Dysfunction in Respiration and Osmotic Regulation of Larval Japanese Flounder Affected by Viral Epidermal Hyperplasia

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(Received December 19, 2007)

ABSTRACT—Flounder herpesvirus (FHV) induces epidermal hyperplasia in larval Japanese flounder Paralichthys olivaceus. We examined the influence of ambient oxygen and salinity concentrations on mortality of flounder larvae by FHV infection. FHV-infected fish showed markedly higher mortality than uninfected fish under normoxia (partial oxygen pressure; \( P_{O_2} \) = ca. 160 Torr), but survived at high rates under hyperoxia (\( P_{O_2} \) = ca. 280 Torr or higher) as in uninfected fish. In the \( P_{O_2} \) range from 100 to 400 Torr, infected fish always displayed lower levels of oxygen consumption (\( \dot{M}O_2 \)) compared to the uninfected fish. Under \( P_{O_2} \) of 260 Torr, the 48 h-survival rate of infected fish in diluted seawater (salinity 8 or 16 ppt) was much higher than that in full-strength seawater (salinity 32 ppt). Whole-mount immunocytochemistry to detect Na+/K+-ATPase and Na+/K+/2Cl– cotransporter of the skin of both infected and uninfected larvae revealed that the infected larvae had a significantly lower number of chloride cells. These results suggest that flounder larvae infected with FHV die of dysfunction in respiration and osmotic regulation.

Key words: flounder herpesvirus, Paralichthys olivaceus, viral epidermal hyperplasia, gas exchange, oxygen consumption, hyperoxia, salinity tolerance, chloride cell

Viral epidermal hyperplasia is a herpesvirus infection causing mass mortality in hatchery-reared larvae of Japanese flounder Paralichthys olivaceus, and the causative virus was designated as flounder herpesvirus (FHV) (Iida et al., 1989; Iida et al., 1991). No cell lines to propagate the virus have found. The affected fish showed epidermal hyperplasia on the fins and skin with vacuolar degeneration of the Malpighian cells, and epidermal necrosis in advanced stages of the infection (Iida et al., 1991). Experimental infection using filtered homogenate of the affected fish revealed that only larval fish are susceptible to FHV (Masumura et al., 1989).

Japanese flounder exhibits drastic metamorphosis from pelagic larvae to benthic juveniles, involving migration of the right eye to the left side of the head. Physiological changes in oxygen consumption accompany the developmental changes during metamorphosis (Kurokura et al., 1995). Japanese flounder shows tolerance to low salinity, and the increased tolerance of low salinity also starts at the beginning of metamorphosis and reaches its maximum at metamorphic climax (Hiroi et al., 1997). It was suggested that larval chloride cells in the epithelia covering the body have an ion-secreting function in seawater (Kaneko et al., 2002). Hiroi et al. (1998) reported that cutaneous chloride cells decrease in size and density at the beginning of metamorphosis and disappear at the metamorphic climax, indicating the shift of ion secretion from cutaneous to branchial chloride cells.

Based on the current knowledge of fish skin in larvae, we hypothesized that hyperplastic epithelium of FHV-infected fish affects cutaneous respiration and ion regulation of chloride cells. To demonstrate this hypothesis, the influence of FHV infection on the oxygen uptake in flounder larvae was analyzed by examining the viability and oxygen consumption under hypoxic and hyperoxic conditions. Furthermore, osmoregulatory
abilities of FHV-infected fish were analyzed by testing the salinity tolerance of the fish and by observing the distribution of chloride cells in the skin.

