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Female-Dependency of Circulating Anodic Antigen Level in *Schistosoma japonicum* Infection

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Introduction

Berggren and Weller (1967) first demonstrated antigen in the sera of mice and hamsters infected with *Schistosoma mansoni.* The antigen has been reported to be heat-stable, anodic on immunoelectrophoresis and with a molecular weight of more than 100,000 (Gold *et al.*, 1969; Nash *et al.*, 1974). Further characterization of the antigen has revealed that it is a proteoglycan (Nash *et al.*, 1977). In *S. japonicum* infection, the presence of an antigen with similar characteristics was confirmed in the circulation of the dd mouse and rabbit and in homogenates of *S. japonicum* and *S. mansoni* (Hirata and Akusawa, 1975; Hirata, 1976). The circulating anodic antigen (CAA) has been reported to be genus specific (Hillyer, 1976).

CAA is easily detected in mammals with schistosomal infection, with relatively sensitive methods such as complement fixation, counter immunoelectrophoresis, indirect haemagglutination, microfluorometric assay, enzyme-linked immunosorbent assay and radioimmunoassay (Bawden and Weller, 1974; Hirata *et al.*, 1977; Deelder and Eveleigh, 1978; Deelder *et al.*, 1978; Ferreira *et al.*, 1979; Carlier *et al.*, 1980). These findings suggest that the detection of CAA is useful in the immunodiagnosis of schistosomiasis. The presence of CAA in the sera of patients with *Schistosoma mansoni* infections has been reported (Hernández-Almenas and Hillyer, 1973; Madwar and Voller, 1975; 1977; Carlier *et al.*, 1980).

The concentration of CAA, however, does not remain constant. During the course of infection concentrations may decrease to undetectable levels (Hirata and Akusawa, 1975; Hillyer, 1976). Anti-CAA antibody is believed to be an important factor contributing to this disappearance. Deelder *et al.* (1978) reported that specific antibodies can be expected to form in experimental infections with *S. mansoni* in the hamster and mouse. Nash (1978) reported that all 49 patients in his series, who had been infected with either *S. mansoni, S. haematobium* or *S. japonicum,* demonstrated anti-CAA antibodies in their sera.

To date, no prior studies have been done which detail the time course of CAA concentration in schistosomes. Because CAA is of schistosome origin, changes in CAA concentration in schistosomes may also be contributory. Synthesis and release of excretory-secretory materials from schisto-

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somes has been studied using labeled precursors (Nash, 1979; Wilson and Barnes, 1979).

In the present study, a time course study of CAA in the sera of rabbits infected with *S. japonicum* was carried out with rocket immunoelectrophoresis. CAA concentration in *S. japonicum* males and females recovered from infected mice and rabbits was examined at timed intervals after infection and compared with the change in CAA titers of infected rabbit sera. Finally, the differences in CAA concentration among schistosome species and among host species were studied.

Material and Methods

Animals, sera and worms

Seven rabbits, weighing 2.5 to 3.2 Kg were infected with about 1,500 *Schistosoma japonicum* cercariae by intraperitoneal injection (i.p.). The cercariae were collected from Kurume and Kofu (Japan) strains of *Oncomelania hupensis nosophora*. The rabbits were bled weekly or biweekly from 3 to 20 weeks after infection. After the blood had been allowed to clot at room temperature for 3 hrs, serum was separated and stored at −20 C. Hirata *et al.* (1977) have noted that decreases in body weight correlated with increases in CAA titers. For this reason, standardization of schistosome burden was calculated as the ratio of the total number of schistosomes to the host's body weight. Rabbit body weights were determined at the time of each bleeding.

Time course of CAA concentration in adult schistosomes was studied. Four rabbits were infected i.p. with approximately 1,000 *S. japonicum* cercariae and sacrificed at 8, 14, 18 and 23 weeks after infection. Sixteen BALB/C mice were infected i.p. with approximately 50 *S. japonicum* cercariae, then sacrificed at weekly intervals between 4 and 10 weeks and at 16 weeks after infection. One hamster was infected i.p. with *S. haematobium* cercariae from *Bulinus globosus* (Kenyan strain) and 4 dd mice were infected i.p. with *S. mansoni* cercariae from *Biomphalaria glabrata* (Puerto Rican strain) (These animals were kindly supplied by the Department of Parasitology, Institute of Tropical Medicine, Nagasaki University, and the Department of Medical Zoology, Faculty of Medicine, Kagoshima University, respectively). The hamster was sacrificed at 35 weeks after infection and all 4 mice at 14 weeks after infection.

