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Nile tilapia reared under full-strength seawater: Hemato-immunological changes and susceptibility to pathogens

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ABSTRACT

Co-culture of Nile tilapia, *Oreochromis niloticus*, with marine shrimp has been an increasing farm practice as evidence has suggested that the co-culture is one of a few ways to mitigate shrimp diseases, especially the acute hepatopancreatic necrosis disease that has affected shrimp culture industry worldwide. Since Nile tilapia are basically freshwater species, having them in co-culture with marine shrimp therefore requires a certain degree of salinity adaptation, in which the fish are readily capable to. The present study was aimed at studying certain hemato-immunological changes of the fish reared under elevated salinity, as well as their susceptibility to pathogens. Nile tilapia (180 g initial BW) were reared under gradually increasing salinity within one month from 0 to 30 ppt at 3 ppt/3 d and maintained for another month in 30-ppt salinity. At the end of the 60-day experimental period, 50% of the fish survived with mortality occurred mainly during the second month when the salinity was at 30 ppt. Their growth rate was retarded to 60% of that of the control fish maintained under freshwater (0 ppt). The fish reared under elevated salinity also had significantly lower hematocrit, higher total white blood cell counts, higher absolute numbers of lymphocytes and thrombocytes and higher serum lysozyme activity, compared to those reared under freshwater. Presence of three representative pathogens: infectious spleen and kidney necrosis virus (ISKNV), *Francisella noatunensis* and *Streptococcus agalactiae*; were determined in the fish reared under freshwater and in those reared under elevated salinity, using polymerase chain reaction (PCR) method. Of the three pathogens, only ISKNV was detected in 1/10 of the freshwater and 6/10 of the elevated-salinity fish. In both groups, the virus was present in the gills, brain, liver, gonads, skin, kidney and spleen, with moderately and severely positive reaction observed in the kidney and spleen. The detection of ISKNV was confirmed by *in situ* hybridization of the gonads and kidney, using loop-mediated DNA amplification with digoxigenin, which clearly showed dark-brown stain of the positive signals in the PCR-positive samples. These results suggest that a substantial number of *O. niltoicus* could survive elevated salinity up to full-strength seawater provided that the salinity had been gradually elevated. And under that situation, changes in hemato-immunological functions of the fish did occur, which probably caused lethal proliferation of pre-existing ISKNV that otherwise would remain dormant.

1. Introduction

In recent decades, Nile (*Oreochromis niloticus*) and red (*Oreochromis* sp.) tilapia have become one of the major aquaculture species around the world, at the production of 4.0 million tons yearly, which is only second to that of carp production (FAO, 2012). With their euryhaline characteristics, some species of tilapia prefer freshwater, while others survive well under brackish or even full-strength (30 ppt) seawater. In general, *O. mossambicus* and red tilapia tolerate wide range of salinity, from 0 to 30 ppt, while *O. niloticus* prefer freshwater although they are

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capable to tolerate elevated salinity to a certain extent (Kamal and Mair, 2005; Luan et al., 2008; Schofield et al., 2011).

For many years, Nile and red tilapia have been co-cultured with marine shrimp, especially the black tiger shrimp *Penaeus monodon* and the Pacific whiteleg shrimp *Litopenaeus vannamei*, in shrimp farms (Yi and Fitzsimmons, 2004; Tendencia et al., 2006; Troell, 2009; Withyachumnarnkul, 2013). The fish consume algae, left-over shrimp feed and shrimp carcass; therefore, the purpose of the co-culture practice is to decrease organic matters, resulting in improving water qualities in shrimp ponds. Following an incidence of a devastating disease called acute hepatopancreatic necrosis disease (AHPND) of cultured marine shrimp, it was found that co-culturing the shrimp with tilapia could prevent or lessen severity of the disease (Tran et al., 2013; Withyachumnarnkul, 2013). Therefore, co-culturing tilapia with marine shrimp has since then served a better purpose than what used to be.

As *O. mossambicus* and red tilapia tolerate wide range of salinity, they should have become species of choice to co-culture with marine shrimp. However, the brightly red, pink or orange color of red tilapia attracts birds that could introduce diseases to shrimp ponds; therefore, they are not a preferred choice for many shrimp growers. *O. mossambicus*, on the other hand, grow slowly, thus not appealing to farmers for being the by-product of shrimp farming. In addition, *O. mossambicus* reproduce freely in the pond as no sex selection has been applied to the fish as in Nile and red tilapia, leading to uncontrollable number of the fish in shrimp pond that consume shrimp feed. Therefore *O. niloticus* has become the best choice among the three provided that they could survive and grow normally in elevated salinity.