Materials and Methods

Fish

Spontaneously spawned eggs of the Japanese flounder were collected from a broodstock of a private facility, and then transferred to the Fisheries & Marine Technology Center, Hiroshima Prefectural Technology Research Institute. Larvae were reared in a tank (1,000 L) with running seawater treated with ultraviolet irradiation. FHV was not detected in the larvae stock by a PCR method previously described in Iida and Nagai (2004). Water temperature was maintained at 18–20°C, and the salinity ranged between 31.5 and 32.5 ppt. Larvae were initially fed on rotifers Branchionus rotundiformis cultivated with Nannochloropsis sp., and later on brine shrimp Artemia spp. nauplii enriched with docosahexaenoic acid. Developmental stages were classified based upon morphological criteria previously described by Minami (1982) and Miwa et al. (1988): premetamorphosis (C–D stages; eyes are bilaterally symmetrical), metamorphosis (E–F stages; the translocating eye is slightly asymmetrical to the non-translocating eye), metamorphic climax (G–H stages; the translocating eye is visible from the opposite side of the head), and post-metamorphic juvenile (I stage; the translocated eye reaches its final position).

Preparation of virus-infected and uninfected fish

Infection was carried out by the method of Iida et al. (1989). Spontaneously FHV-infected fish preserved at −80°C were homogenized with 99 volumes of Hanks’ balanced salt solution (HBSS). The homogenate was centrifuged at 5,000 × g for 15 min (4°C), and the supernatant was filtered through a 0.45 µm membrane filter. The filtrate was used as a virus inoculum. Two plastic tanks (500 L) with closed filtering systems were filled with sand-filtered seawater kept at 20°C, in which apparently healthy flounder larvae, each approximately 1,000 individuals, were introduced. Twenty milliliters of the virus inoculum was added to a tank for FHV infection, and the same volume of HBSS was added to another tank for sham infection. When exposed to the virus in this manner, almost 100% larvae exhibited epidermal hyperplasia in the skin and fins in a week after virus exposure (Iida et al., 1989; Iida et al., 1991). The live fish at 4 days post-infection were used as FHV-infected fish for survival, oxygen consumption and salinity tolerance tests. For whole-mount immunocytochemistry, fish were sampled from the tank at 8 days post-infection, anesthetized with MS-222, fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C for 48 h, and preserved in 70% ethanol. Uninfected normal fish at the same ages were fixed in the same manner and used as controls.

Fish survival under hypoxia and hyperoxia

Glass flasks of 2.450 mL were filled with sand-filtered seawater adjusted to various conditions of PO₂ (90–400 Torr) by bubbling nitrogen or oxygen gas. Oxygen concentration in seawater was determined by oxygen electrode DO-14P (TOA Electronics Ltd., Japan). Fifty uninfected or FHV-infected fish of D stage were transferred by pipettes from the stocking tanks to each flask, and then the flask was sealed with a silicon plug to prevent gas exchange and put into a water bath controlled at 20°C. Survival rates of fish were examined 24 h after transfer.

Oxygen consumption

For oxygen consumption (MO₂) measurement, oxygen saturation levels of seawater were adjusted by bubbling oxygen gas to 200, 150 and 100%, or nitrogen gas to 75 and 50%, equivalent to PO₂ values of 316, 237, 158, 119, and 79 Torr, respectively. Uninfected or infected fish (D stage), 20–30 individuals, were transferred into a glass bottle (300 mL) filled with seawater having different PO₂ values, and the bottle was sealed and kept in a water bath (20°C). The resting rate of MO₂ was measured for 2.0–2.5 h, depending on the fish size, which was long enough to record changes of 0.2–0.5 mL O₂ per bottle without decreasing the dissolved oxygen concentration below 90% of the initial saturation level. The rate of MO₂ (nmol O₂/individual/h) was calculated as MO₂ = δO₂/v/m, where δO₂ is oxygen concentration measured in nmol O₂/L, v is chamber volume without fish volume (assuming 1 g = 1 mL), m is the number of fish employed, and t is time in hours.

Salinity tolerance

To test the salinity tolerance of fish under the normoxic condition, FHV-infected fish at three different developmental stages, premetamorphosis (C or D stage), metamorphosis (E or F stage) and post-metamorphic juvenile (I stage) were transferred into 10 L plastic tanks so that each tank received 25 fish. The tank contained either 1/8-diluted (salinity: 4 ppt), 1/4-diluted (8 ppt), 1/2-diluted (16 ppt) or full-strength seawater (32 ppt). Survival rates were examined at 48 h after the transfer. The experiments with fish at premetamorphic and metamorphic stages were performed twice.

