Iodine disinfection of grouper *Epinephelus coioides* eggs

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Abstract

In this study, the developmental stage, iodine concentration and exposure time that will reduce the bacterial load without decreasing the hatching rate for the disinfection of grouper (*Epinephelus coioides*) egg were established. Results of the study showed that the best stage to disinfect grouper egg is at late neurula stage where the embryo shows twitching movement. The effective and safe concentration that will reduce bacterial load but will not decrease hatching rate for the disinfection of grouper egg is 7.5 ppm free iodine for 10 min. Total bacterial count of eggs disinfected with 7.5 ppm free iodine for 10 min (8.99×10^2 cfu/20 eggs) is significantly lower than the unrinsed/undisinfected eggs (1.99×10^7 cfu/20 eggs).

Introduction

Grouper, *Epinephelus coioides*, is an important food fish in the Philippines. Seed production of this species needs improvement particularly the poor hatching rate. Causes of egg death during incubation are not fully understood. Many studies have been conducted to investigate the correlation between bacteria and egg death. However, except for some preliminary investigation, no study has been made on grouper. Sauter *et al*. (1987) suggested that presence of *Vibrio*, *Listeria*, *Corynebacterium* and *Staphylococcus* spp. are possible contributors to early developmental mortality in chinook salmon egg. In 1989, Barker *et al*. reported a significant correlation between hatching success and numbers of surface bacteria on rainbow trout (*Onchornchus mykiss*) eggs. Barker *et al*. (1991) reported that significantly higher rate of egg death during early stages of incubation was found among rainbow trout eggs exposed to *Pseudomonas fluoresens* at water hardening.

The use of iodophors is well established as a means of disinfecting fish eggs and for aquaculture use. It has been shown to have little toxicity to eggs of several fish but is toxic to fish pathogens. However, there are also reports on the toxicity of iodophor to fish eggs (Fowler and Banks, 1991). Salvesen *et al*. (1991) reported that doses of Buffodine (iodophor: 1.06% free iodine) that reduced the bacterial load were above the toxic level. However, Hirazawa *et al*. (1999) reported that no bacterial growth was recognized in spotted halibut (*Verasper variegatus*) eggs treated with 75 ppm iodide for 15 min and the hatching rate were not significantly different from the control. The same study reported that no bacterial growth was observed in red sea bream (*Pagrus major*) egg treated with 100 and 200 ppm active iodide for 5 min and there is also no significant difference in the hatching rate of treated eggs and the control. Tendencia (2001) reported that at 15 and 20 ppm I_2, total bacterial count of grouper egg at the cleav-

age and eyed stages are significantly reduced; however, the hatching rate is also significantly lowered at these concentrations. Different fish species and different egg stages have different levels of tolerance to iodophor, therefore it is important to establish the developmental stage and iodine concentration and exposure time that are safe and effective in reducing the bacterial load and at the same time increase the hatching rate of eggs of different fish species.

The aim of this study is to establish the appropriate egg developmental stage as well as the concentration and exposure time for the I_2 disinfection of grouper, *Epinephelus coioides*.

Materials and Methods

Eggs

Grouper (*Epinephelus coioides*) eggs were collected from tank-reared broodstock at the Tigbauan Main Station of the Aquaculture Department of the Southeast Asian Fisheries Development Center in Iloilo, Philippines. Only good eggs which are floating were used in the study.

Establishment of the appropriate developmental stage of disinfection

Disinfection was carried out at six different egg developmental stages- cleavage, early blastula, early gastrula, early neurula, eyed stage and stage where the embryo showed twitching movement. For each developmental stage, approximately 100 eggs were placed in 500 ml seawater with iodine (I_2)(Argentyne; 1% free iodine, Argent) to give a final concentration of 5 ppm, for 15 min. The eggs were then placed in I_2 soaked strainer (20 ppm I_2 for 10 min rinsed in autoclaved seawater) and rinsed with flowing autoclaved (121°C for 15 min) seawater for 20 sec. Rinsed eggs were transferred into sterile flasks with 500 ml autoclaved seawater and provided with aeration where they were allowed to hatch. There were two control groups. One control group (treated control) was treated as in the I_2 disinfected eggs but without the disinfectant to investigate the effect of handling stress brought about by the disinfection procedure, the other was the non-treated control. The eggs were placed directly into the flask where they were hatched and hatching rates were determined for each of three replicates for each treatment.

Establishment of suitable exposure time

The safe exposure time for the disinfection of grouper egg was established using eggs at the appropriate developmental stage. Eggs were treated with 5 ppm I_2 solution for 5, 10, and 15 min. The eggs were disinfected, incubated and hatched as earlier described. There were three replicates for each treatment.

