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NOTE

# Strain differences in non-specific immunity of tilapia *Oreochromis niloticus* following challenge with *Vibrio parahaemolyticus*

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ABSTRACT: Red and black strains of tilapia *Oreochromis niloticus* were compared for differences in non-specific immunity. Communally reared, naive fish were challenged with the bacterium *Vibrio parahaemolyticus*. Serum lysozyme, phagocyte activity and differential leucocyte numbers were examined in pre-challenge and post-challenge fish. The percent cumulative mortality in the 2 strains were not significantly different. There was a significant effect of strain on serum lysozyme activity and phagocyte activity. Phagocyte activity increased significantly following the disease challenge. Lymphocyte numbers decreased significantly in the post-challenge sample, while thrombocytes, neutrophils, and monocytes remained unchanged. This study provides the first report of strain differences in non-specific immunity in tilapia.

KEY WORDS: Tilapia · Disease resistance · Non-specific immunity · *Vibrio parahaemolyticus* · Lysozyme · Phagocytic activity · Leucocyte counts

The selection of disease resistant fish strains is one approach to improving the survival of cultured fish. Strain differences in disease resistance have been found for a variety of salmonid diseases (Gjedrem & Aulstad 1974, Bakke et al. 1990, Withler & Evelyn 1990, Ibarra et al. 1991, McGeer et al. 1991). Reports of strain differences in disease resistance of non-salmonid fishes are lacking in the literature. The Nile tilapia *Oreochromis niloticus* is one of the world's most important warm-water cultured fishes in countries such as Malaysia.

Natural selection for enhanced innate, non-specific immunity has been implicated in the evolution of strain differences in disease resistance (Beacham & Evelyn 1992). The objective of this study was to compare the non-specific immune system of red and black strains of the Nile tilapia. These strains of royal (Bangkok Dusit Palace, Thailand) Nile tilapia were chosen for comparison because of their genetic purity (McAndrew & Majumdar 1983). The red colour is an autosomal dominant trait, whereby the development of melanophores is inhibited (McAndrew et al. 1988). The strains of tilapia used in this study were reared under identical environmental conditions; therefore environmental effects are assumed to be negligible. Strain differences in selected components of the non-specific immune system were compared in control and disease-challenged fish. The marine pathogen *Vibrio parahaemolyticus* was used for the disease challenge, to ensure these freshwater fish would be immunologically naive to the bacteria, and therefore allow a more meaningful measurement of non-specific immunity.

**Materials and methods.** ***Fish:*** Approximately 100 fish from each of the communally reared red and black strains of royal tilapia were collected from the Malaysian Department of Fisheries fish breeding station in Jitra, Malaysia. Fish were lightly anaesthetized (40 ppm MS222, tricaine methanesulphonate containing 0.5% NaCl) during transport to the freshwater holding facilities at the Universiti Pertanian Malaysia (UPM). Upon arrival at UPM a prophylactic antibiotic bath (10 ppm furizolidone) was administered to treat a dermal infection caused by abrasions incurred during transport. The tilapia (mean weight 135 g) were placed into a stock tank (29°C) and acclimated for 2 wk. The fish were fed a commercial diet ad libitum.

***Bacterial challenge:*** The challenge bacterium was the Gram-negative pathogen *Vibrio parahaemolyticus*, originally isolated from an infected sea bass *Dicentrarchus labrax* at a cage culture operation in Malaysia (UPM isolate 7/95). The virulence of the pathogen and challenge dose used for this study were determined

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from a preliminary LD50 experiment. Challenge inoculum was prepared in sterile phosphate-buffered saline (PBS, pH 7.2). After 24 h of growth, *V. parahaemolyticus* was aseptically removed from tryptic soy agar (TSA) plates supplemented with 1.5% NaCl. The challenge dose was estimated by measuring the absorbance of the bacterial suspension at 700 nm. To confirm the actual challenge dose, aliquots of the challenge dilutions were inoculated onto TSA plates and the colonies counted after a 24 h incubation at 35°C. The actual challenge dose was then determined as the number of colony forming units (cfu) per ml of PBS.

