Bull. Inst. Zool., Academia Sinica **27**(4): 249-258 (1988)

ON THE *PLISTOPHORA* INFECTION IN EEL
II. The Development of *Plistophora anguillarum* in Experimentally Infected Elvers, *Anguilla japonica*

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(Accepted May 25, 1988)

Wen-Huei T'sui, Chung-Hsiung Wang and Chu-Fang Lo (1988) On the *Plistophora* infection in eel II. The development of *Plistophora anguillarum* in experimentally infected elvers, *Anguilla japonica*. *Bull. Inst. Zool., Academia Sinica* **27**(4): 249-258. In order to investigate the development of *Plistophora anguillarum*, elvers were artifically infected with this Microsporea by immersing them in spore-containing water. Following the histological preparations of infected elvers sacrificed at one day intervals, the developmental stages of *P. anguillarum* were observed. The time of first appearance of schizonts, sporonts, cysts and the free spores was also recorded. The results showed that at 25°C, it was at least 13 days for *P. anguillarum* to complete the life cycle. The sporogony phase began at the 6th day after infection. No inflammatory response was observed until the cysts ruptured (late sporogony phase). According to the present data, it was suggested that the routes of infection of *P. anguillarum* in the elvers were through both skin and digestive tract. Furthermore, the schizonts might reach the infection sites via circulatory system rather than via direct migration through the coelum.

Key words: *Plistophora*, Elver, Development.

The etiology of the "beko disease" of the eel, *Anguilla japonica*, had been known to be a Microsporea, *Plistophora anguillarum*, parasitized in the skeletal muscle (Hoshina, 1951). The histopathology, the ultrastructure and the development of *P. anguillarum* in naturally infected eels had been reported (Akada *et al.*, 1977; Hashimoto and Takinami, 1976; T'sui and Wang, 1988). Experimental induction of eel microsporidiosis was first reported by Kano and Fukui (1982). Artificial infection was done by inoculating orally *Plistophora* spores into juvenile eels or immersing them in water suspension of the fresh spores. Their achievements demonstrated valuable methods for studying the biology of *P. anguillarum*, especially the developmental events of this parasite in their host. Thus, it was attempted in the present studies to observe the artificially infected elvers in detail, in order to understand the precious developmental stages of *P. anguillarum* in early infection.

MATERIALS AND METHODS

I. Purification of spores for artificial infection

The eels, naturally infected with *Plistophora anguillarum* were imobilized by ice and

then dissected. The infected tissues were removed and put into 0.05% (w/v) solution of trypsin in phosphate buffer solution (PBS) at pH 8.0 for digestion. To avoid bacterial growth, penicillin G (400 IU/ml) and streptomycin sulfate (400 μg/ml) were added to the solution. Digestion was at room temperature for 3 to 4 hours with constant stir. The digests were filtered through 5 layers of gauze. The filtrate contained many free spores and cell debris. The filtrate were pelleted at 300×g for 10 minutes. The pellete was washed with 100 ml PBS and then centrifuged at 300×g for 10 minutes. The pellete was resuspended with PBS to make the solution having a concentration of 10^9 spores ml^{-1}. Purification of spores was done by percoll gradient. Percoll (Pharmacia, Fine chemicals) was made iso-osmotic stock solution by add 9 parts (v/v) of percoll to 1 part (v/v) of 1.5 M NaCl. One ml of spore suspension (10^9 spores/ml) were layered onto 9 ml 90% percoll solution in 10.4 ml polycarbonate centrifuge tube and centrifuged at 50,000×g for 30 minutes, in a fixed-angle type 65 motor of a Backman L2-65B ultracentrifuge. The band formed by purified spores was collected with L-shaped spinal needle. The spores were washed with PBS and then pelleted. For artificial infection, the pellete was suspended with sterile water and diluted to have a concentration of 10^7 spores ml^{-1}.

II. Artificial infection and histological observation

Eighty elvers (0.15 g) were immersed in 100 ml spore suspension solution (10^7 spores/ml) at 25°C for 12 hours. The infected elvers were then cultured in aquaria in laboratory and fed with *Tubifix* sp. for 30 days. During this period, the infected elvers were sacrified at one-day intervals and fixed in Zenker's fixative and processing for paraffin embedding. Histological serial sections, 7 μm in thickness, were stained with Masson's triple stain and observed with compound light microscope.

