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ELECTRON MICROSCOPICAL STUDIES OF THE LIFE CYCLE OF

ICHTHYOPHTHIRIUS MULTIFILIIS FOUQUET

By

Mark Geisslinger

B.S. Livingston College, Rutgers University, 1981

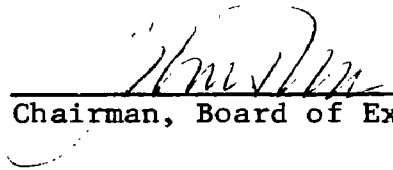
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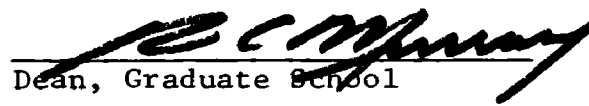
Master of Arts

University of Montana

1984

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Electron Microscopical Studies of the Life Cycle of Ichthyophthirius multifiliis Fouquet (53 pp.)

Director: Dr. John F. Tibbs

Scanning and transmission electron microscopical observations of life cycle stages of the protozoan fish parasite, Ichthyophthirius multifiliis, are presented. Maturing trophozoites can be seen in crevices just below the epidermis of the fish host. The trophozoite exhibits a heterogeneous endoplasm with rough and smooth endoplasmic reticulum, polysomes, food vacuoles, lysosomes, micronucleus and macronucleus. In their cortical region trophozoites possess many mitochondria and extrusive mucocysts as well as somatic cilia and associated structures.

After leaving the host the ciliate swims briefly before settling onto substrate, secreting a cyst and dividing. From the two-cell stage and beyond, two separated masses of cells are seen within the cyst. Ovoid bacteria can be seen within the cyst's wall which becomes thin and fragile in terminal stages. Infective tomites rupture the wall at excystation, the end of the cyst stage.

The emphasis of this study was on the infective tomite and its invasion of the host. Scanning electron microscopy shows tomites to be elongated, banana-shaped, highly ciliated and 38-45 micrometers long. A slight depression leading to a dark oval cavity in the posterior third of the cell can be seen. This may be a rudimentary mouth or cytostomal opening. Transmission electron microscopy shows the presence of an apparently rudimentary but functional cytostome-cytopharyngeal complex. A ciliated buccal cavity leads into a tunnel-like passageway at the end of which is a pouch or vacuole containing bacteria and other debris. The tomite endoplasm contains many bacteria enclosed in vacuoles and apparently not digested. Other endoplasmic features include a large macronucleus and small micronucleus, a contractile vacuole, lipid vesicles and large numbers of mucocysts and mitochondria in the cortical region. Scanning electron microscopy also shows host invasion by the tomite and what may be a boring apparatus at its anterior end. A thick doughnut-shaped structure encircles an opening or cavity. The thickened ring structure possesses eight raised, evenly-spaced struts radiating from the cavity and extending posteriorly over the edge and below the ring. From the cavity protrudes a long, cilium-like structure. As seen with TEM, the apical region possesses a knob-like structure, bounded by the plasma membrane and containing ribosome-free cytoplasm set off from the endoplasm of the tomite. Vesicles can be seen leading to the knob structure, possibly representing packets of hyaluronidase used in lysing host cell binding material.

PERSONALLY RELEVANT QUOTE

A Lunatic

My mind intensely strained,
I sat a little space
Before a man possessed by strange
Hallucinations -- face to face.

-- Mokichi

Acknowledgements

I would like to thank Dr. John Mitchell, Dr. Andrew Sheldon and Dr. C. A. Speer for serving on my graduate committee. I would like to again thank Dr. Speer for initially giving me the opportunity to learn electron microscopy.

I would like to extend a very special thank you to my major professor, Dr. John F. Tibbs, for his support, encouragement and numerous discussions which did much to alleviate my frequent anxieties about graduate school and research.

I would also like to thank William Whitmire and Kamiar Moin for providing assistance on the scanning and transmission electron microscopes when the going got tough.

I will be forever indebted to Ray A. Larsen. Ray was solely responsible for teaching me electron microscopy from sacrificing and fixing specimens right on through to darkroom techniques. He was there when I needed him (which was often). I greatly appreciated his interest, patience and expertise.

I would also like to thank DeWayne Williams for working diligently to provide slides for seminars and a diagram for this thesis.

I will miss the beautiful State of Montana, the University, and above all, the people I've come to know.

This thesis project was completely supported by the Department of Zoology, University of Montana.

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INTRODUCTION

Representatives from many different phylogenetic groups have evolved to take up the parasitic way of life. Of all the groups, helminths, some arthropods and protozoans have been the most successful infecting a wide variety of vertebrate and invertebrate hosts (49). The protozoan phylum Ciliophora features a large number of organisms parasitic on freshwater fish (59). One of these parasitic ciliates is Ichthyophthirius multifiliis.

Ichthyophthirius multifiliis is an obligate ectoparasite of a wide variety of freshwater fish throughout the world (29,32). It invades the epidermis and lamellae of the gills causing white spot disease or ichthyophthiriasis (commonly known as "ich"). The disease is common in fish hatcheries and aquaria causing great losses in some cases (29,32,59).

The life cycle of this parasite is direct, meaning there is a single host. It is not transmitted from fish to fish but undergoes a series of changes, including cystic and free-swimming stages. The adult trophozoite is typically oval, uniformly ciliated and 50 to 1000 micrometers long. It feeds on the fish, stores food reserves and matures. Mature trophozoites have a characteristic U-shaped macronucleus. When fully mature, the parasite leaves the host, migrates to substratum and, in a short period of time, secretes a cyst around itself. At this stage reproduction occurs by transverse binary fission resulting in the release of several hundred to one thousand free-swimming, infective, astomatous tomites approximately 30-45 micrometers long, uniformly ciliated and possessing an anterior boring apparatus or perforatorium (32,47,57). The time required to complete the life cycle is inversely

proportional to temperature within a range of 7°C to 24-26°C (32).

Efforts to control the disease were reviewed by Cross (9). Many of the methods consist of doses or a mixture of doses of chemicals used to treat aquarium, pond or holding tank water. The methods employed destroy the free-swimming, infective tomites. No chemicals are known to be effective against the parasite once it is in the host (9). It appears that nearly all treatments are limited by several factors including cost, environmental conditions of the water and detriment to the fish (9,34). Bauer (4) was one of the first to report a post-invasive immunity in carp to ichthyophthiriasis lasting only a maximum of 7 days. The more recent studies involving Ichthyophthirius multifiliis have dealt with immunity to the disease by means of immunization. Indications are that immunization may be a promising means of control (16,17,63). These studies are discussed further in the literature review.

CHRONOLOGICAL REVIEW OF THE LITERATURE

Hilgendorf and Paulicki (23) were the first to observe Ichthyophthirius multifiliis in 1869 on a number of different species of fish in the aquarium in the zoological gardens in Hamburg, Germany. They described the ciliate as having no mouth or characteristic form. Its body was covered with cilia arranged in longitudinal rows and possessed a nucleus, contractile vacuole, several other vacuole-like structures and granules. When infected fish were placed in a separate aquarium for study, the parasites left their host, fell to the bottom, encysted and multiplied; they postulated that the bodies infected new fish (23).

The first detailed study of the parasite was conducted by Fouquet in 1876 who named Ichthyophthirius multifiliis. Fouquet added much to the observations of Hilgendorf and Paulicki. He recorded the sizes and descriptions of several anatomical features including the cortical layer, horseshoe-shaped nucleus, contractile vacuole, "trichocysts", granules, cytostome (mouth) and esophagus. Fouquet observed that when the adult reached a certain size it left the fish and fell to the bottom where it encysted. The ciliate then divided until approximately 1000 cells were formed. The divisions were completed in 40 to 50 hours, the time varying with the temperature. Fouquet's attempts to infect fish with the young parasites failed (14).

It was not until 1893 when Stiles (60), in a study to determine a solution that would kill the parasite without severely affecting the fish, showed the life cycle to be complete. He observed that young cells

released from the cyst swam to and attacked new hosts. The only possible time to destroy the parasite without detriment to the fish was when it left the host during its free or encysted stage (60).

Nerescheimer (46) conducted the first cytological study of Ichthyophthirius multifiliis. He noted division of the parasite while on the fish as well as within a cyst while off it. This observation was refuted by Haas (18) who observed division only within the cyst and not on the fish in a study which traced the micronucleus throughout the life cycle. Pearson (50) suggested that if division occurs on the host at all it probably happens when mature trophozoites, for some reason, cannot leave the fish at the time they would normally begin encystment (50).

MacLennan (36, 37, 38, 39, 40, 41) conducted a series of studies on the life cycle. He recorded the dedifferentiation and redifferentiation of the neuromotor apparatus, a fibrillar node thought to have control over locomotion, feeding, avoidance, etc. (8, 37), and the origin and function of various cytoplasmic granules. He found that dedifferentiation occurs before division in the cyst and involves absorption of organelles. He suggested that less specialized portions are retained and form the basis of the neuromotor system in the young tomites. Redifferentiation was found to take place during division in the cyst. New fibrils arose from old ones and gave rise to basal bodies (kinetosomes) which gave rise to cilia. A rudimentary cytostome formed in the middle of the ventral side. In the free-living stage ciliary rows became situated around the mouth, which then sank inward. The ciliate

did not differentiate further until it penetrated the host (36). MacLennan (38) also isolated 7 types of cytoplasmic granules functioning in fat, protein and carbohydrate storage, digestion and secretion. He could not identify the granules in the late stages of encystment and concluded that the granules arose de novo (38).

In subsequent studies MacLennan (39, 40) observed growth in the ciliate and described maturity, encystment and volume changes. He found that while on the host, the trophozoite was folded and wrinkled. Upon leaving the fish it swelled, then ceased swimming shortly thereafter, and sank to the bottom where it encysted after undergoing dedifferentiation. The cyst wall is composed of two layers: a sticky ectocyst which anchors the cyst to the substratum and a fine, fibrillar protein endocyst which reinforces the ectocyst. The cyst wall is firm at first, then gelatinous when broken by the newly formed tomites. None of the 7 types of granules MacLennan (38) described earlier was shown to be associated with cyst formation. The only demonstrable function of the cyst was to affix the ciliate to substrate. It was noted, however, that successful reproduction was not possible without the cyst. Efficiency of encystment increased as length of growth period on the fish increased. Thus, the time length necessary for completion of encystment and dedifferentiation decreased (39). Analysis of progressive growth changes during the life cycle indicated a constant instantaneous growth rate of the ciliate. The growth rate as well as the ability to encyst was greater at higher temperature. Variations in the age at which encystment can occur were due to variations in size of infective tomites. Relatively larger tomites were able to encyst after a shorter period of time on

the host than smaller tomites. This resulted in variations in time required to reach minimum size necessary for encystment (40).

MacLennan (41) went on to investigate cytoplasmic granules extracted throughout the life cycle by centrifuging single organisms in individual tubes. He was able to make quantitative studies on the growth and utilization of granular food reserves and on relative densities and changes in viscosity of cyto- and nucleoplasm. MacLennan found that the total volume of reserve granules increased at the same rate as the total volume of the organism (41).

Mugard (44, 45) conducted studies on Ichthyophthirius multifiliis, focusing on the development and multiplication of the longitudinal, functionally integrated rows of kinetosomes and other associated cortical structures, collectively called kineties (8). The pre-division stage, or tomont, typically has double the number of kineties than does the young trophozoite (44).

In 1963 Uspenskaya found Ichthyophthirius multifiliis to possess hyaluronidase, an enzyme that specifically depolymerizes hyaluronic acid, the mucosaccharide which makes up the connective tissue filling intercellular spaces. He found that the appearance of hyaluronidase coincided with the termination of reproduction. It is also at this time that the rudiments of the boring apparatus appear. Uspenskaya suggested that the enzyme was needed for feeding and for short migrations within the skin of the fish. He could not tell with which cellular component hyaluronidase was associated but suggested that it might be linked with a part of the boring apparatus used in penetrating the host (61).

Mosevitch (43) employed transmission electron microscopy to study the contractile vacuole of Ichthyophthirius multifiliis and compared it to others of previously studied ciliates. He found the vacuole to consist of a receptacle, thin injection canals and a discharge canal which opens to the outside via a pore. Fibrils 200-250 Å in diameter were observed in the receptacle's wall. When the vacuole contracts the receptacle shrinks and forms finger-like processes. Mosevitch suggested that the fibrils of the receptacle serve as supporting structures and are not contractile. The fibrils probably promoted expansion of the vacuole after its discharge (43).

Roque et al. (57) conducted a light- and electron microscopic study on the structure and morphogenesis of Ichthyophthirius multifiliis with major emphasis on the buccal apparatus and stomatogenesis (new mouth formation). Ophryoglena, a closely related facultatively parasitic ciliate also of Order Hymenostomatida, was likewise studied. Roque et al. concluded that the generic distinction between the two is justified by morphological differences concerning dimension and form of the buccal apparatus and not by the parasitism of the one versus the facultative existence of the other (57).

Roque and dePuytorac (58) reported the discovery of a new ciliate parasite on guppies (Poecilia reticulata) which they names Ichthyophthirioides browni. It was described to differ from Ichthyophthirius multifiliis due to differences in buccal structure and general ultra-structure. This find was questioned by Canella (6) and Corliss (8) who called for further research into the matter.

Peshkov and Tikhomirova (52, 53) conducted studies on the excretory and nuclear apparatus of Ichthyophthirius multifiliis using light and transmission electron microscopy. Observations recorded on the excretory apparatus (contractile vacuole) added little to ones made by Mosevitch (43) and Roque et al. (57). The nuclei of the trophozoite and cystic stages were studied and compared. Both have a macronucleus surrounded by a two-layered nuclear membrane pierced by pores. The total amount of chromatin is much higher in the pre-cystic tomite than in the trophozoite. The number of nucleoli is smaller in the cystic tomite. The authors suggested that the reason for this was a discharge of RNA-containing material into the cytoplasm observed by Uspenskaya and Ovchinnikova (62) in an earlier study of the changes in the amounts of DNA and RNA during the life cycle. The micronucleus was observed only in the cystic tomite stage. Like the macronucleus it was oval, bounded by a two-layered nuclear membrane and consisted of DNA-containing material (62).

