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BEHAVIORAL AND PATHOLOGICAL CHANGES IN FLAT FISH *(PARALITCHTHYS OLIVACUS)* INFECTED WITH *MIAMIENSIS AVIDUS*

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ABSTRACT

In the present study, experimental infection of Japanese flounders with a scuticociliate, Miamiensis. Avidus (M. avidus), was carried out in Tottori Prefectural Fisheries Experimental Station in Japan using the immersion route on 200 young fries of Japanese flounder of total length: 2.67 cm and six large fishes 2 years old of total length 27-32 cm to study behavioral and pathological changes.

Histopathology showed varying degrees of tissue damage including severe epidermal and dermal necrotic lesions, necrotic myositis, encephalitis and myelitis. Many ciliates engaged in the act of ingestion of red blood cells were observed in the gill, skeletal muscle, skin and brain of the infected flounder. Infection with M. avidus altered swimming behavior (upright and near the surface vs horizontal and near the bottom of aquarium) besides, increasing swimming time and patterns of conspicuous behaviors while, reducing the anti-predation behavior of fish. These behavioral changes may serve as useful indicators for the diagnosis of parasitic infestation. These results lead to the conclusion that the natural route of invasion of the scuticociliate into the host could be through dermal or branchial epithelium, penetration into the periorbital skin or nasal

route, which leads to initial infection of olfactory rosette and subsequent migration along the olfactory nerve to the brain. The cause of death may be due to accumulation of ciliates in the branchial blood vessels leading to obstructive effect on lamellar capillary network, which hinders uptake of oxygen causing asphyxia. Death could also happen due to hypochromic anemia, which results from ingestion of erythrocytes by the ciliate, or neural dysfunction, or systemic disturbance in osmolarity induced by severe ulcerative dermatitis and necrotizing myositis.

Keywords: Fish behavior, Japanese flounder, *Miamiensis avidus*, Scuticociliatosis

INTRODUCTION

Scuiticociliates are free-living ciliates 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 ***(Elston et al., 1999)***, crustaceans and fishes, and continue to live and reproduce within the host tissues. Infections by histophagous scuticociliates (scuticociliatosis) have become one of the most important worldwide parasitological diseases in intensive marine culture of flatfish species, including Japanese flounder (*Paralichthys olivaceus*) ***(Jee et al., 2001)***. 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. *M. avidus* species infects Japanese flounder in Korea ***(Jung et al., 2005)***. Based on morphological and/or genetic analysis, it has been suggested that *M. avidus* and

Philasterides dicentrarchi are synonymous ***(Parama et al., 2003; Jung et al., 2007).*** Where as, in Japan, scuticociliatosis is a serious problem in cultured Japanese flounder. Using morphological characters the causative agent has been identified to be *M. avidus*, and there are at least three *M. avidus* serotypes in Japan, based on immobilization titres and Western blotting profiles ***(Song et al., 2009, in press).***

The natural route of entry of the ciliate into the host remains unknown ***(Jin et al., 2009).*** Recently, a successful immersion infection of Japanese flounder by *Philasterides dicentrarchi* was performed in Korea ***(Jin et al., 2009).*** Experimental infection to Japanese flounder by *M. avidus* using immersion route has been successfully conducted ***(Jung et al., 2007).*** *M. avidus* successfully invades the host directly from seawater, and causes high mortality. The ciliates rapidly invade and proliferate in the skin and gills, as evidenced by the large numbers of the migrating ciliates found at these locations in infected fish. They then consume both host cells and body fluids, and spread to the internal organs in the absence of any additional pathogens such as secondary bacterial invaders. 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 route of entry and dissemination of the scuticociliate.

Most fish in natural populations carry a parasite burden that is likely to impact negatively on their health ***(Huntingford et al., 2006).*** Parasites are thus expected to exert considerable selection pressure on host organisms, and are likely to have played a significant role in the evolution of many aspects of fish behavior and ecology. Fish perform a

wide variety of behaviors over different temporal scales. On day-to-day basis, individuals need to locate and compete for food and avoid predators; over longer time periods, they need to find mates and spawn successfully, which may require long-distance migrations or fighting over a territory or limited resources. Infections potentially affect the ability of individuals to carry out these, and many other, behaviors. Swimming, the most general behavior pattern of fish, involves the integrated effects of numerous physiological processes ***(Schreck, 1990).*** Estimation of swimming ability can provide a sensitive index to general stress and pain in fish. The swimming ability of fish under stressful conditions, compared with that of fish not subjected to stress, is different. The pattern of swimming: swimming into shallow water, swimming lethargically at the surface, lying listlessly on the pond or tank bottom, floating downstream or swimming erratically can be an indicator ***(Plumb, 1994).*** Critical swimming speed and the length of time a certain swimming velocity can be used as an indicator of stress. Parasites are frequently associated with odd host behaviors such as unusual levels of activity, increased conspicuousness, disorientation, and altered responses to stimuli ***(Holmes and Bethel 1972).***

