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Cohabitation Transmission of Infectious Salmon Anemia Virus among Freshwater-Reared Atlantic Salmon

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Abstract.—The present study examined factors affecting horizontal transmission of infectious salmon anemia virus (ISAV) among naive Atlantic salmon *Salmo salar* maintained in freshwater. The ratio of injected to naive cohabitants was varied and mortality was monitored. Whether ISAV transmission in freshwater required contact among salmon was also examined. Pathognomonic histological lesions in fish infected by injection or cohabitation were compared. In trial 1, duplicate tanks containing 0 + 25, 2 + 23, 4 + 21, 6 + 19, or 8 + 17 fish (injected + naive, respectively) were established. Mean days to onset of mortality and mean days to death (mdd) were significantly less among injected fish (16.9 and 18.8 d, respectively) than in fish infected by cohabitation (33.4 and 43.8 d). Cumulative mortality in naive cohabitants in trial 1 was significantly greater in groups containing six injected fish compared with those containing either two or eight injected fish. In trial 2, triplicate tanks of 0 + 25, 4 + 21, and 6 + 19 fish were established. Mean days to onset of mortality and mdd were similar to those in trial 1. Mean cumulative mortalities among naive cohabitants were 93.7% and 93.0% in groups containing four and six injected fish, respectively. In trial 3, salmon exposed to water draining from tanks containing infected fish suffered mortality and ISAV was detected by reverse transcriptase polymerase chain reaction. Pathognomonic lesions in the gut (stomach, caeca, upper intestine) were significantly more frequent in fish infected by cohabitation than in those infected by injection. Lesion scores of gut histology were significantly greater in cohabitation-infected fish than in injected fish whereas those of the liver were significantly greater in injected fish. This study confirmed that ISAV was transmissible among freshwater-reared salmon and that contact among fish was not required for transmission. The reduced frequency and severity of microscopic gut lesions in fish infected by injection may have been related to the more rapid pathogenesis associated with this route of infection.

Infectious salmon anemia (ISA), a rapidly fatal disease of sea-cage-reared Atlantic salmon *Salmo salar*, was first described in Norway (Thorud and Djupvik 1988) and subsequently in eastern Canada (Mullins et al. 1998; Lovely et al. 1999) and Scotland (Rodger et al. 1998). Gross clinical signs include darkening, lethargy, anemia, and abdominal distension with ascites. Vascular damage is indicated by microscopic hemorrhage and congestion associated with the liver, gills, pyloric caeca, and occasionally kidney (Evensen et al. 1991; Jones et al. 1999a). Clinical signs are most commonly associated with profound anemia (Evensen et al. 1991). The etiological agent of ISA is an enveloped virus (ISAV) having a segmented, negative-sense RNA genome and bearing similarities to viruses of the Orthomyxoviridae family (Mjaaland et al. 1997; Krossøy et al. 1999; Ritchie et al. 2001). ISAV replicates in chinook salmon embryo (CHSE-214), salmon head kidney (SHK-1), AS, and Rtgill-W1 cell lines derived from salmonids and causes recognizable cytopathology in the first two lines (Dannevig et al. 1995a; Falk et al. 1997; Sommer and Mennen 1997; Bouchard et al. 1999).

ISA primarily affects farmed salmon after their transfer to the sea, suggesting that virus transmission occurs readily in seawater. Indeed, ISAV was transmitted to naive Atlantic salmon maintained in diluted or undiluted seawater either from infected cohabitants or after filtered tissue homogenates derived from ISA-infected fish were added to the water (Thorud and Djupvik 1988; Nylund et al. 1994; Totland et al. 1996). The virus was detected in neither the ova of naturally infected salmon nor in the resulting progeny, which suggests that vertical transmission of ISAV does not occur (Thorud and Djupvik 1988; Melville and Griffiths 1999). In contrast, the possibility of virus transmission among presmolt salmon held in freshwater has received little attention. Freshwater transmission would provide opportunities for viral

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dissemination in hatcheries from infected broodstock or from migrating anadromous species, as evidently occurred in at least one case (Nylund et al. 1999). The present report builds on earlier observations in which ISA was reproduced experimentally after horizontal transmission of ISAV among freshwater-reared Atlantic salmon parr as a method for evaluating an experimental vaccine (Jones et al. 1999b). This freshwater cohabitation model is now more completely described, and histological changes resulting from infection by injection and cohabitation challenge are compared.

