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Survivability of Infectious Hematopoietic Necrosis Virus in Fertilized Eggs of Masu and Chum Salmon

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Abstract.—The possibility of vertical transmission of infectious hematopoietic necrosis virus (IHNV) was studied with the eggs of masu (cherry) salmon *Oncorhynchus masou* and chum salmon *O. keta*. The surfaces of eggs and sperm were contaminated with IHNV ($10^{3.8}$–$10^{4.8}$ 50% tissue culture infective dose [TCID50]/egg) and then the eggs were fertilized. Eggs just after fertilization and embryonated eggs also were infected by injection with IHNV ($10^{3.8}$ TCID50/egg) directly into the yolk. During incubation, eggs were held in running water at 10°C. Mortality of the eggs or hatched progeny was determined and isolation of IHNV on the surface or inside of the eggs was determined during the incubation period. No mortality occurred and no virus was detected in fertile eggs from contaminated gametes. For injected eggs, IHNV was not detected on the surface of masu and chum salmon eggs after 1 d of incubation. Infectivity of IHNV inside the eggs decreased gradually and could not be detected after 1 month of incubation. This rate of IHNV reduction in the fertilized egg was similar to that found in a mixture of IHNV and homogenized yolk contents. Several individual yolk components also showed anti-IHNV activity. When eyed eggs were injected with IHNV, the embryos of both masu and chum salmon became infected, and the concentration of virus increased rapidly and reached more than $10^{6.5}$ TCID50/fish. The cumulative mortality of eggs injected at the eyed stage for both masu and chum salmon was 90%. The susceptibilities of hatched-out larvae of masu and chum salmon to IHNV were different; cumulative mortality was more than 90% in masu salmon and 20–30% in chum salmon artificially infected with the virus. We concluded that vertical transmission of IHNV is doubtful because the virus is apparently unable to survive in eggs before the eyed stage.

In 1971, the first outbreak of infectious hematopoietic necrosis (IHN) was recorded in Hokkaido, Japan, among kokanee (lacustrine sockeye salmon) *Oncorhynchus nerka* cultured in Hokkaido Fish Hatchery. The next year, IHN broke out again among the same species cultured in Hokkaido Salmon Hatchery (Kimura and Awakura 1977). It has since spread rapidly to Honshu, the main island of Japan, and has been found among rainbow trout *Oncorhynchus mykiss* (formerly *Salmo gairdneri*) produced since 1974 (Sano et al. 1977). Many rivers located in the central part of Honshu, a major district for rainbow trout production, have become contaminated with the IHN virus (IHNV), because several farms use these rivers for their water supply. As a result, there have been substantial losses in rainbow trout production.

For the prevention or control of epizootics of IHN in salmonid fishes, control of the water temperature (Amend 1970, 1976), immunization (Amend 1976), and chemotherapy (Hasobe and Saneyoshi 1985; Kimura et al. 1987) have been studied. The most effective and widely used method is disinfection of eggs at the early eyed stage with iodophor (50 mg/L for 20 min) and subsequent incubation in virus-free water; the resulting fish do not become infected (Amend and Pietsch 1972; Amend 1976; Mulcahy 1983; Yoshimizu et al. 1988).

In Japan, although IHNV infection has been prevented simply by keeping the water temperature at 15°C, most of the rainbow trout farms are able to prevent and control IHN by application of the above methods. At first, eggs at the early eyed stage are treated with iodophor (50 mg/L, 20 min), and then treated eggs are incubated in well water or ultraviolet-irradiated water. Young fish are also kept in well water or ultraviolet-irradiated water until they are no longer susceptible to IHNV (i.e., when fish weigh about 1.5 g). Young fish then are moved to ponds supplied by river water (Kimura 1983). However, a few outbreaks of IHNV have occurred in spite of treatment of the eyed eggs with iodophor before transportation and incubation in well water (T. Yamazaki, Nagano Prefectural Fisheries Experimental Station, personal communication).

