Chapter III. Larval attachment and development of the monogenean *Neoheterobothrium hirame* under low temperature

INTRODUCTION

Infection of *Neoheterobothrium hirame* on olive flounder is widespread in Japanese water. The parasite is highly abundant in a wide geographical area (Mushiake *et al.*, 2001; Anshary *et al.*, 2002), however noticeable declines of flounder catch have been observed only in limited regions (Fig. II-1; Tsutsumi, 2004). Also, variability in the infection levels among different regions has been observed (Mushiake, 2001; Anshary *et al.*, 2002). Our field data obtained from Miyako Bay, Iwate, and Obama Bay, Fukui, also showed a significant geographical difference in the mean intensity (i.e. number of worms per infected fish) of *N. hirame* in those two isolated flounder populations (Chapter II).

Various biotic and abiotic factors are known to influence population dynamics of parasites (Bush *et al.*, 2001). Such biotic factors include host density (Anderson and May, 1978), host immunity (Wassom *et al.*, 1986), and intra/inter-specific competition (Holmes, 1973; Dobson, 1985). Various abiotic factors such as pollution, oxygen content (Koskivaara *et al.*, 1991), salinity (Soleng and Bakke, 1997; Yoshinaga *et al.*, 2000), pH (Soleng *et al.*, 1999), and various metals (Poleo *et al.*, 2004), have been reported to influence the population dynamics of monogenean parasites. Among those, temperature is probably the best known key environmental factor (Andersen and Buchmann, 1998; Jackson *et al.*, 2001). In *N. hirame*, temperature has been reported to affect egg production (Tsutsumi *et al.*, 2002), egg hatching rate (Yoshinaga *et al.*, 2000), growth rate and survival (Tsutsumi *et al.*, 2003). However, these studies have dealt only with narrow ranges of temperature. Miyako Bay, where low intensity of *N. hirame* was observed, is located on the northern Pacific coast of Japan where the water temperature is relatively low. The average annual water temperature at Miyako Bay

in 2001 was 12.0°C, and the average temperature during the winter months (December to March) was only 5.6°C. In this region, the water temperature falls below 10°C during one third, or even more, of a year. To my best knowledge, there has been no report on the infection biology of *N. hirame* under such a low temperature condition. To understand the infection dynamics of *N. hirame* and to interpret our field observation, information on the infection biology of the parasite at low temperatures would be useful.

I hypothesize that the low water temperature is the limiting factor for population growth of *N. hirame* in the regions with low water temperature and underlying mechanisms for the low infection levels observed at Miyako Bay. The aim of the present study is to investigate the effects of low water temperature on the infection of *N. hirame*. Two laboratory experiments were conducted to quantify the attachment rate of *N. hirame* oncomiracidia and the subsequent worm development.

MATERIALS and METHODS

Parasite Eggs and Oncomiracidium Collection

N. hirame oncomiracidia, or hatched larvae, were obtained from experimentally infected young flounder (1-2 years old). Infected flounder were reared in a 2,000 L stock tank with minimal water flow to maintain the infection. Parasite eggs were collected by filtering the drainage water from the stock tank using a nylon net (mesh size 114 μm). Collected eggs were rinsed thoroughly with ozonated seawater, and incubated in a nylon mesh bag (mesh size 63 μm) suspended in a 20 L bucket with running seawater at 21 ± 1°C. Egg samples were observed daily and once wriggling larvae inside the eggs were observed, all eggs were transferred into a 4 L plastic container with still seawater (18°C) with increased aeration. Eggs started to hatch within 24-48 h after the transfer. To obtain only freshly hatched larvae, the container was left undisturbed without aeration so all the eggs settled onto the bottom. Then

the supernatant containing hatched larvae was discarded and replaced with fresh seawater. The remaining eggs were incubated further for 12 h and the oncomiracidia hatched within 12 h were used for the experiments.

Larval Attachment

The attachment of oncomiracidia was investigated *in vitro*, using 6 well plates and gill pieces. The density of freshly hatched larvae was estimated using a standard dilution technique and the number of parasite per volume of water was calculated. Antibiotics (Penicillin 100 iu/mL, Streptomycin 10,000 µg/mL, Amphoterin B 25 µg/mL) were added to the larval suspension to avoid bacterial and fungal contamination. The suspension was then stored at 5°C for 2 h. This was to incorporate the possible negative effects of temperature change onto the larval infectivity by giving all the larvae an equal temperature shock. Ten mL of suspension containing approximately 100 (97-103) larvae and a small gill piece (*ca.* 4 X 4 mm) that were excised freshly from uninfected juvenile flounder and rinsed thoroughly to remove excess blood, were added into each well of 6-well plates (Corning, NY, USA). Each plate was stored in the incubators at 5, 10, or 20°C as a control for 24 h. Considering the degradation of gill tissues, gill pieces were replaced every 3 h with fresh ones. Removed gill pieces were stored in 70% alcohol and later processed to dislodge the parasite using the methods described in the Chapter II. All the material that was filtered by fine nylon mesh was observed under the dissecting scope. All *N. hirame* were counted and the total number of attached parasites (number of attached parasite over 24 h) under the three temperatures were compared.

Parasite Development

To experimentally infect *N. hirame* to juvenile flounder, total of 80 fish, mean total length±SD = 91±4.0 mm, obtained from the local hatchery (Asahi Chigyo ltd.) were

exposed to 4,000 oncomiracidia (*ca.* 50 larvae per fish) in 40 L tanks at 18°C. After exposing for overnight, fish were randomly placed in one of the two experiment tanks. The initial temperature of the tanks were set at 18°C, then increase/decreased 1-1.5°C daily to the final temperature of 20°C or 8°C. It took a total of one week to change the temperature from 18°C to 8°C, and it has to be considered that the parasite development may be facilitated during the acclimation period. Fish were fed with commercial dry pellets and daily monitoring was performed.

Five fish from each tank were sampled at 10 or 15 days interval, except for the last two samplings which were done at longer intervals, to examine parasite development. At each sampling, the following measurements and observations were conducted: total length, body weight, haemoglobin content (Hb) of the peripheral blood and parasite load. Methods for counting the parasite followed the protocol described by Anshary *et al.* (2001) with minor modifications (Chapter II). The worms found from the buccal cavity were considered as adults, and immature parasites on the gill were further categorized into following 5 developmental stages based on the number of clamps as described in the previous chapter. The experiment was terminated at 105 days post exposure (PE).

Statistical Analyses

For all the data, normality was tested by Shapiro-Wilk test. The differences in total number of attached oncomiracidia between the three temperatures were tested using ANOVA followed by Tukey-Kramer HSD multiple comparison. Difference in the parasite densities and Hb between two rearing temperatures was analysed using Wilcoxon rank sum test.

RESULTS

Larval Attachment

Although *N. hirame* oncomiracidia were able to attach the gill pieces in the well-plate at all temperatures, the differences in temperature had significant effect on the cumulative larval attachment rate after 24 h (ANOVA, $F_{2, 17} = 56.54$, $p < 0.0001$; Fig. III-1). The mean (±SD) numbers of attached larvae at 5, 10 and 20°C were 11.7 ± 1.50, 22.5 ± 3.94 and 36.8 ± 5.74 %, respectively and significantly different from each other (Tukey-Kramer HSD). Most larvae attached to the gill tissue within the first 6 to 12 h and reached a plateau, except for 20°C which showed a continuous increase up to 21 h PE (Fig. III-1).

Parasite Development

Both parasite prevalence and mean intensity increased during the first several weeks of infection (Fig. III-2). The maximum observed mean intensities (±SD) at 8 and 20°C were 17.8 ± 7.53 worms at 35 days PE and 31.4 ± 7.33 worms at 21 days PE, respectively and they were significantly different from each other (Wilcoxon, $Z = -2.21$, $p = 0.027$; Fig. III-2). At 20°C, adult worms started to be observed by 35 days PE and their number continued to increase. The adult intensity reached peak at 45 days PE and the worm numbers rapidly decreased thereafter. Parasites were no longer found after 75 days PE. In contrast, the most developed worms on the fish reared at 8°C were stage III observed at 105 days PE. Although the parasite never reached maturity nor migrated to the buccal cavity in 8 °C treatment, gradual decline of the worm number was observed between 35-105 days (Fig. III-2).

A significant fluctuation in Hb was observed on fish reared at 20°C (Fig. III-3). Fish showed anaemic symptoms by 35 days PE, and seemed to be recovering at 60 days PE. At 45 days PE, the Hb was reduced to 94% of that at 1 day PE. There was a significant difference in Hb between the fish reared at two temperature was observed

(Wilcoxon rank sum, $Z = 2.59$, $p = 0.009$). All anaemic fish showed abnormally pale colouration on gills and internal organs, especially the liver. Although Hb of fish at 8°C showed slight reduction over time, no fish with apparent anaemic symptoms were observed.

The mortalities were observed only on fish reared at 20°C, and all were occurred between 41 and 45 days PE. The overall mortality was 12.5% for 20°C, and no fish were died in 8 °C group. None of the dead fish showed obvious signs of particular diseases or trauma, except anaemic symptoms. Because of the mortalities, number of fish sampling at 75 and 105 days PE for 20°C were reduced to 3 and 2 fish, respectively. The average fish size at the end of experiment were 112±0.0 mm for 20°C and 96±5.3 mm for 8°C.

DISCUSSION

The results of the present study confirmed the past studies with other monogeneans that both oncomiracidial attachment and post-larval development of *N. hirame* are temperature dependent. Although *N. hirame* oncomiracidia are still capable to infect fish under the extremely low temperature, 5°C, the attachment rate was greatly reduced. The cumulative attachment rate at 5°C was approximately 30% of that at 20°C. This suggests that the transmission rate of *N. hirame* is greatly reduced in the low temperature environment. However, the larval attachment was investigated *in vitro* using gill pieces in the present study, thus the rate may be underestimated compared to the natural infection level. Regardless of the unnatural experimental condition, reduced parasite infectivity strongly suggests the inhibition of *N. hirame* transmission during the winter in Miyako bay.

The temperature also had a strong influence on the development of *N. hirame*. On the fish reared at 20°C, large numbers of adult worm were observed by 35 days PE and subsequently reduced their number during 45 to 60 days PE. Worms were no longer

found after 75 days PE. The disappearance of the worms was simply due to the worm death or can be due to dislodgment of adults caused by the necrosis of the attaching tissue from the buccal cavity wall (Anshary and Ogawa, 2001). It is estimated that at 20°C, *N. hirame* takes a little less than 35 days to mature, and its lifespan on the host, lies somewhere between 45-75 days, probably around 55-60 days. This estimation is consistent with the past study which showed *N. hirame* reached maturity by 31 days PE at 20°C, and diminished by 66 days PE (Tsutsumi *et al.*, 2003). Yoshinaga *et al.* (2001) also reported a similar developmental pattern using flounder exposed to newly laid eggs.

On the other hand, the parasite development was drastically retarded at 8°C. *N. hirame* never reached maturity during the 105 days of observation period. The gradual reduction of immature worm intensity was also evident during the study. In addition, the maximum worm burdens observed at 8°C was nearly half of that at 20°C. These results indicate that immature parasites may have died or dislodged during unusually long premature period caused by the low water temperature. Such results indicate that parasite mortality is one of the underlying mechanisms causing the reduction of parasite abundance during winter to early spring observed in the field study (Chapter II). The mortality of immature worms is probably due to the unsuitable environmental condition (i.e. low water temperature) rather than host immune responses. Metabolic rate of fish, or most animals, correlates with environmental temperature, and in general, metabolic rate and immune reaction are suppressed under such low temperatures (Nikoskelainen *et al.*, 2004) Although the precise mechanisms are unknown, long period of low temperatures condition may affect *N. hirame* survival through altering the physiological condition of immature worm and cause a reduction in the feeding rate and/or efficiency of energy conversion.

In both the present study and the study by Tsutsumi *et al.* (2003), only small numbers of *N. hirame* could be found shortly after the exposure. However, the

parasite intensity rapidly increases within the next few weeks. Tsutsumi *et al.* (2003) came up with two possible explanations for this extremely low initial parasite intensity; 1) newly settled larvae, namely Stage 0 to Stage I, may have been overlooked because of their small body size, 2) the worms first attach and settled to somewhere other than gills, possibly onto the body surface, and then gradually migrated toward the gills. To overcome the problem of overlooking, I adapted the "stirring method" in this study, an effective method for dislodging the immature worms from the gill filaments (Anshary *et al*, 2001). In addition, I did not encounter any problems of counting oncomiracidia, even smaller, or similar to newly settled worms, during the attachment experiment. Therefore, it is unlikely that oversight is a sole cause for the unusually initial parasite intensity.

To our best knowledge, no study has yet to demonstrate the migration of *N. hirame*, or other gill monogeneans, from the body surface. However, larval *Heterobothrium okamotoi*, a gill parasite of tiger puffer, has been shown to settle onto the body surface, though their fate is unclear (Chigasaki *et al.*, 2000). In my preliminary experiment using CFSE vital stain (5(6)-carboxyfluoroscein deacetate succinimidyl-ester) (Molecular Probes, Inc., USA) and fluorescence microscope, a large number of deciliated *N. hirame* was observed on the body surface, particularly on the pectoral fin, after 12 h or exposure (Shirakashi, unpublished observation). More detailed studies are necessary to understand the initial settlement site of *N. hirame*.

In the present study, parasite maturation, progression of anaemia and occurrence of fish mortality were all coincided. Past studies have shown a strong correlation between the number of adult parasites and the host anaemic symptoms and concluded that the anaemia is mostly due to the adult worms rather than immature ones (Mushiake *et al.*, 2001). However, mass mortality caused by *N. hirame* infection has not been reported, though the fish infected with excessive numbers of worms show some mortality under the specific experimental conditions (N. Tsutsumi, personal

communication). The worm intensity in the present study is within the range of natural infection. For instance, the maximum mean intensity of 0 year old flounder observed in our field study at Obama Bay was 42 in 193 mm fish captured in December, 2002 (Chapter II). Whether *N. hirame* causes mortality in wild flounder is unclear, but this study provided possible effect of *N. hirame* on the survival of juvenile flounder.

In conclusion, the low temperature has negative effects on infection success of *N. hirame* and is likely the key limiting factor for the low parasite infection observed in Miyako Bay. However, considering *N. hirame* is a newly introduced pathogen in Japanese waters, the parasite may still be in progress of adapting to the new environment and there is a possibility of epidemics in the low temperature regions. In addition, recent climatological events including global warming and El Niño seem to initiate the outbreaks of the emerging diseases in various marine animals worldwide (reviewed in Harvell *et al.*, 1999). Long term monitoring of this invader parasite is strongly recommended to prevent further damage to flounder fisheries in Japan.

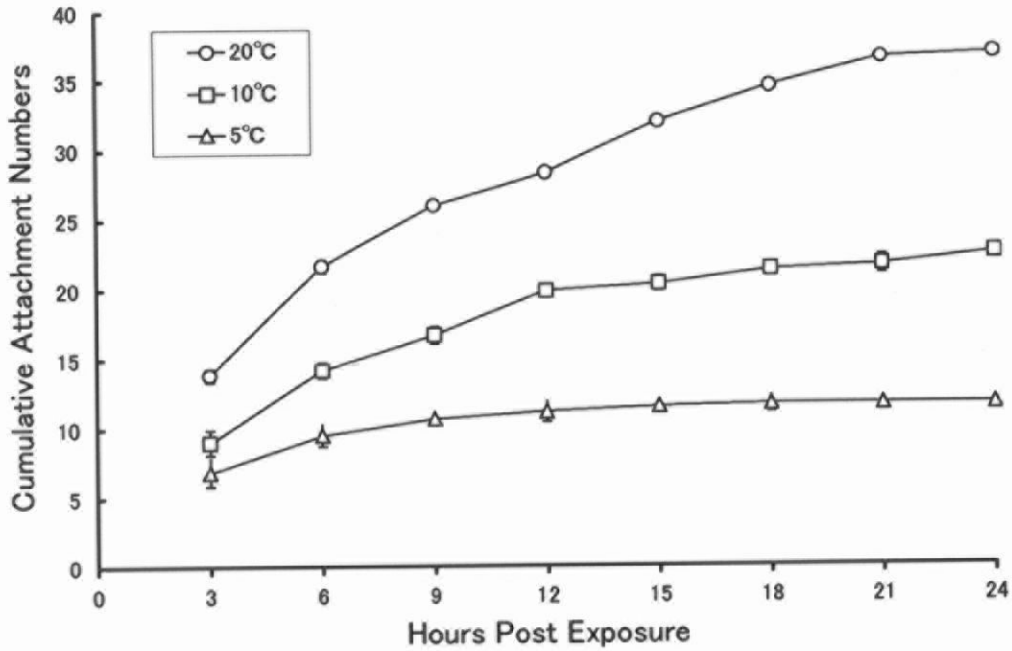

Figure III-1. Changes in average (±SE) cumulative rate of attachment *in vitro* to gill pieces by *Neoheterobothrium hirame* oncomiracidia at 5, 10, and 20℃ over 24 hours.

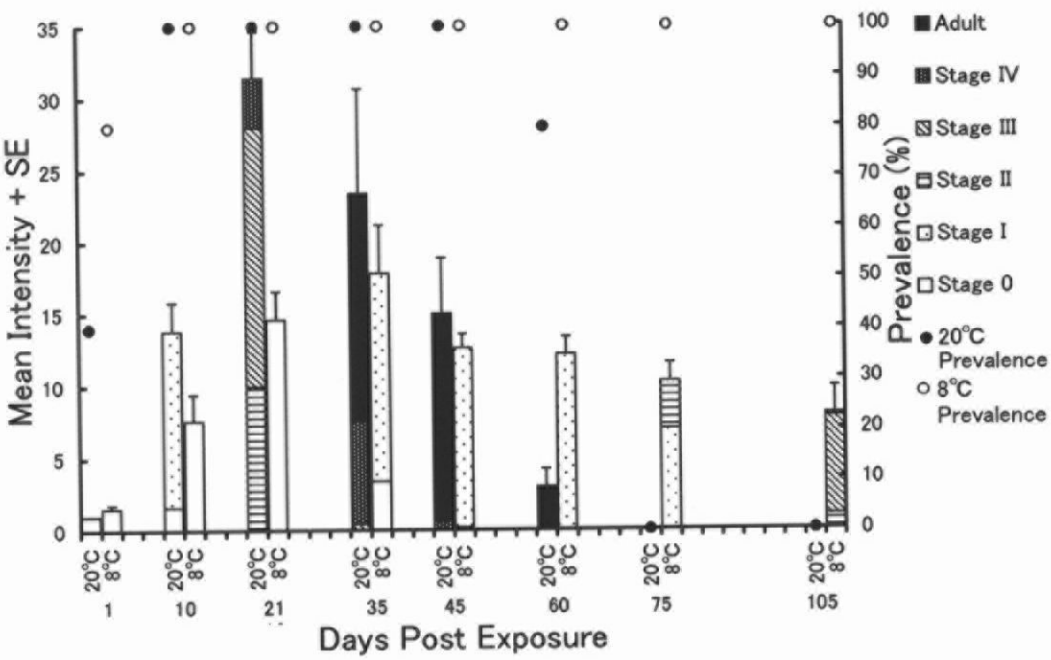

Figure III-2. Development of *N. hirame* on experimentally infected juvenile olive flounder at 8℃ and 20℃. Adult refers to worms inhabit in the buccal cavity. Stage 0 represents worms without clamps, and Stage I – IV, represents ones with 1-4 pairs of clamps, respectively.

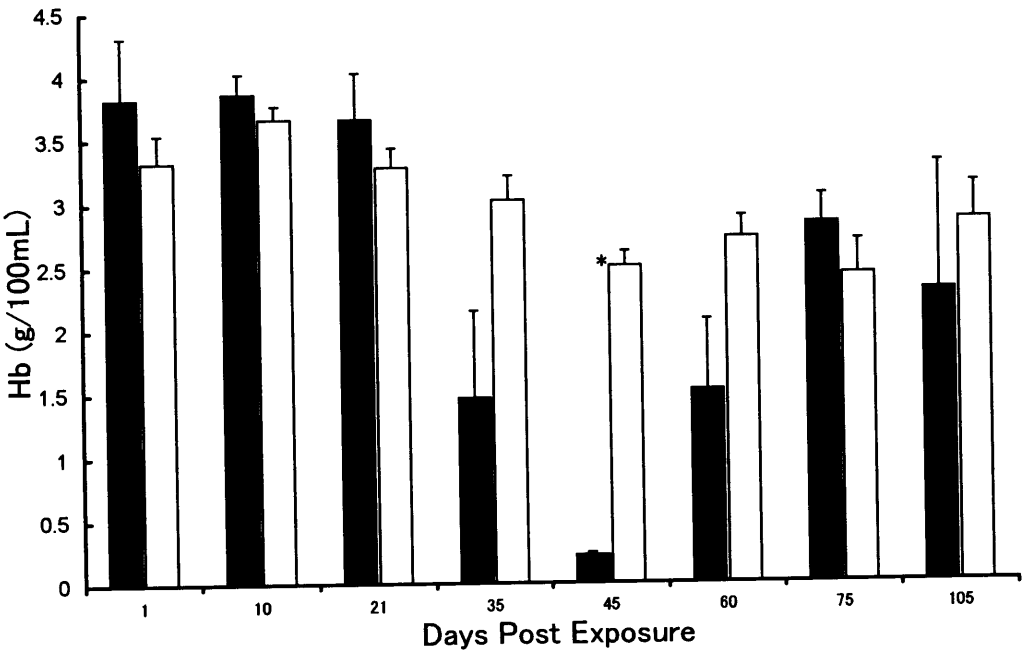

Figure III-3. Change in average (±SE) haemoglobin contents of infected olive flounder reared at 20℃, closed bar and at 8℃, open bar, over the 105 days post exposure. Asterisk indicates statistical differences between the two temperature (p<0.001).

