

Enhancement of Immune Responses in Indian Carp, *Catla catla*, Following Administration of Levamisole by Immersion

H.A.C.C. PERERA¹ and ASOKA PATHIRATNE²

¹National Institute of Fisheries and Nautical Engineering
Crow Island, Mattakkuliya, Colombo 15, Sri Lanka

²Department of Zoology, University of Kelaniya, Kelaniya, Sri Lanka

ABSTRACT

Effects of short term administration of levamisole by immersion on immune responses of the cultured food fish, *Catla catla* were investigated. Sub-adults of *C. catla* were given two hr bath in 1.25 or 2.5 mg/L levamisole solutions and the immune responses were assessed on 14, 21, 28, 42 and 56 days after the treatment in comparison to the controls. Results revealed that leucocrit levels, total leucocyte counts, abundance of leucocytes, total phagocytic activity, phagocytic index, myeloperoxidase activity and oxygen radical production by phagocytes were increased significantly in levamisole treated fish in comparison to the controls. No significant differences in the degree of immuno-stimulation were seen between the fish groups exposed to the two concentrations of levamisole tested. For both exposure levels, most of the parameters tested were greatly elevated on 42 days post exposure to levamisole. Challenge experiments showed that levamisole treated fingerlings of *C. catla* co-habitated with gold fish which had been infected with *Ichthyophthirius* and *Dactylogyrus* exhibited lower infestation levels and mortalities in comparison to the control fish. Levamisole treated *C. catla* which had been challenged with *Aeromonas hydrophila* displayed comparatively less mortalities compared to the controls. The results support the potential use of levamisole as an immunostimulant in culturing this fish.

Perera, H.A.C.C. and Pathiratne, A. 2008. Enhancement of immune responses in Indian carp, *Catla catla*, following administration of levamisole by immersion, pp. 129-142. In Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M. and Subasinghe, R.P. (eds.). Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines. 505 pp.

Corresponding author: A. Pathiratne, asoka@kln.ac.lk

INTRODUCTION

Levamisole is an antihelthic used for the treatment of nematode infections in man and animals (Treves–Brown, 2000). Levamisole has been reported to be an effective immunostimulant for common carp (*Cyprinus carpio*), rainbow trout (*Oncorhynchus mykiss*), gilthead seabream (*Sparus aurata*), and Atlantic salmon (*Salmo salar*) (Siwicki, 1987; 1989; Mulero *et al.*, 1998; Sakai,1999; Findlay and Munday, 2000).

Catla catla (Catla), an Indian carp is a commercial edible fish species cultured in the South Asian region. It has been used for stocking reservoirs and in polyculture systems (Pillay, 1990). *Catla catla* is one of the exotic fish species currently used in culture based fishery in inland reservoirs in Sri Lanka. Catla stocks are maintained in freshwater fish breeding stations of the National Aquaculture Development Authority (NAQDA) of Sri Lanka for continuous seed production for culture systems. However these fish maintained in the breeding stations are highly susceptible to parasitic and bacterial infections (Balasuriya, 1987; Subasinghe, 1992; Wimalawickrama and Pathiratne, 2005).

The aim of the present study was to evaluate the potential of using levamisole as a short-term bath treatment to increase the resistance of Catla to diseases and stress. Specific objectives of the study were to assess the concentration response and time course effects of levamisole treatment through immersion route on selected components of the immune system of Catla and to evaluate the responses of levamisole treated Catla to the experimental bacterial and parasitic challenge. The concentrations of levamisole used in the present study were based on the study carried out by Findlay and Munday (2000) with Atlantic salmon.

MATERIALS AND METHODS

Fish

Catla sub-adults and fingerlings were obtained from the Udawalawa Fish Breeding Station, NAQDA, Sri Lanka. Fish were maintained in outdoor cement tanks filled with aged tap water with continuous aeration under the natural photoperiod for 30 days. During the acclimation period, fish were fed daily with commercial fish food pellets (Prima, Colombo, Sri Lanka). Sub-adult stage of the fish was used in the haematological and serological tests whereas the fingerling stage was used in the experimental challenge tests. The fish used in this study were free of gross lesions or parasitic infections externally and considered as apparently healthy individuals.

Levamisole treatment

Samples of sub-adults of Catla (18-26 cm in total length and 160-210 g in body weight) were bathed in glass aquaria containing 1.25 mg/L or 2.5 mg/L levamisole (Sigma, MO, USA) in aged tap water for two hr. Comparable size fish which were introduced to glass aquaria containing only aged tap water at same biological loads, served as controls. After two hr of exposure, the control fish (aged tap water) and the fish exposed to levamisole

(1.25 mg/L or 2.5 mg/L) were transferred to outdoor cement tanks filled with continuously aerated aged tap water. As the two levamisole treatments could not be tested concurrently due to practical constraints, the two concentrations were tested at two stages. Hence two comparable control groups were used for the two treatments. Fish were provided with commercial food pellets daily at 2% of the body weight. Half of the water in each of the tanks was exchanged with fresh aged tap water every four days. At pre-determined time points (14, 28, 42 and 56 days) after the treatment, levamisole treated and control sub-adults of Catla were killed by pithing to assess the immunomodulatory effects of the treatments based on haematological and serological tests. The blood samples were collected from the fish by bleeding caudal vein. Fish were not tested for the natural immune response prior to the experiment. The responses of the levamisole treated fish were compared with those of comparable controls at the predetered time points. The sub-adult stage was used for haematological and serological tests as adequate blood samples could be obtained.