Mulcahy and Pascho (1984, 1985) reported that IHNV adsorbed onto the surface membrane of sperm from two genera of salmonid fish, and IHNV was isolated from dead eggs and hatched fish. From these findings, they concluded that IHNV is transmitted vertically. Nusbaum and Grizzle (1987) re-

ported that channel catfish virus (CCV) adsorbed onto sperm of channel catfish and Wise et al. (1988) reported the vertical transmission of CCV. We studied the possibility of vertical transmission of IHNV in masu salmon[1] *Oncorhynchus masou* and chum salmon *O. keta*. Susceptibilities of recently hatched fish to the IHNV are different for these two species.

Methods

The virus.—Three strains of IHNV were employed. IHNV strains ChAb and RtTo were isolated from the ovarian fluid of mature chum salmon from the Abashiri River in 1976 and from mature rainbow trout from Aomori Prefectural Fisheries Experimental Station in 1983. Strain ChIw was isolated from moribund chum salmon infected with IHNV in 1986 (Yoshimizu et al. 1988). Viruses were cultured on rainbow trout gonad (RTG-2) cells (Wolf and Quimby 1962) grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 international units (IU) penicillin/mL, and 100 μg streptomycin/mL, and incubated at 15°C for 7 d. Culture fluid was filtered through a 0.45-μm membrane, separated into small amounts in vial tubes, and stored at −80°C until used.

Eggs and fish.—Unfertilized eggs, sperm, and eyed eggs of masu and chum salmon were provided by Hokkaido Salmon Hatchery. Before they were used in our study, fertilized eggs and eyed eggs were disinfected by iodophor treatment (50 mg/L, 20 min). Eggs were incubated in dechlorinated city water at 10–15°C. Progeny of both species were artificially infected just after hatching and at 1 month of age.

Contamination of eggs and sperm.—Four groups of masu and chum salmon eggs were fertilized after treatment with IHNV. For the first group (egg-contaminated), 5 mL of stock IHNV strain ChAb ($10^{6.05}$ TCID50/mL) were added to 100 eggs. The egg–virus mixture was allowed to stand for 30 min, and then the eggs were fertilized. For the second group (sperm-contaminated), 2 mL of virus suspension was added to sperm. The sperm–virus mixture was allowed to stand for 30 min, and then the sperm were used to fertilize 100 eggs. A doubly contaminated group consisted of eggs and sperm that were contaminated with virus by the same methods as described above and then mixed to fertilize the eggs. Finally, a fourth group consisted of a doubly contaminated group that was disinfected with 50 mg iodophor/L for 20 min (disinfected group). These four groups were incubated in dechlorinated city water and subsequent mortality and virus concentrations were recorded.

Yolk-sac injection of eggs.—Eggs at two developmental stages, just after fertilization and after they became embryonated, were experimentally infected by yolk-sac injection. Stock IHNV strain ChAb ($10^{6.05}$ TCID50/mL) was injected into masu salmon eggs, and strains ChAb and ChIw ($10^{6.05}$ TCID50/mL) were injected into chum salmon eggs. Injections were performed with handmade glass needles (diameter = 100 μm) inserted at the vegetative pole and extending into the central part of the yolk. Eagle's minimum essential medium with 10% fetal bovine serum was injected into the control.

Measurement of virus infectivity.—The four groups of eggs contaminated with IHNV were sampled every week, and injected eggs were sampled every 2 d. Ten eggs or five hatched-out fish were pooled for one sample, homogenized with Hanks' balanced salt solution, and filtered through a 0.45-μm membrane. Viruses were counted by the microplate method with RTG-2 cells.