They found correlations between the level of infection and the frequency of conspicuous behaviors such as flashing, contorting, shimmying, and continuous visits to the surface. The heavier the infestation, the more common the odd behaviors were. These odd behaviors could make the fish more susceptible to predation. Success of trophically transmitted parasites depends to a great extent on their ability to manipulate their intermediate hosts in a way that makes them easier

prey for target hosts *(Moore, 2002)*. A large range of host phenotypic traits can be altered by parasites, including morphology, physiology and behavior. Behavioral changes are the most spectacular and diverse examples of manipulation *(Moore, 2002)*. Aggression and territoriality are important in foraging, mating, defense against predators and other vital activities *(Grant, 1997)*. More aggressive individuals could establish individual territories faster, where they are more protected from predators *(Schuett, 1997)*. On the other hand, aggressive individuals could be less vigilant, more conspicuous and vulnerable for predators during contests *(Norrdahl and Korpimäki, 1998)*. Certain behavior changes associated with infection, such as visiting cleaning stations, may be beneficial to hosts since they reduce parasite levels, and these behaviors may be viewed as host adaptations to infection. However, other behavioral changes in hosts may reflect parasite adaptations that increase the probability of successful transmission or otherwise maximize parasite fitness *(Lafferty, 1999)*. Alternatively there remains the possibility that some behavior changes simply reflect inevitable 'side effects' of infection that benefit neither parasite nor host *(Poulin, 1998)*. Differentiating between the various explanations for infection-associated behavioral change, and generating data on its fitness consequences for hosts and parasites under natural environments, remains a key challenge for parasitologists *(Poulin, 2000)*.

The aim of this paper is to clarify the natural possible routes of invasion, dissemination into the body and possible causes of death, as well as, behavioral changes through the experimental infection of a scuticociliate (*Miamiensis avidus*) to Japanese flounder.

MATERIALS AND METHODS

Ciliates isolation and cultivation:

Scuticociliates (*Miamiensis avidus*) 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. The scuticociliates were sub-cultured and maintained in the medium, and the concentration of the scuticociliate was approximately 10^6cells/mL in the culture medium. The isolates were morphologically and genetically identified and classified serologically, using immobilization assays and Western blotting, into "serotype I" and it was named JF05To ***(Song et al, 2009)***.

Aquarium:

The fish in the present study were kept in two acrylic aquariums of 100 L capacity (big enough to allow the fish to behave normally) in the station containing seawater, filtered with cartridge filter (0.5 □m, Millipore). Each of these aquariums contained one perforation at the upper part of a side wall for water drainage. A net was attached to this perforation to prevent the outflow of the young fish.

Fish:

All fish employed for the experiment (200 young fry of Japanese flounder of total length: 2.67 cm and six large olive flounder 2 years old of total length 27-32 cm) were reared at the institution in the same condition as that prevailed in the laboratory in Tottori Prefectural Fisheries Experimental Station in Japan until the 95th day after hatching. Compound fish feeds were fed to the fish fries. Twenty 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 two groups (100 each). Fishes in the first group used as a negative control without infection while in the second group used for experimental infection. Each group was reared in a separate aquarium in the station with a 12 h light: 12 h dark photoperiod. Fish were kept in these conditions for 7 days to acclimatize them. Control and experimental aquariums were located in the same room, providing identical conditions for fish after their transfer. A heater was used to maintain a steady temperature of 19°C. Water in the fish tanks was slightly aerated constantly during the duration of the experiment to keep a constant oxygen concentration in the water (6.5 mg/L).

Experimental infection:

On the first day of infection experiment, each aquarium was filled with 100 L of filtered seawater and fish were released into the tanks in numbers stated above. 10 mL of the culture medium holding 10^7 cells/mL of the scuticociliate was centrifuged at 3,500 rpm for 10 minutes at room temperature. For preparation of concentrate of the ciliates, the supernatant was discarded and the sediment was suspended with 10 mL of filtered seawater were prepared for immersion. This concentrate was added to the second aquarium. The concentration of the ciliate in the immersion aquarium was thus set to become approximately 100 cells/mL. From the next day onwards, half of the water of each aquarium was removed and replaced by the same amount of fresh filtered seawater on a daily basis. The experiment was continued for ten days

after the infection. Just 30 minutes after the start of immersion (infection), five live fish were sampled from each aquarium. Beginning from the first day after the infection till the tenth day, five live fish from both tanks were sampled to examine the possible neural invasion and dissemination routes. Concerning the sampled fish, infection presence, the infested part with the scuticociliate and the dissemination routes were checked by direct micrography of fish from the surface to the interior of the body using stereo microscope before necropsy. Fish were euthanatized after the exposure to eugenol [Fish/Crustacea anesthetic FA100 (TANABE SEIYAKU, Co Ltd, Japan)]. All dead fish of each tank were sampled whenever the death was confirmed regardless of the number of days after 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 substituted by 75% ethanol solution