LITERATURE CITED

Andersen, P. S. & Buchmann, K. 1998. Temperature dependent population growth of *Gyrodactylus derjavini* on rainbow trout, *Oncorhynchus mykiss*. *Journal of Helminthology*, **72,** 9-14.

Anderson, R. M. & May, R. M. 1978. Regulation and stability of host-parasite population interactions. I. Regulatory processes. *Journal of Animal Ecology*, **47,** 219-247.

Anshary, H. & Ogawa, K. 2001. Microhabitats and mode of attachment of *Neoheterobothrium hirame*, a monogenean parasite of Japanese flounder. *Fish Pathology*, **36,** 21-26.

Anshary, H., Ogawa, K., Higuchi, M. & Fujii, T. 2001. A study of long-term changes in summer infection levels of Japanese flounder *Paralichthys olivaceus* with the monogenean *Neoheterobothrium hirame* in the central Sea of Japan, with an application of a new technique for collecting small parasites from the gill filaments. *Fish Pathology*, **36,** 27-32.

Anshary, H., Yamamoto, E., Miyanaga, T. & Ogawa, K. 2002. Infection dynamics of the monogenean *Neoheterobothrium hirame* among young wild Japanese flounder in the western Sea of Japan. *Fish Pathology*, **37,** 131-140.

Bush, A. O., Fernandez, J. C., Esch, G W. & Seed, J. R. 2001. Factors influencing parasite population. In: *Parasitism: The diversity and ecology of animal parasites* (Ed. by Bush, A. O., Fernandez, J. C., Esch, G W. & Seed, J. R.), pp. 331-337. Cambridge: Cambridge University Press.

Chigasaki, M., Nakane, M., Ogawa, K. & Wakabayashi, T. 2000. Standardized method for experimental infection of tiger puffer *Takifugu rubripes* with oncomiracidia of *Heterobothrium okamotoi* (Monogenea: Diclidophoridae) with some data on the oncomiracidial biology. *Fish Pathology*, **35,** 215-221.

Dobson, A. P. 1985. The population dynamics of competition between parasites. *Parasitology*, **91,** 487-498.

Harvell, C. D., Kim, K., Burkholder, J. M., Colwell, R. R., Epstein, P. R., Grimes, D. J., Hofmann, E. E., Lipp, E. K., Osterhaus, A., Overstreet, R. M., Porter, J. W., Smith, G W. & Vasta, G R. 1999. Review: Marine ecology - Emerging marine diseases - Climate links and anthropogenic factors. *Science*, **285,** 1505-1510.

Holmes, J. C. 1973. Site selection by parasitic helminths: interspecific interactions, site segregation, and their importance to the development of helminth communities. *Canadian Journal of Zoology*, **51,** 333-347.

Jackson, J. A., Tinsley, R. C. & Du Preez, L. H. 2001. Differentiation of two locally sympatric *Protopolystoma* (Monogenea: Polystomatidae) species by temperature-dependent larval development and survival. *International Journal for Parasitology*, **31,** 815-821.

Koskivaara, M., Valtonen, E. T. & Prost, M. 1991. Dactylogyrids on the gills of roach in central Finland - Features of infection and species composition. *International Journal for Parasitology*, **21,** 565-572.

Mushiake, K. 2001. Epizootiology of *Neoheterobothrium* infection in wild Japanese flounder. *Fish Pathology*, **36,** 252-253.

Mushiake, K., Mori, K. & Arimoto, M. 2001. Epizootiology of anemia in wild Japanese flounder. *Fish Pathology*, **36,** 125-132.

Nikoskelainen, S., Bylundb, G & Liliusa, E. M. 2004. Effect of environmental temperature on rainbow trout (*Oncorhynchus mykiss*) innate immunity. *Developmental & Comparative Immunology*, **28,** 581-592.

Poleo, A. B. S., Schjolden, J., Hansen, H., Bakke, T. A., Mo, T. A., Rosseland, B. O. & Lydersen, E. 2004. The effect of various metals on *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon (*Salmo salar*). *Parasitology*, **128,** 169-177.

Soleng, A. & Bakke, T. A. 1997. Salinity tolerance of *Gyrodactylus salaris* (Platyhelminthes, Monogenea): laboratory studies. *Canadian Journal of Fisheries and Aquatic Sciences*, **54,** 1837-1845.

Soleng, A., Poleo, A. B. S., Alstad, N. E. W. & Bakke, T. A. 1999. Aqueous aluminium eliminates *Gyrodactylus salaris* (Platyhelminthes, Monogenea) infections in Atlantic salmon. *Parasitology*, **119,** 19-25.

Tsutsumi, N. 2004. The origin and spreads of the monogenean parasite *Neoheterobothrium hirame* infecting wild olive flounder. In: *Department of Aquatic Bioscience*, 157. pp. Tokyo: The University of Tokyo.

Tsutsumi, N., Mushiake, K., Mori, K., Arimoto, M., Yoshinaga, T. & Ogawa, K. 2002. Effects of water temperature on the egg-laying of the monogenean *Neoheterobothrium hirame*. *Fish Pathology*, **37,** 41-43.

Tsutsumi, N., Yoshinaga, T., Kamaishi, T., Nakayasu, C. & Ogawa, K. 2003. Effects of water temperature on the development of the monogenean *Neoheterobothrium hirame* on Japanese flounder *Paralichthys olivaceus*. *Fish Pathology*, **38,** 41-47.

Wassom, D. L., Dick, T. A., Arnason, N., Strickkand, D. & Grundmann, A. W. 1986. Host genetics: a key factor in regulating the distribution of parasites in natural host populations. *Journal of Parasitology*, **72,** 334-337.

Yoshinaga, T., Kamaishi, T., Segawa, I., Yamano, K., Ikeda, H. & Sorimachi, M. 2001. Anemia caused by challenges with the monogenean *Neoheterobothrium hirame* in the Japanese flounder. *Fish Pathology*, **36,** 13-20.

Yoshinaga, T., Segawa, I., Kamaishi, T. & Sorimachi, M. 2000. Effects of temperature, salinity and chlorine treatment on egg hatching of the monogenean *Neoheterobothrium hirame* infecting Japanese flounder. *Fish Pathology*, **35,** 85-88.

Chapter IV. Effects of *Neoheterobothrium hirame* on the behaviours and susceptibility to predation in juvenile olive flounder

INTRODUCTION

Predation is probably the ultimate cause of death in wild animals. Any moribund or abnormal animals are likely to be consumed by others. Juvenile flounder are highly susceptible to predation by both fish and crustacean predators and the high predation pressure causes significant reduction of released seedlings (Yamashita, 1997). Field studies have revealed that released flounder are heavily preyed upon by flathead, *Platycephalus* sp., greeling, *Hexagrammos* sp., various crabs, their larger conspecific 1-2 years old flounder, and several others (Yamashita, 1997; Hossain *et al.*, 2002).

Abnormal behaviours of hatchery-reared (HR) fish are believed to play important roles in their higher susceptibility to predation. Past studies comparing behaviours of HR and wild fish flounder have shown that HR fish tend to spend more time in the water column, have reduced burrowing ability, and have aberrant feeding behaviours (Yamashita, 1997; Furuta, 1998; Kellison *et al.*, 2000). Therefore, it is conceivable that if *N. hirame* infection alters the host's behaviours, infected fish would become more susceptible to predation and resulting in decline of host population.

Helminth parasites are well known for altering their host's behaviour in a way that makes the host more susceptible to predation. Behavioural changes associated with infection are highly diverged, ranging from simple modifications of existing behaviours to expression of the novel behaviours (Moore, 2002). The most commonly reported behavioural change associated with parasite infection is changes in the activity (Poulin, 1994). Infected animals often become hyper/hypo active compared to the uninfected conspecifics. Consequences of such behavioural changes of infected hosts are often ingestion by other animals. For example, killifish (*Fundulus parvipinnis*) infected with a brain-encysting trematode *Euhaplorchis*

californiensis increased the "conspicuous behaviours" such as flashing, contorting, shimmying and jerking, and had higher mortality to bird predators (Lafferty and Morris, 1996). However, such behavioural studies of parasitized animals are mostly conducted using a parasite with a multi-host complex lifecycle as researchers tend to be interested in the "parasite increased trophic transmission"(PITT). Behavioural study involving single-host monogenean parasites are limited.

N. hirame causes anaemia to its host and such physiological impediment could influence various aspects of host's biology. The aim of this chapter is to determine the effects of *N. hirame* on the various behaviour and susceptibility to predation of juvenile flounder. This is the first report of behavioural changes in *N. hirame* infected olive flounder.

MATERIALS and METHODS

Experimental Infection

Uninfected juvenile flounder obtained from the local hatchery were kept in a large stock tank (WxDxH, 230x95x60 cm) laid with 3 to 4cm of beach sand (grain size 1.21±0.31 mm). The stock tank was provided with flow-through ozonated seawater at the ambient water temperature. Fish were maintained with commercial dry pellet for at least one week prior to the experimental infection for acclimation to the laboratory condition and to make certain that they were healthy fish. Fish quickly adapted to the new environment and started to show burrowing behaviour within a week after transfer. Most fish have become completely camouflaged and hidden in the sand by 2 weeks post transfer.

To experimentally infect juvenile flounder with *N. hirame*, the same general protocol was used for all the experiments. Parasite eggs were collected by filtering the drainage water from the tanks containing infected flounder using a fine nylon net (mesh size 114 μm). Eggs were then rinsed thoroughly with ozonated seawater, and

incubated in a mesh bag (mesh size 63 μm) hanged in a 20 L bucket with aeration and running seawater at 24 ± 1°C. To monitor egg development and to estimate the time of egg hatch, a minute portion of eggs were observed daily. Once wriggling larvae inside the eggs were observed, all eggs were transported into a 4 L beaker with still seawater (23 to 25°C) with increased aeration. Majority of eggs hatched within 24-48 hrs. Numbers of oncomiracidium in 1 mL suspension was counted with a few drops of Lugol solution that immobilises and stains the fast-moving larvae. A total number of available oncomiracidia was estimated using the standard dilution technique.

Exposure of juvenile flounder to newly hatched *N. hirame* oncomiracidia was conducted in cylindrical exposure tanks containing 40 L of lightly aerated seawater (18-22°C depending on the room temperature at the time of experiments). Thirty to fifty fish were placed in each tank and estimated volume suspension required to contain 900-1500 oncomiracidia (30 larvae per fish), or an equal amount of seawater for the controls, was added into the tanks. After an overnight exposure, fish were transferred back to the separate stock tanks and kept as described above.

To minimise the human interference and conditioning to the feeding, fish were left undisturbed as much as possible and the food was given at indeterminate time. Once the infected flounder showed the anemic symptoms, namely discolouration of the gills, 10 or 20 fish from each group were sacrificed as an initial samples. Following measurements were conducted for the sampled fish: total length (TL), body weight (BW), haemoglobin contents (Hb), and *N. hirame* infection status. The method for counting adults and immature worms used the same procedures described in the Chapter II.

25h Monitoring Experiment

The monitoring experiment was conducted on September 15, 2003. Ten randomly selected infected or uninfected juvenile flounder (average total length ± SD,

10.67±0.69 cm) were placed in one of the 6 monitoring tanks (WxHxD 49x29x33 cm), 3 tanks for each group, set in the indoor laboratory with controlled photoperiod (12L : 12D, light hours 6:00-18:00). Each monitoring tank was laid with beach sand (4-5 cm in thickness) and provided with running seawater at constant temperature of 20°C. In the laboratory, one 40W incandescent light bulb was placed behind the thick curtain to provide very dim light, so the laboratory was not in a complete darkness when all the fluorescent lights were turned off.

Fish were acclimatised for an overnight in the monitoring tank before starting the observation. Fish in each tank were monitored for their activity and burrowing behaviour at an hour interval for 25 hrs (16:00-17:00). At each monitoring period, numbers of fish showing obvious swimming behaviour (active fish) during the 1 min observation period were recorded. After the observation, each tank was photographed by a digital camera for the image analyses of burrowing behaviour. Night observations were performed with the aid of a dim LED flashlight.

All digital images were analysed to estimate the body surface area of each fish that was exposed from the substrate (exposure area). The exposure area was estimated to the nearest 10% using following criterions: 10%; only eyes are visible, 20%; part of head and operculum, 30%; entire head and operculum, 40-90%; eyes, head and parts of the body depending on the exposed area, 100%; whole head and the body. The exposure area was estimated from the fish in 4 out of 6 monitoring tanks, as other 2 tanks were video monitored and were left undisturbed. After the experiments, each fish was measured for TL, SL, BW, Hb and *N. hirame* infection.

Swimming Performance

Swimming performance of 20 infected- and uninfected juvenile flounder were compared following the general methods described by Hashimoto *et al.* (1996) with some modifications. A randomly selected infected or control fish, average total

length ± SD (here and elsewhere otherwise stated) 10.02±0.65 cm, was placed in the simple flow-water experiment tank and their swimming performance against the water current was measured.

The experiment tank is consisted of a mesh chamber (35x9.5x10 cm, mesh size 1x1 cm), a glass tank (WxHxD = 65x35x30 cm), and 2 electrical pumps which circulate the water in the aquarium (Fig. IV-1). The pumps suck the water from the bottom corner and discharge the water from the square "shower head", a plastic box with numerous holes (2 mm in diameter). The mesh chamber is placed against the shower head in an angle (*ca* 10°) so that the water current pushes the flounder away from the bottom of the chamber, prevents them from holding onto the bottom and forces them to swim. The water temperature of the experiment tank was set at constant temperature of 24°C which was consistent with the temperature of the stock tank at that time. The average water flow at the front, the middle, and the end parts of the chamber were 0.44±0.04, 0.35±0.02, and 0.25±0.03 m/s, respectively.

Each fish was placed in a mesh chamber and video recorded their swimming performance. When fish did not start swimming within the first 30 sec, glass rod stimulation was given to induce swimming. Fish were considered exhausted and video recording was terminated when following conditions persisted for more than 10 seconds: not facing against the water current, part of the body touching the backside of the chamber, not showing obvious fin movements, or combination of those. Fish were considered as "resting" when they regain swimming behaviour from those conditions within 10 sec. and were kept monitored until exhaustion. This "10 sec. rule" was applied based on the preliminary trial in which 95% of fish regain swimming after resting for maximum of 7 sec., and fish rested longer did not. Total swimming time, calculated as total observation time minus resting time, was compared between infected and control fish. After the experiments, TL, SL, BW, Hb measurements and parasite counting were performed on each fish.

Predation Experiment

Total of 4 experimental trials (Trial 1 in July 2002, Trial 2 and Trial 3 in February, 2003, and Trial 4 in September, 2003) were conducted to assess the effect of *N. hirame* on susceptibility to predation of juvenile flounder. At each trial, the survivals of infected and uninfected juvenile flounder were compared after they were cohabitated with predator fish in a large experiment tank for 96 hours. Large flounder (2-3 yeas old, TL 25-33 cm) were used as a model predator because past studies have shown that wild juvenile flounder are frequently preyed upon by their larger conspecifics (Yamashita, 1997).

Each indoor-experimental tank (WxHxD, 200x70x120 cm) was provided with running ozonated seawater (uncontrolled temperature), aeration, and 50 L of beach sand (3-4 cm depth). Lighting was of natural photoperiod. However, the tanks were perhaps not in complete darkness during the night as there were some light sources nearby the building and windows were located by the tanks. The water temperatures at the trial 1, 2, 3, and 4 were 22.5, 14.5, 14.6 and 21.8℃, respectively. Four experimental tanks were used as replicates for each triail, except for trial 1, which used duplicates. Tanks were left undisturbed during the experiment with minimum human interferences.

Ten infected and uninfected prey juvenile flounder were placed in each experimental tank with 3 predator flounder. Mean TL of prey fish used for the trial 1, 2, 3, and 4 were 8.1±0.5, 8.9±0.8, 8.9±0.7, and 9.5±0.7 cm, respectively. The size of predator flounder ranged from approximately 28-32 cm. Prey flounder were acclimatized in the experiment tank for 24 hrs before predator fish were added. Prior to the experiments, 10 or 20 infected and control fish were sacrificed as initial samples to obtain the infection status and Hb contents of each group, as these measurements cannot be conducted after fish were ingested. For easy identification of prey fish, left

or right pelvic fin was clipped with sharp scissors. The side of fin clipping was randomized between the tanks and between the trials.

Statistical Analyses

All the data were tested for normality using Shapiro-Wilk test and transformed to meet the assumptions for parametric analyses if necessary. Swimming time was log-transformed and *t*-test was used for the comparison between the treatments. Spearman correlation test was used for all correlation analyses. A non-parametric Wilcoxon Rank Sum test was performed for the analyses involving Hb. Paired *t*-test was applied for the analyses of the predation experiment as the infected and control survivors from each tank were corresponding to each other.

RESULTS

Experimental Infection

The experimental infection resulted in 100% infection in all the observed fish and the controls remained free of the parasite. The overall mean intensity was 16.34±10.66 worms for the behavioural experiments, and 13.37±8.85 worms for the predation experiment. Almost all the infected fish showed severe anemic symptoms, namely discoloured gills and low Hb values, at the time of the experiments. The mean Hb of infected fish and controls were 0.35±0.27 and 3.69±0.49 g/100mL for behavioural experiments and 0.56±0.34 and 3.60±0.48 g/100mL for the predation experiments, respectively. Infected fish showed significantly lower Hb in both the behavioural (Wilcoxon, $Z = -8.72$, $p<0.0001$) and the predation experiments (Wilcoxon, $Z = -6.66$, $p<0.0001$). The negative correlation between Hb and the parasite intensity was observed in the behavioural experiments (D= -0.88, r = -0.7558, $p <0.0001$, N = 100). None or only minor mortalities were occurred in all experimental infection. When mortality occurred, it was observed from both infected and control fish.

25h Monitoring

Numbers of active fish over 25 h were plotted on Fig. IV-2. Controls showed no activities during the first 3 h, but gradually increased the activity levels thereafter. The activity in control fish was relatively consistent throughout the experiment with the highest average active fish was 1.7 fish per tank. In contrast, infected fish showed a clear peak of activity. The numbers of active fish increased after 22:00, peaked at 3:00 (4 active fish/tank), and rapidly decreased thereafter. The overall average of active fish was significantly higher in the infected fish than the controls (Wilcoxon, Z=2.83, p=0.0046).

Burrowing ability indicated by the exposed body surface area also differed significantly between the groups. Average exposure area at each observation period from each experiment tank is plotted in Fig. IV-3. Fish in the duplicate tanks showed a similar pattern of consistently higher exposure area in infected fish. Overall exposure area in the infected fish was significantly higher than that of controls (Wilcoxon, Z=13.59, p<0.0001). Unlike activity, neither group showed clear temporal pattern in the burrowing activity.

Swimming Performance

The mean (± SD) total swimming time of infected and uninfected juvenile flounder was 451.80±627.60 and 118.60± 222.87 sec, respectively (Fig. IV-4). *N. hirame* infection significantly reduced the swimming time by more than 70 % (t-test, $F_{1, 39}$ = 8.94, p = 0.0049). The swimming time significantly and positively correlated with Hb (Spearman, D= 0.44, r = 0.33, p = 0.0046, N = 40, Fig. IV-5), and negatively correlated with the parasite intensity (D = -0.37, r = -0.30, p = 0.018, N =4 0, Fig. IV-6). Also, the larger fish tended to swim longer time (D = 0.32, r = 0.21, p = 0.0426, N = 40).

Predation Experiment

Juvenile flounder were readily preyed upon by the large flounder under the experimental conditions. Dissections of 2 predator flounder soon after the experiment confirmed the ingestion of juvenile flounder. No carcass or body tissue remaining was found in or around of the experimental tanks, thus all fish disappeared during the experiment were considered as being ingested.

Both *N. hirame* infection and the trials had a significant effect on the numbers of survivors (Table 1, Fig. IV-7). In overall average, *N. hirame* infection reduced the survival rate by approximately 25%. The survival rate differed between the trials, and the Tukey-Kramer HSD test showed that more fish were eaten in the trials conducted in the summer (trial 1 in July and trial 4 in September.) compared to the trials conducted in winter (trial 2 and 3 in February.) ($p < 0.05$). The interaction between the parasite infection and the trial was not significant (Table 1), indicating *N. hirame* affected fish survival regardless of the seasonal differences between the trials.

DISCUSSION

The results of the presents study showed that *N. hirame* infection affects activity, burrowing behaviour, swimming performance, and finally the susceptibility to predation of juvenile flounder. Infected and anaemic flounder tended to be more active with a different activity rhythm, and had the reduced ability to burrow and to swim. In addition, infection made juvenile host more vulnerable to the predation by large flounder. These results strongly suggest that *N. hirame* induces a significant additive mortality in the wild juvenile flounder.