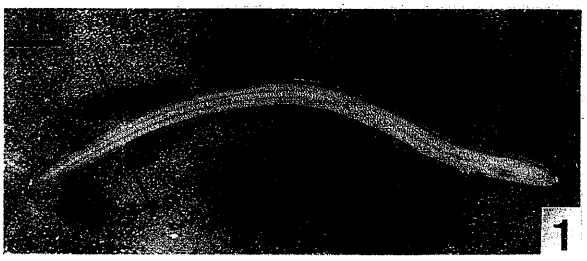
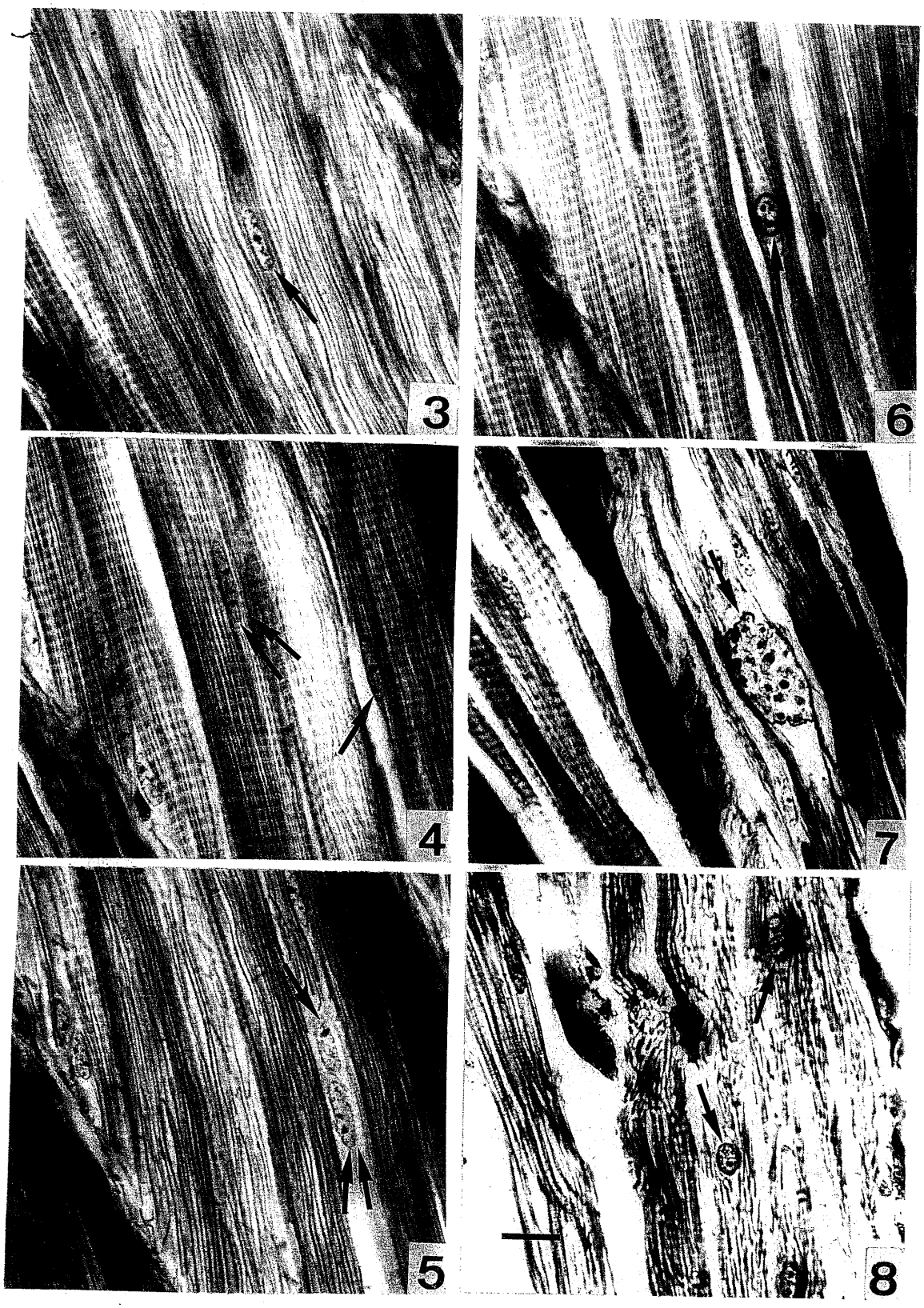


Fig. 1. The infected elver with white spots (arrows) on the body surface. Bar: 1 cm.

Fig. 2. The magnification of part of Fig. 1., showing the white spots (arrows) on the body surface. Bar: 1 mm.