Histopathology:

Once fish behavior was recorded, the sampled fish was captured (with a hand net) and euthanized to determine the gross examination. All necropsied fish were fixed in Davidson's solution and then transferred to 75% ethanol. Fish were submitted to the Veterinary Pathology lab of Tottori University for histopathology. The sampled fish were cut into transverse sections, dehydrated and embedded in paraffin wax. Subsequently, 2-5 μm sections were cut and stained with hematoxylin & eosin (H&E) for light microscopy.

Fish behaviors:

The percentage (%) frequency of fish behaviors were recorded for 30min/day using two video cameras placed in front of two perpendicular tank sides to provide a 3D recording of each fish. The observed behaviors were recorded in three different categories, namely; swimming, conspicuous and anti-predation. Patterns of behavior that characterized as conspicuous were surfacing, flashing, contorting, shimmying, and jerking. We did not assess escape behavior in the tank. Surfacing fish made abrupt dashes up to the tank's surface, flashing fish turned laterally so that one side of the body faced upward (often associated with chafing on the tank's bottom), contorting fish performed a slow, acute, dorsal-ventral bending, usually bending the head and tail in opposite directions, shimmying fish vibrated for a few seconds, and jerking fish moved suddenly forward 3-5 cm. We noticed many of these behaviors in the field as well.

Swimming performance: Swimming endurance against water current was compared between infected (5 days post infection) and uninfected fishes using a small recirculation flow tank adapted from ***Hashimoto et al. (1996).*** The experimental tank consisted of a glass aquarium (60 x 45 x 45 cm) and a mesh chamber (35 x 9.5 x 10 cm, mesh size 1x 1 cm) in which individual fish were placed for monitoring. The pump sucked water from the bottom corner of the aquarium and discharged it from the other end through a "shower head" which consisted of a plastic box with numerous 2 mm diameter holes. The mesh chamber was set in front of the "shower head" at a 10 angle. In this way

the water current forced the fish away from the bottom of the chamber, stimulating it to swim. Average water flows at the front, middle and end of the chamber were 0.44 ± 0.04, 0.35 ± 0.02 and 0.25 ± 0.03 m/s, respectively. The water temperature was held constant at 24 C which was equivalent to that of the stock tanks. Swimming performance of each fish was video recorded and total swimming duration before exhaustion was assessed. A total of 20 infected and 20 uninfected fish were tested. If fish did not start swimming within the first 30 s of monitoring, a glass rod was used to stimulate swimming. Fish were considered exhausted and the trial was terminated when the following conditions persisted for more than 10 s: fish not facing against the water current, part of the body touching the back-end of the chamber, not showing obvious fin and tail movements, or a combination of these criteria. Fish were considered "resting" if they restarted swimming within 10 s. This 10 s constraint was applied based on observations from a preliminary trial. Swimming duration was calculated as the total swimming time before exhaustion minus the resting time ***(Shirakashi et al., 2008)***. Survival against predation (Susceptibility to predation) was compared between infected and uninfected juvenile flounder fish in a cohabitation experiment with predatory fish. Six large olive flounder (2 years old, TL 27-32 cm) were used as a model predator because cannibalism is well documented in flounders ***(Kellison et al., 2002)***. In case of infected fish the test of predation was done 5 days post infection; 10 infected fries transferred into a separate tank containing filtered seawater. After fries were acclimated in this tank for 24 h, three predator flounder were added. The tank was left undisturbed for 96 h, allowing predators to feed on

juveniles. After this time, all the water and substrate were removed from the tanks in order to count the numbers of surviving juveniles in the tank. The same test was done for uninfected fish. Different individual predator fish were recorded. The cumulative mortalities of infected and uninfected fish were recorded.

Statistical analysis: All data obtained were analyzed by the one-way ANOVA in a completely randomized block with the COSTAT program and Duncan multiple range test was used to rank means of treatment (Duncan, 1955).

RESULTS

Cumulative Mortality:

Cumulative mortalities of fish infected with *Miamiensis avidus* by immersion are shown in Figure 1. For our experiments we calculated cumulative mortality by deducting the number of live fish sampled until day 10, which was the end of the period of infection, from the total fish population in the tank. Cumulative mortality initially increased gently to reach 20% on the 5^{th} day since infection (day 5 post-infection). The number of death, however, suddenly leaped from the next day, and the cumulative mortality rose to approximately 80% until day 10 post-infection. The ciliate-infected groups showed significantly higher mortalities ($P<0.05$) than the control group.