Schistosomes were recovered from survivors of infected animals by the method of Radke *et al.* (1961) modified as follows. In the rabbit model, 1 liter of physiological saline containing 1% sodium citrate was injected through the thoracic aorta and schistosomes were collected from blood taken from the portal and mesenteric veins. In the mouse model, 40 ml of physiological saline were injected into the left ventricle and schistosomes were likewise recovered from the portal and mesenteric veins.

Preparation of worm homogenates

Mated schistosomes of *S. jajponicum, S. mansoni* and *S. haematobium* were left at 4 C for approximately 1 hr and then divided into male and female groups. Each group consisted of 13 schistosomes. They were first washed with physiological saline and then with distilled water, and stored at −70 C. The schistosomes were then homogenized by a motor-driven grinder in an acetone-ice bath at 2,500 rpm for 5 min and then lyophilized. The dry material was reconstituted with physiological saline at a ratio of 0.05 ml per schistosome. The solution was incubated at 4 C for 24 hrs. Homogenates (20 mg/ml) of *S. japonicum* recovered from mice were prepared as the standard solution.

Preparation of specific antigen and antiserum

An antigenic solution corresponding to circulating anodic antigen (CAA) was prepared according to Smith (1967), as previ-

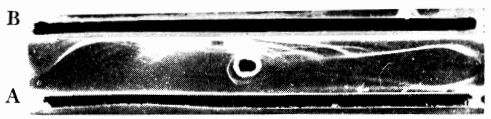

Fig. 1 Immunoelectrophotogram of anti-circulating anodic antigen rabbit serum (A) and anti-*Schistosoma japonicum* rabbit serum (B). *S. japonicum* homogenates (50 mg/ml) were poured into the center well.

ously described (Hirata, 1976). Homogenates of *S. japonicum* adult schistosomes were treated with 80% phenol three times and the resulting aqueous solutions were collected. The phenol was removed with ethylether, and the solution was passed through a Sephadex G-200, 90 cm column.

The phenol extract (4 mg/ml) was mixed with an equal volume of 1% methylated bovine serum albumin, and 2 ml of this mixture were added to Freund's complete adjuvant and injected subcutaneously into the pads and backs of 3 rabbits. Immunization was repeated five times at weekly intervals. The rabbits were bled 10 days after the last injection. The immune sera were examined by immunoelectrophoresis. Antiserum gave a single precipitate against *S. japonicum* homogenates (Fig. 1). The anti-CAA antiserum produced identical reactions with homogenates of *S. japonicum*, *S. mansoni* and *S. haematobium*.

Measurement of antigen titer

CAA titers in infected rabbit sera and in the homogenates of *S. japonicum, S. mansoni* and *S. haematobium* were measured with rocket immunoelectrophoresis (Laurell, 1966). A glass plate (84×94×1 mm), covered with 11 ml of 1% agarose (Nakarai Chem, LTD) containing anti-CAA was used for testing infected rabbit sera and worm homogenates. Preliminary studies showed that female schistosomes contained higher concentrations of CAA. Therefore, 3% anti-CAA was used against female homogenates and 1% anti-CAA against male homogenates and infected rabbit sera. In 15 wells were poured 10 μl aliquots of the samples. Serial 2-fold dilutions of the standard solution were used for comparison of CAA titers between glass plates containing different antiserum concentrations. Electrophoresis was carried out at 6.4 V/cm at 15 C for 20 hrs with barbital buffer pH 8.6, μ=0.05. After electrophoresis, the plates were washed with physiological saline, stained with brilliant blue, and the distances from the central well to the precipitates were then recorded. Electrophoresis was repeated 3 times. The mean values are shown in Figs. 2, 3 and 4. The distance obtained from the agarose plate containing 3% antiserum is expressed as the value obtained for 1% antiserum concentration. The calculation was made as follows: Estimated distance of precipitate in 1% antiserum concentration

$$= \frac{4.01\ (\text{distance of standard solution, 1:16 dilution, in 1\% antiserum})}{1.28\ (\text{distance of standard solution, 1:61 dilution, in 3\% antiserum})} \times$$

measured distance in 3% antiserum.