Fig. 3. The mononucleated schizont (arrow) in skeletal muscle (5 days post infection).
Fig. 4. The mononucleated schizont (single arrow) and bi-nucleated schizont (double arrow) were in the skeletal muscle (6 days post infection).
Fig. 5. The mononucleated schizont (single arrow) and bi-nucleated schizont (double arrow) were in the skeletal muscle (7 days post infection).
Fig. 6. The bi-nucleated sporont (arrow) in the skeletal muscle (7 days post infection).
Fig. 7. The cyst (arrow) in the skeletal muscle (8 days post infection).
Fig. 8. The bi-nucleated sporonts (arrows) in the skeletal muscle (12 days post infection).
Bar in Figs. 3-8 is 10 μm.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RESULTS

I. Artificial infection

In order to get 100% infection, the high concentration spore suspension was used for experimental infection of elvers in the present study. The infection rate of elvers immersed in the spore-suspension (10^7 spores/ml) for 12 hours on the first day of the experiment was 100% as we expected when checked the 20 elvers left at the end of the experiment. The infected elvers 30 days post infection showed many white spots underneath the body surface formed by accumulated cysts when viewed by dissecting microscope (Fig. 1, 2).

II. The development of *P. anguillarum* in the muscle of the experimental infected elvers

Two elvers were sacrificed at one day intervals during the 30 day infection and treated for light microscopy. The daily changes in the tissues of the elvers were carefully observed and recorded. According to the light microscopic observation, the main sequence of the development of *P. anguillarum* in the experimentally infected elvers was divided into three phases: schizogony, sporogony I and sporogony II.

Phase I: schizogony (1–5 days post infection)

The mononucleated schizonts, 10–14 μm × 4–6.5 μm in size, possessed a small nucleus located at the central clear region of sporoplasm (Fig. 3). They were mostly found under the sarcolemma (Fig. 4, 5). The binucleated or tetra-nucleated schizonts were also identified during this phase (Fig. 4, 5). The long axis of schizonts was often parallel to the myofibrils (Fig. 4). The peripheral region of muscle bundles beneath the skin was heavily infected while the interior of muscle fascicle was lightly infected. Only a few schizonts invaded the interior of the muscle fiber. The infected muscle did not show any detectable pathological changes during phase I.

Phase II: sporogony I (6–10 days post infection)

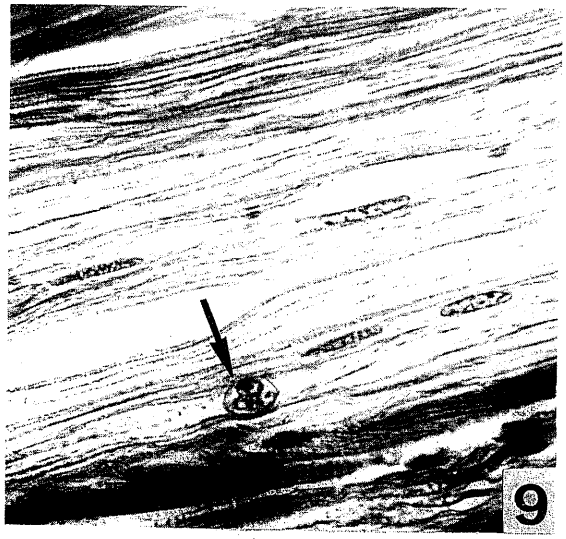
The bi-nucleated sporonts were first discovered 6 days post infection. The oval-shaped sporont found in muscle fiber was surrounded by a thin membrane (Fig. 6). At the same time, schizonts kept on multiplication and infiltration. They increased in number significantly and invaded the interior of muscle fibers. At 7th day after inoculation 4- to 8- nucleated sporonts could be found. The sporonts divided and formed pansporoblasts at 8th day after infection. The oval-shaped cyst, 9.5–13.0 μm × 14.9–24.9 μm in size, contained 4 pansporoblasts which came from the same sporont (Fig. 7). The development of the pansporoblasts in a cyst was not synchronized while the development of sporoblasts in a pansporoblast was synchronized. The microspores in the pansporoblast were observed at the 9th day after infection. There were no significant changes in the infected muscle at the end of this phase, except the increased number of cysts and schizonts.

Phase III: sphorogony II (11–15 days post infection)

The *P. anguillarum* formed the cysts both in the skeletal muscle and the connective tissue at the beginning of this phase. The skeletal muscle were filled with cysts. A few cysts were found in connective tissue. Those cysts in connective tissue were quite small, 3.5–6.2 μm × 5.7–8.4 μm in size, as compared to the cysts in skeletal muscle. And only one or two pansporoblasts were developed within them.

