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Intra-ovum Infection in Salmonid Eggs Artificially Contaminated with Fish Pathogenic Bacteria: *Flavobacterium psychrophilum*, *Renibacterium salmoninarum* and *Aeromonas salmonicida*

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ABSTRACT—Intra-ovum infection of artificially contaminated salmonid (rainbow trout or amago salmon) eggs was studied using three fish pathogens (*Flavobacterium psychrophilum*, *Renibacterium salmoninarum* and *Aeromonas salmonicida*). Artificial contamination was performed using egg-surface contamination or immersion-water contamination. For *F. psychrophilum* and *R. salmoninarum,* infection occurred by the entry of pathogens from the egg surface to the perivitelline space through the micropyle during water-hardening. The infection rate was higher for egg-surface contamination than for immersion-water contamination. The successful concentration of *F. psychrophilum* for the occurrence of intra-ovum infection was 10^7 CFU/mL or more for egg surface contamination, and was 10^9 CFU/mL or more for immersion-water contamination. After the entry into eggs, *F. psychrophilum* increased to 10^7 CFU/egg but *A. salmonicida* steadily decreased and became undetectable.

Key words: intra-ovum infection, micropyle, perivitelline space, salmonid, *Flavobacterium psychrophilum*, *Renibacterium salmoninarum*, *Aeromona salmonicida*

In salmon culture, control of disease in hatcheries has generally been achieved. This is done through health monitoring of brood fish, disinfection of eggs just after fertilization and eyed-egg stages, disinfection of the water supplies, as well as separate rearing of alevins and fry. Despite all these efforts, there are some reports of bacterial cold water disease (BCWD) and bacterial kidney disease (BKD) (Yoshimizu, 2007, 2009). The pathogens that cause these diseases escape control measures by intra-ovum infection. Studies on intra-ovum infection mechanism and preventive measures are therefore important for farming of salmonid fishes.

There have been several studies on intra-ovum infection with *Renibacterium salmoninarum* and *Flavobacterium psychrophilum* (Evelyn *et al*., 1984a, b, 1986a, b; Bruno and Munro, 1986; Lee and Evelyn, 1989, Brown *et al*., 1997; Cipriano, 2005; Kumagai *et al*, 2000; Kumagai and Nawata, 2010a). It was suggested that intra-ovum infection with *R. salmoninarum* occurred in the female body cavity as a result of heavily contaminated coelomic fluid (Evelyn *et al*., 1984a, b, 1986a, b; Bruno and Munro, 1986; Lee and Evelyn, 1989). On the other hand, Kumagai *et al*. (2000) and Kumagai and Nawata (2010a) reported that intra-ovum infection with *F. psychrophilum* occurred during water-hardening of fertilized coho salmon *Oncorhynchus kisutch* and rainbow trout *Oncorhynchus mykiss* eggs. In the same reports, *F. psychrophilum* was observed in the perivitelline space of infected eggs. Although Kumagai *et.al* (2000) reported intra-ovum infection of fertilized coho salmon eggs, Kumagai and Nuwata (2010a) reported intra-ovum infection of rainbow trout eggs contaminated before fertilization. The possibility that infection occurs during fertilization was therefore not eliminated. In order to clarify underlying mechanisms, intra-ovum infection of salmonids eggs was artificially induced using three fish pathogens of *F. psychrophilum*, *R. salmoninarum* and *Aeromonas salmonicida*.

Materials and Methods

Bacterial strains and preparation of bacterial suspensions for challenge test

Three fish pathogenic bacteria were used for the experimental infection by immersion challenge test of eggs. *Flavobacterium psychrophilum* strain 06–003, *R. salmoninarum* strain 09–001 and *A. salmonicida* strain 08–029 were isolated from the kidney of diseased rainbow trout, amago salmon *O. masou isikawae* and brown trout *Salmo trutta*, respectively. *Flavobacterium psychrophilum* was cultured in enriched Anecker and Ordal's broth (tryptone 5 g, yeast extract 0.5 g, beef extract 0.2 g, sodium acetate 0.2 g, fatal bovine serum

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50 mL/L, pH 7.2) at 15°C for 3 days. *Renibacterium salmoninarum* was cultured in KDM-SMRs broth with 5% FBS (Matsui *et al*., 2009) at 15°C for 5 days. *Aeromonas salmonicida* was cultured in trypto-soya broth at 15°C for 2 days. All bacterial cells were collected by centrifugation (1,800 ×*g* for 5 min at 5°C), and rinsed twice with a sterilized isotonic solution (sodium chloride 9.04 g, potassium chloride 0.24 g, calcium chloride 0.26 g/L) before being re-suspended in sterilized isotonic solution which was one-fifth the volume of the culture broth.