Fluorescein antibody technique

Mated *S. japonicum* was studied *in situ* by fluorescein antibody technique. Schistosomes remaining in mouse liver tissue were fixed with Rossman's fixative overnight, embedded in paraffin, and prepared according to Nash (1974). Serial 6 μ section were made and submitted to immunofluorescent examination. The slides were layered with 1:8 dilution of rabbit anti-CAA antiserum and incubated at room temperature for 20 min. After three 5-minute washings with phosphate buffered saline (PBS), pH 7.2, the slides were layered with 1:16 dilution of fluorescein isothiocyanate conjugated goat anti-rabbit IgG (Behring Institute), and incubated at room temperature for 20 min. After 3 5-minute washings with PBS, the slides were observed on an Olympus Vanox AH-RFI microscope, fitted with an Osram HBO 200 W mercury lamp, excitor filter BG12 and DM500, and two O515 barrier filters. Photographs were taken on Fuji 100 reversal film. Control

slides included normal rabbit serum plus conjugate, infected rabbit serum plus conjugate, PBS alone, anti-CAA antiserum alone, and conjugate alone.

Results

Changes in antigen titers of infected rabbit sera during the course of infection

In the sera of *Schistosoma japonicum*-infected rabbits, CAA was first detected 4 weeks after infection. Fig. 2 shows three representative rabbits with changes in CAA titers. In general, CAA titers gradually increased, peaking between 5 and 9 weeks after infection. Thereafter, titers decreased in all but one rabbit for which the CAA titer increased until the animal died. As the infection proceeded, titers increased again in 3 of 6 rabbits, represented as Nos. 1 and 3, and decreased in 2 rabbits, No. 2. The second increase was accompanied by a significant loss in body weight.

Changes in antigen concentration in adult S. japonicum during the course of infection

The changes in CAA concentrations in adult schistosomes recovered from rabbits are shown in Fig. 3. No constant change

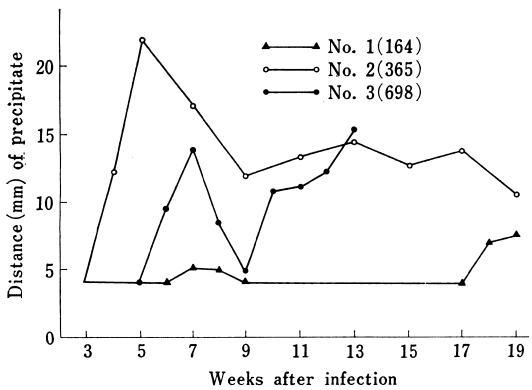

Fig. 2 Changes in circulating anodic antigen titers in the sera of *Schistosoma japonicum*-infected rabbits by rocket immunoelectrophoresis. Three representative cases are shown. Precipitates more than 5 mm are shown as positive. The numbers of worms recovered are given in parentheses.

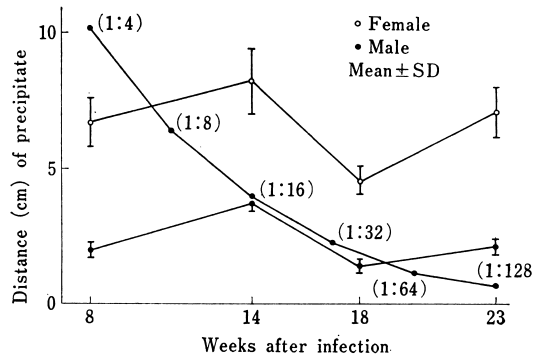

Fig. 3 Time course of changes in circulating anodic antigen concentration by rocket immunoelectrophoresis in *Schistosoma japonicum* males and females obtained from rabbits. Agarose plate containing 1% antiserum was used for male homogenates and 3% for female homogenates. The distance of precipitates developed in 3% antiserum are expressed as values in 1%. The calculation is described in the text. Parentheses show dilutions of a standard solution.