To evaluate the responses of levamisole treated Catla to the experimental challenge, fingerlings (5.5-6 cm in total length and 5-10 g in body weight) were exposed to 1.25 mg/L levamisole in glass aquaria for two hr before conducting bacterial and parasitic challenge tests after 14 days of the treatment. Comparable sized fingerlings were also transferred to glass aquaria containing only aged water concurrently and maintained in these aquaria at comparable densities for two hr. These fish were considered as controls.

Haematocrit and leucocyte counts

For deteration of the haematocrit and leucocrit levels, blood samples were taken into heparinized capillary tubes and centrifuged in the haematocrit centrifuge. Heamatocrit value of each sample was measured using the haematocrit gauge. Height of the leucocyte column was measured under a light microscope using a micrometer scale in order to determine the leucocrit level. Total leucocyte count was detered using Shaw's solutions as dilution fluids following the method of Hesser (1960). Blood smears of the fish were prepared, fixed in 100% methanol and were stained with Wright-Giemsa stain. Different types of leucocytes were identified as described by Hibiya (1982).

Phagocytosis assays

Phagocytic cells were detected using *Staphylococcus aureus* (Sigma, MO, USA) as described by Anderson and Siwicki (1995). A sample (0.1 mL) of blood was placed in a microtiter plate well, 0.1 mL of *Staphylococcus aureus* 1×10^7 cells suspended in phosphate buffered saline pH 7.2, was added and then mixed well. The bacteria-blood solution was incubated for 20 at room temperature. Five μ L of this solution was taken on to a clean glass slide and a smear was prepared. The smear was air dried, then fixed with ethanol (95%) for 5 min and air dried. Then the smear was stained with Giemsa stain for 10 min. The two smears were made from each fish. The total of 100 neutrophils and monocytes from each smear were observed under the light microscope and the number of phagocytizing cells and the number of bacteria engulfed by the phagocyte were counted. Phagocytic activity and phagocytic index were calculated as follows: Phagocytic activity equals the number

of phagocytizing cells divided by the total number of phagocytes counted. Phagocytic index is expressed as the total number of bacteria engulfed by the phagocytes, divided by the total number of phagocytes containing engulfed bacteria.

Nitroblue tetrazolium assay and Myeloperoxidase activity

Production of oxygen radicals from phagocytes in the blood was measured using nitroblue tetrazolium (NBT) dye as described by Anderson and Siwicki (1995). A sample (0.1 mL) of heparinized blood was placed in to a microtiter plate well and equal amount of 0.2% NBT (Sigma, MO, USA) was added, the NBT-blood cell suspension was incubated for 30 min at room temperature. A sample (0.05 mL) of the NBT-blood cell suspension was taken out and added to a glass tube containing 1.0 mL of N,N-dimethylformamide solution. Then the mixture was centrifuged for 5 min at 3000 g. The supernatant was taken into a glass cuvette and absorbance was read at 540 nm using a spectrophotometer.

Myeloperoxidase activity was detected using commercially available kits (The peroxidase kit, catalog no. 391- A, Sigma, MO, USA). The positive cells were counted as described by Anderson and Siwicki (1995).

Lysozyme assay

Lysozyme activity of blood serum was detered as described by Anderson and Siwicki (1995) with some modifications. Blood serum was prepared by centrifuging the blood at 3000g for 5 min. Serum (0.1 mL) was placed in test tubes and 0.9 mL of a 0.75 mg/mL *Micrococcus lysodeikticus* (Sigma, MO, USA) suspension in phosphate buffered saline, pH 6.2 was added and mixed well. The absorbance was measured at 450 nm by a spectrophotometer at 1 min intervals for 10 min after mixing with bacteria and rate of change of absorbance calculated. Lysozyme activities were calculated using hen egg white lysozyme (Sigma, MO, USA) as a standard.

Total protein and total immunoglobulin in plasma

Total protein content in blood plasma was determined using Peterson's modifications of the micro-Lowry method using a protein assay kit (Sigma Diagnostics, P 5656, Sigma, MO, USA). The protein concentrations were determined using a calibration curve prepared using bovine serum albumin as the standard. For the determination of the immunoglobulin in the plasma, immunoglobulins were separated from the plasma by precipitation with polyethylene glycol as described by Anderson and Siwicki, (1995). Plasma (0.1 mL) was placed in plastic serum vial and 0.1 mL of 12% polyethylene glycol was added and incubated at room temperature for 2 hr under constant mixing. After incubation, the solutions were centrifuged at 7000 g for 10 min. The protein content in the supernatant was detered using protein assay kit. The total immunoglobulin content was detered by subtracting the protein content in the supernatant from the total protein content in the plasma.