Effects on Activity

In general, flounder are considered as a diurnal fish because they feed predominantly during the daytime using their visual sensor (Hirota *et al.*, 1990).

However, past studies have shown that flounder can be highly active during the night or in the darkness. Both hatchery-reared and wild juvenile flounder, presumably free of *N. hirame*, showed the high frequency of pelagic swimming during the night while they tended to stay close to the bottom, and buried into the substrate during the daytime (Nashida *et al.*, 1996; Miyazaki *et al.*, 1997,).

Such diel differences in swimming patterns can be a strategy to avoid specific predators (Miyazaki *et al.*, 2004). Benthic swimming and burying behaviour make flounder less conspicuous to the diurnal predators while pelagic swimming may reduce the risk of being captured by nocturnal bottom-feeding predators. Pelagic swimming can also be an efficient mode of migration. Flounder swim upward to the water column and glide so that they can travel a long distance with minimal energy output (Kakimoto *et al.*, 1979; Nashida *et al.*, 1996). In the present study, only infected flounder showed an apparent activity rhythm. Infected fish were highly active between 22:00-6:00 with the peak at 3:00. However, it is unclear whether these fish performed pelagic or benthic swimming during those periods.

Hyper- and hypo-activities are the most commonly observed behavioural changes in parasitized animals (Poulin, 1994). Such alterations in activity are often interpreted as underlying mechanism for the increased predation rate. Kellison *et al.* (2000) showed that HR summer flounder spent more time in the water column compared to the wild flounder and were more vulnerable to the predation by blue crab (*Callinectes sapidus*). Similarly, the present study demonstrated that infected fish become highly active during the night and they were subsequently confirmed as being more vulnerable to the predation. Abnormally active animal may be more conspicuous and may have increased chances for encountering predators. Although the higher activity in the darkness might not make the fish more vulnerable to the visual predation, it is possible that active fish are more likely to be detected by nocturnal crustaceans or spotted by visual predators in the shallow water under the

moonlight.

In the present study, it is unclear whether the infected fish became hyper-active during the night, or controls were generally less active in the unfamiliar experimental conditions. HR juvenile flounder showed more frequent off-bottom swimming in the tank when they were placed in a tank without sand than in a tank with substrate (Miyazaki *et al.*, 1997). This is probably associated with the stress from not having the substrate. The association between stress and presence of substrate was further confirmed by another study in which flounder in the tank with sand substrate showed only a slight change in a heart rate when light stimuli were given, whereas fish in the bare tank significantly increased their heart rate (Zhang *et al.*, 1996). *N. hirame* infection is probably a great stress to the flounder. Irritation and other stress from the infection may be the underlying mechanism for their higher activity level. In addition, anaemia associated with parasitic infection may also increase their activity. For example, Mouristen and Jensen (1997) showed that anaemic amphipod caused by infection by the trematode *Maritrema subdolum* increased the surface activity which made them more vulnerable to the waterfowls. To assess the precise effects of anaemia on the flounder behaviours, experiments using flounder with artificially induced anaemic condition would be ideal.

Effects on Burrowing Ability

The reduced burrowing ability observed in the infected fish could have significant meanings for juvenile flounder in the wild. Flounder are highly adapted to the benthic habitat with their flat body, asymmetrical eye position, and camouflaged body colour. Flounder, and other flatfish, are well known for their burrowing ability, and they have specific preference for the types of substrate (Gibson and Robb, 2000; McConnaughey and Smith, 2000; Stoner and Titgen, 2003).

Their burrowing behaviour is considered to have an important role in both feeding

and predation avoidance. Flounder is a "sit and wait" type predator in which they hide in the substrate being invisible, and wait to ambush until food animals come nearby. In addition, flounder are less likely to be detected by predators (and prey) by being hidden in the substrate. It has been shown that juvenile flounder (Yasunaga and Koshiishi, 1980) and plaice (Ansell and Gibson, 1993) are more vulnerable to the fish predators when they are placed in the tank without substrate than in the tank with sand substrate. Similarly, HR juvenile flounder have shown to have the poorer burrowing ability (Yamashita, 1997 and references therein) and are more likely eaten by *C. sapidus* compared to wild ones (Kellison *et al.*, 2000). Thus, the deficient burying ability could have strong negative effects on feeding success as well as escaping from the fish and crustacean predators. High exposed body surface of infected fish during the night time is likely associated with their increased activity level. However, unlike the activity, there was no clear diel rhythm in the burrowing behaviour. This indicates that the infected fish were exposed from the substrate regardless of their activity level.

The underlying mechanism behind the reduced burrowing is unclear. The infected fish were severely anemic and the energetic loss from reduced blood level may impair their overall physical performances, though they had higher activity level. In the present study, the correlation between Hb and exposed body area could not be analysed as fish were not individually marked. An effective marking method which do not interfere fish behaviour is necessary and should be established for further investigations.

Effects on Swimming Performance

In addition to the altered activity and the reduced burrowing ability, *N. hirame* also had a negative effect on host's swimming performance. Although long lasting swimming performance may be less important for demersal flounder unlike for the pelagic fish, swimming is the fundamental mode of locomotion and is the key aspects

of daily activities. These include migration, reproduction, feeding, and escape responses. Impediment swimming performance or reduced stamina resulted from *N. hirame* infection can affect all of those essential behaviours and could be detrimental to the wild fish.

It is indisputable that reduced swimming performance is caused by depleted haemoglobin concentration caused by hematophagia of *N. hirame*. This is supported by the significant positive correlation between Hb level and total swimming time observed in the present study. Correlations between severity of anaemia and the number of adult *N. hirame* has been reported in both field and laboratory studies (Mushiake *et al.*, 2001; Yoshinaga *et al.*, 2001). Therefore, the wild flounder in *N. hirame* abundant waters are likely to be suffered from impediment swimming performance.

Fast (or burst) swimming is also an important element in flounder survival. Numbers of studies have reported the negative effects of parasites on the swimming performance of various fish species (reviewed by Barber *et al.*, 2000). Fish require extra oxygen during such burst swimming and they can cope with high demand of oxygen by releasing erythrocytes from the spleen (Nanba, 2002). Low Hb of infected fish may hinder this. Moreover, physical disturbance from large worms attached to the buccal cavity wall and gills may interfere the opercular jetting, a discharge of water from the opercular valve, during the fast escape response (Brainerd *et al.*, 1997). However, Miyazaki *et al.* (2004) compared the fast escape swimming between HR and wild flounder and showed no apparent difference.

The present study only assessed the long lasting swimming performance, but it is also important to assess the effects of *N. hirame* on the burst swimming of flounder as well as the cruising behaviour. The preliminary experiments showed that the mechanical stimuli (poked with grass rod, finger, metal stick, etc.) are not an effective method for consistently inducing burst swimming. Fish showed enormously variable

reactions to the stimuli and many showed no response. Therefore, the development of more refined techniques for inducing burst swimming is necessary to further investigate this matter. The cruising behaviour in flounder may play important role in the migration. Further studies are necessary to assess other swimming behaviours of infected flounders.

Predation Experiment

Large flounder readily preyed on juveniles in the experimental condition. Lower survival of the infected flounder was repeatedly observed, except in the trial 3, in which only a few fish were eaten regardless of infection. Predator flounder seemed to be less active, and had reduced appetite during the winter, probably due to the low water temperature. As seawater used for the experiment was of natural temperature, difference in temperature might have caused a difference in feeding activity between the trials.

All of the behavioural changes observed in the present study could have contributed to the higher predation rate of the infected juvenile flounder. Flounder are considered as being most vulnerable to the predators when they are in the water column (Noichi, 1997). Therefore, as mentioned earlier, higher activities and reduced burrowing ability may increase the predation rate because of increased conspicuousness and encountering rate. Furthermore, reduced swimming performance could impair the escape response.

Numbers of studies have shown the behavioural changes of parasitized animals in ways that appear to enhance hosts vulnerability to the predation (Holmes and Bethel, 1972; Barnard and Behnke 1990; Poulin, 1995). However, experimental studies that demonstrated the direct effect of parasite on the host predation rate are rare (Bethel and Holmes, 1977; Brassard *et al.*, 1982; Lafferty and Morris, 1996). Moreover, most of those studies dealt with the parasite species that utilise trophic transmission

(transmission through ingestion of intermediate host) (Poulin, 1994). For parasites with a single-host direct lifecycle like monogenea, death of the host usually means their own death. Therefore, the behavioural changes and higher predation rate observed in the present study cannot be adaptive to *N. hirame*, but rather it is merely a side effect of the infection. Such highly pathogenic traits should be selected against, and the host-parasite interaction should evolve toward more benign outcome (Bush *et al.*, 2001). Hence, the high pathogenicity of *N. hirame* on olive flounder implies their relatively short interaction period and supports that N. hirame has recently been introduced.

Various crustaceans and fishes have been reported to prey upon juvenile flounder, and larger flounder is one of them. Manderson *et al.* (2000) showed that 0-year winter flounder was a dominant prey of summer flounder of age-1+. Field observations suggested that flounder of 1-2 years old are one of the main fish predators of juveniles (Yamashita, 1997 and references therein; Furuta, 1998). Cannibalism in flounders is recognised as a major cause of mortality in both unnatural hatchery and laboratory environment (Burke *et al.*, 1999; Kellison *et al.*, 2002), and in the wild (Noichi *et al.*, 1993; Yamashita *et al.*, 1993; Furuta, 1998). Therefore, a usage of large flounder as predator in the study was relevant and may represent the wild conditions. Flounder predator can also act as a model fish predator, thus *N. hirame* infection probably increase the predation by other piscivorous fish.

There is accumulating evidence that micro- and macro-crustaceans are major predators of juvenile flounder. It was estimated that a crab, *Charybdis japonica*, was responsible for the loss of more than one third of the stocked juvenile flounder within the first week of releasing (Asahi News Paper*). Field studies also demonstrated high carnivorous activity of micro-crustaceans. Flounder left at the ocean bottom in the mesh cage were completely consumed except for the bones within 24 hrs (Satoshi Shiozawa, personal communication). In the experimental condition, the carnivorous

amphipod *Scopelocheirus onagawae* has been shown to be more attracted to the wounded and moriband flounder and consumed them within 24 hours (Ide *et al.*, 2004).

In my preliminary experiment, female swimming crab, *Portunus trituberculatus*, (carapace width approx. 17cm) were shown to be capable of capturing live juvenile flounder. However, the wild swimming crab are apparently very sensitive to the environmental changes and some individuals did not show any appetite under the experimental conditions (Shirakashi unpublished data). Experiment using such sensitive animals raises problems in the repeatability of the experiment. In addition, although I was succeeded in collecting carnivorous micro-crustaceans using specially designed traps, their numbers were insufficient to conduct predation experiment. Successful establishment of the experimental methods in these studies will provide additional information about predation of juvenile flounder in the wild.

The results of the present study suggest that *N. hirame* infection facilitate the predation of juveniles by larger flounder and possibly by other fish predators. As predation is known to be a key biotic factor shaping the size of a prey population, *N. hirame* infection likely reduce the juvenile population. Field studies have revealed that *N. hirame* prevalence can be as high as 100% and the mean intensity could be than 20 worms per fish (Chapter II) at some time of the year. Reduction of the population of juvenile fish due to increased predation can be expected in such heavily infested waters. However, it has to be noted that this study is merely experimental under unnatural laboratory conditions. Experiments under more natural conditions would be useful to clarify the parasite effect on wild fish. This study demonstrated the effect of *N. hirame* on the individual-level, however, it is important to determine the population-level consequences of such effects to understand the relationship between population decline and the *N. hirame* epidemic in Japanese water.

*Asahi News Paper Nov. 13, 2004.

Table 1. Summary of ANOVA statistics for effects of *N.hirame* infection on the survival rate of juvenile flounder from predation.

Source of Variation	D.F.	MS	F	*p*
Trial	3	24.07	23.21	<0.0001
N. hirame Infection	1	23.01	22.19	0.0002
Trials*Infection	3	2.17	2.09	0.1369
Error	18	1.04		

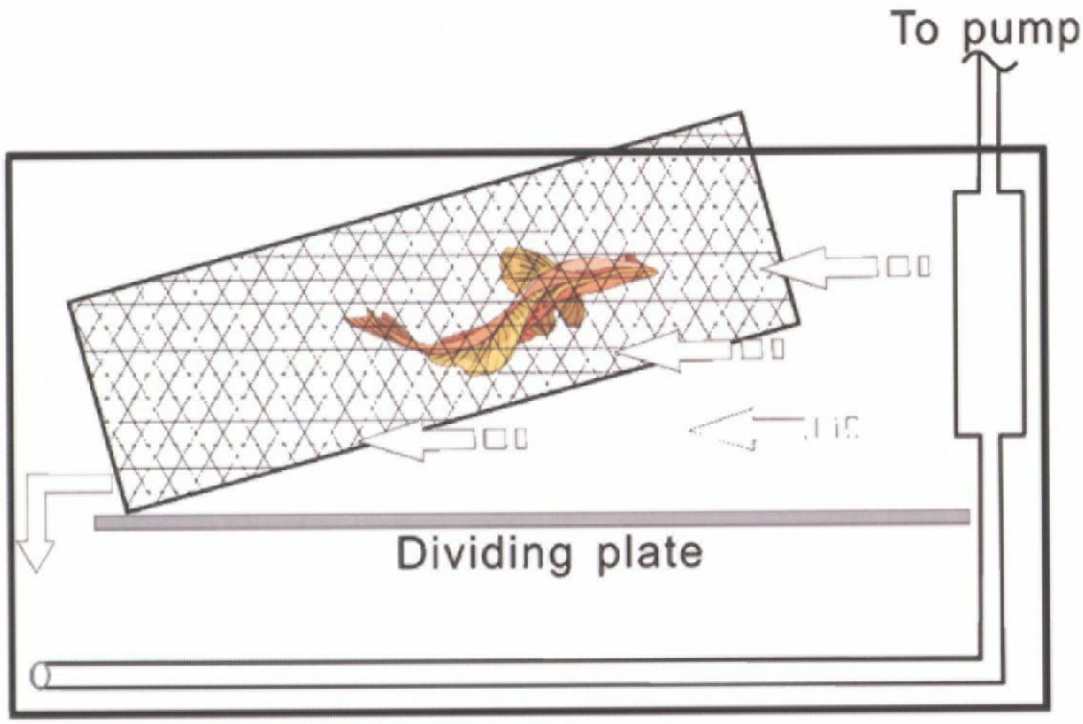

Figure IV-1. Diagram of the experimental tank for monitoring swimming performance of juvenile flounder. Fish are placed in the mesh chamber set in approximately 10°. Allows indicate the direction of water current created by electrical pumps.

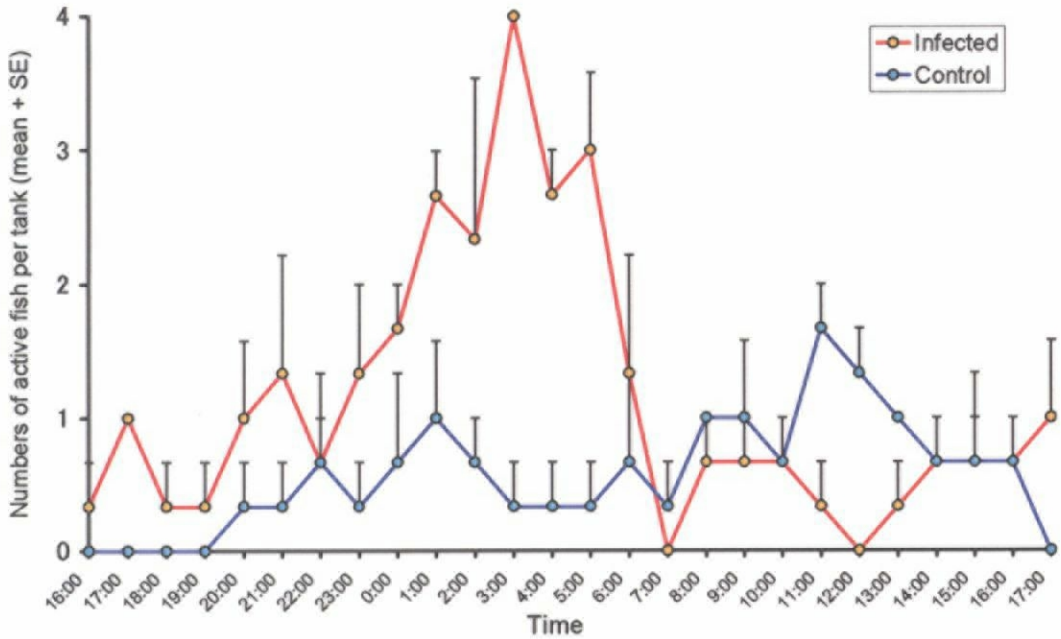

Figure IV-2. Change in the active fish over 25 hrs of monitoring. The red line represents the infected fish and blue line represents the controls. Note the distinct peak of activity in infected fish at 3:00 am

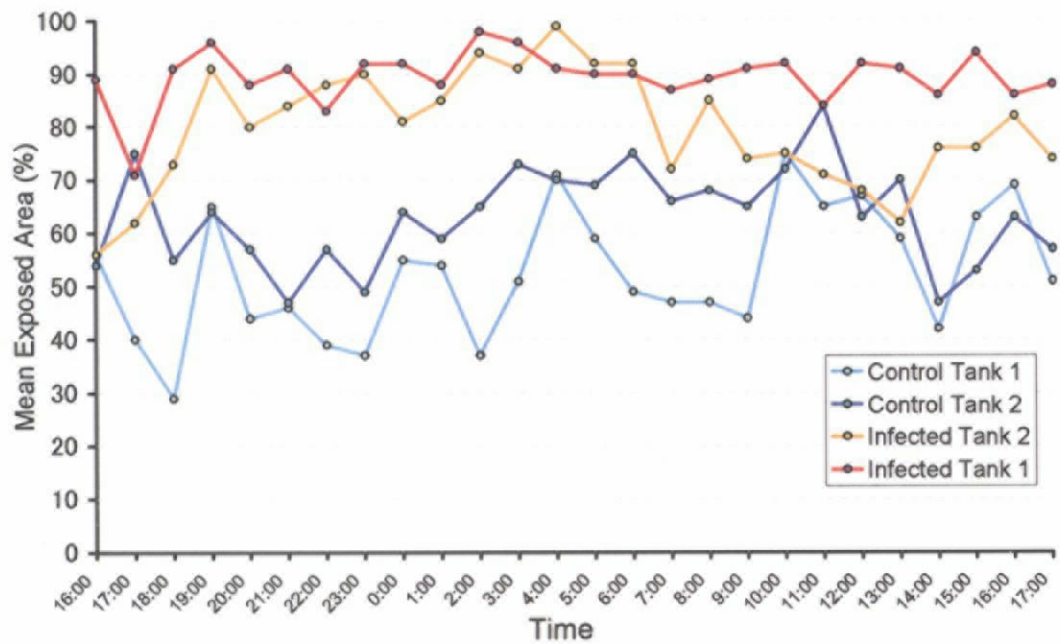

Figure IV-3. Change in the exposed body area over 25 hrs of monitoring. The reddish lines represent the infected fish and bluish lines represent the controls. Each line represents the average of 10 fish in each experimental tank.

