eggs in a drop of egg suspension was counted under microscope. Sampling was repeated at least 5 times and the mean number was calculated. At any time a few percent of degenerated eggs were comprised, and they were excluded from the number of eggs planted. In order to assess, prior to experiment, whether the eggs are damaged by the floatation method or not, the rate of degenerated eggs and the hatching rate of eggs collected by floatation method were compared with those of eggs not treated with saturated NaCl solution. For collecting the eggs without using saturated NaCl solution, the feces of an infected puppy was dissolved in distilled water, passed through a sieve, and washed with distilled

water by centrifugation. The eggs thus obtained and the eggs obtained by floataion method were examined for the rate of degenerated eggs under microscope. Besides, 300–500 eggs in 5 ml of water in petri dishes were held at 28C for 4 hr and examined for hatching rates.

All the egg cultures were carried out by filter paper test tube method. In experiments 1 and 2, feces or fecal dilution of non-infected puppy with this parasite was spread on the upper half of a filter paper strip (2.5×12 cm) and the known number of eggs were placed on it. In experiment 3, on the other hand, the feces of infected puppy was spread on the filter paper. After 24–30 hr incubation at 28C, during which period the free-living males, free-living females and infective larvae were formed, the worms were recovered by the following manner. Each of the test tubes was filled with warm water (40C) up to the upper edge of the filter paper strip. By this procedure, almost all worms on the filter paper migrated into the warm water in a few minutes. Those worms were then collected by centrifugation and counted under microscope.

In experiment 1, in order to estimate the effect of quantity of food (feces) upon the larval development and differentiation, the eggs were cultured with various concentrations of feces. For this purpose, egg-free feces of a puppy was diluted with water up to 1 : 8 (1 g feces in 8 ml water) and on one occasion 1 : 16 (1 g feces in 16 ml water). The known number of eggs (406 in group 1, 178 in 2, and 147 in 3) were cultured with 0.2 ml of those fecal dilutions or 0.2 g of feces by the manner as mentioned above.

In experiment 2, to study the effect of population density of larvae upon their development and differentiation, different number of eggs (3,000, 1,500, 750, 375 and 200) were cultured with 0.2 g of egg-free feces.

In experiment 3, the effect of population density was further studied by culturing 0.2 g of infected puppy's feces, which showed different E.P.G. in the course of infection. Each of four puppies (Nos. 1–4) was given 2,000 infective larvae subcutaneously and the fecal cultures were carried out at various days after infection. The E.P.G. was examined with Stoll's dilution egg-count technic. For the control, 150–200 eggs from the above puppies were cultivated with 0.2 g of egg-free feces.

The puppies used in this investigation were fed on commercial dog biscuit (Oriental Kobo Inc.).

Results

Preliminary experiment. To assess the effect of floatation method upon the eggs, the percentage of degenerated eggs and hatching rate of eggs after 4 hours incubation at 28C, both collected by the floatation method, were compared with those of the eggs collected only by diluting the feces with water (control). As is shown in Table 1, no significant difference was observed in both rates between the eggs obtained by these two procedures. This suggests that the eggs are not damaged by the floatation method.

Experiment 1. To study the effect of quantity of food (feces) on the developmental types of free-living generation, the known number of eggs were cultured with various concentrations of egg-free feces of a puppy.

Table 1 Effects of floatation method on the appearance of degenerated eggs and hatching rate of eggs of *S. planiceps*

% of	Floatation method	Control	Significance of difference tested by χ^2
Degenerated eggs	7.1(325)*	7.8(309)	Not significant
Hatching	88.8(340)	90.2(338)	Not significant

* Number of eggs examined in parenthesis.

The experiments were carried out three times (Group 1–3). The appearance rates of free-living males, free-living females and *infective* larvae, each expressed as percentage of the number of eggs cultured, were shown in Table 2. The total recovery rates were as high as over 80% in lower fecal dilutions up to 1 : 2 or 1 : 4 in each group. The total recovery became lower in higher fecal dilutions, and the larval development was never observed in control cultures which had no fecal element as nutrition. In lower fecal dilutions less than 1 : 2 or 1 : 4, recovery rates of free-living males (M) were almost constant in each group as about 50% in Group 1 and 2, and about 20% in Group 3, though they decreased in higher dilutions proportionally to the decrease of total recovery. On the other hand, the rate of free-living females (F), which was maximum in the lowest dilution (1 : 0), decreased markedly with the increase of dilution. The very reverse of what had been observed in the case of free-living females, occured in the case of infective larvae (f), though sum of the rates of F and f was nearly constant. To indicate the change between free-living females (F) and infective larvae (f), an index, $f/F+f\times100$ (*f-rate*), was set up. As shown in Table 2, the f-rate of cultures given feces not diluted (1 : 0) were 16.5, 42.9 and 52.8% in three groups, respectively. With the increase of dilution the f-rate increased rapidly and in the highest dilution it reached as high as 99%.