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 skin and fins, right from

day 1 post-infection. Scuticociliates were observed in gills, brain and spinal cord from day 3 post-infection, and from day 6 post-infection they were observed in eyes and nostrils. Scuticociliates were observed on lips and mouth from day 8 post-infection (data not shown). The expanse of the infection was checked by direct micrograph of fish from the surface to the interior of the body.

Scuticociliate dissemination inside the body:

Results of histological detection of *M. avidus* organism in experimentally infected fish are summarized in Table 1. The first ciliate infection was observed in skin and gills in individuals of the group sampled on day 0 (30 minutes after the immersion). High densities of scuticociliates were observed in epidermis and gill mucosal epithelium. On day 1 post-infection, the number of fish showing histological evidence of infection and ciliate densities within the affected areas remained unchanged. However, there was an increase in the severity of the infection at the affected tissues e.g. extensive epidermal damage and appearance of epidermal ulcer. Then, the ciliate gradually disseminated within the entire body, and it was observed in muscle tissue on day 2 post-infection, in the periorbital cavity surrounding the eye, brain and spinal cord on day 3 post-infection and in gastrointestinal tract, liver and kidney from days 4 and 5 post-infection onwards. Since then, the infection was generalized and the scuticociliates distribution became more evident, both in terms of ciliate density and tissues colonized, in other words, confirmed in all tissues, which were used for histological observation. The distribution of ciliate in fish became clearer under histological investigation of infected tissues.

Histopathology:

Histopathological examinations on day 1 post-infection revealed extensive epidermal necrosis, subsequent dermal degeneration with lymphocytic, monocytic and eosinophilic granular cell infiltration associated with the presence of a high number of scuticociliates. On day 2 post-infection, muscles showed liquefactive necrosis (Fig. 2) in the presence of the invading scuticociliate underneath the epidermal layer. Scuticociliate were observed inside the muscle nerve bundles and nerve fiber endings (Fig. 3). On day 3 post- infection, heavy infestation of the scuticociliate was observed in the periorbital cavity (Fig. 4) surrounding the eye, brain, muscular vertebra of the spinal cord and in the spinal nerves (Fig. 5). From day 4 onwards, the infection became more systemic, and scuticociliates were noticed in the lamina propria and lamina muscularis of the gastrointestinal tract and in the abdominal cavity. Periglomerular, peritubular and perivascular mononuclear inflammatory cell infiltration in the presence of scuticociliates inside blood vessels were observed in the kidney. Moreover, scuticociliates were observed in the blood vessels of semicircular canal in the cranium (Fig. 6), nasal cavity, optic nerve, olfactory lobe (Fig. 7 A, B), third ventricle and cerebrum. Many ciliates containing red blood cells in the cytoplasm were observed, from the Day 6 onwards post-infection, accompanying hemorrhagic and necrotic lesions in the gills, skeletal muscles, skin and brains of the infected fish.

Fish Behavior:

The infected fish showed abnormal swimming behavior (upright or vertical swimming) near the surface of the aquarium while the control one showed straight or horizontal swimming near the bottom of aquarium. Infection with *M. avidus* increased the swimming time of juvenile flounder (The average swimming duration of uninfected controls fish was 140 s, while that of infected was 290 s (Table 2). Flat fish fries were readily eaten by large predator flounder under the experimental conditions (the percent of predation of infected fish was 90% whereas it was 10% among uninfected fish). This was approved by the dissections of three predator flounder immediately after the period of cohabitation. No carcasses or body remains were observed inside the experimental tank after the cohabitation test, thus all fries which disappeared during the test were considered as being eaten. The survival rate against predation was significantly reduced in the infected group. On average, *M. avidus* infection reduced the survival rate by approximately 60% (it was 80% for uninfected fish vs 20% for infected one). In addition, parasitized fish exhibited conspicuous behaviors more frequently than that occurred among the unparasitized fish. Only parasitized fish contorted, shimmied, or jerked in comparison with unparasitized fish. All behaviors were found to be more frequent in parasitized fish than unparasitized fish except the flashing pattern which most commonly occurred in unparasitized fish. Surfacing was the most common behavior strongly associated with infected fish.

