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Effect of iodine disinfection on the bacterial flora and hatching rate of grouper, *Epinephelus coioides* eggs at the cleavage and eyed stages

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Abstract
In this study, the effect of iodine disinfection on the bacterial flora and hatching rate of grouper egg at two different stages of development were investigated. The eggs (at cleavage and eyed stages) were soaked for 10 min in sterile seawater (control) and at different free iodine concentrations- 2.5, 5, 10, 15 and 20 ppm. Total bacterial and presumptive Vibrio count, as well as the hatching rate of the treated eggs were determined. Results showed that Vibrios are eliminated by iodine disinfection (2.5- 20 ppm) but not by rinsing with sterile seawater. The total bacterial load and hatching rate of eggs decreased as the iodine concentration increased. Iodine concentrations of 15 and 20 ppm were effective in significantly reducing the total bacterial load of grouper egg at both the cleavage and eyed stages. However, at these concentrations the hatching rates were also significantly lower. Results also showed that grouper, *Epinephelus coioides*, eggs tolerate stress better at the eyed stage than at the cleavage stage.

Mass mortalities due to infectious and non-infectious diseases have often occurred in larvae reared in hatcheries (Muroga, 1995). Schachte (1979) reported that bacteria may be responsible for some mortalities of fish eggs incubated in hatchery tanks. According to Barker *et al*. (1989) and Sauter *et al*. (1987), bacteria can influence egg survival. Ogbondenum (1994) and Fernandez *et al*. (1996) reported that the bacterial flora of eggs during incubation reflects those of the environment but could not be correlated with egg mortality.

In the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC AQD) in Tigbauan, Iloilo, Philippines, opaque spawned eggs of grouper, (Epinepheluis coioides) in cage-reared broodstock are encountered while opaque and multicolored eggs are often observed in tank-reared fish, and could be one of the reasons for the poor hatching rate. Many studies on the use of different antimicrobials to treat fish eggs to improve hatching rate have been reported (Harboe *et al*., 1994; Hirazawa *et al*., 1999). Iodine, one of the disinfectants has been shown to have relatively little toxicity to eggs of several fishes but is highly toxic to fish pathogens such as bacteria (MacFadden, 1969) and viruses (Goldes and Mead, 1995; Batts *et al*., 1991). This study aims to investigate the effect of different iodine concentrations on the hatching rate and bacterial flora of grouper egg.

Grouper eggs that are in the early cleavage (stage where the egg divide into smaller cells; 30 min after fertilization) and eyed (stage where the optic vessicles are developed; 12 h after fertilization) stages were collected from tank-reared broodstock at SEAFDEC AQD.

Egg stage	Iodine concentration (ppm)	Bacterial count (cfu/20 eggs)*		Hatching rate (%)*
		Presumptive *Vibrio*	Total bacteria	
Cleavage	0	5.8×10^{1}	6.66×10^{4} cde	73.5 bc
	0.25	0	1.36×10^{4} bcde	80.2 bc
	5	0	3.04×10^{3} bcd	74.5 bc
	10	0	1.95×10^{3} bc	72.9 bc
	15	0	7.24×10^{2} ab	60.5 ab
	20	0	2.95×10^{2} ab	43.9 a
Eyed	0	7.16×10^{1}	1.35×10^{5} cde	94.2 c
	0.25	0	3.72×10^{5} e	92.0 c
	5	0	1.48×10^{5} cde	90.9 c
	10	0	1.99×10^{5} de	83.9 bc
	15	0	4.57×10^{3} ab	84.5 bc
	20	0	5.80×10^{1} a	68.3 bc

Table 1. Bacterial count and hatching rate off eggs disinfected with different iodine concentrations for 10min. *Mean of three spawning batches. Values in the same column with the same superscripts are not significantly different (P>0.01). cfu = colony forming units, ppm = parts per million.

Only the fertilized eggs, which are floating and transparent (Hussain and Higuchi, 1980) were processed for bacterial count and viability test. Eggs from three spawnings were processed in this study.

To determine the effect of Iodine on the hatching rate, one hundred eggs were placed in aerated beakers with 500 ml iodine (Argentyne) solution with the following concentrations- 2.5 ppm, 5 ppm, 10 ppm, 15 ppm and 20 ppm (free I_2). The eggs were soaked in the iodine for ten minutes and transferred to an iodine disinfected strainer (20 ppm for 10 min rinsed in sterile seawater for 30 sec) and rinsed with flowing autoclaved (121 ^{O}C for 15 min) seawater for 30 seconds to remove the disinfectant. For the control (0 ppm I_2), the eggs were aerated in 500 ml autoclaved seawater and rinsed with flowing autoclaved seawater as in the disinfected eggs. Rinsed eggs were placed in sterile flasks with 500 ml autoclaved seawater provided with aeration. The eggs were incubated until hatched and the number of eggs that hatched were counted. There were three replicates for each treatment.