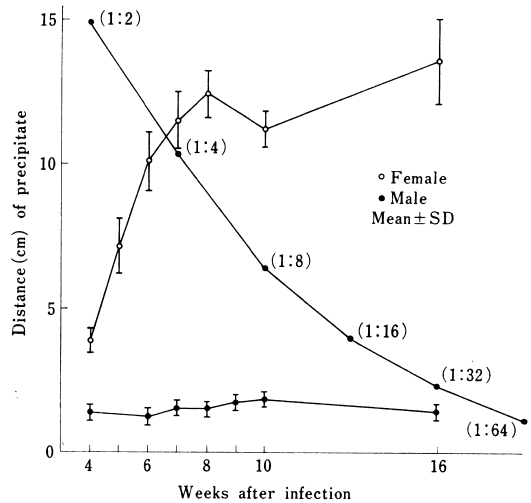

Fig. 4 Time course of changes in circulating anodic antigen concentration by rocket immunoelectrophoresis in *Schistosoma japonicum* males and females obtained from mice. Agarose plate containing 1% antiserum was used for male homogenates and 3% for female homogenates. The distance of precipitates developed in 3% antiserum are expressed in values corresponding to distance for 1% antiserum. The calculation is described in the text. Parentheses show dilutions of a standard solution.

was detected during the period of observation. CAA concentration in adult schistosomes recovered from mice was investigated with particular attention paid to changes in the earlier stages. CAA concentrations in female schistosomes increased steadily from the 4th to 7th week (Fig. 4). Thereafter, fluctuations were observed between the 8th and 16th week in the mouse, though marked decreases, as seen in the sera of infected rabbits, were not detected. In contrast, no notable changes were observed in males.

Difference in antigen concentration between males and females

It is noteworthy that the CAA concentration in *S. japonicum* males was less than that in females, as is shown in Figs. 3 and 4. This work observed at all stages examined as well as for the schistosomes from different hosts (mouse and rabbit). The female to male ratios of the CAA concentration for mice increased progressively from 3.2 to 8.5 between the 4th and 8th week after infection as a result of the striking increase in CAA concentration in the female. The female reaches maturation approximately 7 weeks after infection. The female to male ratio remained at approximately 13 from the 7th to 16th week after infection. The time course for female to male ratios for CAA concentrations in rabbits was less dramatic. From the 8th to 23rd week after infection, the ratio remained at approximately 4. The sex differences for *S. mansoni*-infected mice and the *S. haematobium*-infected hamster were similarly small. The female to male ratio was 1.7 for *S. mansoni* and 2.1 for *S. haematobium* (Fig. 6).

Fluorescein antibodies were employed to demonstrate qualitative differences in CAA by sex. Specific fluorescence was observed along the borders of the male and female caecum, though with variable intensity. In addition to the caecum, the excretory canals were stained. No staining was observed in the tegument or parenchyma of the schistosome, nor in any of the controls. The only difference observed between sexes was that the bulk of the female caecum was considerably larger than that of the male caecum (Fig. 5).

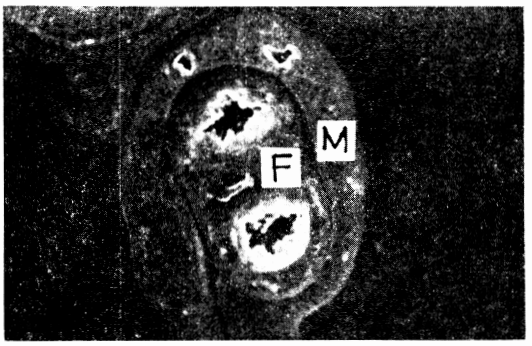

Fig. 5 Specific fluorescence at the caecum of *Schistosoma japonicum* male and female. Autofluorescence of egg shell is seen at the center. Note the difference of the caecum bulk between male (M) and Female (F).