Challenge test by cohabitating test fish with parasitic infested fish

Fingerlings of Catla which were treated with 1.25 mg/L levamisole were subjected to a parasitic challenge by cohabitating the fish with a sample of gold fish *Carassius auratus* infested with *Ichthyophthirius* sp and *Dactylogyrus* sp in glass aquaria. As significant concentration specific difference with respect to the degree of immunostimulation was not clearly seen between the two exposure levels of levamisole, it was decided to use 1.25 mg/L levamisole for challenge tests. After the introduction of parasite infested fish, mortality and abnormal signs of fish were observed daily and parasitological survey was carried out as described by Kabata (1985) on 7, 14, 21, 28, 35, 42 and 49 days after levamisole treatment.

Challenge test with bacteria

Fingerlings of Catla which were treated with 1.25 mg/L levamisole were subjected to a bacterial challenge. A virulent strain of *Aeromonas hydrophila* (donated by Mr. C. Hettiarachchi, Confifi Aquaculture Pvt., Ltd., Sri Lanka), that has been isolated from characteristic lesions in an ornamental fish *Trichogaster leeri* (pearl gouramy), was used in the challenge tests. Fingerlings of control Catla (n=30 individuals) and Catla treated with levamisole (n = 30 individuals) were injected intraperitoneally (i.p.) with *Aeromonas hydrophila* using sterile syringes at a dose of 0.1 mL, containing 10⁹ Colony Forming Units in 0.85% NaCl per fish. Fish were observed for 49 days after the i.p. injection.

Statistical analysis

Haematological and serological data are presented as mean \pm standard error of the mean for 6 fish per group. Data were analyzed using one-way analysis of variance (ANOVA). Where differences are significant, differences among the mean parameters of fish exposed to different levamisole concentrations were compared using Tukey's test. The intensities of parasitic infestations were analyzed using non-parametric Mann-Whitney U test (Zar, 1999). The accepted level of significance was $P \leq 0.05$.

RESULTS

All control and levamisole treated sub-adults of *C. catla* survived during the experimental period. No significant difference was found between levamisole treated fish and respective control fish in relation to the haematocrit levels in the blood (results not shown, $P > 0.05$). Leucocrit levels and the total leucocyte counts of levamisole treated fish were significantly higher than that of the respective control fish on 14 days of post exposure onwards. Maximum increase in these parameters occurred on 42 days of post exposure to levamisole (Figure 1). The leucocrit levels and leucocyte count in the blood increased by nearly two folds on 42 days of levamisole post exposure compared to the respective controls. However, no concentration specific significant difference was detected at each time point ($P > 0.05$).

