Pathological Study on the Scuticociliatosis of Japanese Flounder (*Paralichthys olivaceus*) in Japan

日本のヒラメのスクーチカ症に関する病理学的研究

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ABSTRACT

Occurrences of the scuticociliatosis have been reported in both natural and farmed fishes throughout the world including Japan, Korea and Spain. In Japan and Korea, outbreaks of scuticociliatosis have frequently occurred in Japanese flounder (*Paralichthys olivaceus).* However, detailed pathology on scuticociliatosis occurring in Japan has not yet been reported. In addition, very little is known about the way by which the ciliate invades the host tissue and the evasive mechanism of the scuticociliate against the host cellular defense response. Abraded skin and/or gills are reported to be the main routes of entry, and blood vessels as the main dissemination route. Connective tissue is also reported as a possible quick way of dissemination of the scuticociliate to the body in turbot. This study has two aims. The first aim is to demonstrate the pathological findings of naturally infected flounders affected with scuticociliatosis in Japan. The second aim is to elucidate the possible neural invasion and dissemination routes of the present scuticociliate into the fish body in flounder.

In the first study, pathological findings of farmed Japanese flounder obtained during an outbreak of scuticociliatosis in Japan were described. Ten moribund fishes, farmed in Tottori Prefectural Fisheries Experimental Station, showed cutaneous ulcers, darkened skin, fin and tail rot, exophthalmia and alterations in swimming behaviour. Histopathological examination revealed severe epidermal degeneration and necrosis, hyperplasia of branchial epithelium, myositis, myelitis, encephalitis associated with heavy accumulation of scuticociliates in the periorbital cavity and optic nerve fiber. Moreover, masses of ciliates were found to feed on the host tissues such as skeletal muscles, gills and brain, causing severe degenerative changes associated with abundant neutrophilic and lymphocytic infiltration. These findings suggest that the present scuticociliate, *Miamiensis avidus,* is a

highly invasive and destructive pathogen infecting Japanese flounder and capable of developing systemic fatal infection.

In the second study, Japanese flounder *(Paralichthys olivaceus)* were experimentally infected with the highly pathogenic scuticociliate *Miamiensis avidus (*syn. *Philasterides dicentrarachi)* using the immersion method to clarify/identify the possible neural invasion routes and the possible neural ways of dissemination that enable rapid expanse of the scuticociliate into the fish body. Affected fish showed varying degrees of tissue damage including severe epidermal and dermal necrotic lesions, necrotic myositis, encephalitis and myelitis. Brain lesions were observed in most of the examined fish and considered to be the cause of the sudden increase in mortality. Scuticociliates were frequently observed along the optic and/or olfactory nerve in the fish accompanied by severe brain lesions but by minimum lesions in the gills and skin, suggesting that in addition to skin and/or gills, neural routes including periorbital and nasal routes may play a role in scuticociliate invasion to the brain. Scuticociliates were also observed in the peripheral nerve fibers in the muscle tissue, cranial and spinal nerves, cranial cavity and in the vertebral canal, suggesting that nerve fibers and/or cerebrospinal fluid circulation may be involved in the spread of the scuticociliate throughout the body in addition to the blood circulation and connective tissue.

From these results, it can be concluded that the current scuticociliate, *M. avidus*, is a highly invasive and destructive pathogen infecting Japanese flounder and capable of developing systemic fatal infection. The pathogen can invade the fish body not only through skin and/or gill epithelium as previously hypothesized, but also through the neural routes, periorbital and nasal routes. In addition, the dissemination of the scuticociliate into the fish body can be not only through blood stream and/or connective tissue, but also through either nerve fibers and/or cerebrospinal fluid circulation.

Contents

GENERAL INTRODUCTION AND BACKGROUND

1. Japanese flounder

Japanese flounder (*Paralichthys olivaceus*) is a temperate marine fish species cultured in coastal areas of Korea, Japan and China. Flounders are known for their unique and spectacular transformation from a normal-looking fish with an eye on each side of the head to one with both eyes on the same side of the head. This metamorphosis occurs while they are still larvae and is one of the more fascinating transformations among fishes.

1.1. Appearance and life cycle

Adult flounders are asymmetrical in appearance. Instead of swimming through the water column like other fishes, flounders rest on the bottom with a dark pigmented side upwards and a white unpigmented side facing down. Both eyes and nostrils are on the upper side of the head. The mouth is also slightly twisted toward the upper side.

Larval flounders look like other fish until they reach metamorphosis. During metamorphosis, which begins about 30 to 40 days post-hatching, the right eye slowly migrates to the left side of the head, the jaw twists slightly, and the fish a side-to-side swimming motion and to an up-and-down motion. When metamorphosis is complete in about 2 to 3 weeks, the fish resemble adults and thereafter rest on the bottom most of the time (Figure 1) (Daniels, 2000).

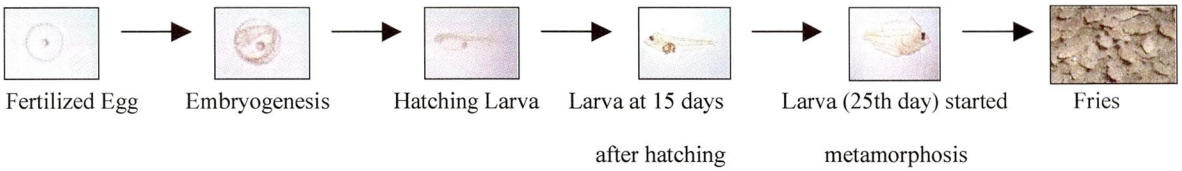


Figure 1: Life cycle of Japanese flounder

1.2. Feeding habit

Flounders feed by ambushing passing prey in a rapid upward lunge, accompanied by a vacuum action of the mouth, to capture and ingest the food in one swift motion. Wild flounders consume mainly shrimp and small fish. After feeding, they immediately glide back down to the bottom. Cultured flounders feed in the same way, but can be trained to come to the water surface to eat dry, pelleted feed (Daniels, 2000).

1.3. Growth rates

Little is known about Japanese flounder growth rates and feed conversion values, but it has been determined that flounder females grow approximately three times faster than males. As with other flatfish, the sex of the fish is not determined until after metamorphosis; the precise time is not known for flounders. In Japanese flounder, the sex of the fish can be influenced by temperature. The optimum temperature for producing the highest percentage of females is approximately 70 °F (21 °C). Temperatures significantly higher or lower than that likely will result in a higher percentage of males in the overall

population. High stocking densities may also shift the population towards males. To achieve optimum growth and profitability of the cultured flounder, hatcheries should produce all female fingerlings.

Japanese flounder are cannibalistic and feed aggressively at the surface. This aggressive behavior leads to uneven growth rates, so they must be graded often to increase survival. Fingerlings may need to be graded three or four times during the few months it takes them to grow from 2 to 10 g. Larger fish do not require such frequent grading (Daniels, 2000).

1.4. Classification

Scientific classification	
Kingdom :	Animalia
Phylum :	Chordata
Class :	Actinopterygii
Order :	Pleuronectiformes
Family :	Paralichthyidae
Genus :	Paralichthys
Species :	***P. olivaceus***

Table 1: Classification of Japanese flounder

2. Japanese flounder production

2.1. In Korea

The most important marine fish cultured in Korea is olive flounder (*Paralichthys olivaceus* Temminck & Schlegel, 1846), also commonly known as bastard halibut and Japanese flounder. The olive flounder (*Paralichthys olivaceus*) is an economically important food fish in Korea with 98% of the domestic demand met through indigenous farming in land based tanks (Jung et al., 2001). Although the aquaculture for the olive flounder in Korea started from late 1980s, its commercial production commenced in the beginning of the 1990s when wild stocks of *P. olivaceus* were exhausted. Soon after the industrialized production, the Korean production maximized by the year 1997 when over 25000 metric tonnes were produced; more than double the quantity of the second-most produced species (black rockfish *Sebastes schlegeli*) (Kim, 2000). Currently, olive flounder culture is a major activity with about 300 commercial farms situated in Jeju Island, South Korea. Reported annual production was 21,368 metric tonnes in 1998 (Figure 2A & 2B) (Statistical Year Book of Maritime Affairs and Fisheries, 2000; Korea National Statistical Office, 2001).

Olive flounder is highly esteemed in NE Asia for raw consumption in thin slices, and commands a good market price for farmers. However, this species is also known to be susceptible to many pathogens (Kim, 2000).

Recently, mass mortality of adult flounder showing ascites occurred at many aquaculture farms in southern Korea (Korea National Statistical Office, 2001; Oh et al., 2006).

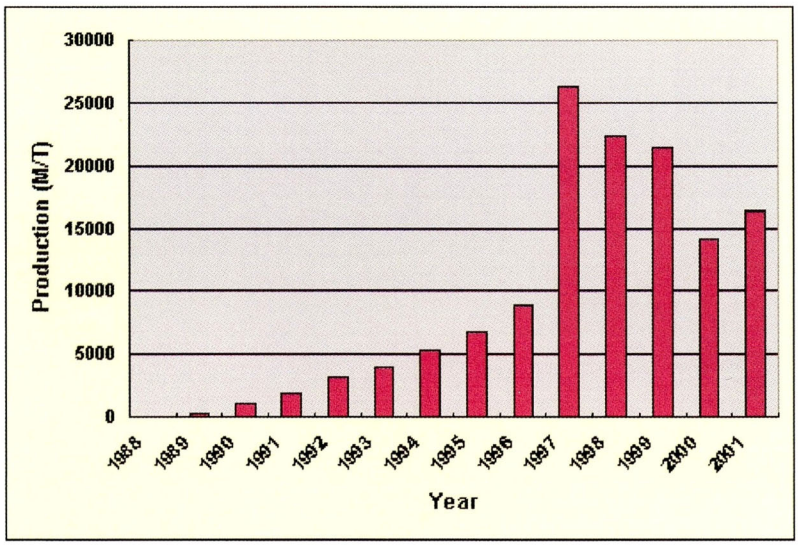


Figure 2 A: Production trend of farmed olive flounder in Korea. Data from Korea National Statistical Office (2001)

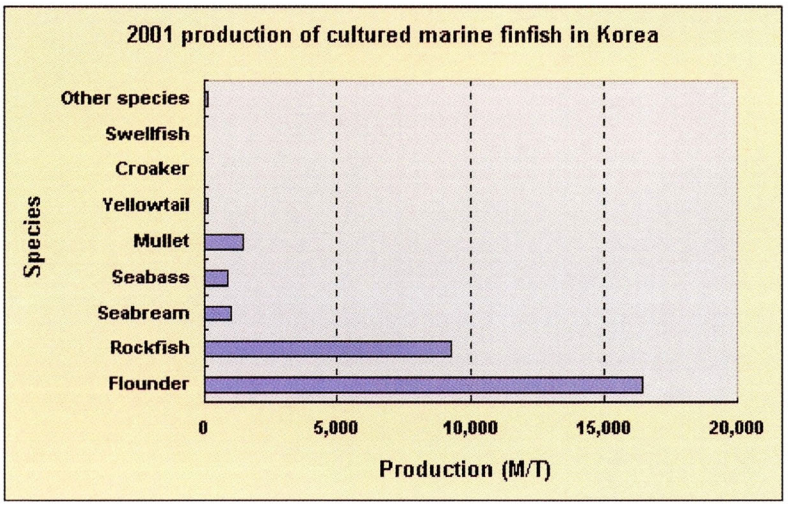


Figure 2 B: Production of cultured marine finfish in Korea 2001

2.2. In Japan

The Japanese flounder (*Paralichthys olivaceus*) is one of the most important commercial fish species among Japanese pleuronectiforms and widely distributed from Kyushu to Hokkaido, being particularly abundant in the Japan Sea. On the Wakasa Bay area, at the central part of the Japan Sea, its spawning season is known to be from March to May (Seikai et al., 1986). Production of Japanese flounder ranked the 4th among marine cultured finfish in 1995. Being carnivorous, it requires a high percentage of protein in the diet (Kikuchi et al., 1999). Interest in the culture of flatfish worldwide has increased recently because of its good growth characteristics and high market price.

3. Marine parasites

Fish and fisheries products are important sources of protein and contribute a great deal to available food resources worldwide. Over-fishing and environmental degradation are already threatening most of the larger fish stocks, and a further increase in fisheries production seems to be dependent on the cultivation of aquatic organisms within semi-extensive and intensive mariculture. An intensive culture leads to an increasing risk of infection by disease causing agents, such as fungi, viruses, bacteria and parasites.

Parasitic diseases are a major problem for aquarium fishes. Almost all marine fish have parasites to some degree. Protozoan, small microscopic animals generally invisible to the naked eye, are the most serious types of parasites. There are numerous parasites

responsible for diseases of marine fish. Parasites as disease causing agents can have various effects on the fish's health; they can infest all organs. Depending on the site and intensity of infestation, they are more or less harmful.

Parasites in marine fish are of public concern if they are found dead or alive within food products that are intended for human consumption. Besides infection from living parasites, pathogens that are already dead or their remains within the fish tissue might harm the consumer by causing allergic reactions. The disgusting appearance of heavily parasitized fish can prevent them from being sold on local and international markets as their presence in the musculature can offend potential consumers. Thus, parasites can significantly contribute to financial losses for the fisheries industry.

4. Economic importance of parasitic diseases in marine fishes

Economic effects of disease in marine fishes may be categorized as follows: reduction in numbers of food fish available to the fishery; weight loss of individuals; rejection of diseased fish by dealers or consumers and subsequent loss of interest in fishery products as food; and indirect effects, either favorable or unfavorable, on survival of other species in a food chain.

Certainly, the scuticociliates are common inhabitants of marine and brackish water which is conceivably the reservoir of infection for the flounders. So, the high mortality rates involving scuticociliatosis outbreaks should not be so surprising and suggests that these emergent protozoa may pose a potential serious economic problem for Japanese

aquaculture in the near future.

Fish diseases act as a great problem in cultured fishes especially protozoan infections which lead to heavy losses. Subsequently, cause a great drop in fish production and industry. Most of protozoan diseases of intensively reared marine water farms are of high mortality nature. The intensification of fish production has led to major changes in the host-pathogen relationships, whereby the protozoan load of the environment increases to such levels that minor factors weakening the resistance of the host result in outbreaks of disease. It has been suggested that the production of fish is often carried out under suboptimal conditions allowing the early establishment of the pathogens.

5. Scuticociliatosis

5.1. Causative agents

Infections by histophagous scuticociliates (scuticociliatosis) have become one of the most important worldwide parasitological diseases in the intensive marine culture of flatfish species, including Japanese flounder (*Paralichthys olivaceus*) (Jee et al., 2001). Scuticociliates are free-living organisms in sea-water, feeding on suspended particulate matters (bacteria, microalgae & protozoa). Under certain circumstances, however, these ciliates may behave as opportunistic histophagous parasites, and actively feed on cells and tissue residues of certain mollusks, crustaceans and fishes, and continue to live and reproduce within the host tissues (Elston et al., 1999). These ciliates are characterized by

their high potential for systemically invading and destroying tissues, and cause significant mortalities in cultured fish (Jin et al., 2009).

Several scuticociliate species have been reported as agents that cause scuticociliatosis in farmed marine fish. The species of *Philasterides dicentrarachi* infects European sea-bass (*Dicentrarchus labrax*) (Dragesco et al., 1995), turbot (*Scophthalmus maximus* = *Psetta maxima*) (Iglesias et al., 2001; Iglesias et al., 2002; Puig et al., 2007), weedy sea dragon (Umehara et al., 2003), Japanese flounder (*Paralichthys olivaceus*) (Kim et al., 2004a; Jin et al., 2009) and population of both leafy and weedy sea dragons (*Phycodurus eques* and *Phyllopteryx taeniolatus*) (Rossteuscher et al., 2008). *Uronema nigricans* species infect subadult Southern bluefin tuna (*Thunnus maccoyii*) (Munday et al., 1997), where as *Uronema marinum* infects many species of aquarium fishes (Cheung et al., 1980; Bassleer, 1983), Japanese flounder (Jee et al., 2001; Kwon et al., 2003) and silver pomfret (*Pampus argenteus Euphrasen*) in Kuwait (Azad et al., 2007). *Uronema*-like ciliates infected turbot (Sterud et al., 2000) and *Tetrahymena corlissi* infected guppy fish (*Poecilia reticulata*) (Imai et al., 2000). Moreover, a mixed infection of the microsporidian *Tetrarnicra brevifjlum* together with a histophagus ciliate infecting turbot was reported (Dyková & Figueras, 1994).

5.2. Mortality from scuticociliatosis

Scuticociliates are serious pathogens in worldwide marine aquaculture, causing mass mortalities in fish and crustaceans (Armstrong et al., 1981) such as the Japanese flounder (*Paralichthys olivaceus*) (Yoshinaga & Nakazoe, 1993; Jee et al., 2001; Kwon et

al., 2002; Kim et al., 2004a; Kim et al., 2004b; Jung et al., 2005; Jung et al., 2007; Jin et al., 2009), turbot (*Scophthalmus maximus*) (Dyková & Figueras, 1994; Sterud et al., 2000; Iglesias et al., 2001), European sea bass *Dicentrarchus labrax* (Dragesco et al., 1995), Cultured striped trumpeter (*Latris lineata*) (Langdon, 1992; Watts et al., 1996), subadult Southern bluefin tuna (*Thunnus maccoyi*) (Munday et al., 1997), American lobster (*Homarus americanus*) (Cawthorn et al., 1996; Cawthorn, 1997), blue crab (*Callinectes sapidus*) (Messick & Small, 1996), seahorse (*Hippocampus erectus*) (Thompson & Moewus, 1964) and silver pomfret (*Pampus argenteus*) in Kuwait (Azad et al., 2007).