Establishment of the effective iodine concentration

Eggs at the appropriate stage for disinfection were exposed for 10 min to different levels of I_2 solution in triplicate- 0, 0.5, 1.0, 1.5, 2.0, 5.0, 7.5, 10.0, 15.0, and 20.0 ppm- to determine the safe concentration. The eggs were disinfected, incubated, and hatched as previously described.

Investigation of the bactericidal effect

The bactericidal effect of the safe I_2 concentration on the appropriate stage of development was determined. A preliminary investigation to determine the effect of 7.5 ppm I_2 for 5, 10, 15 and 20 min on the bacterial flora of grouper eggs at the late neurula stage

Stage	Hatching Rate (%)		
	I_2 Disinfected	Treated Control	Non-treated Control
cleavage	81.00[a]	52.00[a]	51.33[a]
early blastula	76.33[a]	40.33[ab]	14.00[b]
early gastrula	90.67*[a]	67.67[b]	65.00[b]
early neurula	62.00[a]	50.67[a]	38.00[a]
eyed	80.00[a]	53.00[a]	35.33[a]
twitching	89.33*[a]	87.33[b]	68.00[a]

Table 1. Hatching rate of different developmental stages of grouper (*Epinephelus coioides*) eggs disinfected with 5.0 ppm I_2 for 10 min. *values in the same column are significantly high at P<0.05. Values in the same row with the same superscript are not significantly different (P<0.05)

where the embryo showed twitching movement was done. Approximately 20 eggs were placed in I_2 soaked strainer, soaked in 7.5 ppm I_2 for 5, 10, 15, and 20 min and rinsed in flowing autoclaved seawater for 20 sec. Disinfected and rinsed eggs were homogenized in autoclaved seawater and diluted serially 10 fold. Portions, 0.1 ml, of the diluted homogenate was spread in duplicates onto Zobell's marine agar (ZM)(Aaronson, 1970) to determine the total number of bacteria present, and onto thiosulfate citrate bilesalt sucrose agar (TCBS)(BBL), to determine the presumptive *Vibrio* count. Inoculated plates were counted after incubation at 30°C for 72 and 24 h , respectively. There were three replicates for each treatment.

Viability test and bactericidal effect

The appropriate egg developmental stage, I_2 concentration and exposure time based on the hatching rate and bactericidal effect were used in this study. For the viability test, the eggs were disinfected, rinsed and incubated until hatched as previously described. For the bactericidal effect, the eggs were disinfected, rinsed and processed as described earlier. Ten spawning batches with three replicates each were processed for this study.

Statistical Analysis

Results were analyzed using the Analysis of Variance (ANOVA) and Duncan's multiple range test (DMRT) using the SPSS program. Correlation between the hatching rate and the bacterial count was analyzed using scatter graph. Bacterial levels were log transformed before analysis. Likewise, all values expressed in percent were arcsine transformed.

Results

The hatching rate of grouper egg disinfected at early gastrula (90.67%) and late neurula when the embryo showed twitching movement (89.33%) were significantly higher (P<0.05) than those disinfected at other stages (Table 1). Hatching rate of the non-treated control group at the early blastula and early gastrula stage was significantly lower than the I_2 treated eggs. Results also showed that there was no significant difference (P>0.05) in the hatching rate of grouper eggs exposed to 5 ppm I_2 for 5 (92%), 10 (97%) and 15 (94%) min.

Table 2 shows the hatching rate of eggs at the stage where the embryo showed twitching movement, exposed to different I_2 concentra-

Conc. (ppm)	HR (%)
0	40.00
0.5	55.00
1.0	74.67
1.5	72.67
2.0	53.00
5.0	78.00*
7.5	78.33*
10	71.00
15	63.33
20	44.67

Table 2. Hatching rate (HR) of grouper, *Epinephelus coioides*, eggs disinfected with different I_2 concentrations. * significantly high at P<0.05

Treatment	Count (cfu/20 eggs)
Non-treated control	1.00×10^{7d}
Treated control	7.36×10^{4c}
I_2 treated for:	
5 min	3.89×10^{2a}
10 min	1.51×10^{2a}
15 min	1.51×10^{3ab}
20 min	9.70×10^{3bc}

Table 3. Bacterial count of grouper (*Epinephelus coioides*) eggs disinfected with 5.0 ppm I_2 at different exposure time. Values in the same column with the same superscript are not significantly different (P>0.05)

tions for 10 min. Results showed that the hatching rate of eggs disinfected with 5 (75%) and 7.5 (75.33 %) ppm I_2 were significantly higher (P<0.05) than those exposed to other concentrations.

Bacterial load of eggs exposed to 7.5 ppm I_2 for 5 (3.89×10^2 cfu/20 eggs) and 10 min (1.51×10^2 cfu/20 eggs) were significantly lower (P<0.05) than the other treatments (table 3). Bacterial level of eggs exposed to 7.5 ppm I_2 for 20 min (9.7×10^3 cfu/20 eggs) is not significantly different from the treated control (7.36×10^4 cfu/20 eggs)(table 3).