For salinity tolerance under a hypoxic condition, 25 FHV-infected or uninfected fish at premetamorphosis (D stage) were transferred to plastic tanks containing dilute or undiluted seawater, as described above. Oxygen gas was introduced into the tank with injection needles to adjust PO₂ to 260 Torr. Water temperature was maintained at 20–21°C. Survival rates of fish were
examined at 48 h after the transfer.

**Whole-mount immunocytochemistry**

Rabbit polyclonal antiserum was raised against a synthetic peptide corresponding to part of the highly conserved region of the Na⁺/K⁺-ATPase α-subunit (NAK121; Katoh et al., 2000), which was based on the method described by Ura et al. (1996). The specific antibody was purified by affinity chromatography and conjugated to Alexa Fluor 546 using a Protein Labeling Kit (Molecular Probes, USA). For detection of Na⁺/K⁺/2Cl⁻ cotransporter (NKCC), a mouse monoclonal antibody (T4) obtained from the Developmental Studies Hybridoma Bank (University of Iowa, USA) was used. This T4 antibody, which was raised against human colonic NKCC, proved to react specifically with NKCC from many vertebrates including teleost fish (McCormick et al., 2003). The antibody was directly labeled with Alexa Fluor 488 using a Zenon Mouse IgG Labeling Kit (Molecular Probes). These antibodies were diluted in phosphate-buffered saline containing 0.2% Triton X-100 (PBST), 0.02% keyhole limpet hemocyanin, 0.1% bovine serum albumin, 10% normal goat serum and 0.01% sodium azide.

The fixed fish were rinsed in PBST for 1 h, and then incubated simultaneously with the labeled anti-Na⁺/K⁺-ATPase (diluted at a ratio of 1:250), anti-NKCC (0.8 mg/mL; Molecular Probes) overnight at 4°C. After washing in PBST for 1 h and fixation with 4% paraformaldehyde for 15 min, the sample was mounted on a slide with SlowFade-Light antifade reagent (Molecular Probes) and examined by a Zeiss LSM 510 META confocal laser scanning microscope (Carl Zeiss, Germany). Confocal images on abdominal skin of the left (future ocular) side of fish were taken with 10-μm intervals to generate Z-stacks. The area and density of immunopositive chloride cells were measured using the NIH Image program (http://rsb.info.nih.gov/nih-image/).

**Statistical analyses**

All measurements were analysed using the JMP (SAS Inc., USA). For oxygen consumption, data were analyzed using regression analysis and analysis of covariance (ANCOVA). The relationships between PO₂ and MO₂ for uninfected and FHV-infected larvae were tested for logarithmic linearity of response. For salinity tolerance of fish after logit transformation ($\log_{10}(p/(100-p))$, $p =$ % survival rate), significant differences of survival rates were tested by ANOVA, the Tukey-Kramer test, the $\chi^2$-test, and Cochran-Mantel-Haenszel test. Two-sample t-test was used for analysis in immunocytochemistry. We considered differences significant at $P < 0.05$.

**Results**

**Fish survival under hypoxia and hyperoxia**

Most flounder larvae (D stage), both uninfected and FHV-infected, died under hypoxia (100 Torr) in 24 h. Under conditions below approximately 240 Torr, the survival rates of FHV-infected fish were always lower than those of uninfected fish. Under the hyperoxia of more than 280 Torr, however, both fish groups showed survival rates higher than 85% (Fig. 1).

**Oxygen consumption**

The oxygen consumption (MO₂) of uninfected and FHV-infected flounder larvae (D stage) under different conditions was measured. The relationship between ambient partial oxygen pressure (PO₂) and oxygen consumption (MO₂) of FHV-infected and uninfected (normal) flounder larvae (premetamorphic D stage) was analyzed. Fig. 2 shows the relationship between PO₂ and MO₂.