The micronuclei and the macronuclei of Ichthyophthirius multifiliis were subjects of studies conducted by Hauser (19, 20). He observed the events of micronuclear mitosis, emphasizing premetaphase spindle formation in I. multifiliis and the suctorian Paracineta limbata in one study (19). In another study, pre-divisional intranuclear movements were described in the macronucleus (20). These studies were important at that time because studies of nuclear division in protozoans were limited to concern over stages of metaphase and telophase (20).

To gain an understanding of the disease, ichthyophthiriasis, Hines and Spira (24, 25, 26, 27, 28) conducted a five-part series of studies on parasitized mirror carp (Cyprinus carpio). In the first study (24) two groups of carp were infected with Ichthyophthirius multifiliis at initial doses of 40 parasites or 400 parasites per fish and the course of infection was observed and compared between the two groups. When infected with small doses (40 parasites/fish) the fish undergo a mild form of the disease and recover within three weeks. Recovered carp were free of the parasite. In large doses (400 parasites/fish) I. multifiliis induced severe disease symptoms with high mortality in 22 to 25 days. In both cases the parasite was not found to be evenly distributed over the body of the host. Parasite density was highest in the area of the gills. They were also three times more prevalent in the dorsal region than on the lateral or ventral surfaces for reasons unknown. Differences in skin structure were ruled out as a possible explanation (24).

The next study in the series by Hines and Spira (25) involved an analysis of the types and counts of leukocytes in normal and infected carp. Young lymphocytes were found to be more numerous in uninfected fish. Pro-neutrophil numbers increased sharply while old neutrophils decreased during the course of the infection. There was no change in the overall leukocyte count but proportions of types of white cells changed. Leukocyte counts were low in blood films of fish in the terminal stages of infection. A drop in neutrophil numbers was not accompanied by maturation of young neutrophils along with a rise in precursor cells, indicating the "exhaustion" of the granulocytic system.

The white blood cell changes observed were found to be similar to changes seen in other diseases and certain stress conditions in fish (25).

The pathology of ichthyophthiriasis was the subject of the next study by Hines and Spira (26). Carp were infected with heavy doses (400 parasites per fish) and the effect on body organs observed. Analysis of skin revealed epithelial cell division around parasites at days 8-12. Mucous cells became more numerous in the epidermis throughout the whole body of the fish. As a result, the epidermis became as much as four times thicker than normal by days 12-14 of infection. Blood and lymph vessels became congested with increased numbers of leukocytes. Late in the infection, cell proliferation subsided and the epithelium regained its normal thickness. In the final days of the infection (days 19-26) epithelial destruction deepened and extended to the basal layer. Underlying muscle became inflamed, resulting in swelling. Subsequent exposure of the vascular network of the dermis interfered with osmoregulation. In the gills, adhesion of lamellae and mucous cell proliferation were observed in the early stages. In the later stages, lamellar spaces were occluded by epithelial cell proliferation. These changes severely reduced the surface area of the gill lamellae. Lamellar atrophy and filament necrosis were characteristic of the terminal stages. The liver was the site of progressive devacuolization of hepatocytes followed by swelling. Autopsy revealed a pale and blotched liver. These changes were related to the mobilization of fat reserves induced by the extra energy drain on the fish. The kidney, partially hemopoietic in

fish, eventually became enlarged and pale due to the sharp increase in leukocyte production and the eventual collapse of the blood-forming tissues. This exhaustion was accompanied by an increase in blood-forming tissue in the spleen which became enlarged (26).

The fourth part of the study by Hines and Spira (27) analyzed physiological changes during the course of infection. Ichthyophthirius multifiliis caused significant osmoregulatory dysfunction in carp. Serum Na^+ ion concentrations declined due to the reduced surface area of the gills caused by irritation and cell proliferation. The active transport of Na^+ by certain branchial cells was impeded. Serum Mg^+ concentrations dropped and K^+ concentrations increased due to loss of mucus and skin, both important barriers to ion loss and water entry. Ichthyophthiriasis decreased the rate at which carp absorbed oxygen. Consequently, tolerance of carp to low O_2 concentration was reduced. Hemoglobin concentrations were inexplicably unaffected. A build-up of blood urea-ammonia concentrations was also observed. It was suggested that gill pathology may have been the cause of this since Na^+ is usually exchanged for NH_4^+ by the gills (27).

The final part of the series of studies conducted by Hines and Spira (28) dealt with acquired immunity. Tests confirmed an acquisition of a post-invasive immunity in carp. Fish recovering from heavy infections remained refractory to normally lethal doses of the parasite for up to eight months. Free-swimming tomites were immobilized by the serum of recovered carp. The barrier to reinfection was apparently the result of a qualitative change in the mucous coatings since amounts of

mucus were found to be the same in normal and recovered fish. The mucus is primarily glycoprotein with albumin and other serum proteins. It was suggested that the presence of serum proteins released from the mucus into the water surrounding the recovered fish may immobilize the parasite before it reaches the fish's skin (28).

Lom and Cerkasovova (35) made some interesting observations in a study of the response of Ichthyophthirius multifiliis tomites to certain attractants of fish. They treated cubes of agar with possible attractants, namely mucus, urea, cyclic AMP and serum. Tomite showed the strongest positive reaction to serum. If tomites came close enough to the emitting surface in their random movements, they crept at the agar cube's edge and attempted to penetrate. Results indicated that the ciliate did not identify its host by long-range chemotaxis. Substances derived from the blood serum of the fish may attract or stimulate the parasite when in proximity (35). This is interesting in light of what Hines and Spira (28) reported about the effects of serum of recovered fish on tomites.

Nigrelli et al. (47) drew on reports from previous authors and from their own observations at the New York Aquarium to present arguments for the possible existence of physiological races and/or other species of Ichthyophthirius. They pointed out that because various stages of the life cycle are temperature dependent, and because of the parasite's wide distribution, there must be several physiological races related to the temperature tolerance of the host species. The authors cited Roque and dePuytorac (58) who described Ichthyophthirioides browni with its buccal and morphological differences from I. multifiliis, including a rod-shaped

macronucleus. In addition, they mentioned a ciliate observed on a species of catfish, and on a cyprinid imported from the Congo to the New York Aquarium, which exhibited encystment and tomite stages and possessed a spiral-shaped macronucleus. Other examples studied by the authors on a wide variety of freshwater and tropical fishes showed variations in macronuclear morphology distinct from the typical kidney- or U-shaped macronuclear form. From these data they concluded that more research is needed to determine whether or not more than one species of Ichthyophthirius, or even more than one genus of Ichthyophthirius-like ophryoglenids, exists (47).

Recent studies done on Ichthyophthirius multifiliis have been concerned with the dynamics of and immunization against ichthyophthiriasis. McCallum (42) studied infection dynamics of the parasite in the black mollie (Poecilia latipinna). In his experiments all newly emerging tomites successfully located a host. There appeared to be no density-dependence correlated with parasitic establishment. Variability between hosts in proportional parasitic establishment was observed and may have been due to previous exposure of the fish to the disease. Qualitative changes in the mucus of the previously infected fish, as described by Hines and Spira (28), may account for this resistance and the resulting variability in host susceptibility. The author called for more studies on how host genetics, environmental factors and past infection experience affect host susceptibility (42).

In an effort to immunize the fish against ichthyophthiriasis, Goven et al. (16, 17) used preparations of cilia and of whole cells of

Ichthyophthirius multifiliis. Channel catfish (Ictalurus punctatus) were injected with the preparations and challenged with doses of I. multifiliis. Cilia and whole cell preparations of a closely related ciliate, Tetrahymena pyriformis, were used as well to see if they elicited a protective response. Results of the experiment showed T. pyriformis ciliary antigens to be the most protective. This indicated to the authors that an antigenic relationship between the two ciliates exists (16). Further studies by this group confirmed this and showed that the cross-reacting antigens were located on the cilia (17).

Wolf and Markiw (63) had similar results using whole cells and cilia of Tetrahymena thermophila. The fish used in these experiments (rainbow trout, Salmo gairdneri) were not injected but immersed in baths containing the ciliate antigen and then challenged with Ichthyophthirius multifiliis. These preliminary studies have shown immunization to be a very promising means of dealing with ichthyophthiriasis.

The latest studies done on Ichthyophthirius multifiliis have been conducted using electron microscopy. Chapman and Kern (7) described the ultrastructure of the contractile vacuole and somatic cortex of the trophont (trophozoite) stage of the life cycle. Their findings, particularly the description of the contractile vacuole complex, added much to the observations of previous authors who used electron microscopy (43, 54, 57). The somatic cortex and contractile vacuole systems were compared to those of Paramecium aurelia and Tetrahymena pyriformis. Chapman and Kern concluded that I. multifiliis was more closely related to T. pyriformis. They also concluded that I. multifiliis as well as

other ciliates may prove to be useful in experiments to determine methods for combating parasitic infections through the manipulation of synthesis and assembly of microtubules (7).

Ewing et al. (11, 12) have done studies using light and electron microscopy on life cycle stages of Ichthyophthirius multifiliis. Their study on cyst wall morphology added significantly to the light microscopic observations of MacLennan (39). Although what role the cyst plays is not completely understood, it is believed by the authors that it may be some sort of barrier to the passage of particles in and out of the cell (11). The other study done by this group has been abstracted recently (12). This study dealt with the invasion of the fish host (channel catfish) by free-swimming, infective tomites.

Until very recently, few studies using electron microscopy have been carried out on I. multifiliis. Previous studies, presented in the literature review, have been concerned with the ultrastructure of the nuclear apparatus (19, 20, 52, 54), the contractile vacuole (7, 43, 54, 7), the cortex (57, 8), buccal structure and new mouth formation (stomatogenesis) (57), morphology of the cyst wall and host invasion (11, 12). It is surprising that, with the exception of the study on the cyst wall by Ewing et al. (11), there have been no studies conducted using scanning electron microscopy. Also, with the exception of Ewing et al. (12), the free-swimming, infective tomite stage has not been studied in any detail using electron microscopy. It is the purpose of this thesis project to study the life cycle of Ichthyophthirius multifiliis with transmission and scanning electron microscopy emphasizing the tomite stage and invasion of the host.

MATERIALS AND METHODS

I used channel catfish fingerlings (Ictalurus punctatus) provided by Carolina Biological Supply Company (Burlington, NC) as host species for this study. The fingerlings measured from 4.0 to 6.0 cm in length. The fish were maintained in two 10-gallon aquaria which I set up, each equipped with bottom filter, heater, floating thermometer and gravel. I fed the fish daily with commercial fish food (Hartz) and maintained water temperature at approximately 20°C. I designated one aquarium as a stock tank for uninfected fish. These fish were eventually transferred to the other tank which I designated as the infection tank. I maintained the infection by introducing stock fish into the infection tank as needed. An ample supply of stock fish was maintained throughout the study. Many of the fish I received from the supplier already had white spot disease, thereby making the problem of initiating the infection quite simple. To identify Ichthyophthirius multifiliis I followed some suggestions put forth by Beckert (5) to isolate it for inspection. I sacrificed a heavily infected fish by decapitation with a razor blade and put it into a histological staining dish filled with aquarium water. Trophozoites could be seen leaving the fish and swimming about freely within five minutes. I collected several of the ciliates, easily distinguishable by their size and appearance, and examined them using a compound microscope. By means of keys found in Hoffman et al. (31) and Pennak (51), I was able to identify I. multifiliis with certainty.

Preparation of Life Cycle Stages for Transmission Electron Microscopy

(TEM)

For transmission electron microscopy (TEM) I fixed specimens in 2.5% or 1.0% (v/v) glutaraldehyde in Millonig's phosphate buffer (pH 7.2) for 30 minutes. The tissue was then washed in Millonig's buffer (3 x 15 min.) followed by post-fixation in 2.0% (w/v) OsO_4 in Millonig's buffer for one hour. I then washed the specimens in Millonig's buffer (3 x 15 min.). I dehydrated the specimens in a graded series of ethanol (50%, 70%, 95%, 100%, 100%) for 10 to 15 minutes in each concentration. The protocol up to this point was done in a styrofoam bucket filled with ice. The specimen in absolute alcohol was then brought to room temperature. I used Spurr's low viscosity embedding medium (epoxy resin) to infiltrate the specimens stepwise in 1:1 embedding medium to ethanol for one hour, 3:1 embedding medium to ethanol for three hours and 100% embedding medium for at least eight hours. The specimens were infiltrated in a Pelco Infiltron rotator. I then put specimens into molds with fresh embedding medium and polymerized them at 70°C for 16 to 18 hours. I cut thick and thin sections with glass knives on a Sorvall MT-2 or Sorvall MT-5000 ultramicrotome. I stained thick sections with Toluidine blue stain and examined them under a compound microscope for the presence of the parasite. Gold- and silver-colored thin sections were picked up with 150-200 mesh copper grids and stained with saturated solutions of uranyl acetate for 1.5 minutes and lead citrate for 2.5 minutes. I viewed them in a Zeiss EM 9S-2 transmission electron microscope operated at 50 kV.

Trophozoites

For maturing trophozoites I cut infected skin, gill and fin tissue into approximately 1 mm³ pieces on dental wax with a razor blade. The tissues were then put in glutaraldehyde and prepared for TEM.

Cyst Stages

For cyst stages I collected free-swimming, pre-cystic trophozoites in the same manner described earlier for identification of the parasite. I transferred these to separate mold capsules with a Pasteur pipette, waited for them to settle to the bottom, encyst and then begin division. I placed several trophozoites in each capsule to ensure that at least one or two would encyst at the very bottom. I then replaced the aquarium water with glutaraldehyde. The cyst's outer sticky wall (ectocyst) firmly adhered to the bottom of the mold capsule, allowing for easy removal and replacement of fluids used in TEM preparation. I did all medium changes and polymerization in the same mold capsule.

Tomites

I maintained tomites by collecting free-swimming, pre-cystic trophozoites with a Pasteur pipette and transferring them to plastic tissue culture wells filled with aquarium water. Approximately 24 hours after settlement, free-swimming tomites could be seen swimming about in the well. I collected the tomites with a Pasteur pipette, transferred them to small plastic centrifuge capsules and centrifuged the tomites at a gravitational force of 400g for 10 minutes. I then removed the capsule from the centrifuge, pipetted the supernatant off, pipetted more tomites into the same centrifuge capsule and centrifuged

again for 10 minutes. I repeated the procedure at least twice to attain a pellet of adequate size at the bottom of the capsule. I then replaced the aquarium water in the capsule with glutaraldehyde and prepared it for TEM study as previously described.

