Iodophor Disinfection of Eggs of Spotted Halibut *Verasper variegatus* and Red Sea Bream *Pagrus major*

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The suitable egg developmental stage and effective iodophor (Povidon-iodide) concentration for disinfection of spotted halibut *Verasper variegatus* and red sea bream *Pagrus major* were investigated. In spotted halibut, the suitable egg stage was the beginning of heartbeat, a day before hatching. The eggs were disinfected successfully by treatment with seawater containing iodophor at 75 ppm active iodide for 15 minutes. The hatching rate was decreased at higher concentrations. In red sea bream, the morula stage was most suitable for the treatment. The most successful concentration for disinfection was 100–200 ppm active iodide applied for five minutes. Furthermore, in spotted halibut, iodophor treatment did not have any harmful influence on survival, growth and occurrence of deformity. In the case of red sea bream, when eggs were obtained from the early spawning period, iodophor treatment seemed to reduce the survival rate of the larvae at an early stage, presumably due to egg quality. Growth and occurrence of deformity were not affected. The results suggest that the conditions for disinfection shown above are suitable for seed production. Moreover, the optimum conditions for iodophor disinfection of eggs differed between these two species. It is suggested that conditions for disinfection of eggs should be determined separately for each fish species.

Key words: *Verasper variegatus*, *Pagrus major*, egg, iodophor, disinfection, bactericidal effect

In Japan, seed production techniques for many species of marine fish have been developed to a commercial level. However, viral and bacterial diseases often occur in larvae reared in hatcheries resulting in significant economic losses.¹⁾ It has been proved that some of the causative agents of these diseases were transmitted vertically from spawners to their offsprings.^{2,3)}

To prevent the vertical transmission of causative agents, prophylactic treatment of the egg surface with antimicrobial agents has been investigated. Iodophor, one of the antimicrobial agent, has been shown to have relatively little toxicity to eggs of several fresh water fishes but to be highly toxic to fish pathogen.^{4–7)} Therefore, especially in salmonids, iodophor disinfection of eggs has been developed and applied widely. However, there have been few studies on the use of iodophor to disinfect marine fish eggs.

On the other hand, it has been reported that treatment with iodophors to disinfect trout and salmon eggs during water hardening has often resulted in increased egg losses.⁸⁾ Also, too much iodophor is toxic to fish eggs.⁹⁾ Thus, the developmental stages and concentrations that are safe and effective are needed to be elucidated.

In this study, we examined the suitable developmental stage and concentration of iodophor, Povidon-iodide, to disinfect the eggs of two marine fish cultured in Japan, the spotted halibut *Verasper variegatus* and the red sea bream *Pagrus major*. Furthermore, we investigated the influence of Povidon-iodide treatment on survival, growth and frequency of deformity of larvae together with the bactericidal effect on the surface of eggs.

Materials and Methods

Eggs

Spotted halibut: Eggs were collected from three-year-old mature fish. Eggs were fertilized artificially and incubated in a 200 l tank supplied with seawater. Only buoyant eggs were used for experiments. The temperature of the seawater was kept at $10 \pm 0.5^\circ\text{C}$ during experiments.

Red sea bream: Eggs were obtained from three- to five-year-old mature fish. Natural spawned eggs were collected and incubated in a 200 l tank supplied with seawater at $18 \pm 1^\circ\text{C}$. Eggs were collected early in the spawning period for rearing experiments and in the middle through to the late spawning period for other experiments. Only buoyant eggs were used for experiments.

Seawater

Sand filtered and UV irradiated seawater of salinity of about 34‰ was used in the experiments.

Conditions for Disinfection

1. Evaluation of developmental stages

Spotted halibut: Disinfection was carried out and evaluated at seven different stages i.e., one hour after fertilization, four cell stage, morula stage, appearance of embryo, appearance of optic and Kupffer's vesicles, appearance of auditory vesicles and beginning of heartbeat, a day before hatching. Approximately 100 buoyant eggs were transferred to a cylindrical steel mesh (volume approximately 150 ml) and placed in an incubator supplied with fresh sea-