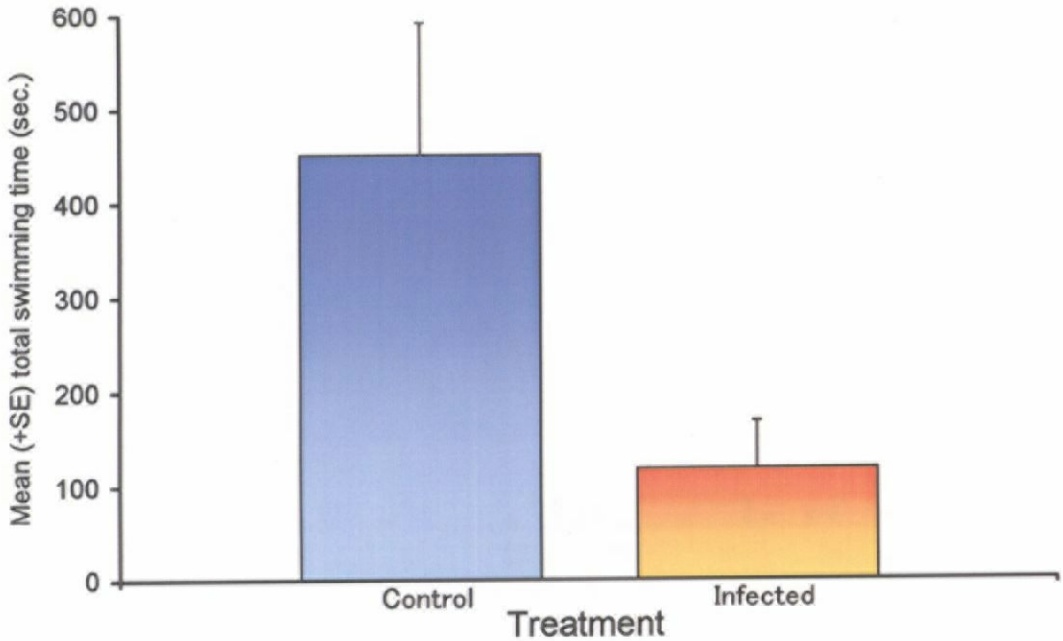

Figure IV-4. Total swimming time before exhaustion for uninfected controls (blue) and *N. hirame* infected fish (orange)

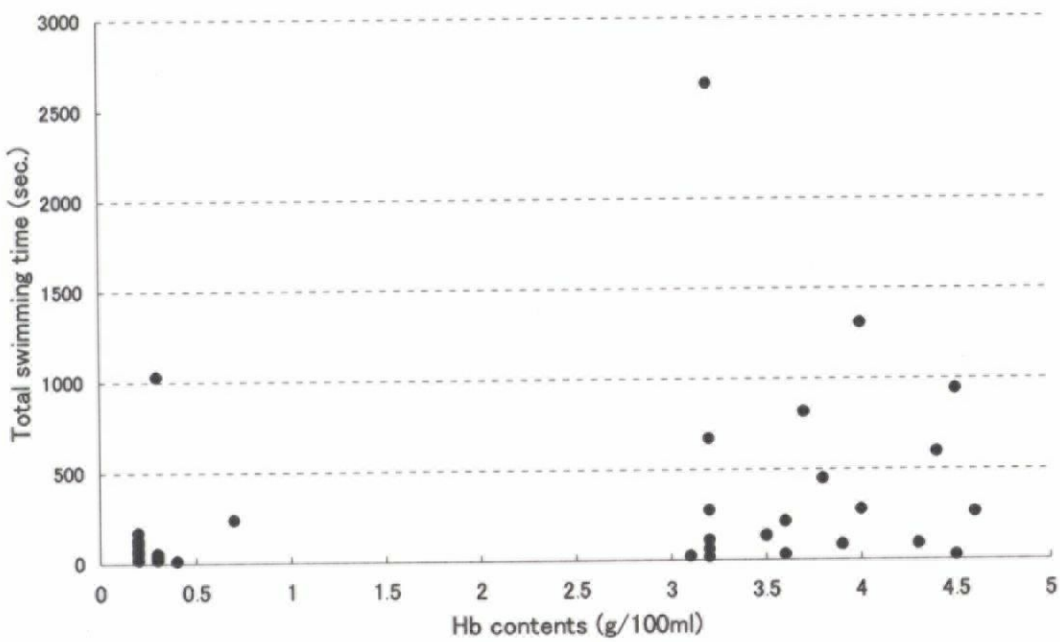

Figure IV-5. Relationship between the total swimming time (Y axis) and Hb (X axis) in juvenile flounder. Spearman rank correlation test indicates a significant positive correlation ($p<0.005$).

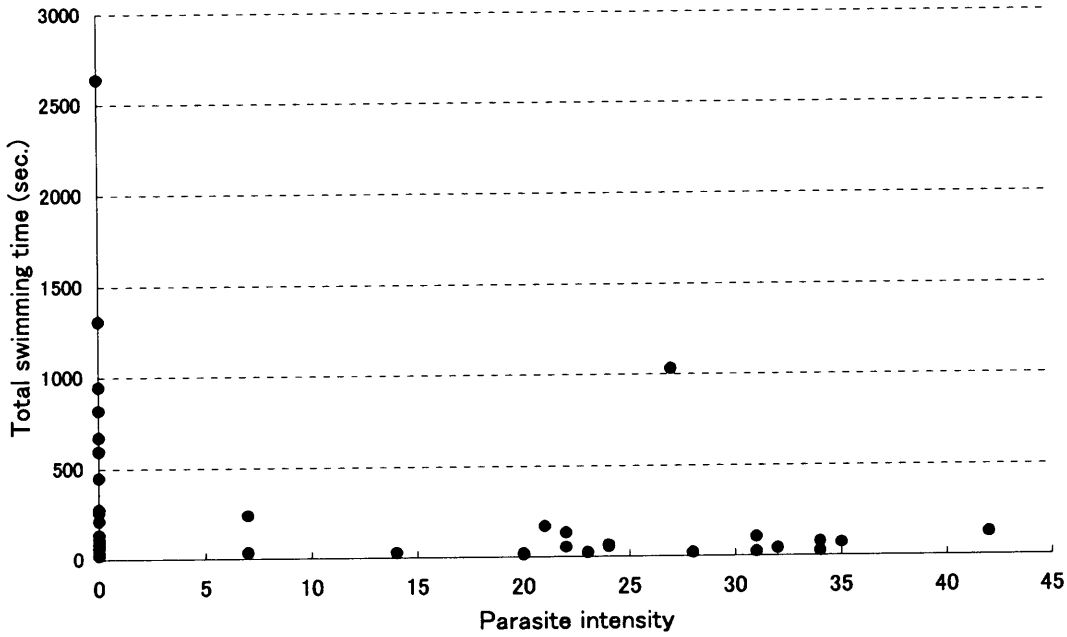

Figure IV-6. Relationship between the total swimming time (Y axis) and parasite intensity (X axis). Spearman rank correlation test indicates a significant negative correlation (p=0.018).

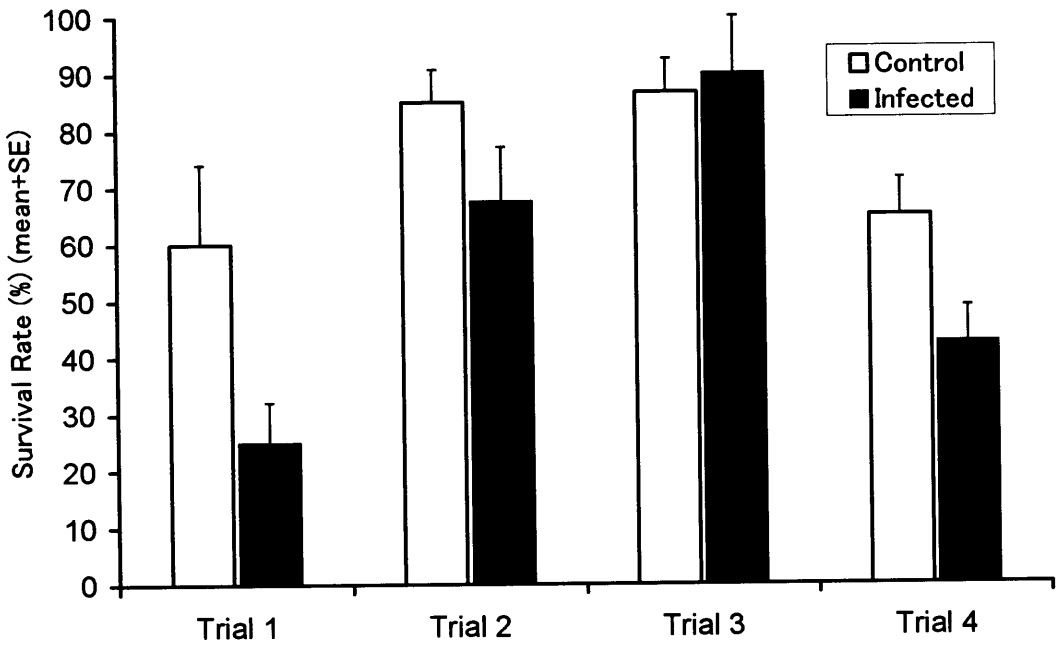

Figure IV-7. Mean survival rate of uninfected (open bar) and infected (closed bar) juvenile flounder against predation by large flounder at each experiment trial. Each bar represents the survivor from total of 40 fish, except for the bars from the trail 1 which represents 20 fish.

LITERATURE CITED

Ansell, A. D. & Gibson, R. N. 1993. The effect of sand and light on predation of juvenile plaice (*Pleuronectes platessa*) by fishes and crustaceans. *Journal of Fish Biology*, **43,** 837-845.

Barber, I., Hoare, D. & Krause, J. 2000. Effects of parasites on fish behaviour: a review and evolutionary perspective. *Reviews in Fish Biology and Fisheries*, **10,** 131-165.

Barnard, C. J. & Behnke, J. M. 1990. *Parasitism and Host Behaviour*. London: Taylor & Francis.

Bethel, W. M. & Holmes, J. C. 1977. Increased vulnerability of amphipods to predation owing to altered behavior induced by larval acanthocephalans. *Canadian Journal of Zoology*, **55,** 110-115.

Brainerd, E. L., Page, B. N. & Fish, F. E. 1997. Opercular jetting during fast-starts by flatfishes. *Journal of Experimental Biology*, **200,** 1179-1188.

Brassard, P., Rau, M. E. & Curtis, M. A. 1982. Parasite-induced susceptibility to predation in diplostomiasis. *Parasitology*, **85,** 495-501.

Burke, J. S., Seikai, T., Tanaka, Y. & Tanaka, M. 1999. Experimental intensive culture of summer flounder, *Paralichthys dentatus*. *Aquaculture*, **176,** 135-144.

Bush, A. O., Fernandez, J. C., Esch, G W. & Seed, J. R. 2001. *Parasitism: The diversity and ecology of animal parasites*. Cambridge, UK: Cambridge University Press.

Furuta, S. 1998. Comparison of feeding behavior of wild and hatchery-reared Japanese flounder, *Paralichthys olivaceus*, juveniles by laboratory experiments. *Nippon Suisan Gakkaishi*, **64,** 393-397.

Gibson, R. N. & Robb, L. 2000. Sediment selection in juvenile plaice and its behavioural basis. *Journal of Fish Biology*, **56,** 1258-1275.

Hashimoto, S., Hiraishi, T., Suzuki, K., Yamamoto, K. & Nashimoto, K. 1996. Swimming ability of bastard halibut *Paralichthys olivaceus* at the bottom of net cage. *Nippon Suisan Gakkaishi*, **62,** 12-16.

Hirota, Y., Koshiishi, Y. & Naganuma, N. 1990. Size of mysids eaten by juvenile flounder *Paralychthys olivaceus* and diurnal change of its feeding activity. *Nippon Suisan Gakkaishi*, **56,** 201-206.

Holmes, J. C. & Bethel, W. M. 1972. Modification of intermediate host behavior by parasites. In: *Behavioral aspects of parasite transmission* (Ed. by Canning, E. U. & Wright, C. A.), pp. 123-149. New York: Academic Press.

Hossain, M. A. R., Tanaka, M. & Masuda, R. 2002. Predator-prey interaction between hatchery-reared Japanese flounder juvenile, *Paralichthys olivaceus*, and sandy shore crab, *Matuta lunaris*: daily rhythms, anti-predator conditioning and starvation. *Journal of Experimental Marine Biology and Ecology*, **267,** 1-14.

Ide, K., Sasaki, K. & Ohmori, Y. 2004. Funiku shokusei kogata koukakurui *Scopelocherius onagawae* no shokumotsu senkousei. In: *The Annual Meeting of the Japanese Society of Fisheries Science*, pp. 44. Korimoto Campus, Kagoshima University.

Kakimoto, H., Ookubo, H. & Itano, H. 1979. Migratory behavior of adult Japanese flounder; bio-telemetry observation. *Bulletin of Niigata Prefectural Fishery Experimental Station*, **8,** 13-35.

Kellison, G T., Eggleston, D. B. & Burke, J. S. 2000. Comparative behaviour and survival of hatchery-reared versus wild summer flounder (*Paralichthys dentatus*). *Canadian Journal of Fisheries and Aquatic Sciences*, **57,** 1870-1877.

Kellison, G T., Eggleston, D. B. & Tanaka, M. 2002. Density-dependent predation and implications for stock enhancement with Japanese flounder. *Journal of Fish Biology*, **60,** 968-980.

Lafferty, K. D. & Morris, K. A. 1996. Altered behavior of parasitized killifish increases susceptibility to predation by bird final hosts. *Ecology*, **77,** 1390-1397.

Manderson, J. P., Phelan, B. A., Stoner, A. W. & Hilbert, J. 2000. Predator-prey relations between age-1 + summer flounder (*Paralichthys dentatus* Linnaeus) and age-0 winter flounder (*Pseudopleuronectes americanus* Walbaum): predator diets, prey selection, and effects of sediments and macrophytes. *Journal of Experimental Marine Biology and Ecology*, **251,** 17-39.

McConnaughey, R. A. & Smith, K. R. 2000. Associations between flatfish abundance and surficial sediments in the eastern Bering Sea. *Canadian Journal of Fisheries and Aquatic Sciences*, **57,** 2410-2419.

Miyazaki, T., Masuda, R., Furuta, S. & Tsukamoto, K. 1997. Laboratory observation on the nocturnal activity of hatchery-reared juvenile Japanese flounder *Paralichthys olivaceus*. *Fisheries Science*, **63,** 205-210.

Miyazaki, T., Seikai, T., Kinoshita, I. & Tsukamoto, K. 2004. Comparison of escape behavior of wild and hatchery-reared juvenile Japanese flounder *Paralichthys olivaceus*. *Fisheries Science*, **70,** 7-10.

Moore, J. 2002. *Parasites and the Behavior of Animals*. New York: Oxford University Press.

Mouristen, K. N. & Jensen, K. T. 1997. Parasite transmission between soft-bottom invertebrates: temperature mediated infection rates and mortality in *Corophium volutator*. *Marine Ecology Progress Series*, **151,** 123-134.

Mushiake, K., Mori, K. & Arimoto, M. 2001. Epizootiology of anemia in wild Japanese flounder. *Fish Pathology*, **36,** 125-132.

Nanba, K. 2002. Kokyu & Jyunkan. In: *Gyorui Seirigaku no Kiso* (Ed. by Aida, K.), pp. 45-66. Tokyo: Koseisyakoseikaku.

Nashida, K., Nato, Y., Nakano, K. & Iwao, K. 1996. Behavior analysis of free ranging Japanese flounder *Paralichthys olivaceus* by using micro data logger. *Nippon*

Suisan Gakkaishi, **62,** 132-133.

Noichi, T. 1997. Early life history. In: *Biology and stock enhancement of Japanese flounder* (Ed. by Minami, T. & Tanaka, M.), pp. 25-40. Tokyo: Koseisyakouseikaku Inc.

Noichi, T., Kusano, M., Kanbara, T. & Senta, T. 1993. Predation by fishes on larval and juvenile Japanese flounder at Yanagihama beach, Nagasaki, Japan. *Nippon Suisan Gakkaishi*, **59,** 1851-1855.

Poulin, R. 1994. Meta-analysis of parasite-induced behavioural changes. *Animal Behaviour*, **48,** 137-146.

Poulin, R. 1995. "Adaptive" changes in the behaviour of parasitized animals: A critical review. *International Journal for Parasitology*, **25,** 1371-1383.

Stoner, A. W. & Titgen, R. H. 2003. Biological structures and bottom type influence habitat choices made by Alaska flatfishes. *Journal of Experimental Marine Biology and Ecology*, **292,** 43-59.

Yamashita, Y. 1997. Ecology and releaseing techniques. In: *Biology and Stock Enhancement of Japanese Flounder* (Ed. by Minami, T. & Tanaka, M.), pp. 117-126. Tokyo: Koseisyakouseikaku Inc.

Yasunaga, Y. & Koshiishi, Y. 1980. Basic studies of problem on the propagation of plaice, *Paralichthys olivaceus*-I. Acclimatization to low salinity, feeding and gathering behaviour. *Bulletin of the Japan Sea Regional Fisheries Research Laboratory*, **31,** 17-31.

Yoshinaga, T., Kamaishi, T., Segawa, I., Yamano, K., Ikeda, H. & Sorimachi, M. 2001. Anemia caused by challenges with the monogenean *Neoheterobothrium hirame* in the Japanese flounder. *Fish Pathology*, **36,** 13-20.

Zgang, X., Fujita, K. & Watanabe, T. 1996. The effects of environmental changes on the heart rate of Japanese flounder (*Paralichthys olivaceus*). *Technical Report of the National Research Institute of Fisheries Engineering*, **8,** 233-238.

Chapter V. Effects of *Neoheterobothrium hirame* on feeding behaviour and starvation tolerance of juvenile flounder.

INTRODUCTION

Alterations of the various fundamental behaviours and increased predation risk in juvenile flounder associated with *N. hirame* infection were demonstrated in the previous chapter. The present chapter focuses on the effects of the parasite on feeding efficiency and starvation resistance of juvenile flounder.

Starvation often causes significant impact on the wild fish. Yamashita (1997) stated that predation and inanition are the two main contributing factors for mortality of juvenile flounder in the wild. Depletion of food resources, both directly and indirectly, could cause death. It is easily conceivable that if fish are fasted for a longer period than they can no longer recover (the point of no return, PNR), the result will be death. The tolerance limit for starvation is 5-15 days for the 40-50 mm flounder, and the limiting period increases as they grow (Yamashita, 1997).

Starved fish often show abnormal behaviours to compensate for the reduced energy input (McFarlane *et al.*, 2004). For instance, fish that are fasted for a number of days become less active to conserve energy. Alternatively, they can become more active due to increasing demand for foraging. Although flounder being a sit-and-wait type of predator and expected to have relatively higher endurance for starvation, even a short period of food depletion could cause significant change in their behaviours. Miyazaki *et al.* (2000) showed that juvenile flounder starved for 4 days spent significantly longer time in the water column during their feeding activity compared to well fed fish. Similarly, predatory attack patterns toward live mysids gradually changed in association with 1-7 days of starvation (Furuta, 1998). Such behavioural changes are considered to increase the risk of predation, thus indirectly cause mortality in juveniles. Laboratory experiments showed that juvenile flounder are more easily

preyed upon by large flounder when starved for a 3-7 days (Furuta, 1998) and by crab, *Matuta lunaris* after only 24hr of fasting (Hossain *et al.*, 2002).

Infected hosts often show abnormal feeding behaviours to meet their energetic requirements. For instance, three-spined sticklebacks, *Gasterosteus aculeatus*, harbouring a large cestode, *Schistocephalus solidus*, in their abdomen increased foraging time in expense of anti-predatory behaviours (Barber *et al.*, 2000). As parasites derive nutrition from their hosts, energetic losses owning to parasite infection may cause similar effects as starvation, or further enhance the effects of starvation. Severe anaemia exhibited by *N. hirame* infected flounder indicates a significant loss of energy through the loss of blood. It is conceivable that anaemic flounder have different feeding regime and/or become less tolerant to starvation. Parasitic infection is also known to impair competitive ability of fish hosts by reducing swimming speed, manoeuvrability, and/or prey detection (reviewed by Barber *et al.*, 2000). Reduction of swimming performance and other maladaptive behaviours observed in the flounder infected with *N. hirame* could also have significant effect on the prey capturing and competitive ability for feeding (Chapter IV).

In the present chapter, feeding efficiency, feeding behaviour towards live prey and starvation tolerability are compared between *N. hirame* infected and control juvenile flounder to determine the effect of the parasite on the host's feeding.

MATERIALS and METHODS

Feeding Competition

The feeding efficiency for capturing live mysids, *Neomysids* sp. was compared between experimentally-infected and uninfected juvenile flounder. Experimental infection was conducted in exactly the same manner described in the previous chapter (Chapter IV). A total of 10 fish (TL = 9.30±0.56 cm), 5 infected and 5 controls, which were starved for 48 hrs, were placed together in a 45 L glass aquarium (WxDxH

45x30x35 cm) and were competed against each other for mysids. The experiment tanks were laid with beach sand (4-5 cm) and to minimise the stress to both fish and mysids, the sides and the back of the tank were covered with blue acrylic boards. Fish were acclimatised to the experiment tank for 2 hrs prior to adding mysids. The tanks were provided with gentle flow of ozonated seawater at 20℃ during the acclimation. Water flow was stopped during the experiment to avoid accidental loss of mysids. After the acclimation, 100 live mysids (*Neomysids* sp.), 5-12 mm in length, were gently added to the tanks. Infected and control fish were allowed to freely feed live mysids for one hour. Human interaction was minimised during the experiment, except for the occasional observation which was made behind a screen curtain. Two experiment tanks were used for each experiment trials and a total of 4 trials were conducted (8 replicates in total).

After one hour of feeding competition, all the fish were removed from the tanks and measured for total length (TL; cm), standard length (SL; cm), body weight (BW; g), and haemoglobin level (Hb; g/100mL) as well as their parasite loads. Every fish was dissected and numbers of mysids in the digestive tract were counted.

Feeding Behaviour

Feeding behaviour of infected and uninfected flounder was compared using video monitoring technique. Ten infected or uninfected fish that were fasted for 24 hrs were placed in a separate glass aquarium and acclimatised for 6 hrs prior to the video monitoring. The setting of the aquarium is the same as described above. Two hundred live mysids were added to the tank and feeding behaviour of each treatment group was video recorded (Sony Digital Handycom-8) for 30 min.

Video footage were analysed frame-by-frame (1/30sec) for the following parameters for every feeding attempt based on the general methods described by Furuta (1998) and Miyazaki *et al.* (2000); duration of time during the feeding (i.e. time from the

beginning of aiming until fish become completely re-settled on the bottom), numbers of "attacks" per feeding attempt, maximum height fish reached during the feeding, and feeding patterns (Fig. V-1). Any behaviour with obvious "aiming" and/or "creeping" are considered as a feeding attempt (Miyazaki *et al.*, 2000).

Each feeding pattern was categorised into one of five patterns (pattern A - E) following the basic categorisation by Miyazaki *et al.*, (2000) with some modifications; A; fish returned close to the departure point, B; fish showed some inversion motion, but did not return to the departure point, C; fish did not show any inversion motion and swam straight forward, D; fish remained at the original position and captured mysids just by raising head, E; fish did not return to the bottom and "hovered around" (Fig. V-1). Some fish showed quick but short distance (less than 1 body length) forwarding feeding motion and those were further categorised as a "short distance feeding". Any behaviour distracted by glass wall or pipes was excluded from the analyses.