Source of fish eggs and sperm

Fish eggs and sperm were obtained from *F. psychrophilum*-, *R. salmoninarum*- and *A. salmonicida*-free ripe rainbow trout or amago salmon reared at Nagano Prefectural Fisheries Experimental Station and private fish farms in Nagano Prefecture. Eggs and sperm were collected from three to five fish, and pooled for use. Newly spawned eggs were washed with sterilized isotonic solution (Kohara *et al.*, 2010) before the contamination experiments.

The effect of fertilization on intra-ovum infection with F. psychrophilum *introduced by surface contamination*

To determine the effect of fertilization on intra-ovum infection, two trials each having three experiments were performed. The sequence of treatments were surface contamination, fertilization and water-hardening; fertilization, surface contamination and water-hardening; as well as surface contamination, no fertilization and water-hardening. All the experiments were done in 200 mL conical flasks, each containing 400 eggs. Surface contamination was done by adding 2 mL of *F. psychrophilum* suspension to the eggs and gently shaking. Bacterial doses were 0, $10^{4.3}$ and $10^{8.3}$ CFU/mL for the first trial. For the second trial, bacterial doses were 0, $10^{4.4}$ and $10^{8.4}$ CFU/mL. Where necessary, fertilization was done by adding 0.2 mL of a sperm suspension. Finally water-hardening was done by suspending the eggs in 200 mL of sterilized water for 1 h without agitation.

Water-hardened eggs were disinfected with 50 ppm povidone-iodine solution for 15 min, and incubated in a small aquarium (2 L) using un-chlorinated flowing tap water at 8 to 12°C until eyed-egg stage.

The effect of water-hardening time on the entry of F. psychrophilum *and sperm*

Six time intervals (0, 2, 5, 10, 30, 60 min) were used to determine the effect of water-hardening time on the entry of *F. psychrophilum*. Fertilized eggs were divided into six conical flasks, each flask containing 400 eggs. For all the groups of eggs (except eggs for the 0 min time interval), water-hardening was induced by adding 200 mL of water. After time intervals of 2, 5, 10, 30, 60 min the water was decanted and eggs were artificially contaminated using 2 mL of *F. psychrophilum* ($10^{8.9}$ CFU/mL) suspension with gentle agitation. After artificial contamination, 200 mL of sterilized water was added and hardening was allowed to continue for a cumulative period of 1 h. After hardening, all the egg surfaces were disinfected with povidone-iodine, and subsequently incubated until the eyed stage.

To determine effect of water-hardening time on sperm entry, pre-fertilized eggs were divided into five conical flasks, each flask containing 400 eggs. Each group of eggs received 200 mL of sterilized water for water-hardening. After time intervals of 0, 2, 5, 10, 30 min, the water in the flask was removed and eggs were fertilized with 0.2 mL of sperm. After fertilization, 200 mL of sterilized water was added and hardening was allowed to continue for a cumulative period of 1 h. After hardening, all the egg surfaces were disinfected with povidone-iodine, and subsequently incubated until the eyed stage.

The difference in F. psychrophilum *infection rates between surface and immersion-water contaminated eggs*

Two different artificial contamination methods were used to determine their effect on infection rates. The two methods were surface contamination and immersion-water contamination. Experiments were performed in two trials. The doses of *F. psychrophilum* suspension for artificial contamination were $10^{9.9–5.9}$ and $10^{9.9–3.9}$ CFU/mL for the first and second trials respectively. Surface contamination was achieved by adding 2 mL of *F. psychrophilum* suspension to 400 eggs in a flask. The contaminated eggs were then fertilized with 0.2 mL of sperm, and gently mixed by agitation. The fertilized eggs were water-hardened in 200 mL of sterilized water for 1 h. On the other hand, for immersion-water contaminated eggs, 400 fertilized eggs were suspended in 200mL of water containing 2 mL of *F. psychrophilum*, and allowed to water-harden for 1 h. Total *F. psychrophilum* contamination doses for both contamination methods were equal. The water-hardened eggs were disinfected with povidone-iodine, and incubated until eyed stage.