DISCUSSION

Scuticociliatosis is a common disease in aquaculture fish ***(Song et al., 2009)***, and in the last few years there have been several reports on fatal outbreaks of systemic infection by *Miamiensis avidus* in Japanese flounder in Korea ***(Jung et al., 2005 and Jung et al., 2007)*** as well as in Japan as demonstrated in our previous study (data not yet published). Pathogenicity of *Miamiensis avidus* in Japanese flounder was previously confirmed ***(Jung et al., 2005; Jung et al., 2007 and Song et al., 2009).*** *Miamiensis avidus* is the only highly pathogenic scuticociliate to Japanese flounder and can induce primary infection even in the absence of abraded skin. In the present study, successful experimental infection of Japanese flounder indicates that *Miamiensis avidus* is a highly virulent endoparasite and can induce primary infection. These findings are in accordance with the previous reports ***(Jung et al., 2007 and Jin et al., 2009).*** The ciliates rapidly invade and proliferate in the skin and gills, as evidenced by the large number of scuticociliate found at these locations in the infected fish. They then consume both host cells and body fluids, and spread to the internal organs in the absence of any additional pathogens such as secondary bacterial invaders. Histopathological examinations demonstrated extensive epidermal damage, dermal degeneration, hyperplasia of branchial epithelium, necrotic myositis, encephalitis and myelitis; all of which are in agreement with previous studies on the nature of infections by scuticociliates in fish ***(Iglesias et al., 2001; Jung et al., 2005; Jung et al., 2007; Azad et al., 2007 and Jin et al., 2009).***

Currently it is not known as to how exactly *Miamiensis avidus* invades the host. Results of the present study suggest 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).*** In the affected fishes, the first skin lesions were noticed by direct micrography of the fish as early as 30 minutes 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 are produced during parasitic infections and involved in host pathology. They moreover, 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 and Joachim, 2000).*** Also, it was demonstrated that *Philasterides dicentrarchi* (syn. *Miamiensis avidus*) produces a cysteine-proteinase type toxin. The role of proteinases in pathogenesis includes 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 ***(Parama et al., 2007)***, activation of inflammation ***(McKerrow et al., 2006)*** and the degradation of host proteins during the course of feeding ***(Rosenthal, 1999).*** The enzyme is also known to have a role in the histophagus digestibility producing severe skin lesions in silver pomfret *(Pampus argenteus Euphrasen)* ***(Azad et al., 2007).*** 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, 1993 and 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 and kwon et al., 2003).***

The strain of ciliate used in this study was serotype I (among three serotypes in Japan) based on immobilization titres and Western blotting profiles ***(Song et al., 2009).*** As they reflect the differences of the protein structure, this serotype may be related to the difference of the property of the protease. The result of a detailed study, which is awaited, may clarify as to whether serotypic differences reflect different symptoms.

Natural possible routes of entry are yet unknown. The presence of ciliates in the gills, dermal and muscular layers suggests that the natural route of entry of the parasite could be through the skin or gill epithelium (dermal or branchial epithelium), which then spreads quickly via blood vessels. This indicates that once the parasite breaks the skin-blood barrier, it can enter into the internal organs through blood stream, as has been hypothesized previously by ***Paramá et al. (2003), Sterud et al. (2000), Jung et al. (2007), Ramos et al. (2007) and Jin et al. (2009).*** The presence of scuticociliates in the bloodstream, nerve bundles, nerve fiber endings, and in perivascular and perineural connective tissues indicates that *Miamiensis avidus* uses these tissues as routes for accessing almost all organs of the body. Therefore, it is thought that progress of the infection and the expansion to adjacent organs are enabled through the connective tissues ***(Puig et al., 2007).***

The scuticociliate lesions in the eye or the periorbital skin may likewise facilitate their entry. In the present experiment, brain infection was observed in most of the fish, but systemic infection existed only in 60% of the population. This fact, together with the microscopically confirmed presence of ciliates in the optic nerve, suggests that *Miamiensis avidus* is able to reach the brain not only via the bloodstream but also via nervous tissues. This would explain why many fish showed abundant ciliates in the periorbital region as hypothesized by ***Paramá et***

al., (2003).The presence of ciliates in the olfactory bulb in the brain, nasal cavity and spinal cord suggests that the infection could spread through the nasal route. The starting point of infection may be traced from nostrils into the olfactory rosette. From there, the ciliates moved up to the olfactory nerve to the brain causing locomotor dysfunction that eventually led to death. These observations have also been recorded by ***Munday et al. (1997).***

In view of the presence of ciliate in the subepithelial connective tissue of the digestive tract of turbot, an oral route of entry was suggested for an unidentified scuticociliate parasite ***(Dykova and Figueras, 1994)***. In the present study, however, it is unlikely that *Miamiensis avidus* invades the host via the oral route because the low pH of stomach lumen would likely provide a sufficient barrier to the infection. In addition, the mucosal epithelium of the digestive tract in the infected fishes is not damaged, and the ciliates were found predominantly in the higher vascular lamina propria. This also supports the idea that the ciliate infect mainly through the skin and/or gills and then spread via the blood stream ***(Jung et al., 2007).***