From these results it is said that the developmental course either to free-living females or to infective larvae is affected by

Table 2 Effect of fecal dilutions on the developmental types of free-living generation of *S. planiceps*

Group No.	Fecal dilution 1 :	Number of eggs planted	Mean % total recovery	Mean % recovery			F+f (%)	f-rate (%)
				M	F	f		
1	0*	406	93.7	53.0	34.0	6.7	40.7	16.5
	1	〃	94.7	55.5	21.0	18.2	39.2	46.4
	2	〃	89.5	51.1	18.0	20.4	38.4	53.1
	4	〃	87.5	50.9	12.5	24.1	36.6	65.8
	8	〃	75.1	36.2	4.2	34.7	38.9	89.2
	16	〃	51.5	18.5	0.2	32.8	33.2	99.4
	C**	〃	0	0	0	0	—	—
2	0	178	95.3	52.4	24.5	18.4	42.7	42.9
	1	〃	95.4	51.1	7.6	36.7	44.3	82.8
	2	〃	83.4	51.3	2.0	30.0	32.1	93.8
	4	〃	70.2	33.9	1.5	34.8	36.3	95.9
	8	〃	47.2	18.9	0.4	27.9	28.3	98.6
	C	〃	0	0	0	0	—	—
3	0	147	92.6	23.3	32.7	36.6	69.3	52.8
	1	〃	90.8	25.4	10.6	54.8	65.4	83.8
	2	〃	85.1	20.8	5.6	58.7	64.3	91.3
	4	〃	85.0	19.9	1.4	63.7	65.1	97.8
	8	〃	69.7	7.1	0.2	62.4	62.6	99.7
	C	〃	0	0	0	0	—	—

Triplicate tubes were observed in each dilution. M : free-living males. F : free-living females. f : infective larvae. f-rate $=\frac{f}{F+f}\times100$. * Feces not diluted. ** Control culture which had no fecal element.

the concentrations of feces given, while free-living males appear rather constantly.

Experiment 2. To study the relations between density of eggs and the developmental types, various number of eggs (200, 375, 750, 1,500 and 3,000) were cultured with 0.2 g of egg-free feces. The rate of free-living males, females and infective larvae were expressed as percentage of the number of eggs planted. As shown in Table 3 the total recovery rates were constant (89.2–94.0%) between egg population densities 200 and 3,000. The rates of free-living males were also constant (47.2–50.6%) within those egg densities. On the other hand, the rate of free-living females which was maximum (41.5%) in the lowest egg density, decreased gradually according to the increase of egg density, and reached minimum (15.6%) in the highest egg density. While on the infective larvae, being minimum (3.0%) in the lowest egg density, the rate increased to 26.3% in the highest egg density. The f-rate increased according to the increase of egg densities. Therefore, the development either to free-living females or to infective larvae was influenced by egg density in cultures, while free-living males were not.

Experiment 3. The fecal cultures of infected puppies were performed at various days after infection and examined whether the developmental types varied according to E.P.G. or not.

The changes of E.P.G., composition of worms and f-rate during the infection course of puppies Nos. 1–4 were shown in Fig. 1 and 2. In puppy No. 1, the rates of free-living males showed some fluctuation from day to day around the level of 30% but it had no relationship to E.P.G. values (Graph 2 of Fig. 1). On the other hand, the rates of free-living females and infective larvae showed a certain relationship to E.P.G. values. Until 50 days post infection, during which period E.P.G. were more than 3,000, many infective larvae and a few free-living females were formed. However, on day 61 and 70 when E.P.G. decreased to 1,500 and 300, many free-living females and conversely very few infective larvae were formed. The f-rate in the fecal cultures showed transition parallel to E.P.G. as shown in the Graph 3 of Fig. 1 with solid line. When E.P.G. was more than 3,000, the f-rate was more than 80%, but as E.P.G. decreased to 1500 and 300, the f-rate decreased to 11.4 and 5.8%, respectively. The broken line in the same graph represents the f-rate in control cultures, in which 150–200 eggs from the puppy No. 1 were cultured with 0.2 g of egg-free feces. The density of eggs used in control cultures is equivalent to that of the fecal cultures with 750–1,000 E.P.G.. The f-rates of control cultures were at most 33.0% (22 days post infection) throughout the infection course, while the f-rates in the fecal cultures were more than 80% until late stage of infection. These results suggest that the rate of free-living females and infective larvae have a certain relationship to E.P.G. values rather

