Larval Attachment and Development of the Monogenean *Neoheterobothrium hirame* under Low Water Temperature

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ABSTRACT—Effects of low water temperature (below 10°C) on the oncomiracidial attachment and its subsequent development of *Neoheterobothrium hirame* were investigated. The cumulative attached larvae to the gill pieces from olive flounder *Paralichthys olivaceus* was reduced by 30% at 5°C when compared with 20°C. At 8°C the parasite development on flounder was significantly retarded compared at 20°C, and considerable number of worms disappeared from the host before reaching maturation. These results suggest low water temperature is a factor limiting the population growth of *N. hirame* in cold temperature regions.

Key words: *Neoheterobothrium hirame*, *Paralichthys olivaceus*, water temperature, survival, development

The blood-feeding monogenean *Neoheterobothrium hirame* is a widespread parasite among olive flounder, *Paralichthys olivaceus*, in the Japanese waters¹⁾. Although high prevalence of *N. hirame* infection has been observed in a wide geographical area, regional variability in the infection level has been observed^{1,2)}. Our ongoing field observations in Miyako Bay, Iwate and Obama Bay, Fukui also showed that infection level in Miyako was significantly lower than that in Obama. Miyako Bay is located in the northern Pacific coast of Japan, and the average annual water temperature in 2002 was 12.0°C, with average water temperature during the winter months (December to March) of 5.6°C. This made the water temperature in one third of a year below 10°C. We hypothesize that such low water temperature is a factor limiting population growth of *N. hirame*. Although past studies have showed effects of water temperature on egg production³⁾, egg hatching

rate⁴⁾, growth rate and survival⁵⁾ in *N. hirame*, there have been no report on the infection biology of *N. hirame* below 10°C. Two laboratory experiments were conducted to quantify the larval attachment and the parasite development under low water temperature conditions.

Materials and Methods

Collection of parasite eggs and oncomiracidia

Neoheterobothrium hirame eggs were collected from a tank with infected olive flounder and incubated at 21°C. Freshly hatched larvae were used for the following experiment.

Larval attachment to host gills in vitro

The attachment of oncomiracidia to the host at different temperatures was investigated *in vitro*, using gill pieces of juvenile olive flounder. To avoid contamination, antibiotics (Penicillin G potassium 100 units/mL, Streptomycin sulphate 1000 µg/mL, Amphotericin B 25 µg/mL) were added to the larval suspension. The suspension was then stored at 5°C for 2 h to incorporate possible negative effects of temperature change onto the attachment by giving all the larvae an equal temperature shock. The suspension containing approximately 100 (97–103) larvae and a small piece of gill (ca. 4 × 4 mm) that were excised freshly from uninfected juvenile flounder were added into each well of 6 Well Microplate (Corning, USA). Each plate was stored at 5, 10 or 20°C for 24 h. Considering the degradation of the gill tissue, gill piece was replaced with a fresh one every three hours and numbers of larvae attached to the removed gill pieces were counted. Thus, total of seven gill pieces were used for each well. The cumulative attachment rate over 24 h among the three temperature treatments were compared at the end.

Parasite development

For the experimental infection, total of 80 juvenile olive flounder, mean total length (± SD, here and elsewhere otherwise stated) of 91 ± 4.0 mm, were exposed to 4,000 oncomiracidia (ca. 50 larvae per fish) at 18°C. Fish were then randomly assigned to a rearing tank kept at 20 or 8°C. Five fish from each tank were sampled at 10 or 15 day intervals, except for the last two samplings which were done at longer intervals. At each sampling, the following measurements and observations were conducted: total body length, body weight, haemoglobin content (Hb) of the peripheral blood, coloration of the gills and internal organs, and parasite load. Worms found from the buccal cavity were considered as adults, and immature parasites on the gills were further categorized into the following five developmental stages based on the number of clamps; Stage 0: no obvious developed clamps, Stages I–IV: 1–4 pairs of clamps accordingly⁵⁾. The experiment was terminated at 105 days post expo-

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sure (PE).