Possible causes of death would be as follows. The accumulation of ciliates in the principal branchial blood vessels, particularly in the capillaries of the secondary lamellae can be expected to induce a marked obstructive effect on the lamellar capillary network, hindering uptake and transport of oxygen causing asphyxia, which is probably one of the major causes of death in the affected fish. Also, 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. Additionally,

some reports demonstrated that severe ulcerative dermatitis and necrotizing myositis lesions induced by scuticociliates result in a systemic disturbance of osmolarity that cause death. Since the primary function of the skin in fish is osmoregulation and sustenance of a waterproof barrier, such severe lesions result in a systemic disturbance of osmolarity, which causes death as previously hypothesized ***(Rossteuscher et al., 2008 and Umehara et al., 2003)***. In addition, it was suggested that heavy infestation of nerve bundles and nerve fibers might be one cause of death due to neural dysfunction ***(Jin et al., 2009).***

There are consequently three main reasons why it is important to link the role of parasites in an exploration of the relationships between welfare and behavior in fish. Firstly, the captive housing of fishes being used for research, display or in aquaculture may restrict the expression of normally adaptive behavior patterns, and this could lead to elevated infection levels. Secondly, many of the documented effects that parasites have on the behavior of their hosts may exacerbate welfare problems that are normally associated with the captive housing of fish. A third reason is that fish harboring high parasite loads sometimes exhibit characteristic behaviors that might serve as useful indicators for the diagnosis of infection status.

This result supports the hypothesis that parasites modify the behavior of their intermediate hosts and make them more susceptible to predation. The upright swimming behavior is explained by that disease make respiratory distress so; the fish try to get more oxygen by engulfing the air or swim near the top of aquarium. Surfacing was the most common behavior associated with parasitism. Parasitized fish might be going to the surface to feed more, to help meet increased metabolic

demands caused by parasitism. ***Milinski (1985)*** observed this effect with parasite that places a high metabolic demand on their hosts. Knowing a little about the mechanisms parasites use to alter host behavior, but some evidence exists for increased oxygen demand partially explains why parasitized fish frequently surface ***(Smith and Kramer, 1987).*** In this study, the physical presence of hundreds of parasite cysts in the brain case might be sufficient to alter fish behavior. In spite of these explanations, it is always possible that there are other factors associated with parasitism and behavior that not taken in the consideration.

The parasite indirectly affects the survival of the fish. Very low post infection mortality was observed for all the experimentally infected fish. This indicates that the parasite is not a direct cause of mortality, at least within this experimental design. However, infection can indirectly lead to host death through changing behavior(s) of the host that may increase their susceptibility to predation. This experiment demonstrated that the parasitic infection increased the general activity level, by increasing swimming performance of the host and consequently made them more vulnerable to predation. Although behavioral changes of parasitized fish are a well known phenomenon ***(Moore, 2002)***, fish infected by *M. avidus* appeared to be more active than uninfected fish. Hyperactivity is one of the most commonly observed behavioral changes in parasitized fish ***(Poulin, 1994 and Moore, 2002).*** It is often identified as an explanation for increased predation rate because active individuals are more conspicuous and have a greater chance of encountering predators. Increased activity of infected fish may be related to infection stress. The irritation and physical disturbance caused by the parasite may act as stressors and affect the fish in a similar manner. High activity

levels of infected fish may be the key to their higher susceptibility to predation because flounder are most vulnerable to predators when they are actively swimming in the water column ***(Noichi, 1997)***. The low activity levels observed in control fish may be their strategy for risk avoidance while the infected fish were insensitive to the risk and failed to modify their behavior to adapt to the unfamiliar experimental conditions. Regardless of whether the parasite makes the host hyperactive or insensitive, it likely has negative effects on their survival, particularly against predation. Cannibalism is well documented in wild flounder. The larger flounder, typically 1-2 years old, fish are recognized as one of the major predators of yearlings ***(Kellison et al., 2002)***. The present result indicates that *M. avidus* can be an additional factor facilitating cannibalism. We showed that *M. avidus* makes juvenile flounder on average approximately 25% more vulnerable to predation.

Parasites can affect the normal patterns of fish behavior in three main ways. First, because animals are expected to have evolved behavioral mechanisms to limit contact with infective stages to reduce the demand placed on the immune system ***(Hart, 1990)***, Second, parasitized fish may perform behaviors that reduce levels of infection with already□acquired parasites. Such behaviors range from simple ''flashing'' against the substratum or rubbing against other structural components of their environments to dislodge ectoparasites ***(Urawa, 1992)***. Third, parasite infections may cause host behaviors to alter in ways that serve to mediate the detrimental effects of infection. Because parasites cause local pathology to host tissues by their attachment, movements, growth, or development, the specific sites they occupy may have important consequences for the type and extent of host behavioral

change ***(Holmes and Zohar, 1990)***. Another explanation from the above pathological changes as, many parasites have a predilection sites within their hosts, leading to damage of sensory tissues or occlusion of sensory organs (brain or eyes) is sufficient to impair vision or chemotransmitters. All these may be sufficient to bring about changes to host behavior.