To determine the effect of the different iodine concentrations on the microflora of grouper eggs, five replicates of 20 eggs each were soaked in different free iodine concentrations (2.5, 5, 10, 15, 20, ppm for 10 min) in a petri dish. The eggs were then transferred into an iodine disinfected strainer (20 ppm for 10 min) and rinsed with autoclaved seawater as described above. The control was also processed as described. The control and the disinfected and rinsed eggs (20 pcs) were homogenized in autoclaved sterile seawater (1ml) in sterile test tubes using a sterile glass rod using and five serial ten-fold dilutions were made. Aliquots, 0.1 ml, of each dilution were plated in duplicate onto Thiosulphate Citrate Bile salt Sucrose Agar (TCBS)(BBL), a presumptive Vibrio medium, and Zobell's medium

(ZM)(Aaronson, 1970), a general medium. Colonies were counted after 24 h and 72 h incubation at 35 ^{O}C, respectively.

Results were analyzed using analysis of variance (ANOVA) and Duncan's Multiple Range Test (DMRT) and correlation analysis using the Statistical Program for the Social Sciences (SPSS).

Presumptive *Vibrio* spp were recovered from the sterile seawater rinsed eggs and not from eggs that were disinfected with the I_2 solutions (2.5- 20 ppm)(Table 1). This implies that the presumptive Vibrio recovered in these two stages are found on the egg surface only and I_2 disinfection (2.5 - 20 ppm) is effective in reducing the number of *Vibrio* spp. on the egg surface. Table 1 shows the bacterial level and hatching rate of grouper eggs disinfected with different iodine concentrations for 10 minutes. The bacterial level was lowest in eggs disinfected with 20 ppm. I_2. Generally, it is observed that as the I_2 concentration increases the bacterial count decreases, as does the hatching rate. Results showed that I_2 disinfection reduced the presence of bacteria on grouper eggs and reduced bacterial growth was observed in eggs disinfected with higher I_2 concentrations (Table 1). This suggests that iodine was effective in disinfecting grouper eggs. At the cleavage stage bacterial level in eggs disinfected with 15 and 20 ppm I_2 was significantly lower than the control. However, disinfection with 15 and 20 ppm I_2 also significantly decreased the hatching rate ($P<0.01$)(Table 1). This shows that the use of 15 and 20 ppm I_2 for 10 min is not practical for disinfection of grouper eggs at cleavage stage. This is similar to the observation of Hirazawa *et al*. (1999) in spotted halibut eggs, where hatching in eggs disinfected one hour after fertilization was significantly lower than the control group. At eyed stage, disinfection of *E. coioides* eggs with 20 ppm I_2 significantly lowered the bacterial count ($P<0.01$). However, the hatching rate was also significantly lower than the control ($P<0.01$).

Fowler and Banks (1990) reported that adverse effect of iodophor treatment in fall chinook salmon eggs was most evident at the eyed stage of egg development. In red sea bream eggs, bacterial load of those treated with iodophor was significantly lower than the control group at all developmental stages although, it is at the morula stage where the highest hatching rate was observed (Hirazawa *et al*., 1999). Results also showed that there was a significant difference in the hatching rate of eggs disinfected with different iodine concentrations at the cleavage and eyed stage ($P<0.01$). Higher hatching rate was observed in the eyed stage. This suggests that eggs can tolerate mechanical stress better at the eyed stage. Holmefjord and Bolla (1988) reported that Atlantic halibut eggs can tolerate mechanical stress after blastopore closure. Bacterial levels in eggs at the cleavage and eyed stage were not significantly different ($P>0.01$) although those at the eyed stage did appear to have a slightly higher bacterial load. A similar observation was reported by Barker *et al*. (1989), who reported that bacterial numbers increased with progressive incubation periods.

Results showed that a correlation exists between the bacterial count and I_2 concentration at both the cleavage and the eyed stage. At the cleavage stage, the hatching rate was correlated with the I_2 concentration but not with

the bacterial level. At eyed stage, hatching rate was not correlated with the bacterial count or with the I_2 concentration. This again shows that eggs at the eyed stage are more tolerant to stress.

This is the first report on the disinfection of grouper, *E. coioides* eggs, therefore, the results reported here serve as baseline data for future research on the treatment of grouper egg to improve hatching rate and decrease bacterial load. Further studies are needed to establish the appropriate egg developmental stage and effective concentration for the disinfection of *E. coioides* eggs, taking into consideration the hatching rate and bactericidal effect.

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