

Effect of *Renibacterium salmoninarum* levels in the ovarian fluid of spawning chinook salmon on the prevalence of the pathogen in their eggs and progeny

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ABSTRACT: Chinook salmon, *Oncorhynchus tshawytscha*, eggs were exposed to ovarian fluid inoculated with various concentrations of *Renibacterium salmoninarum* (Rs), the causative agent of bacterial kidney disease of salmonids, in an attempt to infect them. None of the eggs exposed to 1.7×10^3 or fewer Rs cells ml^{-1} of ovarian fluid proved to be infected intraovum. However, intraovum infections occurred in eggs exposed to Rs cells at concentrations of 1.7×10^5 or higher ml^{-1} . Smolts derived from eggs exposed to Rs cells at concentrations of 1.7×10^3 and higher were Rs-infected. As with eggs, the highest prevalence of infection in smolts occurred in those derived from eggs exposed to the highest concentration of Rs tested. In addition, smolts derived from eggs that were not deliberately exposed to Rs, but which were obtained from ovarian fluid naturally infected with as few as 28 to 113 Rs cells ml^{-1} , were also infected. The implications of these findings are discussed.

INTRODUCTION

Bacterial kidney disease (BKD) is an important chronic disease of wild and propagated salmonids. The disease can be transmitted horizontally (Mitchum & Sherman 1981, Bell et al. 1984) as well as vertically (Bullock et al. 1978, Evelyn et al. 1986a). Vertical transmission of the causative bacterium *Renibacterium salmoninarum* (Rs) is mediated by the intraovum bacterium (Evelyn et al. 1984a, b, Lee & Gordon 1987a), and ovarian fluid has been implicated as an important source of infection for eggs (Evelyn et al. 1986a).

In an effort to minimize vertical transmission of Rs, salmon farmers in British Columbia (B.C.) have undertaken costly broodstock screening programs to identify and avoid Rs-infected females that might yield infected eggs (Lee 1986, Lee and Gordon 1987a). Screening was accomplished by examining ovarian fluid for the presence of Rs using the indirect fluorescent antibody technique (IFAT) and culture. Eggs taken from fish found to be infected were destroyed. Unfortunately, the

screening program appears not to have worked. The screening procedures, as applied, often resulted in contradictory results (Armstrong et al. in press), making it difficult to decide which batches of eggs were safe and suggesting that many Rs-infected eggs may have been passed as acceptable.

Recently, Elliott & Barila (1987) developed a membrane filtration-fluorescent antibody technique for detecting and quantifying Rs cells in ovarian fluid. The method proved very sensitive, and a modified version of it – the membrane filtration-indirect fluorescent antibody technique (MF-IFAT) (Lee & Gordon 1987b) – was successfully used for investigating the numbers of Rs cells in ovarian fluid. The results indicated that the numbers of Rs cells found in ovarian fluid using MF-IFAT were dramatically higher than those found using conventional methods of culture or IFAT. The MF-IFAT was therefore used in the present study, the objective of which was to determine the effect of Rs cell numbers in ovarian fluid of chinook salmon, *Oncorhynchus tshawytscha*, on the prevalence of Rs infections in their eggs and progeny.

MATERIALS AND METHODS

Source of eggs and milt. Approximately 5000 eggs were collected from 10 apparently healthy chinook salmon that had returned from the sea to spawn at Robertson Creek Hatchery, Vancouver Island, B.C. The eggs from each fish were collected in clean, separate containers (500 per container) and were kept on ice until needed. Milt from 3 apparently healthy chinook salmon was also collected at this hatchery and stored on ice until required.

General. Eggs were used in 2 experiments immediately on their arrival in the laboratory (within 6 h of collection). In the first experiment, equal portions of eggs from Spawners 1 to 5 and Spawners 6 to 10 were pooled to form 2 egg lots: Lots I and II, respectively. Groups of eggs from these lots were exposed to various concentrations of Rs cells to determine what effect this would have on the prevalence of infection in eggs and the progeny derived from these eggs. In the second experiment, eggs remaining from Spawners 1 to 5 were processed separately, and without exposure to Rs, to permit observations on the prevalence of background Rs infection conferred on young fish by individual spawners. Eggs from Spawners 6 to 10 were not processed in this manner due to a shortage of egg incubation trays.