In farmed Japanese flounder, mortality has been reported through the infection with unidentified scuticociliate (Yoshinaga & Nakazoe, 1993), *Uronema marinum* (Jee et al., 2001), *Pseudocohnilembus persalinus* (Kim et al., 2004b), *Philasterides dicentrarachi* (Kim et al., 2004a; Jin et al., 2009) and *Miamiensis avidus* (Jung et al., 2005; Jung et al., 2007). However, Song et al., (2009a) has reported in a comparative pathogenicity study that *Miamiensis avidus* is the main cause of scuticociliatosis in Japanese flounder in a series of reports (Jung et al., 2005; Jung et al., 2007; Song et al., 2009a). Additionally, based on morphological and/or genetic analysis, it has been suggested that *Miamiensis avidus* and *Philasterides dicentrarachi* are synonymous (Paramá et al., 2006; Jung et al., 2007).

5.3. Occurrence of scuticociliatosis worldwide

In Australia, a syndrome characterized by atypical swimming behaviour followed by rapid death was first reported in captive subadult Southern bluefin tuna (*Thunnus*

maccoyii) in the winter of 1993. The cause of this behaviour was found to be parasitic fatal encephalitis due to the scuticociliate; *Uronema nigricans* (Munday et al., 1997).

Cawthorn et al., (1996) reported that "Bumper car" disease, caused by an anophrys-like ciliate (*Anophryoides haemophila*); was first documented from Eastern Canada as a pathogen of captive adult and third-stage larval American lobsters (*Homarus americanus*) held in a flow through system at 0 to 10 °C (at cold temperatures). During winter 1972-1973, infected adult lobsters from Nova Scotia and Prince Edward Island also died and third-stage larval lobsters infected with ciliates died. The 'bumper car' disease problem was apparent from November through March or April in these tank held, laboratory stocks of lobsters (Aiken et al., 1973). More recently, a ciliate mistakenly identified as *Mugardia* spp. (Morado & Small, 1994, 1995) caused significant mortalities in lobster impoundments of Eastern North America (Loughlin & Bayer, 1991; Sherburne & Bean, 1991; Loughlin et al., 1993). 'Bumper car' disease was well documented in winter and spring in Maine (1990, 1991, 1992) and New Brunswick (1989, 1992, 1993), with mortalities up to 25% (Sherburne & Bean, 1991; Loughlin et al., 1993, 1994). Additionally, affected lobsters have reduced muscle mass, poorer meat quality and unsavoury flavour (Loughlin et al., 1993). Survivability during shipping can be highly reduced (Sherburne & Bean, 1991). On the other hand, Elston et al. (1999) discovered the presence of a primary invasive orchitophryid ciliate which caused high morbidity and mortality in cultures of early stage juvenile Pacific oysters (Kumomoto oysters, *Crassostrea gigas* and *Crassostrea sikamea*).

In Kuwait, the disease in the cultured silver pomfret (*Pampus argenteus Euphrasen*) was reported in 2004 under the laboratory (both outdoor and indoor facilities) conditions (Al-Marzouk et al., 2004; Durumdez et al., 2004) in which infections due to *Uronema spp.*

were also mentioned. Recently, Azad et al. (2007) has reported valuable information on the clinical and histopathological manifestations of scuticociliatosis in sub-adults of the silver pomfret reared indoors with high mortalities from 2005 mainly due to *Uronema spp.*

In Spain, scuticociliatosis has been reported as a result of many ciliates; *Ichthyophthirius multifiliis* and *Cryptocarion irritans* which were recognized for long time as the causes of significant economic losses in aquaculture. However, the scuticociliate responsible for the two outbreaks of scuticociliatosis detected in a turbot (*Scophthalmus maximus*) farm in Galicia (northwest Spain) was classified into *Philasterides dicentrarachi* through the morphological and biometric characteristics of the organism (Iglesias et al., 2001). Systemic scuticociliatosis outbreaks have been well described in the turbot industry, substantially increasing production costs due to mortality and morbidity (Iglesias et al., 2002). This protozoan generates severe systemic infection invading internal organs such as brain, gills, liver and intestine that generally results in the death of the host. Several epidemiological, clinical and pathological descriptions of outbreaks have been published (Dyková & Figueras, 1994; Dragesco et al., 1995; Sterud et al., 2000; Iglesias et al., 2001). The taxonomy of this group has been recently revisited (Lynn & Small, 2000), though the variation in the criteria used has left it rather confusing (Álvarez-Pellitero et al., 2004). Besides the strategies of prophylaxis and treatment, which were under study (Lee et al., 2004; Leiro et al., 2004; Paramá et al., 2004), a better understanding of the pathogenesis of the disease was reported through an experimental infection in turbot; that may be a useful approach for improving the control of the disease (Puig et al., 2007; Paramá et al., 2003).

In Switzerland, severe systemic scuticociliate infection with *Philasterides dicentrarachi* has been reported in a population of both leafy and weedy sea dragons (*Phycodurus eques* and *Phyllopteryx taeniolatus*) (Rossteucher et al., 2008).

In Norway, systemic infection with Uronema-like ciliate in farmed turbot was reported by Sterud et al., (2000).

In Portugal, scuticociliate infection has been reported during the years 2004 and 2005 in turbot (*Scophthalmus maximus*) from a fish farm grow-out facility located in the north of Portugal, where fish were held in a flow-through supply of seawater supplemented with oxygen in circular concrete tanks (Ramos et al., 2007).

5.4. Occurrence of scuticociliatosis in Korea

Commercially significant occurrences of scuticociliatosis in olive flounder in Korea were first noted in 1990, and quickly became a challenging disease with no effective control (Chun, 2000). The ciliates occurred in the gills, skin, heart, brain, muscles and visceral organs including the intestine. Scuticociliatosis is highly histophagous and destroys infected tissues. After 2000, the disease inflicted severe mass mortality in many commercial farms in Jeju Island (Oh et al., 2006). Not only mass mortalities of fry but also high cumulative moralities of juveniles caused by infection with ciliates have occurred frequently in many farms (Jee et al., 2001). Although various silver impregnation methods have been used to identify Scuticociliatida (Corliss, 1953; Foissner, 1991), there was still some confusion in the identification of certain species (Song & Wilbert, 2000). The causative agents of scuticociliatosis in the olive flounder in Korea with the same clinical

signs mentioned above have been isolated and identified as *Uronema marinum* (Jee et al., 2001), *Pseudocohnilembus persalinus* (Kim et al., 2004b), *Philasterides dicentrarachi* (Iglesias et al., 2001; Kim et al., 2004a; Jin et al., 2009) and *Miamiensis avidus* (Jung et al., 2005; Jung et al., 2007; Song et al., 2009a). Using small subunit ribosomal RNA gene (SSU rRNA) and morphological characteristics, it was confirmed *Miamiensis avidus* as a senior synonym of *Philasterides dicentrarachi* (Jung et al., 2007).

Flounder culture mainly begins in March and ends in December in a land-based raceway culture system with an average mortality of 15-20% (Lee, 2007). The incidence of the disease begins in May (Jin, 2006) with a marked withering of scales followed by imbalanced swimming behavior, anemia, weight loss, brownish skin patches, necrotic lesions, enteritis, excessive body mucus and yellowish intestinal mucus.

5.5. Occurrence of scuticociliatosis in Japan

Although Korea is the dominant producer of olive flounder (Hambrey, 2000), its diseases have been much better documented in Japan. A new condition, characterized by severe anemia leading to mortality in both wild and cultured flounder, was reported in Japan by Miwa & Inouye, (1999). More recently, however, Ogawa, (1999) and Yoshinaga et al., (2000a, 2001) concluded that haematophagia by a large monogenean, *Neoheterobothrium hirame*, was very strongly associated with anemia in both wild and cultured fish. Similarly, Ogawa, (2000a, b) suggested that the sharp decline in the catch of wild flounder in Tottori Prefecture in Japan may have been due to anemia induced by infections of *Neoheterobothrium hirame*. Because *N. hirame* has emerged as a serious

pathogen in Japan, the biological characteristics of the worm are now being investigated in detail, with a view to eventual control (Yoshinaga et al., 2000b, c). However, *N. hirame* may have already reached Korean waters and become established, because of the remarkable proliferation and spread of the pest within Japan, after its initial discovery in the NW of the country (Anshary et al., 2001).

Ogawa & Yokoyama, (1998) list a total of 8 ecto-parasites afflicting cultured Japanese flounder in Japan: a flagellate (*Ichthyobodo* spp.), 3 ciliates (*Trichodina* spp., *Cryptocaryon irritans* and an un-described scuticociliate), a gill myxozoan (*Kudoa shiomitsui*) and three monogeneans [*Neobenedenia melleni*, *Benedenia epinepheli* and *Neoheterobothrium hirame* (Hayward et al., 2001)]. In addition, a ninth ectoparasitic pathogen is known in Japan the copepod *Lepeophtheirus paralichthydis* (Ho, 2000).

Ototake & Matsusato, (1986) recorded scuticociliatosis in cultured flounder for the first time in Japan. It was suggested that the ciliates of the order Scuticociliatida are facultative parasites, opportunistically invading fish seriously stressed under unfavorable environments (Otatake & Matsusato, 1986; Lom & Dyková, 1992). The culture characteristics of the ciliates, including optimal cultivation conditions, have been well established (Yoshinaga & Nakazoe, 1993, 1997).

From February to May of 2001, high mortality of weedy sea dragons (*Phyllopteryx taeniolatus*) was observed at an aquarium in Kanagawa Prefecture of Japan. Two weedy sea dragons showing sloughing of the epidermis were used for pathological examination. Based on the arrangement and shape of the buccal structure and the number of somatic ciliature, ciliates obtained from the infected weedy sea dragons belonged to the order *Scuticociliatida* (Umehara et al., 2003).

In July 2005, a huge outbreak of scuticociliatosis in Japanese flounder had occurred in Tottori prefecture Fisheries Experimental Station; in which the mortality of fishes reached 70-80%. Although the cause of scuticociliatosis in Japanese flounder was considered to be unidentified ciliate (Yoshinaga & Nakazoe, 1993) for many years, it has recently become clear that the main cause is *Miamiensis avidus* as demonstrated in the present study. Using morphological characters, Song et al., (2009b) proved that there are at least three *M. avidus* serotypes in Japan, based on immobilization titres and Western blotting profiles. Therefore this ciliate becomes currently the greatest target for this disease.

6. Classification and morphology of scuticociliates

6.1. Classification of scuticociliates

Scuticociliatia is recognized as a subclass within the class Oligohymenophorea (Table 2) (Lynn, 2008) and is characterized by a paroral membrane that is divided into three segments; anterior, middle and posterior or scutica. The scutica, named for its hook-like or "whiplash" configuration taken during stomatogenesis in some forms, is the major synapomorphy for the group. (Lynn & Strüder-Kypke, 2005; Miao et al., 2004; Shang et al., 2003). Shang and Song, (2005) have successfully used RAPD fingerprinting (PCR-based Randomly Amplified Polymorphic DNA) to identify and separate marine scuticociliate species. Subdivision of the subclass is recognizing three included orders: order Philasterida, order Pleuronematida and order Thigmotrichida. The order Philasterida appears to be strongly supported as a group by molecular phylogenetics (Lynn & Strüder-Kypke, 2005;

Shang et al., 2003). 16 families are included in the order Philasterida: the Cinetochilidae, Cohnilembidae, Cryptochilidae, Entodiscidae, Entorhipidiidae, Loxocephalidae, Orchitophryidae, Paralembidae, Parauronematidae, Philasteridae, Pseudocohnilembidae, Chizocaryidae, Thigmophryidae, Thyrophylacidae, Uronematidae and Urozonidae.

Scientific classification	
Kingdom	Animalia
Phylum	Protozoa, Ciliophora
Subphylum	Intermacronucleata
Class	Oligohymenophorea
subclass	Scuticociliatia
Order	Philasterida
Family	Philasteridae
Genus	Philasterides
Species	***P. dicentrarachi***

Scientific classification	
Kingdom	Animalia
Phylum	Protozoa, Ciliophora
Subphylum	Intermacronucleata
Class	Oligohymenophorea
subclass	Scuticociliatia
Order	Philasterida
Family	Uronematidae
Genus	Uronema
Species	***U. marinum***

Scientific classification	
Kingdom	Animalia
Phylum	Protozoa, Ciliophora
Subphylum	Intermacronucleata
Class	Oligohymenophorea
subclass	Scuticociliatia
Order	Philasterida
Family	Parauronematidae
Genus	Miamiensis
Species	***M. avidus***

Table 2: Classification of scuticociliates

6.2. Morphology of scuticociliates

Noga, 1996 reported that *Uronema marinum* has a characteristic tear drop shape which is narrow anteriorly, rounded posteriorly and the cilia is distributed allover the body. In addition, the closely related and morphologically similar *Miamiensis avidus* has only been reported once from nodular lesions on sea-horses (Thompson & Moewus, 1964). Both Uronema and Miamiensis can live in an aquarium without fish and the pathology is similar. A Uronema-like ciliate has caused disease in cultured Japanese flounder and red sea bream in Japan (Yoshinaga & Nakazoe, 1993).

7. Predisposing factors for scuticociliate infection

Frequent predisposing factors are reported to be key factors for developing of scuticociliatosis among fishes:

1. The oxygen super-saturation sometimes obtained in aquaculture systems may lead to small lesions of the skin, eyes and branchae through which ciliates may penetrate at least during the early stage of infectious outbreaks (Sterud et al., 2000).
2. Stress factors including nutritional, environmental and/or physical conditions. The immunosuppression in the fish is considered as a key factor for developing scuticociliatosis. Munday et al., (1997) discussed both diet and stress immunosuppression. Dragesco et al., (1995) focused on the weakness caused by wound or stress. Environmental stressors like high water temperature, reduced water change and/or overcrowdness were believed to play an essential role for the infection of *Uronema marinum* in a variety of marine water fishes held in aquarium (Cheung et al., 1980). The higher water temperatures to above 20 °C probably caused the high mortalities observed during the summer months; however, other factors could contribute to the high mortalities observed during this period. On the other hand, reduced water change and/or overcrowdness may lead to respiratory distress; resulting in rapid movement of operculum and consequently the scuticociliate can get entry through gills epithelium.
3. Hypo-salinity of the water; the lower salinity conditions can be a key factor for outbreaks of scuticociliatosis in Japanese flounder. The avoiding the use of hypo-

saline seawater may lead to reduction of mortalities of Japanese flounder by scuticociliatosis in aquaculture farms (Takagishi et al., 2009).

4. Primary bacterial infection. Bacterial diseases producing mainly skin lesions like *Flexibacter maritimus* may help to develop a good media for scuticociliate invasion particularly through skin epithelium (data not yet published).

8. Possible causes of death

There are many reports about the different causes of death of the fish due to infection with scuticociliatosis:

1. The accumulation of ciliates in the principal branchial blood vessels and particularly in the capillaries of the secondary lamellae can be expected to have a marked obstructive effect on the lamellar capillary network, hindering uptake and transport of oxygen, so that asphyxia is probably one of the major causes of death in the affected fish (Iglesias et al., 2001; Moustafa et al., 2008).
2. The presence of numerous ciliates in the blood stream during the systemic phase of the infection clearly constitutes a severe problem, since erythrocytes are massively ingested by the ciliates causing hypochromic anaemia as reported in the infection of sea bream by *Philasterides dicentrarachi* (Dragesco et al., 1995).
3. The sloughing of the epidermis induced by the ciliate infection might be a major contributory factor to the death of the sea dragons. (Umehara et al., 2003)
4. Some reports demonstrate that severe ulcerative dermatitis and necrotizing myositis

lesions induced by scuticociliates result in a systemic disturbance of osmolarity and death as the primary function of the skin in fish is osmoregulation and sustaining of a waterproof barrier (Rossteucher et al., 2008).

5. Locomotor dysfunction associated with invasion of the olfactory nerve and severe brain lesions (Munday et al., 1997).
6. Respiratory and excretory dysfunction due to the presence of numerous scuticociliate on gills along with complete necrotisation of gill tissues; resulting in interference in its function as a respiratory and/or excretory organ (Cheung et al., 1980; Dragesco et al., 1995; Jin et al., 2009) .

OBJECTIVE OF THE THESIS

The overall objective of this thesis was to describe the pathological changes associated with scuticociliatosis infection of Japanese flounder (*Paralichthys olivaceus*) which were not described in Japan before. In addition, to clarify the possible neural routes of invasion and possible neural ways of dissemination into the fish body.

