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Effect of β glucan immersion on the survival of mud crab *Scylla serrata* (Portunidae) larvae

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Abstract. This paper evaluates the use of immunostimulant in mud crab larval rearing. Mud crab larvae, stocked at density of 50 pcs L^{-1}, were subjected to continuous immersion in various concentrations of β glucan (30 ppm, 60 ppm and 120 ppm). Set-up with antibiotics (2 ppm oxytetracycline) served as positive control while set-up without antibiotics and without β glucan served as negative control. Administration of β glucan and oxytetracycline followed hatchery protocol. Survival from the first zoea stage (z1) and the second zoea stage (z2) were obtained. Significantly higher survival ($P<0.05$) were obtained in the positive control and 30 ppm β glucan in z1. Survival was highest in the 30 ppm β glucan in z2. This was significantly higher ($P<0.05$) than the rest of the treatments. Immune fatigue might have caused the 60 ppm and 120 ppm β glucan concentrations to become ineffective in improving larvae survival. This paper reports the effective use of immunostimulant in the larval rearing of mud crab from up to z2.
Key Words: Antibiotics, oxytetracycline, immunostimulant, mud crab larvae, larval rearing.

Introduction. Crab larval stages are characterized by rapid development of parts or organs and frequent metamorphosis or molting. These cause the mud crab larvae to be susceptible to pathogenic organisms present in the rearing environment. These pathogens include the bacteria and fungi associated with the use of rotifers as food during the most vulnerable period of development, the first zoea (z1) and second zoea (z2) stages (FAO 2011). While the use of antibiotics in the larval rearing has improved survival, it also poses a problem on food safety, may cause impaired growth of cultured organism and foster the development of drug resistant pathogens when used repeatedly (Bachere 2000; Smith et al 2003; FAO 2011).

An alternative currently being considered is the use of immunostimulants. Immunostimulants are naturally occurring compounds that modulates the immune system by increasing the host's resistance against diseases that in most circumstances are caused by pathogens (Bricknell & Dalmo 2005). Among the immunostimulants currently used in aquaculture, β glucan has one of the most widespread application (Ganguly et al 2010; Ringø et al 2012; Meena et al 2013). β glucans are naturally occurring polysaccharides, with glucose linked by β glycosidic bonds, found in the cell wall of many plants, fungi, bacteria as well as seaweeds (Meena et al 2013). While immunostimulants, particularly β glucan, have long been successfully applied in grow-out culture of crustaceans, there is limited information on its use in the larval rearing phase. Thus, this study evaluates the use of β glucan in the hatchery rearing of mud crab larvae during the most susceptible period of development, the z1 and z2 stages.

Material and Method

Experimental animals. Mud crab larvae were obtained from eye-stalk ablated spawner at the Multi-species Hatchery of the Institute of Aquaculture, College of Fisheries and

Ocean Sciences, University of the Philippines Visayas. These were stocked in plastic containers at a density of 50 larvae L^{-1} of water (100 larvae 2 L^{-1}). Rotifers (*Brachionus sp*) were fed at 10,000 to 15,000 inviduals L^{-1}. *Nannochloropsis* were added at a density of 1 x 10^8 cells L^{-1} to serve as food for the rotifers.

Hatchery protocol. The hatchery protocol for mud crab developed by Quinitio & Parado-Estepa (2003) was followed in the experiment. The application of immunostimulant (MacroGard, β glucan with 60 % purity) was incorporated into the hatchery protocol. Water exchange was done every three to five days, with 50 % of water being replaced, corresponding to crab larval development.

A water bath was used to prevent fluctuations in the temperature of the set-up. Moderate aeration was provided for each container. Filtered, UV treated water was used in the experiment. Salinity of the water was within the range of 28 - 30 ppt. A 12-hour light/dark cycle was maintained in the set up.

Experimental design. The experiment included five treatments: negative control (no β glucan and no antibiotics), positive control (2 ppm oxytetracycline antibiotics), 30 ppm, 60 ppm and 120 ppm of β glucan, respectively. A total of 20 dark-colored containers were used in the experiment (four replicates for each treatment). These containers were randomly distributed in the water bath. β glucan and oxytetracycline were dissolved in one liter of seawater added during the water change. To ensure that β glucan was dissolved in seawater, it was subjected to heavy aeration 30 min. to 1 hr prior to being introduced to mud crab larvae culture basins.

Determination of survival and data analysis. During water exchange, the mud crab larvae were counted. Those alive were retained in the container and the dead were discarded. Data with 100 % survival were converted (100-1/4n, where n = larvae stocked per container) prior to being transformed (arcsine$\sqrt{x}$, where x = percent survival) following the methods of Gomez & Gomez (1984). The survival data were subjected to ONE-WAY ANOVA. Significant differences among treatment means were determined through the Duncan Multiple Range Test (DMRT). SPSS version 16 was used for data analysis.

Results and Discussion. Results of the experiment are shown in figure 1. Data obtained (mean percent survival ± SD) for z1 showed significantly higher survival ($P<0.05$) in the positive control (85.25 ± 10.05 %) and the treatment with 30 ppm β glucan (82.75 ± 6.95 %) when compared with the rest of the treatments (negative control 52 ± 5.94 %, 60 ppm β glucan 52.5 ± 3.42 % and 120 ppm β glucan 60.75 ± 4.75 %) (Figure 1a). Figure 1b shows survival data in z2. Mean survival was highest in the treatment with 30 ppm β glucan (52 ± 2.58 %). This was significantly higher ($P<0.05$) than the rest of the tretments (negative control 33 ± 10.95 %, positive control 35 ± 13.56 %, 60 ppm β glucan 21 ± 6.16 % and 120 ppm β glucan 32.25 ± 2.99 %).