All tested fish were later measured for TL, SL, BW, Hb as well as their infection status. However, as fish used for this experiment were subsequently used for a different experiment, number of mysids in the digestive tract was not counted. It also has to be noted that Hb measurement was conduced a week after the trial.

Starvation Tolerance

To investigate the effect of *N. hirame* on the starvation tolerability of juvenile flounder, mortality during the 3 months of food depletion was compared compared between the juvenile flounder infected with different intensity of *N. hirame*. A total of 3 treatment groups were prepared by exposing one of the following *N. hirame* doses; high dose (50 larvae / fish), low dose (10 larvae / fish), or control (no larva / fish). The method for exposing the parasite was following the basic method previously described. Two rearing tanks were prepared for each treatment group and an additional tank was set to reared 50 fish which were exposed the high dose of

parasites and fed to satiation every other day (fed-control group). Fifty five fish (TL = approx. 9.0 cm) were placed in each rearing tank (WxDxH, 79x44x46 cm) which was provided with ozonated seawater, aeration and an acrylic cover sheet to prevent any food materials to accidentally fall into the tanks. The water temperature was maintained at 17 ± 1℃.

Mortality was observed daily and 5 fish from each group (2 or 3 fish per tank) were sampled at a 10 day interval. All sampled and dead fish were measured for TL, SL, BW and Hb (for periodic samples only) as well as their infection status. The condition factor (K) was calculated as BW/SL^3 x 100 for all fish, where BW is in g and SL is in cm.

Statistical Analyses

Chi-square test was used for comparing the proportion of fish with empty-stomach in the competition experiment, and to compare feeding pattern between the groups in the behavioural experiment. T-test was used to test the differences in numbers of captured mysids, TL and BW between the groups. Analyses involving feeding height, feeding duration and number of attacks, a non-parametric Wilcoxon rank sum test was used. Spearman's rank correlation test was used for all correlation analyses. Kaplan-Meier survival probabilities were computed for starvation experiment, and differences in mortality between three groups were tested using the log-rank test.

RESULTS

Feeding Competition

The result of feeding competition and the conditions of tested animal were summarised in the Table V-1. The numbers of captured mysids did not differ between the groups (Fig. V-2, t-test, $p>0.1$) and there was no statistical difference (Chi-square, $p>0.1$) in the rate of the empty stomach between the groups. Total number of

ingested mysids at each trial was approximately 80, thus majority of given mysids were eaten during the experiment. Although no correlation between numbers of captured mysids and Hb was observed (p = 0.69), there was a negative and significant correlation between the numbers of ingested mysids and adult worms intensity (r = -0.33, p = 0.033, N=40; Fig. V-3). At the time of the experiment, all worms had developed into adults and all fish from infected group harboured adult parasite (i.e 100% prevalence). The mean parasite intensity was 5.3, ranging from 1 to 14 worms per fish. All control fish were confirmed as free of *N. hirame*. Majority of infected fish exhibited pale gills indicating severe anaemia. The Hb value of the infected fish was significantly lower than that of the controls (Wilcoxon, p<0.001). There was no difference in the fish size between the treatments.

Feeding Behaviour

During the acclimation, all fish were burrowing into the substrates or lying quietly on the bottom until mysids were added to the tank. However, once the mysids were added, majority were ingested during the first 30 min and less than 10% (20 /200 mysids) were left at the end of the experiment. A total of 872 feeding attempts were observed from two groups and were summarised in the Table V-2. Number of feeding attempt observed from infected fish was 48% higher than that from controls. Infected fish spent significantly longer time at each feeding attempts, and had less numbers of attack per attempts. Infected fish also tend to swam higher in the water column, though the difference was not significant. In addition, the proportion of feeding attempts in which fish swam up close to surface (< 3cm from the surface), was significantly higher in infected fish than controls (Table V-2).

The feeding pattern of infected fish differed significantly from that of uninfected controls (Chi-square, p<0.0001). Most frequently observed feeding pattern for both groups was C, the straight forward catch-and-land pattern (Fig. V-4). However,

proportion of C-pattern feeding in controls (62%) was significantly higher than that of infected fish (42%) (Chi-square, $p<0.0001$). Uninfected fish showed more frequent short-distance swimming than infected fish. Furthermore, infected fish showed higher proportion of E pattern ("hover around" type) (Chi-square $p=0.0044$) and also had considerably more feeding attempts without any attacks comparing to the controls. To summarise this, feeding pattern of infected fish tend to be wander around type without no feeding, while that of controls is more agile and efficient.

Starvation Tolerance

All fish have died from all treatment groups, except for the 2 fish from Low-intensity group, during the 93 days of the experiment period. Only 1 fish died in the fed and heavily infected group. The mortality curve from each tank was plotted in Fig. V-5. The first mortality was observed from most tanks at around 42-50 days post exposure (pe) followed by a sharp linear increase after 56 days pe. The Kaplan-Meier survival analysis indicated no statistical differences in the survival time between the groups ($p = 0.164$).

The mean condition factors (K) of periodic samples fluctuated significantly over time (ANOVA; $F_{8,134} = 11.08$, $p < 0.0001$, Fig. V-6). The K-value of sampled fish from all groups showed U-shaped curve with the lowest observed mean of 0.76 ± 0.06 at 44 dpe, and apparently increased thereafter (Fig. V-6). No statistical differences between the groups were observed in K-value of the dead fish (ANOVA; $F_{2,175} = 0.26$, $p < 0.7713$, Fig. V-6).

All sampled fish from Low- and Hi- infection groups harboured parasites between 12 and 44 days pe (100% prevalence). The prevalence was lower in the fish sampled soon after the exposure and also after 52 days pe. The worm intensity continued to increase by 44 days pe and declined thereafter (Fig. V-7). The reduction of worm intensity associated with the appearance of adult worms and is consistent with the

earlier study (Chapter III). Significant differences in the parasite intensity between low- and hi- intensity group were observed at 12, 23, 32, and 44 days pe (Mann-Whitney U-test; $p < 0.05$). The Hb levels in High- and Low- infection groups were significantly lower than that of controls during 44 and 83 days pe, and no obvious recovery of Hb was observed at the end of the experiment in the two infection groups (ANOVA, $p<0.05$) (Fig. V-7).

DISCUSSION

Feeding Competition

Infected flounder were shown to be equally efficient in capturing live mysids as their uninfected competitors. However, it is unclear whether the given mysids density was too high to cause high competition between the groups. To further enhance the competition, the experimental condition should be reconsidered. Trials with reduced mysids density, or experiment using larger experimental tank may provide different results.

Number of captured mysids was highly variable, ranging from 0 to over 20, among infected fish. Negative correlation between parasite intensity and captured mysids suggests the potential effect of *N. hirame* on host's feeding. As all infected fish suffered from anaemia, with similar Hb values, factors other than anaemic condition may have contributed to the lower feeding rate of heavily parasitized individuals. Anshary and Ogawa (2000) reported occasional necrosis of the host tissue around the infection site of adult worms. Such physical damages, mechanical destruction and irritation caused by the large adult worms hanging in the buccal cavity wall may hinder feeding behaviour of infected fish.

Feeding Behaviour

The differences in feeding behaviours observed in the present experiment could have

significant consequences in the survival of wild flounder. Flounder faces most predation risk when they are away from the bottom. Therefore, wild juvenile flounder quickly return to the initial departure points after capturing the prey to minimise the risk. In contrast, HR flounder tend to spend longer time in the water column during the feeding (Furuta, 1998). Such behavioural differences between wild and HR fish are considered to associate with higher rate of predation of HR flounder after releasing. Although the fish used in the present experiments are all HR and their behaviour could be different from the wild fish, the results indicate strong effects of *N. hirame* on the feeding behaviour of juvenile flounder. Longer swimming duration and frequent "wandering" behaviour observed in infected fish probably increase their predation risk as suggested for HR fish.

Interestingly, infected fish showed frequent off-bottom swimming which did not associate with any attacking behaviour. This non-attacking swimming behaviour was also indicated by their considerably greater number of feeding attempts during the same observation period as in controls. It seemed as those fish lost sight from the aimed prey or "gave up" on chasing. Such unrewarded behaviours increase the energetic loss and risk of predation. In contrast, controls often show more than one attacks during a single feeding attempts, but feeding time is generally shorter. These differences between infected fish and uninfected controls suggest that *N. hirame* has strong negative effect on the feeding efficiency of juvenile flounder. If such changes of feeding behaviour is consistent in the wild-infected fish under natural conditions, *N. hirame* could have significant effect on the fish survival by making them more susceptible to predation and by increasing energetic losses.

Starvation Tolerance

The results of the starvation experiment were unexpected. Despite the progression in anaemic symptoms, survival between all treatment groups did not differ. *N. hirame*

infection also did not affect the host's condition factor. This suggests that the loss of blood by the worm affect neither endurance against inanition nor body condition in flounder. Mortality started after 40-50 days and rapidly increased after 55 days of starvation. This indicates that the tolerance limit for juvenile flounder (length approximately 10.0 cm) lies somewhere around those periods at the given experimental condition (17℃). However, the limit can be shorter for the wild flounder. In the wild, oceanic current and other environmental factors causes extra energetic losses. Throughout the starvation experiment, fish remained almost motionless at the bottom, probably for conserving energy. Experiment under more natural condition, or field experiments may provide different result and is useful to understand the precise effects of *N. hirame* on the starvation tolerability of wild fish.

The U-shaped change in K-value observed in sampled fish seems unrealistic. It appeared as the fish regained body weight after long period of starvation. However, this can be explained by the death of fish which had lower K-values. Fish with the worst condition died earlier in the experiment and survivors with higher K-value remained in the tank. By sampling those higher K-value survivors causing apparent increase of the condition factors. However, the measurement in the dead fish indicates that the condition of juvenile fish cannot simply be assessed by the K-value. Fish with low K-value probably died first, but mortality of fish with high K-value was also commonly observed at the end of experiment. This suggests that minimum threshold of K-value below which fish no longer viable, does not exist. Regardless of K-value, juvenile flounder can no longer survive after a certain period of inanition. Other physiological changes associating with inanition may play more important role in flounder survival.

Starvation has shown to change the blood composition in fish. More than 50 days of food depletion causes reduction in serum glucose, cholesterol, total protein, urea nitrogen, and total phosphorus in brook trout (Heming and Paleczny, 1987). Similar

changes in blood composition may have occurred in flounder. However, *N. hirame* did not seem to have suffered from the inanition of the host as their development and longevity was similar to the ones observed on the well fed fish (Chapter III). The nutrition requirement of *N. hirame* is not known. To investigate this matter further, more precise physiological analysis in both hosts and the parasite is necessary.

In the present experiment, 2 exposure doses successfully resulted in different infection intensity at least for the certain period. Although the intensities of immature worms differed significantly, the density of adult worms evened out at the end. This indicates the density-dependent regulation of the parasite intensity. Higher parasite intensity may increase the competition among the adult worms toward nutrition and attachment site. Nonetheless, this study successfully demonstrated the experimental infection methods used here is capable for controlling parasite intensity at some level. This opens door way to the future research investigating the intensity-dependent effect of *N. hirame*.

In the summary, this chapter demonstrated the negative effect of *N. hirame* on the feeding behaviour and feeding success of juvenile flounder. However, further studies are necessary to understand the implication of this matter in the wild fish.

Table V-1. Summary of competition experiment. P value less than 0.05 was considered significant

	Control	Infected	*p* value
Feeding Competition			
No. mysis / fish	8.18 ± 6.32	8.70 ± 8.66	0.750 t-test
Rae of empty stomach	4/40 (10%)	9/40 (21.5%)	0.129 Chi-square
Total mysis eaten / trial	81.75±30.92	87±40.05	0.842 t-test
Host Status			
T.L. (cm)	9.35±0.45	9.27±031	0.546 t-test
B.W. (g)	4.05±0.69	3.97±0.98	0.722 t-test
Parasite Intensity	0.00±0.00	5.13±3.09	
Hb (g/100mL)	3.90±0.5±3	0.42±0.46	<0.0001 Mann-Whitney

Table V-2. Summary of the feeding behaviour experiment. P value less than 0.05 was considered significant

	Control	Infected	*p* value
Total feeding attempts	351	521	
Attempt disturbed by wall/pipe	80	127	
Average feeding duration (sec)	3.57±5.67	7.76±17.39	<0.0004 Mann-Whitney
Average No. attacks / attempt	1.00±0.81	0.87±0.99	<0.0013 Mann-Whitney
Average max. reached height (cm)	4.67±6.69	5.97±8.67	0.2846 Mann-Whitney
No. of "short-distance" feedings	87 (32.10%)	81 (20.45%)	<0.0007 Chi-square
No. of feeding attempt without attack	44 (16.24%)	123 (31.06%)	<0.0001 Chi-square
No. of feeding attempt in which fish swam close to the surface	4 (1.48)	18 (4.55%)	0.0217 Chi-Square

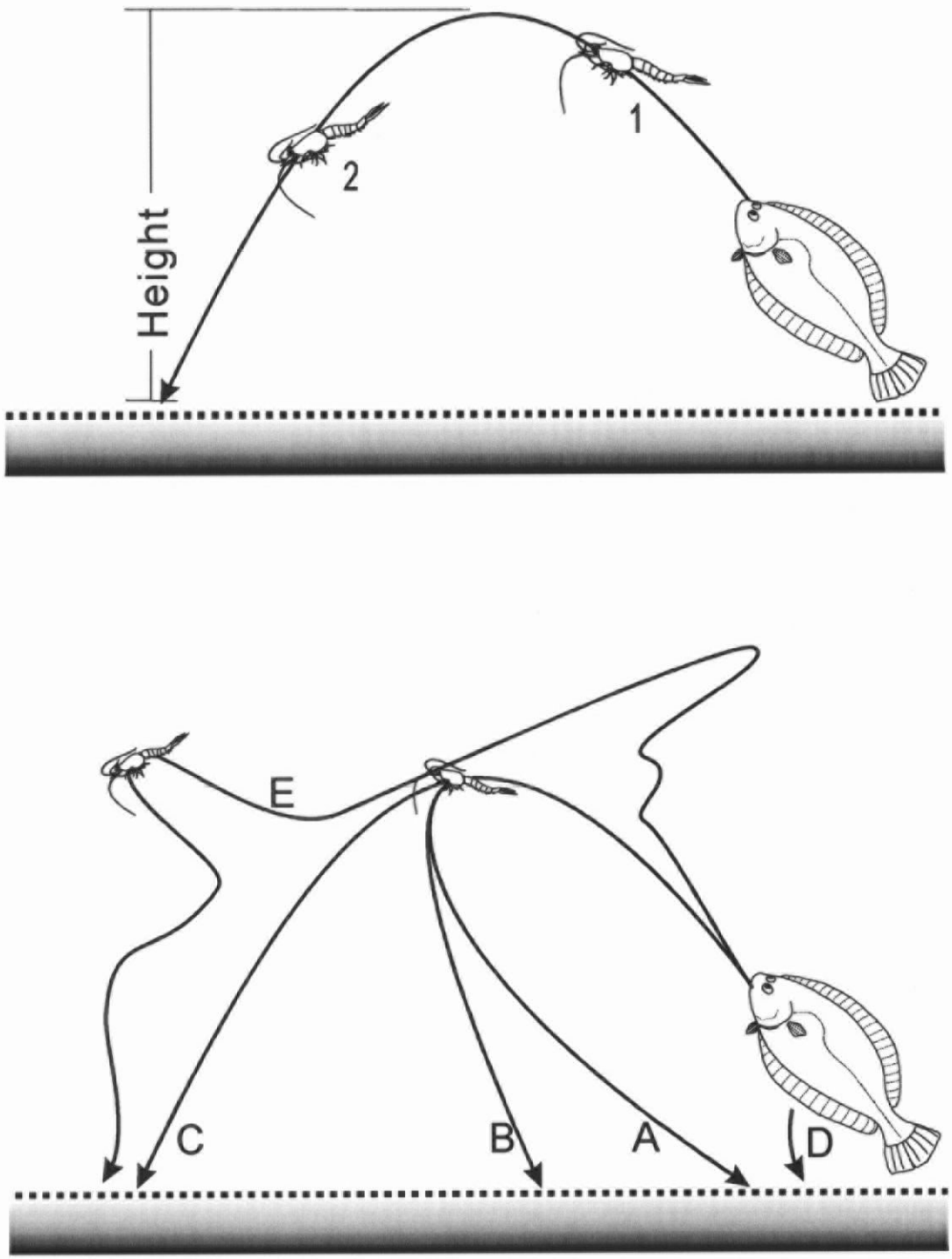

Figure V-1. Feeding height and attacking of juvenile flounder (top) and five patterns (A-E) of feeding behaviour observed in the feeding trail with live mysids.

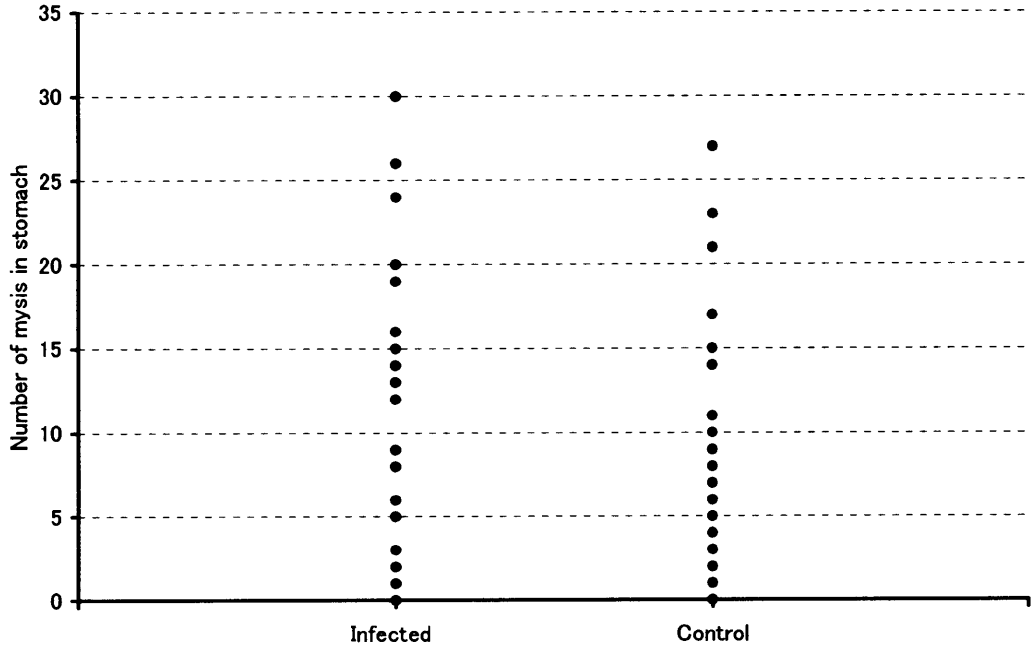

Figure V-2. Numbers of mysids in the stomach after competing for 1 hr. No statistical difference was observed between the fish infected with *N. hirame* and uninfected controls.

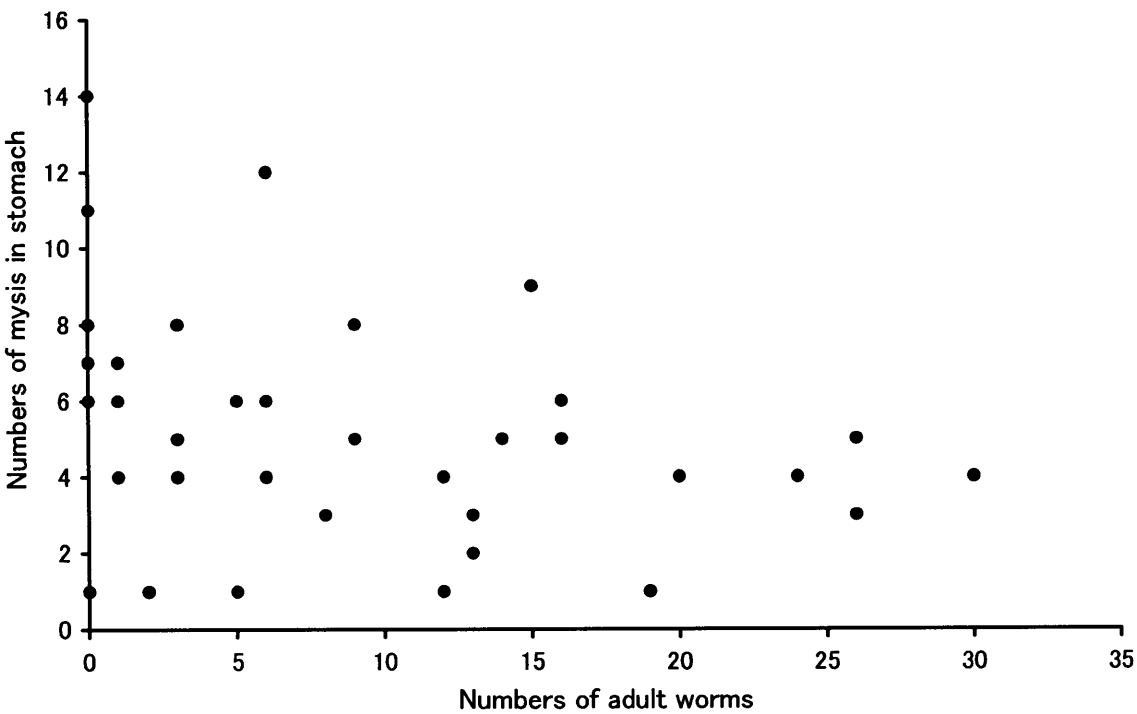

Figure V-3. Relationship between the numbers of captured mysids and numbers of adult *N. hirame*. Spearmans test indicates the negative correlation.