In addition to the cysts, a few membrane bound bi-nucleated sporonts underneath the sarcolemma of the skeletal muscle were distinguished (Fig. 8). It implied that the

Fig. 9-14. The microphotographs of the muscle of elvers 12-13 days post infection with *P. anguillarum* showing the early to late developing stages of sporonts (arrows). Bar: 10 μm.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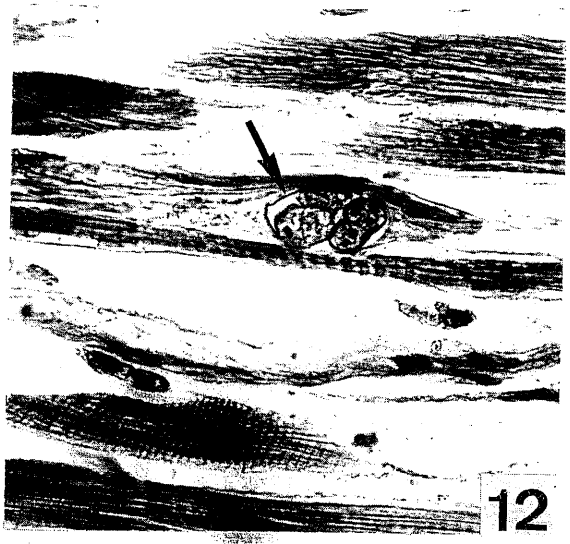
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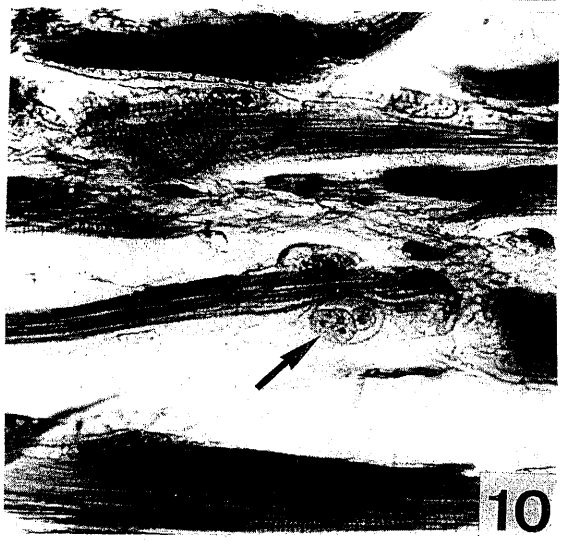
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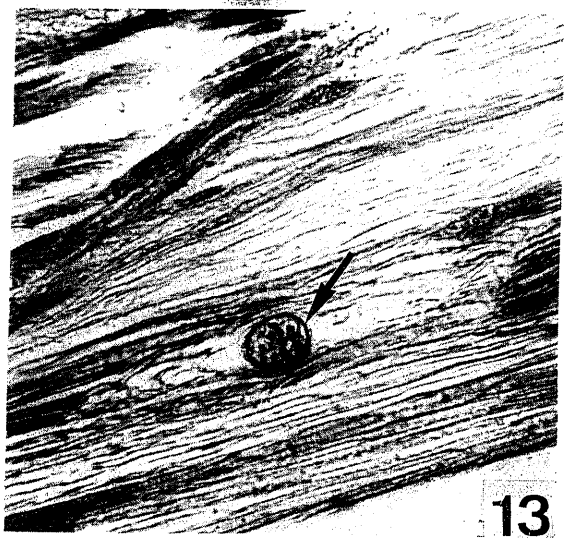
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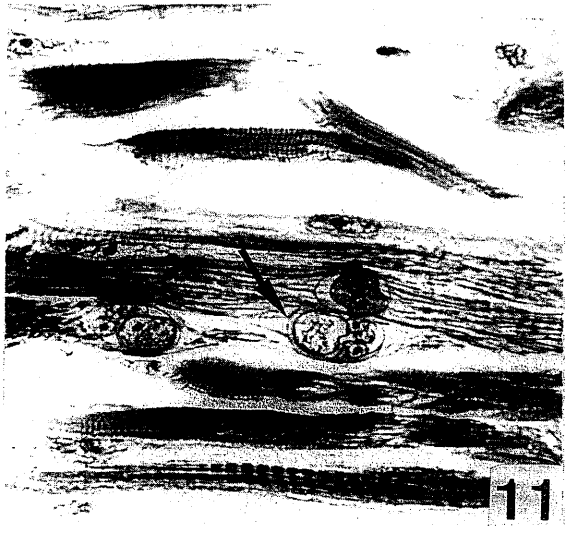
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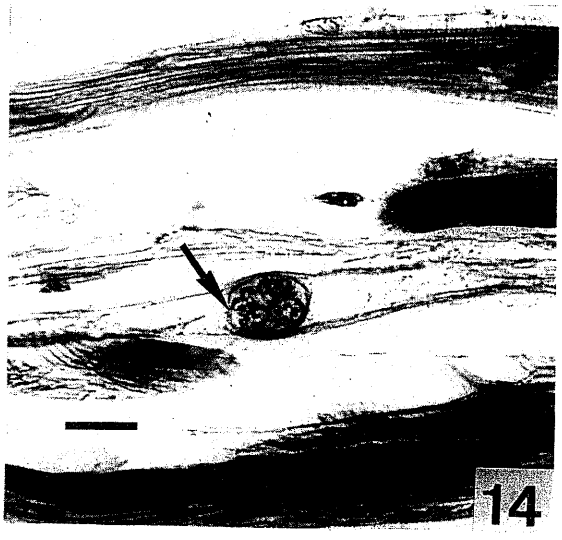
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second sporogony stage start to develope. Membrane-bound sporonts with 2-8 nuclei appeared in the skeletal muscle 12 days post-infection (Fig. 9, 10, 11, 12, 13 and 14). These multi-nucleated sporonts eventually splited through cytokinesis into 4-8 pansporoblasts (Fig. 15, 16). At the end of this, 2-4 sporoblasts were observed.