Methods

Fish.—Atlantic salmon parr of 5–35 g were obtained from a certified disease-free hatchery. Tissue homogenates from naive salmon were screened either by culture on the SHK-1 cell line or by reverse transcription polymerase chain reaction (RT-PCR; see below) and found to be negative for ISAV. Fish were maintained in well water (total hardness, 230 mg/L; pH, 7.6; Na, ~40 mg/L; Cl^-, ~80 mg/L; O_2, 9–10 mg/L; total gas saturation, ~100%; temperature, 10 ± 1°C) flowing at 1.5 L/min, and they were fed a daily pelleted diet at 1.5% body weight.

Virus.—Strain NBISAV01 of ISAV was isolated from farmed Atlantic salmon in New Brunswick, Canada, as previously described (Jones et al. 1999b). The virus was maintained by serial passage in CHSE-214 cells in Leibovitz medium (L-15) supplemented with 5% fetal bovine serum and incubated at 15°C. Inocula, prepared from an infected culture supernatant (six passages since isolation), were stored at −80°C in 0.5-mL aliquots. Viral titer in the inoculum was estimated by calculating the TCID50 in CHSE cells by the method of Kärber (1931).

Cohabitation trials.—Salmon were anesthetized by immersion in 50 mg/L benzocaine and infected by intraperitoneal (ip) injection with 0.1 mL of inoculum containing approximately $10^{4.5}$ TCID50 of ISAV. The anal fin of injected fish was clipped, and fish were randomly assigned to trial tanks immediately after recovering from anesthetic. In all trials, fish were monitored three times daily and any that died were removed. Homogenates, prepared in balanced salt solution from kidney, spleen, and liver taken from at least three of the dead fish from each tank, were stored at −80°C for analysis.

Trials 1 and 2 were conducted in 15-L tanks and examined the effects of varying the numbers of injected fish. In trial1, duplicate tanks of zero, two, four, six, and eight injected fish were established with sufficient naive cohabitants to bring the total number per tank to 25. In trial 2, triplicate tanks of zero, four, or six injected naive fish were established, each with sufficient naive animals to give a total of 25. Twelve to 14 moribund fish from each infection category in trial 2 were sampled for histological assessment when clinical signs (darkening, listlessness, exophthalmia, or abdominal distension) were displayed. Fish were killed by immersion in benzocaine, the ventral abdominal musculature was removed, and the remaining tissues were fixed in buffered 10% formalin. Samples were coded and processed routinely for histology and stained with hematoxylin and eosin. The following scale was adopted for assessment of gastrointestinal lesions: 1, mild, multifocal congestion of the lamina propria and submucosal vasculature, often with evidence of erythrophagia; 2, moderate, diffuse congestion of the lamina propria and submucosal vasculature, with evidence of intravascular hemolysis and erythrophagia; and 3, marked, diffuse congestion as above, but with evidence of mucosal necrosis and sloughing into the gut lumen. The following scale was adopted for liver: 1, mild sinusoidal congestion, usually multifocal or regional and often randomly distributed; 2, sinusoidal congestion, coagulative necrosis, or vasculitis, characterized by a mixed leukocytic infiltrate of larger blood vessel walls or perivascular cuffing of smaller vessels; 3, congestion and necrosis. In all tissues, a score of zero was assigned when no microscopic changes were detected.

Trial 3 was designed to assess whether fish-to-fish contact was required for transmission. Two pairs of 60-L tanks were established, each pair consisting of an upper and a lower tank. The upper tanks contained two groups of 10 fish: those that were injected ip with ISAV (and marked by fin clipping) and those that were untreated and unmarked (contact cohabitants). The lower tanks of each pair contained 20 untreated, unmarked fish (noncontact cohabitants). All tanks received freshwater at 1.5 L/min. In addition, water from each upper tank drained into the one beneath at approximately 400 mL/h.