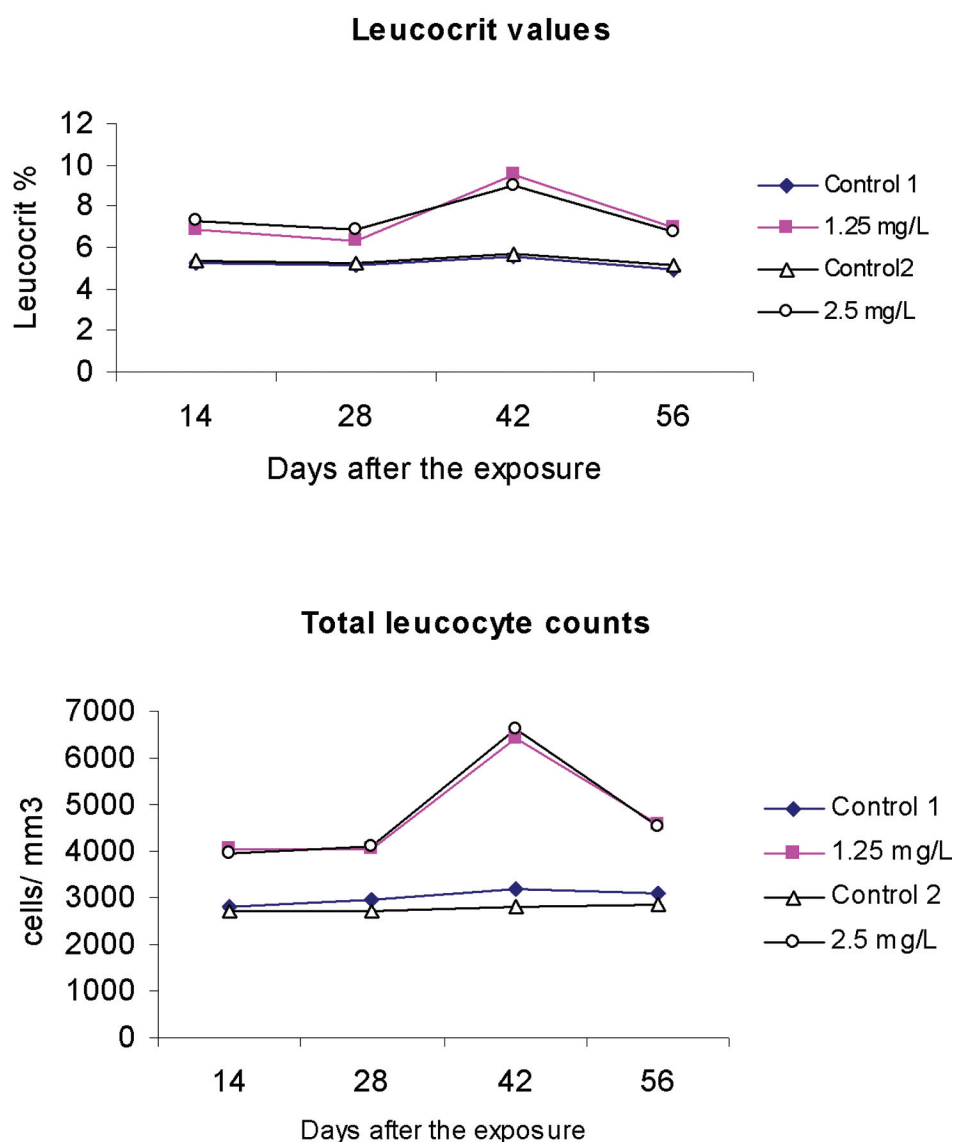


Figure 1. Time-course elevation pattern in the mean leucocrit levels and total leucocyte counts in the blood of *Catla catla* following levamisole exposure (1.25 mg/L or 2.5 mg/L for 2 hrs) through immersion route. At each time point, the parameters in the blood of levamisole treated fish were significantly different from those of the controls (ANOVA, Tukey's test, $P < 0.05$)

Percentages and absolute numbers of populations of neutrophils and monocytes in the blood of fish increased significantly after levamisole treatment. Maximum increase in these parameters occurred on 42 days of post exposure to levamisole. On the 42nd day of the post immersion to 1.25 and 2.5 mg/L levamisole, abundance of neutrophils increased by 2.5 and 3.2 folds respectively whereas populations of monocytes in the blood of levamisole treated fish increased by 2.7 and 3.6 folds compared to the respective controls (Table 1).

Populations of neutrophils in the blood of levamisole treated fish remained increased by nearly two folds even on the 56th day of post levamisole administration. Even though percentage abundance of leucocytes in the blood of levamisole treated fish decreased in comparison to the controls (results not shown), absolute numbers of lymphocytes in the blood of fish treated with 2.5 mg/L levamisole were significantly increased during the study period (1.6–2.6 folds) compared to the respective controls (Table 1). However, a significant increase in the abundance of lymphocytes in the blood of fish treated with 1.25 mg/L levamisole was seen only on 42nd and 56th day of post treatment.

Table 1. Concentration–response and time-course effects of levamisole adistration through immersion route on different leucocyte populations (in cells mm⁻³) in the blood of sub-adults of *Catla catla*.

Leucocyte type and fish group	Days after levamisole treatment			
	14 days	28 days	42 days	56 days
Neutrophils				
Control 1	1368 ± 103 ^a	1469 ± 82 ^a	1599 ± 156 ^a	1546 ± 119 ^a
Levamisole 1.25 mg/L	2323 ± 260 ^b	2435 ± 123 ^b	3926 ± 318 ^b	2605 ± 207 ^b
Control 2	1276 ± 230 ^a	1258 ± 200 ^a	1273 ± 94 ^a	1362 ± 202 ^a
Levamisole 2.5mg/L	2459 ± 170 ^b	2584 ± 166 ^b	4073 ± 528 ^b	2308 ± 204 ^b
Monocytes				
Control 1	185 ± 60 ^a	186 ± 43 ^a	240 ± 63 ^a	207 ± 64 ^a
Levamisole 1.25 mg/L	389 ± 83 ^b	378 ± 59 ^b	652 ± 63 ^b	369 ± 56 ^b
Control 2	138 ± 30 ^a	167 ± 72 ^a	216 ± 39 ^a	176 ± 64 ^a
Levamisole 2.5mg/L	410 ± 28 ^b	407 ± 93 ^b	776 ± 137 ^b	398 ± 59 ^b
Lymphocytes				
Control 1	1342 ± 135 ^a	1286 ± 114 ^a	1369 ± 143 ^a	1388 ± 188 ^a
Levamisole 1.25 mg/L	1314 ± 225 ^a	1160 ± 194 ^a	2008 ± 507 ^b	1880 ± 50 ^b
Control 2	1274 ± 112 ^a	1226 ± 114 ^a	1298 ± 169 ^a	1273 ± 86 ^a
Levamisole 2.5mg/L	2005 ± 168 ^b	2116 ± 72 ^b	3317 ± 177 ^b	2204 ± 303 ^b

Fish were exposed to levamisole at 1.25 mg/L or 2.5 mg/L for 2 h as immersion and different types of leucocytes were evaluated at different times of post exposure. Results are expressed as mean ± SEM for 6 fish per group. For a specific type of lymphocyte, the data in a column with different superscripts are significantly different from each other (ANOVA, Tukey’s test, P < 0.05)

Total phagocytic activity, phagocytic index, NBT activity and myeloperoxidase activity in the blood of fish exposed to levamisole are compared with the respective controls in Figure 2. Total phagocytic activity and phagocytic index of fish exposed to different concentrations of levamisole were significantly higher than that of the control fish. NBT activity and myeloperoxidase activity in the blood of fish exposed to levamisole were also significantly higher than that of the respective control values at each time point. Time course pattern showed that levamisole induced elevation of NBT activity in the blood of fish were maximum on 42 days of post treatment. There were no concentration related significantly difference between the fish exposed to 1.25 mg/L and 2.5 mg/L levamisole with respect to the above four parameters tested.