Expt 1. Egg infection protocol: Egg infections were accomplished by immersing soft (non-water-hardened) eggs in ovarian fluid containing various concentrations of Rs cells: 0 (control group), 1.7×10^3 , 1.7×10^5 , 1.7×10^7 , or 1.7×10^9 Rs cells ml^{-1} . From each of the 2 lots of pooled eggs, groups of ca 500 eggs were immersed in 25 ml of ovarian fluid containing one or the other of the above Rs cell concentrations. The exposure was conducted at 4 to 5°C in cotton-stoppered 1 l Erlenmeyer flasks for 12 h, the flasks being rotated slowly (30 to 40 rpm) on a Labline Orbit Shaker.

Ovarian fluid used for egg exposures was freed of particulate material (host cells, tissue debris, bacteria) by centrifugation ($3000 \times g$ for 10 min) and by passage through a 0.45 μm pore diameter filter (Nalgene sterilization filter unit, Type S; Sybron/Nalge, Rochester, NY). The clarified fluid was then inoculated with sufficient Rs cells to achieve the Rs cell concentrations mentioned. The Rs cells used in the egg exposures (Rs Isolate 384; Evelyn et al. 1986a) were grown on KDM2 (Evelyn 1977) for 14 d at 15°C and were suspended in phosphate buffered saline (pH 7.2) to give a Rs cell count of 1.7×10^{10} ml^{-1} using MF-IFAT. This suspension was appropriately diluted with sterile, clarified ovarian fluid to give the desired egg challenge suspensions. Homogeneous distribution of Rs cells in the suspensions was achieved by repeated trituration using a 3 ml syringe fitted with a 22 gauge 1 in. hypodermic needle.

Detection of intra-ovum Rs infections: Following the challenge of eggs, they were immediately dry-fertilized with milt pooled from 3 apparently healthy chinook salmon, the kidneys of which proved Rs-free on the basis of (IFAT). Eggs were then rinsed free of milt with several changes of dechlorinated Vancouver City water (4°C) and surface-disinfected for 15 min with 500 ppm iodine (supplied as Ovadine®). Duplicate groups (100 eggs group^{-1}) of eggs from each lot and from each challenge treatment were then tested for the presence of intra-ovum Rs. In this test, eggs were incubated in KDM2 broth (1 egg per tube of 5 ml broth) at 15°C for 2 mo to enrich the numbers of any intra-ovum Rs cells present (Evelyn et al. 1984b). An egg was considered surface-sterile (and fit, therefore, for intra-ovum sampling) if the broth surrounding it remained clear and if no Rs cells were found in the broth using MF-IFAT.

To detect Rs cells within eggs, the contents of those eggs shown to be surface-sterile were aseptically removed (Lee & Gordon 1987a). For any given challenge treatment, the egg contents were pooled (10 eggs pool^{-1} , or in a few instances 5 to 14 eggs pool^{-1}). The pooled samples were each centrifuged ($3000 \times g$ for 10 min) to yield a pellet from which a smear was prepared for examination by IFAT. Fifty microscopic fields per smear were read (magnification = 1000). Details of the egg content screening procedure are given in Lee & Gordon (1987a). The egg content pellets were also cultured for Rs on KDM2 (at 15°C for 4 wk).

Surface-disinfected eggs from the variously challenged groups that were not sampled for intra-ovum Rs were incubated, according to the treatment received, in separate Heath trays. Trays were supplied with dechlorinated Vancouver City water at 4 to 8°C, the dechlorination having been accomplished in our laboratory, just prior to use. On hatching, the resultant fry were immediately transferred to 1000 l tanks, 1 treatment tank⁻¹, where they were raised to the smolt stage on a dry, pelleted, commercial ration in the same water supply (8 to 15°C). (The above water supply was used to reduce the likelihood that Rs infections would be introduced with the water.) At this stage (195 d post-hatching), 100 smolts (4.45 g a piece) from each treatment group were collected at random and examined for Rs to determine whether vertical transmission had occurred (MF-IFAT, Lee 1989). In the procedure, kidney tissue samples were homogenized in peptone (0.1%) - saline (0.85% NaCl) using a stomacher. The suspensions were then filtered (5 μm pore diameter filter) and trypsinized to facilitate their examination by MF-IFAT. Fifty microscopic fields (magnification = 1000) were examined per kidney sample.

Expt 2. Approximately 150 eggs, retained from each of Spawners 1 to 5, were dry-fertilized with the same

batch of milt used in the first experiment. Eggs were then rinsed free of milt and surface-disinfected as already described. They were then incubated, according to spawner, in separate Heath trays until hatching. Resultant fry were immediately transferred to 200 l tanks (progeny of 1 spawner tank⁻¹) where they were reared to the smolt stage. At this stage, 100 smolts were collected at random and their kidneys examined for Rs - to determine whether vertical transmission had occurred. The egg incubation and fry rearing conditions, the timing of smolt sampling, and the procedure used for detecting Rs in smolts were the same as those already described in connection with Rs-challenged eggs.