Detection of antigens in blastomeres.—The surface of each of 20 eggs was treated with 10% formaldehyde for 2 h at 1, 2, 3, and 4 d after injection with IHNV. For controls, virus-free culture fluid of IHNV prepared by tangential flow filtration (Pellicon Cassette System, Millipore Corporation) was injected. Blastodiscs were picked up and two specimens were pooled. Pooled blastodiscs were treated with 0.1% trypsin in Versene solution for 5 min. Suspended blastomeres were washed with distilled water, smeared on glass slides, and fixed at 100°C for 3 h. The presence of IHNV antigen in the blastomere was detected by immunoperoxidase stain (Watanabe 1981). Fixed blastomeres were treated with 0.3% H_2O_2 at room temperature for 20 min and washed with distilled water. Subsequently, bovine serum solution (5% fetal bovine serum, 0.1% NaN_3, and 0.1% $MgCl_2$ in phosphate buffered saline solution; pH = 7.2) was added, and the specimens were incubated at 37°C for 20 min. After the blastomeres were washed with distilled water, anti-IHNV monoclonal antibody RB/B5 (provided by J. R. Winton, National Fisheries Research Center, Seattle, Washington) was added, and the specimens again were incubated at 37°C for 20 min. After washes, we added rabbit anti-

[1] The common name preferred by the American Fisheries Society for *Oncorhynchus masou* is cherry salmon.

mouse immunoglobulin G conjugated to horseradish peroxidase (Daiko Co., Ltd.) diluted 1:100 in phosphate buffered saline solution. After additional distilled water washes, 3,3'-diaminobenzidine (Wako Co., Ltd.; 0.25 mg/mL in 0.05 M tris buffer, pH = 8.3, containing 0.015% H_2O_2) was added and allowed to react for 30 min, and then the slide was washed with distilled water and examined by light microscopy.

Pathogenicity of virus strains.—Fifty chum salmon eleutheroembryos were infected immediately posthatching with IHNV strains RtTo, ChAb, and ChIw by water-borne exposure to a virus concentration of $10^{3.75}$ TCID50/mL for 60 min. Fifty 1-month-old masu and chum salmon also were infected with IHNV strain RtTo by the water-borne route with a dose of $10^{2.0}$ TCID50/mL for 60 min.

Survivability of virus in yolk contents.—Each of 20 masu and chum salmon eggs were sampled immediately after fertilization and at 5-d intervals for 25 d. The eggs were disinfected with iodophor, and egg membranes and blastodiscs or embryos were removed with forceps and sterile gauze. The same volume of stock IHNV strain ChAb ($10^{5.05}$ TCID50/mL) that was injected into yolk sacs of intact eggs was added to homogenized yolk contents and allowed to stand at 5°C. Virus infectivity was measured 1, 7, 14, and 21 d later.

Effect of egg components on virus infectivity.—The effect of egg components of salmonid fishes reported by Ando (1962a, 1962b) on the infectivity of IHNV was tested. The amino acids L-aspartic acid, L-glutamic acid, DL-phenylalanine, L-tryptophan, L-tyrosine, L-arginine, L-lysine, L-histidine, L-valine, DL-alanine, L-leucine, DL-isoleucine, DL-threonine, L-serine, DL-methionine, L-cysteine, and L-proline and vitamins ascorbic acid, pyridoxal phosphate, biotin, and folic acid (Wako) were dissolved in Hanks' balanced salt solution at 10 mg/mL. Lecithin, cephalin, sphingomyelin, and cerebrosides (Sigma) were suspended in Hanks' balanced salt solution at 1 mg/mL and homogenized by ultrasonication (Otake Seisakusho). Antiviral activity of these compounds was measured by three methods: (1) stock IHNV strain ChAb was mixed with the compounds and left to react for 30 min at 15°C; (2) the compounds were added to the cell culture medium on RTG-2 cells that were cultured for 24 h, washed twice with Hanks' balanced salt solution, and immediately inoculated with virus; and (3) the components were added to overlay medium above cells already infected with IHNV and subsequently cultured. Final concentrations of these compounds were adjusted to one-tenth of stock solutions. Antiviral effects were measured by plaque assay (Kamei et al. 1987), and the plaque reduction rates were compared with those of an untreated control group (Kamei et al. 1988).