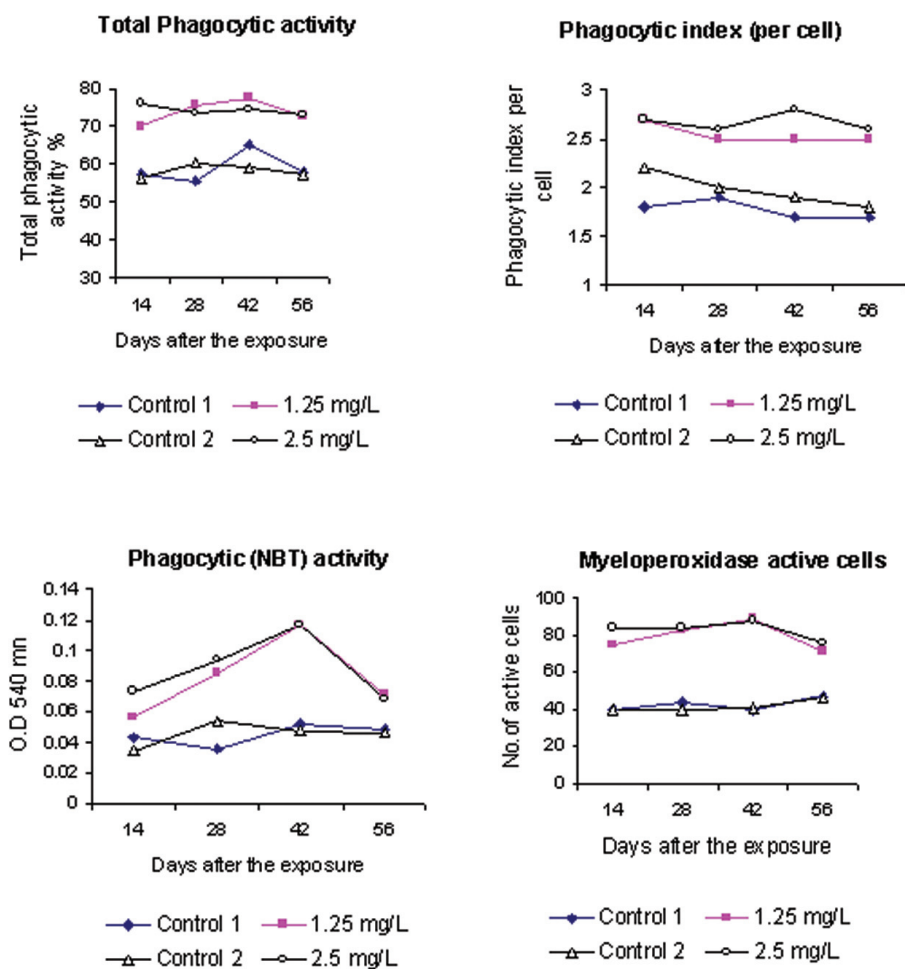


Figure 2. Time course elevation pattern in the total phagocytic activity, phagocytic index, NBT activity and myeloperoxidase activity (mean values) in the blood of *Catla catla* following levamisole exposure (1.25 mg/L or 2.5 mg/L for 2 hr) through immersion route. At each time point, the parameters in the blood of levamisole treated fish were significantly different from those of the controls (ANOVA, Tukey's test, $P < 0.05$).

Lysozyme activity in the blood serum of the fish exposed to both concentrations of levamisole was significantly higher only on 28th day of post exposure in comparison to the controls. No significant difference was found between levamisole treated fish and control fish during the other study periods probably due to large individual variations (Table 2). Total protein levels in the circulating blood of fish exposed to levamisole were significantly higher than that of the control values (Figure 3). Time course stimulation pattern showed that levamisole induced elevation of protein levels in circulating blood of fish persist at least 56 days post exposure. Maximum increase in the blood protein levels was seen on the 42nd day of the post exposure. However, immunoglobulin level in the blood of fish exposed to levamisole was significantly not different from the control fish (Figure 3).

Table 2. Concentration–response and time-course effects of levamisole adistration through immersion route on serum lysozyme activities (in µg/ml serum) of sub-adults of *Catla catla*.

Fish	Days after exposure			
	14 days	28 days	42 days	56 days
Control 1	4.8 ± 1.2 ^a	2.7 ± 0.4 ^a	3.8 ± 1.3 ^a	2.3 ± 1.2 ^a
Levamisole 1.25 mg/L	4.0 ± 1.0 ^a	5.1 ± 0.9 ^b	4.3 ± 1.4 ^a	2.4 ± 0.7 ^a
Control 2	2.5 ± 1.6 ^a	2.7 ± 0.5 ^a	4.2 ± 1.7 ^a	2.6 ± 0.3 ^a
Levamisole 2.5mg/L	3.6 ± 1.9 ^a	4.8 ± 1.3 ^b	4.8 ± 1.5 ^a	3.2 ± 0.3 ^a

Fish were exposed to levamisole at 1.25 mg/L or 2.5 mg/L for 2 hr as immersion and serum lysozyme levels were evaluated at different times of post exposure. Results are expressed as mean ± SEM for 6 fish per group. The data in a column with different superscripts are significantly different from each other (ANOVA, Tukeys’ test, P > 0.05).