RESULTS AND DISCUSSION

Table 1 presents results of the efficacy of the egg surface disinfection procedure. Among the 20 groups of

Table 1. *Renibacterium salmoninarum*. Efficacy of surface disinfection of eggs exposed to various concentrations of Rs in ovarian fluid. Unfertilized non-water-hardened eggs were challenged at 4 to 5°C by immersing them for 12 h under continuous shaking at 30 to 40 rpm in ovarian fluid inoculated with various dosages of viable Rs cells. Eggs were fertilized with fresh milt pooled from 3 healthy males. Prior to incubation in KDM2 broth at 15°C, fertilized eggs were rinsed with water and surface-disinfected by immersion for 15 min in 500 ppm iodine (Ovadine®). Egg survival: no. disinfected eggs with surviving surface-borne bacteria or Rs cells per 100 eggs tested; Sterile: % eggs found surface-sterile

Rs cells ml ⁻¹	Lot	Replicate	Egg survival		Sterile
			Bac- teria	Rs cells	
0	I	1	8	0	92
0		2	11	0	89
0	II	1	7	0	93
0		2	5	0	95
1.7 × 10 ³	I	1	10	0	90
1.7 × 10 ³		2	11	0	89
1.7 × 10 ³	II	1	12	0	88
1.7 × 10 ³		2	15	0	85
1.7 × 10 ⁵	I	1	9	0	91
1.7 × 10 ⁵		2	16	0	84
1.7 × 10 ⁵	II	1	7	0	93
1.7 × 10 ⁵		2	13	0	87
1.7 × 10 ⁷	I	1	8	0	92
1.7 × 10 ⁷		2	12	0	88
1.7 × 10 ⁷	II	1	11	0	89
1.7 × 10 ⁷		2	9	0	91
1.7 × 10 ⁹	I	1	14	0	86
1.7 × 10 ⁹		2	9	0	91
1.7 × 10 ⁹	II	1	7	0	93
1.7 × 10 ⁹		2	11	0	89

surface-disinfected eggs (100 per group), the rate of successful surface sterilization ranged from 84% to 95%. Surface-sterile eggs were deemed fit for intra-ovum sampling because the risk of contaminating egg content samples with surface-borne bacteria was non-existent. Significantly, the only bacteria surviving the egg surface disinfection procedure were fast-growing (unidentified) species. Viable Rs was not found on the surface of the disinfected eggs. Thus, the presence of Rs in smolts derived from eggs used in our 2 experiments (see later) must be accounted for by intra-ovum bacterium.

The hatch success of the various groups of eggs used in the 2 experiments are indicated in Table 2. Hatch rates were higher (70 to 85%) in those eggs that were not subjected to the handling procedures involved in attempting to infect them with Rs, i.e., the eggs used in the second experiment. Not surprisingly, eggs in the first experiment, which experienced the Rs challenge procedure exhibited lower hatch rates (38 to 62%). Notwithstanding this, and as will be shown later, enough Rs-challenged eggs hatched to permit comparisons of the effect of the challenge level on the rate of Rs infections in the resulting smolts.

Table 2. *Renibacterium salmoninarum*. Hatching success of eggs challenged with various concentrations of Rs. Eggs were immersed in ovarian fluid inoculated with a calculated number of Rs cells ml⁻¹ ovarian fluid (based on a MF-IFAT count made on the Rs cell suspension used to inoculate the ovarian fluid). Spawner: 2 replicate groups of eggs from each Rs challenge level were combined and incubated to hatching in trays according to the challenge received. In addition, eggs from individual Spawners 1 to 5 were incubated in trays according to spawner. Actual nos. of Rs cells ml⁻¹ in ovarian fluid were determined using MF-IFAT

Lot	Calculated Rs cells ml ⁻¹	Spawner	Actual Rs cells ml ⁻¹	No. fry hatched/ no. eggs incubated	Eggs hatched (%)
	None	1	0	105/149	70
	None	2	0	119/150	79
	None	3	28	132/155	85
	None	4	56	120/152	79
	None	5	113	115/148	77
I	None	1-5, pooled	28	159/256	62
	1.7 × 10 ³	1-5, pooled	1.8 × 10 ³	117/248	47
	1.7 × 10 ⁵	1-5, pooled	2.0 × 10 ⁵	101/220	46
	1.7 × 10 ⁷	1-5, pooled	1.9 × 10 ⁷	108/261	41
	1.7 × 10 ⁹	1-5, pooled	2.1 × 10 ⁹	105/245	43
II	None	6-10, pooled	56	108/285	38
	1.7 × 10 ³	6-10, pooled	2.0 × 10 ⁴	142/270	53
	1.7 × 10 ⁵	6-10, pooled	1.6 × 10 ⁵	112/291	39
	1.7 × 10 ⁷	6-10, pooled	1.5 × 10 ⁷	129/235	55
	1.7 × 10 ⁹	6-10, pooled	1.9 × 10 ⁹	109/231	47