The difference between intra-ovum infection rates of R. salmoninarum *and* A. salmonicida

To compare infection rates, rainbow trout eggs were used for *R. salmoninarum* contamination experiments while amago salmon eggs used for *A. salmonicida* contamination experiments. Depending on the experiment, eggs (n = 400) were contaminated with 2 mL of *R. salmoninarum* ($10^{5.2–10.2}$, $10^{5.3–10.3}$ CFU/mL) or *A. salmonicida* ($10^{5.0–9.0}$ CFU/mL) suspension. Where necessary, fertilization was done after artificial contamination. All eggs were water-hardened for 1 h, disinfected with povidone-iodine, and then incubated

until the eyed stage.

Intra-ovum growth patterns of F. psychrophilum *and* A. salmonicida

To understand the intra-ovum growth patterns of *F. psychrophilum* and *A. salmonicida*, 1,200 unfertilized rainbow trout eggs were contaminated with 6 mL suspension of either pathogen ($10^{10.0}$ CFU/mL for *F. psychrophilum* or $10^{9.5}$ CFU/mL for *A. salmonicida*) and water-hardened for 1 h. After disinfection with povidone-iodine, eggs were divided into six groups of 200 eggs each and incubated until eyed stage (21 days). Five out of six groups of eggs were examined and one group was reserved for temporary isolation. After 0, 3, 7, 14, 21 days for incubation, intra-ovum viable bacterial counts for 60 randomly selected eggs were determined.

Isolation of bacteria from egg contents

Eyed-eggs and normal unfertilized eggs (n = 60–80 eggs) were collected from aquarium at eyed stage, and disinfected with povidone-iodine. After disinfection, each egg was rolled on the agar plate to detect bacteria on egg surface and placed in each well of micro-plate (96 wells). As a result of disinfection prior to isolating bacteria from egg contents in every experiment, any viable bacteria were not isolated from egg surface.

Eyed-egg in a well was cracked with a sterilized needle (23G) of syringe and homogenized with a sterilized cotton swab in a well, and homogenized egg contents were smeared on the agar plate. In the case of unfertilized eggs, egg contents were collected by sucking with a sterilized syringe (1 mL) with needle, and smeared. For viable bacterial counts, egg contents collected were suspended in 1 mL of PBS. The suspension was serially diluted with PBS and 100 μL of each dilution was spread on the agar plate. Viable bacterial counts were calculated from the number of colony on the agar plate.

Flavobacterium psychrophilum, *R. salmoninarum* and *A. salmonicida* were isolated using Anecker and Ordal's agar plate at 15°C for 7 days, KDM-SMRs agar plate at 15°C for 30 days and Coomassie brilliant blue (CBB) agar plate (Cipriano and Bertolini, 1988) at 15°C for 5 days, respectively. *Flavobacterium psychrophilum* was identified by agglutination test with antiserum against *F. psychrophilum* strain FPC-840 and PCR (Yoshiura *et al*, 2006). *Renibacterium salmoninarum* was identified by agglutination test or indirect immunofluorescent antibody technique (IFAT) with antiserum against *R. salmoninarum* strain ATCC33209. These antisera were provided by Japan Fisheries Resource Conservation Association. *Aeromonas salmonicida* was identified by the growth of blue-violet colonies on the CBB agar plate, production of soluble brown pigment in nutrient agar plate, Gram-staining and motility test.

Results

The effect of fertilization on intra-ovum infection with F. psychrophilum

When contamination doses were high ($10^{8.3–8.8}$ CFU/mL) intra-ovum infection with *F. psychrophilum* occurred in both unfertilized and fertilized eggs. No infections occurred at low contamination doses (0–$10^{4.8}$ CFU/mL) (Table 1).

The effect of water-hardening time on the entry of F. psychrophilum *and sperm*

Flavobacterium psychrophilum was isolated from the contents of eggs which were contaminated after water-hardening times of 0, 2, 5, 10 min. No entry occurred after water-hardening times of 30 and 60 min. Similarly the entry of sperm was only successful (eyed eggs formed) for water-hardening times of 0, 2, 5, 10 min, but was not successful after 30 min of water-hardening (Table 2). The fertilization rates (fraction of eyed-eggs) decreased rapidly with the passage of water-hardening time.