Preparation of Life Cycle Stages for Scanning Electron Microscopy (SEM)

I prepared specimens for scanning electron microscopy (SEM) by fixing them in 2.5% (v/v) glutaraldehyde in Millonig's phosphate buffer (pH 7.2) for at least 30 minutes, followed by washings in Millonig's buffer (3 x 15 min.). I post-fixed specimens in 2.0% (w/v) OsO_4 in Millonig's buffer for one hour followed by washings in Millonig's buffer (3 x 15 min.). I then dehydrated the specimens in a graded series of ethanol (50%, 70%, 95%, 100%, 100%, 100%) for a duration of 15 minutes in each concentration and brought it to room temperature. I dried the specimens in a Tousimis Samdri Regulated Critical Point Dryer, mounted them on nickel studs with double adhesive tape and coated them with 300 Å gold/paladium in a SPI Sputter. I viewed specimens in a Novascan 30 scanning electron microscope operated at 15 kV.

Trophozoites

For SEM study of maturing trophozoites I decapitated a heavily infected fish and cut the rest of its body into three or four sections. I then put the body sections into glutaraldehyde and carried out the remaining SEM protocol as described previously.

Cyst Stages

To generate cyst stages I collected free-swimming, pre-cystic trophozoites as before and transferred them with a Pasteur pipette to

glass culture wells, each containing a 10.0 mm wide glass or plastic coverslip and filled with aquarium water. By observing through a dissecting microscope I waited for trophozoites to settle onto coverslips, encyst and begin to divide before I replaced the aquarium water with glutaraldehyde. Again, cysts readily and firmly adhered to the coverslips, making preparation relatively trouble-free.

Free-swimming and Invading Tomites

I collected free-swimming and invading tomites using the same methods as those for TEM study. I used a millipore filtering device which consisted of a moistened millipore filter (13 mm wide, 0.6 micrometers pore size) placed in a plastic filter chamber. I attached a 10 cc syringe without a plunger on the top and 10.0 cc syringe with a plunger on the bottom. I then pipetted 2.0 cc of tomite-infested aquarium water into the top syringe followed by 2.0 cc of glutaraldehyde. By pulling the plunger of the syringe on the bottom, I was able to draw the tomite medium through the filter and fix the tomites onto the filter at the same time. I then removed the filter from the chamber and placed it in glutaraldehyde.

To prepare invading tomites for SEM study I took an uninfected fish from the stock tank, decapitated it and cut it into three or four trunk pieces. I immediately placed the pieces into plastic tissue culture wells containing aquarium water infested with tomites. I removed the pieces after two to five minutes and placed them in glutaraldehyde. For this procedure to be successful, the tissue must be from a freshly-killed fish; otherwise, tomites will not attempt to invade it.

RESULTS

Maturing trophozoites, exhibiting a high degree of somatic ciliature, are found situated between or within the gill lamellae or just below the epidermis of the fish host (Figs. 1 and 2). In heavy infections the ciliates are seen close together. Trophozoites appear wrinkled or undulant when viewed with SEM. Large crevices in host skin are sites for finding trophozoites (Figs. 3 and 4). The crevices may be indicative of extensive tunneling by the parasite. Comparisons made with healthy fish skin reveal the same sinuous cracks, although not as wide open as in infected fish. As seen through the light microscope trophozoites are heavily ciliated and contain a light brownish endoplasm that appears vacuolated. Transmission electron microscopy shows the trophozoite to possess a heterogeneous endoplasm (Fig. 5). Rough and smooth endoplasmic reticulum as well as free ribosomes are scattered throughout the cell (Fig. 6). Food vacuoles are also a prominent feature (Fig. 7). A typical food vacuole is bounded by a double membrane and contains dark, electron-dense, myelin-like material among other debris. Lysosomes, bounded by a single membrane, can be seen near many of the vacuoles apparently converging on them (Fig. 8). The endoplasm also features the large macronucleus (Fig. 5) and smaller micronucleus (Fig. 10) typical of most ciliates. In the cortical region cilia are abundant. A longitudinal section through this area (Fig. 9) shows a single cilium sitting in a slight depression in the cortex. The cilium is lined with the plasma membrane and, at its proximal end, is composed of a central axosome from which an axoneme arises. The

Fig. 1- Ichthyophthirius multifiliis trophozoites (Tr) under epidermis (E) of fish host. (X100).

Fig. 2- I. multifiliis trophozoites (Tr) under epidermis (E) of fish host. (X220).

Fig. 3- Ichthyophthirius multifiliis trophozoite (Tr) in crevice-like channel of fish host epidermis. (X40).

Fig. 4- I. multifiliis trophozoite (Tr) under epidermis of fish host. Note relatively large crevice below the feeding ciliate (arrow). (X150).

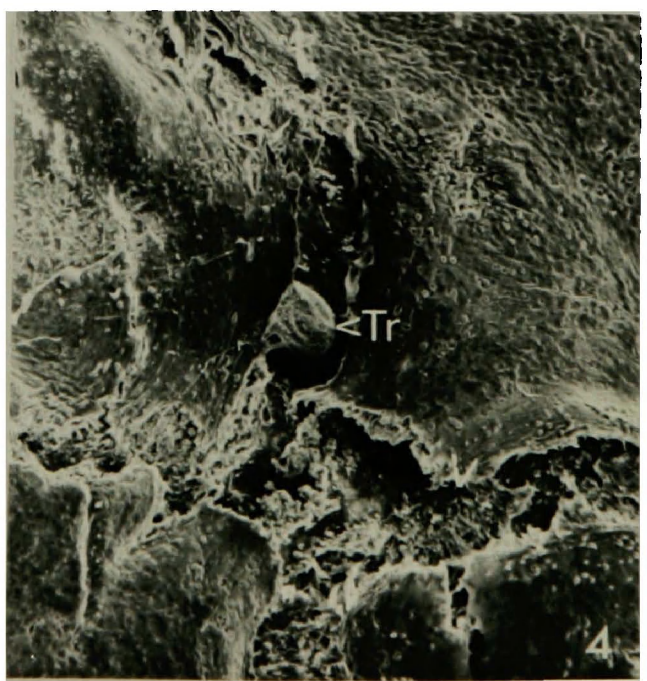
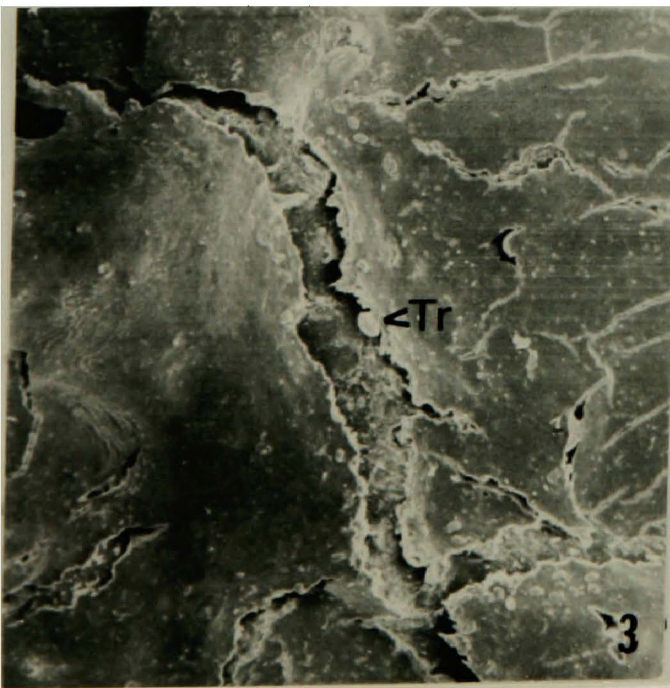
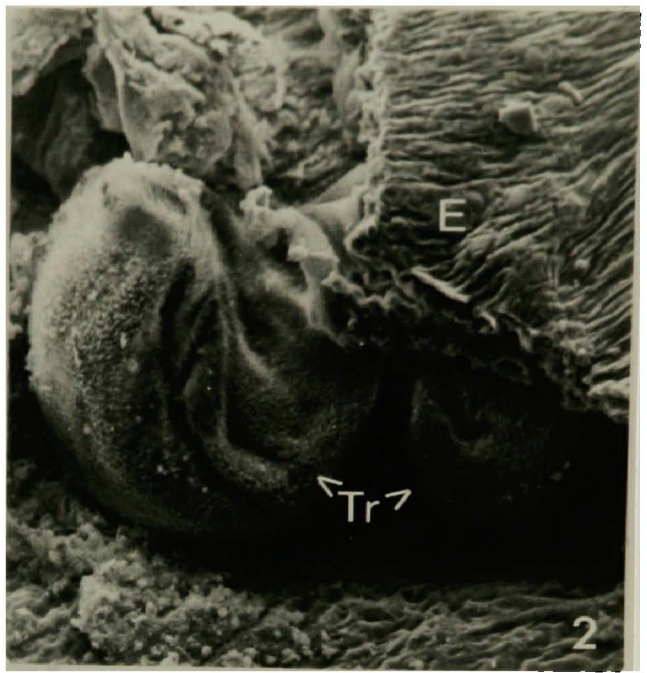


Fig. 5- Ichthyophthirius multifiliis trophozoite in gill of fish host exhibiting heterogeneous endoplasm. Macronucleus (MA), food vacuoles (FV), lysosome (Ly). (X3000).

Fig. 6- Cross-section through cortical area of trophozoite infecting gill tissue (G) of host. Note mitochondria (Mit), rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER). (X9400).

Fig. 7- Longitudinal section of trophozoite showing food vacuoles (FV), macronucleus (Ma). (X11,860).