***In chapter 1*:**

* The specific aim of the study was to describe the pathological changes associated with scuticociliatosis caused by *Miamiensis avidus* in farmed Japanese flounder (*Paralichthys olivaceus*) in Japan.

***In chapter 2*:**

* The specific objectives were to clarify/identify the following:

- The possible neural invasion routes.
- Possible neural ways of dissemination of the ciliates into the fish body

Through the pathological study of Japanese flounder (*Paralichthys olivaceus*) experimentally infected by the immersion method with a scuticociliate, *Miamiensis avidus*.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications:

- Moustafa, E. M. M., Naota, M., Morita, T., Tange, N. and Shimada, A. (2010). Pathological Study on the Scuticociliatosis Affecting Farmed Japanese Flounder (*Paralichthys olivaceus*) in Japan. *J. Vet. Med. Sci.* **72(10),** 1359-1362.
- Moustafa, E. M. M., Tange, N., Shimada, A. and Morita, T. (2010). Experimental Scuticociliatosis in Japanese Flounder (*Paralichthys olivaceus*) Infected with *Miamiensis avidus*: Pathological Study on the Possible Neural Routes of Invasion and Dissemination of the Scuticociliate Inside the Fish Body. *J. Vet. Med. Sci.* **72(12),** 1557-1563.

CHAPTER 1

Pathological Study on the Scuticociliatosis Affecting Farmed Japanese Flounder (*Paralichthys olivaceus*) in Japan

Abstract

Pathological findings associated with scuticociliatosis in farmed Japanese flounder in Japan are described. Ten moribund fishes, farmed in Tottori Prefectural Fisheries Experimental Station, showed cutaneous ulcers, darkened skin, fin and tail rot, exophthalmia and alterations in swimming behaviour. Histopathological examination revealed severe epidermal degeneration and necrosis, hyperplasia of branchial epithelium, myositis, myelitis, encephalitis associated with heavy accumulation of scuticociliates in the periorbital cavity and optic nerve fiber. Moreover, masses of ciliates were found to feed on the host tissues such as skeletal muscles, gills and brain, causing severe degenerative changes associated with abundant neutrophilic and lymphocytic infiltration.

The current scuticociliate used in the present study was confirmed to be *Miamiensis avidus*; not only on the basis of the morphology of scuticociliate under both light microscopy and electron microscopic but also, on the PCR results obtained by one of the co-authors. Electron microscopy demonstrated that the buccal apparatus of the present suticociliate had three oral polykinetids with membranelles and two paraoral membranes separated by a narrow gap. In addition, the ciliate contained many digestive vacuoles in the posterior part of the body, macronucleus and micronucleus in the center of the body. These findings suggest that the present scuticociliate, *Miamiensis avidus,* is a highly invasive and destructive pathogen infecting Japanese flounder and capable of developing systemic fatal infection and has strong phagocytic activity especially for ingestion of erythrocytes.

KEY WORDS: Histopathology, *Miamiensis avidus*, *Paralichthys olivaceus*, Scuticociliatosis.

1. Introduction

Scuticociliates are free-living in sea-water, and typically scavengers that feed on suspended particulate matters but, under certain circumstances, these ciliates may behave as opportunistic histophagous parasites and actively feed on cells of certain mollusks, crustaceans and fishes, and continue to live and reproduce within the host tissues (Álvarez-Pellitero et al., 2004). Infections by histophagous scuticociliates (scuticociliatosis) have become one of the most important worldwide parasitological diseases in the intensive marine culture of flatfish species including Japanese flounder (*Paralichthys olivaceus*) (Jee et al., 2001).

A variety of scuticociliates have been isolated and/or detected in diseased fish. For example, *Philasterides dicentrarchi* causes scuticociliatosis in turbot (*Scophthalmus maximus*) (Iglesias et al., 2001). Other pathogenic scuticociliates infecting farmed aquatic animals are *Uronema nigricans* in southern bluefin tuna (*Thunnus maccoyii*) (Munday et al., 1997). These ciliates infect the gills, skin, muscles and visceral organs including the intestine, heart and brain. The disease is frequently observed in Japanese flounder, one of the major farmed fish species in Asian countries including Japan, Korea and China. Etiologic agents of the disease include an unidentified scuticociliate (Yoshinaga & Nakazoe, 1993), *Uronema marinum* (Jee et al., 2001), *P. dicentrarachi* (Kim et al., 2004a) and *Miamiensis avidus* (Jung et al., 2005).

In Japan and Korea, outbreaks of scuticociliatosis have frequently occurred in Japanese flounder (Song et al., 2009b). Ototake and Matsusato, (1986) recorded scuticociliatosis in farmed flounders for the first time in Japan. Morphological

observations and small subunit rRNA gene sequences have shown that the causative agent of scuticociliatosis in the flounder is *M. avidus* (Jung et al., 2005; Song et al., 2009b). However, detailed pathology on scuticociliatosis occurring in Japan has not yet been reported. In addition, very little is known about the route through which the ciliate invade the host tissue and the evasive mechanism of the scuticociliate against the host cellular defense response.

The aim of the present study is to describe the pathological changes associated with scuticociliatosis caused by *M. avidus* in farmed Japanese flounder in Japan.

2. Materials and Methods

Ten moribund farmed flounders (total body length ranged from 12 to 17 cm) were obtained from Tottori Prefectural Fisheries Experimental Station in Japan (Table 1). These fishes were collected during an outbreak of scuticociliatosis in July 2005; the mortality of fishes reached 70-80%. Six thousands fishes reared in one indoor reinforced fiberglass tank supplied with 16.5 °C underground water were moved to 75 ton round concrete tank supplied with 22 °C filtered seawater. Then, antibacterial drug was added to the tank as a prophylaxis against *Flexibacter maritimus*. Three days later, the clinical signs of scuticociliatosis began to appear in all ages of the fishes in the tank. The affected fishes showed alteration in swimming behaviour, darkened skin (Figure 1 A),, a dark patch at the base of the operculum, areas of depigmentation and exophthalmia (Figure 1 B), as well as fin and tail rot (Figure 1 C), and skin ulcers surrounded by a whitish halo (Figure 1 D) especially on the abdomen. The entire body of the obtained fishes were preserved in 10% formalin and then transported to Veterinary Pathology laboratory at Tottori University.

After the gross examination, the skin, gills, eyes, brain, anterior spinal cord, muscles and internal organs were collected and fixed in 10% neutral buffered formalin. After fixation, preserved fish/tissues were processed, embedded in paraffin wax by a routine method, and sliced into 2-5 μm sections. Sections were then stained with hematoxylin and eosin (H&E) and examined by light microscopy.

Electron microscopy was performed on the muscle tissues fixed in formalin. The muscles were cut into 1-2 mm^3 cubic blocks. The small blocks were rinsed in 0.1 M

phosphate buffer saline at pH 7.4, postfixed for 1 hour in 1% osmium tetroxide in 0.1 M phosphate buffer at pH 7.4, dehydrated in alcohol, and embedded in epoxy resin. Semithin (1 μm thick) sections were stained with 1% toluidine blue in distilled water to select and locate interesting areas for electron microscopic examination. Ultrathin sections stained with 3 % uranyl acetate in distilled water and Reynolds' lead citrate were examined under JEM-100CX electron microscope (Japan Electron Optical Laboratory, Tokyo, Japan).

3. Results

Histologically, masses of ciliates were found feeding the host tissues, such as muscles, gills and brain, causing focal degeneration and necrosis (Table 1). In the ulcerated skin, losses of epidermal epithelial cells associated with inflammation of the underlying hypodermis and musculature were observed (Figure 2). Degeneration and necrosis of the gill tissue was observed. Scuticociliates along with mononuclear inflammatory cells infiltration were observed in and between the gill filaments and in the gill lamellae; these lesions were associated with hyperplasia of the branchial epithelium. The extensive proliferation of the gill epithelial cells in fusion with the gill filaments was observed with clubbing appearance (Figure 3 A & 3 B). Skeletal muscles showed severe degenerative and necrotic changes together with presence of the scuticociliate in and between the muscle bundles with heavy lymphocytic infiltration (Figure 4 A). Ciliates containing numerous erythrocytes in the cytoplasm (Figure 4 B) were observed in the hemorrhagic lesions in the skeletal muscle. Scuticociliates along with mononuclear cells infiltration was also observed in the lamina propria of the gastrointestinal tract. No inflammatory changes were observed in the liver and spleen. Heavy accumulation of the scuticociliate was observed in the periorbital cavity, optic nerve fiber (Figure 5), meninges, optic lobe in the brain, in the white matter of the spinal cord mainly in the peripheral parenchyma (Figure 6) and in the vertebral canal. Scuticociliates were occasionally observed inside and around the blood vessels in the brain parenchyma.

The observed scuticociliate was generally elongated with a rounded posterior and bluntly pointed anterior end characterized by tear-drop shape (Figure 7 A & 7 B).

Electron microscopy demonstrated that the buccal apparatus of the present scuticociliate had three oral polykinetids with membranelles and two paraoral membranes (Figure 8) separated by a narrow gap. In addition, the ciliate contained many digestive vacuoles (Figure 9) in the posterior part of the body, macronucleus and micronucleus (Figure 10) in the center of the body.

Table 1. Summarized pathological findings in the fishes affected with scuticociliatosis

Case number	Body length (cm)	Macroscopic findings	Histological findings									
				Skin	Gills	Eye	Muscle	Gastro-intestinal tract	Liver	Kidney	Brain	Spinal cord
1	13	Skin ulcers Exophthalmia Dark skin	Lesions	+	+	-	+	+	-	-	+	+
			Parasite	+	+	-	+	+	-	-	+	+
2	12	Fin rot Dark skin Exophthalmia	Lesions	-	+	+	+	+	-	+	+	-
			Parasite	-	-	+	+	+	-	+	+	-
3	13.8	Dark skin Tail rot Depigmentation areas	Lesions	-	+	-	+	-	-	+	-	-
			Parasite	-	-	-	+	-	-	+	-	-
4	12.6	Dark patch at the operculum base Depigmentation areas Exophthalmia	Lesions	+	+	+	+	+	-	-	-	+
			Parasite	+	-	+	+	+	-	-	-	+
5	14	No	Lesions	-	-	-	+	-	-	+	+	-
			Parasite	-	-	-	+	-	-	+	+	-
6	16	Dark skin Exophthalmia	Lesions	-	-	-	+	+	-	-	+	-
			Parasite	-	-	-	+	+	-	-	+	-
7	14.6	No	Lesions	-	+	+	+	+	-	-	-	-
			Parasite	-	+	+	+	-	-	-	-	-
8	15.3	No	Lesions	-	+	+	+	+	-	-	+	+
			Parasite	-	+	+	+	-	-	-	+	+
9	17	Fin rot	Lesions	+	-	-	+	-	-	+	-	-
			Parasite	+	-	-	+	-	-	-	-	-
10	14.5	No	Lesions	-	+	-	+	-	-	-	-	-
			Parasite	-	-	-	+	-	-	-	-	-

4. Discussion

Scuticociliates, previously regarded as exclusively free-living ciliates, have been recognized as a serious pathogen (Cheung et al., 1980). Several species of scuticociliates (*Philasterides dicentrarachi, Uronema marinum* and *Miamiensis avidus*) are known to be histophagus parasites causing severe infections in olive flounder (Jee et al., 2001; Jung et al., 2007; Kim et al., 2004a). All the aforementioned species of scuticociliates can exist in an aquarium rearing no fishes. Pathology of fishes affected with Uronema and Miamiensis are reported to be similar to the present cases (Dyková & Figueras, 1994; Noga, 1996). Moreover, it was reported that *Philasterides dicentrarachi* and *Miamiensis avidus* are conspecific based on careful morphological description and a wide range of reference comparisons (Jung et al., 2007). Although, a detailed morphological study of the present ciliate is beyond the scope of this paper, some interesting findings were observed. The observed scuticociliate was generally elongated with a rounded posterior and bluntly pointed anterior end characterized by tear-drop shape; the morphological features strongly resembled those of the Uronematid ciliates, *Uronema marinum* and *Miamiensis avidus* (Dyková & Figueras, 1994; Noga, 1996). Electron microscopy demonstrated cilia carried by oral and paraoral membranelles and presence of many digestive vacuoles inside the body indicating strong phagocytic activity. Presence of two separated paraoral membranes suggests that this ciliate may belong to either Philasterides or Miamiensis (Álvarez-Pellitero et al., 2004). PCR analysis for the gene of the current ciliate obtained from the fish suggests that the present scuticociliate is *Miamiensis avidus* (data obtained from our coauthor).

In the present fishes, macroscopic skin lesions including cutaneous ulcers and dark patches were observed; these findings are similar to those reported before in turbot (Iglesias et al., 2001), Japanese flounder (Jin et al., 2009; Jung et al., 2007) and in silver pomfret in Kuwait (Azad et al., 2007). Exophthalmia has not been reported in Japanese flounder before.

In the present study, histological analysis of the affected fishes showed masses of ciliates feeding on the host tissues such as muscles, gills and brain, causing degenerative and necrotic changes. Severe epidermal degeneration and necrosis, myositis, encephalitis and myelitis were the most frequent changes observed in the present study. These histological findings are in accordance with those reported in marine fishes (Cheung et al., 1980), turbot (Dyková & Figueras, 1994), Japanese flounder (Jee et al., 2001; Jin et al., 2009; Jung et al., 2007) and in silver pomfret (Azad et al., 2007); skeletal muscle was the most severely damaged tissue out of all tissues affected suggesting that the muscle is the most predisposed organ to the infection (Cheung et al., 1980). The research results indicating that the scuticociliates invade the musculature and internal organs (Cheung et al., 1980; Bassleer, 1983; Gill & Calinan, 1997; Munday et al., 1997; Iglesias et al., 2001; Lee et al., 2003; Azad et al., 2007) suggest on the seriousness of scuticociliate infestations in fish. Ciliates containing numerous erythrocytes in the cytoplasm were observed in the hemorrhagic lesions in the skeletal muscle (Azad et al., 2007; Jee et al., 2001). Heavy accumulation of scuticociliates in the periorbital cavity and optic nerve fiber was also observed in this study.

Environmental stress is believed to play an essential role for the occurrence of scuticociliatosis in a variety of marine water fishes held in aquarium (Cheung et al.,

1980). Stress factors including sudden increase in the water temperature and/or antibiotic exposure may be considered as triggers for the present outbreak of the scuticociliatosis (Cheung et al., 1980; Munday et al., 1997).

The presence of ciliates in the gill, skin, skeletal muscle and blood vessel suggests that the initial route of entry of the parasite could be the skin and/or gill lesions and its subsequent spread into the body was perhaps *via* blood vessels (Iglesias et al., 2001; Jin et al., 2009; Paramá et al., 2003; Sterud et al., 2000). Scuticociliate may also enter through the periorbital route and migrate through the optic nerve to the brain (Paramá et al., 2003); this would explain the occurrence of the severe brain lesions in association with exophthalmia with abundant ciliates in the periorbital cavity in this study. High densities of scuticociliates observed in brain and peripheral parenchyma of the spinal cord together with accumulation of scuticociliate in the periorbital cavity (Figure 11); this observed result enabled us to focus on the significance of neural routes as possible invasion routes.

Based on these pathological findings, it is elucidated that the present scuticociliate is a highly invasive and destructive pathogen to the host tissue and the scuticociliate could achieve rapid expanse inside the host organs and tissues.

Figures and figure legends

Figure 1: **A**. Naturally infected fish showing darkened skin (a consolidated arrow). Skin; Fish No. 2.

Figure 1: **B**. Skin shows a dark patch at the base of the operculum (a consolidated arrow), areas of depigmentation (an unconsolidated arrow) and exophthalmia (an arrow head). Skin; Fish No. 4.

Figure 1: **C.** Naturally infected fish showing tail rot (a consolidated arrow). Tail, Fish No. 3.

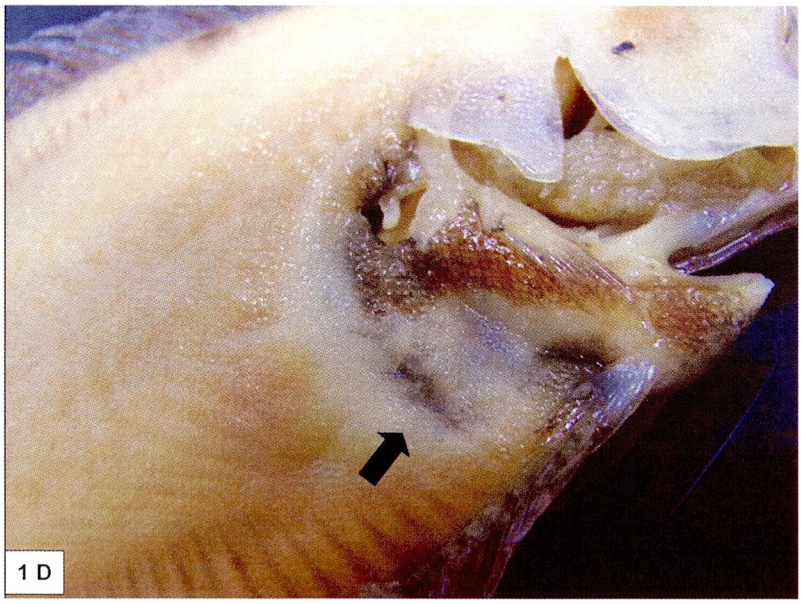


Figure 1: **D.** Skin ulcer (a consolidated arrow) in the abdomen surrounded by a whitish halo. Skin; Fish No. 1.