water constantly. The eggs were immersed in 1.2 l of seawater in which Povidon-iodide (Isodine for aquaculture; Meiji Seika CO., LTD.) was added to give a final concentration of 50 ppm active iodide at each developmental stage. After 15 minutes, the steel mesh was moved and placed in a 1 l plastic beaker supplied with fresh seawater at the flow rate of 400 ml/minute for 20 minutes to remove Povidon-iodide. The steel mesh was then placed in the incubator again. The day before hatching, eggs were transferred from the steel mesh to a 1 l plastic beaker containing 1 l of seawater and incubated till hatching. Two control groups were set up. One was treated as above but without disinfectant at each developmental stage to investigate the effect of the mechanical stress of the disinfection procedure (treated control). The other was non treated (control). Hatching and normally-hatching rates were measured and compared. Experiments were performed three times with eggs from different lots.

Red sea bream: Iodophor treatment was applied and evaluated at five different stages i.e., morula stage, appearance of embryo, appearance of optic and Kupffer's vesicles, appearance of heart and beginning of heartbeat. The procedures for treatment and analysis were performed as for the spotted halibut eggs.

2. Evaluation of exposure time for red sea bream eggs

A safe exposure time of disinfection was determined with red sea bream eggs at the most suitable stage of development as determined from developmental stage experiments. Eggs were treated with 50 ppm active iodide for 0, 5, 10 and 15 minutes by the procedure mentioned above.

3. Evaluation of iodophor concentrations

Spotted halibut: Safe concentrations of Povidon-iodide were determined in eggs at the most suitable stage of development as determined from developmental stage experiments. Eggs were treated with different Povidon-iodide concentrations i.e., 0, 30, 50, 75, 100 and 150 ppm active iodide. The exposure time was 15 minutes. Disinfection was carried out as already described. Viability of eggs was evaluated starting from the developmental stage at which the experiment began. Therefore, hatching and normally-hatching rates are calculated for post-treatment.

Red sea bream: Eggs were treated with different Povidon-iodide concentrations i.e., 0, 50, 100, 200 and 500 ppm for five minutes. The treatment time was determined by exposure time experiments. The procedures for treatment and analysis were as already described.

4. Bactericidal effect

Spotted halibut: Eggs (approximately 20,000 eggs) were transferred to a cylindrical steel mesh (diameter of 34 cm × depth of 18 cm) and treated with iodophor under the conditions determined by developmental stage and Povidon-iodide concentration experiments. The eggs were disinfected at a density of 2,000 eggs/l and agitated gently every minute during disinfection. After the treatment with Povidon-iodide, the eggs in the steel mesh were moved to a 30 l tank supplied with seawater at a flow rate of 10.5 l/minute for 20 minutes. Fifty treated eggs were sampled and incubated in 5 ml of brain heart infusion (BHI) medium (Nissui pharmaceutical CO., LTD.) supplemented with 1% NaCl at 25°C for 24 hours with continuous agitation. Subsequently, optical densities of cultured medium

were measured at 600 nm. Two controls were set. One was treated by the same procedure but without disinfectant (treated control) and the other was un-treated (control). Each was performed in triplicate and values were compared.

Red sea bream: Approximately 300,000 eggs were treated under the conditions determined by developmental stage, exposure time and Povidon-iodide concentration experiments. The eggs were disinfected at a density of 10,000 eggs/l. The procedures for treatment and analysis were as for the spotted halibut eggs.

Rearing of Larvae Derived from Disinfected Eggs

Spotted halibut: Approximately 2,100 eggs at the beginning of the heartbeat stage were disinfected with 75 ppm active iodide for 15 minutes at a density of 2,000 eggs/l. Approximately 2,000 eggs were transferred to a 100 l plastic tank for rearing and about 100 eggs to a beaker containing 1 l of seawater to calculate hatching and normally-hatching rates. Non treated eggs served as a control. Experiments were performed twice with eggs from different lots; group A fertilized on January 24, 1996 and group B fertilized on January 26, 1996. Experiments were carried out for 80 (group A) or 100 (group B) days. Water was supplied at a flow rate of 55–550 ml/minute throughout the experiment. Water temperature was 12–17°C during the experiment. The tanks were cleaned using a siphon system every two days. The larvae were fed *Artemia* nauplii from two days after hatching to the end of the experiment. From 50 days after hatching, larvae were also fed an artificial diet, 700–1000 µm in diameter. Survival, total length and frequency of deformity were measured at the end of the experiment.