Differences in cumulative mortalities and in the proportion of histological samples with lesions were compared by chi-square analysis; differences in mean time to onset of mortality or mean days-to-death were compared by Student's *t*-tests. Differences in mean lesion scores were compared by using the nonparametric Kruskal–Wallis one-way analysis of variance (ANOVA). In all analyses,

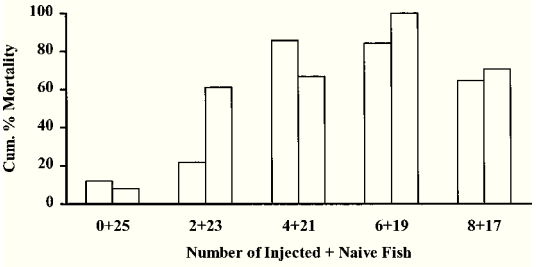

FIGURE 1.—Cohabitation infection of Atlantic salmon with infectious salmon anaemia virus (ISAV; trial 1): effect of the ratio of injected to naive salmon on cumulative mortality among naive cohabitants. The indicated number of injected fish ($10^{4.5}$ TCID50) and naive fish were introduced to duplicate tanks on day 0, and the indicated cumulative mortality was calculated to day 55. Each bar represents the cumulative mortality in one tank.

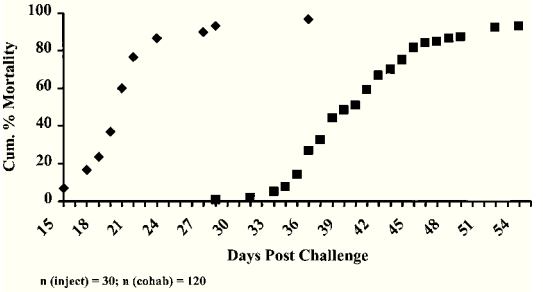

FIGURE 2.—Cohabitation infection of Atlantic salmon with ISAV (trial 2): mortality curves for 30 injected (diamonds) and 120 naive cohabitant (squares) fish. Injected and naive fish were introduced to tanks (see text) at time 0.

differences were considered significant if $P \leq 0.05$.

Reverse transcriptase polymerase chain reaction (RT-PCR).—Total RNA was extracted from 300 μL of clarified tissue homogenate (centrifuged for 20 min at 800 × gravity) with Trizol (Gibco-Life Technologies) and chloroform, followed by precipitation in 100% isopropanol. A one-step RT-PCR protocol was adopted, performed with Ready-to-Go RT-PCR beads (Pharmacia). Briefly, 43 μL of sterile distilled water, 1 μL of RNA, 2 μL of $pd(N)_6$ (Pharmacia), and 2 μL each of primers FA-3 (GAA GAG TCA GGA TGC CAA GAC G) and RA-3 (GAA GTC GAT GAA CTG CAG CGA; Devold et al. 2000) were added to each reaction tube. Complementary DNA (cDNA) was synthesized at 42°C for 10 min and reverse transcriptase was denatured at 95°C for 5 min. Complementary DNA was amplified for 35 cycles (94°C, 30 s; 59°C, 45 s; 72°C, 90 s), followed by a 10-min extension at 72°C. Controls included sterile water and RNA extracted from uninfected tissue. Reaction products were resolved (60 min, 120 V) in 1% agarose containing ethidium bromide. A 221-bp amplicon was diagnostic for ISAV (Devold et al. 2000).

Results

Mortalities occurred in all treatment groups in trial 1, on average beginning in ip-injected fish 16.9 d (range, 14–19 d) after injection. Mean days-to-death (mdd) of ip-injected fish were similar among treatment groups, ranging from 17.5 to 20.0 d. Mean time to onset of mortality in cohabitant fish was 33.4 d (range, 27–39 d). The mdd of cohabitants were similar among treatment groups, ranging from 42.3 (cohabiting with six injected fish) to 45.5 d (with two injected). When values were pooled, the mdd of all injected fish were significantly less than those of all cohabitants (18.8 d vs. 43.8 d; $P < 0.01$). Cumulative mortality of cohabitants was significantly greater in groups containing six injected fish than in those containing two ($\chi^2 = 23.4$; $P < 0.01$) and was significantly less in groups containing eight injected fish than in those containing six ($\chi^2 = 6.85$; $P < 0.01$; Figure 1). Variation in tank-to-tank cohabitant mortality, measured by the coefficient of variation, ranged from 6.2% in tanks with eight injected fish to 67.1% in those with two injected fish. A total of 5 of 50 fish died in the two control tanks (0 + 25 [injected + naive]).

In trial 2, mean time to onset of mortality in injected fish was 16.8 d (range, 15–19 d). The mdd of injected fish in each treatment group (four and six injected fish) were 21.5 and 20.0 d, respectively, and were not significantly different ($P = 0.54$). In all, 29 of 30 (96.7%) injected fish died (Figure 2). Mean time to onset of mortality among cohabitant fish was 32.8 d (range, 28–38 d). The mdd of cohabitant groups were 39.0 and 41.7 d, respectively ($P = 0.39$), and their mean cumulative mortalities were 93.7% and 93.0%, respectively ($P = 0.91$; Figure 2). No mortalities occurred in the three uninfected control tanks.