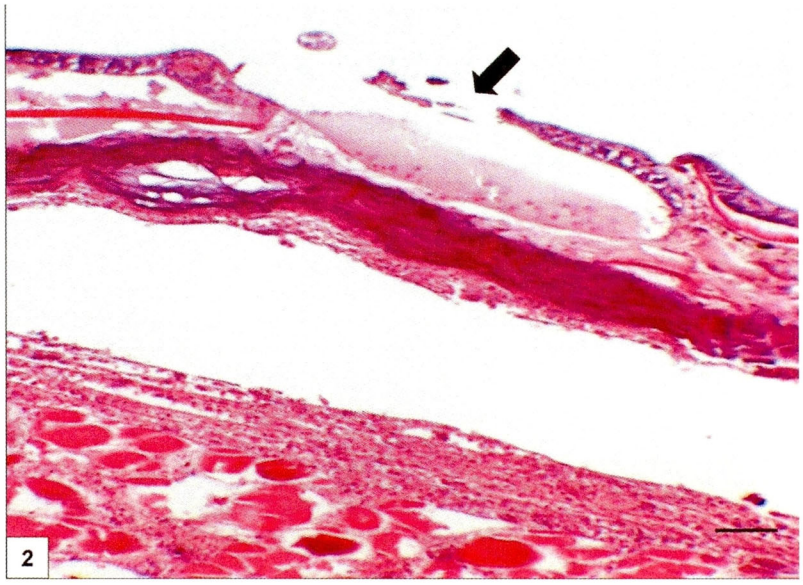


Figure 2: Loss of epidermal epithelial cells (an arrow) with inflammation of the underlying hypodermis and musculature. Ulcerated skin, Fish No. 1, Bar = 60 μm.

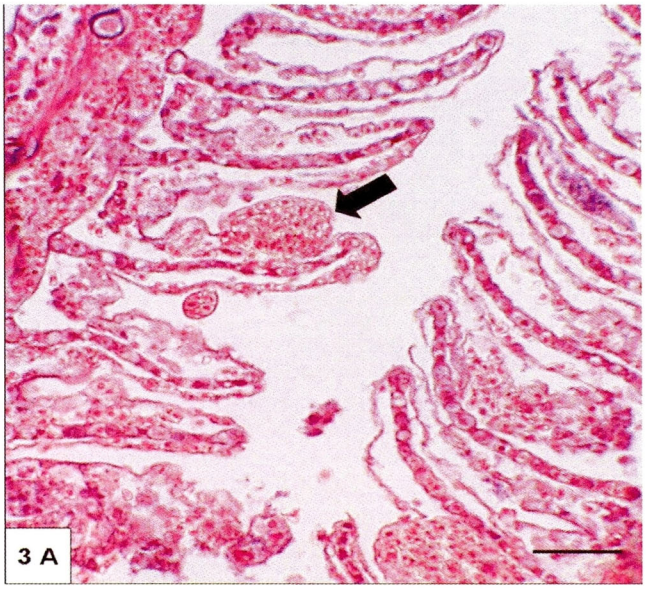


Figure 3 A: Degeneration and hyperplastic changes with clubbing appearance (an arrow) of the branchial epithelium. Gill, Fish No.1, Bar = 30 μm.

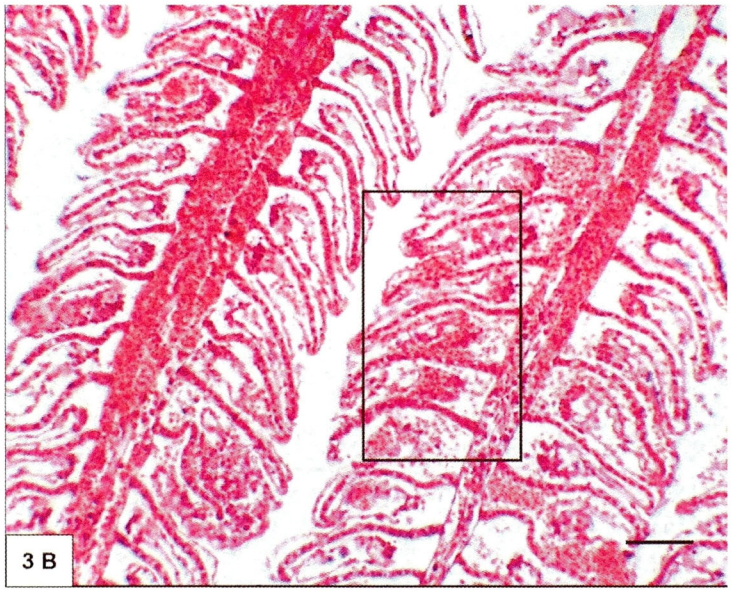


Figure 3 B: Necrotic and degenerative changes in both the gill filament and gill lamellae (inside the rectangular) of the branchial epithelium. Gill, Fish No.1, Bar = 30 μm.

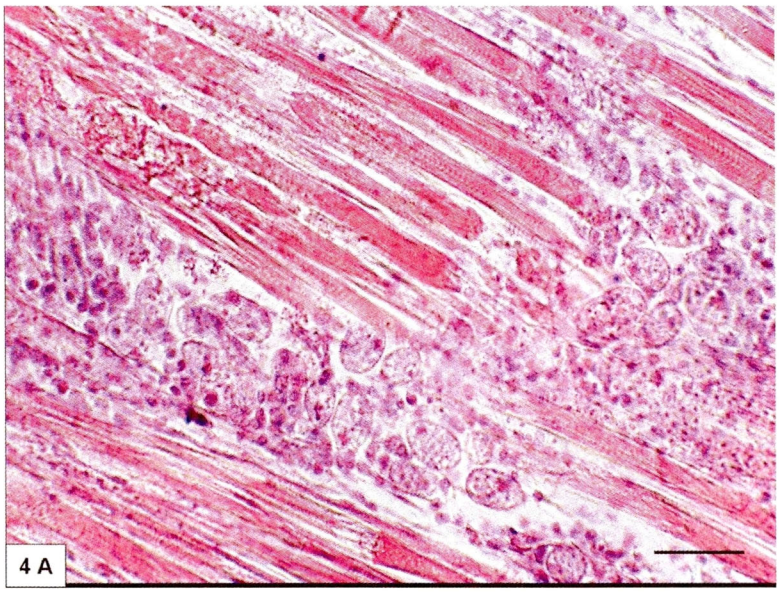


Figure 4: A. Severe degeneration and necrosis of the striated muscle and presence of scuticociliates in and between the muscle bundles. Muscles, Fish No. 7. Bar = 30 μm.

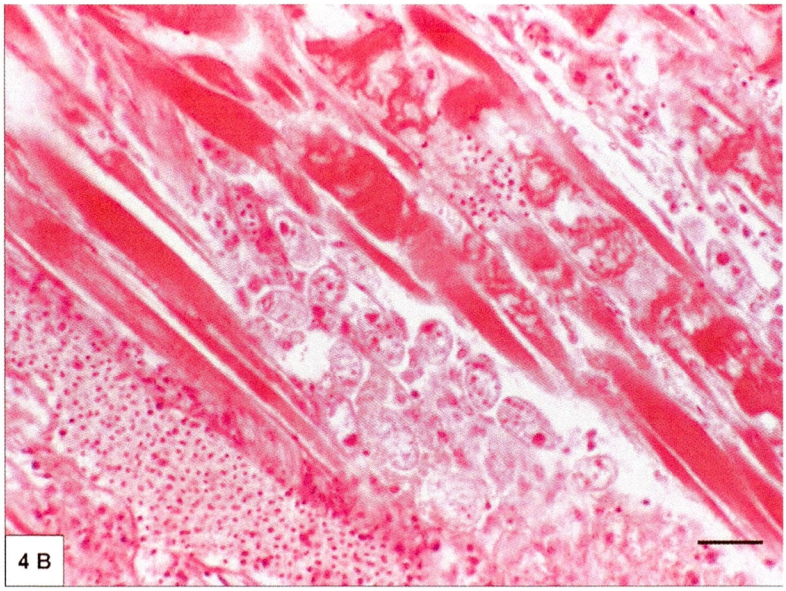


Figure 4: B. Scuticociliates containing engulfed red blood cells. Muscles, Fish No. 7. Bar = 30 μm.

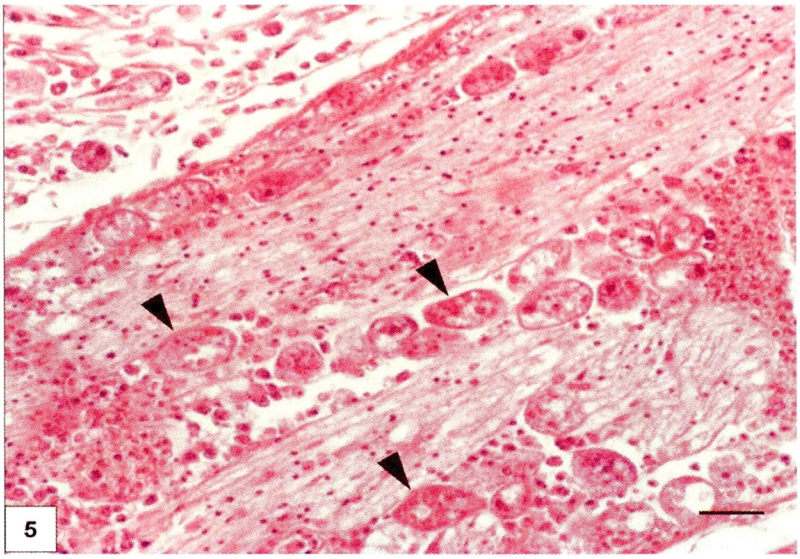


Figure 5: Scuticociliates (arrow heads) associated with inflammatory cell infiltration are observed inside the optic nerve fiber. Optic nerve, Fish No. 4, Bar = 30 μm.

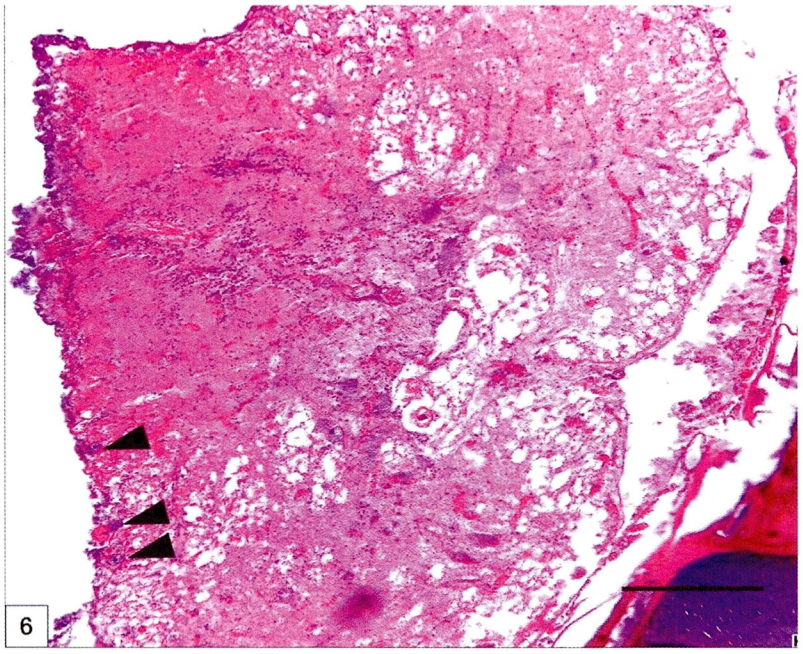


Figure 6: Scuticociliates (arrow heads) are observed in the spinal cord. Anterior spinal cord, Fish No. 4, Bar = 200 μm.

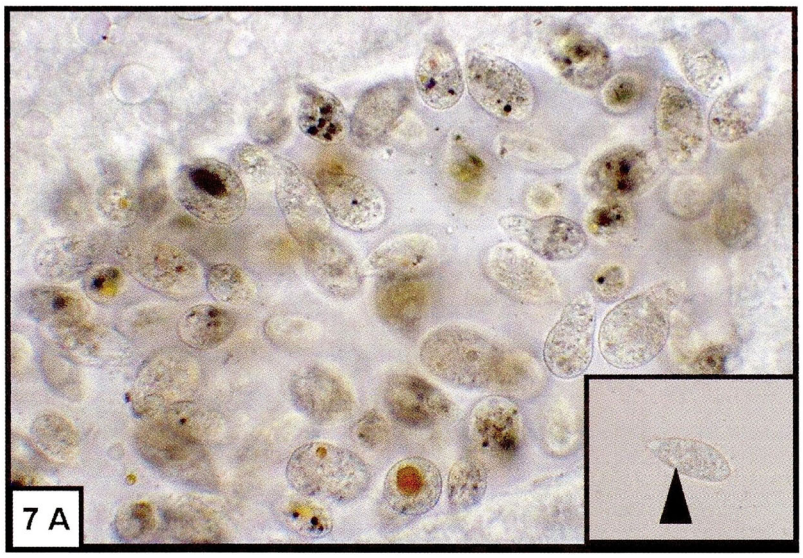


Figure 7: A. Skin smear showing high number of movable scuticociliates.

Inset: The scuticociliate (arrow head) morphology; tear drop shape with cilia surrounding the whole body.

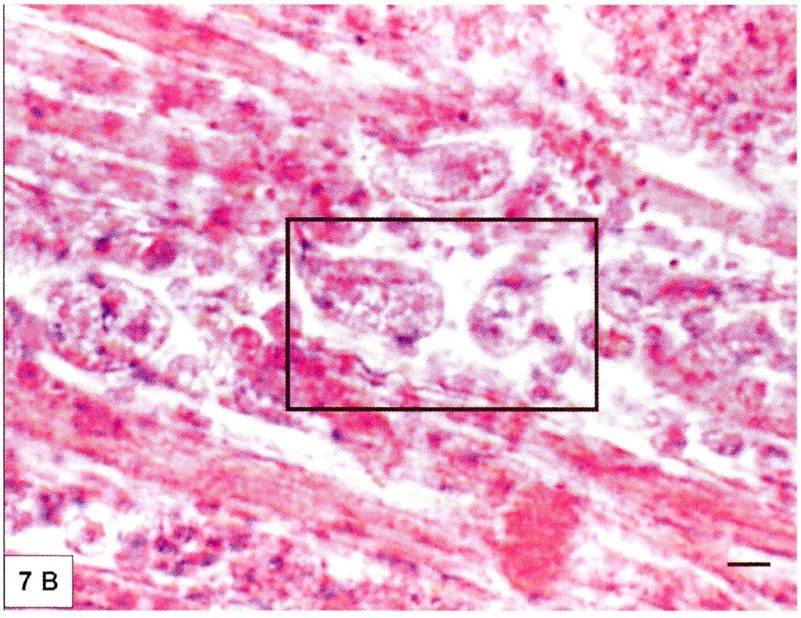


Figure 7: B. The scuticociliate (inside rectangular) morphology inside the muscle tissues (tear drop shape). Muscle. Bar= 30 μm.

Figure 8: Electron micrograph showing three oral polykinetids with membranelles (M1, M2, M3) and two paraoral membranes (PM1, PM2) of the present scuticociliate. Bar = 4 μm.

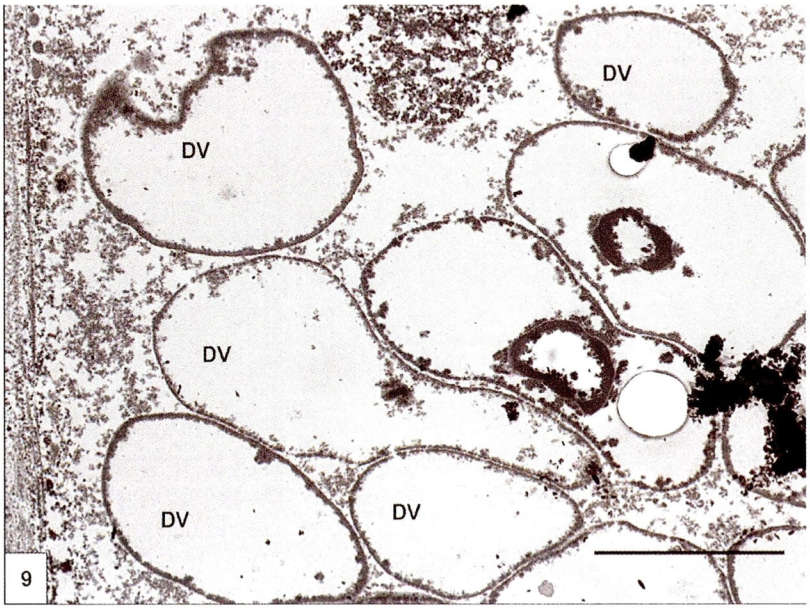


Figure 9: Electron micrograph showing many digestive vacuoles (DV) contained within the ciliate. Bar = 3 μm.