The development 15-30 days after infection

Since the *P. anguillarum* could complete their developmental cycle through phase I to phase III within 13 days, many of them might enter the second cycle of the development at least 13 days after infection. But it should be mentioned that some of schizonts formed in phase I of first cycle delayed their development and entered the phase II at any time later than what was shown in previous paragraphs. As a results, a mix of many developmental stages was actually revealed after phase I, especially 15 days post-infection. The increase of sporonts in the tissue resulted from the development of the mononucleated schizont at the 14th day after infection. The larger cysts, 24.3-37.8 μm×38.2-51.4 μm in size, with 8 or more pansporoblasts were observed. The number of sporoblasts in each pansporoblast was usually more than 8 (Fig. 17). Most of the matured cysts were filled with many spores. The infected muscle fibers were distorted and distended due to the development of cysts and depressed the adjacent muscle fibers (Fig. 18, 19). The serious infected muscle fibers showed a Zenker's symptom and lost their striation characteristics and stainability. At last, the muscle bundles were completely eliminated. The cysts eventually ruptured and the spores of cysts disposed in intercellular space or connective tissue (Fig. 20, 21).

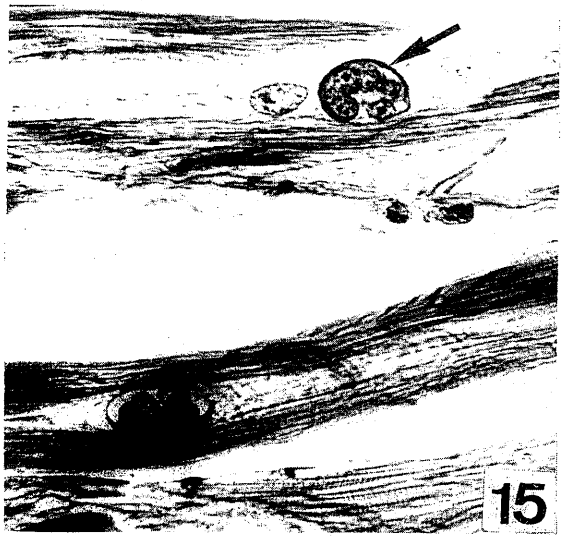
III. The development of *P. anguillarum* in the digestive tract of the experimentally infected elvers

Only a few *P. anguillarum* were occasionally found in digestive tract because the experimental infection was done by immersion method. Thus, it is impossible in present study to describe the developmental sequence in digestive tract according to the time table. However, some sporonts could be found in muscular and submucosa layer of the digestive tract. The cysts found in both layers were quite small, 14.9-23 μm×16.8-25.7 μm in size (Fig. 22, 23, 24, 25 and 26). No detectable pathological changes were observed in the mucosa layer of the epithelium.

DISCUSSION

Hashimoto and Takinami (1976) had described the ultrastructure of *P. anguillarum*, but they did not interpret the time table of the developing stages in the life cycle. In the present study, the experimental infection was carried out by immersion the elvers in water suspensions of the fresh spores at 25°C. It was successfully to induce the same symptom, beko disease, as that of naturally infected eel. Early occurrence of schizonts, sporonts, cysts and the free spores were observed at the 1st, 6th, 8th and 14th days after inoculation, respectively. It was suggested that *P. anguillarum* takes at least 13 days to complete the life cycle in elvers at