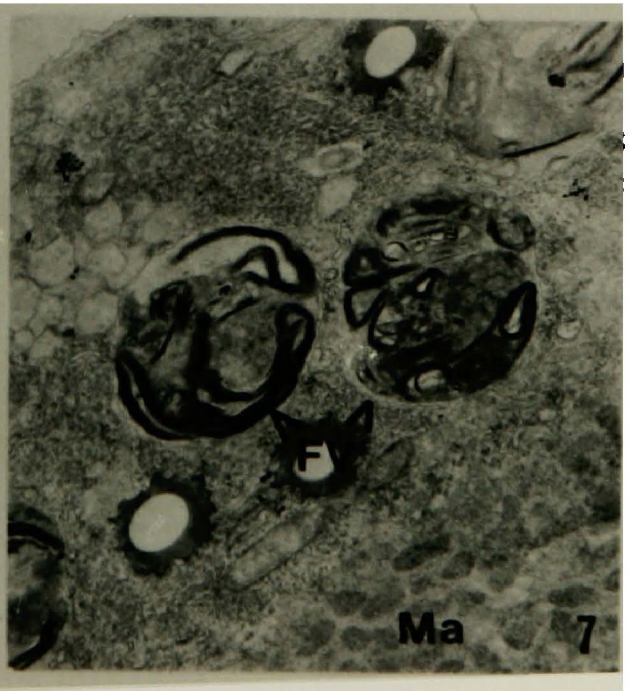
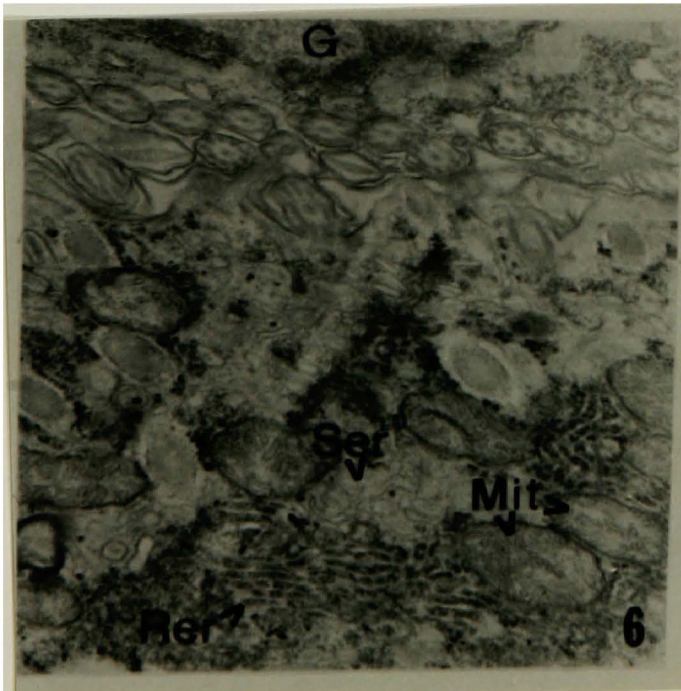
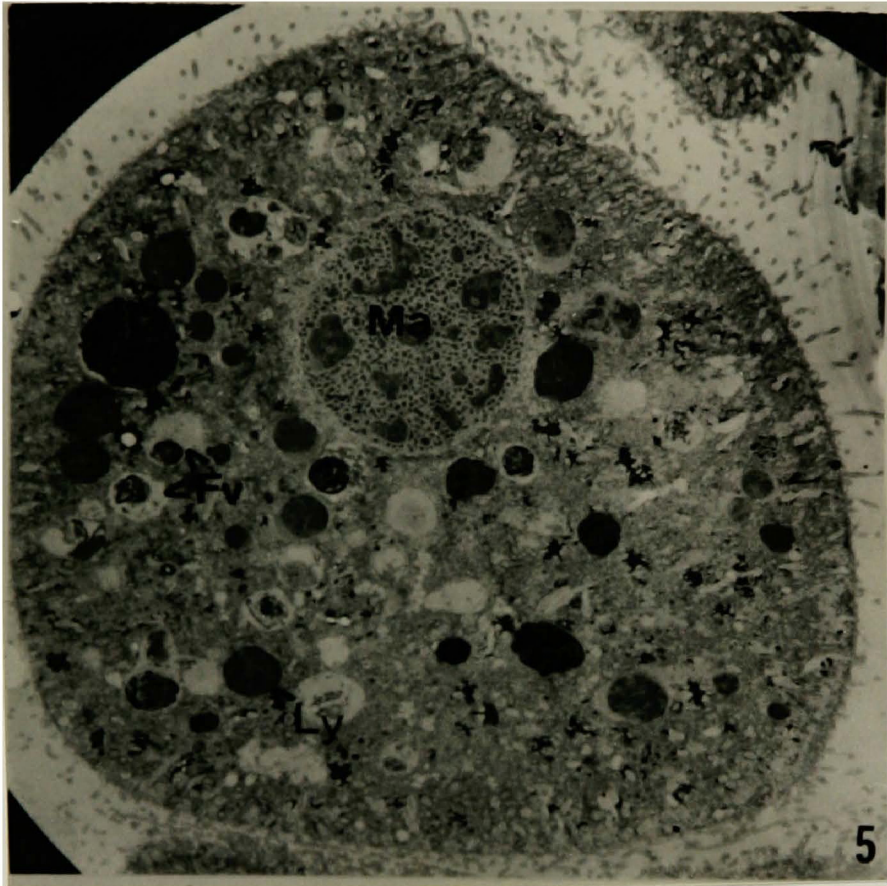
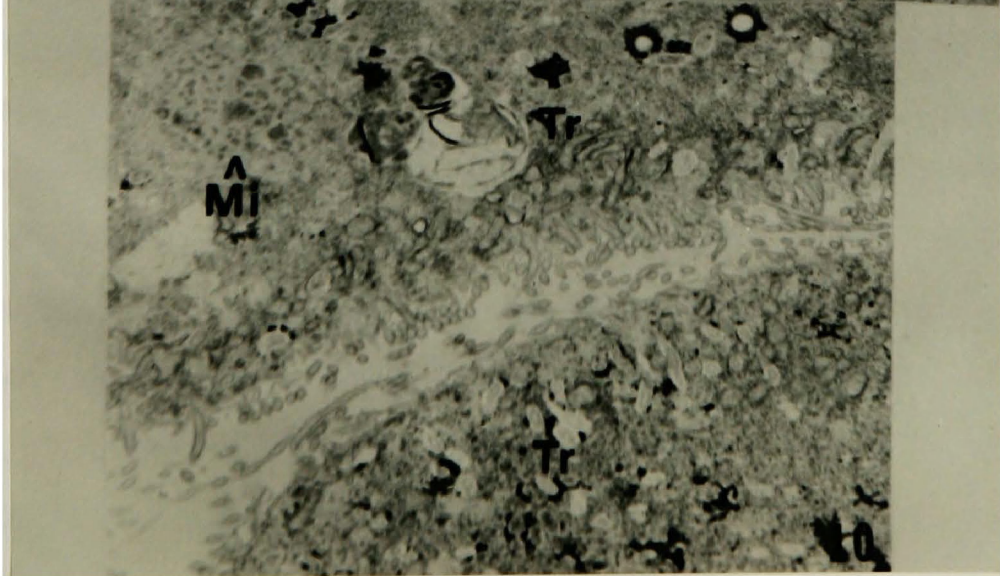
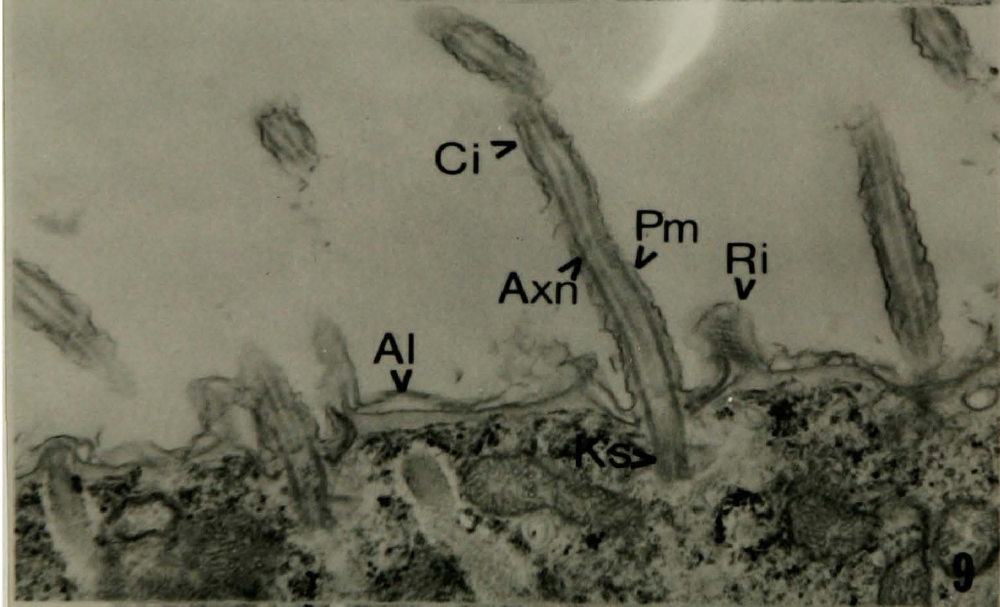
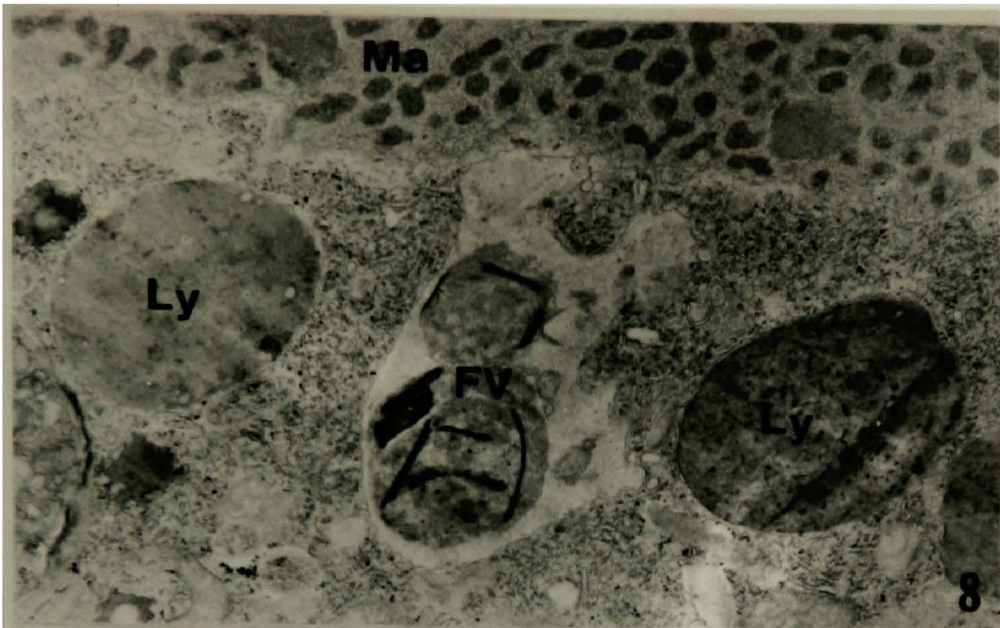


Fig. 8- Section of trophozoite endoplasm showing a food vacuole (FV) and two closely associated lysosomes (Ly), macronucleus (Ma). (X10,960).

Fig. 9- Longitudinal section of somatic cilia (Ci) of trophozoite. Ridge (Ri), plasma membrane (Pm), axoneme (Axn), alveolus (Al), kinetosome (Ks). (X22,800).

Fig. 10- Two adjacent trophozoites (Tr) in gill of fish host. Note micronucleus (Mi). (X4500).



axoneme shows the familiar 9+2 microtubular arrangement. Below the cortex lies the kinetosome from which the cilium arises. Closely associated to the cilium's left, connected by a pair of transverse tubules, is a ridge composed of a ribbon containing approximately seven parallel microtubules. A pellicular alveolus, a sac composed of two membranes below the plasma membrane, can be seen adjacent to this ridge (Fig. 9). Mitochondria are located in the vicinity of the cell cortex as well as extrusive mucocysts in longitudinal and cross-section showing a definite core surrounded by denser material (Fig. 11). Mucocysts are also located further within the endoplasm.

After leaving the host the trophozoite is observed to swim slowly about for a brief period before sinking to the bottom of the glass staining dish. Shortly thereafter (within 10 to 15 minutes) it secretes a transparent, mucoid cyst around itself and adheres firmly to the glass substrate. Relatively strong suction is required to free the cyst from its site of attachment. Once attached, the cell begins to divide (Fig. 12) and from the 2-cell stage on, two separated masses of cells appear within the cyst (Fig. 13). At times the two masses appear to have separate rates of fission, giving the cyst an asymmetrical appearance (Fig. 14). Due to the adherent nature of the cyst, much debris can be seen sticking to it. Transmission electron microscopy reveals large numbers of mucocysts in the developing tomites within the cyst. The mucocysts are closely associated with or locked into position in the cortex of the cell (Fig. 15). Figure 16 illustrates how mucocysts are distributed in this region. They are irregularly arranged in bunches of two to

Fig. 11- Section of cortical region of trophozoite featuring mucocysts (Mu) in longitudinal and cross-section. Mitochondrion (Mit). (X9400).

Fig. 12- Two-cell stage of cyst. (X205).

Fig. 13- Four- to eight-cell stage of cyst. Note debris sticking to cyst wall. (X195).

Fig. 14- Eight-cell stage of cyst. Note debris sticking to cyst wall. (X170).

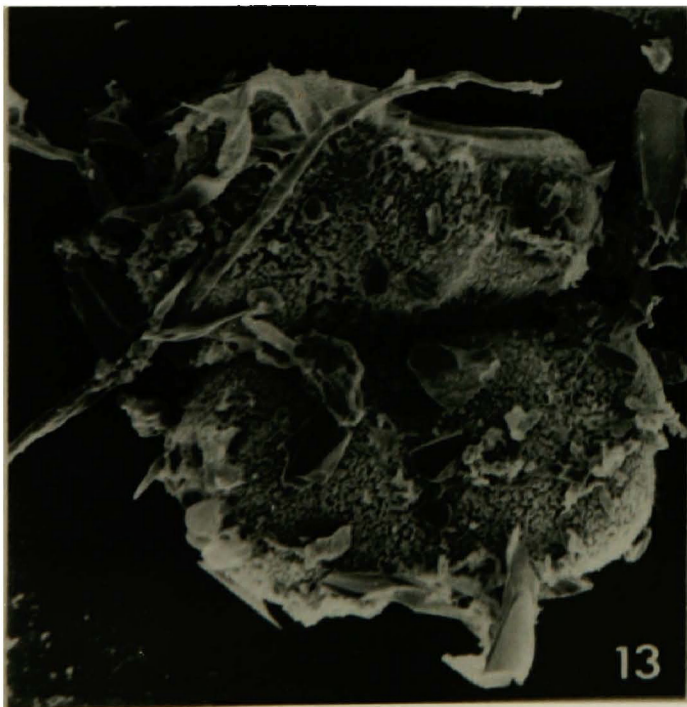
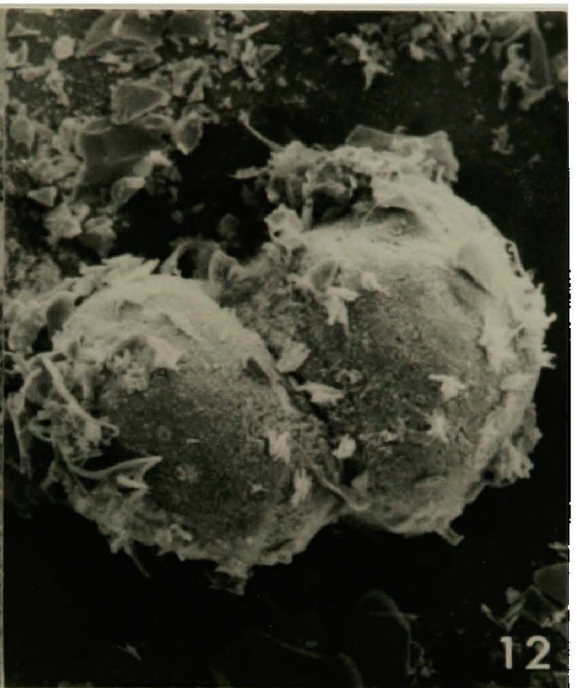
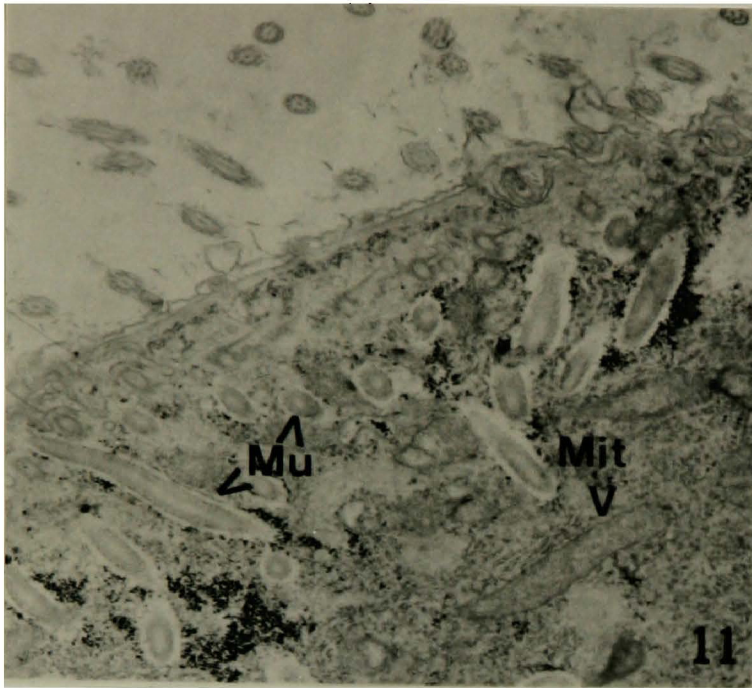
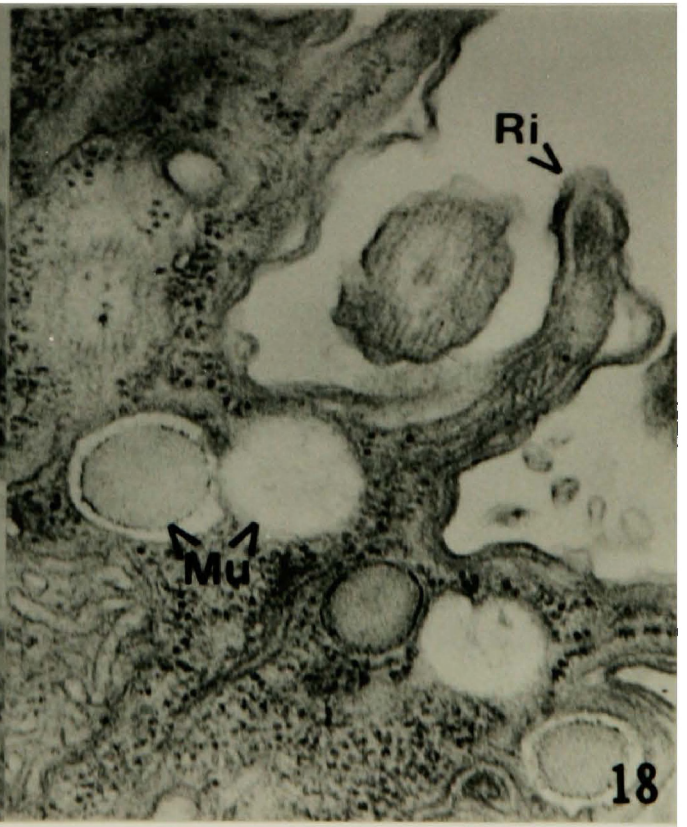
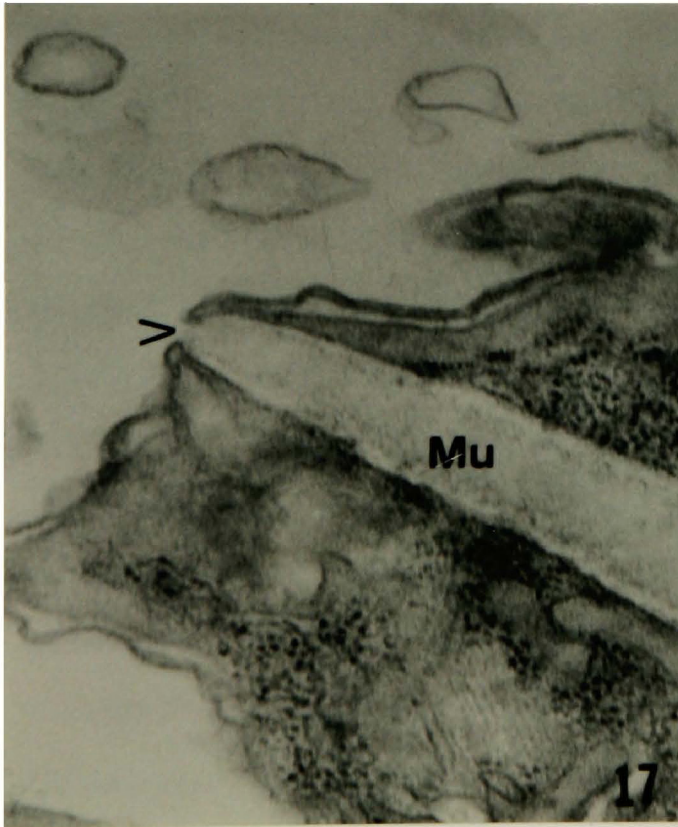