Further investigation showed that the total bacterial count of grouper eggs disinfected with 7.5 ppm I_2 for 10 min (8.99×10^2 cfu/20 eggs) was significantly lower than the treated control (9.38×10^4 cfu/20 eggs) which was significantly lower than the non-treated control (1.99×10^7 cfu/20 eggs). No presumptive *Vibrio* spp. were recovered from the disinfected eggs, however, this is not significantly different from the treated control (1.02×10^1 cfu/20 eggs) but was significantly lower than the non-treated control (4.26×10^2 cfu/20 eggs). Hatching rate of disinfected eggs (92.8%) was higher than, but not significantly different from, the treated control (88.27%) nor from the non-treated control (85.77 %)(Table 4). No correlation existed between the hatching rate and the bacterial level.

Discussion

Results of the study showed that grouper, *Epinephelus coioides*, eggs can be disinfected with iodine at the early gastrula and at the late neurula stage where the embryo shows twitching movement. However, the latter was chosen because of the ease in collecting the eggs, early gastrula takes place at night while the stage where the embryo shows twitching movement takes place in the morning. Furthermore, Caberoy and Quinitio (1998) reported that egg viability, hatching rate and percentage of normal larvae are lowest in eggs collected and handled at the early cleavage through gastrula stages. However, results of this study showed that disinfection procedures significantly improved the hatching rate of eggs at the early blastula and early gastrula stages but not at the other developmental stages, thus, in grouper, iodine disinfection influences the survival of the embryo during the early stages (i.e. early blastula and early gastrula) of egg development. Results also showed that stress brought about by handling during disinfection did not adversely affect grouper eggs at all stages. This is in contrast with the observations of Ramnarine (1996) in cascadu eggs that the critical periods for handling eggs are the cleavage and early embryo phase. Similarly, Piper *et al*. (1982) reported that stages preceding the eyed stages are generally considered critical and the eggs should not be disturbed. In spotted halibut, hatching rate of eggs at all developmental stages

Treatment	Bacterial count (cfu/20 eggs)		
	Total	Presumptive Vibrio	Hatching rate (%)
Disinfected	8.99×10^{2a}	0^{a}	92.80^{a}
Treated Control	9.38×10^{4b}	1.02×10^{1a}	88.27^{a}
Non-treated Control	1.99×10^{7c}	4.26×10^{2b}	85.77^{a}

Table 4. Bacterial count and hatching rate of eggs treated with 7.5 ppm I_2 for 10 min compared with the treated and non-treated control. Values in the same column with the same superscript are not significantly different (P>0.01)

were not influenced by iodophor treatment (Hirazawa *et al*., 1999).

Exposure time did not affect the hatching rate of grouper egg. This is contrary to Douillet and Holt (1994) who reported that the time of egg exposure to hydrogen peroxide should not be extended beyond 5 min to avoid increased mortality. Although exposure time had no effect on the hatching rate of grouper eggs, it affected the bacterial load. Bacterial level in grouper eggs exposed to 75 ppm I_2 for at least 15 min was significantly higher than those exposed for 5 or 10 min. This may be explained by breakown of I_2 over periods in excess of 10 min.

Bacterial count of all bacterial taxa among the I_2 treated, treated control and non-treated control groups were significantly different. Highest bacterial count was observed among those which were not treated with either autoclaved seawater or I_2. Results also showed that rinsing with autoclaved seawater was not effective in significantly reducing the total bacterial count. No presumptive *Vibrio* bacteria were recovered from eggs disinfected with 7.5 ppm I_2. This implies that 7.5 ppm I_2 is effective in reducing the bacterial load of grouper eggs. Hirazawa *et al* . (1999) reported that no bacterial load was recognized in spotted halibut eggs treated with 75 ppm I_2 for 15 min. In contrast, Salvesen *et al*. (1991) observed that 100 to 200 ppm free iodine had no effect on the bacterial load of plaice eggs.

Although the bacterial load of grouper eggs disinfected with 7.5 pm I_2 for 10 min was significantly lower than the controls, the hatching rate of I_2 treated eggs was not significantly different from the treated control and the non-treated control. This implies that in grouper eggs, hatching rate is not affected by the bacterial load.

Results of this study showed that the best stage to disinfect grouper eggs was at late neurula stage where the embryo shows twitching movement. The effective (indicated by reduced bacterial load) and safe (indicated by no effect on hatching rate) concentration for iodine disinfection of grouper eggs would appear to be 7.5 ppm for 10 min. Disinfection with 7.5 ppm I_2 for 10 min significantly lowered the bacterial load. These results indicate that iodine can be used as a prophylactic treatment and form a hygiene barrier between the broodstock and the larvae. Further studies are needed to evaluate the efficacy of I_2 against viruses.

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