Enhanced z1 survival in treatment with 30 ppm β glucan is an indication of a positive response to immunostimulation. This suggests that the immune system develops early in the crustaceans. Although very little is known regarding the ontogeny of the immune system of crustacean larvae because of their small size (Smith et al 2003), a study on marine fish larvae indicated that immunocompetence develops early at a time around metamorphosis (Skjermo & Vadstein 1999). While fish is a vertebrate, the fish larvae is similar with crustaceans in being dependent on the innate immune system until its adaptive immune system is fully developed (Bricknell & Dalmo 2005). Further, low quantities of β glucan have been shown to activate the immune response in crustaceans (Sritunyalucksana & Soderhall 2005). β glucan elicits this response because the pattern in the microbial cell wall is recognized by the crustacean immune system in the same manner as it recognizes the invading pathogens as non-self compound (Lee & Soderhall 2002).

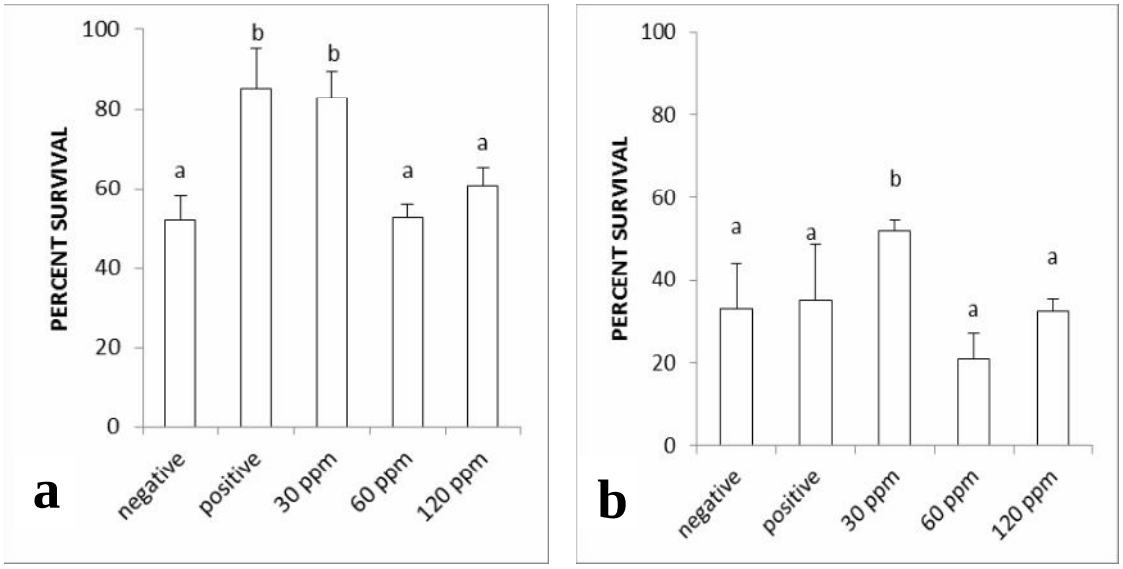

Figure 1. Survival percent (Mean ± SD) of mud crab larvae from (a) first zoea stage (z1) and (b) second zoea stage (z2). Different superscripts indicate significant differences among means (ONE WAY ANOVA, α = 0.05). Negative is the control set-up without β glucan and oxytetracycline addition, positive is the control set-up with 2 ppm oxytetracycline addition.

Results obtained for z2 showed enhanced survival of mud crab larvae only in the treatment with 30 ppm β glucan. The reduced survival in the treatment with higher β glucan concentrations could be due to immune fatigue. The crustaceans, having only the innate immune system, exhibits similar inflammatory response to disparate classes of pathogens (Arala-Chavez & Sequeira 2000).

The general innate immune response in crustaceans involves a cascade of processes and substances are produced intended to eliminate pathogens. The proPO (pro Phenoloxidase) cascade, which performs crucial role in the crustacean immune defense, has built-in checks and balances that ensure that its amplification will proceed if stimulation is appropriate and sustained (Smith et al 2003). However, it has to be controlled and regulated to avoid the deleterious effect of active compounds in the system, and in particular PO, which can produce toxic intermediaries (Lee & Soderhall 2002). There are several proteinase inhibitors of the proPO cascade. In general, crustaceans have a plasma containing alpha 2-macroglobulin- a highly conserved molecule with a characteristic bait region that 'cages' proteases and renders them ineffective (Smith et al 2003). It also serves to restrict damage by self proteases and block the activity of parasite or pathogen proteases. The period in which these are activated in the crustacean larvae is not fully elucidated. Thus, mud crab larvae may not have yet enough of these inhibitors, or are yet to develop these, which limit their capacity to control intense immune response.

Immune fatigue also impairs the ability of mud crab larvae to overcome subsequent infection. After stress, caused by immunostimulation, the defense capabilities are reduced at different level (Le Moullac & Haffner 2000). It would mean that for a short time after antigenic challenge the size of the defense armamentarium is reduced and the ability of the host to deal with any subsequent infection is diminished (Smith et al 2003). The final phase of the innate immune response is the humoral and cellular stage recovery (Bachere 2000). In this phase, the immune system fully recovers and is again ready to respond to pathogen invasion. However, the process of recovery is delayed when there is immune fatigue. From this perspective, it can be added that immune fatigue due to high concentrations of β glucan does not only cause immunosuppression and self damage. Immune fatigue can also render some aspects of the host defense system susceptible to

pathogenic invasion, thereby canceling any beneficial effect caused by such highly immune response.

Conclusions. The study reports the use of immunostimulants in the larval rearing of mud crab larvae. The administration of β glucan at 30 ppm concentration through continuous immersion can enhance survival of mud crab larvae up to z2.

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