Following the acclimation period, the tilapia were removed from the stock tank and randomly distributed into 4 tanks as follows: tank 1: 18 red, 18 black; tank 2: 17 red, 17 black; tank 3: 18 red, 18 black; tank 4: 17 red, 17 black. The following day, the fish were anaesthetized (MS222, 200 ppm) and challenged by intraperitoneal (ip) injection. Fish from tanks 1 and 2 (disease group) were injected with $7.35 \times 10^8$ cfu *Vibrio parahaemolyticus* (actual dose) in 1 ml sterile PBS. Fish from tanks 3 and 4 (control group) were injected with 1 ml sterile PBS. Mortalities were collected daily for 1 wk. Each dead fish was necropsied, and kidney and liver tissue cultured onto TSA plates supplemented with 1.5% NaCl. The cause of death was presumed to be *V. parahaemolyticus* if the culture was found to be a growth of Gram-negative rods, sensitive to the vibriostat 0/129 (2,4-diamino-6,7-diisopropylpteridine), and producing green colonies when subcultured onto thiosulfate-citrate bile sucrose (TCBS) agar.

***Sampling:*** A pre-challenge sample (10 red and 10 black) was taken from the stock tank, 1 d prior to the challenge. At 18 to 24 h post-challenge, 7 red and 7 black tilapia were randomly removed from each tank, for a total of 14 fish per strain for each group (disease and control). The remaining 10 or 11 fish in each of the tanks were left and mortality monitored for the following week.

***Blood/serum collection:*** The sampled fish were euthanized in a lethal dose of MS222, weighed and blood taken from the caudal vessel using a syringe. Blood smears were prepared onto cleaned glass slides for differential leucocyte counts. The remaining blood was placed into test tubes, left for 1 h at room temperature, then 4 h at 4°C. The tubes were then centrifuged at $2000 \times g$ at 5°C, for 3 to 4 min. The serum was collected and frozen at –20°C for later analysis of lysozyme activity.

***Assays of non-specific immunity:*** The respiratory burst activity of anterior kidney phagocytes was determined using a modified nitroblue tetrazolium (NBT) assay (Anderson 1992). Briefly, the anterior kidney was aseptically dissected from each fish and placed into 250 µl Leibovitz medium (L-15). The kidney was homogenized and 50 µl aliquots were dispensed into 4 wells on a multiwelled pre-cleaned glass slide. The slides were incubated in a moist chamber at 25°C for 30 min. Non-adherent cells were gently rinsed off the slide with PBS. NBT (0.2% in 0.85% sterile saline) was dropped (approx. 50 µl) onto each well, and the slide incubated for 1 h in the moist chamber. After incubation, a coverslip was placed over the slide and the slides examined under oil immersion (1000×). A total of 200 adherent phagocytes were counted per sample (50 per well), and the proportion of blue (i.e. active) phagoctyes of the total was calculated.

The lysoplate method was used to determine serum lysozyme activity (Osserman & Lawlor 1966, modifications by Lie et al. 1986). Lysozyme activity was expressed as µg hen egg white lysozyme per ml serum. The serum samples were assayed in triplicate, and the mean used for all statistical analyses.

Blood smears prepared from fresh blood were air dried, fixed in 95% methanol for 10 min, and stored at 5°C. The slides were stained in May-Grunwald (0.25% w/v in methanol) for 10 min, and 15 min at half strength (diluted with deionized distilled water, $ddH_2O$). They were then counter-stained in Giemsa (1:10 in $ddH_2O$) for 30 min. The slides were rinsed in $ddH_2O$ for 5 min and left to air dry. The slides were examined under oil immersion (1000×). Approximately 100 leucocytes were counted and classified as lymphocytes, thrombocytes, neutrophils and monocytes. The relative percent of each leucocyte type was calculated.