Red sea bream: Approximately 600,000 morula stage eggs were disinfected with 100 ppm active iodide for five minutes at a density of 10,000 eggs/l. Approximately 200 eggs were transferred to a beaker containing 1 l of seawater. The others were transferred to a 30 t concrete tank for rearing test and at 50 days after hatching, they were transferred to an open-sea net-pen. Non treated eggs served as a control. The experiment was carried out over 93 days. No water supply was given during the first two post-hatching days after which the flow rate was gradually increased to 6.7 t/hour till 50 days after hatching. Water temperature was 17–20.5°C during the experiment. The larvae were fed rotifer from three to 45 days after hatching, *Artemia* nauplii from 26 to 50 days after hatching and an artificial diet from 12 days after hatching to the end of the experiment. Survival rate and total length were measured periodically and frequency of deformity was measured at the end of the experiment.

Statistical Analysis

As the quality of eggs used for the experiments of evaluation of developmental stages of spotted halibut were unstable, the data were tested by χ^2 analysis between the treated group and control at each development stage for each trial. For other experiments, only the eggs of good quality (more than 75% cleavage rate) were used and the data of the treated group was calculated as relative value from the control which was corrected to 100. These data were transformed to radian with arcsine transformation and tested

by ANOVA to determine significant differences between the experimental groups. In the rearing experiments, data of hatching and normally-hatching rates, survival rate and occurrence of deformity were tested by χ^2 analysis and data of growth were tested by t test to determine significant differences between the experimental groups. A probability level of $p \leq 0.05$ was considered significant.

Results

Conditions for Disinfection of Spotted Halibut Eggs

There were no significant differences between control and treated control in hatching and normally-hatching rate (Fig. 1). In the case of experiment 1 and 2, when the

percentages of buoyant and cleaved eggs were high, hatching and normally-hatching rates of the group treated with iodophor were not significantly different from the control group. In the case of experiment 3, when the percentages of buoyant and cleaved eggs were low, normally-hatching rates in disinfected eggs at one hour after fertilization and the appearance of optic and Kupffer's vesicles were significantly lower than the control group. However, disinfected eggs at the four cell stage, morula stage, appearance of embryo, appearance of auditory vesicles and beginning of heartbeat were not significantly different from the control group.

To investigate the tolerance of eggs for iodide, eggs at the beginning of heartbeat were treated with 30–150 ppm active iodide for 15 minutes. There were no significant differences between the control (0 ppm) and groups treated with 30, 40, 50 and 75 ppm active iodide in hatching and normally-hatching rates. However, hatching and normally-hatching rates of groups treated with 100 and 150 ppm active iodide were significantly low (Fig. 2).

No bacterial growth was recognized in eggs treated at 75 ppm active iodide for 15 minutes (Fig. 3).

Fig. 1. Influence of Povidon-iodide treatment on hatching at each stage in spotted halibut eggs.

Hatching and normally-hatching rates were determined from buoyant eggs after fertilization. Eggs were treated with 50 ppm active iodide for 15 min. Experiments were done three times and each bar represents the value for each developmental stage. Significant differences from the control values are indicated by * ($p < 0.05$). Developmental stages are described as: A; one hour after fertilization, B; four cell stage, C; morula stage, D; appearance of embryo, E; appearance of optic and Kupffer's vesicles, F; appearance of auditory vesicles, G; beginning of heartbeat a day before hatching.

Fig. 2. Influence of Povidon-iodide concentration on hatching in spotted halibut eggs when the treatment was applied at the heartbeat stage.

Hatching and normally-hatching rates were measured at the post-heartbeat stage. Eggs were treated for 15 min. Experiments were done three times and values are means and standard deviations. Significant differences from the control values are indicated by * ($p < 0.05$) and ** ($p < 0.01$).

Fig. 3. Bactericidal effect of iodide treatment on bacteria adhering to eggs of spotted halibut.

Values are means and standard deviations of the triplicates for each procedure.

Fig. 4. Influence of Povidon-iodide treatment at stage of red sea bream eggs on hatching.