Fig. 15- Section of cortical region of developing tomite within cyst. Mucocyst (Mu), bacterium (B). (X19,000).

Fig. 16- Tangential section of developing tomite in late cyst state. Note location and distribution of mucocysts (Mu) between ciliary rows or kineties (Kn). (X9,600).

Fig. 17- Mucocyst (Mu) of developing tomite within a cyst initiating extrusion of contents (arrow). (X66,200).

Fig. 18- Cortical region of developing tomite within a cyst exhibiting mucocysts (Mu) prior to and after expulsion. Cortical ridge (Ri). (X66,200).



several between adjacent ciliary rows or kineties ready to be ejected. Mucocysts can be seen beginning the process of expelling their contents (Fig. 17) or having already been expelled, appearing as an empty vesicular chamber (Fig. 18). Bacteria were observed within the cyst wall, sometimes very closely associated with the developing tomite's cortex (Fig. 15). The ovoid bacteria are bounded by a single membrane or cell wall and contain a granular cytoplasm possessing electron-luscent vacuole-like structures. In late stages of development the wall of the cyst appears thin and fragile. Bumps representing nearly mature tomites are evident at this time (Fig. 19). At excystation, tomites rupture out of the cyst and become free-swimming, infective forms (Fig. 20).

Tomites swim through the water with a boring motion like that of Paramecium. Scanning electron microscopy shows tomites to be somewhat elongated and banana-shaped and slightly tapered at the anterior end (Fig. 21). Tomites measure 38 to 45 micrometers long. Like maturing trophozoites they are heavily ciliated. Situated in the posterior third of the cell is a slight depression leading into a deep oval cavity measuring approximately 2.0 micrometers long by 1.0 micrometers wide (Fig. 22). This may be the outermost part of a rudimentary mouth or cytostome. Transmission electron microscopy reveals the presence of a cytostome that opens into a buccal cavity (Fig. 23). Embedded in one of the walls of this cavity is an ovoid, electron-dense body measuring approximately 1.8 micrometers long by 1.3 micrometers wide known as Lieberkühn's organelle (Figs. 23 and 24). The cytostomal opening is ciliated on the wall opposite Lieberkühn's organelle. Cross-sectioned,

Fig. 19- Late cyst stage. Note bumps representing nearly mature tomites (Tm) and weakening of the cyst wall (arrow). (X225).

Fig. 20- Excystation. The end of the cyst stage. (X250).

Fig. 21- Free-swimming, infective tomite stage (Tm). (X2,500).

Fig. 22- Higher magnification of depression (rudimentary cytostomal opening?) seen towards posterior end of tomite seen in Figure 21. (X13,150).

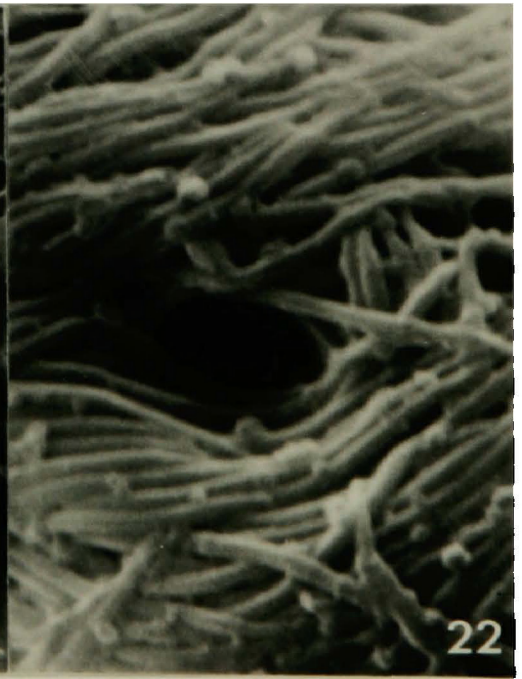
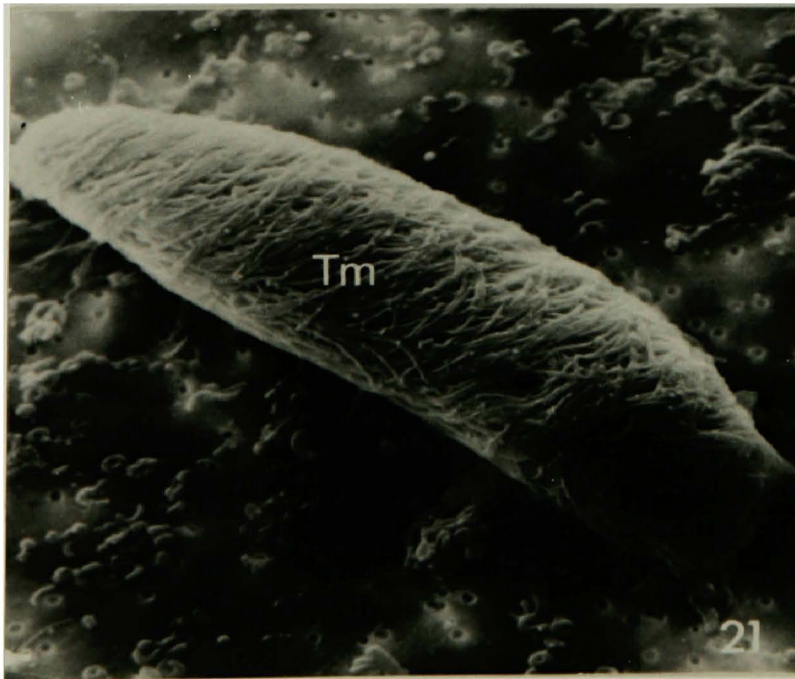
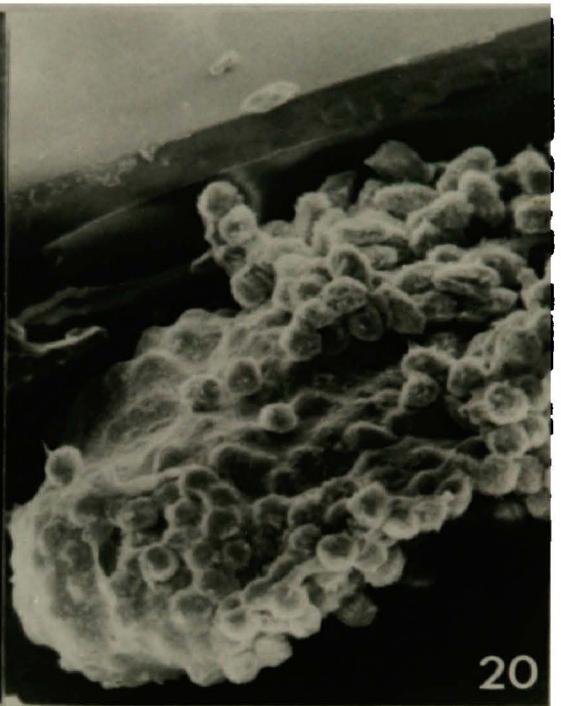
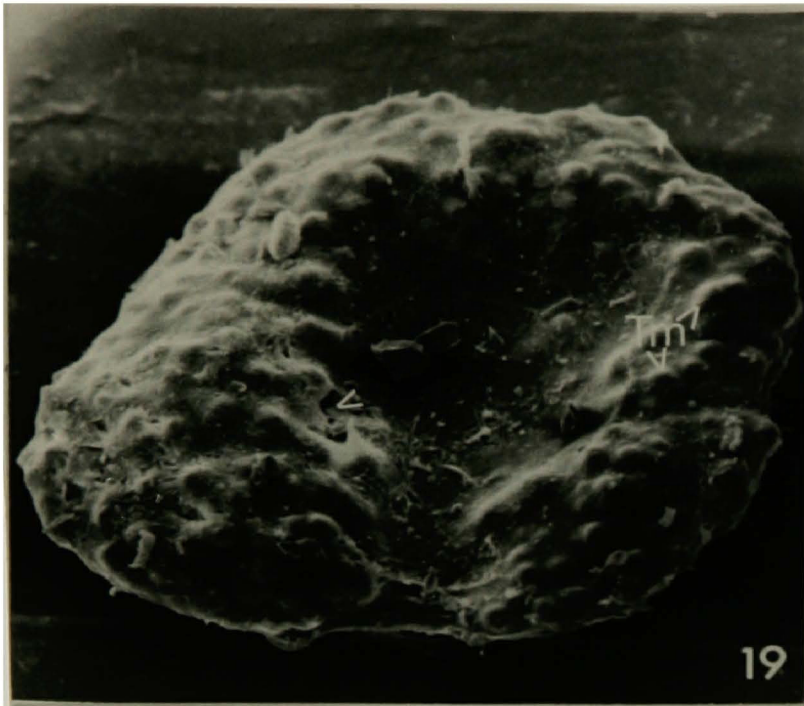