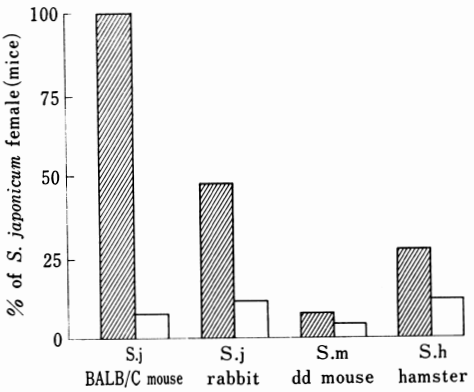

Fig. 6 Comparison of circulating anodic antigen (CAA) concentrations by rocket immunoelectrophoresis between *Schistosoma japonicum* (S.j.), *S. mansoni* (S.m) and *S. haematobium* (S.h) males (white), and females (hatched). The distance of precipitate of *S. japonicum* females is shown as 100%. CAA concentrations of *S. japonicum*, obtained from mice or rabbits 7 weeks after infection, were averaged (see also Figs. 3–4).

Comparison of antigen concentration in three schistosome species

S. japonicum were recovered from BALB/

C mice and rabbits. CAA concentrations of mature schistosomes (more than 7 weeks old) are shown in Fig. 6 (see also Figs. 3 and 4). *S. mansoni* (14 weeks old) were recovered from 4 dd mice and *S. haematobium* (35 weeks old) were recovered from a hamster. Fig. 6 demonstrates notable differences among these 3 species. The differences are most apparent in females; *S. japonicum* shows the highest CAA concentration and *S. mansoni* the lowest. It is noteworthy that the concentration in *S. japonicum* females differs depending upon the host, mouse or rabbit.

Discussion

Several antigens have been detected in the sera of mammals infected with schistosome species (Berggren and Weller, 1967); Nash *et al.*, 1977; Calier *et al.*, 1978; Bout *et al.*, 1978; Santro *et al.*, 1979; Deelder *et al.*, 1980). Hirata and Tsutsumi (1978) showed that of all schistosome antigens, only CAA was detectable by immunoelectrophoresis in the sera of mice at 48 hours after injection with *S. japonicum* homogenates. All other antigens were undetectable after 24 hours. Furthermore, CAA was detectable at 24 hours after a second, booster inoculation one week later, while all other antigens were undetectable after 2 hours. Thus, CAA remains the most easily studied antigen.

Anti-CAA was produced in the rabbit by immunization with the phenol extract of CAA from *S. japonicum*. The anti-CAA gave a single precipitin band against the homogenates of the 3 schistosome species tested. Further support for the specificity of the anti-CAA is provided by the fluorescein antibody test, which demonstrated in *S. japonicum* clearly positive caecal and excretory organelles and distinctly negative tegument, parenchyma and controls.

Recently, Deelder *et al.* (1980) investigated the nature of two polysaccharide antigens in the trichloroacetic acid extract of *S. mansoni* schistosome antigen. They found a cathodic antigen and an anodic antigen on immunoelectrophoresis. Antibodies against the cathodic antigen could not be induced in the rabbit by immunization; however, high titers of antisera were produced against the anodic antigen. It is likely that the phenol extraction method tested in my experiments also included the cathodic antigen. However, only a single anodic precipitin line was observed when the extract was reacted with rabbit antiserum, thus confirming Deelder's finding of a single, inducible antibody, that is, anti-CAA.

CAA has been detected in the sera of *S. japonicum*-infected rabbits, chimpanzees and mice (Hirata and Akusawa, 1975; Hillyer, 1976; Hirata, 1976) and *S. mansoni*-infected hamsters and mice (Bawden and Weller, 1974). In their study, Bawden and Weller reported that titers increased steadily during the 42 to 44 day period of observation. In the present study, CAA titers in *S. japonicum* infected rabbits peaked between the 5th and 9th week, consistent with Bawden and Weller's findings.

Comparing the first phase of the time courses of CAA titers in infected rabbits with the CAA concentrations of female *S. japonicum* shows a remarkable degree of agreement. These data suggest that the female *S. japonicum* is responsible for the first phase of increased CAA titers in infected rabbits. However, subsequent to the first phase, no consistent host response pattern emerges. CAA titers decreased in 5 of 6 rabbits, then increased again in 3 of these 5 rabbits, The decrease in CAA titer was independent of CAA concentration in female schistosomes. The decrease may be attributed to the development of anti-CAA antibodies (Deelder *et al.*, 1978; Nash, 1978; Carlier *et al.*, 1980) or possibly lethal IgG antibodies (Clegg and Smithers, 1972; Murrell and Clay, 1972).