Table 1. Comparison of *F. psychrophilum* intra-ovum infection rates for artificial contamination experiments with rainbow trout eggs

Exp.	Contamination dose ($\log_{10}$ CFU/mL)	Artificial contamination stage		
		before fertilization (infected eggs/examined eggs)	after fertilization (infected eggs/examined eggs)	without fertilization (infected eggs/examined eggs)
1	8.3	16/60	24/60*	11/60*
	4.3	0/60	0/60	0/60
	0	0/60	0/60	0/60
2	8.8	2/60	5/60	4/60
	4.8	0/60	0/60	0/60
	0	0/60	0/60	0/60

Rainbow trout eggs were artificially contaminated with *F. psychrophilum* suspension before, after or without fertilization, and water-hardened. The eggs were then disinfected with povidone-iodine, incubated until eyed-egg stage and examined for *F. psychrophilum* intra-ovum infection.

* Significant difference (χ^2-test, $P < 0.01$) in intra-ovum infection ratio between the two groups of eggs.

Difference between F. psychrophilum *infection rates for surface and immersion-water contaminated eggs*

Flavobacterium psychrophilum intra-ovum infection rates were significantly higher for surface contaminated eggs than for immersion-water contaminated eggs (χ^2-test, $P < 0.01$). Infection occurred with surface contamination doses above $10^{7.9}$ CFU/mL. On the other hand contaminated immersion-water only caused *F. psychrophilum* infection at contamination doses of $10^{9.9}$ CFU/mL (Table 3). For both trials no infection was caused when contamination doses were below $10^{6.9}$ CFU/mL.

Difference between intra-ovum infection rates of R. salmoninarum *and* A. salmonicida

Intra-ovum infection with *R. salmoninarum* was observed in both unfertilized and fertilized eggs at high contamination doses ($10^{10.3}$ and $10^{10.2}$ CFU/mL). However intra-ovum infection with *A. salmonicida* was not observed even with relatively high contamination doses (Table 4).

Intra-ovum growth patterns of F. psychrophilum *and* A. salmonicida

The intra-ovum infection rates and changes in viable bacterial counts for rainbow trout eggs artificially contaminated with *F. psychrophilum* or *A. salmonicida*

Table 2. Effect of water-hardening time on *F. psychrophilum* intra-ovum infection and fertilization rates of rainbow trout eggs

Water-hardening time (min)	Intra-ovum infection rate (infected eggs/examined eggs)	Fertilization (Eyed-egg) rate (%)
0 (before)	5/80	48.6
2	3/80	15.0
5	1/80	3.9
10	1/80	1.4
30	0/80	0
60	0/80	—*

Water-hardening of rainbow trout eggs was initiated. After 0,2,5,10,30,60 min water-hardening was interrupted and, depending on the experiment, eggs were contaminated with *F. psychrophilum* ($10^{8.9}$ CFU/mL) suspension or fertilized. Water-hardening was then resumed for a cumulative period of 60 min. Eggs were then disinfected with povidone-iodine and incubated until eyed-egg stage. Finally, depending on the experiment, eggs were examined for intra-ovum infection or formation of eyes. The respective rates were calculated.
* Fertilization experiment was not done.

Table 3. Effect of contamination method on intra-ovum infection rate of rainbow trout eggs with *F. psychrophilum*

Exp.	Contamination dose ($\log_{10}$ CFU/mL)	Method used for contamination of eggs: surface contamination (infected eggs/examined eggs)	Method used for contamination of eggs: immersion-water contamination (infected eggs/examined eggs)
1	9.9	21/60*1	1/60*1
	8.9	3/60	0/60
	7.9	1/60	0/60
	6.9	0/60	0/60
	5.9	0/60	0/60
	0	0/60	0/60
2	9.9	14/60*2	2/60*2
	7.9	1/60	0/60
	5.9	0/60	0/60
	4.9	0/60	0/60
	3.9	0/60	0/60
	0	0/60	0/60