![Fig. 1. Survival rates of FHV-infected and uninfected (normal) flounder larvae (premetamorphic D stage). Fish were kept for 24 h under graded partial oxygen pressures (PO₂).](image)

![Fig. 2. Relationship between ambient partial oxygen pressure (PO₂) and oxygen consumption (MO₂) of FHV-infected and uninfected (normal) flounder larvae (premetamorphic D stage). Longitudinal bars show standard deviations from two measurements.](image)
PO2 conditions is shown in Fig. 2. In the PO2 range from 100 to 350 Torr, MO2 in both fish groups increased in proportion to PO2, with the same slope of 0.884 (ANCOVA), but the infected fish always displayed lower levels of oxygen consumption compared to uninfected fish. The intercepts (= MO2 / PO2^0.884) of the regression lines were significantly different with respective values for uninfected and infected larvae of 1.38 and 0.60 (ANCOVA).

**Salinity tolerance**

The results of salinity tolerance test for FHV-infected flounder larvae under normoxia (about 160 Torr) are shown in Fig. 3. Post-metamorphic juveniles (I stage) showed high resistance against all salinity levels for 48 h. All prometamorphic larvae (E or F stage) died in salinity of 4 ppt (1/8 diluted seawater) but fish more than 70% survived in 8, 16, and 32 ppt (full-strength seawater) salinities without significant differences in the survival rates (Tukey-Kramer test). In contrast, premetamorphic larvae (C or D stage) were highly sensitive to all test waters and all the survival rates were less than 20%.

Fig. 4 shows the salinity tolerance of FHV-infected and uninfected premetamorphic larvae (D stage) under hyperoxia (about 260 Torr). Uninfected fish showed survival rates higher than 80% in salinities of 8, 16, and 32 ppt, but only 20% in 4 ppt salinity. FHV-infected fish displayed a prominently lower survival rate in 32 ppt compared to those in 16 and 8 ppt, and no FHV-infected fish survived at 4 ppt. Neither infected nor uninfected fish showed a difference in survival rates between 16 and 8 ppt salinities (Cochran-Mantel-Haenszel test).

**Whole-mount immunocytochemistry**

As shown in Fig. 5, the pattern of immunoreactivity for NKCC was nearly identical to that of Na+/K+-ATPase in both FHV-infected and uninfected premetamorphic larvae (D stage). Chloride cells in the skin of both infected and uninfected fish were located only on the surface (Fig. 5, d and h). Size/frequency distributions of immunopositive chloride cells in FHV-infected and uninfected larvae (D stage) are shown in Fig. 6. Chloride cells were significantly less dense in FHV-infected fish (137 cells/mm²) compared to uninfected fish (371 cells/mm²) (two-sample t-test), although the size of immunopositive chloride cells was quite similar in both fish (177 μm² in FHV-infected fish versus 179 μm² in uninfected; two-sample t-test).

**Discussion**

FHV infection under normoxia was fatal to Japanese flounder larvae in the present experimental infection, as it is in spontaneous infection (Iida et al., 1989). The present study, however, showed that the infected larvae exhibited high survival rate under hyperoxia at approximately 280 Torr or higher PO2. Because of the importance of skin in respiratory gas exchange during larval development before gills become functional (Burggren and Pinder, 1991), this suggests that the increased dissolved oxygen compensated for poor cutaneous gas exchange across the hyperplastic epidermis caused by FHV infection. Cutaneous epithelia are only one or two cells thick in larval fish, and thus diffusion distances for ambient oxygen to underlying tissues are generally short (less than 10 μm) (Roberts et al., 1973; Steffensen and Lomholt, 1985; Seikai, 1992; Wells and Pinder, 1996). The epidermis of normal flounder larvae is two cells thick, while that of FHV-infected larvae is about four cells thick in average (Iida et al., 1991), indicating that
Fig. 5. Immunoreactivity of Na⁺/K⁺-ATPase (red) and Na⁺/K⁺/2Cl⁻ cotransporter (NKCC; green) on abdominal skin of the left (future ocular) side of uninfected (a–d) and FHV-infected (e–h) flounder larvae (premetamorphic D stage). Nuclei were stained with TO-PRO-3 iodide (blue). Double-immunostaining and nuclear staining were performed on the same sections. Images d and h (horizontal X–Y plane optical sections) were merged from a–c and e–g, respectively, to highlight the co-localization of Na⁺/K⁺-ATPase and NKCC cotransporter. Co-localization of green and red signals appears yellow-orange. Vertical (X–Z and Y–Z plane) optical sections perpendicular to the plane of the epidermis were digitally compiled; these are shown in the narrow images at the edges of d and h. The scale bar represents 20 μm.
Physiological dysfunction of VEH-affected larvae