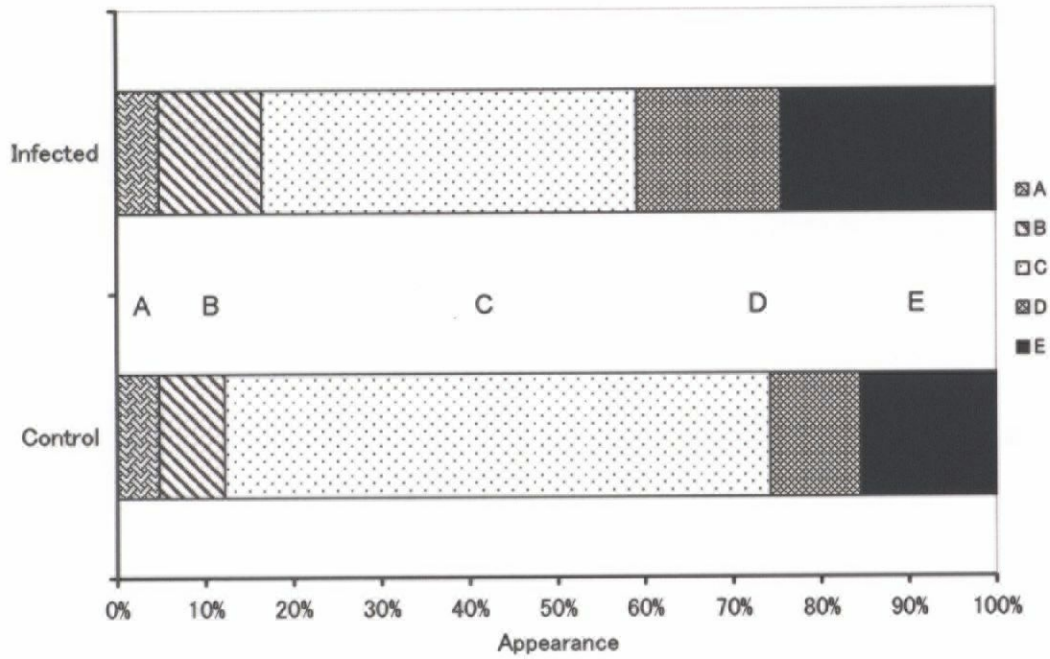

Figure V-4. Composition of the feeding pattern observed in *N. hirame* infected- and control fish during the 30 min observation.

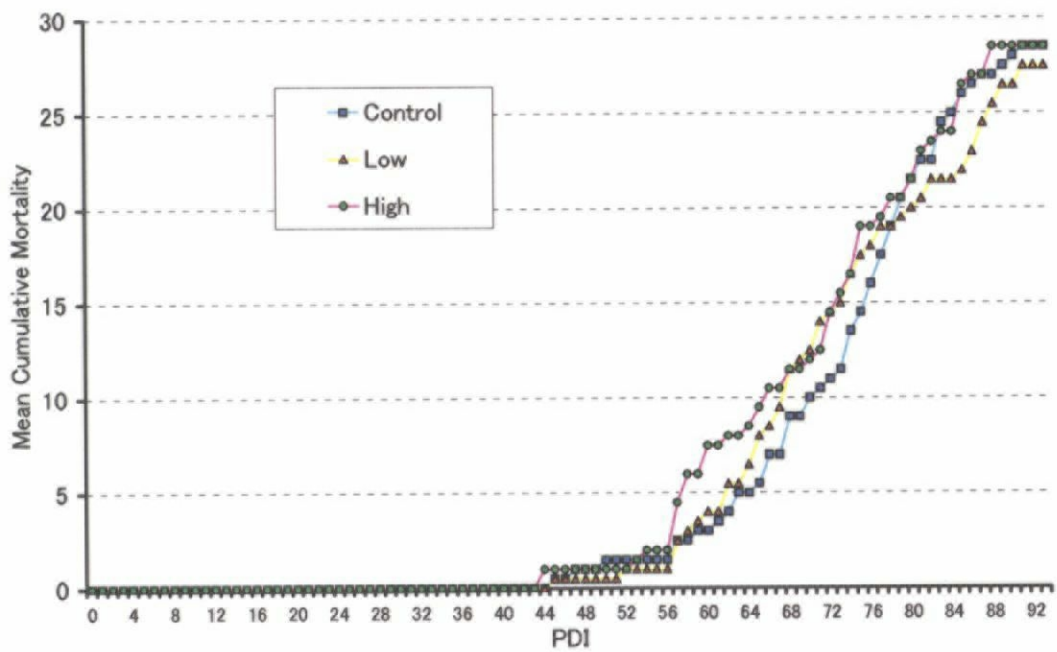

Figure V-5. Average cumulative mortality due to starvation in juvenile flounder infected with high dose (red line), low dose (yellow) of *N. hirame* and uninfected controls (blue).

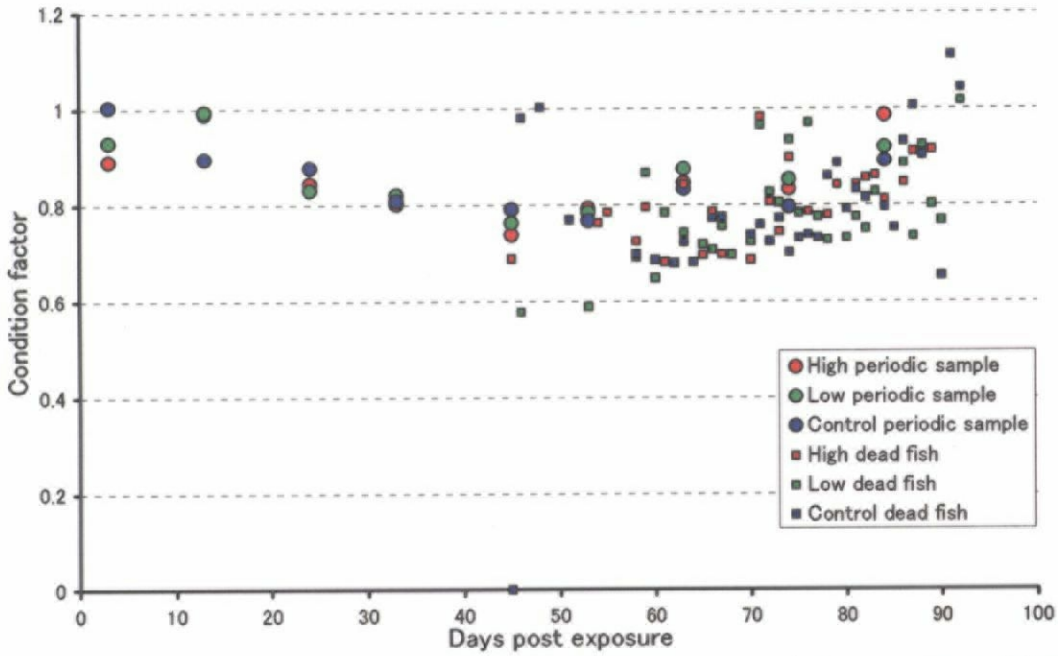

Figure V-6. Change in the mean condition factors (K) from periodic samples and dead fish. Notice the U-shaped change in K for periodic samples. Each point for dead fish represents the mean of all the dead fish on the same day.

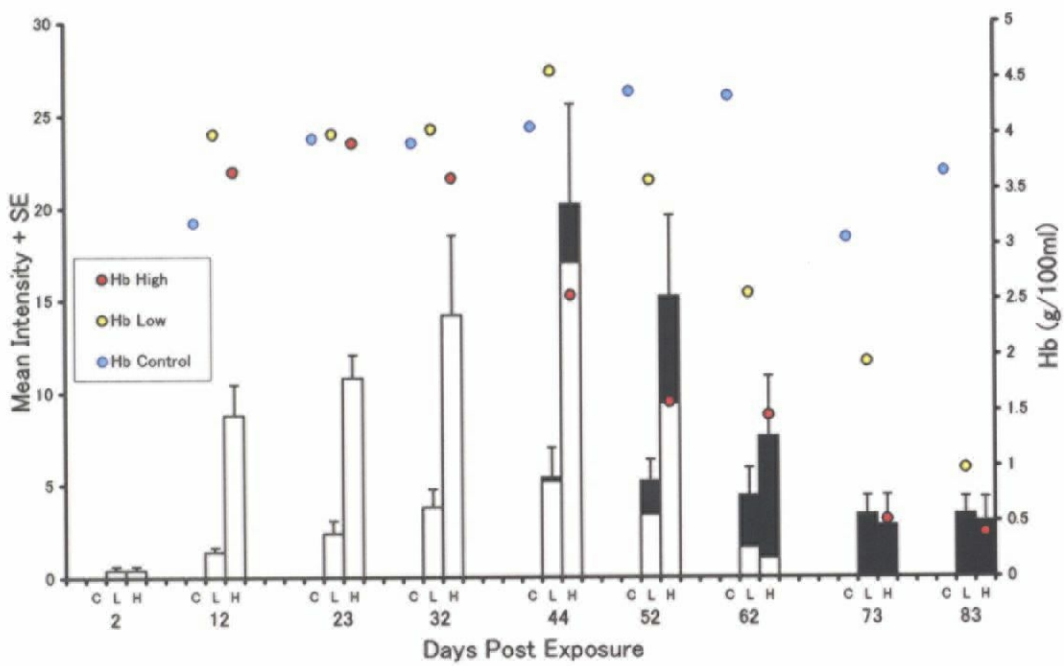

Figure V-7. Change in the mean intensity of immature (open bar) and adult (closed bar) worms. Each coloured circle represents mean haemoglobin contents of 3-5 fish from each treatment group.

LITERATURE CITED

Barber, I., Hoare, D. & Krause, J. 2000. Effects of parasites on fish behaviour: a review and evolutionary perspective. *Reviews in Fish Biology and Fisheries*, **10,** 131-165.

Furuta, S. 1998. Basic studies on release techniques of hatchery-reared Japanese flounder. V - Effects of starvation on feeding behavior and predation vulnerability of wild Japanese flounder juvenile. *Nippon Suisan Gakkaishi*, **64,** 658-664.

Heming, T. A. & Paleczny, E. J. 1987. Compositional changes in skin mucus and blood serum during starvation of trout. *Aquaculture*, **66,** 265-273.

Hossain, M. A. R., Tanaka, M. & Masuda, R. 2002. Predator-prey interaction between hatchery-reared Japanese flounder juvenile, *Paralichthys olivaceus*, and sandy shore crab, *Matuta lunaris*: daily rhythms, anti-predator conditioning and starvation. *Journal of Experimental Marine Biology and Ecology*, **267,** 1-14.

McFarlane, W. J., Cubitt, K. F., Williamsb, H., Rowsellb, D., Mocciac, R., Gosineb, R. & McKinleya, R. S. 2004. Can feeding status and stress level be assessed by analyzing patterns of muscle activity in free swimming rainbow trout (*Oncorhynchus mykiss Walbaum*)? *Aquaculture*, **239,** 467-484.

Miyazaki, T., Masuda, R., Furuta, S. & Tsukamoto, K. 2000. Feeding behaviour of hatchery-reared juveniles of the Japanese flounder following a period of starvation. *Aquaculture*, **190,** 129-138.

Yamashita, Y. 1997. Ecology and releasing techniques. In: *Biology and stock enhancement of Japanese flounder* (Ed. by Minami, T. & Tanaka, M.), pp. 107-116. Tokyo: Koseisyakouseikaku Inc.

Chapter VI. Co-infection of *Neoheterobothrium hirame* and viral haemorrhagic septicaemia virus (VHSV) in juvenile olive flounder

INTRODUCTION

Parasitic infections have been shown to facilitate the progression of diseases caused by other pathogens. Effects of parasitic infection on other infectious diseases are well studied in human pathogens. For instance, infection of *Schistosoma mansoni*, a trematode parasite, delays the resolution of cutaneous lesions and parasitaemia in mice caused by infection from protozoan parasite, *Leishmania major* (La Flamme *et al.*, 2002). Similarly, *Leishmania* infection enhances both transcription and production of HIV in *in vivo* experiment using mice (Zhao *et al.*, 2004). Such negative parasite effects on other infectious diseases can be a significant threat in the areas where various pathogens are co-exist and widespread.

Epidemiological information about the diseases of flounder is mostly restricted in hatchery and farming environments and such data on wild fish are limited. Viral hemorrhagic septicaemia virus (VHSV) is one of a few pathogens on which field studies have been conducted. Viral hemorrhagic septicaemia (VHS) was originally considered as a disease of rainbow trout causing extensive losses in farming industries in European countries. However, since the first isolation of VHSV, the virus has been found in more than 20 marine fishes (Meyers *et al.*, 1999; Smail, 1999; Brudeseth and Evensen, 2002).

First occurrence of VHS in Japanese water was recorded in 1996 from the flounder farm (Isshiki *et al.*, 2001) at an island on the Sea of Japan. It then rapidly spread and become an outbreak among cultured flounder. In the wild, the virus was isolated from 10% of wild flounder collected from 8 coastal areas in Japan (Watanabe *et al.*, 2002). Another study found that 6.5% of wild flounder captured in Obama Bay, where *N. hirame* is highly abundant, were infected with VHSV. These results

indicate that VHSV and *N. hirame* co-occur in the same areas and there is a high possibility that a single individual could be infected with both pathogens.

The aim of this chapter is to determine the effects of co-infection with *N. hirame* and VHSV on juvenile flounder. Two experiments were conducted in the study. First was to challenge VHSV on the flounder previously infected with *N. hirame*. The second one is to expose *N. hirame* onto the flounder that survived from VHSV injection.

MATERIALS and METHODS

Fish, the Parasite and the Virus

Juvenile olive flounder (total length approximately 10 cm) obtained from a local hatchery were kept in a 500 L stock tank with ozonated seawater for at least two weeks prior to the experiment for ensuring their health. Ten randomly selected fish were sacrificed for the pre-experiment examination for both *N. hirame* and VHSV infections.

Collection of *N. hirame* oncomiracidia was followed the same protocols described in the earlier chapter (Chapter IV). Briefly, parasite eggs collected from the infected fish were incubated in a net hanged in a bucket with running ozonated water at 20℃ for 5 days. Eggs were then transferred to a plastic container with increased aeration to induce hatching. Numbers of hatched oncomiracidia were estimated and suspension required to contain desired numbers of larvae was calculated. Larvae used for all the experimental infection were within 24 hrs post-hatch.

The VHSV isolate used for all the experiments was Obam25 (North American strain; Nishizawa *et al.*, 2002) originally isolated from the wild olive flounder captured in Wakasa Bay in 1999 (Takano *et al.*, 2000). The virus was propagated using fathead minnow (FHM) cell line maintained in Minimum Essential Medium (MEM; GIBCO, Carlsbad, CA, USA.) supplemented with 10% (V/V) Fetal Bovine Serum (FBS) and

antibiotics (Penicillin 100 iu/mL, Streptomycin 10,000 μg/mL, Amphoterin B 25μg/mL) at 20℃. After incubated for 7 days, virus was harvested by centrifugation at 1,300X *g* for15 min and the supernatant was stored at -120℃. The isolate was of 3rd passage from the original isolation.

VHSV Challenge to N. hirame *Infected Fish*

To determine the effect of *N. hirame* on the susceptibility to VHSV in juvenile olive flounder, the similar experiments were repeated twice at the different time period using different batch of fish. Two trials were performed in February 2003 and November, 2004. Fish were initially infected with *N. hirame* and then exposed to VHSV (co-infection group) for comparing the mortality with the fish infected with only *N. hirame* (*N. hirame* group), fish challenged with VHSV alone (VHSV group), and uninfected controls (Control group).

Methods for experimentally infecting juvenile flounder to *N. hirame* followed the procedures described in the earlier chapter (Chapter IV). A total of 160 fish (TL 9.14±0.9 cm for trial 1, 9.56±0.7for trial 2) were randomly placed in one of four 60 L exposure tanks. Forty fish were placed in each exposure tank and the suspension containing 1,200 oncomiracidia (*ca.* 30 larvae per fish), or equal amount of sea water for controls, were added. Fish were then reared in separate rearing tanks until they showed apparent anaemic symptoms.

Once fish started to show anaemic symptoms, namely discoloration of the gills, fish were assigned to one of 8 experiment tanks (WxHxD 79x44x46 cm) provided with ozonated seawater at 18℃. Twenty fish were placed in each tank and duplicate tanks were prepared for each treatment groups (total of 8 tanks). Prior to the VHSV challenge, water temperature of the experiment tank were lowered 1℃ per day to 13℃ (± 1℃), as VHS outbreaks generally occur at low temperatures (Takano *et al.*, 2000; Iida *et al.*, 2003). Twenty fish were sampled prior to VHSV challenge to

confirm *N. hirame* infection and determine the severity of anaemia.

Fish were challenged with VHSV through immersion, a method mimicking the viral transmission in the natural environment. Water level of each experimental tank was lowered to 20 L and virus suspension ($10^{4.0}$ $TCID_{50}$ / tank) for VHSV and co-infection groups, or equal amount of MEM for *N. hirame* and control groups was added. Fish were immersed for 1 hr, and then fresh seawater was supplied.

Mortalities from each tank were monitored daily. Experiment was terminated when the no fish was died for 7 days. All the dead fish were taken out from the tank as soon as spotted and placed on the ice until all the measurements and samplings were performed. Each dead fish was measured for total length, body weight, and parasite counting was conducted. Head kidney was also excised from all the dead fish, stored at -80℃, and later used for virus titration.

N. hirame Infection to VHSV Carriers

To investigate whether *N. hirame* infection induce VHS in the virus carrier fish, we assessed mortality of juvenile flounder that had been survived from VHSV injection and were subsequently infected with *N. hirame*. A total of 265 juvenile flounder obtained from the local hatchery and confirmed free of *N. hirame* and VHSV were used. Fish were kept in the stock tank for 1 week prior to the experiment, and randomly separated into two groups. A group of 165 fish were intramuscularly injected with VHSV ($10^{3.5}$ $TCID_{50}$/fish) at dorsal muscle of the eyed side. Remaining 100 fish were injected in the same manner as other group but with MEM as a mock infection controls. Fish injected with VHSV were further divided into 6 groups of 20 fish and reared to monitor progression of VHS in separate experimental tanks at 20℃ (± 1℃). All controls were kept in a 500L tank.

At 1 and 5 days post (dp) injection, 6 randomly selected fish were sampled from VHSV injected group for the virus quantification from kidney and heart. Mortality

after VHSV injection was recorded daily and all dead fish were measured for TL, SL, BW, and observed for any VHS symptoms such as haemorrhage in dorsal muscle, internal organs or abdominal fluid. When mortality was ceased for more than two weeks, survivor fish were considered as "VHSV carriers", and further challenged to *N. hirame* infection. Parasite infection was delayed until 45 dp virus injection due to unavailability of the parasite larvae.

Fish were exposed to *N. hirame* larvae using the same protocols described above. Prior to the experimental infection, 8 fish from VHSV-injected group and MEM-injected groups were sampled to quantify the initial virus titre. All the VHS survivors were once pooled together, and divided randomly into 2 groups. Those groups of fish were exposed to a suspension which was estimated to contain 30 *N. hirame* larvae per fish, or equal amount of the sea water for the controls. The same numbers of MEM-injected controls were exposed to *N. hirame*.

Therefore, following 4 groups with duplicates (a total of 8 tanks) were created at the end; VHS survivors infected with *N. hirame* (co-infection group), VHS survivors (VHSV carrier group), MEM injected fish infected with *N. hirame* (*N. hirame* group), and MEM injected fish without parasite infection (controls group). Mortalities from each tank were monitored daily for the next 58 days. Water temperature was maintained at 20℃ throughout the experiment for faster development of *N. hirame*. All fish were equally fed 3 to 4 times a week. All dead fish were processed as previously described. Virus titration was attempted from the head kidney of all dead and survivor fish. The hearts from selected fish were also sampled. To determine if the survivors from the virus injection have indeed become the virus carrier, stress test was conducted. As drastic change of water temperature has been shown to increase the virus titre (Iida *et al.*, 2003), five fish from VHS survivor group were immersed to 13℃ seawater for an hour then immediately transferred back to 20℃. Kidneys and hearts from the tested fish were sampled in the following day to assess the virus titre.

Virus Titration

Infective titres of VHSV in the kidney of all dead and randomly chosen survivors were quantified using the conventional methods by FHM cell line (Takano *et al.*, 2000). The 0.45 μm-filtered kidney homogenate was inoculated to the FHM cells seeded in 96-well plates. The plates were incubated at 20℃ and cytopathogenic effects (CPEs) were monitored daily for the following 2 weeks. $TCID_{50}$ for each sample were calculated following the Behrens-Karbere method. Confirmation of the VHSV induced CPEs were performed by the morphological characteristics of the CPEs and standard RT-PCR on selected samples (Isshiki *et al.*, 2001).