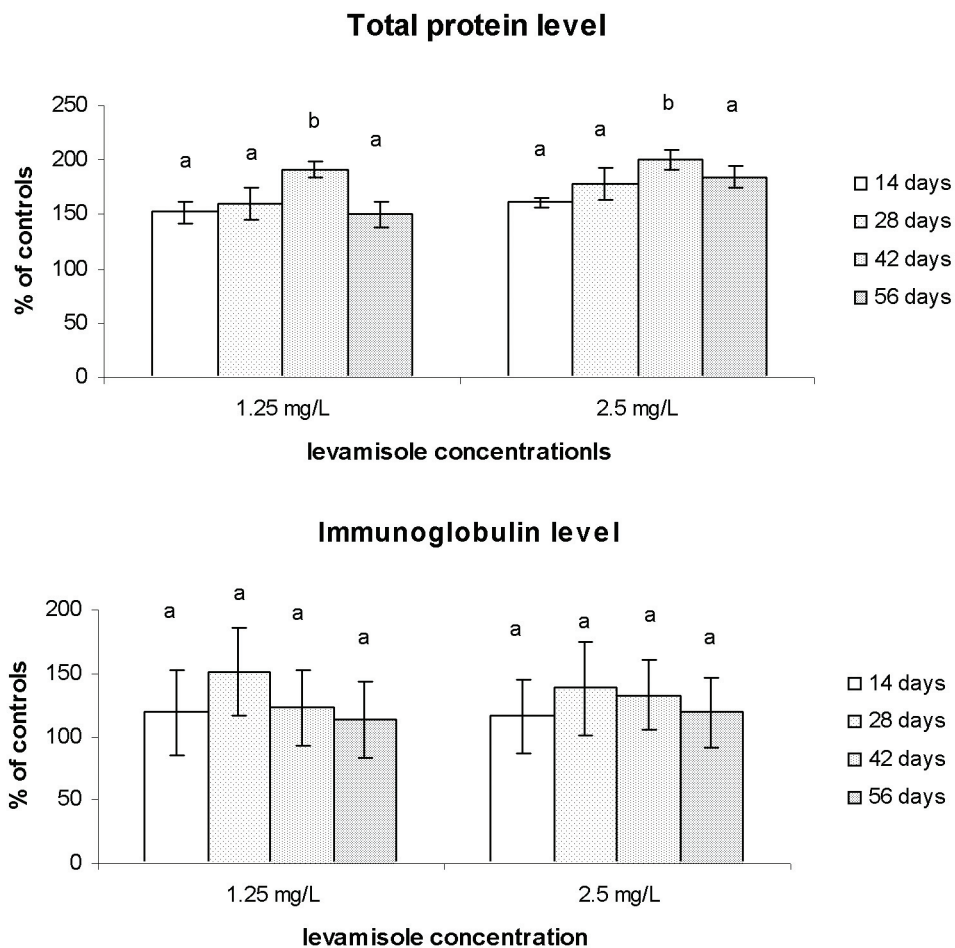


Figure 3. Effects of adistration of levamisole (1.25 mg/L and 2.5 mg/L for 2 hr) through immersion route on total protein levels and immunoglobulin levels in *Catla catla*. For each concentration, bars with the different letters are significantly different from each other (ANOVA, Tukey’s test, P < 0.05)

Occurrence of *Ichthyophthirius* and *Dactylogyrus* on fingerlings of *Catla* cohabitated with the gold fish infested with *Ichthyophthirius* sp. and *Dactylogyrus* sp. are presented in Table 3. Numbers of parasites on the gill filaments were higher in control fish cohabitated with parasites in comparison to the fish treated with levamisole. The cumulative mortality of the control fish cohabitated with gold fish was 7% where as none of the fish treated with levamisole died during the study period. The results of the challenge experiment show that cumulative mortality of control fingerlings challenged with *Aeromonas hydrophila* were higher than that of the levamisole treated fish (Figure 4). No mortality in the control fish or levamisole treated fish which were not challenged with the bacteria (negative control groups) was observed during this period. In the control fish challenged with the bacteria, injected site turned into a reddish patch within two days of the challenge and it was spread to a larger area with time. The injection site of the fish treated with levamisole was completely healed by 8 days.

Table 3. Occurrence of parasites in the gills of *Catla catla* after cohabitation with gold fish infested with *Ichthyophthirius* sp and *Dactylogyrus* sp.