Fig. 15. The early developing cyst with thin wall in the skeletal muscle (14 days post infection).
Fig. 16. The early developing cyst with thick wall in the skeletal muscle (14 days post infection).
Fig. 17. The mature cyst (arrow) with spores in the skeletal muscle (22 days post infection).
Fig. 18. The mature cyst (arrow) with many spores in the skeletal muscle. The myofibril was still intact (23 days post infection).
Fig. 19. The mature cysts (single arrow) aggregated in the skeletal muscle. The ruptured cyst resulted in the dissolution of myofibrils (double arrow). (23 days post infection).
Fig. 20. The cyst ruptured and the free spores (arrow) was observed in the intercellular space. (23 days post infection).
Bar in Figs. 5-20 is 10 μm.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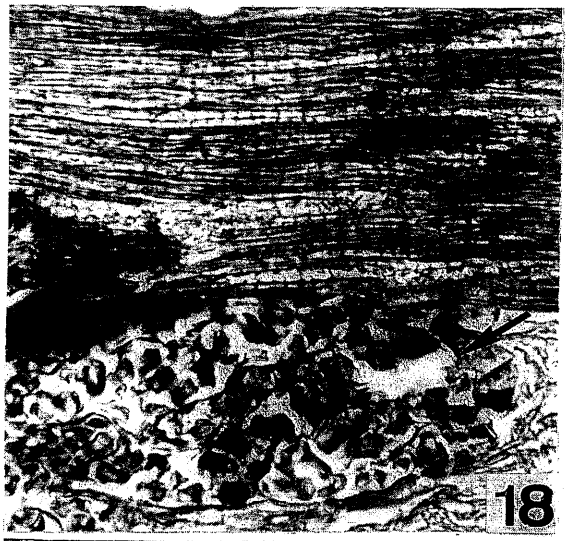
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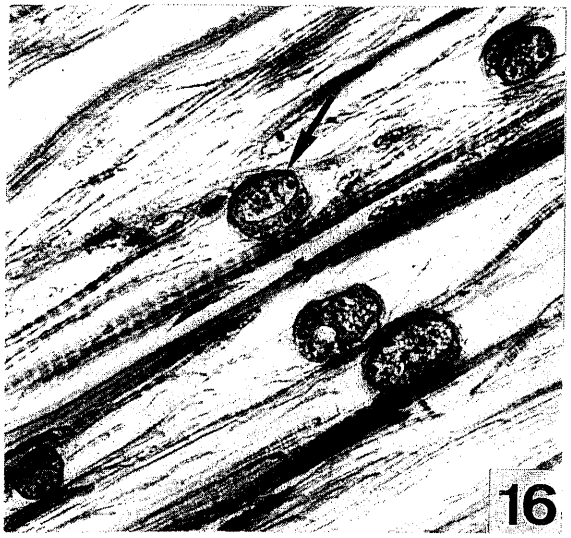
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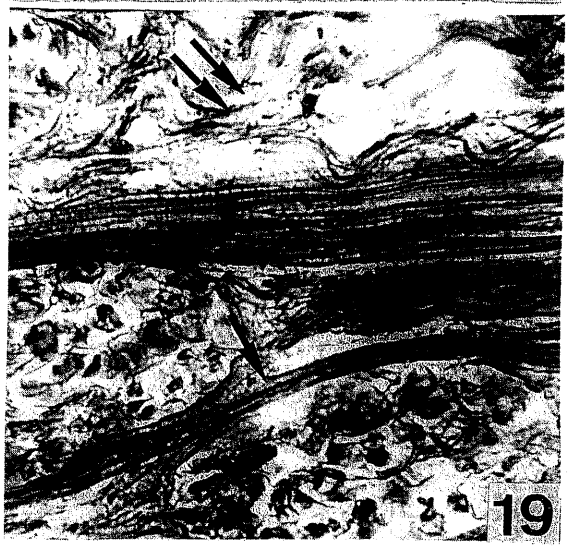
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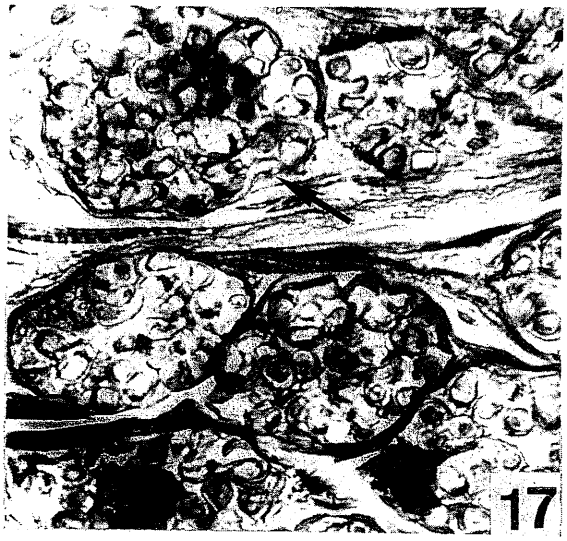
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25°C. This result is different from that reported by Kano & Fukui (1982).