Results

Mortality of Infected Eggs

The four gamete-contaminated egg groups (egg-contaminated, sperm-contaminated, doubly contaminated, and disinfected) were incubated under IHNV-free conditions. No deaths were recorded for the four groups and virus was not detected on the surface or inside of fertilized masu and chum salmon eggs.

Detection of Virus in Injected Eggs

Although the mortality of the eggs of masu and chum salmon injected into the yolk sac just after fertilization reached 100% in the following 5 weeks, IHNV was not detected at 1 week in masu salmon or at 5 weeks in chum salmon. The mortality of the control masu and chum salmon reached 42.9 and 41.4%, respectively. IHNV was not isolated from either of the controls (Figure 1).

No IHNV antigen was detected 1 d after injection, but detection rates of infected blastomeres stained by immunoperoxidase were 50, 70, and 50% after 2, 3, and 4 d, respectively. Blastomeres of control eggs were all negative for IHNV antigen.

Mortality of masu salmon eggs injected at the eyed stage reached 100% in the following 7 d, and the concentration of IHNV reached more than $10^{6.50}$ TCID50/fish. Cumulative mortalities reached 30.0 and 22.9%, respectively, in the MEM-10-injected and needle-pricked controls (Figure 2). The masu salmon eggs began to hatch after 1 d and all eggs had hatched out 5 d after injection.

Cumulative mortalities of chum salmon eggs injected with IHNV strains ChAb and ChIw were 91.1 and 88.9%, respectively. The IHNV was recovered and 9 d later the infectivity reached more than $10^{6.50}$ TCID50/fish (Figure 3). The chum salmon eggs began to hatch after 3 d and all eggs had hatched 13 d after injection.

Pathogenicity of Virus Strains

Cumulative mortalities of 1-month-old masu and chum salmon artificially infected with IHNV RtTo strain were 90 and 20%, respectively, and for the sac fry (i.e., fry with yolk sac remaining) of chum salmon infected with IHNV strains RtTo,

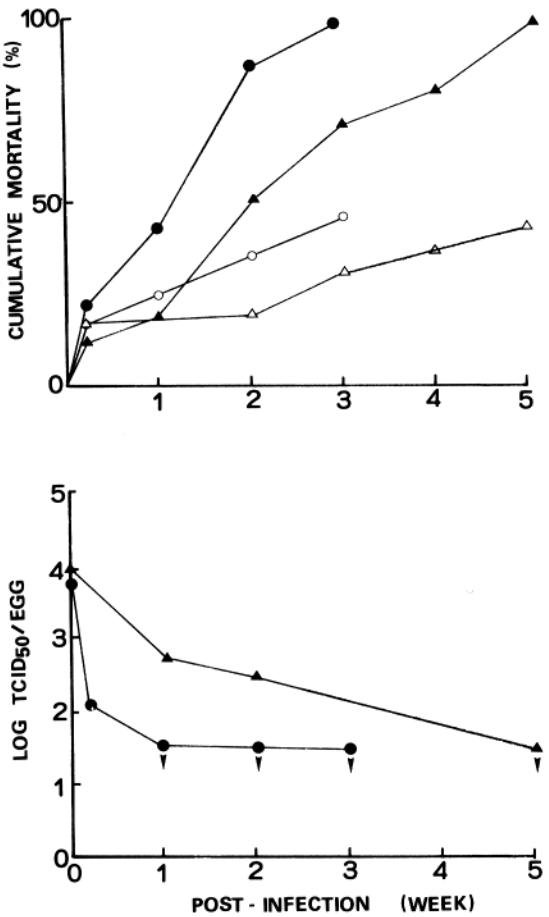

FIGURE 1.—Infectivity of infectious hematopoietic necrosis virus (IHNV, strain ChAb) and cumulative mortalities in eggs of masu and chum salmon after injection of IHNV (dose, $10^{3.75}$ TCID50/egg) directly into the yolk of eggs immediately after fertilization. Control eggs were injected with Eagle's minimum essential medium with 10% fetal bovine serum and no virus. Key: ●, masu salmon eggs injected with IHNV; ▲, chum salmon eggs injected with IHNV; ○, masu salmon eggs without IHNV (controls); △, chum salmon eggs without IHNV (controls); ↾, actual value below detectable limit.

