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Bacterial microbiota of eggs from cage-reared and tank-reared grouper, *Epinephelus coioides*

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ABSTRACT

At SEAFDEC AQD, opaque spawned grouper eggs are observed during collection in cage-reared brood stock; while opaque and multi-colored eggs are often observed in tank-reared fishes. This study aimed to investigate the occurrence of these opaque and multicolored eggs and at the same time to compare the bacterial microbiota of eggs from brood stock reared in cages, to those from fish reared in concrete tanks. Grouper eggs from brood stocks reared in cages and tanks were processed for bacterial count and identification. Results showed that total bacterial count (on MA) and presumptive *Vibrio* count (on TCBS) of eggs from brood stock reared in concrete tanks were lower than those from cage-reared fishes. Aeromonads (for tank-reared) and Pseudomonads (for cage-reared) were the dominant bacteria in the good eggs; while Vibrios were dominant in the bad eggs for both egg sources. Total bacterial count of the egg-incubating medium from the brood stock tanks (10^4 cfu/ml) was lower than the total bacterial count of water from the cages (10^7 cfu/ml). Presumptive *Vibrio* counts of water from the tanks (10^2 cfu/ml) were lower than those from the cages (10^6 cfu/ml). The Aeromonads dominated the water from the tanks; while Vibrios dominated those from the cages. Good eggs that did not hatch, turned yellow after 3 days, and pink after 5 days.

Grouper, *Epinephelus coioides*, is an important food fish in Southeast Asia. At the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC AQD) in Iloilo (Philippines), groupers are spawned in concrete tanks and in floating net cages. Opaque spawned grouper eggs are observed during collection in cage-reared brood stock; while opaque and multi-colored eggs are often observed in tank-reared fishes. This study compares the bacterial microbiota of eggs from brood stock reared in cages and those from fish reared in concrete tanks and investigates the occurrence of multi-colored eggs.

Grouper, *Epinephelus coioides*, eggs at the late neurula stage (20h after fertilization) used in this study were spawned from grouper brood stock reared in cages at the Igang Marine Substation (IMS) and in concrete tanks at the Tigbauan Main Station (TMS) of the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC AQD) in Iloilo, Philippines. Water temperature in the cages ranged from 27°C to 29°C. Salinity ranged from 33 to 34 ppt. In

the tanks, temperature ranged from 27°C to 29°C and salinity from 32 to 33 ppt. Eggs spawned from IMS were transported to TMS (2h travel time) 18 h after spawning.

A total of 10 spawning periods of grouper egg from IMS and 9 from TMS were processed for bacterial analysis. There were five replicates for each spawning period.

Good (transparent and floating) and bad eggs (colored, opaque, settled) were separately processed to compare their bacterial load. Each type of egg was processed in two ways to compare the number of external and internal microbiota.

External microbiota. Egg samples (20 pcs) were placed in iodine-disinfected strainer and rinsed with flowing autoclaved seawater (121°C)(SSW) for 20 sec to remove any detritus or loosely adhered bacteria. Washed eggs were homogenized in SSW and serially diluted 10-fold until 10^{-6}. Portions, 0.1 ml, of each dilution were plated in duplicates onto 2261E Zobell's Marine Agar (MA)(Aaronson, 1970) and thiosulfate citrate bilesalt sucrose agar (TCBS)(BBL). Colonies were incubated at 30°C and counted after 72 h and 24 h, respectively.

Internal microbiota. Eggs samples (20 pcs.) were place in iodine-disinfected strainer and soaked in 20 ppm free iodine for 15 min (Barker et al., 1989). Disinfected eggs were rinsed with flowing SSW for 20 sec to remove any trace of the disinfectant. Eggs were homogenize and processed as in external microflora.

Water samples. Samples of the egg incubating water (100 ml) were also collected using autoclaved bottles. The sample was serially diluted in SSW and processed as for the egg samples.

Representative bacterial colonies were selected from MA from all treatments. Isolates were purified on nutrient agar, an all purpose medium which supports the growth of a wide range of bacteria, and identified using conventional biochemical tests methods described by MacFaddin (1990). Isolates were identified to genus level based on Bergey's Manual (Holt et al., 1993).

To investigate the occurrence of multicolored eggs, disinfected and not disinfected good eggs were incubated for five days. Eggs used in this study were taken from tank-reared brood stock at the neurula stage. Disinfected (20 ppm free iodine for 15 min) and not disinfected good eggs (125 pcs; Duray et al., 1997) were placed in sterile flasks with 500 ml SSW provided with aeration. Hatched larvae were counted and removed 42 h after spawning (to ensure that all viable eggs has hatched). Unhatched eggs were left in the flask and monitored daily for 5 days. Nine batches of eggs with three replicates each were processed for this study.

Table 1 shows the result of the bacterial count. Total bacterial count (on MA) and presumptive *Vibrio* count (on TCBS) of the incubating water were higher in samples from the cages than in samples from the tanks. Rearing tanks at the TMS have a recirculation water system which continuously dilutes the incubating water by replacing the water with freshly filtered re-circulated water; and removes suspended organic matter which could result in a lower bacterial count. In the natural envi-

	Cage*	Tank*
All kinds of bacteria		
EW	1.8×10^7	3.7×10^4
DG	8.4×10^1	2.1×10^0
NG	9.8×10^3	3.6×10^3
DB	3.3×10^5	1.8×10^5
NB	1.7×10^6	2.3×10^7
Ext(GE)	9.7×10^3	3.6×10^7
Ext(BE)	1.4×10^6	2.3×10^7
Presumptive Vibrio		
EW	1.1×10^6	7.4×10^2
DG	3.6×10^0	0
NG	1.5×10^1	2.3×10^0
DB	3.7×10^2	5.3×10^0
NB	2.6×10^4	3.5×10^2
Ext(GE)	1.1×10^1	2.3×10^0
Ext(BE)	2.6×10^6	3.4×10^2

EW: Egg incubating water
DG: Disinfected (with 20 ppm I_2) good eggs; internal microbiota
NG: Not disinfected good eggs
DB: Disinfected (with 20 ppm I_2) bad eggs; internal microbiota
NB: Not disinfected bad eggs
Ext(GE): External microbiota of good eggs; NG minus DG
Ext (BE): External microbiota of bad eggs; NB minus DB
*: Cfu/ml for the incubating water; cfu/20eggs for the egg samples

Table 1: Bacterial count of the different egg samples.

ronment or in cages, bioturbation and the presence of other cages in the area may contribute to tan increase in the bacterial load.