Rainbow trout eggs were artificially contaminated with *F. psychrophilum* using two different methods (surface contamination and immersion-water contamination). One group of eggs were contaminated on the surface with *F. psychrophilum* suspension before fertilization and water-hardened, another group of eggs were water-hardened in the contaminated water after fertilization. After disinfection with povidone-iodine eggs were incubated until eyed-egg stage and examined for *F. psychrophilum* intra-ovum infection.
*1,2 There was the statistical significance ($P < 0.01$) in the intra-ovum infection rate between the two groups of eggs by χ^2-test.

are shown in Fig. 1. *Flavobacterium psychrophilum* intra-ovum infection rates were 33.3–50.0% during the incubation period of 21 days. The viable bacterial counts of *F. psychrophilum* were $10^{0–1.5}$ CFU/egg ($10^{1.0}$ CFU/egg on average) at the end of water-hardening, and gradually increased to $10^{5.2–7.8}$ CFU/egg ($10^{6.9}$ CFU/egg on average) 21 days later. On the other hand, *A. salmonicida* was only isolated at the end of water-hardening (10.0%) and 3 days later (5.0%). The viable bacterial counts of *A. salmonicida* were in the range of $10^{0–1.8}$ CFU/egg at the end of water-hardening, but dropped to undetectable levels 7 days later.

Discussion

The essential factors to explain the mechanism of intra-ovum infection are the time of entry, the entry site, the condition of contamination, and the growth of bacteria in the egg. Kumagai *et al*. (2000) and Kumagai and Nawata (2010a) suggested that *F. psychrophilum* possibly entered eggs during water-hardening. In the present study, *F. psychrophilum* equally entered unfertilized eggs as well as fertilized eggs contaminated before or after fertilization. The result clearly showed that intra-ovum infection with *F. psychrophilum* was not significantly correlated to sperm entry (fertilization). This result was in agreement with the experimental infection of steelhead trout *O. mykiss* and coho salmon eggs with *R. salmoninarum* by Evelyn *et al.* (1986b). Consequently, there is a fair possibility that *F. psychrophilum* entered eggs during water-hardening.

In the experiment to compare *F. psychrophilum* and sperm entry during water- hardening, it was found that neither occurred after 10 min of water-hardening.

Table 4. Intra-ovum infection rates of *R. salmoninarum* or *A. salmonicida* for artificial contamination experiments with rainbow trout or amago salmon eggs

Pathogen	Contamination dose (log_{10} CFU/mL)	Artificial contamination stage	
		before fertilization (infected eggs/examined eggs)	without fertilization (infected eggs/examined eggs)
R. salmoninarum	10.2	—*	11/60
	5.2	—	0/60
	0	—	0/60
	10.3	14/60	31/60
	5.3	0/60	—
	0	0/60	—
A. salmonicida	9.0	—	0/60
	7.0	—	0/60
	5.0	—	0/60
	0	—	0/60

Rainbow trout and amago salmon eggs were contaminated with *R. salmoninarum* and *A. salmonicida* suspension respectively before or without fertilization, and water-hardened. The eggs were then disinfected with povidone-iodine, incubated until eyed-egg stage and examined for *R. salmoninarum* and *A. salmonicida* intra-ovum infection.
* Contamination experiment was not done.