The second increase in CAA titer corre-

sponded to a loss in body weight. It is known that *S. japonicum* is more virulent than *S. mansoni* or *S. haematobium* (Noble and Noble, 1976). The loss in body weight may be directly attributable to the increase in CAA titer. Schistosome burden correlated well with the second increase in CAA titer (Data not shown). Recently, Ferreira *et al.* (1979) reported that significantly higher second phase CAA titers also occurred in mice infected with *S. mansoni* male and female. However, they attributed the increase to impairment of glomerular filtration of the parasite antigen.

The CAA concentration in adult *S. japonicum* schistosomes shows a marked contrast between male and female. The preliminary studies by the author demonstrated a several-fold difference, raising technical problems in measuring distances for the precipitates. This necessitated a differential in the anti-CAA antiserum concentration, 1% for males and 3% for females. A series of controls to standardize the distances suggested an approximately linear ratio (Data not shown).

Throughout the experiment, CAA concentrations remained constant in male schistosomes. These data are consistent with the findings of Ferreira *et al.* (1979) who demonstrated a constant, low concentration of CAA for 27 weeks in mice infected with *S. mansoni* male alone.

The presence of the male in the sexual maturation of the female schistosome has been appreciated since the early studies of Severinghaus (1928). He showed that unmated schistosomes reached only 20% of the body length of mated females. Armstrong (1965) reported that a pheromone produced by the *S. mansoni* male schistosome was essential for the initiation and maintenance of the female's sexual maturation. The increase in CAA concentration may also reflect maturational changes.

Lichtenberg *et al.* (1974) and Nash *et al.* (1977) have speculated a protective role for CAA, to minimize the effects of host digestive enzymes and/or host defense immune mechanisms. Female schistosomes have higher metabolic requirements for host materials to support egg production. The larger female caecal bulk may, therefore, reflect the need for a higher CAA concentration to provide protection of the caecum.

The variation in CAA concentrations among the females of the three schistosome species is notable. No such previous studies are available for comparison. The difference is especially clear for *S. japonicum* and *S. mansoni*; CAA concentration of *S. japonicum* female recovered from BALB/C mice is about ten times the concentration of *S. mansoni* female recovered from dd mice. Less dramatically, CAA concentrations in *S. japonicum* female varied between host species, in the present experiment, BALB/C mice and rabbits. Significant differences have also found among geographical strains and substrains of *S. mansoni* in terms of egg numbers and distribution in mouse tissue (Kassim, 1979). It, therefore, remains for future studies to elaborate the roles of host and schistosome in experimental infections.

Summary

Female worms play an important role in determining the level of circulating anodic antigen (CAA) in *Schistosoma japonicum* infections. CAA concentration in female worms increased during the period between 4 to 7 weeks of infection while it generally remained constant in males. The pattern of increase corresponded to that of the first increase in CAA titers of infected rabbit sera. CAA was considered to be sex-dependent, because CAA concentration of females was significantly higher than that of males in all cases, although female to male ratio of CAA concentration varied with the duration of infection and host species. Furthermore, it was noteworthy

that CAA concentration in *S. japonicum* females differed with host species, mouse and rabbit.

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日本住血吸虫感染における循環抗原の雌虫体依存性

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日本住血吸虫感染動物の血液中にみられる循環抗原(CAA) は顕著に雌に多い物質であった．雌：雄のCAA 含有割合は週令で異なり，感染4〜16週のマウス (BALB/C) からえた虫体で 3.2 から 13.0 と変化した．また，感染8週以後のウサギで約 4.0 であった．．これらの週令および宿主による含有割合の変化は主に雌虫体内の CAA 量に原因することが認められた．

ビルハルツおよびマンソン住血吸虫で虫体内の CAA 含有量をしらべた結果，雌に多いことは日本住血吸虫と同様であるが，雌：雄の割合は前者で 2.1，後者で 1.7 と日本住血吸虫の場合程顕著な差は認められなかった．

感染ウサギ血清中の CAA レベルは感染後約7週前後でピークとなるが，この初期の増加パターンは雌虫体内の CAA 量の増加と一致していた．

以上のことから，CAA は性依存性抗原であり，感染血清中の CAA レベルは寄生している雌虫体数と深く関係していると考えられる．