***Data analysis:*** Student *t*-tests were performed to compare the pre-challenge data with the control, saline challenge data. Analysis of variance (2-way ANOVA) tests were used to examine strain (red and black strains) and disease (control and disease groups) effects (Sokal & Rohlf 1981). Student-Newman-Keuls multiple comparisons tests were used to identify which groups were different. The proportion data (NBT and leucocyte counts) were arcsin square-root transformed and lysozyme data were log transformed, before the above analyses were carried out. Percent cumulative mortality for the 2 strains were compared using Fischer's exact test. Statistical significance was noted for all tests where $p < 0.05$.

**Results and discussion.** This study documents the first report of significant strain-related differences in non-specific immunity in Nile tilapia.

Mortality began to occur in the disease group shortly after the post-challenge samples had been taken (at approximately 24 h post-challenge). The percent cumulative mortality for each of the 2 strains did not differ significantly ($p > 0.05$). The black strain had 28.9% mortality (6 dead/21 total), while the red strain 14.3% mortality (3 dead/21 total). No mortality occurred in any of the control fish.

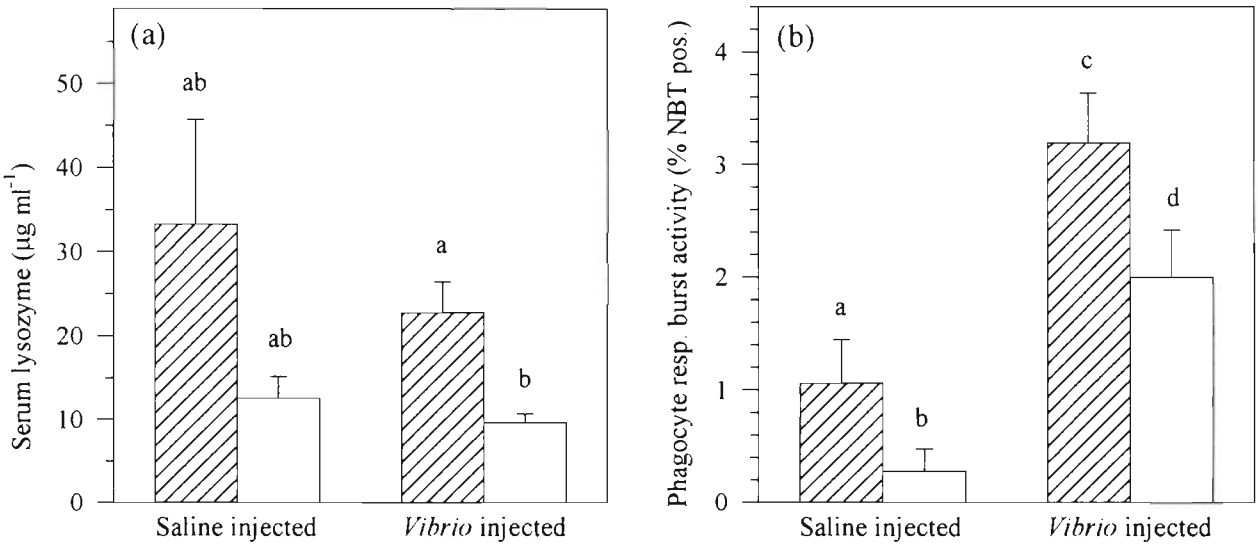


Fig. 1. *Oreochromis niloticus*. (a) Mean (± SE) serum lysozyme activity, and (b) anterior kidney phagocyte respiratory burst activity, in the red (hatched bars) and black (open bars) strains of Nile tilapia, 18 to 24 h after intraperitoneal injection with saline (0.85% NaCl) (control group) or $7.38 \times 10^8$ *Vibrio parahaemolyticus* (disease group). Different letters indicate a significant difference ($p < 0.05$) between groups following ANOVA Student-Newman-Keul multiple pairwise comparison tests

The pre-challenge values for lysozyme, phagocyte respiratory burst activity, and leucocyte numbers were not significantly different from the control (saline challenge) group. The data analyses were then focused on analysis of variance tests examining disease (control vs disease groups) and strain (red vs black) effects.