Table 3 Effect of egg density on the developmental types of *S. planiceps*

Number of eggs planted	Mean % total recovery	Mean % recovery			F+f (%)	f-rate (%)
		M	F	f		
200	91.8	47.3	41.5	3.0	44.5	6.7
375	90.2	47.2	37.7	5.3	43.0	12.3
750	94.0	50.0	35.0	9.0	44.0	20.5
1,500	89.2	50.3	24.0	14.9	38.9	38.3
3,000	92.5	50.6	15.6	26.3	41.9	62.8

Triplicate tubes were observed in each egg density.

M: free-living males. F: free-living females. f: infective larvae. f-rate $=\frac{f}{F+f}\times 100$.

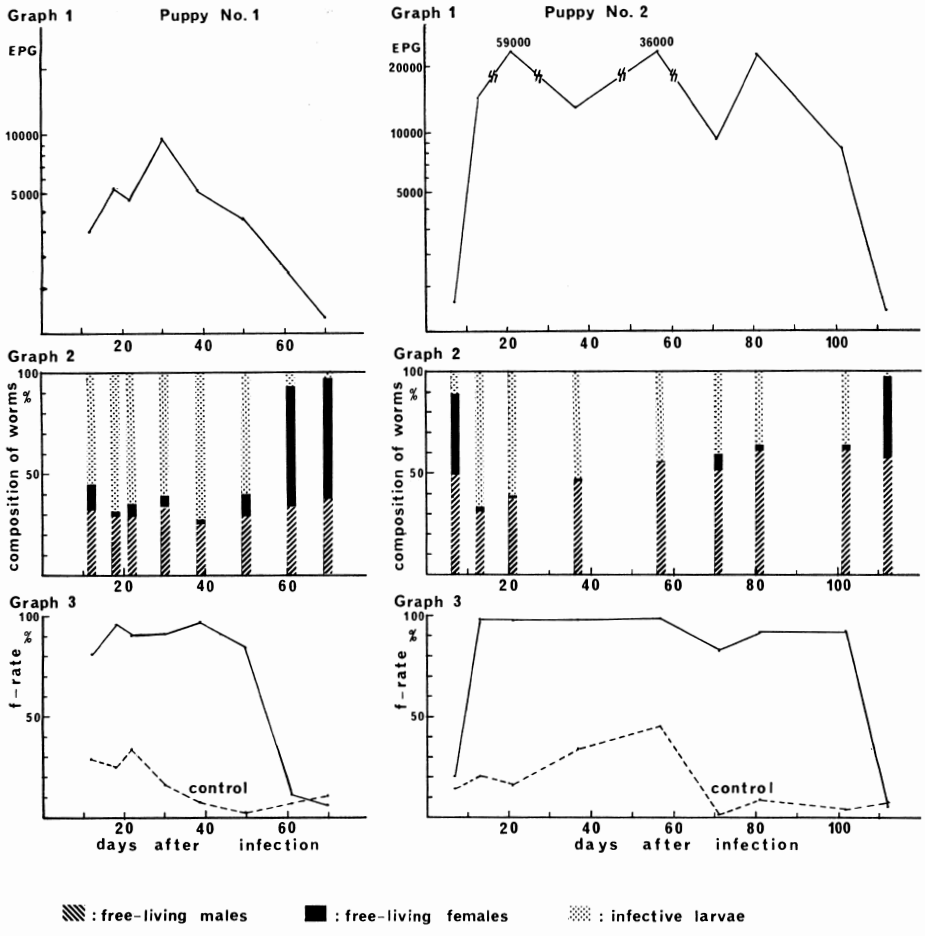

Fig. 1 Changes of E.P.G. (Graph 1), composition of worms (Graph 2), and f-rate (Graph 3) during the course of infection with *S. planiceps* (Puppy Nos. 1 and 2).

than age of parasites.

In puppies Nos. 2–4, the relationship of f-rates to E.P.G. values was similar to that in puppy No. 1. Especially in No. 3, the E.P.G. temporarily decreased to 800 and 2,300 on day 45 and 52, respectively in the middle course of infection without known reasons (Graph 1 of Fig. 2). At that time, the f-rates also decreased to 12.8 and 2.8 %, and then recovered to 83.1 % on day 61 when the E.P.G. increased to 4,400.