FHV-infected larvae have a longer diffusion distance in the skin. Masumura et al. (1989) reported that FHV can induce epidermal hyperplasia in benthic juveniles, but this hyperplasia was not fatal. Since metamorphosed flounder juveniles have well-developed gills, the tolerance of benthic juveniles to FHV infection is likely to be attributable to the shift of major respiratory organs into the gills.

$\dot{M}O_2$ in both FHV-infected and uninfected larvae groups was raised in proportion to ambient $PO_2$ (Fig. 2). $\dot{M}O_2$ of FHV-infected fish, however, was consistently lower than that of the uninfected fish. The regression lines in log scale between FHV-infected and uninfected fish were statistically parallel, and these regression lines had different intercepts, indicating the same $O_2$ uptake mechanism in both groups. The survival rate of uninfected larvae increased under $PO_2$ more than 100 Torr (Fig. 1), where the $\dot{M}O_2$ for uninfected larvae was approximately $\geq 90$ nmol O$_2$/larva/h (Fig. 2). In contrast, FHV-infected fish could secure this critical $\dot{M}O_2$ only in hyperoxia approximately above 290 Torr ($PO_2$) (Fig. 2). This calculated critical $PO_2$ value of FHV-infected larvae is close to the actually observed minimum $PO_2$ required for high viability of the FHV-infected larvae (about 280 Torr) (Masumura et al., 1989). The present high survivability of FHV-infected larvae under hyperoxia suggests that introduction of more oxygen into the rearing water would be effective to control the disease.

FHV-infected larvae at premetamorphic C or D stage, irrespective of salinity, showed fairly high mortality under normoxia, but fish at premetamorphic E or F stage and post-metamorphic juvenile I stage survived at high rates in seawater with salinity higher than 8 ppt (Fig. 3). An interesting finding on the salinity tolerance was obtained in the experiment under hyperoxia using larvae of D stage (Fig. 4). High survival rates was observed in FHV-infected fish in 8 and 16 ppt salinities, but that in 32 ppt salinity was significantly decreased. Furthermore, the results of the immunocytochemistry revealed that the number of skin chloride cells in the FHV-infected fish were substantially decreased (Figs. 5 and 6), suggesting poorer osmoregulatory ability for these fish. In addition, suppression of the expression of NKCC in chloride cells may be involved in the reduced osmoregulatory function, as is reported by Maglova et al. (1998) where MRC-5 cell line (human lung fibroblast) was infected with human cytomegalovirus (HCMV, a herpesvirus). Thus, it is reasonable that the survival rate of FHV-infected fish was higher in seawater with lower osmolarities that are closer to the physiological osmolarity in the fish body.

From these results, it is most likely concluded that Japanese flounder larvae affected by viral epidermal hyperplasia die of dysfunction of respiration and osmotic regulation.
Acknowledgements

We thank Y. Sakakura (Nagasaki University, Japan) for his valuable advice during the progress of this work. Thanks are extended to T. Mitsuyasu, T. Kakomura and A. Kawaoaka of the Fisheries & Marine Technology Center, Hiroshima Prefectural Technology Research Institute for painstaking help.

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