There were significant strain effects on serum lysozyme activity ($p < 0.005$) (Fig. 1a). Our ability to detect significant strain effects in lysozyme activity in tilapia supports the conclusions of Lund et al. (1995) and Røed et al. (1989, 1993), who suggested a genetic basis for variation of lysozyme in salmonids. There were no significant effects of disease on lysozyme activity ($p > 0.05$). It is possible that there was no significant disease effect on lysozyme activity because the number of neutrophils did not increase significantly (Table 1). Neutrophils synthesize and secrete lysozyme (Murray & Fletcher 1976), and increases in serum lysozyme activity have been associated with increases in their numbers (Muona & Soivio 1992). There were no significant differences in the relative numbers of thrombocytes and monocytes, between either strains or disease treatments. The percent of circulating lymphocytes from both strains showed a significant effect of disease (Table 1). Lymphopenia has been reported in fish infected with *Vibrio anguillarum*, and has been attributed to the migration of lymphocytes to tissues and the destruction of lymphocytes by the bacteria (Harbell et al. 1979, Lamas et al. 1994)

The respiratory burst activity of the anterior kidney phagocytes demonstrated significant disease ($p < 0.001$) and strain ($p < 0.005$) effects (Fig. 1b). The effect of the disease challenge was observed for both tilapia strain, by a significant increase in phagocyte respiratory burst activity. Respiratory burst is one of the most important bactericidal mechanisms in fish (Secombes & Fletcher 1992), and may reflect the ability of the fish to protect itself against infection. Similar increases in phagocyte activity have been found to occur in coho salmon *Oncorhynchus kisutch* during the initial stages of infection (Balfry et al. 1994).

In this study, significant strain differences in non-specific immunity were demonstrated. Despite the strain differences in serum lysozyme and phagocyte respiratory burst activity, there was no statistically significant correlation between these non-specific

Table 1 *Oreochromis niloticus*. Means (± SE) of different leucocyte types in black and red strains of Nile tilapia 18 to 24 h after intraperitoneal injection with saline (control group) or $7.38 \times 10^8$ *Vibrio parahaemolyticus* (disease group)

| | Saline injected (n = 14) | | *V. parahaemolyticus* injected (n = 14) | |
|---|---|---|---|---|
| | Red strain | Black strain | Red strain | Black strain |
| % Lymphocytes | 36.1 ± 5.57 | 38.5 ± 4.08 | 24.9 ± 4.26* | 26.4 ± 3.93* |
| % Thrombocytes | 56.0 ± 5.31 | 53.7 ± 4.12 | 63.0 ± 5.78 | 63.6 ± 3.61 |
| % Neutrophils | 3.21 ± 1.43 | 3.76 ± 1.77 | 6.66 ± 2.08 | 7.12 ± 1.83 |
| % Monocytes | 4.68 ± 2.25 | 4.02 ± 1.70 | 5.38 ± 2.09 | 2.87 ± 1.32 |

*Significant differences due to disease ($p < 0.05$)

immune parameters and disease resistance (as measured by mortality). Perhaps with a larger sample, and higher post-challenge mortality, a correlation between these parameters and disease resistance would have been statistically significant. Other researchers have reported a positive correlation between lysozyme and mortality (Fevolden et al. 1991, Lund et al. 1995). Further research is needed to compare these and other tilapia strains for differences in non-specific immunity and disease resistance before selection programs could be utilized. The significant strain differences in serum lysozyme activity and phagocyte activity reported here suggest there may be strain differences in disease resistance in Nile tilapia that may help to improve its survival under culture conditions.

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