Previous studies have shown that *O niloticus* could survive in elevated or even full-strength seawater under gradually increasing salinity but growth rate was generally lower than those reared under freshwater (Popma and Lovshin, 1996; Schofield et al., 2011). Osmolarity stress resulting in reduced survival and growth rate has been reported in many fish species, such as in the marine flatfish *Solea senegalensis* or in common seabream *Pagrus pagrus* being reared under low salinity (Arjona et al., 2009; Vargas-Chacoff et al., 2011). Long-term survival rate of *O. niltoicus* in elevated salinity has not been reported, although being suspected to be lower than those being reared under freshwater because of osmolarity stress. This study was therefore aimed at finding out the survival and growth rate of *O. niltoicus* being reared under gradual increase salinity, from 0 to 30 ppt. Since it is likely that the fish under elevated salinity may be defective in their defense mechanisms, certain hemato-immunological parameters were therefore determined. With recent findings that a large proportion of tilapia though appears healthy contain multiple pathogens (Suebsing et al., 2013, 2015, 2016; Pradeep et al., 2016), it is possible that those hidden pathogens that would otherwise reside in the fish without causing diseases may replicate and induce clinical manifestation while being under salinity stress. Therefore, in this study, the presence of certain pathogens in various tissues of *O. niloticus* reared under elevated salinities was also studied and compared to that of the fish reared under 0 ppt.

2. Materials and methods

2.1. Experimental procedure

Nile tilapia *O. niloticus* were stocked in two sets of 2-ton canvas round tanks (2-m diameter × 1-m high). Each set was composed of three replicates; each of which contained 0.8-m deep water and 20 fish (initial BW 180 g). The first set was the freshwater control group, where the fish were reared under 0-ppt water throughout the 60-day experimental period (0-ppt fish), while the second set was the fish reared under elevated salinities, where the water salinity was increased stepwise at 3 ppt/3 d until reaching 30 ppt and the fish were further kept in that salinity level for another 30 days (30-ppt fish). Freshwater was from underground water and seawater from the Gulf of Thailand, being passed through sand-filter and 1-μm cartridge filter before use.

The fish were acclimatized in freshwater for 3 days, with adequate aeration provided by sand stones. Water qualities: dissolved oxygen (DO), total ammonia nitrogen (TAN), total nitrite, alkalinity, pH and temperature, were monitored daily. To ensure optimum conditions for fish rearing, TAN and total nitrite were monitored at < 1.0 ppm and DO at > 4.0 ppm. Water exchange (80%) was performed whenever any of the parameters was not at optimum.

The fish were given commercial pellets (containing 25% crude protein) *ad libitum* (approx. 3.5% biomass/d), three times a day (08.00 h, 13.00 h and 18.00 h) and were individually weighed at every 5 days. As the salinity rose to 18 ppt and above, some fish died. Surviving fish, however, looked healthy, active and fed well. The healthy fish from both groups were randomly sampled, anaesthetized under 0.01 mg L^{-1} of phenoxyethanol, and their blood withdrawn from tail veins for the determinations of hematological parameters. The serum was also isolated for determinations of certain immunological and biochemical parameters. In a separated set of samples, ten 0-ppt fish and ten weak or moribund 30-ppt fish were randomly sampled and internal organs isolated for determination of the presence of pathogens, using polymerase chain reaction (PCR) and *in situ* hybridization methods.

To compare growth rate of the 0-ppt and 30-ppt fish without being bias on stocking density, the 0-ppt fish in the freshwater tanks (C1, C2 and C3 replicates) were taken away from the tanks to make the numbers equal to those of the 30-ppt fish in the respective tanks (E1, E2 and E3 replicates). Therefore, at all time, the number of fish in C1 tank was equal to that in E1 tank, and so on. The taken-away fish were continuously observed in separate tanks and taken into account for recording the survival rate until the end of the experiment.

2.2. Blood preparation for determinations of hematological parameters and serum enzyme levels

Blood, 100 μL, withdrawn from tail vein was immediately mixed with heparin (200 U/mL) and immediately determined for hematocrit (Hct), total red blood cell (RBC) counts, total and differential white blood cell (WBC) counts and mean corpuscular volume (MCV). Hematocrit was determined by placing well-mixed blood into capillary tubes and centrifuged at 12,000 rpm for 5 min, and calculated as percentage of total RBC volume against whole blood volume. The RBC and WBC counts were determined by adding Yokoyama staining solution (Yokoyama, 1947) into the whole blood and placing well-mixed stained blood under Neubauer chamber and counted. Mean corpuscular volume was determined by using the formula: MCV (fL) = (Hct × 10) / total RBC ($\times 10^6$) per μL. WBC differential counts were performed by smearing whole blood onto microscopic slides, stained by Wright–Giemsa and examined under light microscopy for neutrophil, lymphocyte, monocyte and thrombocyte. At least 200 cells were counted for differential WBC determinations. From the total WBC count and percentage of each cell types, absolute counts of neutrophil, lymphocyte, monocyte and thrombocyte were calculated.

For the determinations of serum enzyme levels, 1-mL of blood was withdrawn from the tail vein without anti-coagulant and transferred to 1-mL Eppendorf tube. Serum was isolated by centrifugation at 4,000 × *g* for 10 min and stored at − 20 °C until analysis.

2.3. Serum enzyme levels

2.3.1. Lysozyme activity

Lysozyme activity of serum was determined following the procedures described by Chang et al. (2012) with some modifications. Briefly, 100 μL serum was mixed with an equal volume of 0.3 mg·mL^{-1} suspension of *Micrococcus lysodeikticus* (Sigma-Aldrich, USA) in phosphate buffered saline (PBS). Optical density was measured at 540 nm using a microplate reader (iMark Microplate Reader, Bio-Rad, USA) at