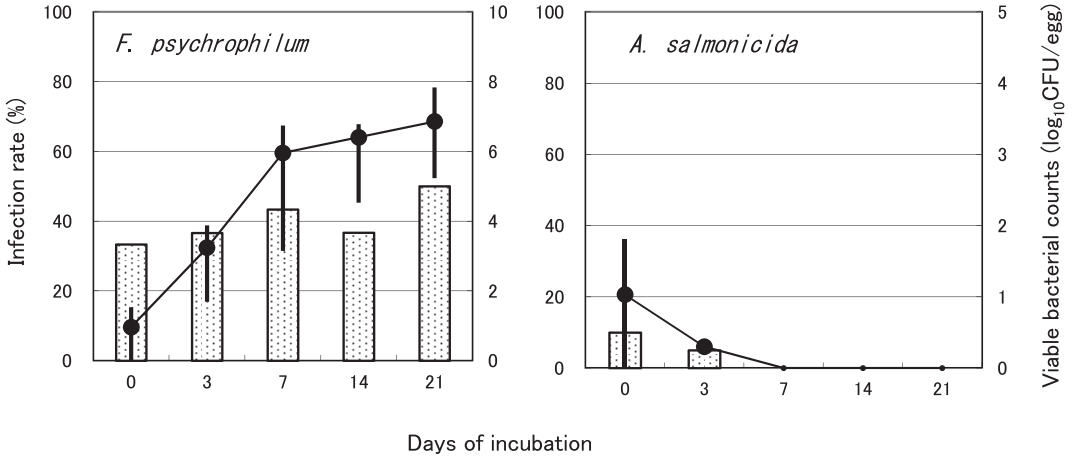

Fig. 1. Intra-ovum changes in viable bacterial counts (—: range, ●: average) and infection rates (▨) in rainbow trout eggs artificially contaminated with *F. psychrophilum* or *A. salmonicida* suspension. Contamination doses were $10^{10.0}$ CFU/mL for *F. psychrophilum* and $10^{9.5}$ CFU/mL for *A. salmonicida*.

It is possible that *F. psychrophilum* physically entered eggs through the micropyle during the water-hardening. This conclusion is supported from the evidence that during water-hardening, the micropyler canal in chum salmon eggs closed after 10 min (Kobayashi and Yamamoto, 1993). Kumagai and Nawata (2010a) detected *F. psychrophilum* in the perivitelline space by the observation of frozen sections. In general, fish eggs are activated by hypotonic solutions and begin to form the perivitelline space around the micropyle. It has been reported that the surplus sperm entered the perivitelline space during artificial fertilization of fish (Iwamatsu, 2004). Therefore, it seems reasonable to conclude that *F. psychrophilum* entered the perivitelline space through the micropyle during water-hardening.

The results in this study also show that the presence of *F. psychrophilum* on the egg surface before water-hardening significantly increases intra-ovum infection rates. This was evidenced by higher rates of infection due to surface contamination as compared to contamination via immersion-water. It is likely that surface contamination enhanced adherence of *F. psychrophilum* to the egg surface, thus increasing chances of entry. The minimum *F. psychrophilum* dose that caused intra-ovum infection was 10^7 CFU/mL. A similar contamination dose has been reported by Kumagai and Nawata (2010a).

If the entry during water-hardening through the micropyle was the only factor regulating intra-ovum infection, other kinds of bacteria should be able to cause such infection. However, *A. salmonicida* did not cause intra-ovum infection (Kumagai *et al.*, 2000). In this study, *A. salmonicida* did not cause intra-ovum infections as *R. salmoninarum.* This inter-specific difference in intra-ovum infection was emphasized by results from experiments on the viable bacterial counts in eggs contaminated with *F. psychrophilum* and *A. salmonicida*. It was shown that viable bacterial counts of *F. psychrophilum* increased to $10^{5.2-7.8}$ CFU/egg at eyed stage, a result similar to that of Kumagai and Nawata (2010a). *Aeromonas salmonicida* entered eggs but, unlike *F. psychrophilum,* viable bacterial counts gradually decreased. The presence of antimicrobial agents in the perivitelline space and egg membrane of fertilized egg has been suggested in several reports (Kudo, 1983, 2000; Iwamatsu, 2004; Shiina *et al*., 2002; Tateno *et al*., 1998). Furthermore, it has been reported that *R. salmoninarum* and *F. psychrophilum* were resistant to lysozyme but *A. salmonicida* was not (Yousif *et al*., 1994; Brown *et al*., 1997). It was postulated that intra-ovum infection was related to lysozyme-resistance of the infecting pathogen. Kumagai *et al*. (2000) supports this idea based on artificial contamination experiments with *F. psychrophilum* and *A. salmonicida*. From the above results, however, it can be concluded that any bacterium can enter the egg, only bacterial pathogens which are resistant to the antimicrobial agents in the egg can cause intra-ovum infections. There is a possibility that intra-ovum viral infection occur through a similar pattern.

In salmonids, ovulated eggs are spawned with coelomic fluid. It is general knowledge that ripe females may have pathogenic bacteria and viruses in the coelomic fluid. Therefore intra-ovum infection may occur as a result of contaminated coelomic fluid. It has been reported that egg-washing with isotonic solution (Kohara *et al*., 2010) and the disinfection with povidone-iodine (Kumagai and Nawata, 2010b) before fertilization were effective for the prevention of intra-ovum infection. The wide use of egg-washing and disinfection with povidone-iodine remains an important step in the artificial fertilization of salmonids.

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