Hatching and normally-hatching rates were determined from buoyant eggs obtained from natural spawning. Eggs were treated with 50 ppm active iodide for 15 min. Experiments were done three times and values are means and standard deviations. Significant differences from the control values are indicated by * ($p < 0.05$) and ** ($p < 0.01$). Developmental stages are described as: A; morula stage, B; appearance of embryo, C; appearance of optic and Kupffer's vesicles, D; appearance of heart, G; beginning of heartbeat.

Fig. 5. Influence of Povidon-iodide exposure time on hatching of red sea bream eggs when the treatment was applied at the morula stage.

Hatching and normally-hatching rates were determined at the post-morula stage. Eggs were treated with 50 ppm active iodide. Experiments were done three times and values are means and standard deviations. Significant differences from the control values are indicated by * ($p < 0.05$).

Rearing of Larvae Derived from Disinfected Eggs of Spotted Halibut

The hatching and normally-hatching rates of all control group A, B and disinfected group A, B exceeded 98% and 84%, respectively, showing that the quality of eggs used for these experiments was good. The survival rates of control group A and B were 24.3% and 22.0% and those of disinfected group A and B were 29.4%, and 28.9%, respec-

Fig. 6. Influence of each Povidon-iodide concentration on hatching of morula stage red sea bream eggs.

Hatching and normally-hatching rates were determined from morula stage eggs. Eggs were treated with each concentration of active iodide for 5 min. Experiments were done three times and values are means and standard deviations. Significant differences from the control values are indicated by ** ($p < 0.01$).

Fig. 7. Bactericidal effect of iodide treatment on bacteria adhering to eggs of red sea bream.

Values are means and standard deviations of the triplicates for each procedure.

tively, and survival after hatching was higher in the disinfected group ($p < 0.05$). The total length at the end of rearing experiment was 26.7 ± 5.5 mm (mean $\pm$ S.D.) and 36.2 ± 11.6 mm for control group A and B and 26.7 ± 6.1 mm and 37.6 ± 12.3 mm for disinfected group A and B, respectively. There were no remarkable differences in the number or type of deformed fish between the disinfected and control in either group of A and B.

Conditions for Disinfection of Red Sea Bream Eggs

There were no significant differences between control and treated control in hatching and normally-hatching rates. Hatching and normally-hatching rates of eggs treated with iodophor were significantly lower than the control group at all developmental stages. Among developmental stages, the morula stage showed the highest hatching and normally-hatching rates in treated eggs (Fig. 4). As the exposure time was shortened, the hatching and normally-hatching rates increased with no significant differences observed between the eggs treated for five minutes and the control in either rate (Fig. 5).

There were no significant differences between the groups treated with 50–200 ppm active iodide and the control in hatching and normally-hatching rates. However, eggs treated at 500 ppm did not hatch (Fig. 6).

Bacterial growth in BHI medium containing eggs of the

control group and eggs treated with 50 ppm active iodide was observed. On the other hand, no bacterial growth was observed in the eggs treated with 100 and 200 ppm active iodide for five minutes (Fig. 7).

Rearing of Larvae Derived from Disinfected Eggs of Red Sea Bream

Hatching and normally-hatching rates of the control were 97.1% and 92.4% and those of disinfected eggs were 77.7% and 73.0%, respectively. Hatching and normally-hatching rates of disinfected eggs were significantly lower than the control ($p < 0.05$). The survival rate at 31 days was 56.4% for the control group and 35.2% for the disinfected group. At 94 days, the survival rate was 29.9% for the control group and 24.2% for the disinfected group, showing that the survival of larvae derived from disinfected eggs was also low ($p < 0.05$). However, there were no remarkable differences in growth (total length of control group; 52.8 ± 5.0 mm, disinfected group; 55.6 ± 4.22 mm at the end of experiment) and number or type of deformed fish.

Discussion

We have established the safe and effective conditions for Povidon-iodide disinfection of spotted halibut and red sea bream eggs. Ideally, the eggs of spotted halibut should be used at the beginning of the heartbeat stage and treated with 75 ppm active iodide for 15 minutes. In red sea bream eggs, the most successful conditions for disinfection are eggs at the morula stage and treatment of 100–200 ppm active iodide for five minutes. In spotted halibut eggs, under these conditions, the agent did not have any harmful effect on survival, growth and occurrence of deformity after hatching. In the case of red sea bream eggs obtained at the early spawning season, iodophor treatment seemed to influence the survival of the larvae at the early stage of before 31 days after hatching, presumably due to egg quality. However, growth and occurrence of deformity were not influenced.