Statistical analyses

For the analysis comparing the survival between the treatment group, Kaplan-Meier survival probabilities were computed, and differences were tested using the log-rank test. Hb content between the groups was compared using Mann-Whitney U test. For the comparison of $TCID_{50}$, t-test was used.

RESULTS

VHSV Challenge to N. hirame *Infected Fish*

All the observed fish that were exposed to *N. hirame* and checked prior to the VHSV infection harboured adult and/or immature worms with total mean intensity (± SD otherwise stated) of 12.15±7.34 for the trial 1 and 14.68±9.81 worms for the trail 2. Anaemic symptoms, namely discoloration of the gills, were presented in the all observed fish. The Hb values of *N. hirame* infected fish were significantly lower (0.28±0.17 for trial 1, 0.35±0.07 mg/100mL for trial 2) than that of controls (3.31±0.63 for trial 1 and 3.58±0.46 mg/100mL for trial 2) (Mann-Whitney U test, $p<0.001$).

The mortality after VHSV infection was highest in the co-infected group at both

trials. In the trial 1, mortality was observed only from the fish infected with VHSV (i.e. co-infection and VHSV group) (Fig. VI-1, Table VI-1). The highest cumulative mortality in the first trial was 35% noted from one of the tank in co-infected group, while that in the replicate tank was only 5%. Co-infection by *N. hirame* and VHSV significantly decreased survival of juvenile flounder compared to fish from other groups ($p<0.05$ log-rank test). VHSV was detected from all dead fish except for two; the fish from VHSV group which died at 16 dp virus exposure, and the fish from co-infected group which died at 1 dp virus exposure (Fig. VI-1). However, almost none of the dead fish showed the typical VHS symptoms. The virus titre of the dead fish ranged from$10^{6.3}$ to $10^{10.1}$ with the mean value of $10^{8.42}$ $TCID_{50}$/g. Adult *N. hirame* were found in all co-infected dead fish with mean intensity of 11.38±7.07 (range 3-27). No worms were found from VHSV or Control groups.

In the second trial, the mortality curves from replicate tanks showed a relatively similar pattern (Fig. VI-2). Again, co-infection by both *N. hirame* and VHSV significantly reduced flounder survival compared to other groups (all $p<0.001$ log-rank test) (Table VI-1). However, unlike the first trial, mortalities was also observed in the fish infected only with *N. hirame*, and the survival of *N. hirame* group did not differ from that of VHSV group ($p=0.06$ log-rank test). Consistent with the first trial, most of the co-infected dead fish showed no or only minor physical VHS symptoms. In contrast, dead fish from the VHSV group showed the prominent symptoms including haemorrhage and extended abdomen filled with fluid.

The result of the virus detection in the second trial was somewhat complicated. Overall mortalities from co-infected, VHSV and *N. hirame* groups were 36, 20 and 4 fish (see Table VI-1), and of those, proportion of fish with positive VHSV detection were 54.3, 100.0 and 46.7%, respectively. This means that all the dead fish from VHSV group were confirmed with VHSV infection, but the virus could not be detected

in nearly the half of co-infected dead fish. Moreover, the virus was present in the fish infected only with *N. hirame* which were supposedly VHSV free. All the VHSV positive fish in *N. hirame* group were from the same tank in which rapid increase in mortality was observed after 24 dp infection (Fig. VI-2), indicating the virus contamination to this particular tank. Thus, the data obtained from this contaminated tank was excluded from the rest of analyses. Most of VHSV negative co-infected fish were concentrated within a first week of the experiment (Fig VI-2). The virus titre in kidney of the dead fish from VHSV group ($10^{9.49} \pm 10^{0.96}$ $TCID_{50}$/g) was significantly higher than that from co-infected group ($10^{8.28} \pm 10^{2.34}$ $TCID_{50}$/g; t-test, p=0.0405).

N. hirame *Challenge to VHSV Carriers*

After the VHSV injection, approximately 45 % have died in total with typical VHS symptoms. The mortality in each rearing tanks ranged from 33 to 63 % (Fig. VI-3). The mortality started by 4 dp virus injection, ceased by 13 days, and no further mortality was observed thereafter. Both virus titre and rate of virus detection from both in heart and kidney increased at between 1 and 5 dp injection (Table VI-2). However, the difference in virus titre between the two sampling periods was not statistically significant, probably due to the small sample size (N=5). Virus was not detected from the fish sampled prior to the *N. hirame* infection (i.e. 45 dp virus injection). No mortality, except for 2 fish, was observed from fish injected with MEM.

After exposing to *N. hirame*, major mortalities were observed between 18 and 29 dp exposure from the "VHS carrier" fish, regardless of *N. hirame* infection (Fig. VI-4). Co-infected fish showed the second increase of mortality at around 40 dp parasite infection. Corresponding to this second increase, major mortality from the *N. hirame* group was observed. However, death was noted only from one of the replicate tanks. No subsequent death in VHS group was observed after the initial mortality. Average

cumulative mortality from the two tanks for co-infected, VHSV carrier, *N. hirame* and control group were 54.4, 34.9, 25.1 and 0%, respectively (Table VI-1). Log-rank analysis showed significantly lower survival of co-infected group compared to other treatment groups ($p < 0.01$), except for VHSV carrier group. There was no difference in the survivals between *N. hirame* and VHSV carrier groups (log-rank test $p=0.18$).

Surprisingly, VHSV was not detected by either FHM cell lines or RT-PCR method from any of the dead fish, survivor fish, or the fish given temperature stress. Fish did not show the apparent signs of anorexia throughout the experiment. Some of VHS symptoms, mainly haemorrhage on the skin and accumulation of abdomen fluid, were present in the some of the dead fish, but were not apparent in many others. Worms were found from almost all the dead fish from the co-infected and *N. hirame* groups. However, some worms were degenerated. Mean worm intensities in dead fish from *N. hirame* group was 17.11±2.67 and it was significantly higher than that from co-infected group with 11.33±5.15 worms (Mann Whitney U-test; $p=0.008$). No controls died throughout the experiment.

DISCUSSION

The results of these experiments showed that the mortality of juvenile flounder were most prominent when they were co-infected with *N. hirame* and VHSV. It seemed that *N. hirame* infected fish are more easily became manifested by VHS. The result of the first trial clearly demonstrated this. In the first trial, almost all the mortalities were observed from co-infected group and all the dead fish were identified as VHSV-positive. Co-infections by two pathogens have are known to increase the mortality in flounder. Pakingking *et al.* (2003) showed the higher mortalities in flounder dually injected with aquabirnavirus and *Edwardsiella tarda*, or *Streptococcus iniae* than the fish with single infection. However, the inconsistency in mortality between the replicate tanks cast a question to the duplicability of the experiment.

Moreover, VHSV was not detected from nearly half of the co-infected dead fish in the second trial. Therefore, in the second trial, the cause of death cannot be identified from some of the dead fish. Whether this was owed to the inaptitude in the sample processing or death caused other than VHS is unclear. In the same trial, 20% mortality was observed from fish singly infected with *N. hirame*. It is possible that deaths in co-infection group were the result of additive mortality caused independently by VHSV and *N. hirame*.

The parasite density used for the present study was not outstandingly high compared to the natural infection level (Chapter II). The mortality of *N. hirame* infected fish was also observed in the earlier study (Chapter III). Hence, this result further confirmed that *N. hirame* could cause mortality in the juvenile flounder at least under the specific experimental conditions. To determine the effect of *N. hirame* on the replication of VSHV in flounder, further studies with more refined methods are necessary.

Most dead fish dually infected with *N. hirame* and VHSV did not exhibit typical VHS symptoms. This is probably due to the low haemoglobin contents caused by *N. hirame*. As blood of *N. hirame* infected fish often become almost colourless due to low haemoglobin contents, haemorrhage can be cryptic to our eyes. This can be a problem in the area in which *N. hirame* is abundant as one could overlook the VHS infection.

In the VHSV carrier experiment, more than half of fish survived from intramuscular injection of VHSV of $10^{3.5}$ $TCID_{50}$ virus titre. Virus was detected from more than 80% of fish sampled at 5 dp injection, but completely diminished by 45 days. This indicates that most of fish have once become infected with virus but the virus has diminished, or decreased to the undetected level afterward. This is consistent with the study by Iida *et al.* (2003) who showed that after giving the flounder an immersion

challenger, VHSV was first detected from various organs but become no longer detected in any organs by 7 weeks post challenge. They also showed the increase in the virus titre after giving fish the temperature stresses. In the present study, virus was not detected from any of dead, survivors or fish given the temperature stress by both cell line and PCR methods. This was an unpredicted outcome and the reason is still unclear. As the virus was not detected, the cause of death cannot be identified. Possible involvement of the third pathogen other than *N. hirame* and VHSV is highly unlikely because no mortality was observed in uninfected controls. One possibility is that the trauma from VHSV injection and subsequent replication of the virus in the target organs caused physical damage to the fish and the stress caused by *N. hirame* infection led the weaken fish to death. Nonetheless, further studies are mandatory, with possible application of more sensitive detection methods, such as nested-PCR, to clarify the fate of the virus.

In the summary, these two experiments showed that co-existence of two pathogens, *N. hirame* and VHSV reduced the survival of juvenile flounder. However, more studies are needed to understand the underlying mechanisms for this phenomenon.

Table VI-1. Mortality rate (numbers of dead/total) in each of replicates from co-infected, VHSV group *N. hirame* group, and controls at each experiment trial. Experiment 1: VHSV Challenge to *N. hirame* Infected Fish, Experiment 2: *N. hirame* Infection to VHSV Carriers. NA: data is not available due to the virus contamination.

	Co-infection			VHSV alone			*N. hirame* alone			Controls		
	Tank 1	Tank 2	Total	Tank 1	Tank 2	Total	Tank 1	Tank 2	Total	Tank 1	Tank 2	Total
Experiment 1												
Trial 1	35% (7/20)	5% (1/20)	20%	5% (1/20)	0% (0/20)	2.5%	0% (0/20)	0% (0/20)	0%	0% (0/20)	0% (0/20)	0%
Trial 2	80% (16/20)	100% (20/20)	90%	45% (9/20)	55% (11/20)	50%	NA	20% (4/20)	20%	0% (0/20)	0% (0/20)	0%
Experiment 2	50% (8/16)	59% (10/17)	54.5%	28% (5/18)	42% (8/19)	35.1	45% (9/20)	5% (1/19)	25.64	0% (0/17)	0% (0/18)	0%

Table VI-2. Virus titre in the kidney and heart from fish sampled at 1 and 5 days post VHSV injection. Both proportion of VHSV positive fish and virus titre increased over time. ND indicates negative detection by inoculating in FHM cell line.

	Virus Titre (log10 $TCID_{50}$/g)	
Samples	Kidney	Heart
1 days		
1	6.30	ND
2	6.80	ND
3	5.55	ND
4	ND	ND
5	ND	ND
6	ND	ND
Mean	6.22	--
Prevalence	50%	0%
5 days		
1	7.99	8.55
2	4.30	4.05
3	9.25	8.05
4	6.49	4.55
5	7.05	6.05
6	ND	ND
Mean	7.02	6.25
Prevalence	83%	83%

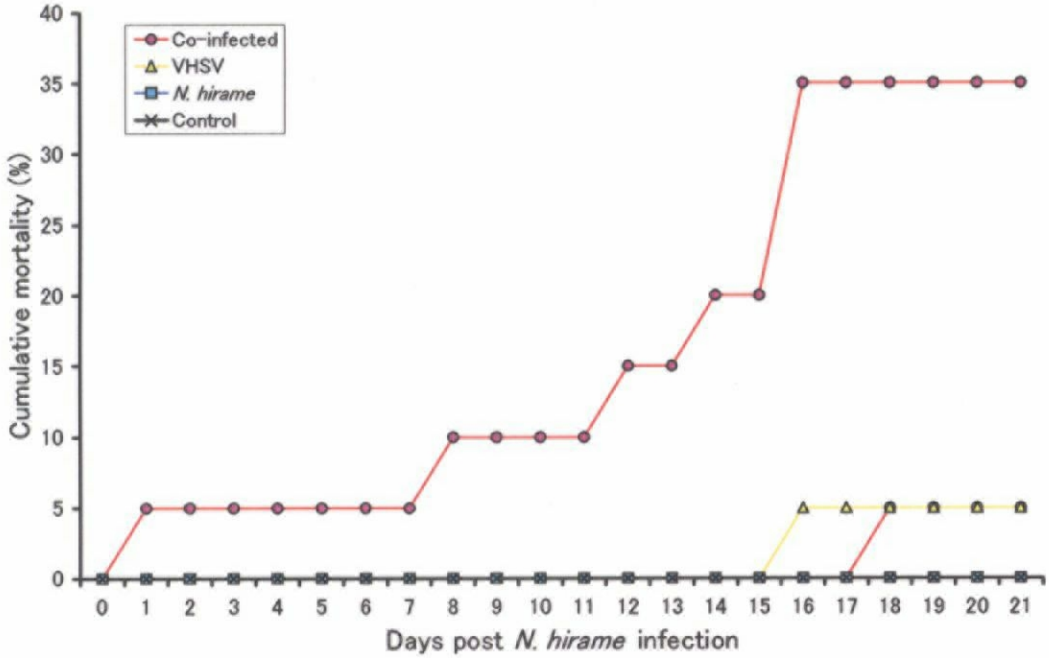

Figure VI-1. Cumulative mortality from the co-infected, VHSV, *N. hirame* and control group at the trial 1 of the "VHSV Challenge to *N. hirame* infected fish" experiment

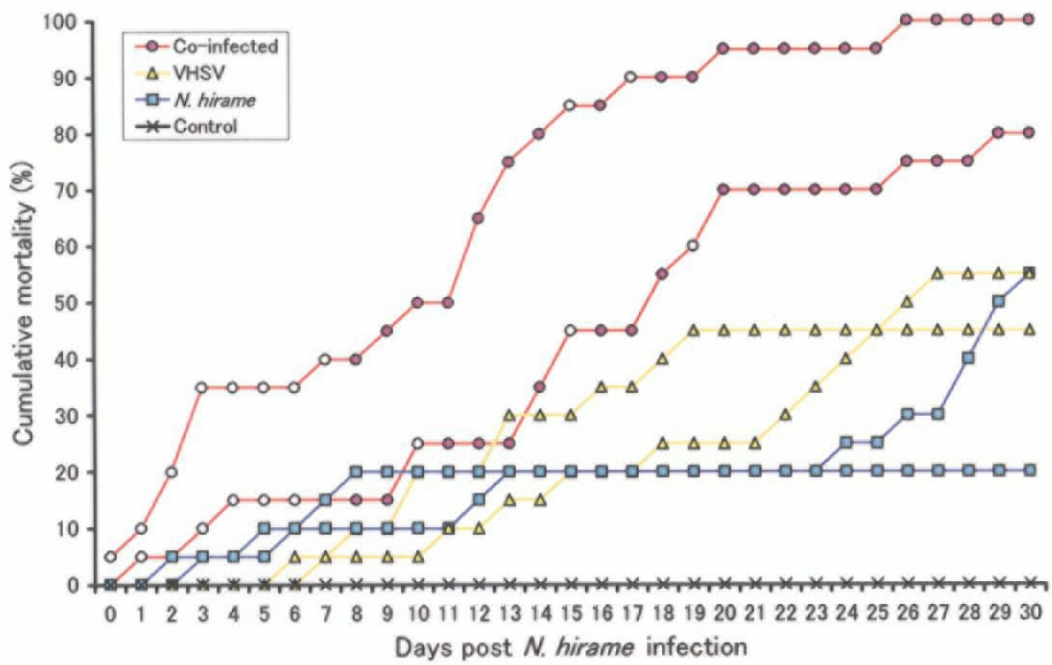

Figure VI-2. Cumulative mortality from the co-infected, VHSV, *N. hirame* and control group at the trial 2 of the "VHSV Challenge to *N. hirame* infected fish" experiment. Open circle indicates the presence of fish from which VHSV was not detected.

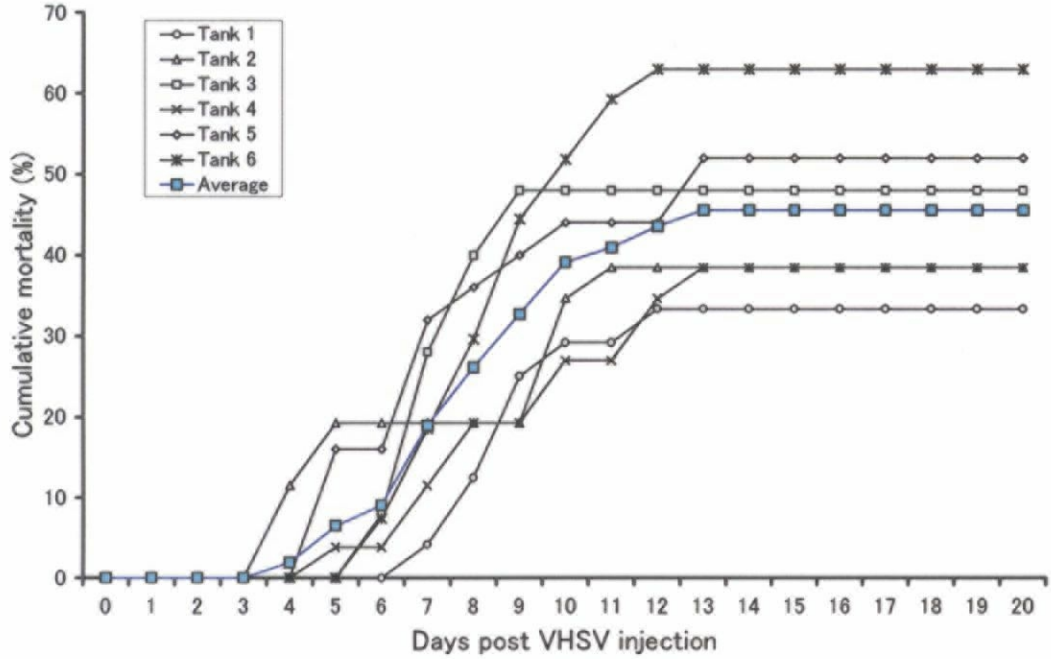

Figure VI-3. Cumulative mortality in juvenile flounder following VHSV injection. Mortality ceased in all the replicate tanks by 13 days post injection.

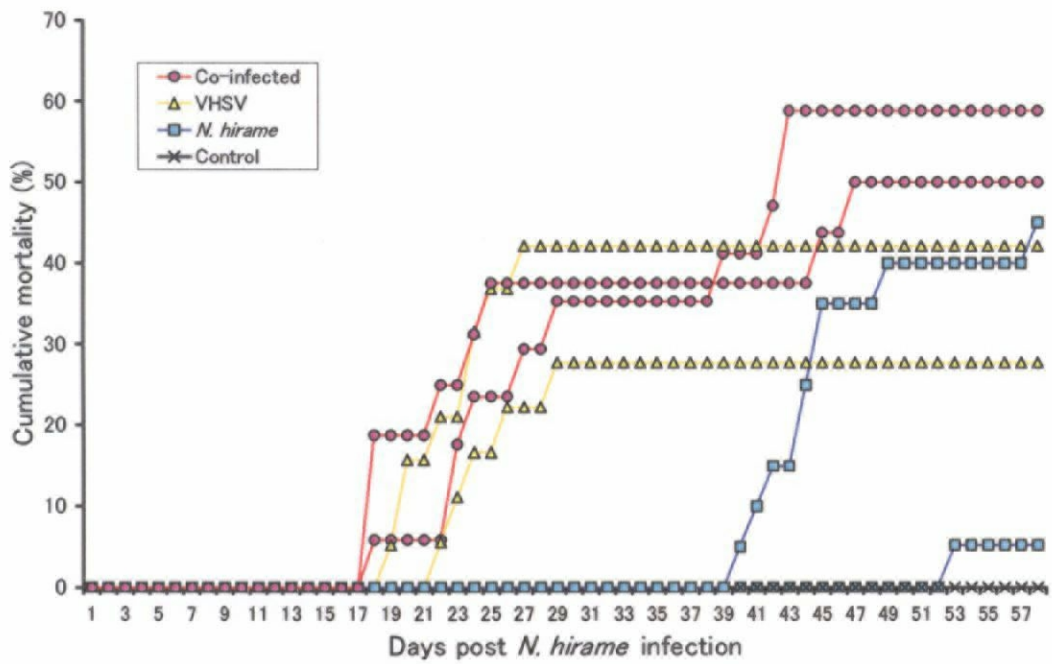

Figure VI-4. Cumulative mortality in fish that survived from VHSV injection and subsequently infected with *N. hirame*.

LITERATURE CITED

Brudeseth, B. E. & Evensen, O. 2002. Occurrence of viral haemorrhagic septicaemia virus (VHSV) in wild marine fish species in the coastal regions of Norway. *Diseases of Aquatic Organisms*, **52,** 21-28.

Iida, H., Mori, K., Nishizawa, T., Arimoto, M. & Muroga, K. 2003. Fate of viral hemorrhagic septicemia virus in Japanese flounder *Paralichthys olivaceus* challenged by immersion. *Fish Pathology*, **38,** 87-91.

Isshiki, T., Nishizawa, T., Kobayashi, T., Nagano, T. & Miyazaki, T. 2001. An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. *Diseases of Aquatic Organisms*, **47,** 87-99.