Table 3 summarizes the consequences of incubating eggs in ovarian fluid containing various concentrations of Rs cells. As expected, eggs incubated in Rs-free ovarian fluid proved to be free of intra-ovum Rs (none of the 37 pooled egg content samples tested were infected), indicating that if natural Rs infections occurred in the eggs used in these experiments the infections must have been rare occurrences. No intra-ovum infections occurred when eggs were exposed to 1.7×10^3 Rs cells ml^{-1} of ovarian fluid, but intra-ovum infections did occur (one in 35 or 36 egg pools) when 1.7×10^5 or 1.7×10^7 Rs cells ml^{-1} were present. The prevalence of intra-ovum infections was highest (8 of 36 egg pools) in eggs exposed to Rs cell concentrations of 1.7×10^9 ml^{-1} , a result consistent with that obtained by Evelyn et al. (1986a) and one that simulates the situation found in nature where egg infections were always found in fish with ovarian fluid containing 10^9 or more Rs cells ml^{-1} (Evelyn et al. 1984a, b, 1986b). Unfortunately, precise information on the prevalence of intra-ovum infections

Table 3. *Renibacterium salmoninarum*. Effect of incubating non-water-hardened, unfertilized eggs in ovarian fluid containing various concentrations of Rs cells on the prevalence of intraovum (Rs) as judged from pooled egg content samples. Eggs immersed in ovarian fluid inoculated with number Rs cells ml^{-1} . Eggs: no. surface-sterile eggs tested; Rs-infected eggs/no. tested: number of pooled. Egg contents (10, rarely 5 to 14 eggs) infected by Rs - detected, following enrichment by culture, by IFAT and cultural examinations of the particulate fraction present in the samples (details in text) - per number tested

Rs cells ml^{-1}	Lot	Repl-icate	Eggs	Rs-infected eggs/no. tested
0	I	1	92	0/9
0		2	89	0/9
0	II	1	93	0/9
0		2	95	0/10
1.7×10^3	I	1	90	0/9
1.7×10^3		2	89	0/9
1.7×10^3	II	1	88	0/9
1.7×10^3		2	85	0/9
1.7×10^5	I	1	91	0/9
1.7×10^5		2	84	0/8
1.7×10^5	II	1	93	1/9
1.7×10^5		2	87	0/9
1.7×10^7	I	1	92	1/9
1.7×10^7		2	88	0/9
1.7×10^7	II	1	89	0/9
1.7×10^7		2	91	0/9
1.7×10^9	I	1	86	0/9
1.7×10^9		2	91	1/9
1.7×10^9	II	1	93	2/9
1.7×10^9		2	89	5/9

is not available because pooled samples were used to reduce the number of egg samples that had to be assayed.

Table 4 records the findings of the first experiment in which smolts resulting from eggs exposed to various levels of Rs were monitored for BKD and Rs. None of the smolts developed clinical signs of BKD and none, of the few specimens that died and were examined within the last 2 mo of rearing, were infected with Rs. The groups of smolts resulting from the experimentally challenged groups of eggs were infected with Rs, indicating that vertical transmission of Rs had occurred. This held true even for the group that appeared to be uninfected following exposure to the lowest challenge level (1.7×10^3 Rs cell ml^{-1}), suggesting that some intra-ovum infections must, after all, have resulted from this challenge. The prevalence of infection in smolts may have been inflated because of the possibility of cross-infections during the 195 d rearing period. Notwithstanding this, the conclusion that vertical transmission had occurred is supported by the fact that the prevalence of infection tended to show a trend: smolts derived from eggs exposed to the lowest challenge