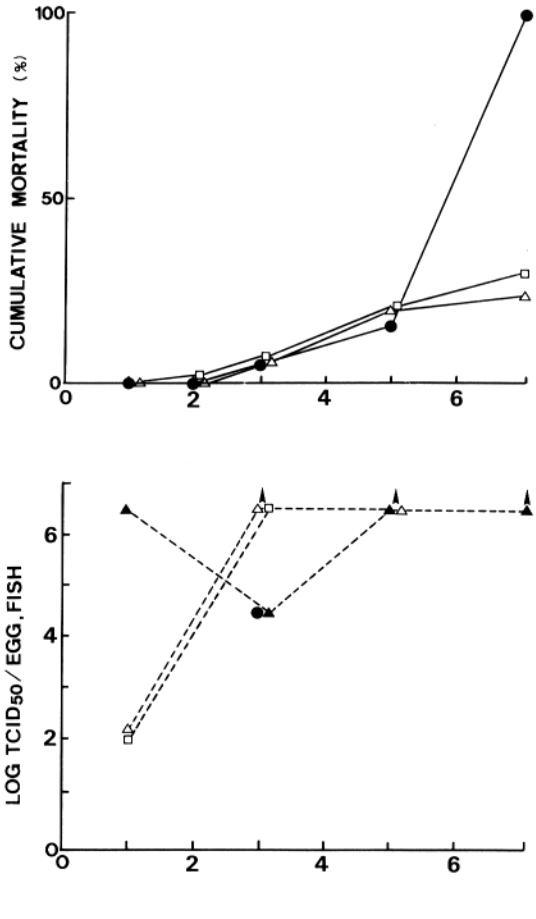

FIGURE 2.—Infectivity of infectious hematopoietic necrosis virus (IHNV, strain ChAb) and cumulative mortalities in eyed eggs and sac fry of masu salmon after injection of IHNV (dose, $10^{3.75}$ TCID50/egg) directly into the yolk of eyed eggs. Control eggs were injected with Eagle's minimum essential medium with 10% fetal bovine serum and no virus (group 1) or needle-pricked only (group 2). Key: ●——●, test group; □——□, control group 1; △——△, control group 2; ●-----●, dead egg; ▲-----▲, dead fingerling; □-----□, live egg; △-----△, live fingerling; ↿, actual value higher than detectable limit.

ChAb, and ChIw were 26, 6, and 28%, respectively (Figure 4).

Effect of Egg Components on IHNV

Infectivity of the IHNV strain ChAb decreased rapidly when the virus was mixed with homogenized yolk contents from eggs just after fertilization. This rapid rate of IHNV reduction in the fertilized eggs was observed before the eyed stage. However, the infectivity of the virus mixed with yolk components from eyed-stage eggs continued to decrease gradually (Figure 5). The masu and chum salmon eggs in this study reached the eyed stage 15 and 20 d after fertilization, respectively.