La Flamme, A. C., Scott, P. & Pearce, E. J. 2002. Schistosomiasis delays lesion resolution during *Leishmania major* infection by impairing parasite killing by macrophages. *Parasite Immunology*, **24,** 339-345.

Meyers, T. R., Short, S. & Lipton, K. 1999. Isolation of the North American strain of viral hemorrhagic septicemia virus (VHSV) associated with epizootic mortality in 2 new host species of Alaskan marine fish. *Diseases of Aquatic Organisms*, **38,** 81-86.

Nishizawa, T., Iida, H., Takano, R., Isshiki, T., Nakajima, K. & Muroga, K. 2002. Genetic relatedness among Japanese, American and European isolates of viral hemorrhagic septicemia virus (VHSV) based on partial G and P genes. *Diseases Of Aquatic Organisms*, **48,** 143-148.

Parkingking, R., Takano, R., Nishizawa, T., Mori, K., Iida, Y., Arimoto, M. & Muroga, K. 2003. Experimental coinfection with aquabirnavirus and viral hemorrhagic septicemia virus (VHSV), *Edwardsiella tarda* or *Streptococcus iniae* in Japanese flounder *Paralichthys olivaceus*. *Fish Pathology*, **38,** 15-21.

Smail, D. A. 1999. Viral haemorrhagic septicaemia. In: *Fish diseases and disorder* (Ed. by Woo, P. T. K. & Bruno, D. W.), pp. 123-147. Wallingford, U.K.: CABI Publishing.

Takano, R., Mori, K., Nishizawa, T., Arimoto, M. & Muroga, K. 2001. Isolation of viruses from wild Japanese flounder *Paralichthys olivaceus*. *Fish Pathology*, **36,** 153-160.

Takano, R., Nishizawa, T., Arimoto, M. & Muroga, K. 2000. Isolation of viral haemorrhagic septicaemia virus (VHSV) from wild Japanese flounder, *Paralichthys olivaceus*. *Bulletin of the European Association of Fish Pathologists*, **20,** 186-192.

Watanabe, L., Pakingking, R., Iida, H., Nishizawa, T., Iida, Y., Arimoto, M. & Muroga, K. 2002. Isolation of aquabirnavirus and viral hemorrhagic septicemia virus (VHSV) from wild marine fishes. *Fish Pathology*, **37,** 189-191.

Zhao, C. Q., Papadopoulou, B. & Tremblay, M. J. 2004. *Leishmania infantum* promotes replication of HIV type 1 in human lymphoid tissue cultured ex vivo by inducing secretion of the proinflammatory cytokines TNF-alpha and IL-1 alpha. *Journal of Immunology*, **172,** 3086-3093.

Chapter VII. General Discussion

The aim of this research was to elucidate the causal relationship between the decline of populations of olive flounder, *Paralichthys olivaceus*, and recent epidemic of the invaded parasite, *Neohetrobothrium hirame*. Two main emphases of the study were to investigate the underlying mechanism for the spatial variability in decline of flounder catch in association with the parasite infection, and to determine the effect of *N. hirame* on the survival of juvenile flounder. Through observational field studies and the laboratory experiments, I believe this study successfully demonstrated the causal effect of *N. hirame* onto the host survival, and provided the indirect but strong evidence for the parasite-mediated mortality in wild juvenile flounder. Significant advances have been made to understand the impacts of this pathogen on the olive flounder resources.

Effects of N. hirame *on Host Behaviours and Predation*

One significant advance was the demonstration that *N. hirame* affects various behaviours of juvenile flounder and subsequently makes them more susceptible to the predation. Infected flounder showed marked reduction in swimming performance and burrowing ability in addition to the maladaptive activity and feeding behaviour (Chapters IV and V). All of these behavioural changes may contribute to the lower survival rate of infected fish against fish predators (Chapter IV).

Numerous studies have reported the behavioural changes in various animal hosts infected with parasites from a wide range of taxa (reviewed by Holmes and Zohar, 1990; Moore and Gottelli, 1990; Poulin, 1994; Moore, 2002). Fish are frequently used in such behavioural studies as a model animal because they are convenient vertebrates to study (reviewed in Barber *et al.*, 2000). Although many of these studies speculated the association between the parasite-induced behavioural changes

and increased predation risk of infected host, empirical studies demonstrating the link between the two are rare. The present study provided a comprehensive and valuable example of a parasite-mediated induction of predation in a fish-monogenean system.

It is the logical thought that flounder population would face significant reduction in the area with high parasite intensity if mortality due to predation increases in association with the parasite infection. However, the present study has only assessed the effect of *N. hirame* in the individual level, and the effect on population-level and its ecological consequences are still unclear. All the experiments were conducted in unnatural laboratory conditions, and were not representing the natural environments. In addition, all fish used in the experiments were hatchery-reared juveniles with similar size class (ca. 10 cm in body length). Juvenile flounder were used for all the experiments as *N. hirame* has been suspected to cause mass mortality on 0 year flounder in the wild (Anshary *et al.*, 2002). Fish with this specific size class were used mainly because they were easy to obtain. However, wild fish of larger size classes are often heavily infected with *N. hriame* and their behaviour could be different from that of the smaller ones. Thus, studies using different sizes of fish, or wild fish are also important. Repeating the similar experiments using fish from various size classes, wild fish, and/or under more natural environment will provide more definitive evidence that *N. hirame* causes the reduction in the flounder population.

Worm Intensity and its Effects

Demonstration of the positive association between the magnitude of behavioural changes and *N. hirame* intensity is another significant advance of the present study. Negative correlations between the number of adult worms and swimming performance (Chapter IV), and prey capturing ability (Chapter V) have been clearly shown. This is an important finding because the association between worm intensity and decline of the host population size was indicated by the field study (Chapter II). The mean

parasite intensity at the peak infection season in Obama Bay was nearly five-fold higher than that in Miyako Bay. As flounder catch has been reduced in Obama, but not in Miyako Bay, the observed differences in parasite intensity is probably one of the important contributing factors for the geographical differences in the degree of flounder population decline.

Intensity-dependent pathogenicity is a fundamental characteristic in parasite (*sensu* Anderson and May, 1978). Epidemiological models distinguish parasites (macroparasite) from other pathogens by their intensity-dependent effects (Anderson and May, 1978). Bacteria, protozoans, viruses and fungi propagate within a host, hence their numbers in an individual host are less important when one considers their pathogenicity. On the other hand, pathological effects increase in association with the number of individual macroparasitic worms in a single host. For instance, a marine amphipod, *Paracalliope novizealandiae,* showed significantly higher mortality when they were infected with 25 or 125 *Maritrema novaezelandensis* metacercariae than with 5 (Fredensborg *et al.*, 2004). Similarly, a significant negative correlation between the intensity of *Uvulifer ambloplitis* metacercariae and survival of its host, *Lepomis macrochirus*, against temperature change has been shown (Lemly and Esch, 1984).

N. hirame is a typical macroparasite with a direct lifecycle and the intensity-dependent pathogenicity of *N. hirame* has been demonstrated through the positive correlation between the intensity of adult *N. hirame* and the severity of anaemia (Mushiake *et al.*, 2001; Yoshinaga *et al.*, 2001). The present study also showed the increase in the magnitude of maladaptive behavioural change in association with worm intensity. Therefore, the effects of *N. hirame* are considered to be greater in the area with high worm intensity. This finding provides further support that *N. hirame* plays an important role in the decline of flounder populations observed in the heavily parasitised water.

The present study also demonstrated that *N. hirame* cause direct mortality to the juvenile flounder, at least under the specific experimental conditions (Chapter III and VI). Past studies have also showed mortality of infected flounder, but the fish were infected with unnaturally high numbers of worms (Tsutsumi, 2004). The parasite intensity used for the present experiments were within the range of natural infection observed in Obama Bay, thus the direct mortality caused by the parasite is possible in the wild. However, infected fish used for behavioural studies that were kept in a large rearing tank with sand substrate, more natural environment, showed none, or very low mortality (Chapter IV and V). Therefore, the stress from the specific rearing condition can be an important factor for mortality of *N. hirame* infected fish. Nonetheless, the fact that *N. hirame* cause the death to the flounder indicate the strong pathogenicity of the parasite on the host's survival.

Infection of N. hirame *under Low Temperature*

The field observation and experimental study extended our knowledge on the basic biology of *N. hirame*. It was shown that the parasite is more abundant during the winter in the wild conditions, yet the low temperature (below 10℃) reduced the infectivity and survival (Chapter III). These seemingly contradictory results raise some questions. One possibility is that the higher infection level during winter is associated with lower immunity of the host. Wild flounder have reduced condition factor during winter (Chpater II), hence it is conceivable their physiological functions is also reduced during this period. This is supported by the past study that showed relatively lower antibody titre in the *N. hirame* infected-flounder reared at 15℃ than that at 20℃ or 25℃ (Tsutsumi *et al.*, 2003). Therefore, flounder are generally more susceptible to the infection during winter.

Another possibility is the seasonal change of the host density. Transmission success of monogenean parasites is a function of host density. In the other words,

infection level is should be high when there are more numbers of susceptible host in the given geographical area. If the flounder density is high during the winter and extremely high transmission is occurring, higher infection level can be expected even though the parasite infectivity is reduced in under the low temperature. Tsutsumi (2004) have attempted to determine the effect of host density on *N. hirame* infection. However, the result did not show any clear tendency, largely due to the experimental design. He also attempted to estimate the life-time egg production of *N. hirame* under three different temperatures. According to his estimation, life time fecundity is greatest at 15℃, then 25℃ followed by 20℃ (*cf.* daily egg production; 20℃> 25℃ >15℃). From this estimation, higher parasite transmission may be occurring during the winter period because of higher numbers of eggs are present at the ocean bottom during this period. However, these are merely speculations and more precise experiments and more information on the flounder ecology are necessary to determine the effect of water temperature and host density on the *N. hirame* population.

N. hirame *and VHSV Co-infection*

The fourth advance of the present study is the demonstration of increased mortality in flounder co-infected with *N. hirame* and VHSV (Chapter VI). As VHS outbreaks often occur during winter (Isshiki *et al.*, 2001), and VHSV has been isolated from the wild flounder in the same geographical area where the *N. hirame* is abundant (Takano *et al.*, 2000), there is a great possibility that both *N. hirame* and VHSV coexist in the same fish. Moreover, increased cannibalism (and predation) associated with *N. hirame* infection (Chapter III) may facilitate VHSV transmission among the flounder population and to other fishes. Therefore, the infection from these two pathogens could have a significant impact on the flounder population.

Despite the clear tendency of high mortality in co-infected fish, there was some obscureness in the experiment. The main problem was the low detection rate of

VHSV from the dead fish. VHSV was detected from only a half of dead fish that have been infected with *N. hirame* and subsequently challenged with VHSV. Moreover, VHSV could not be detected from any of fish in the VHSV carrier experiment (Chapter VI). Because of such a low VHSV detection rate, definitive cause of death could not be identified. This was the major weakness of the experiment, and there is a need to refine the experimental design and the virus detection techniques.

Although the present study only looked at the interaction between VHSV and *N. hirame*, co-infection experiments using various pathogens are of great interest. In the natural environment, it is rare to find individual fish infected only with a single pathogen. Single fish often harbours a variety of organisms, whether pathogenic or not. These organisms may have complex interactions as they are competing for the same resources in the same environment, the host, and trying to maximize their fitness. In the field study, high numbers of unidentified trematode parasites were found in the fish infected with *N. hirame* (Chapter II). Determining the effect of multiple infections on a host is often a difficult task, but it is essential for understanding the effect of a specific parasite on the host population under the natural environment. Co-infection experiment with *N. hirame* and other monogeneans, trematodes, protozoans, and opportunistic bacteria will open the door way to the future researches.

Future Perspectives

All of the results from the present study only provided partial idea on how and to what degree *N. hirame* influences the host population. Much more studies are needed to have more definitive and conclusive ideas about the effect of *N. hirame* on wild flounder. The foremost important future study will be the continuous and longitudinal monitoring of *N. hirame* in the wild fish. Since its first appearance, the spatial dispersion of the parasite has been well documented. This is a very rare

opportunity for the epidemiological study of an introduced macroparasite pathogen. I believe that *N. hirame*-flounder system is an excellent model system to work on. The longitudinal monitoring of the fate of this parasite is an important task for Japanese parasitologists.

In my personal speculation, *N. hirame* will expand its distribution range and their abundance will be increased within the next decade, until olive flounder can cope with this newly introduced parasite. As parasites are capable to adapt to the vast environmental changes, *N. hirame* may adapt to the low water temperature and flounder population in the northern region may suffer in the near future. However, the impact of *N. hirame* onto the host population will eventually be lowered as host cope with this parasite. It has only been a decade since the first report of *N. hirame* in the Japanese water, and it may take some more time until the epidemic settles down. Researchers, fishermen, and national/local government bodies should continuously monitor this parasite to prevent and foresee the potential future problem.

Various experimental researches are also needed to have more comprehensive idea about the effect *N. hirame* on flounder under the natural environment. Predation experiments can be conducted using other predators, including other piscivorous fishes as well as micro- and macro-crustaceans. The effects of *N. hirame* on other important behaviours, such as migratory behaviour, escape response, and learning should also be investigated. Furthermore, the information about the effect on adult flounder are completely lacking and need more studies. Assessing the clutch size, reproductive behaviour, maturation and other reproductive characteristics on *N. hirame* infected flounder also helps understand the parasite effect on host fitness.

It is a general tendency that when epidemics of a specific disease are ceased out, or effective treatment methods are developed, researchers seem to lose interest and shift their interests to a new disease. However, to fully understand the biology and ecology of pathogens, and their effect on other organisms, we should not shift our

focus and continuous research is necessary. I truly hope that the *N. hirame*-flounder system is continuously being a subject for future research.

Prevention and Control for Invasive Pathogens

Recent globalisations of the economies substantially increase the biological invasion and causing outbreaks of emerging diseases (Daszak *et al.*, 2000; Torchin *et al.*, 2002). In addition, climate changes and climatic events also facilitate disease outbreaks (Harvell *et al.*, 1999). In a marine system, infectious diseases can be a significant threat to the marine lives as the rate of spread of pathogens are generally higher than those in the terrestrial environment (McCallum *et al.*, 2003). Eradication or control of disease is a much more difficult task in the marine systems compared to the terrestrial system mainly due to its inaccessibility.

The total eradication of pathogens is nearly impossible once they enter the system. However, there are limited cases in which epidemic is successfully controlled in the wild marine animals. Abalone, *Haliotis rufescens*, in California suffered from shell deformation caused by sabellid polychaete, *Terebrasabella heterouncinata*, which was accidentally introduced through aquaculture practices. The removal of the most susceptible host species, the black turban snail, *Tegula funebralis*, and susceptible size class reduced the propagation of the polychaete and successfully eliminated the pest from the area (Calver and Kuris, 2000).

Purposefully reducing the host density below the thresholds for parasite persistence is one way to control parasitic diseases in wild fish. The culling can be an effective control method for *N. hirame*. For instance, reduction of the stocking size, development of effective stocking techniques, or lowering the density of a local young flounder will probably reduce the rate of the parasite transmission. However, reducing fish density, even for a temporarily, could have a significant economical impact on local fisheries. Therefore, careful planning is mandatory to practise such

controlling methods.

Prevention is of course the fundamental method in disease control. In 2003, Japan imported total of nearly 1.6 trillion yens worth of fishes and fishery product from all over the world (The Ministry of Agriculture, Forestry and Fisheries of Japan), and many of them are imported as live or fresh. Unfortunately, Japanese quarantine does not have an effective communicable disease control measure. Recent epidemic of KHV (Koi hepers virus) among both wild and cultured carp, *Cyprinus carpio,* revealed the flaw in the disease prevention and control practices in Japan.

N. hirame has introduced to Japanese water most likely via transportation of live fish for aquaculture purposes, and translocation of infected farmed fish or seedlings might have facilitated the spread of the parasite. However, the conclusive evidence for this hypothesis is still lacking and we still do not have definite idea on where the *N. hirame* came from, how it entered Japan, and how it spread in such a fast rate. To develop the effective prevention methods for potential pathogens yet to be introduced, investigation of the origin and route of entry of *N. hirame* is an important task.

There can be many other pathogens that unknowingly entered Japanese waters. Some may be affecting indigenous animals, both directly and indirectly without our realization. Preventing pathogens, or any unwanted organisms, from entering Japanese waters is essential to protect our unique ecosystems. Also, early detection of any signs of disease epidemics is mandatory for controlling the spread of disease in the wild. Unfortunately, Japanese government pays only limited attention to the environmental and ecological-related issues. The problem of *N. hirame* is probably only a tip of an iceberg. Immediate and major revision of the control measures is required to prevent further epidemics of invasive pathogens in Japan.

LITERATURE CITED

Anderson, R. M. & May, R. M. 1978. Regulation and stability of host-parasite population interactions.1. Regulatory processes. *Journal of Animal Ecology*, **47,** 219-247.

Anshary, H., Yamamoto, E., Miyanaga, T. & Ogawa, K. 2002. Infection dynamics of the monogenean *Neoheterobothrium hirame* among young wild Japanese flounder in the western Sea of Japan. *Fish Pathology*, **37,** 131-140.

Barber, I., Hoare, D. & Krause, J. 2000. Effects of parasites on fish behaviour: a review and evolutionary perspective. *Reviews in Fish Biology and Fisheries*, **10,** 131-165.

Culver, C. S. & Kuris, A. M. 2000. The apparent eradication of a locally established introduced marine pest. *Biological Invasions*, **2,** 245-253.

Daszak, P. 2000. Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science*, **287,** 1756.

Fredensborg, B. L., Mouritsen, K. N. & Poulin, R. 2004. Intensity-dependent mortality of *Paracalliope novizealandiae* (Amphipoda: Crustacea) infected by a trematode: experimental infections and field observations. *Journal of Experimental Marine Biology and Ecology*, **311,** 253-265.

Harvell, C. D., Kim, K., Burkholder, J. M., Colwell, R. R., Epstein, P. R., Grimes, D. J., Hofmann, E. E., Lipp, E. K., Osterhaus, A., Overstreet, R. M., Porter, J. W., Smith, G W. & Vasta, G R. 1999. Review: Marine ecology - Emerging marine diseases - Climate links and anthropogenic factors. *Science*, **285,** 1505-1510.

Holmes, J. C. & Zohar, S. 1990. Pathology and host behaviour. In: *Parasitism and Host behaviour* (Ed. by Barnard, C. J. & Behnke, J. M.), pp. 34-63. London: Taylor & Francis Ltd.

Isshiki, T., Nishizawa, T., Kobayashi, T., Nagano, T. & Miyazaki, T. 2001. An outbreak of VHSV (viral hemorrhagic septicemia virus) infection in farmed Japanese flounder *Paralichthys olivaceus* in Japan. *Diseases of Aquatic Organisms*, **47,** 87-99.

Lemly, A. D. & Esch, G W. 1984. Population biology of the trematode *Uvulifer ambloplitis* (Hughes, 1927) in juvenile bluegill sunfish, *Lepomis macrochirus*, and largemouth bass, *Micropterus salmoides*. *Journal of Parasitology*, **70,** 466-474.

McCallum, H., Harvell, D. & Dobson, A. 2003. Rates of spread of marine pathogens. *Ecology Letters*, **6,** 1062-1067.

Moore, J. 2002. *Parasites and the Behavior of Animals*. New York: Oxford University Press.

Moore, J. & Gotelli, N. J. 1990. A philogenetic perspective on the evolution of altered host behaviours: a critical look at the manipuation hypothesis. In: *Parasitism*

and Host Behaviour (Ed. by Barnard, C. J. & Behnke, J. M.), pp. 193-229. London: Taylor & Francis Ltd.

Mushiake, K., Mori, K. & Arimoto, M. 2001. Epizootiology of anemia in wild Japanese flounder. *Fish Pathology*, **36,** 125-132.

Poulin, R. 1994. Meta-analysis of parasite-induced behavioural changes. *Animal Behaviour*, **48,** 137-146.

Takano, R., Nishizawa, T., Arimoto, M. & Muroga, K. 2000. Isolation of viral haemorrhagic septicaemia virus (VHSV) from wild Japanese flounder, *Paralichthys olivaceus*. *Bulletin of the European Association of Fish Pathologists*, **20,** 186-192.

Torchin, M. E., Lafferty, K. D. & Kuris, A. M. 2002. Parasites and marine invasions. *Parasitology*, **124,** S137-151.

Tsutsumi, N. 2004. The origin and spreads of the monogenean parasite *Neoheterobothrium hirame* infecting wild olive flounder. In: *Department of Aquatic Bioscience*, 157. pp. Tokyo: The University of Tokyo.

Tsutsumi, N., Yoshinaga, T., Kamaishi, T., Nakayasu, C. & Ogawa, K. 2003. Effects of water temperature on the development of the monogenean *Neoheterobothrium hirame* on Japanese flounder *Paralichthys olivaceus*. *Fish Pathology*, **38,** 41-47.

Yoshinaga, T., Kamaishi, T., Segawa, I., Yamano, K., Ikeda, H. & Sorimachi, M. 2001. Anemia caused by challenges with the monogenean *Neoheterobothrium hirame* in the Japanese flounder. *Fish Pathology*, **36,** 13-20.