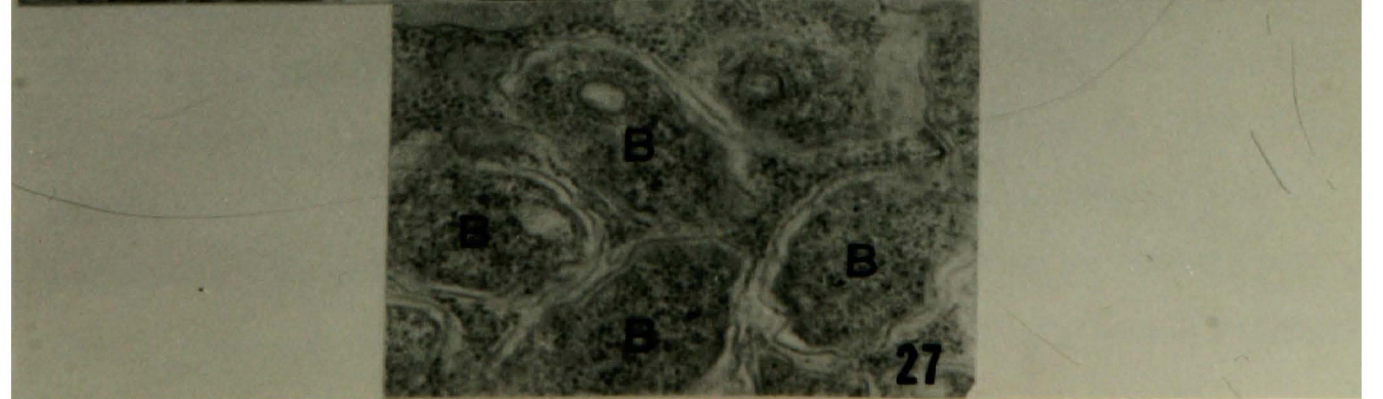
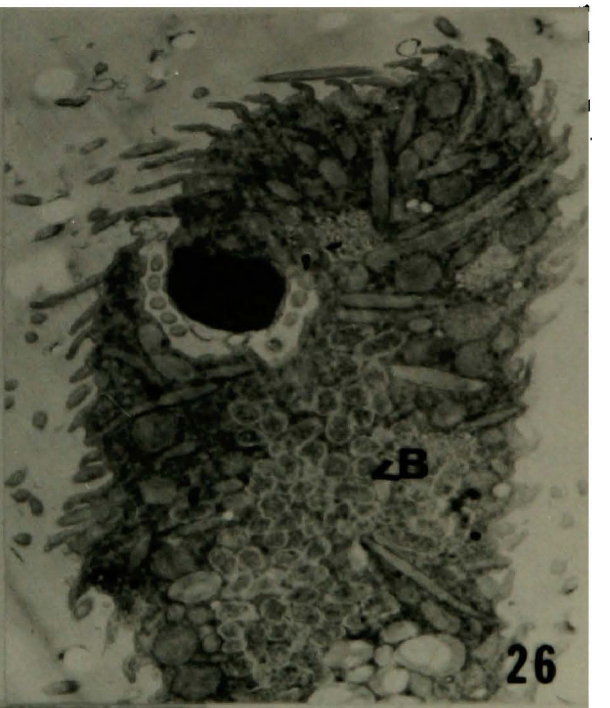
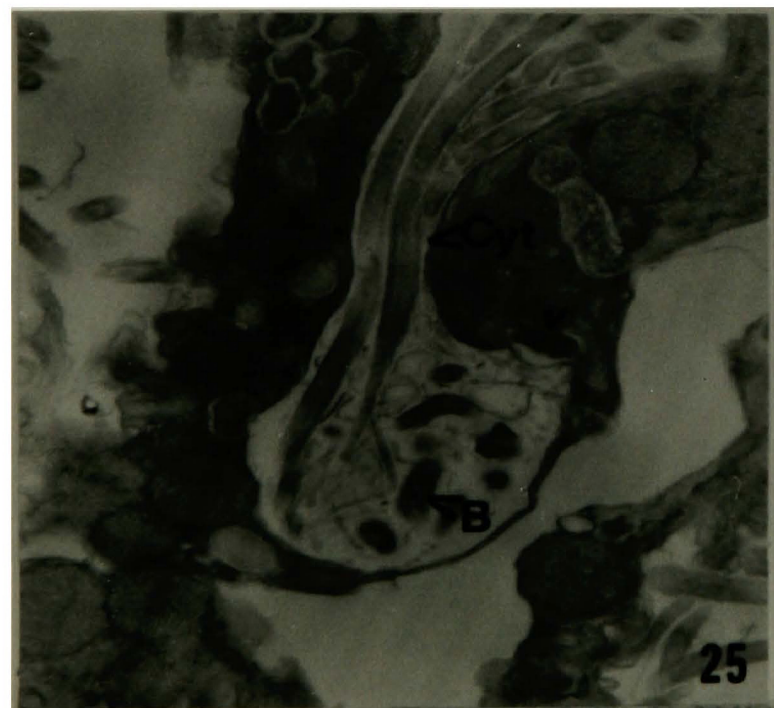
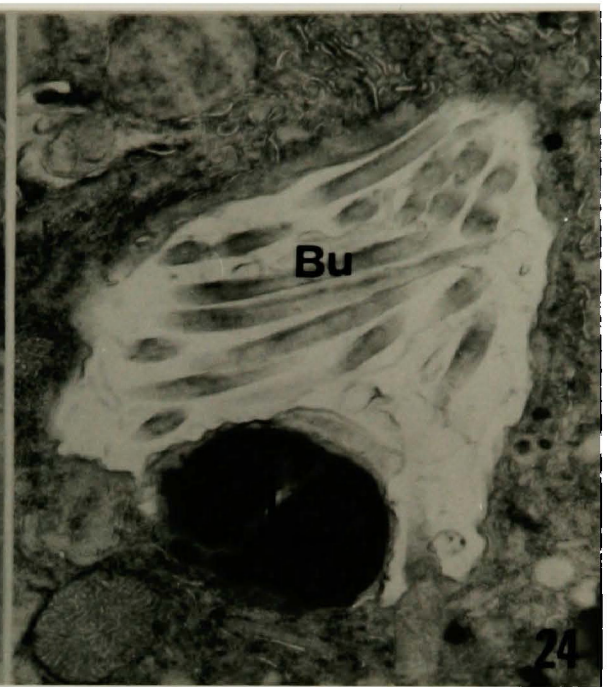
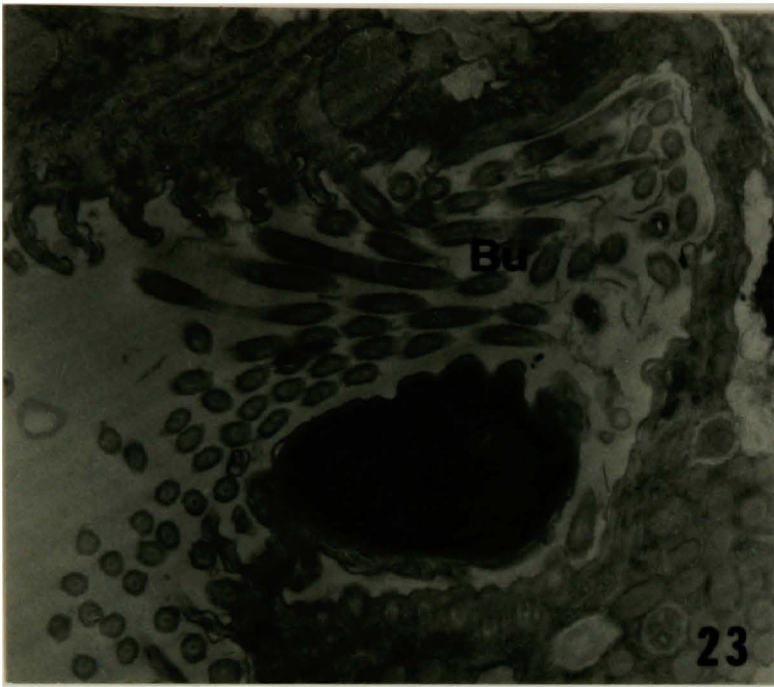
Fig. 23- Longitudinal section of cytostome and ciliated buccal cavity (Bu) of tomite. Note Lieberkühn's organelle (L). (X17,100).

Fig. 24- Cross-section of buccal cavity (Bu) with Lieberkühn's organelle (L) imbedded in one side of the cavity in the tomite. (X17,100).

Fig. 25- Longitudinal section of tomite showing tunnel-like cytopharyngeal passage (Cyt) leading into enlarged pouch or vacuole containing bacteria (B) and other debris. Note bacterium in vacuole apparently starting to pinch off at upper right (arrow). (X17,100).

Fig. 26- Cross-section of tomite showing many closely associated bacteria (B) in separate vacuoles. Lieberkühn's organelle (L). (X7,850).

Fig. 27- High magnification of bacteria (B) found in tomite endoplasm. (X44,800).



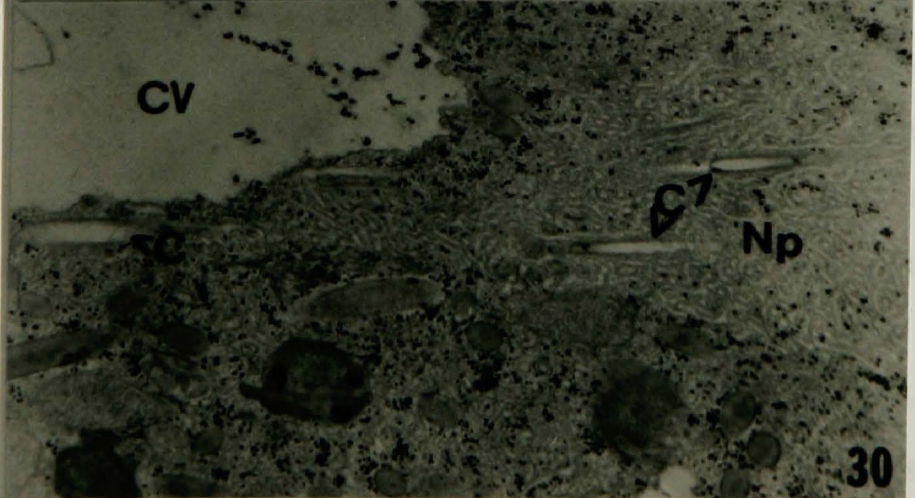
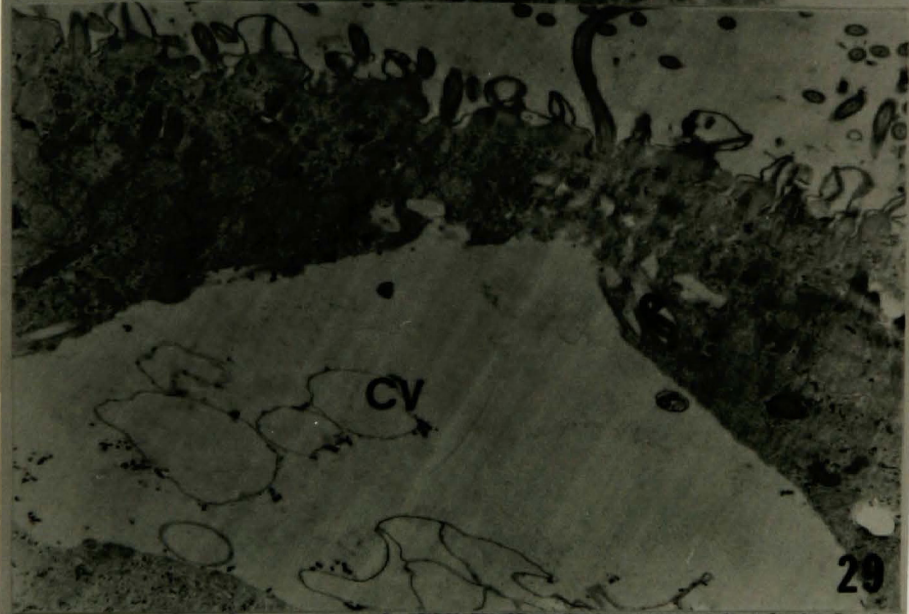
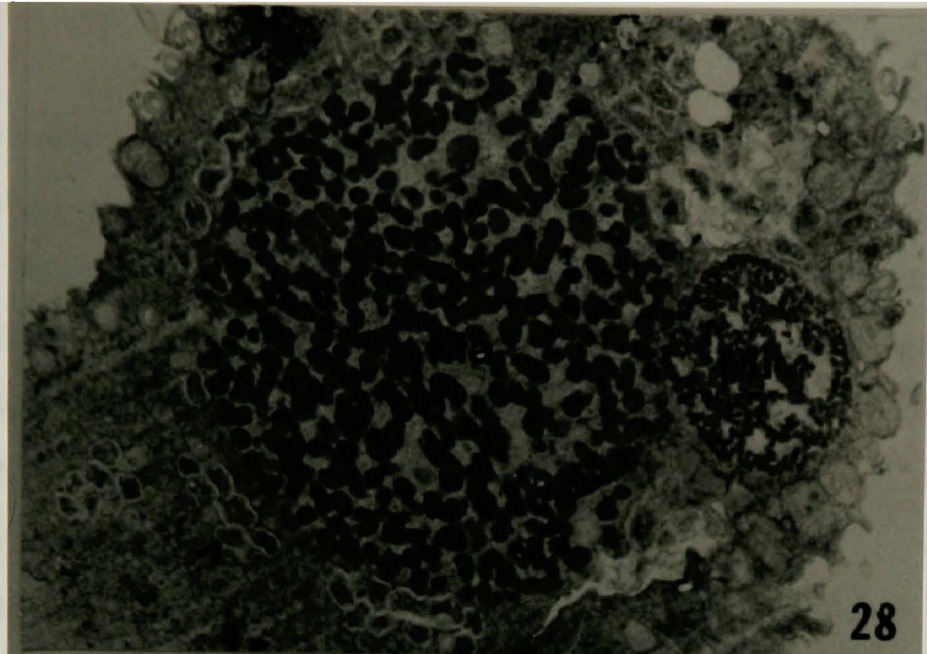
obliquely-cut and longitudinally-cut cilia are seen within the cavity adjacent to the organelle and outside the cavity as well (Fig. 23). A tunnel-like passageway extends from the cytostomal cavity into an enlarged pouch within the endoplasm (Fig. 25). Three or four longitudinally cut ciliary structures lead into this pouch or vacuole which contains what appears to be bacteria and other debris (Fig. 25). A single bacterium appears to be pinching off from the upper right side of the pouch. Figure 26 shows many bacteria grouped closely together within the endoplasm of the cell. All are intact within separate vacuoles and it appears that none of them are being digested. All of the bacteria are bounded by a vacuolar membrane and two cell membranes of their own (Fig. 27). The cytoplasm of the bacteria contains numerous light and dark granules, resembling the ribosome-riddled endoplasm of the tomite. There are no other distinctive characteristics within the bacterium cytoplasm. These microorganisms do not closely resemble the bacteria seen in the cyst during the reproductive stage of the life cycle.

Other endoplasmic features of the tomite include the large macronucleus and small micronucleus (Fig. 28). The micronucleus contains a more condensed chromatin material than that within the macronucleus to which it is abutted. Bacteria can also be seen around the periphery of the macronucleus. The contractile vacuole just below the cell cortex in Fig. 29 communicates with a cytoplasmic tubular network, the nephridioplasm (Fig. 30). The nephridioplasm consists of several canals seen cut obliquely in Figure 30, the walls of which are reinforced

Fig. 28- Section of tomite showing macronucleus (Ma) and closely associated micronucleus (Mi). Note bacteria around periphery of macronucleus. (X15,800).

Fig. 29- Section of tomite showing contractile vacuole (CV) near cell cortex. (X16,150).

Fig. 30- Section of tomite showing nephridioplasm (Np) with canals (C) running through it and towards the contractile vacuole (CV). (X16,150).



with sets of microtubules running parallel to the lumen. The cortical region of the cell contains large numbers of mucocysts and mitochondria (Fig. 31). Lipid vesicles, at times grouped closely together, are also prominent (Fig. 32), and are distinguishable by their electron-lucency.

In this study free-swimming tomites perished two to three days after excystation if they were unable to infect a fish. If, however, freshly killed fish tissue was introduced into a plastic tissue culture well filled with tomite-invested aquarium water the parasites responded to it almost immediately. At first, they adhered to the host and appeared to creep along the epidermis very briefly before penetrating. In the first stage of invasion of the host, somatic cilia can be seen adhering to the host's epidermis (Fig. 33). After a short period of creeping the tomite begins to invade the "desired" area of the epidermis (Fig. 34). Following penetration (Figs. 35 and 37), the tomite is eventually covered by a transparent layer of proliferated epidermis and mucus, the pustule.