According to our result, the sporogony phase began at the 6th day after inoculation and most of the schizonts developed into the sporogony phase at the 11th day, but some schizonts were still found in the tissue. It is implied that some schizonts kept on progressing schizogony reproduction throughout the infection period. It is not clear in the present studies that when the schizogony phase will be ended. The mechanism which triggers the parasites to enter the sporogony phase is still needed to be elucided.

Although some scientists had shown that the infection route of *P. anguillarum* passes through the digestive trace (Egusa 1978). Kano and Fukui (1982) inoculated successfully the juvenile eels with fresh spores by both immersion and oral methods, and suggested that the early stages of this microsporazoa reach the muscular tissue via gut wall and peritoneal fluid or skin, rather than via the blood vascular system. Using the similar immersion method in this study, the appearance of this Microsporea in the elvers was from peripheral tissue to internal tissue with a time-dependent pheromenon. Therefore it is now fairly certained that the infection routes of *P. anguillarum* in the elvers is regarded as through both skin and digestive tract. Furthermore, there were no Microsporea in the coelum of the infected elvers. This result indicated that the schizont might be invading the internal tissue via circulatory system rather than via direct migration through the coelum. In the present study, there was no signals of inflammatory response occurred before 22 days post infection of the elvers. Our previous reports had indicated that the harm done to the eel is by mechanical damage in early infections and chemical damage in late infection (T'sui and Wang, 1988). Now we had proved again that the rupture of the cyst and the release of its content led to the dissolution of the muscle as well as the infiltration of the lymphocytes into the infective sites. It was therefore concluded here that the Zenker's degeneration of muscle must result from the chemical destruction and the lymphocyte infiitration may be a chemotaxic action of lymphocytes.

Acknowledgements: The research was supported by Council of Agriculture.

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Fig. 21. The mononucleated schizont (single arrow) and the cyst (doublearrow) observed in the skeletal muscle. (23 days post infection).

Fig. 22. The sporont in the muscle layer of the digestive tract. (9 days post infection).

Fig. 23. The bi-nucleated sporont (arrow) observed in the muscular layer of the digestive tract. (12 days post infection).

Fig. 24. The 4-nucleated sporont (arrow) observed in the muscular layer of the digestive tract. (13 days post infection).

Fig 25. The early developing stages of the cyst (arrow) observed in the muscular layer of the digestive tract. (22 days post infection).

Fig. 26. The late developing stages of the cyst (arrow) observed in the muscular layer of the digestive tract. (23 days post infection).