There are several reports on effective conditions of iodophor for bactericidal and virucidal effects, e.g. viral haemorrhagic septicemia virus at 8 ppm for 2.5 minutes,⁴⁾ infectious hematopoietic necrosis virus at 8 ppm for 0.5 minute,⁵⁾ infectious pancreatic necrosis virus at 10 ppm for 0.5 minute,⁶⁾ *Renibacterium salmoninarum* at 25 ppm for five minutes and *Yersinia ruckeri* at 25 ppm for 0.25 minute.⁷⁾ Considering that the treatment time and iodide concentrations exceeded the conditions reported above and no bacterial growth was observed on the surface of eggs, our conditions for disinfection for both species were effective. Additionally, survival rates were higher in disinfected groups in the rearing experiments of spotted halibut and we suggest that this was due to the bactericidal effect. Disinfection of Atlantic halibut *Hippoglossus hippoglossus* eggs with glutaric dialdehyde or iodophor demonstrated similar results and the authors supposed this is due to the elimination of possibly harmful bacteria on eggs by disinfection.^{10,11)}

In the case of spotted halibut eggs with high percentages of buoyancy and cleavage, hatching and normally-hatching rates of eggs at all developmental stages examined were

not influenced by iodophor treatment. For low percentages of buoyancy and cleavage, hatching and normally-hatching rate of eggs at one hour after fertilization and appearance of optic and Kupffer's vesicles were influenced. All other stages were considered suitable for disinfection, however, eggs at the beginning of heartbeat were chosen due to ease of discriminating the stage. In red sea bream, evaluation of developmental stages was started from the morula stage because the eggs had already reached the morula stage when natural spawned eggs were first collected from the egg collection tank. In regard to red sea bream eggs, the morula stage was most suitable among the five stages. These results show that the suitable stage for red sea bream eggs is more restrictive than that for spotted halibut eggs. It is important to note that there might be a difference between species in available range of developmental stages. Additionally, our results show that morula-stage eggs were tolerant to Povidon-iodide in both species. In marine fish eggs, sensitivity at different developmental stages to iodophor had been investigated in Atlantic halibut.¹⁰⁾ This experiment was performed on 0.8, 5, 15, 30, 45, and 60 day-degrees and the authors suggested that 45 day-degrees were the most suitable for disinfection. On the other hand, Holmefjord and Bolla¹²⁾ have reported that the tolerance for mechanical stress rose above 50 day-degrees and at this stage, embryos undergo blastophore closure. These results suggest that the stage most tolerant of mechanical stress does not always reflect the most suitable stage for iodophor disinfection.

In our results, red sea bream eggs were more sensitive to Povidon-iodide than spotted halibut eggs. However, on shortening of the exposure time of disinfectant treatment to five minutes, hatching and normally-hatching rates attained an acceptable level. It has been reported that yellow snapper *Ocyurus chrysurus* and spotted seatrout *Cynoscion nebulosus* were more sensitive to hydrogen peroxide than red drum *Sciaenops ocellatus*.¹³⁾ There also seems to be a difference in sensitivity to disinfectant between species.

In the rearing of red sea bream eggs obtained early in the spawning period, treatment with iodide had a harmful influence on the survival of the larvae at the early stage. We observed that sensitivities of eggs to iodide changed during the spawning season with eggs at the early spawning season the most sensitive.¹⁴⁾ Eggs obtained at the early spawning season were used for the rearing experiments which may explain the relatively low survival rates. It is important to know the sensitivity of the eggs before applying the disinfectant, especially for mass production. In spotted halibut eggs, disinfection had a harmful influence on hatching when eggs in which the percentages of buoyancy and cleavage were low were used (Fig. 1). Consequently, egg quality is important.

These matters show that safe and effective conditions for disinfection of eggs should be determined separately for each fish species. The strategies outlined in this paper should be useful in determining safe and efficient conditions for disinfection of eggs from other species.

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