In trial 3, mortality among injected fish in the two upper tanks began 6 and 17 d after injection. Mean days to death among these fish were 15.4 and 26.5, respectively, and all 10 injected fish in each tank eventually died. Onset of mortality among contact cohabitants in the two tanks occurred 20 and 28 d after introduction of injected fish to each tank; their mdd were 30.1 and 36.5 d,

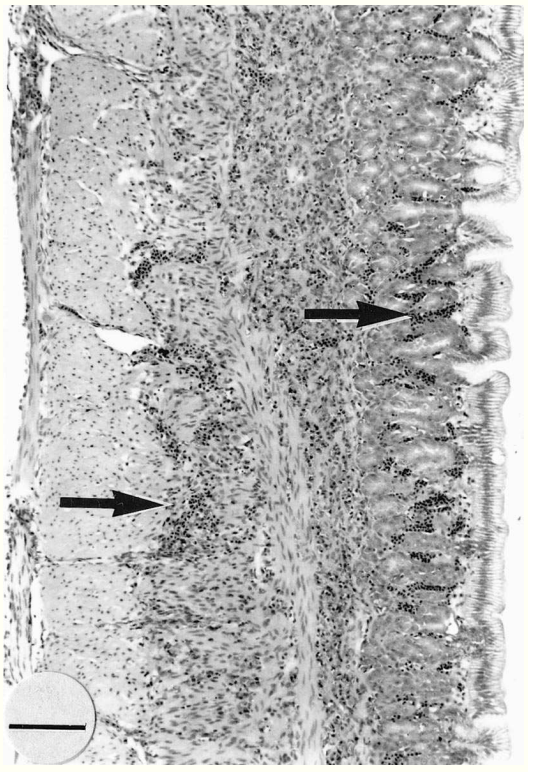

FIGURE 3.—Transverse section of a stomach showing congestion within the lamina propria and muscularis (arrows). Hematoxylin and eosin were used as stains; the bar = 133 μm.

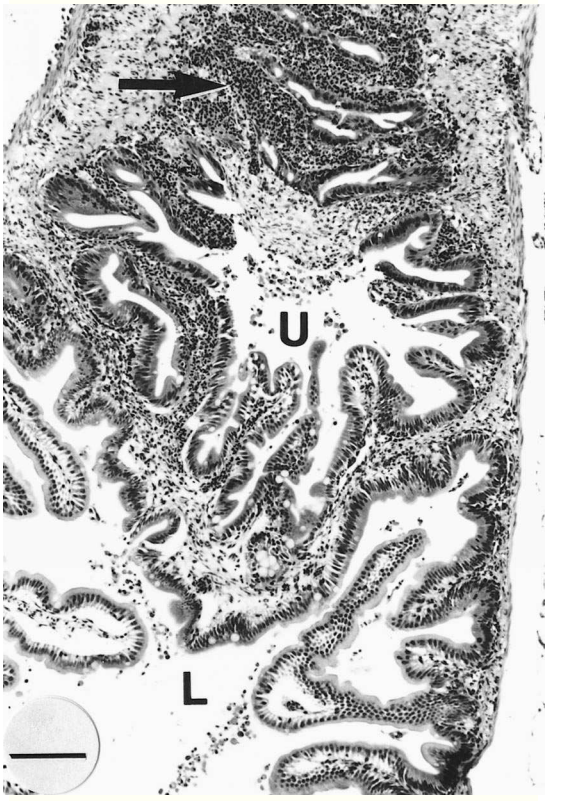

FIGURE 4.—Sagittal section of an intestine at the midintestinal valve showing congestion of the lamina propria (arrow) in the upper intestine (U) and the absence of congestion in the lower intestine (L). Hematoxylin and eosin were used as stains; the bar = 133 μm.

with total mortalities of 10/10 and 8/10, respectively. Onset of mortality among noncontact cohabitants (in the two lower tanks) occurred 37 and 35 d after addition of injected fish to the upper tanks, with mdd of 47.6 and 49.9 d, respectively. Cumulative mortalities among noncontact cohabitants to day 60 were 25% and 35%, respectively.