Fish	Intensity of infestation						
	7 days	14 days	21 days	28 days	35 days	42 days	49 days
<i>Ichthyophthirius</i> sp							
Control fish	7-8	10-14	7-9	9-11	9-10	7-10	1-12
Levamisole treated fish	0-2	0	0	0	0	0-1	0
<i>Dactylogyrus</i> sp.							
Control fish	21-26*	24-28*	29-31*	34-38*	30-36*	34-41*	3-34*
Levamisole treated fish	5-6	3-5	1-4	1-2	3-4	1-2	2-4

Results are presented as ranges (n=30 fish per group).

* Significantly different from levamisole (1.25 mg/L for 2 hr) treated fish cohabitated with gold fish infested with parasites (Mann-Whitney U test, $\alpha = 0.05$)

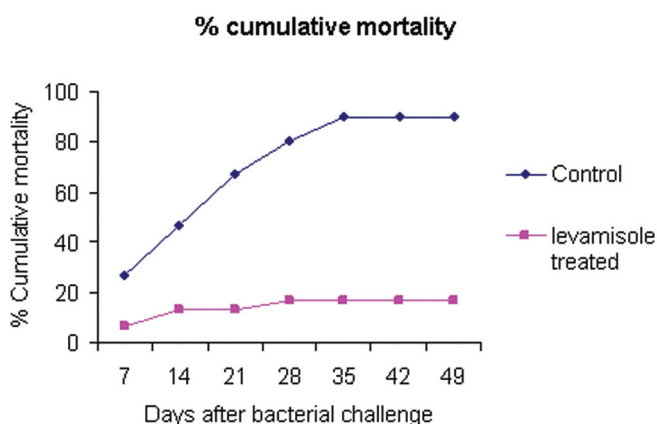


Figure 4. Mortality of fingerlings of *Catla catla* challenged with *Aeromonas hydrophila*.

DISCUSSION

The use of immunostimulants in fish culture is opening new opportunities to improve fish health and prevent losses due to diseases. Levamisole has been shown to act as an immunostimulant in number of fish species: by oral administration or i.p. injection for *Cyprinus carpio* (Siwicki, 1987; 1989; Sakai, 1999) by oral administration for *Sparus aurata* (Mulero *et al.*, 1998) and for *Labeo rohita* (Wijendra and Pathiratne, 2004), by immersion for *Salmo salar* (Findlay and Munday, 2000) and by immersion and injection for *Oncorhynchus mykiss* (Anderson *et al.*, 1995). The present study demonstrated immunostimulatory properties of levamisole administered through immersion route on an Indian carp, *C. catla*.

Findlay and Munday (2000) used 2.5 mg/L levamisole as a 2 hr bath treatment for *Salmo salar* to increase immunostimulation. In this study, levamisole was tested as a 2 hr bath treatment at two different concentrations (1.25 and 2.5 mg/L) to assess concentration–response and time-course effects on the immune system of Catla. The results revealed that both concentrations of levamisole could enhance the immune system especially some components of the non specific immune system. Leucocrit levels, total leucocyte counts, abundance of neutrophils, monocytes and lymphocytes, total phagocytic activity, phagocytic index, NBT activity, myeloperoxidase activity and total protein level in the blood were increased significantly in levamisole treated fish in comparison to the control fish on 14-56 days post exposure to levamisole. It appears that proliferation of leucocytes increases several days after exposure to levamisole. No significant concentration specific differences was found between 1.25 and 2.5 mg/L levamisole treatments with respect to above parameters except the time course stimulation pattern of lymphocyte populations. Time-course pattern of immune stimulation revealed that enhanced immune responses persist at least 56 days whereas for most of the parameters tested, maximum response was observed at 42 days post administration.

Transiently lower haematocrit values have been shown in gilthead seabream (*Sparus aurata*) fed diets containing higher levels (125-500 mg/kg body weight) of levamisole (Mulero *et al.*, 1998). In the present study, no significant difference was found between levamisole treated Catla and respective control fish for haematocrit values in the blood corroborating the findings of Anderson *et al.*, (1995), Findlay and Munday (2000) and Wijendra and Pathiratne (2004) for *Oncorhynchus mykiss*, *Salmo salar*, and *Labeo rohita* respectively. The haematocrit levels serve as a general indicator of fish health. It is often used as a confirmation that an immunostimulant is not disturbing the profile of erythrocytes. Thus, levamisole does not appear to modify the erythrocyte levels in the blood of the fish.

Elevated leucocyte numbers, enhanced phagocytic activity, and myeloperoxidase activity in neutrophils and elevated lysozyme levels have been reported in *Cyprinus carpio* following levamisole injections. Oral administration of levamisole has also increased the number of leucocytes, lysozyme levels in the serum and the phagocytic index and NBT reduction by phagocytes in carp (Siwicki, 1987; 1989). In this study, treatment of fish with levamisole bath at 1.25 and 2.5 mg/L for 2 hr significantly increased leucocrit levels, total leucocytes, populations of neutrophils, monocytes and lymphocytes in the blood of Catla nearly by 2-3 folds during the study period. Monocytes and neutrophils which are

the main cells of the non-specific defence system, are phagocytic and capable of killing a variety of pathogens including bacteria. The primary function of fish lymphocytes seems to be to act as the cells of specific immune system via antibody production (Evelyn, 2002). Hence enhancement of immune responses could be expected in *Catla* treated with 1.25 and 2.5 mg/L levamisole through its stimulatory effects on different leucocyte populations.