A reduction of 81 and 60% in the number of IHNV plaques resulted when the virus was mixed with 100 μg of cephalin or 1,000 μg of phenylalanine for only 30 min (Table 1). No plaques appeared when IHNV was cultured with the overlay medium containing 1,000 μg of either glutamic

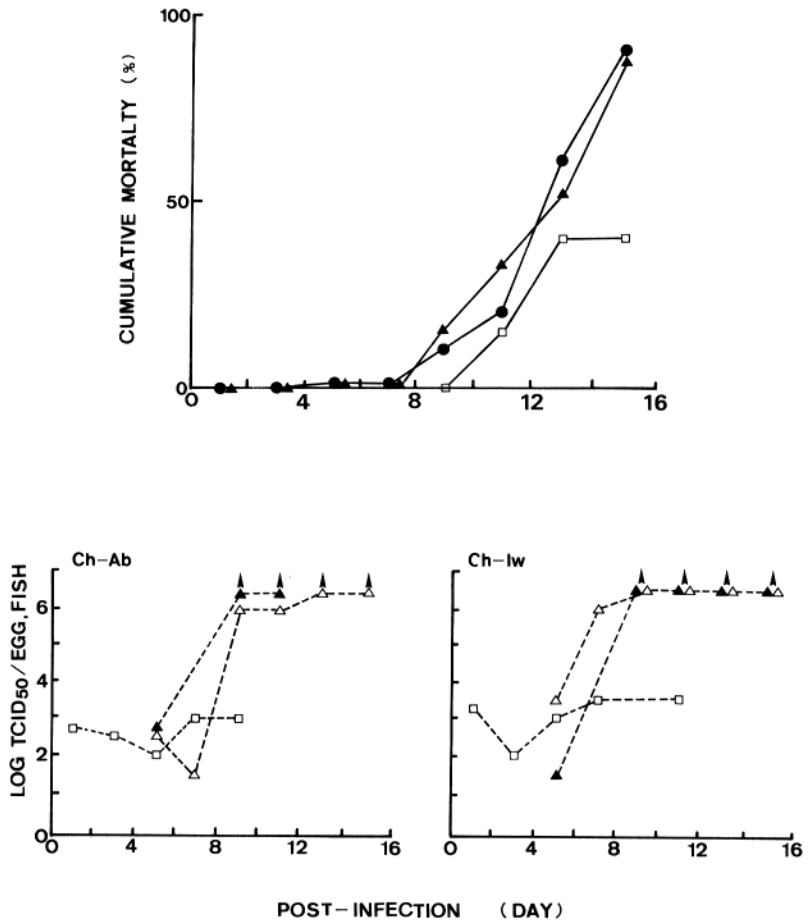

FIGURE 3.—Infectivity of infectious hematopoietic necrosis virus (IHNV, strains ChAb and ChIw) and cumulative mortalities in eyed eggs and sac fry of chum salmon after injection of IHNV (dose, $10^{3.75}$ TCID50/egg) directly into the yolk of eyed eggs. Control eggs were injected with Eagle's minimum essential medium with 10% fetal bovine serum and no virus (group 1) or needle-pricked only (group 2). Key: ●——●, test group for ChAb; ▲——▲, test group for ChIw; □——□, control group; ▲-----▲, dead fingerling; □-----□, live egg; △-----△, live fingerling; ↿, actual value higher than detectable limit.

acid, pyridoxal phosphate, or folic acid, and the number of plaques was reduced 69 and 44% when the overlay medium contained 1,000 μg of tryptophan or ascorbic acid, respectively. Other compounds did not reduce plaque compared with the controls.

Discussion

We previously reported that susceptibilities of masu and chum salmon fry to IHNV were different (Kumura et al. 1982). In this study, we quantified the mortality from infection by one strain of IHNV in both of these salmon species and by each of two additional strains in chum salmon.

From our results, it appears that IHNV is inactivated when the virus enters the egg with the sperm. The lack of mortality among the four groups of IHNV-contaminated eggs and our failure to detect the virus in or on fertilized eggs corroborate the findings of Amend et al. (1972) and Amend (1975), although our study should be repeated with a larger sample size. This explains the success of the combined iodophor treatment (to kill virus outside the egg) and subsequent incubation of eggs in virus-free water to prevent IHN. Fish hatched from eggs treated in this manner have no problems with IHNV infection if they are maintained in virus-free conditions until they are past the IHNV-susceptible stage.