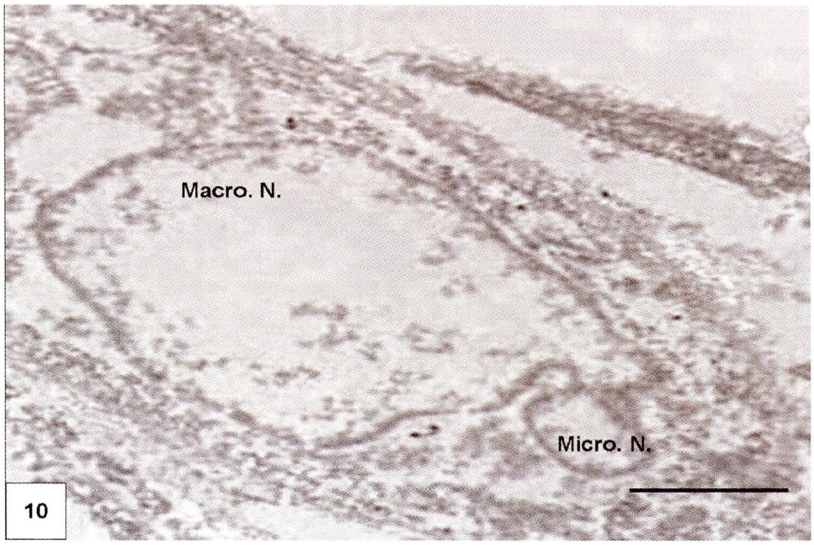


Figure 10: Electron micrograph showing big macronucleus (Macro. N.) and small micronucleus (Micro. N.) contained within the center part of the ciliate body. Bar = 3 μm.

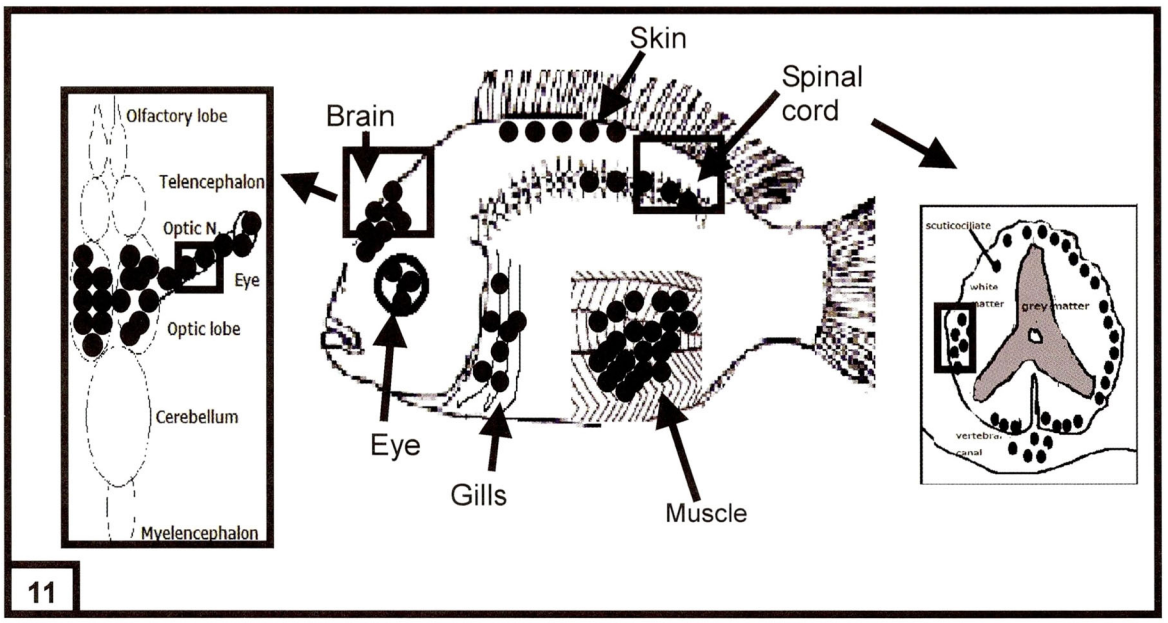


Figure 11: Schematic diagram showing scuticociliate (dark black circle) distribution inside the fish tissue with special clarification about sites of distribution in the brain and spinal cord of the infected fish.

CHAPTER 2

Experimental Scuticociliatosis in Japanese Flounder (*Paralichthys olivaceus*) Infected with *Miamiensis avidus*

Pathological Study on the Possible Neural Routes of Invasion and Dissemination of the Scuticociliate Inside the Fish Body

Abstract

Japanese flounder (*Paralichthys olivaceus*) were experimentally infected with the highly pathogenic scuticociliate *Miamiensis avidus* (syn. *Philasterides dicentrarachi*) using the immersion method to clarify/identify the possible neural routes of entry and ways of dissemination of the scuticociliate in the fish body. Scuticociliates were observed on the skin and gills right from day 0-1 post-infection, muscle tissue on day 2 post-infection, reached the brain, and spinal cord from day 3 post-infection and systemic infection was prominent afterwards. Systemic infection was noted in 30% of the fish population; however, brain lesions were observed in most of the examined fish. Cumulative mortalities initially increased gently to reach 16% on the day 5 post-infection, however, suddenly leaped from the next day, and the cumulative mortality of the infected fish rose up to approximately 80% until day 10 post-infection and were significantly high when compared with control group. This sudden increase in mortalities was considered to be due to severe brain lesions, which appeared frequently from day 3 and day 4 post-infection. Affected fish showed varying degrees of tissue damage including severe epidermal and dermal necrotic lesions, necrotic myositis, encephalitis and myelitis. Whereas, scuticociliates were frequently observed along the optic and/or olfactory nerve in the fish which were accompanied by severe brain lesions but by minimum lesions in the gills and skin, suggesting that in addition to skin and/or gills, neural routes including periorbital and nasal routes may play a role in scuticociliate invasion to the brain. Scuticociliates were also observed in the peripheral nerve fibers in the muscle tissue, cranial and spinal nerves, cranial cavity and in the vertebral canal, suggesting that nerve

fibers and/or cerebrospinal fluid circulation may be involved in the spread of the scuticociliate throughout the body in addition to the blood circulation and connective tissue.

Keywords: Experimental infection, Japanese flounder, *Miamiensis avidus*, Neural routes, Scuticociliatosis.

1. Introduction

Scuticociliates are free-living organisms in sea-water, feeding on suspended particulate matters (bacteria, microalgae & protozoa). Under certain circumstances, however, these ciliates may behave as opportunistic histophagous parasites, and actively feed on cells and tissue residues of certain mollusks, crustaceans and fishes, and continue to live and reproduce within the host tissues (Elston et al., 1999). Several scuticociliate species have been reported as agents to cause scuticociliatosis in farmed marine fish; species of *Philasterides dicentrarachi* infects sea-bass (Dragesco et al., 1995), and population of sea dragons (Rossteuscher et al., 2008), *Uronema nigricans* species infect bluefin tuna (Munday et al., 1997), and *Uronema marinum* infects many species of aquarium fishes (Cheung et al., 1980).

In Korea, scuticociliatosis in cultured Japanese flounder (*Paralichthys olivaceus*) was reported to be due to *Uronema marinum* (Jee et al., 2001), *Philasterides dicentrarachi* (Kim et al., 2004a), *Pseudocohnilembus persalinus* (Kim et al., 2004b) and *Miamiensis avidus* (Jung et al., 2005). It was suggested that *Miamiensis avidus* is the main cause of scuticociliatosis in Japanese flounder in a series of reports (Jung et al., 2005; Jung et al., 2007; Song et al., 2009a).

Recently, a successful experimental immersion infection of Japanese flounder (*Paralichthys olivaceus*) by *Philasterides dicentrarachi* was performed in Korea. It was suggested that *P. dicentrarachi* is a strong pathogen that can cause a primary infection in flounder by penetrating the gills and skin, followed by its travel *via* the blood stream to the other parts of the body (Jin et al., 2009). Jung et al., (2007) demonstrated that *M.*

avidus successfully invades the host directly from seawater, and causes high mortality. The ciliates rapidly invade and proliferate in the skin and gills, and spread to the internal organs in the absence of any additional pathogens such as secondary bacterial invaders. These reports demonstrated abraded skin and/or gills as the main routes of entry and blood vessels as the dissemination route (Jung et al., 2007; Jin et al., 2009). Based on the results from the experimental infection study by intracoelomic injection of the ciliates to the turbot, Puig et al., (2007) suggested the connective tissue as a possible quick way of dissemination of the scuticociliate to the body.

Munday et al., (1997) proposed that the scuticociliate *Uronema nigricans* entered its host blue fin tuna by the nasal route; the starting point of infection was traced to the olfactory rosettes, from which the parasite moved up the olfactory nerve to the brain, leading to locomotor dysfunction and final death. Experimental infections by *Philasterides dicentrarachi* using different routes were successfully attempted in turbot by Paramá et al., (2003); after periorbital inoculation of *P. dicentrarachi*, all fish died within 5-13 days post-inoculation and the infection lesions ware largely localized in brain (100% of the fish). Brain infection was observed in all the fish together with the microscopically confirmed presence of ciliates in the optic nerve, suggesting that *P. dicentrarachi* is able to reach the brain not only via the blood stream but also via nervous tissues (Paramá et al., 2003).

In Japan, though the cause of the outbreak of scuticociliatosis in Japanese flounder (*Paralichthys olivaceus*) was considered to be unidentified ciliate (Yoshinaga & Nakazoe, 1993) for many years, it has recently become clear that the main cause of the scuticociliatosis is *Miamiensis avidus* as demonstrated in our previous study (Moustafa et

al., 2010). Exophthalmia, protrusion of eye ball, was detected in many affected fish accompanied by brain lesions; the findings enabled us to focus on the significance of the neural routes of entry and dissemination of the scuticociliate.

The aim of this study is to clarify/identify the possible neural invasion routes and possible ways of dissemination of the ciliates in the fish body through the pathological study of Japanese flounder (*Paralichthys olivaceus*) experimentally infected by immersion method with a scuticociliate, *Miamiensis avidus*.

2. Materials and Methods

2. 1. Ciliates isolation and cultivation

Scuticociliates were isolated from the brain of naturally infected Japanese flounders reared at Tottori Prefectural Fisheries Experimental Station in Japan during an outbreak of the disease in 2005. A small piece of the brain of Japanese flounder containing active ciliates was inoculated into Epithelioma Papillosum Cyprini (EPC) cells previously cultured in Minimum Essential Medium (Invitrogen), supplemented with 3% fetal bovine serum and 1% Antibiotic-Antimycotic, liquid (Invitrogen, Tokyo, Japan). Scuticociliates multiplied with EPC were then kept at 25 °C for a period of overnight to 3 days. For subculture, 1 μl of the culture supernatant including the scuticociliates was added to 10 ml of Yeast Extract Horse Serum liquid medium [2% 'Lab-lemco' powder (Oxoid, Tokyo, Japan), 0.5% Bacto^{TM} Yeast Extract (Difco 0.5% glucose, 0.8% sodium chloride and 5% inactivated horse serum)] (personal communication), and incubated at 18 °C. The scuticociliates were sub-cultured and maintained in the medium, and the concentration of the scuticociliate in the culture medium was approximately 10^5 cells/ml.

The isolates were morphologically and genetically identified as *Miamiensis avidus*, and classified serologically, using immobilization assays and Western blotting, into "serotype I" and it was named JF05To (Song et al., 2009b).

2. 2. Aquarium

In the present study, 11 acrylic tanks of 30 L capacity containing seawater, filtered with cartridge filter (0.5 μm, Millipore, Tokyo, Japan), were employed. Each of these tanks contained one perforation at the upper part of a side wall for water drainage. A net was attached to the perforation in the tank to prevent the outflow of the young fish.

2. 3. Fish

All fish used in the experiment (143 young fry of Japanese flounder of total length: 27.7 mm) were reared until the 94th day after hatchering in the institution controlled under the same conditions as those in the laboratory in Tottori Prefectural Fisheries Experimental Station in Japan. Compound fish feeds were fed to the fish fries. Two to three days before initiation of the experiment, fish were stopped feeding. Fifteen randomly selected fish were examined under a stereo microscope before necropsy to ensure that they were free from the parasites in the brain, gills, muscle and epidermal skin mucus. Fish were divided into 11 experimental groups and each group was reared in a separate tank. The first 10 groups contained 13 (Tank 1), 10 (2), 10 (3), 10 (4), 10 (5), 15 (6), 15 (7), 15 (8), 15 (9) and 20 (10) fish, respectively and the 11th group contained 10 fish as the negative control. The fish in each tank were sampled in the manner to ensure the minimum number for the investigation and to avoid an intentional selection of weakened individuals. Because of the predicted increased risk of mortality of the fish associated with the progress of the infection, the initial numbers of fish in latter tanks

were increased so that the total number of the fish examined in each tank would be equal (Miwa & Nakayasu, 2005).

All tanks were placed in a big water bath provided with a titanium heater to maintain a steady temperature of 20 °C. Water in the fish tanks was slightly aerated constantly during the experiment.

2. 4. Experimental infection

On the first day of infection experiment, each tank was filled with 10 L of filtered seawater and fish were released into the tanks in numbers stated above.

10 ml of the culture medium holding 10^6 cells of the scuticociliate was centrifuged at 3,500 rpm for 10 min at room temperature. For preparation of concentrate of the ciliates, the supernatant was discarded and the sediment was suspended with 1 ml of filtered seawater. Ten equal concentrates of the scuticociliates were prepared for immersion. These concentrates were added to each of the tanks from tank 1 to 10. The concentration of the ciliate in the immersion tanks was approximately 100 cells/ml. At 24 hrs later, a further 20 L of filtered seawater were added to each tank. From the next day onwards, half of the water of each tank was removed and replaced by 15 L of filtered seawater on a daily basis. The fish were observed for maximally ten days after the infection without feeding.

Just 30 minutes after the start of immersion (infection), five live fish were sampled from tank 1. From the first day to the tenth day after the infection, five live fish were sampled from each of tank 1 to 10 on each day and examined for the possible neural routes of entry and ways of dissemination of the ciliates (Table 1). The fish were checked

from the surface to the interior of the body using stereo microscope before necropsy in order to examine the evidence of infection, the infested part with the scuticociliate and the dissemination routes. Fish were euthanatized with an exposure of eugenol [Fish/Crustacea anesthetic FA100 (TANABE SEIYAKU, Co Ltd, Osaka, Japan)]. All dead fish of each tank were sampled upon confirmation of the death without consideration about the duration after the infection. All the sampled fish, live and dead, were fixed in Davidson's solution (330 ml of 95% ethanol, 220 ml commercial formaldehyde solution containing 35% formaldehyde and 8% methanol, 115 ml glacial acetic acid and 335 ml distilled water). After one week, Davidson's solution used for fixation was switched to 75% ethanol solution.

2. 5. Histopathology

After the gross examination, all fish stored finally in 75% ethanol, previously passed through the fixation by Davidson's solution, were transported to the laboratory of Veterinary Pathology of Tottori University. The preserved fish were cut into transverse sections, dehydrated and embedded in paraffin wax. Subsequently, 2-5 μm sections were stained with hematoxylin & eosin (H&E) for light microscopy.

2. 6. Calculation of cumulative mortality

In the present experiment, cumulative mortality was calculated by summation of the number of daily dead fish. The significance of cumulative mortality was calculated using F test.

3. Results

3. 1. Cumulative mortality

Cumulative mortalities of fish infected with *Miamiensis avidus* by immersion are shown in Figure 1. Cumulative mortality initially increased gently to reach 16% on the 5th day since infection (day 5 post-infection). The number of death, however, suddenly leaped from the next day followed by rise of the cumulative mortality up to approximately 80% until day 10 post-infection. Pathological examination of the dead fish showed frequent severe brain lesions without severe lesions in the other tissues and organs. The scuticociliate infected groups showed significantly higher mortalities ($P < 0.005$) than the control group.

The cumulative mortality of control group reached 30% on day 10 post-infection. Few fish in the control group were found to be infected individually with ciliates, which were confirmed as *Miamiensis avidus* negative by PCR (data prepared for submission).

3. 2. Clinical symptoms

Bleached spots on the skin and dermal necrotic lesions were the first observable clinical symptoms, which appeared just 30 minutes after the infection. Scuticociliates were observed on the skin and fins from day 1 post-infection, and detected to spread to the organs as follows; gills, eyes, brain and spinal cord from day 3 post-infection; nostrils from day 4 post-infection; lips and mouth from day 8 post-infection (data not shown).