Transmission and scanning electron microscopy reveal what may be the boring apparatus used in penetrating the host. In a head-on view of the anterior end of a free-swimming tomite, a modification of the cortex can be seen at the apex (Figs. 37-39). A thick doughnut-shaped structure, measuring 1.5 micrometers dorsoventrally and 1.2 micrometers laterally, encircles a dark opening or cavity. The thickened ring structure possesses what appear to be eight raised, evenly spaced struts radiating from the cavity and extending posteriorly over the edge of, and below, the ring. Projecting from the apical cavity is a long, thin structure which was thought to be an artifact of some kind. However, after

Fig. 31- Tangential section of tomite showing cortical region with large amounts of mucocysts (Mu) and mitochondria (Mit). (X15,500).

Fig. 32- Section of tomite showing lipid storage vesicles (Lp). (X17,100).

Fig. 33- SEM of tomite adhering to host epidermis with its somatic cilia (arrows). (X2,900).

Fig. 34- Tomite about to penetrate host. (X2,400).

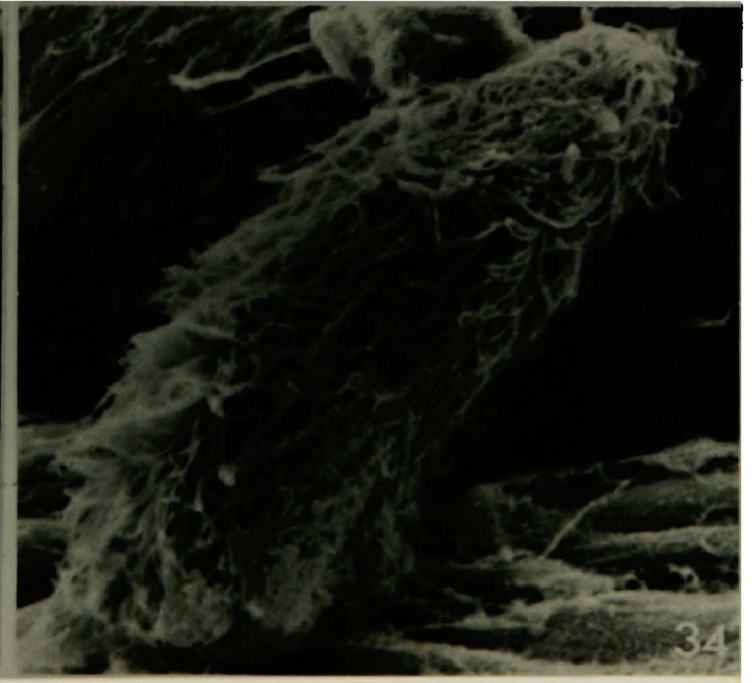
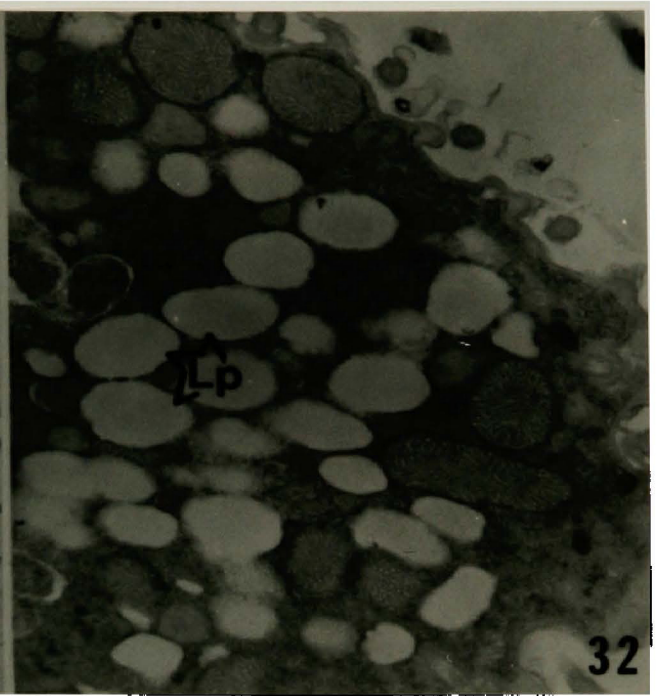
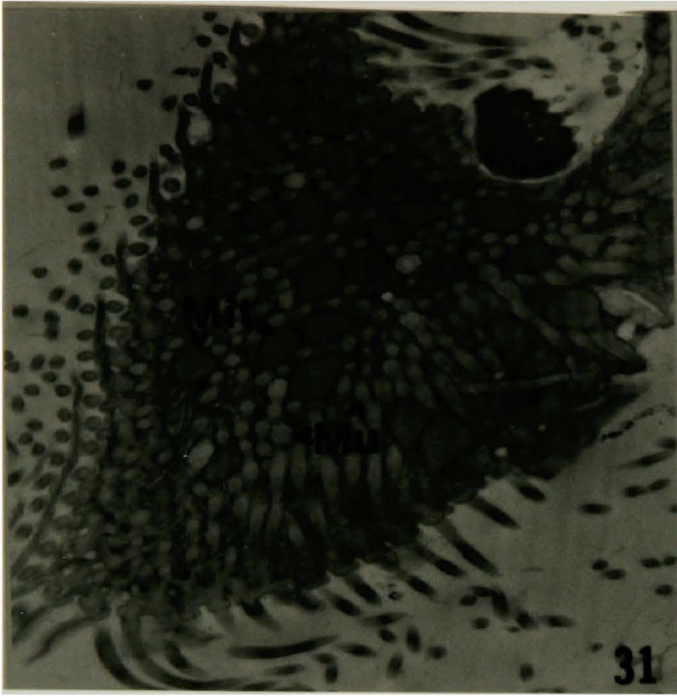
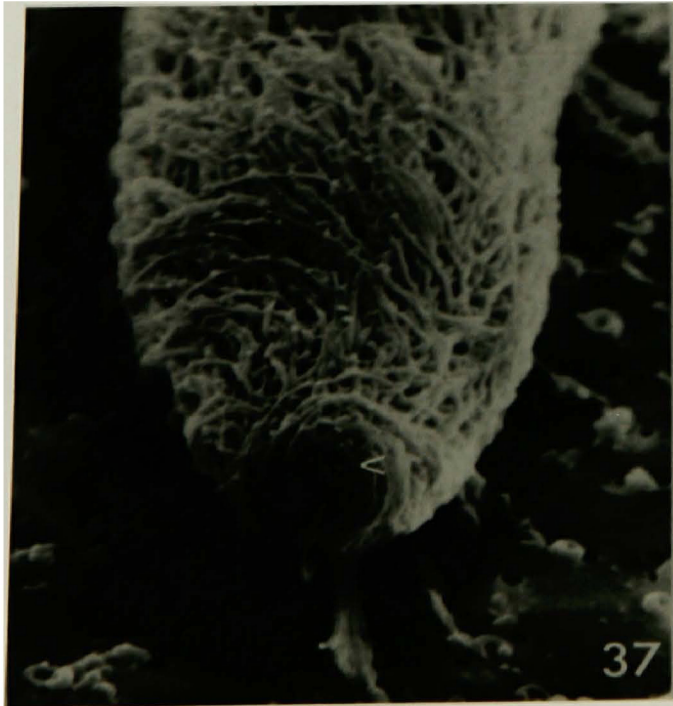


Fig. 35- Tomite penetrating host. (X2,400).

Fig. 36- Tomite towards end of penetration of host. (X2,500).

Fig. 37- Head-on view of anterior end of free-swimming tomite.
Arrow points to possible boring apparatus used in host
penetration. Refer to text for explanation. (X2,400).

Fig. 38- Higher magnification of Figure 37. (X5,000).

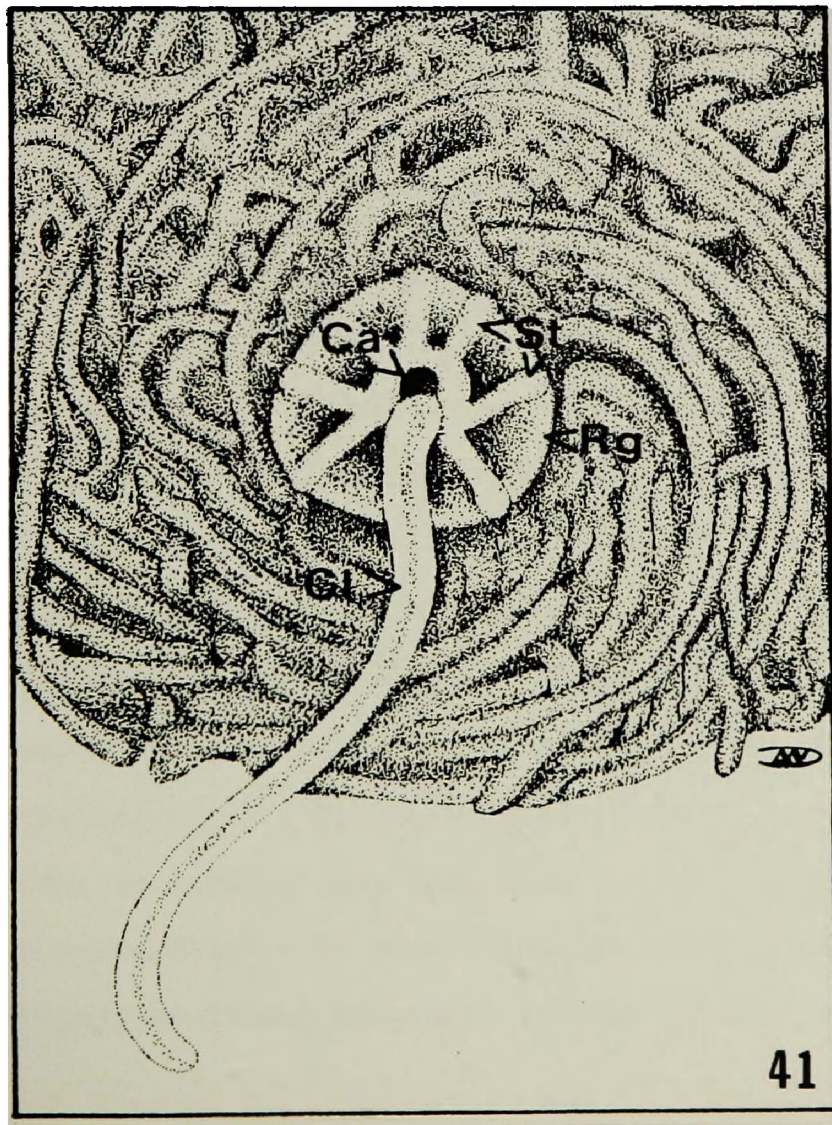
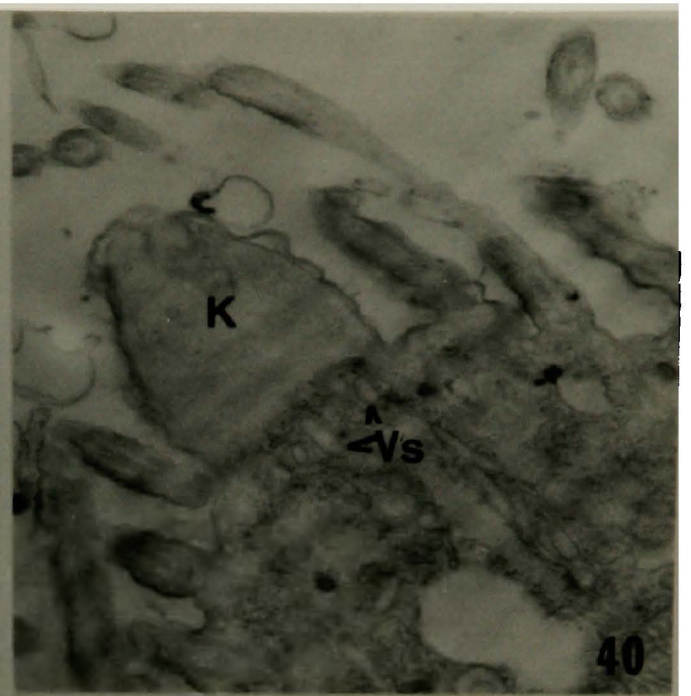
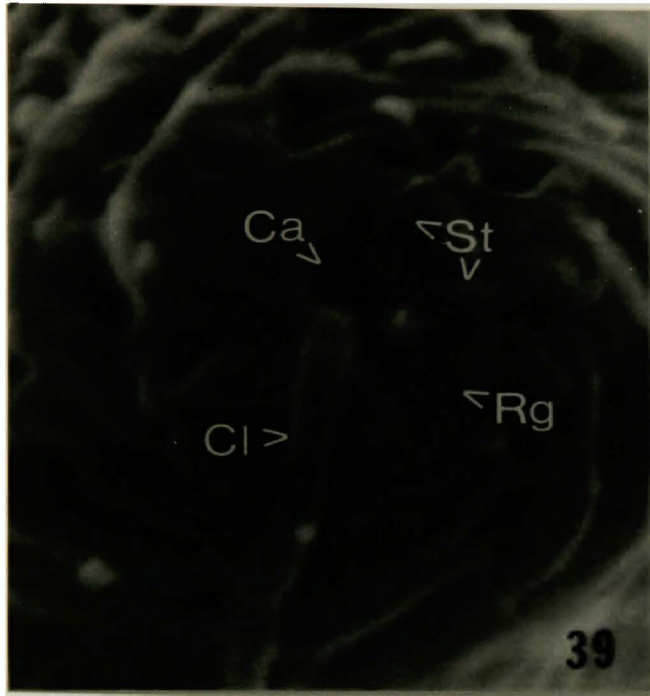


examination of other tomites, I concluded that it was part of this apical modification. All tomites examined possessed this cilium-like structure protruding and extending downward from the apical cavity. As seen with TEM, the apical region of the tomite possesses a knob-like structure bounded by the plasma membrane continuous with that covering the rest of the cell (Fig. 40). The structure contains what appears to be ribosome-free cytoplasm abruptly set off from the endoplasm of the cell proper. A series of vesicles can be seen on the endoplasmic side of this boundary separating the knob-like structure from the cell (Fig. 40).

Fig. 39- Higher magnification of Figure 38. Note thickened ring structure (Rg) possessing radially arranged struts (St) and cilium-like structure (Cl) protruding from cavity (Ca) in center. (X24,000).

Fig. 40- Longitudinal section of anterior end of free-swimming tomite. Note vesicles (Vs) apparently leading to knob-like structure (K) at apex. (X25,650).