All of the relationships between E.P.G. and f-rate of each fecal culture in puppies Nos. 1–4 were summarized in Fig. 3 irrespective of the days after infection. The correlation coefficient ($r=0.796$) was highly significant ($p<0.001$). Generally speaking, as E.P.G. was lower than 1,000, f-rate was less than 30 %, while under the overcrowding condition such as more than 10,000 E.P.G., it increased to nearly 100 %.

Discussion

Beach (1936) studied on the develpment of *S. simiae* and stated that the infective larva was believed to be potential free-living male or female. On the other hand, Premvati (1958) studied on *S. fülleborni* and stated, without adequate data, that only those

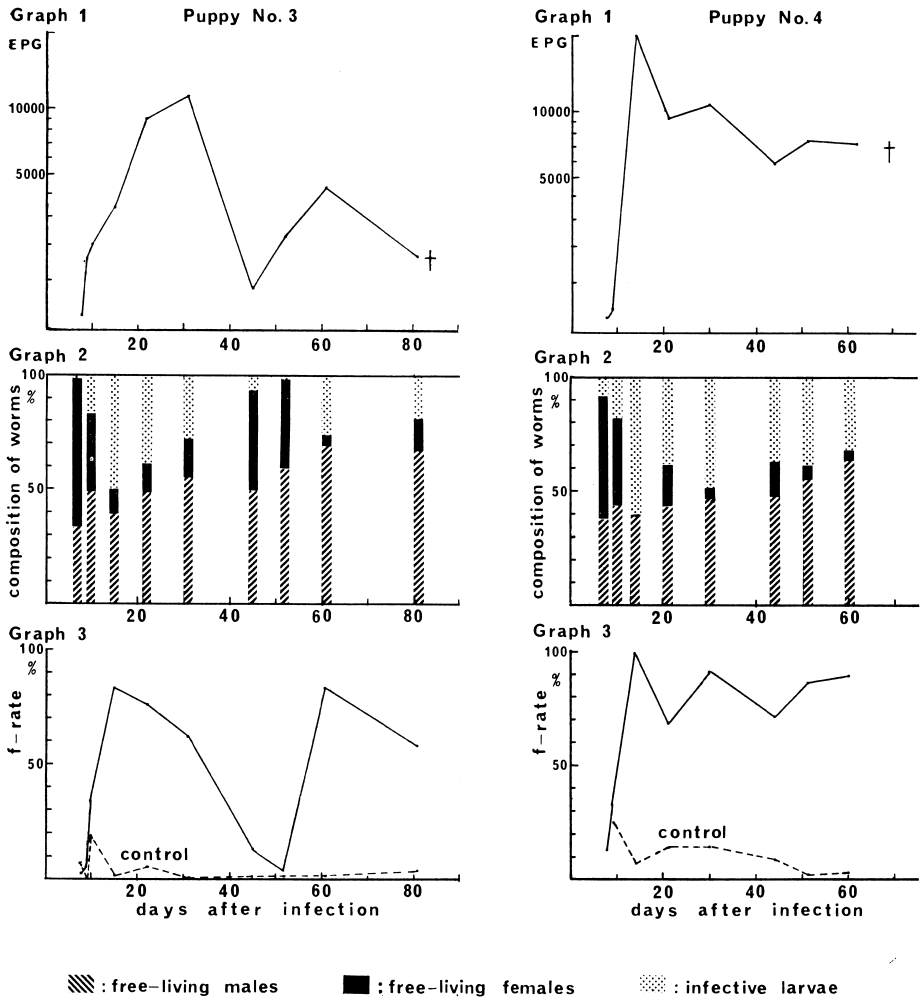

Fig. 2 Changes of E.P.G. (Graph 1), composition of worms (Graph 2), and f-rate (Graph 3) during the course of infection with *S. planiceps* (Puppy Nos. 3 and 4).

larvae which would develop into free-living females were able to change into infective larvae. Little (1962) also observed the changes of composition between free-living females and infective larvae of *S. fülleborni*.

In the present study it was shown that the free-living females and infective larvae appeared in a converse way according to the two factors, quantity of food (feces) and egg population density. Namely, many free-living females with a few infective larvae were formed in low population density cultures with sufficient feces as food. But in high population density cultures or in cultures with a very small amount of feces, free-living females became a few in number and conversely many infective larvae were formed.