3. 3. Scuticociliates dissemination inside the body

Results of the histological detection of *Miamiensis avidus* organism in the experimentally infected fish are summarized in Table 1. The first ciliate infection was observed in the skin and gills in individuals of the group sampled on day 0 (30 minutes after the immersion). High densities of scuticociliates were observed in the epidermis and gill mucosal epithelium. On day 1 post-infection, the number of fish showing histological evidence of infection and scuticociliates densities within the affected areas remained unchanged. There was an increase in the severity of the lesions as demonstrated by the extensive epidermal damage associated with epidermal ulcer. Then, the scuticociliates gradually disseminated within the entire body and appeared in subcutaneous connective tissue, muscle tissue and peripheral nerve endings on day 2 post-infection, in the periorbital cavity surrounding the eye, optic nerve, brain, spinal nerve and spinal cord on day 3 post-infection, in the nasal cavity disseminating into the olfactory nerve towards the brain from day 4 post-infection and in the gastrointestinal tract, liver and kidney from days 4 and 5 post-infection onwards. Since then, the infection was generalized and systemic infection was noted in 30% of the fish population; brain infection was observed in most of the daily examined fish. The scuticociliates distribution became more evident by both ciliate density and numbers of the lesions in the affected organs and tissues.

Table 1. Histological detection of experimentally infected fish with *Miamiensis avidus* by immersion method

Tissue		Days post-immersion										
		0	1	2	3	4	5	6	7	8	9	10
Skin	Scuticociliate	+	+	+	+	+	+	+	+	+	+	+
	No. of fish/5	5	5	5	5	5	5	5	5	4	4	3
Gills	Scuticociliate	+	+	+	+	+	+	+	+	+	+	+
	No. of fish/5	3	3	2	2	3	3	5	5	3	3	2
Muscle	Scuticociliate	-	-	+	+	+	+	+	+	+	+	+
	No. of fish/5	0	0	4	5	5	5	5	5	5	5	5
Eye	Scuticociliate	-	-	-	+	+	+	+	+	+	+	+
	No. of fish/5	0	0	0	3	3	3	3	3	2	3	2
Brain	Scuticociliate	-	-	-	+	+	+	+	+	+	+	+
	No. of fish/5	0	0	0	4	4	4	5	5	4	4	3
Nasal cavity	Scuticociliate	-	-	-	-	+	+	+	+	+	+	+
	No. of fish/5	0	0	0	0	1	3	5	5	3	3	2
Spinal cord	Scuticociliate	-	-	-	+	+	+	+	+	+	+	+
	No. of fish/5	0	0	0	4	4	4	5	5	4	4	3
Gastro-intestinal tract	Scuticociliate	-	-	-	-	+	+	+	+	+	+	+
	No. of fish/5	0	0	0	0	3	3	3	3	3	2	2
Liver	Scuticociliate	-	-	-	-	-	+	+	+	+	+	+
	No. of fish/5	0	0	0	0	0	3	3	3	2	2	1
Kidney	Scuticociliate	-	-	-	-	-	+	+	+	+	+	+
	No. of fish/5	0	0	0	0	0	4	4	3	3	2	2

3. 4. Histopathology

Histopathological examination on day 1 post-infection revealed extensive epidermal destruction, subsequent dermal degeneration with lymphocytic, monocytic and eosinophilic granular cell infiltration associated with the presence of a high number of the scuticociliates (Figure 2 A & 2 B). On day 2 post-infection, there was liquifactive necrosis in the muscles (Figure 3) together with the scuticociliates invading underneath

the epidermal layer and in the subcutaneous connective tissue. The scuticociliates were also observed in the nerve bundles (Figure 4 A) and nerve fiber endings (Figure 4 B) in the muscle tissues affected. On day 3 post- infection, severe infection of the scuticociliates was observed in the periorbital cavity (Figure 5 A), eye, optic nerve accompanied by inflammatory cells infiltration (Figure 5 B). The scuticociliates were also observed in the brain (Figure 6), spinal cord, spinal nerve (Figure 7) and in the vertebral canal (Figure 8). From day 4 onwards, the scuticociliates were observed in the blood vessels of a variety of tissues and/or organs (Figure 9), nasal cavity, along the olfactory nerve and in the olfactory lobe, where severe inflammatory changes were detected (Figure 10 A & 10 B) and third ventricle. Since then, the infection became more systemic and scuticociliates were observed in the lamina propria and lamina muscularis of the gastrointestinal tract (Figure 11), and in the abdominal cavity (Figure 12). Periglomerular, peritubular and perivascular mononuclear inflammatory cells infiltration along with the presence of the scuticociliates inside blood vessels were observed in the kidney. From the Day 6 onwards post-infection, many scuticociliates containing red blood cells in the cytoplasm were observed in the gills, skeletal muscles, skin and brains of the infected fish.

4. Discussion

Miamiensis avidus is a highly invasive and destructive histophagous endoparasitic scuticociliate infecting Japanese flounder. In the present study, an experimental infection of Japanese flounder was successfully achieved by immersion method to clarify/identify the possible neural routes of entry and ways of dissemination of the scuticociliate into the fish body.

After daily sampling the five alive fish, density between tanks used for current immersion experiment became slightly different. This might have an impact on the pathogenicity. However, much attention was paid to clarify/identify possible additional neural routes of entry and ways of dissemination of the scuticociliate in the host tissue. The same experimental design was used by Miwa and Nakayasu, (2005).

The cumulative mortality of control group reached 30% on day 10 post-infection. This might be due to some stress factors, lack of nutrients, and/or some infectious agents; either bacteria or parasite. Some fish were found to be infected individually with ciliates, which were PCR negative for *Miamiensis avidus* (data prepared for submission). However, the scuticociliate infected groups showed significantly higher mortalities ($P < 0.005$) than the control group.

In the present study, the scuticociliates were firstly observed in the dermal and/or branchial epithelium from day 0 post-infection and then disseminated to different body tissues, suggesting that the first possible invasion route may be through the dermal and/or branchial epithelium (skin and/or gill) (Dyková & Figueras, 1994; Paramá et al., 2003; Jung et al., 2007; Jin et al., 2009). Whereas, from Day 3 post-infection, high densities of

scuticociliates were frequently observed in the periorbital cavity, along the optic nerve and in the optic lobe in the brain. From Day 4 post-infection, high densities of scuticociliates were frequently observed in the nasal cavity, along the olfactory nerve and in the olfactory lobe in the brain which were accompanied by severe brain lesions but by minimum lesions in the gills and skin suggesting that optic nerve and/or olfactory nerve (the periorbital and nasal routes) may play a role in the current scuticociliate invasion to the brain (Paramá et al., 2003 and Munday et al., 1997). Heavy accumulation of scuticociliates in the cranial canal in the brain might suggest the dissemination of the scuticociliate through cerebrospinal fluid. On the other hand, on day 3 post-infection, heavy accumulation of scuticociliates could be observed in the spinal nerves, white matter of the spinal cord especially in the peripheral parenchyma and in the vertebral canal; suggesting that peripheral nerve fibers and spinal nerves are considered ways of dissemination of the current scuticociliate into the host tissue.

Extensive epidermal damage, dermal degeneration, hyperplasia of branchial epithelium, necrotic myositis, encephalitis and myelitis were observed in the present study; these changes are in agreement with previous studies on the pathology of the outbreaks and experimental cases of scuticociliatosis in fish (Iglesias et al., 2001; Jung et al., 2005; Jung et al., 2007; Azad et al., 2007; Jin et al., 2009). Systemic infection was noted in 30% of the fish population; brain infection was, however, observed in most of the daily selected fish examined, suggesting that the brain is one of the most susceptible organs. Pathological examination of the dead fish taken from day 6 post-infection onwards, when sudden leap in mortality detected, showed extensive damage in the brain without severe lesions in the other tissues and organs. These findings suggest the

significance of the brain lesions as the cause of death of the infected fish.

Blood stream has been regarded as a major way of dissemination into the other tissues (Paramá et al., 2003; Sterud et al., 2000; Jung et al., 2007; Ramos et al., 2007 and Jin et al., 2009). In addition, the connective tissue was previously suggested as a possible quick way of dissemination of the scuticociliate to the body (Puig et al., 2007). In the present study, the scuticociliates were observed in and along the peripheral, spinal and cranial nerve fibers, cranial cavity and in the vertebral canal, suggesting that the ciliate could spread from the entry sites not only through blood stream and connective tissue but also through nerve fibers and/or cerebrospinal fluid circulation (Figure 13).

Currently it is not known as to how exactly *Miamiensis avidus* naturally invades the host. Results of the present study suggested that this ciliate penetrates the surface membrane of host cell to achieve entry into the host and subsequently manages rapid dissemination throughout the body. Therefore, it is thought that the metabolism of membrane phospholipids plays an important role in host cell penetration of parasite (Seo et al., 2005). Some important virulent factors involved in the invasion process were suggested; in the affected fish, the first skin lesions were noticed early after infection that grew large, perhaps as a result of the production of toxin-like eicosanoids (particularly prostaglandins) secreted by the scuticociliate (Jin et al., 2009). Eicosanoids enhance the pro-inflammatory responses and may even play an important role in penetration, immune-suppression, inflammation, parasitic invasion and establishment within the body of the fish (Daugschies & Joachim, 2000). Also, it was previously demonstrated that *Philasterides dicentrarachi* produces a cysteine-proteinase type toxin which play a role in pathogenesis; including their involvement in invasion of the host by parasite migration

through tissue barriers, degradation of hemoglobin and other blood proteins, destruction of mediators of immune responses (Paramá et al., 2007), activation of inflammation (McKerrow et al., 2006) and the degradation of host proteins during the course of feeding (Rosenthal, 1999). Another factor that could have contributed to the severity of the infestation is the ability of the parasite to produce protease. Proteases have been shown to play important roles in host-tissue invasion, digestion of host proteins, and protection against immunological attacks by the host (McKerrow, 1989; McKerrow et al., 1993; North, 1992). For example; *Uronema marinum* has been known to produce metalloproteases that have high potentials for destroying the host tissue (Lee et al., 2003; kwon et al., 2003). The functions of metalloproteases in the pathogenesis and in the degradation of the host tissue have been well documented for *Cryptobia salmositica*, a haemoflagellate parasite of fishes (Zuo & Woo, 1997a, b, 1998a, b, 2000). Because *Miamiensis avidus* has been suggested to be synonym of *Philasterides dicentrarachi* (Paramá et al., 2006; Jung et al., 2007), it is thought that it might produce that enzyme, cysteine-proteinase, and various symptoms that appeared as a result of infection related to its role in nature of protein degradation.

Presence of scuticociliates ingesting red blood cells in the skin, gills and muscles, supports the hypothesis that once the parasite breaks the skin-blood barrier, it can enter into the internal organs through blood stream, as has been previously hypothesized (Paramá et al., 2003; Sterud et al., 2000; Jung et al., 2007; Ramos et al., 2007; Jin et al., 2009). Besides, in the present study, it was proved that the current ciliate, *Miamiensis avidus*, has a strong phagocytic activity; represented first by cilia allover its body which help to capture erythrocytes and engulf it, and second by many digestive vacuoles

observed inside the body of the ciliate by electron microscopy. The presence of scuticociliates in the bloodstream, nerve bundles, nerve fiber endings, and/or in perivascular and perineural connective tissues hypothesized that *Miamiensis avidus* uses these tissues as routes for accessing almost all organs of the body.

Figures and figure legends

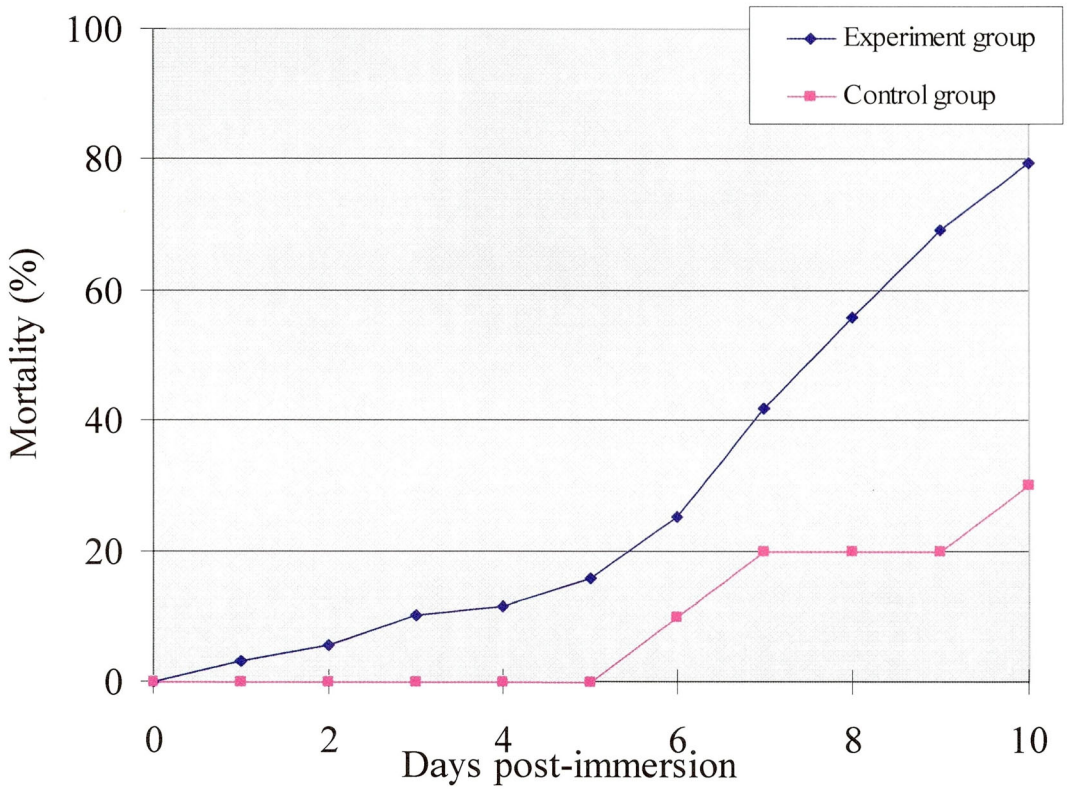


Figure 1. Cumulative mortality of fish infected with *Miamiensis avidus* by immersion.

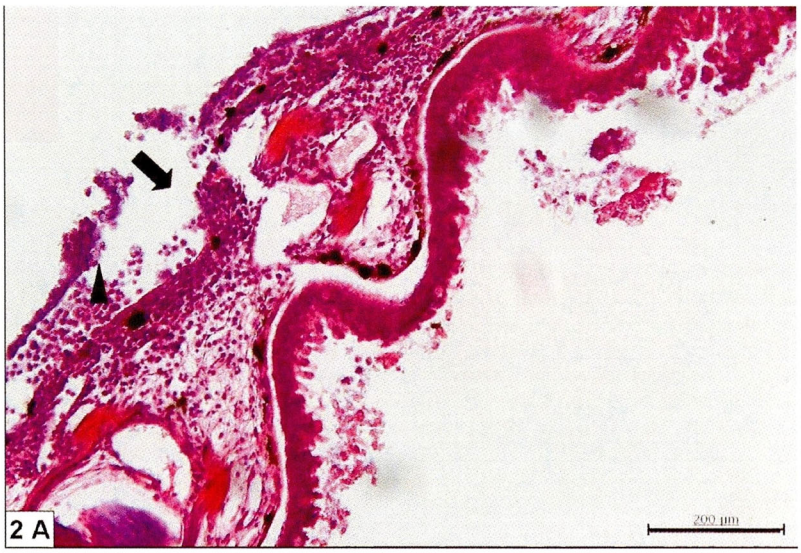


Figure 2: **A**. Extensive dermal degeneration (arrow) is observed at the area of epidermal ulcer. Scuticociliates (arrow head) and mononuclear inflammatory cells are observed. Skin, Bar = 200 μm.

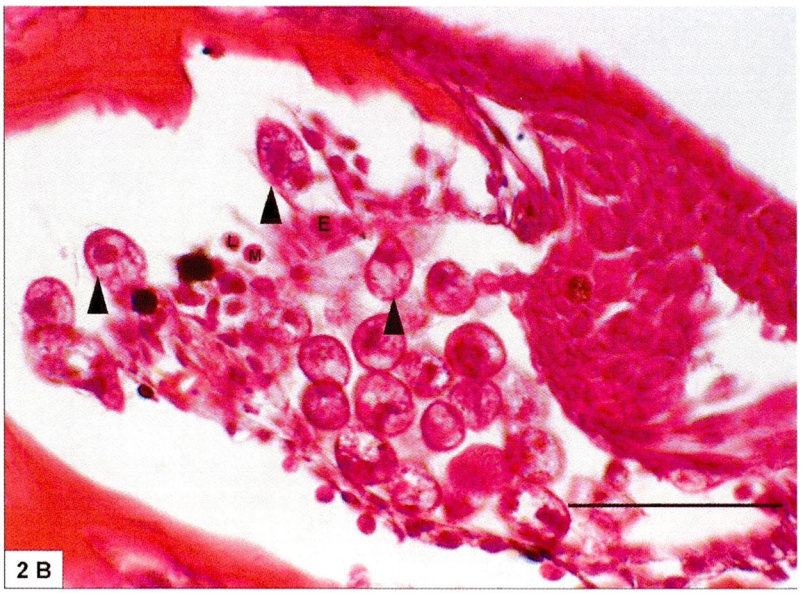


Figure 2: **B.** Inflammatory cell infiltration with presence of scuticociliates (arrow heads) in the dermis. Skin, Bar = 50 μm.