Fig. 41- Diagrammatic representation of the anterior end of a tomite as seen in Figures 37-39. Note apical cavity (Ca) from which a cilium-like structure protrudes (Cl), a thickened ring structure (Rg) surrounding the cavity and raised struts (St) radiating from the cavity. (Courtesy of DeWayne Williams, Department of Zoology, University of Montana.)



DISCUSSION

As revealed by SEM study, trophozoites occur just below the epidermis of the fish host. All trophozoites seen on the host with SEM had a wrinkled appearance. This observation was also reported in the light microscopic studies of MacLennan (39) who also noted that the trophozoite swelled when it left the host before sinking to attach to substrate to encyst (39). The wrinkling may be due to the hyperosmotic environment of the fish skin causing the parasite to lose internal fluids by osmosis. When trophozoites leave the host they enter a hypo-osmotic environment causing them to swell, another observation made by MacLennan (39). In this study I attempted culturing cyst stages by using fresh tap water which failed. Trophozoites invariably burst before they could encyst.

Whether or not feeding trophozoites tunnel laterally under host epidermis has been a subject of debate in the literature. Uspenskaya (61) believed the parasite undertook short migrations within the host skin. Reichenbach-Klinke and Elhan (56) reported that trophozoites burrow laterally near the surface. Hines and Spira (26) reported that the ciliates did not burrow laterally but remained in one place, encapsulated by surrounding epithelial cells. In this study, SEM revealed trophozoites located in crevices (Figs. 3 and 4) that may have been the result of the parasites tunneling laterally beneath the epidermis and so weakened it that it cracked wide open when it was prepared for study. Reichenbach-Klinke and Elhan (56) also suggested that in its progress a burrowing ciliate may be joined by others so that parasites may be

found close to one another. Pairs of trophozoites were observed on many occasions in SEM study (Figs. 1 and 2).

In this study the mouth, or cytostome, of the maturing trophozoite was not readily visible through SEM due to the cell's much ciliated appearance. Studies using light microscopy in conjunction with silver impregnation staining and transmission electron microscopy have been conducted on cytostome and buccal structure and its formation (stomatogenesis) by Roque et al. (57). The cytostomal opening is situated at the very bottom of the anterior third of the ciliate. This opens into a wide vestibular cavity which leads into a narrow cytopharynx that extends posteriorly in the endoplasm, terminating in a digestive vacuole (57). Ciliated buccal structures can be seen extending over a vestibular cavity. These structures, called peniculi, are long bands of fused cilia forming undulating membranelles (8). Two peniculi run along the wall on one side of the vestibular cavity and encircle a refractive structure called the Lieberkühn's organelle, the function of which remains unknown although a sensory capability has been suggested (8). A third peniculus runs along the anterior wall of the vestibule and extends downward into the cytopharynx (57). The peniculi are important along with other cytostomal cilia in the feeding process, creating water currents that pull in and drive particles down the cytopharynx to the formation of food vacuoles.

Food vacuoles are a prominent feature in the maturing trophozoite stage (Figs. 5 and 7). The formation and fate of food vacuoles has been much studied in other hymenostome ciliates, namely Paramecium and

and Tetrahymena (10, 48, 1, 2). Generally, the food vacuole, or phagosome, forms in an endocytotic fashion at the distal end of the cytopharynx. Allen et al. (1, 2) terms it a nascent digestive vacuole at this point. Digestive vacuole membrane comes from cytoplasmic vesicles, some of which are recycled from previous vacuoles (2). The food vacuole then pinches off and moves posteriorly into the endoplasm. Primary lysosomes, formed in the lumen of the rough endoplasmic reticulum, fuse with the vacuole and release acid phosphatases that digest vacuolar contents (10). Nutrients are extruded from the vacuole in blebs that pinch off the vacuolar membrane (10). Residual material is expelled through the cell anus, or cytoproct (10, 48, 2). The membrane of the remaining emptied vacuole collapses into small vesicles which are recycled into newly forming food vacuoles (10, 48, 2).

Roque et al. (57) and, to a greater extent, Chapman and Kern (7) conducted studies using transmission electron microscopy on the somatic cortex of Ichthyophthirius multifiliis. Both studies reported the presence of numerous somatic cilia. This was further illustrated in this study with SEM. Cilia feature the 9+2 arrangement of microtubules and sit in cortical depressions. Each cilium is triangularly supported at its base or kinetosome by two microtubular bands (post-ciliary microtubules and transverse microtubules) and a long striated fiber (kinetodesmal fiber) that extends towards the anterior part of the cell (7). Figure 9 reveals a ridge closely associated to the right of the cilium containing parallel microtubular bands. Chapman and Kern (7) reported ridges containing up to 24 ridge microtubules. Similar ridges

were found in this study, as seen close-up in a cyst stage tomite (Fig. 18) but none were perpendicularly cut enough to reveal the microtubules clearly. The ridge in Figure 9 may be a cut at a different orientation of the cell revealing a different perspective of the ridge microtubules. The tri-membraned pellicle (plasma membrane plus aveolus) is seen to be well developed as is generally the case in the hymenostome ciliates, according to Corliss (8). Mucocysts are not only present in the trophozoite stage but in the cystic and tomite stages as well (Figs, 15, 16, 31). According to Hausmann (21) ciliates feature resting mucocysts in predictable positions in the cortex. This is definitely not the case in I. multifiliis (Figure 16). Mucocysts are found randomly distributed, many times in groups, between kineties. Chapman and Kern (7) also made note of this pattern. The origin and development of mucocysts, one of several types of extrusive organelles found in protozoans, has been studied by others (21). Mucocysts, as in Tetrahymena, are believed to be derived as a vesicle from the endoplasmic reticulum. The vesicle contains granular, then crystalline material and grows to a certain size. It then migrates through the cytoplasm and finds its way to the cortex to be discharged (21). Mature mucocysts can be seen in the cytoplasm of I. multifiliis (Figs. 5 and 11), probably en route to the cortex.

After leaving the fish the trophozoite secretes a cyst after a brief free-swimming existence. Ewing (11) described the ultrastructure of the cyst wall and its formation. Initially, the wall is of varying thickness, being thickest at its point of attachment. As reported by

MacLennan (39) the wall is composed of two layers, the ectocyst (outer) and the endocyst (inner). According to Ewing et al. (11) the inner wall is homogeneous in nature, closely resembling contents secreted by tomites within the cyst. Overlying the inner wall is a less dense layer (ectocyst) containing debris. This is illustrated in Figures 13 and 14. Much debris can be seen attached to the cyst wall. Ewing et al. (11) also reported the presence of bacteria in the ectocyst. This was also the case in this study although it could not be determined whether or not they occurred in the inner or outer layer. Bacteria, however, were closely associated with maturing tomites while in the cyst (Fig. 15). Near the time of tomite maturation the cyst wall appears to become thinner in areas enabling the mature tomites to rupture through it and go on to infect a host (11). This is also evident in a SEM of the late cyst stage (Fig. 19). Ewing et al. (11) concluded that the probable function of the cyst wall is to enable the cyst to remain in a fixed position for division.

Free-swimming tomites of Ichthyophthirius multifiliis have hardly been examined, particularly with electron microscopy. The average size of tomites found in this study fell within size ranges reported by others (cf., e.g., 57, 47, 32, 59). As a result of TEM and SEM study I concluded that the tomite possesses at least a rudimentary but functional cytostome-cytopharyngeal complex. The cytostomal opening can be seen at the anterior part of the lower, posterior third of the ciliate at this stage. This is in contrast to where the cytostome is located in the trophozoite (57). At this stage the cytostome is found

at the posterior end of the anterior third of the ciliate lying midway between the equator and the apex (57). There also appears to be some disagreement in the literature as to whether or not the tomite possesses a mouth. For example, Nigrelli et al. (47) reported that they are astomatous and not able to feed. MacLennan (36) reported that the new mouth started to form after division but still within the cyst. It formed in the very middle of the ventral side. The tomites then emerged from the cyst with a mouth rudiment that developed further during their brief free-swimming stage. The mouth opening then sank inward. No further development occurred nor did the mouth become functional until after the tomite penetrated its fish host (36). Roque et al. agreed with these findings (57). In this study, TEM revealed the presence of bacteria at the base of the cytopharynx in what appeared to be a forming food vacuole (Fig. 25). Also, bacteria could be seen in separate vacuoles in the tomite endoplasm (Fig. 26). This is clear evidence that the mouth is functional to some extent, but perhaps not functional enough to keep the free-swimming tomite alive for more than a very short time. The bacteria ingested did not appear to be digested. There is also a conspicuous lack of food vacuoles in the endoplasm of tomites observed with TEM. Tomites apparently carry enough reserve material in them, much of which appears to be lipid (Fig. 32), to survive long enough to find a host.

The question arises as to what is the significance of the bacteria, especially if they are not being digested by the ciliate. Endo- and ectosymbiotic bacteria have been reported in protozoans in the past (3,

55, 13). The bacteria observed in this study are somewhat similar in structure to endosymbiotic forms found in ciliates in previous studies using electron microscopy (15, 35, 22, 13). Endosymbiotic bacteria apparently can play several roles. In Crithidia oncepelti, a flagellate, bacteria provide their host with biosynthetic capability and thus some nutritional independence (15). The ciliate Euplotes aediculatus cannot divide in the absence of the bacteria it normally harbors (22). Certain strains of Paramecium aurelia harbor endosymbiotic bacteria that produce and release toxins that kill other strains, giving the host a competitive advantage, particularly since they are immune to the toxins (55). In return, the bacteria receive necessary nutrients for growth and a "home" protecting them from the external environment (55, 13). In Ichthyophthirius multifiliis bacteria have also been reported in the trophozoite but their role was not discussed (57). It is not clear why I. multifiliis may harbor endosymbiotic bacteria but it seems likely that they function in a capacity similar to ones mentioned above.

Several of the features of the tomite's contractile vacuole seen with TEM are practically identical to those of the trophozoite as reported by Chapman and Kern (7). Both possess tubular and vesicular nephridioplasm with injection canals reinforced by microtubules. Excess water and electrolytes exit the cell via a microtubule-supported discharge canal, or pore (7). It is not known specifically how many contractile vacuoles are in the tomite but there are many in the trophozoite,

possibly increasing in number as the maturing cell increases in volume while feeding on the fish host (7).

Mucocysts are very prominent in the tomite stage (Fig. 31). It is already felt that mucocysts are instrumental in secreting the cyst wall of Ichthyophthirius multifiliis (11) and other protozoans (21). In a recent abstract Ewing (12) reported that when approaching the fish host, tomites became surrounded by an "amorphous material secreted by secretory mucocysts" and that it was this material that contacted the fish host first.

No detailed studies have been published on host invasion by tomites, although one based on an abstract by Ewing (12) is pending (Ewing, personal communication). The present study provided visualization and some details of this phenomenon through SEM but unfortunately not TEM. Scanning electron microscopy revealed that the tomite apparently attaches to the fish host in the first stage of host invasion. Amorphous material surrounding the surface of the cell, as was reported by Ewing (12), could not be distinguished. However, cilia could be seen touching the surface of host epidermis (Fig. 33). It is possible that the tomite possesses thigmotactic ciliature that functions in adhering. According to Corliss (8), thigmotactic ciliature refers to a zone of specialized somatic cilia that has a possible sensory-tactile or adhering function. The tomite appears to creep briefly before penetration, an observation also made by Lom and Cerkasovova (35) in their study of tomite attractants of fish. They concluded that thigmotaxis to a favorable substance-emitting surface probably

plays a role in finding a host and subsequent invasion (35). Thigmotactic ciliature is most notably found in the ciliate suborder Thigmotrichina, a member of the same class as Ichthyophthirius multifiliis, Oligohymenophora (8). The majority of these ciliates occur in the mantle cavity of lamellaebranch molluscs and use an antero-dorsally located zone of cilia not only to attach to, but to crawl on the host, as in Ancistrumina nuellae (33,8).

Scanning and transmission electron microscopical analysis of the apical end of the tomite was also done in this study in an effort to shed some light on what the infective stage uses to bore into its host. The literature has been vague as to the structure of the boring apparatus. Several authors, including MacLennan (36), reported the presence of a hyaline cap or knob at the anterior. Roque et al. (57) reported that the tomite's anterior end tapered and possessed a "perforatorium" in its ectoplasm in addition to an "armed rostrum." A diagram in Roque et al. (57), apparently based on a silver impregnation preparation, shows a rostrum with a spike-like structure, the perforatorium, in it. The rostrum shown in the diagram appeared "unarmed" to me. Hoffman (32) reported the presence of an anterior knob or perforatorium but did not elaborate further. Schmidt and Roberts (59) reported a pointed, unciliated anterior end used in boring. There is no doubt that the anterior end of the infective tomite is employed in host invasion. Scanning electron microscopy revealed, in a head-on view, the presence of a thickened, doughnut-shaped structure with its edges traversed by raised struts (Figs. 37-39 and 41). From the center of this structure a long

cilium-like structure protrudes. The thick ring may serve as a rigid and strong boring mechanism with the struts acting as small blades to cut through host tissue. Other ciliate fish parasites, of the order Peritrichida, use radially arranged scleroprotein denticles on the ventral side of their body to bore into the skin and gills of their host (32 8, 59). The supposed boring apparatus of Ichthyophthirius multifiliis tomites is not as elaborate. But if the tomite does secrete hyaluronidase, as was reported by Uspenskaya (61), the structure may need not to be elaborate. Hyaluronidase is secreted by several parasites that bore into or within their hosts. These include trematode cercariae, monogenetic trematodes, larval nematodes, Balantidium coli (a ciliate), and the amoeba Entamoeba histolytica (61). The enzyme lyses hyaluronic acid, the matrix material that binds cells together. According to Uspenskaya (61), it is not known with which cellular component the enzyme is associated but it was postulated that the component in question may be linked to the boring apparatus in some way. Transmission electron microscopical study of the apical end of the tomite revealed the presence of vesicles apparently leading to the apex of the tomite, seen as a knob-like structure in Figure 40. These vesicles may represent packets of hyaluronidase originating from within the cell (the endoplasmic reticulum and Golgi apparatus ?) which enter and pass out of the apical cavity of the boring apparatus. The cilium-like structure observed is puzzling. It may be a cilium with some sensory (thigmotactic and/or chemotactic) function allowing the tomite to specifically locate the "right" spot to penetrate. However, this is

speculation because this phenomenon was not observed. All free-swimming tomites observed with SEM possessed this structure. It must play some role in host invasion that only further study will elucidate.

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