In all trials, ISAV was detected by RT-PCR in tissue homogenates prepared from dead fish removed from the tanks: in 4 of 6 injected fish, in 47 of 50 contact cohabitants (trials 1 and 2), and in 7 of 9 noncontact cohabitants (trial 3). No ISAV was detected in tissues obtained from a control mortality (trial 1).

Blinded histological assessments were made of tissues sampled from fish with clinical disease in cohabitant (n = 14) and injection (n = 12) infection groups in trial 2. Microscopic histological changes consistent with ISA were observed in samples of stomach (Figure 3), caeca, upper intestine (Figure 4), kidney, or liver from all fish examined. Lesions in samples from the three gastrointestinal sites were similar among individual fish. These included mild, multifocal congestion of the lamina propria and submucosal vasculature, congested blood vessels, and occasional evidence of erythrophagia in mild and moderate cases; more severely affected samples displayed nearly uniform distributions of congestion and necrosis in the mucosal epithelium. Comparison of upper and lower intestinal mucosae revealed that lesions were localized to above the intestinal valve (that is, restricted to the upper intestine; Figure 4). Microscopic lesions in the liver consisted primarily of sinusoidal congestion of various intensities; histological changes ranged from dilation or peliosis of the vessels in mild cases to a greater frequency of coalescing coagulative necrosis of hepatocytes or vascular inflammation, or both, in more severe cases.

A significantly greater proportion (P = 0.019) of caecal, upper intestinal, and stomach samples from cohabiting fish (12 of 14) had demonstrable

lesions than did those from injected fish (5 of 12). Mean gut lesion scores for all cohabited samples were significantly greater than those from all injected samples: caeca, 1.21 and 0.50, respectively ($P = 0.017$); upper intestine, 1.50 and 0.50, respectively ($P = 0.002$); and stomach, 0.93 and 0.42, respectively ($P = 0.017$). When scores were compared only among samples with lesions (i.e., not including values of zero), the differences were not significant. Liver lesions occurred in all 12 samples from injected fish and in 12 of 14 samples from cohabitation-infected fish ($P = 0.173$). In contrast to gastrointestinal samples, mean lesion scores for all cohabitation liver samples were significantly less than those from all injected liver samples (1.36 and 2.08, respectively; $P = 0.047$). Differences in mean lesion scores were not significant when samples with a score of zero were excluded from the analysis. No significant differences were observed in lesion scores between infection categories in kidney, spleen, gill, pancreas, eye, or heart. Similarly, the proportions of these tissues in which histological lesions were detected were similar between infection categories. No evidence of significant morphological changes or inflammation was evident in stained sections of gastrointestinal tissue from eight uninfected salmon.

Discussion

Infectious salmon anamia (ISA) was reproduced in naive Atlantic salmon parr by ip injection with a strain of ISAV originally isolated from farmed salmon in New Brunswick, Canada (Jones et al. 1999a). The virulence displayed in this study confirmed the earlier work, in which a marked decline in hematocrit and and the appearance of pathognomonic histological lesions accompanied acute mortality. The kinetics of mortality in the ip-injected salmon parr shown here was similar to that of seawater-acclimatized salmon after injection with Norwegian strains of the ISAV (Dannevig et al. 1995b; Sommer and Mennen 1997), perhaps indicating that progress of the disease is similar in freshwater- and seawater-reared salmon. Mortality in Atlantic salmon injected with a similar dose of another Canadian isolate of ISAV, however, occurred between 41 and 60 d after injection (Simko et al. 2000), two to three times longer than was observed here. Although these studies were conducted in different laboratories, this apparent difference in virulence may support the argument for phenotypic variation among Canadian ISAV isolates (Kibenge et al. 2000).