CONCLUSION

As demonstrated in the present study, it can be concluded that the scuticociliate, *Miamiensis avidus* can naturally invade Japanese flounder through three routes; the penetration into the skin and/or gills epithelium, the penetration of the periorbital skin and/or through the nasal route which leads to initial infection of olfactory rosette and then migration to the brain along the olfactory nerve. Subsequently the scuticociliates can disseminate within the body of the fish through blood stream, nerve fibers and/or connective tissue. Four possible causes of death are suggested which are as follows: i) asphyxia caused by accumulation of ciliates in the principal branchial blood vessels that leads to an obstruction effect on lamellar capillary network, which hinders the uptake of oxygen; ii) hypochromic anaemia caused by the ingestion of erythrocytes by the scuticociliates; iii) neural dysfunction evidenced by heavy infestation of nerve bundles or nerve fibers; iv) and/or systemic osmolarity disturbance induced by severe ulcerative dermatitis and necrotizing myositis. Lastly, the behavioral results indicated that *M. avidus* indirectly reduces the survival rate of juvenile P. olivaceus by altering various fundamental behaviors (swimming, conspicuous and anti-predation) under experimental conditions. The impact of *M. avidus* on its host is substantial enough to help explain recent depletion of flounder populations in Japan.

Table (1): Histological detection of experimentally infected fish with *M. avidus* by immersion method.

Tissues	No. Fish//day	Days post-immersion										
		0	1	2	3	4	5	6	7	8	9	10
Skin	5	5	5	5	5	5	5	5	5	4	4	3
Gills	5	3	3	2	2	3	3	5	5	3	3	2
Muscle	5	0	0	4	5	5	5	5	5	5	5	5
Eye	5	0	0	0	3	3	3	3	3	2	3	2
Brain	5	0	0	0	4	4	4	5	5	4	4	3
Nasal cavity	5	0	0	0	0	1	3	5	5	3	3	2
Spinal cord	5	0	0	0	4	4	4	5	5	4	4	3
Intestine	5	0	0	0	0	3	3	3	3	3	2	2
Liver	5	0	0	0	0	0	3	3	3	2	2	1
Kidney	5	0	0	0	0	0	4	4	3	3	2	2

Table (2): Effect of experimentally infection with *M. avidus* on flat fish behaviors.

Behaviors		Parasitized	Unparasitized	SE	Significant
Swimming time (sec)		290	140	0.862	<0.001
Conspicuous behavior (%)	Flashing	18.08	71.66	0.622	<0.001
	Surfacing	53.37	28.34	0.418	<0.001
	Contorting	4.28	0	0.045	<0.001
	Shimmying	10.47	0	0.036	<0.001
	Jerking	13.80	0	0.098	<0.001
Predation (%)		90	10	0.042	<0.001
Survival rate (%)		20	80	0.051	<0.001

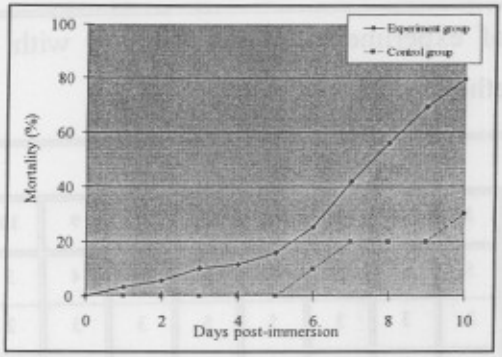

Fig. (1): Cumulative mortality of fish infected with *M. avidus* by immersion.

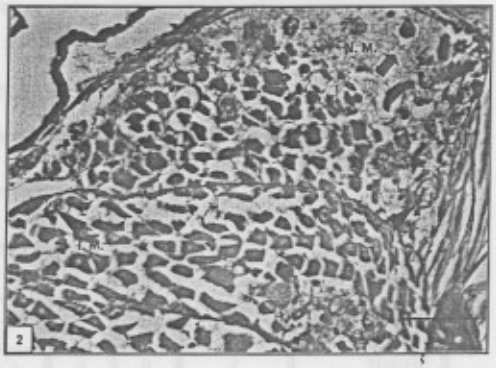

Fig. (2): Degeneration and necrosis (arrow) of the muscle tissue with the presence of scuticociliates (arrow heads). Muscle, Bar = 100 □m. (N. M. = necrotized muscle & I. M. = intact muscle)