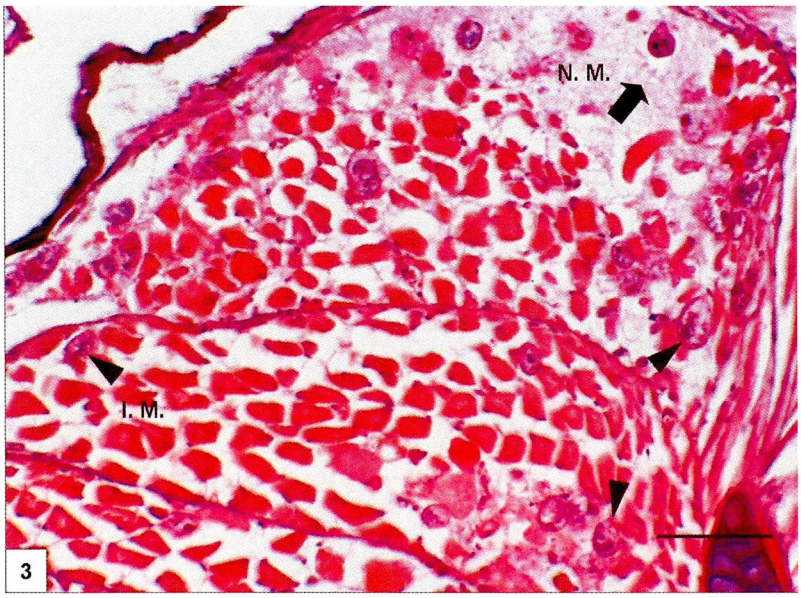


Figure 3: Degeneration and necrosis (arrow) of the muscle tissue with the presence of scuticociliates (arrow heads). Muscle, Bar = 100 μm. (N. M. = necrotized muscle & I. M. = intact muscle)

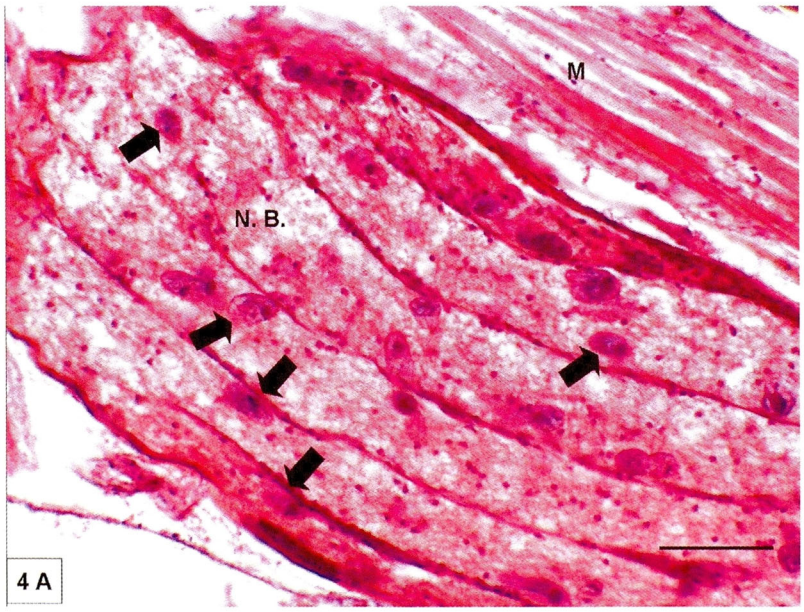


Figure 4: **A.** Scuticociliates (arrows) inside the nerve bundles (N. B.) in the muscle tissue (M). Muscle tissue from infected fish taken on day 2 post-infection, Bar = 100 μm.

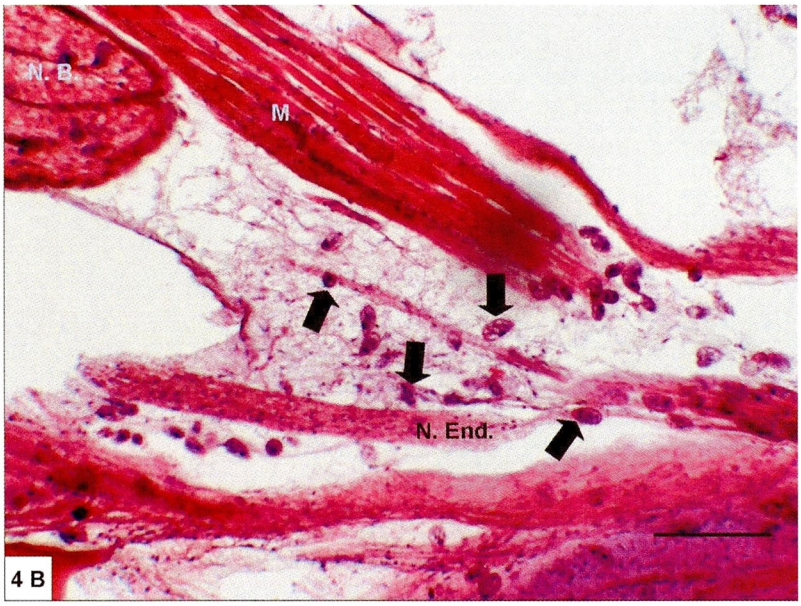


Figure 4: **B.** Scuticociliates (arrows) in the nerve endings (N. End.) in the muscle tissue (M). Muscle tissue from infected fish taken on day 2 post-infection, Bar = 100 μm.

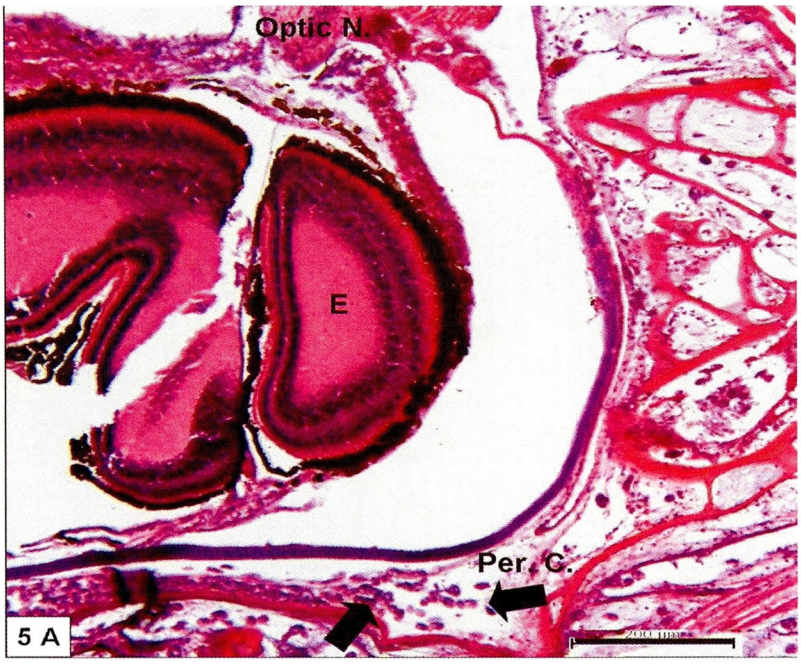


Figure 5: **A.** Eye (E) showing severe infection of the scuticociliates (arrows) in the periorbital cavity (Per. C.). Bar = 200 μm.

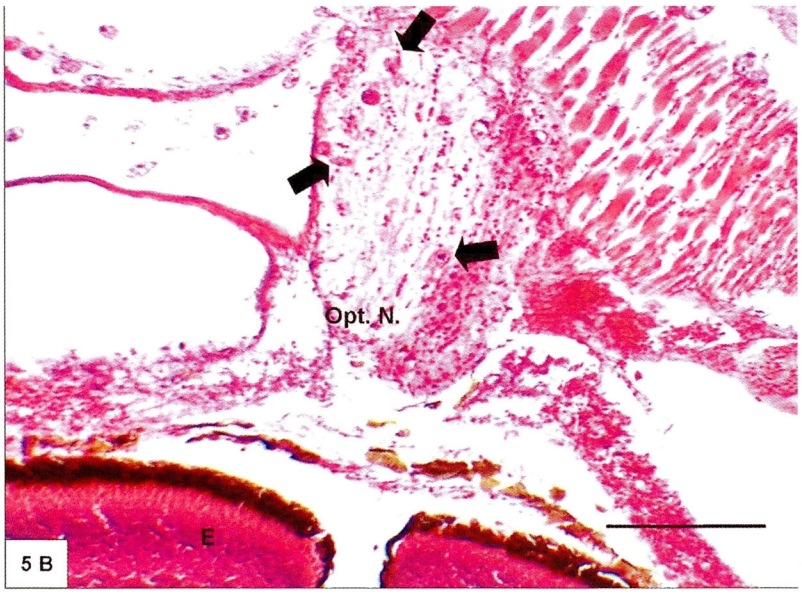


Figure 5: **B.** Optic nerve (Opt. N.) showing scuticociliates (arrows) with inflammatory changes. Optic nerve from infected fish taken on day 3 post-infection, Bar = 50 μm.

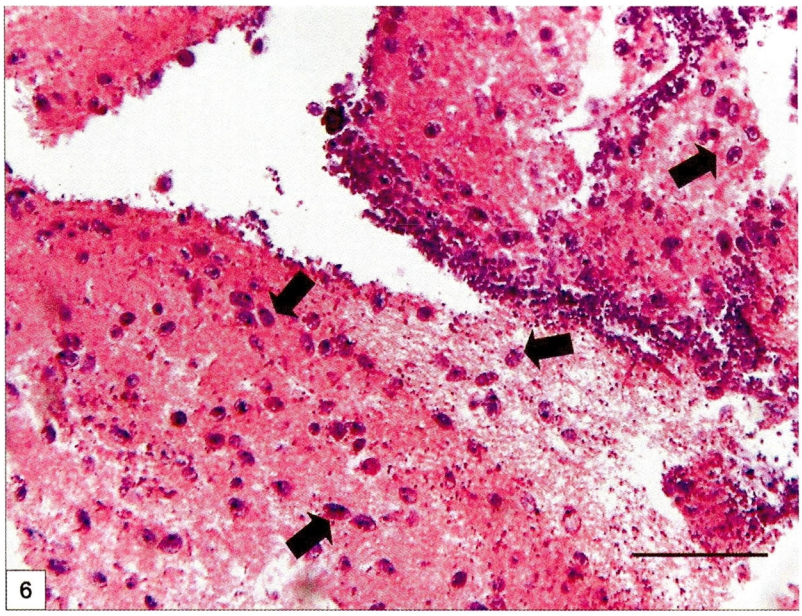


Figure 6: Scuticociliate (arrows) inside the brain. Brain, Bar = 200 μm.

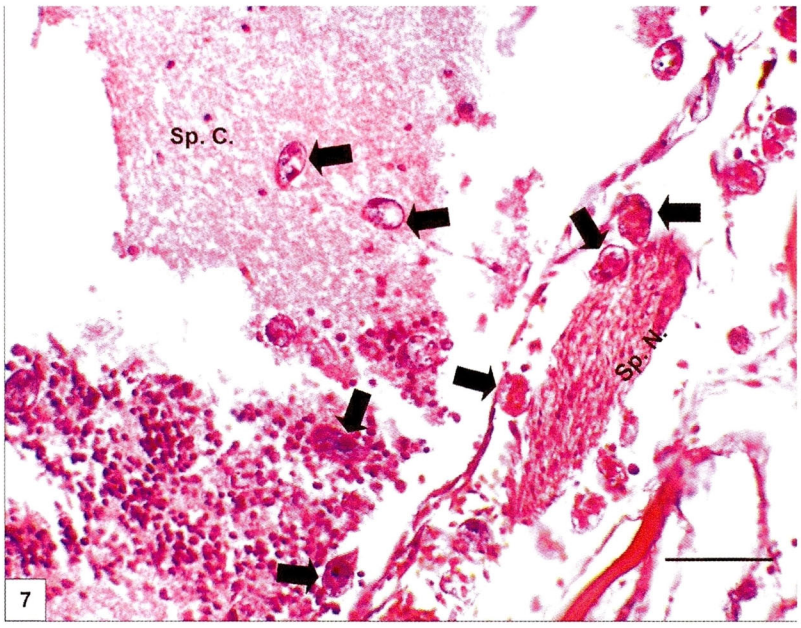


Figure 7: Scuticociliates (arrows) in the spinal cord (Sp. C.) and in the spinal nerve (Sp. N.) with severe inflammatory changes. Spinal cord from infected fish taken on day 3 post-infection, Bar =100 μm.

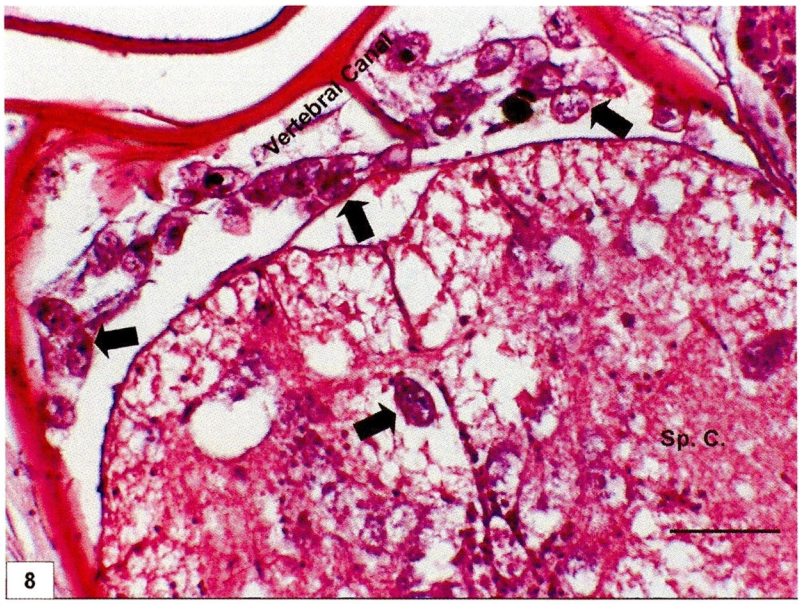


Figure 8: Scuticociliates (arrows) in the spinal cord (Sp. C.) accompanied by minimum inflammatory changes and in the vertebral canal. Spinal cord from infected fish taken on day 4 post-infection, Bar =100 μm.

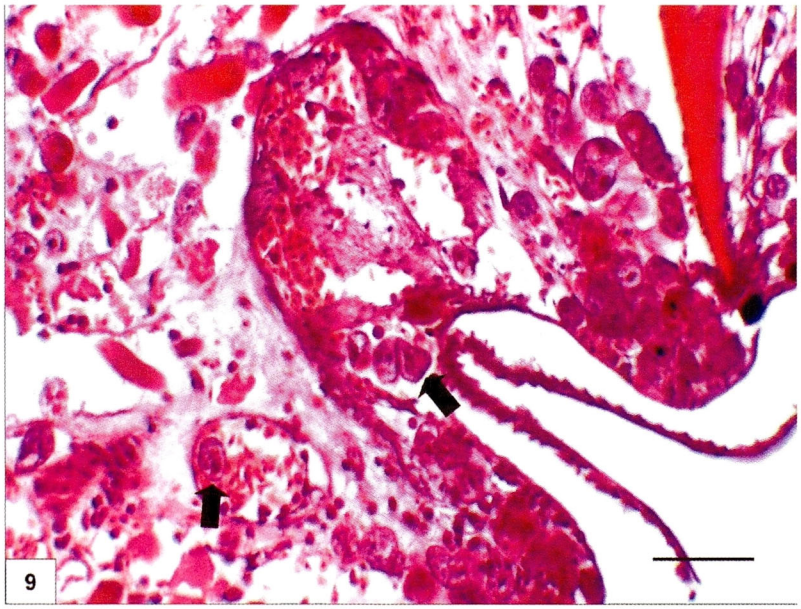
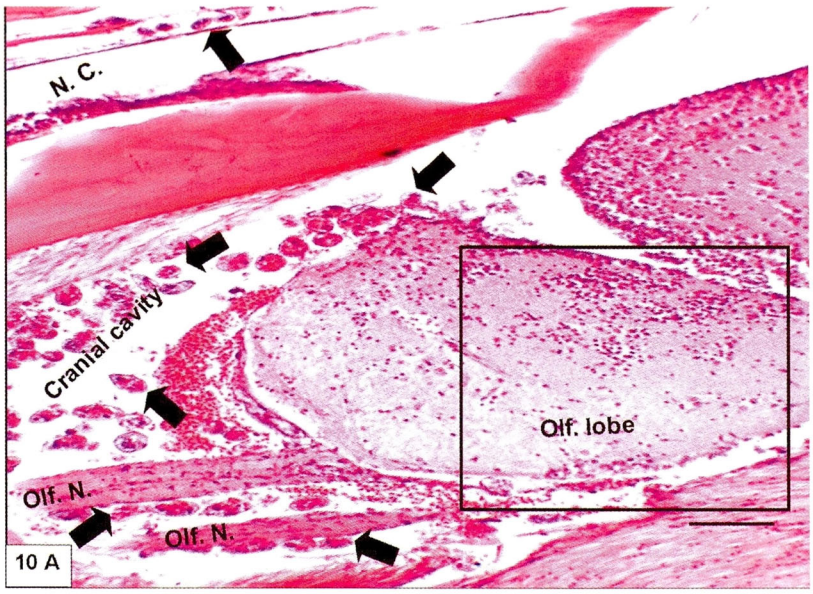


Figure 9: Scuticociliates (arrows) inside the blood vessels of semicircular canal in the cranium. Cranium from infected fish taken on day 5 post-infection, Bar = 100 μm.