The kinetics of mortality among injection- and cohabitation-infected fish differed such that the former began to die after approximately 17 d, whereas mortalities among the latter ensued after approximately 33 d. Similarly, the difference between mean time to onset of mortality and mdd was considerably greater in cohabitants than in injected fish. Injected salmon transmit ISAV in quantities capable of inducing mortality from day 7 onwards, and virus particles were detected in tank water containing infected fish as early as 3 d after infection (Totland et al. 1996). Thus ISA progresses more gradually among cohabitation-infected fish probably because of exposure to a relatively low but continuous virus challenge. A delayed onset of mortality and a reduction in mortality rate was also observed among Atlantic salmon infected by cohabitation with *Aeromonas salmonicida* compared with those infected by injection (Nordmo and Ramstad 1997). In the present study, total cumulative mortality among naive cohabitants was greater in tanks containing six injected fish instead of two, suggesting that the infectious dose was related to the number of injected fish and presumably, therefore, to the number of virus-shedding fish. A similar dose effect on cumulative mortality was obtained following ip injection of salmon parr with diluted culture supernatants (Jones et al. 1999b). The reduced mortality in groups containing eight injected fish, therefore, could not be explained by this hypothesis. Stocking density plays an important role in susceptibility to disease within fish populations (Reno 1998). Perhaps the stocking density among cohabitants after the rapid mortality of the eight ip-injected fish (trial 1) was sufficiently low to reduce the proportion of susceptible fish in these tanks. Despite the foregoing, similarities in cohabitation mdd among infection groups in trial 1 indicated that pathogenesis of ISA within susceptible fish was independent of challenge severity. Furthermore, given that some injected fish survived, it is possible that not all injected fish were equally infectious; this may explain the greater tank-to-tank variation in mortality among groups that contained fewer injected fish.

The relative contribution to infection of virus acquired by direct physical contact with infected fish and that acquired from the water column after shedding from infected fish was not determined in trials 1 and 2. However, detection of ISAV in tissue homogenates obtained from the noncontact cohabitant fish that died in trial 3 demonstrated for the first time that ISAV retains infectivity in freshwater after being shed from infected fish. The find-

ings that skin mucus is almost as infectious as blood homogenates in naive salmon (Totland et al. 1996) and that the virus can be detected in skin mucus by RT-PCR (Melville and Griffiths 1999) support the hypothesis that sloughed mucus is a vehicle for virus transmission. Feces from infected fish may also facilitate virus transmission (Nylund et al. 1994; Totland et al. 1996). While it is not known how long ISAV particles remain infectious after being shed in freshwater, cultured ISAV retains infectivity for up to 24 h in freshwater at 10°C and pH 7.2 (Dr. L. Brown, National Research Council of Canada, personal communication). That shed virus may be sheltered within sloughed mucus or feces suggests that a longer duration of infectivity is possible. To properly assess the risk of dissemination of ISAV in freshwater, it is important that further study establish the duration of infectivity of virus-contaminated water and, by implication, the risk of dissemination of the virus in freshwater.

Histological assessments were made of various tissues obtained from moribund fish after infection by injection or cohabitation. Microscopic changes seen in these tissues were qualitatively similar to those described earlier for this strain of ISAV (Jones et al. 1999b) as well as those in fish infected naturally with viral strains of Canadian, Norwegian, or Scottish origin (Evensen et al. 1991; Mullins et al. 1998; Rodger et al. 1998; Simko et al. 2000). To our knowledge, histological lesions associated with ISAV have not previously been reported in the stomach. Moderate to severe congestion associated with necrosis of the gastric mucosal endothelium was similar to lesions described earlier for intestine and pyloric caeca in naturally and experimentally infected salmon (Evensen et al. 1991; Rodger et al. 1998; Jones et al. 1999b; Simko et al. 2000). Furthermore, the apparent differential pathology between upper and lower intestine has previously gone unreported in ISAV-infected salmon. Our finding of hepatic lesions in a majority of liver samples in injection- or cohabitation-infected salmon concurred with observations in experimentally (Simko et al. 2000) and naturally infected salmon (Evensen et al. 1991). Furthermore, the low occurrence of gastrointestinal lesions in salmon infected by injection supported observations of Simko et al. (2000). We do not know why exposure by cohabitation elicited gastrointestinal lesions in a significantly greater proportion of samples than injection did. The gastrointestinal epithelium is unlikely to be an important portal of virus entry in cohabitation-infected fish, because intubation of virus fails to result in clinical disease (Totland et al. 1996). Rather, the pathogenesis of ISA may be viewed as a sequence of pathognomonic changes, beginning with reduced hematocrit and followed by the development of congestion, hemorrhage, and necrosis in most organs. While renal and hepatic involvement appear relatively early (Dannevig et al. 1994), it is possible that gastrointestinal involvement occurs later. If so, the relatively rapid pathogenesis and mortality following injection exposure would tend to preclude the development of microscopic gastrointestinal lesions. This hypothesis may be tested by examining the sequence of histological changes that occur during infections of varying severity induced by injection.

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