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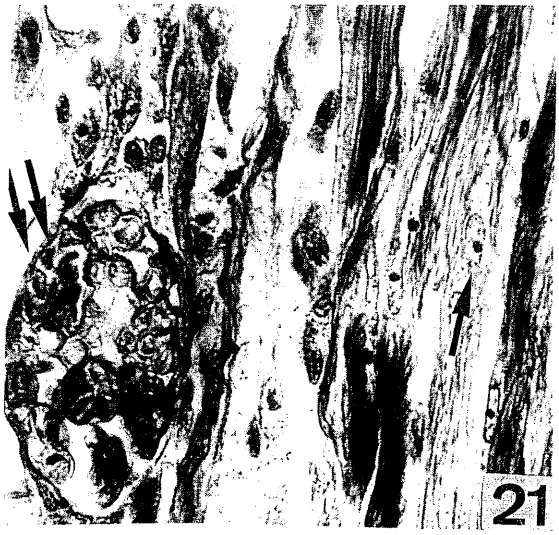
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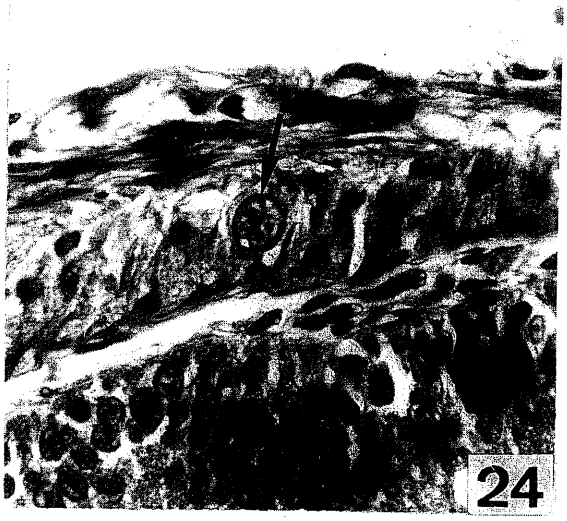
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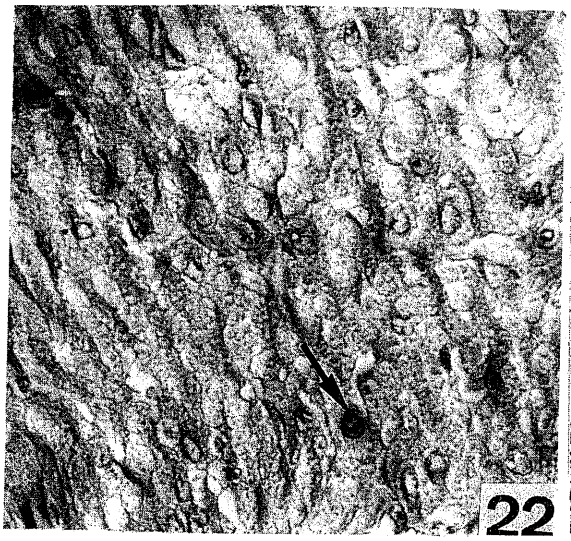
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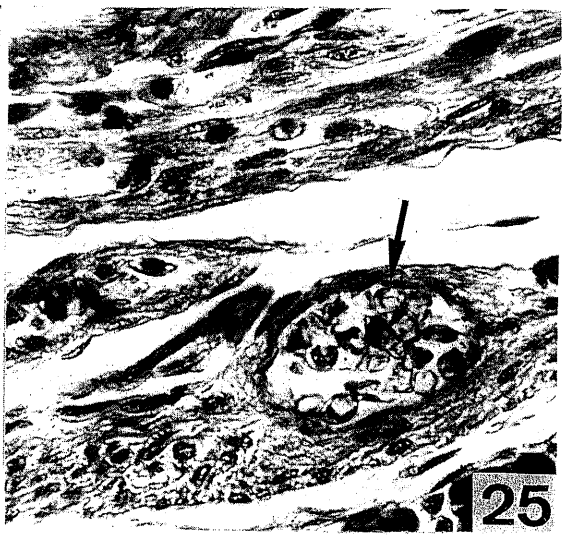
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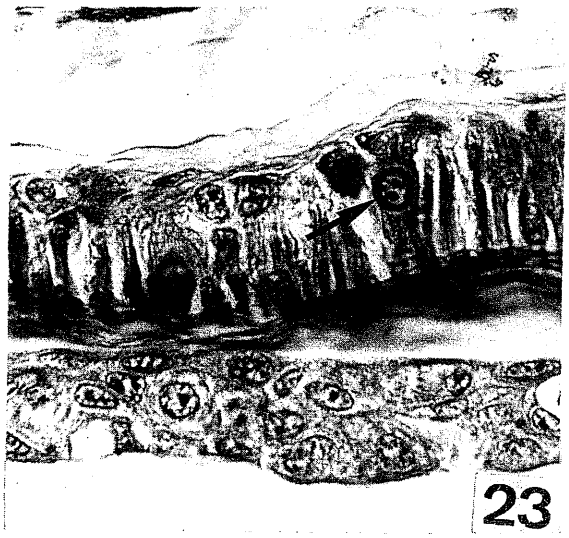
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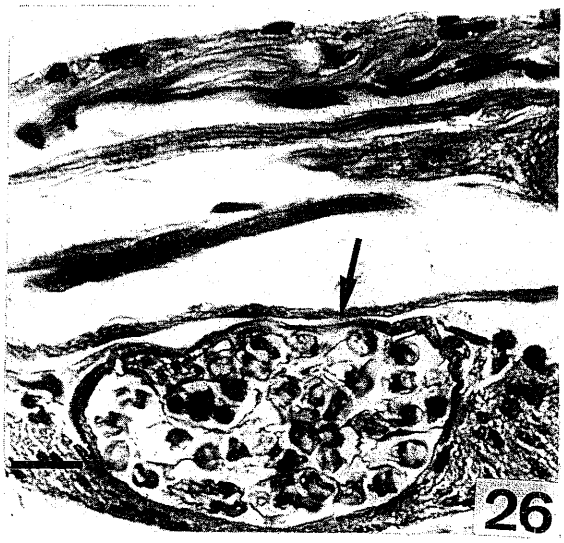
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鰻魚凹凸病之研究

II. 實驗感染鰻線體內：日本鰻凹凸病原蟲之發育

崔文慧　王重雄　羅竹芳

利用人工感染法，將鰻線浸泡於含鰻魚微孢子蟲 (*Plistophora anguillarum*) 的浸液中，由魚體中營養體、母孢子、囊胞與游離孢子的最早出現時間，推算 25°C 下鰻魚微孢子蟲在鰻線體內，需時 13 天完成其生活史，孢子體形成時期始於接種後第 6 天。鰻魚微孢子蟲經由皮膚及消化道進入鰻體，營養體復藉循環系統而非經體腔內之遷移方式入侵其他組織。

又囊胞破裂前（孢子體形成晚期）宿主無任何發炎反應。但囊胞破裂後，即發生細胞浸潤現象。