Table 4. *Renibacterium salmoninarum*. Determination of Rs prevalence in 195 d-old smolts derived from eggs exposed to various dosage levels of the pathogen in ovarian fluid. Prior to experimental exposure to Rs, the non-water-hardened eggs in Lots 1 and 2 were drained of ovarian fluid and then re-immersed in the clarified fluid (controls) or in fluid inoculated with various dosages of Rs. Exposure was conducted at 4 to 5°C with slow shaking for 12 h. Eggs were then dry-fertilized with Rs-free milt (pooled from 3 healthy fish. Actual Rs cell concentrations varied slightly from the expected values (see Table 2). Also, eggs in Lots I and II were inadvertently exposed to ovarian fluid containing 28 and 56 Rs cells ml^{-1} , respectively, prior clarification of the fluid because one or more of the spawners carried Rs in their ovarian fluid. Rs infected smolts were detected using kidney samples and a modification of the MF-IFAT (see text). Dead fish were collected in the 62 d period prior to sampling

Treatment	Rs conc.	Lot	Spaw-ners	Infected smolts/100 tested	Dead in-fected smolts/dead smolts collected
Control	0	I	1-5	1	0/0
		II	6-10	3	0/5
Inoculated	1.7×10^3	I	1-5	10	0/0
		II	6-10	8	0/0
	1.7×10^5	I	1-5	20	0/1
		II	6-10	9	0/4
	1.7×10^7	I	1-5	15	0/2
		II	6-10	29	0/0
	1.7×10^9	I	1-5	44	0/2
		II	6-10	24	0/4

level of Rs showed lower infection rates (8 to 10%) than those derived from the most severely challenged eggs (24 to 44%).

Of particular interest was the fact that smolts derived from eggs not experimentally exposed to Rs were also infected. The prevalence of infection in these smolts was low (1 to 3%) and, again, may have been inflated by cross-infections during the smolts' 195 d rearing period. The presence of infection in these fish was, however, significant because smolts were derived from pooled eggs held in pooled ovarian fluid that, prior to clarification, contained very low concentrations of Rs cells (28 and 56 Rs cells ml⁻¹ of fluid for Lots I and II, respectively). The source of Rs cells in the pooled (unclarified) ovarian fluid of Lots I and II was obviously one or more of the seemingly healthy spawners that provided the eggs and ovarian fluid for the lots. Data relating to this, for the 5 spawners of Lot I are given in Table 5. It can be seen that 2 of the 5 spawners yielded Rs-free ovarian fluid and Rs-free smolts. The other 3 spawners, however, produced ovarian fluid containing low concentrations of Rs cells (28 to 113 ml⁻¹), and 1 to 2% of their progeny carried subclinical Rs infections.

The question to be answered is how the low levels of Rs cells in the ovarian fluid of the foregoing spawners resulted in infected progeny, particularly since it has not been possible in this and one other study (Evelyn et al. 1986a) to establish egg infections using higher concentrations of Rs cells (e.g. ca 10³ Rs cells ml⁻¹). One possibility is that intra-ovum infections also occur directly from infected ovarian tissue prior to ovulation. Data suggesting that this may sometimes occur have already been reported (Lee & Gordon 1987a). Another possibility is that the infections came from the ovarian

fluid but that non-homogeneous distribution of the bacterium in the fluid occurs, resulting in 'hot spots' of infection for eggs – 'hot spots' that are not readily apparent using current ovarian fluid sampling methods. Both possibilities are now being investigated.

In summary, the present findings confirm that vertical transmission occurs as a result of intra-ovum infections and that such infections are likely to be most frequent when eggs are exposed to heavily infected ovarian fluid. The results also show that Rs infections in smolts are most frequent when smolts are derived from eggs exposed to ovarian fluid containing high Rs concentrations. Disconcertingly, however, low level infection in ovarian fluid also resulted in vertical transmission of Rs. These low level infections would almost certainly have gone undetected using the IFAT procedure routinely used in the aforementioned broodstock anti-BKD screening program, perhaps explaining why the broodstock screening program in B.C. has not been successful in producing Rs-free smolts for fish farmers. Clearly, a more sensitive anti-BKD screening procedure is needed and a better understanding of how eggs acquire their infections is required.

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Table 5. *Renibacterium salmoninarum*. Rs prevalence in offspring of 5 spawners in Lot I. Spawners: eggs (not experimentally exposed to Rs), obtained from each of the 5 spawners, were fertilized with Rs-free milt. Eggs from each fish were incubated separately and the resulting progeny were reared in separate tanks to the smolt stage. Rs concentration in the ovarian fluid was determined using MF-IFAT and Rs infections detected using kidney samples and a modification of MF-IFAT (see text). Dead fish were collected in the 62 d period prior to sampling

Spawner	Rs cells ml ⁻¹	Infected smolts/100 tested	Dead infected smolts/dead smolts collected
1	0	0	0/0
2	0	0	0/1
3	28	1	0/0
4	56	1	0/2
5	113	2	0/4

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