When the virus was injected directly into the yolks of fertilized eggs, the high mortality among the test groups was attributed to virus infection,

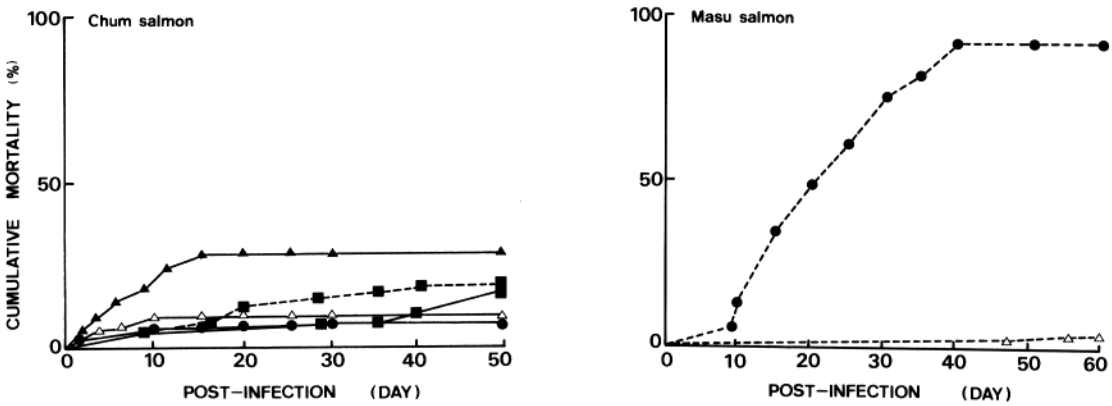

FIGURE 4.—Cumulative mortalities of masu and chum salmon fry infected with three strains, RtTo, ChAb, and ChIw, of infectious hemopoietic necrosis virus (IHNV). Dose was $10^{3.75}$ TCID50/mL for 60 min for ChAb and ChIw and $10^{2.00}$ TCID50/mL for 60 min for RtTo. Treatment was by the immersion method. Key: ■——■, sac fry infected with RtTo; ●——●, sac fry infected with ChAb; ▲——▲, sac fry infected with ChIw; ■----■ and ●----●, 1-month-old fry infected with RtTo; △——△, sac fry controls; △----△, 1-month-old fry controls.

but conditions inside the egg apparently were not suitable for the continued survival of IHNV. Burke and Mulcahy (1983) reported that IHNV infectivity was stable in egg homogenates of sockeye salmon but the infectivity of IHNV decreased when the virus was mixed with homogenized yolk contents from fertilized masu and chum salmon eggs in our study. Furthermore, we were able to demonstrate the ability of seven salmon egg components individually to decrease the infectivity of IHNV substantially; we have yet to determine whether these components act individually or synergistically in ovo.

The ability of yolk homogenate to reduce IHNV infectivity was greatest for eggs immediately after fertilization and decreased as eggs approached the eyed stage. The rate of decrease relative to stage of incubation was similar in intact fertilized eggs. The infectivity of the virus did not decrease when IHNV was mixed with homogenized yolk contents from eyed eggs. These results suggest that anti-IHNV activity in salmon eggs becomes too low by the eyed stage to thwart the virus because the amounts of yolk components with anti-IHNV activity have decreased. Ando (1962a, 1962b), Suyama and Ogino (1958), and Takama et al. (1969) reported that the concentration of fatty acids, especially acetone-insoluble lipids (e.g., cephalin, lecithin, sphingomyelin, and cerebrosides) decrease rapidly during embryogenesis.