Therefore, it can be said that the two factors affect strongly the determination of developmental course either to free-living females or to infective larvae. The free-living males, on the other hand, are not affected by the factors but seems to be

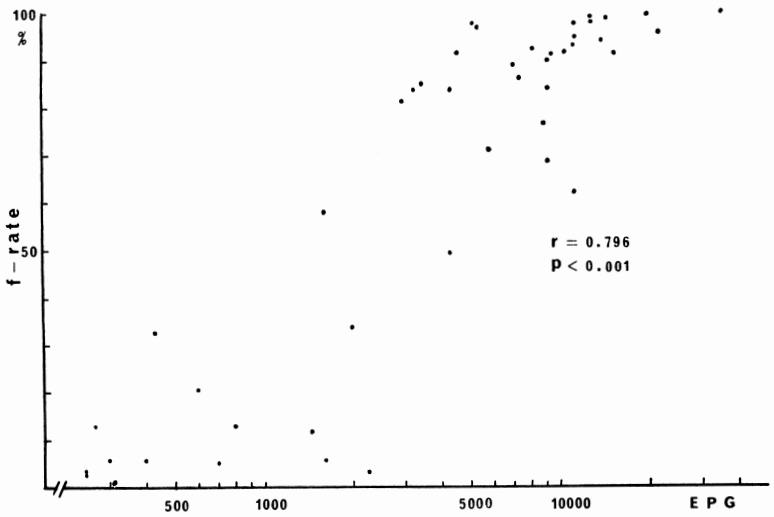

Fig. 3 Correlation between E.P.G. and f-rate.

already fixed as male in the egg stage.

The population density effect was also observed in fecal cultures along the infection course. That is, the developmental course either to free-living females or to infective larvae was affected by the E.P.G. level of the feces.

On the other hand, Varju (1966) observed the changes of developmental types along the infection course of swine *Strongyloides*, and pointed out the relationship of host immunity to it. Namely, in the first 2 or 3 months of infection, many infective larvae were formed, while in more advanced stage when host immunity was established, many free-living adults were produced instead of infective larvae. But in the graphs of his paper, a certain correlation is seen between E.P.G. and the developmental types although he did not take it into consideration. Though immune status of the host puppies was not studied in the present investigation, the relationship of the density of eggs (E.P.G.) and developmental types were clearly proved in experiment 3.

These population density effects might be caused by the competition for food between worms. Because, in the cultures with a very small amount of feces, many infective larvae were produced similarly to the over-crowding condition.

Summary

Studies on the effects of quantity of food (feces) and population density upon the differentiation into three types of development (free-living males, free-living females and infective larvae) were carried out using *Strongyloides planiceps*. Many free-living females with a few infective larvae were formed in low population density cultures with sufficient feces as food. But in high population density cultures or in cultures with a very small amount of feces, free-living females became a few in number and conversely many infective larvae were formed. In the cultures of feces of infected puppies, the relationship between the E.P.G. level and the developmental course were also observed, namely, many free-living females with a few infective larvae in low E.P.G. level, and reverse proportion in high E.P.G. level. These results indicate that the differentiation into either free-living female or infective larva is determined at least by two factors, quantity of food and population density. On the other hand, the free-living males were not affected by these two factors but produced in constant ratio. These suggest that the free-living male is

already fixed in the egg stage.

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Strongyloides planiceps の自由生活世代の研究
I. 食物の量と個体密度が発育型に及ぼす影響について

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Strongyloides planiceps の宿主体外発育における自由生活雄成虫，同雌成虫および感染幼虫の三者への分化に及ぼす食物としての便量，および個体密度の影響を調べた．その結果自由生活雄虫は，食物としての便の稀釈度，および個体密度にかかわらず一定比率で出現したが，自由生活雌虫と感染幼虫はこれら両要因の影響を受けて相補的に出現することが明らかとなつた．即ち，十分な量の便（0.2 g の非稀釈便）に低密度（約200）の虫卵を置いて培養した場合には，自由生活雌虫が多数出現し感染幼虫は少数であるが，卵数を一定にして便を稀釈して与えた場合，あるいは便量を一定にして卵数を増していつた場合には，自由生活雌虫の出現が減じ感染幼虫が増え，ついには大多数が感染幼虫に発育する．これらの現象はまた，感染犬の便培養における発育型とE.P.G. の高低との関連としても明らかにされた．感染初期あるいは末期の E.P.G. が1,000以下の時期には多数の自由生活雌虫が出現し，感染幼虫は少数であつたが，感染中期の E.P.G. が10,000以上の時期にはその比が逆転する．

以上の結果から，自由生活雄成虫は虫卵の時期に既にその発育方向が固定しているが，一方，自由生活雌成虫あるいは感染幼虫への分化は，少くとも二つの環境要因，即ち食物としての便量と個体密度によつて，そのいずれに分化するかが決定されるものと考えられる．