Total bacterial count of egg samples from the two egg sources is generally comparable. Both internal and external microbiota of the bad eggs is greater than those of the good eggs for the two sources. This suggests that bacterial colonization adversely affect grouper eggs. Death of eggs is probably the result of a complex relationship among the embryonated egg, its microbiota and the water (Bell et al., 1971). However, Kjørsvik et al. (1990) reported a negative correlation between bacterial colonization and the physical strength of fish eggs. There is also no apparent correlation between the number of bacteria and egg loss among turbot (Omnes et al., 1993) and milkfish (Fernandez et al., 1996).

Presumptive *Vibrio* count of eggs was highest in samples from the cages. This is because Vibrios are abundant in water samples from the cages. The microbiota of the seawater may also influence the composition of the egg epibionts (Olafsen, 2001). Good eggs, from both sources have lower internal and external *Vibrio* count than the bad ones. This suggests that Vibrios are dominant in dead aquatic organisms.

Results of the bacterial identification (table 2) showed that Vibrios are the dominant microbiota of the incubating water medium from the cages, which supports the results of the bacterial count. Pseudomonads dominated the internal microbiota of the good eggs from the cages. These results suggest that in large bodies of water such as were the cages, the microbiota of the water does not always affect those of the egg. This is in contrast with Fernandez et al. (1996) who reported that in milkfish egg, aerobic microbiota is largely influenced by those in the incubating water even in cages. In samples from the tanks, *Aeromonas* was the dominant bacteria in both

		Percentage Recovery				
		DG	DB	NG	NB	EW
Vibrio	Tank	17	23	15	11	9
	Cage	38	51	35	45	41
Pseudomonas	Tank	0	6	26	16	9
	Cage	50	30	32	30	12
Aeromonas	Tank	83	26	39	27	32
	Cage	4	0	0	3	12
Other bakteria	Tank	0	45	20	47	50
	Cage	8	17	33	22	24

DG: Disinfected (with 20 ppm I_2) good eggs. DB: Disinfected (with 20 ppm I_2) bad eggs.
NG: Not disinfected good eggs. NB: Not disinfected bad eggs. EW: Egg incubating water.

Table 2. Percentage composition of bacteria in the different samples.

the good eggs and its incubating water. Internal microbiota of grouper eggs from tank-reared fishes reflects the normal flora of the incubating water. This observation is similar to that of Omnes et al. (1993) among turbot eggs and Fernandez et al. (1996) in milkfish eggs. Fernandez et al. (1996) further reported that *Pseudomonas* was the dominant bacteria of good eggs and in incubating water in samples from both cage-reared and tank-reared milkfish brood stocks.

Results of the study also indicated that the microbial composition of the bad egg reflects those of the environment for both egg sources. This is because the bad eggs are dead and are therefore prone to invasion by bacteria, which are predominant in the environment. Bacteria thrive well on dead organic matter. Presumptive *Vibrio* count in the bad egg is higher than in the good egg. This implies that Vibrios are dominant in dead aquatic organisms. On the other hand, good eggs from cage-reared brood stocks harbor more Pseudomonads, and Aeromonads in tank-reared fishes, which imply that *Aeromonas* and *Pseudomonas* bacteria are the normal microbiota of grouper egg. Pseudomonads are also the normal microbiota of milkfish of egg (Fernandez et al., 1996).

Results of egg incubation showed that the hatching rate of not disinfected eggs (52%) is higher than disinfected (21%) ones. This implies that 20 ppm is not a suitable free I_2 concentration for the disinfection of grouper egg. Results also showed that disinfection does not guarantee that eggs will be bacteria free since bacteria colonized even the good and disinfected eggs that did not hatch. It is also possible that Vibrios present in the egg proliferate faster when the egg dies. *Vibrio* levels of dead disinfected and undisinfected good eggs were comparable. *Vibrio* count of good eggs that did not hatch after the 5 d incubation was 10^6 cfu/20 eggs for both the disinfected and not disinfected. Total bacteria count on MA was 10^8 cfu/egg for the disinfected and 10^{10} cfu/egg for the not disinfected. Unhatched good eggs turned yellow after three days and became pink after 5 days. The same color changes were observed by Alapide-Tendencia and

Dureza (1997) in shrimp infected with *Vibrio parahaemolyticus*. This implies that color changes from normal to yellow to pink are manifestations of *Vibrio* infection in marine organisms.

In summary, this study has shown that good eggs from cage-reared brood stock has higher bacterial load than eggs from tank-reared fishes. Pseudomonads are the normal microbiota of eggs from cage-reared fishes and Aeromonads for those from tank-reared. Vibrios are the dominant bacterial microbiota of the bad eggs from both cage-reared and tank-reared brood stocks. Unhatched disinfected/not disinfected good eggs become yellow in color after 3 days and turn pink after 5 days.

This study is the first report on the microbiota of grouper egg. This study serves as baseline information on the normal bacteria of grouper eggs from cage- and tank-reared brood stocks as well as their incubating water medium.

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