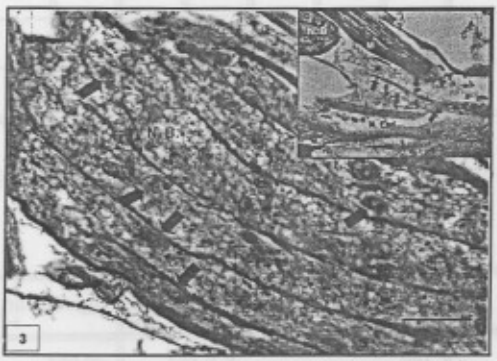

Fig. (3): Scuticociliates (arrows) inside the nerve bundles (N.B.) in the muscle tissue (M). Inset: Scuticociliates (arrows) in the nerve endings (N. End.) in the muscle tissue (M). Muscle tissue from infected fish taken on day 2 post-infection, Bar = 100 □m.

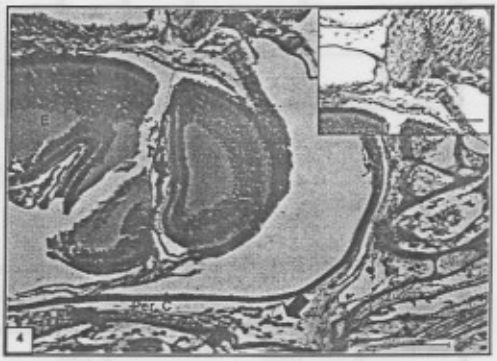

Fig. (4): Eye (E) showing severe infection of the scuticociliates (arrows) in the periorbital cavity (Per. C.). Bar = 200 □m. B. Optic nerve (Opt. N.) showing scuticociliates (arrows) with inflammatory changes. Optic nerve from infected fish taken on day 3 post-infection, Bar = 50 □m.

Fig. (5): 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 □m.

Fig. (6): Scuticociliates (arrows) inside the blood vessels of semicircular canal in the cranium. Cranium from infected fish taken on day 5 post-infection, Bar = 100 □m.

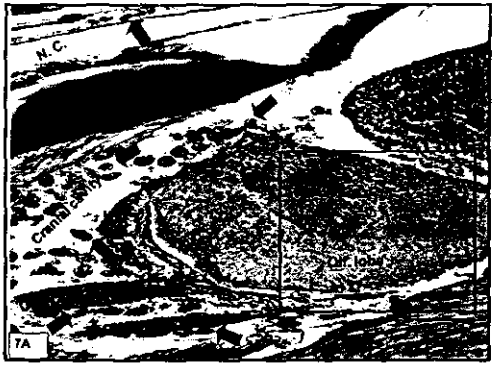

Fig. (7) A: Scuticociliates (arrows) in the nasal cavity (N.C.), cranial cavity, and along the olfactory nerve (Olf. N.). Inflammatory changes in the olfactory nerve, meningitis and severe hemorrhage in the cranial cavity are shown. Bar = 50 □m.

Fig. (7) B: High power magnification of rectangular area of Fig. 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□m.

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التغيرات المرضية والسلوكية للأسماك المفلطحة المعدية بالميامينسيس أفيداس

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أجريت هذه الدراسة على عدد 200 زريعة أسماك فلاوندر المفلطحة وعدد 6 من السمكات الكبيرة للفلاوندر فى محطة التجارب السمكية بمقاطعة توتورى باليابان لدراسة التغيرات المرضية و السلوكية لهذه الاسماك بعد العدوى بطفيل الميامينسيس أفيداس بطريقة الغمس

أظهرت التجارب تغيرات هستوباثولوجية بالأنسجة مثل موت بخلايا الجلد الخارجية – التهاب بالخلايا العضلية و الخلايا المخية و الحبل الشوكى بالأسماك المعدية

سبحت أو عامت الأسماك المعدية رأسيا واقتربت من سطح ماء الحوض – وكانت هناك زيادة ملحوظة فى وقت السباحة والعوم – بينما قل سلوك مقاومة الافتراس بين الأسماك المعدية مقابل السبح افقيا قرب قاع الحوض للأسماك الغير معدية

ويمكن أن نستخلص من هذه الدراسة وصول الطفيل للخلايا العصبية وإحداث الوفاة عن طريق:

1- تجمع الطفيل فى الشريان وانسداده و منع وصول الدم للأعضاء الحيوية.

2- ابتلاع الطفيل لكرات الدم الحمراء مما يؤدى الى حدوث انيميا ونقص فى الاوكسجين.

3- خلل فى وظيفة الجهاز العصبى والحركى.

وأخيرا يمكن تشخيص الإصابة بالطفيليات فى الأسماك من خلال دراسة التغيرات فى سلوكها.