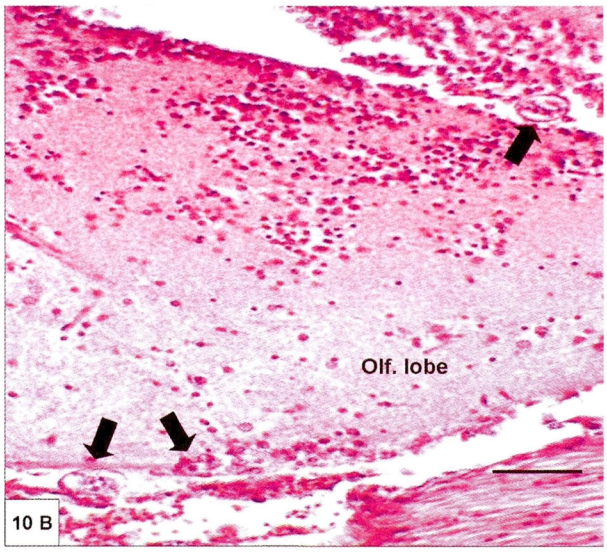


Figure 10: A. Scuticociliates (arrows) in the nasal cavity (N.C.), cranial cavity, and along The olfactory nerve (Olf. N.). Inflamatory changes in the olfactory nerve, menegitis and severe hemorrhage in the cranial cavity are shown. Bar = 50 μm. **B.** High power magnification of rectangular area of Figure A. showing the scuticociliates (arrows) with inflammatory cell infiltration in the olfactory lobe of the brain. Olfactory lobe from infected fish taken on day 4 post-infection, Bar = 100 μm.

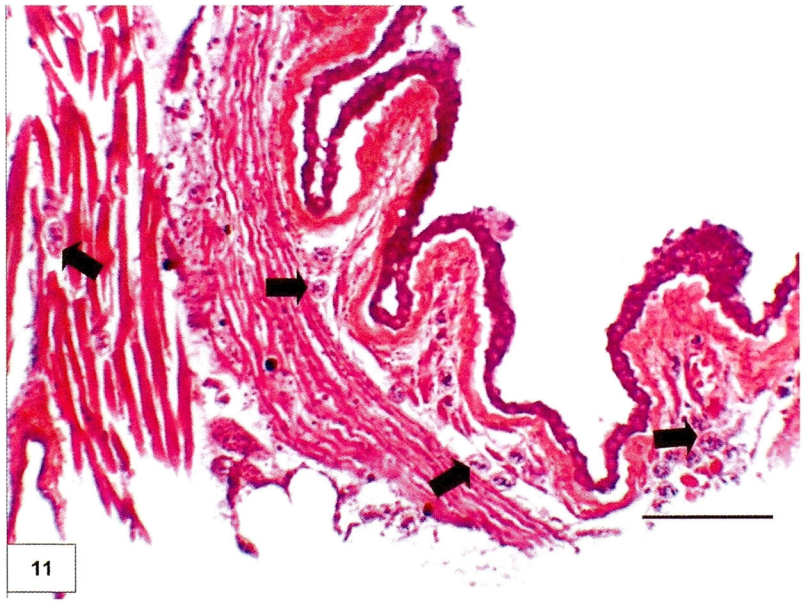


Figure 11: Scuticociliate (arrows) inside the lamina propria and lamina muscularis of gastrointestinal tract. Gastrointestinal tract, Bar = 100 μm.

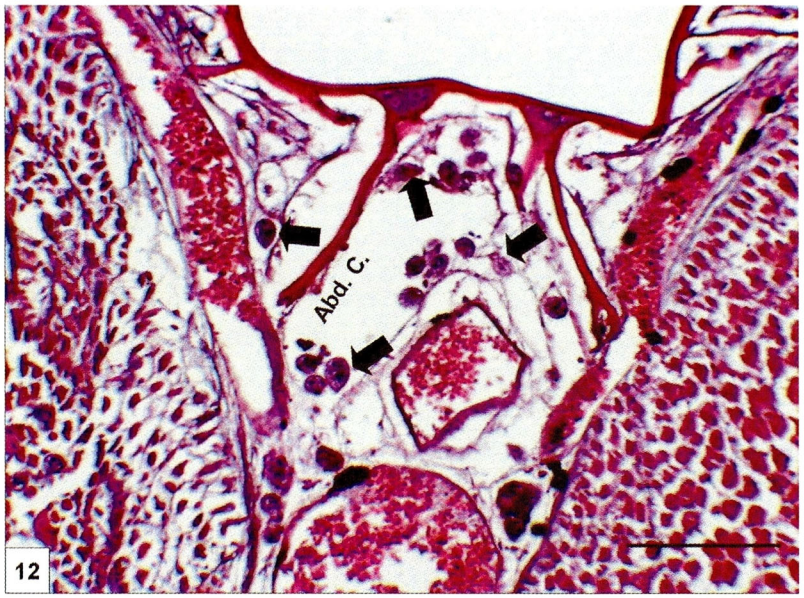


Figure 12: Scuticociliate (arrows) inside the abdominal cavity (Abd. C.). Abdominal cavity, Bar = 100 μm.

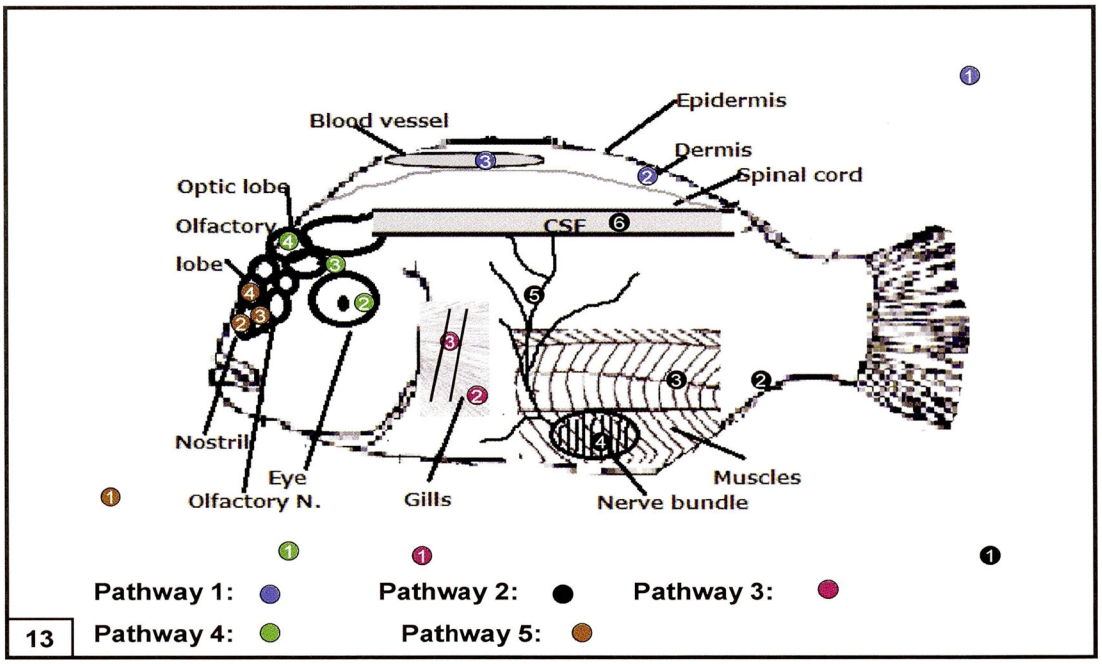


Figure 13: Schematic diagram showing all possible pathways for scuticociliate entry and dissemination into fish tissue. Pathway 1 (blue circle; 1→ 2→3): Scuticociliate invade the epidermal epithelial layer into dermis, break blood skin barrier, get into dermal blood vessels and spread through blood stream. Pathway 2 (black circle; 1→ 2→ 3→ 4→ 5→ 6): Scuticociliate invade the epidermal epithelial layer into dermis, spread through dermal nerves or connective tissue to muscles, localize in nerve bundles, spread into peripheral nerves including spinal nerve and then spread through cerebrospinal fluid. Pathway 3 (violet circle; 1→ 2→ 3): Scuticociliate invade the gill branchial epithelium, penetrate blood vessels and spread through blood stream. Pathway 4 (green circle;1→2→3→ 4): Scuticociliate invade the periorbital cavity surrounding the eye into optic nerve and optic lobe in the brain. Pathway 5 (brown circle; 1→2 →3 → 4): Scuticociliate invade through the nasal cavity, go along olfactory nerve to the olfactory lobe in the brain.

GENERAL CONCLUSION

In the first chapter, the study aimed to demonstrate the pathological findings of the naturally infected flounders (*Paralichthys olivaceus*) with scuticociliatosis in Japan. To our knowledge, there is no detailed pathological report on the pathological changes induced by scuticociliatosis in Japanese flounder in Japan before. Ten moribund fishes, farmed in Tottori Prefectural Fisheries Experimental Station, showing cutaneous ulcers, darkened skin, fin and tail rot, exophthalmia and alterations in swimming behaviour were collected. Histopathological examination revealed severe epidermal degeneration and necrosis, hyperplasia of branchial epithelium, myositis, myelitis, encephalitis associated with heavy accumulation of scuticociliates in the periorbital cavity and optic nerve fiber. Moreover, masses of ciliates were found to feed on the host tissues such as skeletal muscles, gills and brain, causing severe degenerative changes associated with abundant neutrophilic and lymphocytic infiltration. The current scuticociliate used in the present study was confirmed to be *Miamiensis avidus*; not only on the basis of the morphology of scuticociliate under both light microscopy and electron microscopy but also, on the PCR results obtained by one of the co-authors. Electron microscopy demonstrated that the buccal apparatus of the present suticociliate had three oral polykinetids with membranelles and two paraoral membranes separated by a narrow gap. In addition, the ciliate contained many digestive vacuoles in the posterior part of the body, macronucleus and micronucleus in the center of the body. These findings suggest that the present scuticociliate, *Miamiensis avidus,* is a highly invasive and destructive pathogen infecting Japanese flounder and capable of developing systemic fatal infection and has strong

phagocytic activity especially for ingestion of erythrocytes.

In summary, the study in the first chapter showed the detailed pathology of severe degenerative and necrotic changes associated with abundant neutrophilic and lymphocytic infiltration within most of organs such as skeletal muscles, gills and brain. Moreover, it can be concluded that the present scuticociliate, *Miamiensis avidus,* is a highly invasive and destructive pathogen infecting Japanese flounder and capable of developing systemic fatal infection and has strong phagocytic activity especially for ingestion of erythrocytes.

Abraded skin and/or gills are reported to be the main routes of entry, and blood vessels as the main dissemination route. Connective tissue is also reported as a possible quick way of dissemination of the scuticociliate to the body in turbot.

In the second chapter, the study aimed to clarify/identify the possible neural routes of entry and the neural ways of dissemination that enable rapid expanse of the scuticociliate into the fish body. Japanese flounder (*Paralichthys olivaceus*) were experimentally infected with the highly pathogenic scuticociliate *Miamiensis avidus* (syn. *Philasterides dicentrarachi*) using the immersion method. Scuticociliates were observed on the skin and gills right from day 0-1 post-infection, muscle tissue on day 2 post-infection, reached the brain, and spinal cord from day 3 post-infection, and systemic infection was prominent afterwards. Brain lesions were observed in most of the daily selected fish examined. Cumulative mortalities initially increased gently to reach 16% on the day 5 post-infection, however, suddenly leaped from the next day, and the cumulative mortality of the infected fish rose up to approximately 80% until day 10 post-infection and were significantly high when compared with control group. This sudden increase in

mortalities was considered to be due to severe brain lesions, which were observed to be more frequent since day 3 and day 4 post-infection. Affected fish showed varying levels of tissue damage including severe epidermal and dermal necrotic lesions, necrotic myositis, encephalitis and myelitis. Scuticociliates were frequently observed along the optic and/or olfactory nerve in the fish which were accompanied by severe brain lesions but by minimum lesions in the gills and skin, suggesting that in addition to skin and/or gills, neural routes including periorbital and nasal routes may play a role in scuticociliate invasion to the brain. Scuticociliates were also observed in the peripheral nerve fibers in the muscle tissue, cranial and spinal nerves, cranial cavity and in the vertebral canal, suggesting that nerve fibers and/or cerebrospinal fluid circulation may be involved in the spread of the scuticociliate throughout the body in addition to the blood circulation and connective tissue.

From the retrospect results of our study, it is concluded that the current scuticociliate, *Miamiensis avidus*, is a highly invasive and destructive pathogen infecting Japanese flounder and capable of developing systemic fatal infection. The pathogen can invade the fish body not only through skin and/or gill epithelium as previously hypothesized, but also through the neural route, periorbital and nasal routes. In addition, the dissemination of the scuticociliate into the fish body can be not only through blood stream and/or connective tissue, but also through either nerve fibers and/or cerebrospinal fluid circulation.

From our experience in the present study, scuticociliate infection in Japanese flounder is considered to be a stress-related disease. The pathogen is normal inhabitant of marine waters. Stress factors; nutritional, environmental and/or physical are a key factor

for developing scuticociliatosis. Sudden increase in the water temperature and/or antibiotic exposure may be considered as triggers for the present outbreak of the scuticociliatosis. Other predisposing factors are hypothesized; hypo-salinity of the water, oxygen super-saturation and/or primary bacterial infection. On the other hand, for prevention of the parasite invasion, it is recommended to use previously either filtered seawater and/or UV treated seawater for fish culture. However, this method will be so difficult to be applied in case of intensive fish culture system. We prospect, in a future study, to find a way to promote the fish defensive mechanism (immunity) through improvement of bait charges and the rearing environment for the fish and preparing an effective vaccine against *Miamiensis avidus*.

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要　旨

魚の原虫性疾患であるスクーチカ症の自然界の魚および養殖魚での発生は日本、韓国およびスペインを含めた世界各国で報告されている。特に、日本および韓国においては、ヒラメに本症がしばしば大発生した。しかしながら、日本におけるヒラメのスクーチカ症の詳細な病理学的研究はこれまでされてこなかった。また、原虫がどのような経路で宿主に侵入し、その後、宿主の防御系を突破してどのように体内で広がっていくかについてはあまりよく知られていない。傷ついた皮膚や鰓が本原虫の体内への第一の侵入経路であり、侵入後は主に血管や組織中の結合組織内を介して体内にひろがるものと報告されてきた。本研究では、養殖ヒラメに自然発生したスクーチカ症の病理所見を明らかにすることおよび実験的にスクーチカ症に感染させたヒラメにおける本原虫の脳への侵入経路および体内での移行経路を明らかにすることを目的とした。

鳥取県旧栽培漁業センター（現水産試験場）で養殖されていたヒラメに大量発生したスクーチカ症罹患魚を病理学的に解析した。罹患魚は、皮膚の潰瘍および黒変、鰭および尾の壊死、眼球突出や異常遊泳像を示した。 組織学的には、表皮の重度の変性・壊死、鰓の上皮細胞の変性・過形成、筋炎および多数の原虫の眼球内や視神経周囲への集積を伴う脳脊髄炎が認められた。また、おびただしい数の原虫による骨格筋や鰓および脳組織の破壊・捕食巣は多数の好中球およびリンパ球浸潤を伴っていた。 これらの所見は、今回のスクーチカ症の原因原虫である *Miamiensis avidus* が ヒラメの体内に侵入し多数の臓器を破壊する強い病原体であ

り、個体の死を引き起こすことを示している。

ヒラメにスクーチカ症の原因原虫である *Miamiensis avidus* を浸漬法により実験的に感染させたところ、様々な程度の皮膚の潰瘍・壊死、壊死性筋炎、壊死性脳脊髄炎が見られた。感染魚のほとんどに脳病変が認められ、それが死亡率増加の主要な原因病変であることが示唆された。原虫がしばしば視神経および嗅神経に沿って認められ、これらの個体では重度の脳病変を伴っていた。一方、これらの固体の中には皮膚や鰓に病変が見られないものもあった。よって、一般に主要な経路として考えられている皮膚や鰓からのみでなく、視神経や嗅神経などの神経系を経路とした脳組織への侵入経路も本疾患発生に深くかかわることが示唆された。また、本原虫が骨格筋組織内の末梢神経束内、脳および脊髄神経束内および頭蓋腔や脊柱管内に見られたことから、これまでに報告されている血管系や結合組織に加え、神経束や脳脊髄液循環系も本原虫の体内移行に関与することが示唆された。

以上の結果から、スクーチカ症の原因原虫の一つである *Miamiensis avidus* は魚の体内への侵入性および組織破壊力が大変強く、ヒラメの全身諸臓器・組織に傷害をもたらす大変致死性の高い病原体であることが示された。また、魚個体への侵入やその後の体内移行に際しては、神経系も関与することが示唆された。