Statistical analyses

The normality of the data was tested by Shapiro-Wilk test. The differences in total number of attached oncomiracidia among the three temperatures were tested using ANOVA followed by Tukey-Kramer HSD multiple comparison. The parasite densities and Hb

between the two rearing temperatures were analysed using Wilcoxon rank sum test.

Results and Discussion

Although *N. hirame* oncomiracidia still maintained its infectivity at low water temperature, the cumulative attached rate was significantly affected by the tempera-

Fig. 1. Changes in average ($\pm$ SD) cumulative number of larvae attached to the gill pieces of olive flounder at 5, 10 and 20°C over 24 hours. Approximately 100 larvae were placed in each well and each gill piece was exposed to larvae for 3 hours.

Fig. 2. Development of *N. hirame* on experimentally infected juvenile olive flounder at 8°C and 20°C. Adult refer to the worms inhabiting the buccal cavity, and Stage 0 and Stages I–IV refer to those with 0 and with 1–4 pairs of clamps, respectively. Bars indicate mean intensity $\pm$ SD. Closed circle and open circles indicate prevalence at 20°C and 8°C, respectively. □ Stage 0, ▤ Stage I, ▨ Stage II, ▩ Stage III, ■ Stage IV, ■ Adult

ture (ANOVA, $F_{2,17} = 56.54$, $p < 0.0001$; Fig. 1). The mean ratios of attached larvae after 24 h at 5, 10 and 20°C were $11.7 \pm 1.50\%$, $22.5 \pm 3.94\%$ and $36.8 \pm 5.74\%$, respectively and were significantly different from each other (Tukey-Kramer HSD, $p < 0.05$). The attachment rate at 5°C was less than 30% of that at 20°C. Although the attachment rate may be underestimated as it was investigated *in vitro* using the gill pieces, the low attachment rate in lower water temperature groups strongly suggests the inhibition of *N. hirame* transmission among wild olive flounder in cold water regions.

Water temperature also had a strong influence on the development of *N. hirame* (Fig. 2). At 20°C, adult worms were first observed at 35 days PE, but no longer found at 75 days PE, probably because all the adults had died or dislodged. In contrast, the most advanced stage during the 105 days of experimental period at 8°C was only stage III. This indicates the drastic retardation in the parasite development. Moreover, at 8°C, numbers of immature worms declined over time. Furthermore, the highest worm intensity observed at 8°C (17.8 ± 7.5 worms at 35 days PE) was significantly lower than that at 20°C (31.4 ± 7.3 worms at 21 days PE) (Wilcoxon, $Z = -2.21$, $p = 0.027$; Fig. 2). These results suggest that the immature parasites in the 8°C group had died or dislodged⁶⁾ during the prolonged premature period. Such mortality of immature worms is probably due to the unsuitable low water temperature for *N. hirame*.

Haemoglobin contents in the fish reared at 20°C significantly fluctuated. At the beginning of the experiment, mean Hb was 3.8 ± 1.1 g/100 mL, but fish showed anaemic symptoms at 35 (1.5 ± 1.5 g/100 mL) and 45 days PE (0.2 ± 0.04 g/100 mL). However, the anaemia seemed to be recovering at 75 days PE (mean Hb 2.8 ± 0.4 g/100 mL). At 45 days PE, the Hb of fish reared at 20°C was significantly lower than that at 8°C (Wilcoxon rank sum, $Z = 2.59$, $p = 0.009$). All anaemic fish had abnormally pale coloured gills and internal organs, espe-

cially the liver. No anaemic fish were observed at 8°C. Past studies have shown a strong correlation between the number of adult parasites and the host anaemic signs^{1,7)}. The appearance of anaemic flounder in the present study also coincided with the parasite maturation. Thus, this evidences that the adult *N. hirame* is responsible for the anaemia of olive flounder. However, mass mortality of wild flounder due to *N. hirame* infection has never been reported. In the present study, 12.5% mortalities were observed at 20°C between 41 and 45 days PE, associated with anaemia. The worm intensity in the present study was within the range of infection level in wild juvenile flounder in Obama Bay (Shirakashi, unpublished data). Therefore, the present study provided potential evidence that *N. hirame* may cause mortalities of juvenile flounder in the wild as previously suggested²⁾.

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