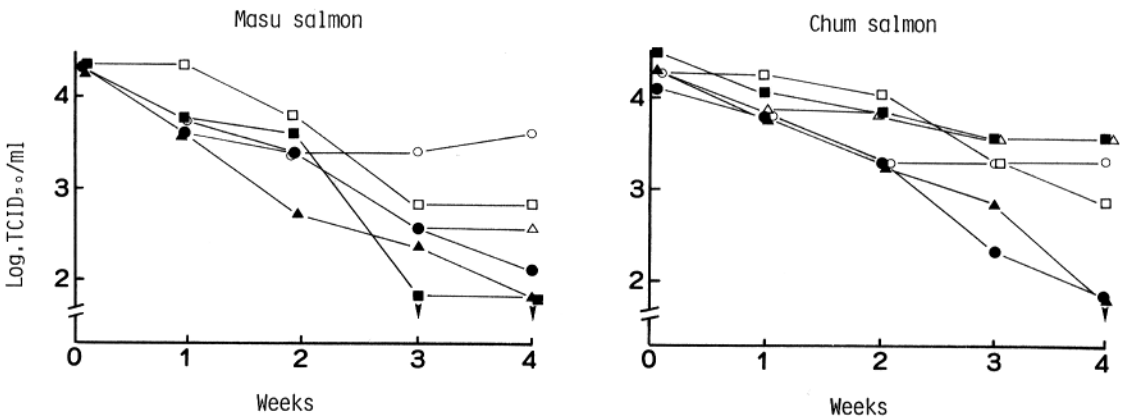

FIGURE 5.—Survivability of infectious hematopoietic necrosis virus (IHNV, strain ChAb) in the yolk contents of masu and chum salmon at different stages after fertilization. Key: ●, immediately after fertilization; ▲, 5 d after fertilization; ■, 10 d after fertilization; ○, 15 d after fertilization; △, 20 d after fertilization; □, 25 d after fertilization; and ▼, value less than detectable limit.

TABLE 1.—Effects of salmon egg components on the infectivity of infectious hematopoietic necrosis virus (IHNV).

Egg component	Concentration (μg/mL)	Plaque reduction (%)		
		Direct reaction[a]	Pretreatment[b]	Mixed with overlay[c]
Amino acid				
L-Aspartic acid	1,000	14	12	3
L-Glutamic acid	1,000	3	17	100
DL-Phenylalanine	1,000	60	−3	8
L-Tryptophan	1,000	11	12	69
L-Tyrosine	1,000	34	18	−12
L-Arginine	1,000	8	14	0
L-Lysine	1,000	7	10	−5
L-Histidine	1,000	0	3	0
L-Valine	1,000	6	2	−6
DL-Alanine	1,000	6	22	2
L-Leucine	1,000	6	1	−22
DL-Isoleucine	1,000	9	6	−8
DL-Threonine	1,000	15	10	−3
L-Serine	1,000	−3	−7	8
DL-Methionine	1,000	23	4	3
L-Cysteine	1,000	8	10	−7
L-Proline	1,000	19	−6	33
Vitamin				
Ascorbic acid	1,000	7	−2	44
Pyridoxal phosphate	1,000	−10	21	100
Biotin	1,000	9	−1	11
Folic acid	1,000	−43		100
Lipid				
Lecithin	100	−7	14	0
Cephalin	100	81	60	16
Sphingomyelin	100	34	10	0
Cerebrosides	100	35	7	−3

[a] IHNV was mixed with egg component and allowed to react for 30 min at 15°C.

[b] Egg component was added to cell culture medium and removed by two successive washes of Hanks' balanced salt solution just before virus inoculation.

[c] Component was added to overlay medium after virus inoculation.

Mulcahy and Pascho (1985) isolated IHNV from dead eggs and suggested, therefore, that eggs play an important role in the vertical transmission of IHNV in salmon. We confirmed the susceptibility of salmon eggs and fry to IHNV, but only from the eyed egg stage onward. When IHNV was introduced onto the surface of either male or female gametes, or both, it had no effect on embryo mortality, and IHNV could not be detected in the eggs after fertilization. Therefore, we concluded that direct vertical transmission of IHNV to salmon progeny is doubtful.

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