Fish phagocytes have potent bactericidal and larvicidal activity and thus, presumably possess both intracellular, and extra cellular killing mechanisms (Secombes, 1996). In the present study, total phagocytic activity, phagocytic index, NBT activity and myeloperoxidase activity in *Catla* treated with 1.25 and 2.5 mg/L levamisole were significantly higher in comparison to the respective control fish at least 56 days post-levamisole treatment. In seabream, *Sparus aurata* leucocyte functions including phagocytosis have also been enhanced by dietary intake of high levels of levamisole (Mulero *et al.*, 1998). It is known that oxygen free radicals are produced by fish phagocytes during the respiratory burst (Evelyn, 2002). The production of oxidative radicals (detected by NBT) was enhanced in *Cyprinus carpio* fed with levamisole (Siwicki, 1989). In the present study, NBT activity in the blood of *Catla* also rose after the levamisole treatment through immersion route.

Lysozyme is found in a wide range of vertebrates including fish and is one of the defensive factors against invasion by microorganisms (Evelyn, 2002). Lysozyme is an important enzyme in blood that actively lyses bacteria. Findlay and Munday (2000) found that bath treatment of fish with levamisole can induce increased activities of both mucus and serum lysozyme of Atlantic salmon, *Salmo salar*. In the present study, even though significantly elevated levels of serum lysozyme were found on 28 days of post exposure, there was no significant difference between levamisole treated fish and respective control fish with respect to the serum lysozyme levels in the other time periods. Immunoglobulins are a major humoral component of the specific immune system. In the present study, total immunoglobulin levels of levamisole treated fish were slightly increased in comparison to the control fish but the difference was not statistically significant probably due to large individual variations. Nevertheless, total protein level in the blood of levamisole treated fish were significantly higher than that of the respective control fish.

Challenge experiments have shown that levamisole increased protection against pathogenic bacteria in carp and trout specially through activation of the non-specific defense mechanisms (Anderson *et al.*, 1995; Sakai, 1999). In the present study, *Catla* not treated with levamisole (controls) and subsequently cohabitated with fish infested with *Ichthyophthirius* and *Dactylogyrus* exhibited significantly high intensity of parasitic infestation than the fish treated with levamisole. The cumulative mortality of the control fish cohabitated with gold fish was 7% where as none of the fish treated with levamisole died during the study period. The results also showed that levamisole treated *Catla* had developed some resistance to *Aeromonas hydrophila* bacterial challenge. Survival of the levamisole treated fish experimentally challenged with *Aeromonas hydrophila* was higher in comparison to the control fish challenged with the bacteria. Infection experiments showed the *in vivo* immune responses of levamisole treated *Catla* to *Ichthyophthirius*, *Dactylogyrus* and *Aeromonas hydrophila*. Levamisole bath used in the present study (1.25 mg/L for 2 hr) was effective enough to provide *Catla* a considerable resistance to parasitic and bacterial challenge.

CONCLUSIONS

The results revealed that administration of levamisole (1.25 or 2.5 mg/L for two hr) through immersion route is a potential method in Catla culture for enhancing the resistance of fish to diseases and stress. Both concentrations of levamisole could enhanced Leucocrit levels, total leucocyte counts, abundance of neutrophils, monocytes and lymphocytes, total phagocytic activity, phagocytic index, NBT activity, myeloperoxidase activity and total protein level in the blood on 14 - 56 days post exposure to levamisole. Time-course pattern of immune stimulation revealed that enhanced immune responses persist at least 56 days whereas for most of the parameters tested, maximum response was observed at 42 days post administration. As significant concentration specific difference with respect to the degree of immunostimulation was not clearly seen between the two exposure levels of levamisole, it would be economically more advantageous to use 1.25 mg/L levamisole for *Catla catla* culture for potential immunostimulation.

REFERENCES

- Anderson, D.P and Siwicki, A.K. 1995. Basic haematology and serology for fish health programs. pp. 185-202. *In* M. Shariff, J.R. Arthur and R.P. Subasinghe. (eds.) Diseases in Asian Aquaculture II, Fish Health Section, Asian Fisheries Society, Manila, Philippines.
- Anderson, D.P., Siwicki, A.K. and Rumsey, G.L. 1995. Injection or immersion delivery of selected immunostimulants to trout demonstrate enhancement of nonspecific defense mechanisms and protective immunity. pp. 413-426. *In* M. Shariff, J.R. Arthur and R.P. Subasinghe. (eds.). Diseases in Asian Aquaculture II, Fish Health Section, Asian Fisheries Society, Manila, Philippines.
- Balasuriya, L.K.S.W. 1987. Current fish disease problems in Sri Lanka. pp 36-40. *In*: J. R. Arthur (ed.). Fish Quarantine and Fish Diseases in South and South East Asia:1986 update, Asian Fisheries Society Special Publication, No. 1, Manila.
- Evelyn, T.P.T. 2002. Finfish immunology and its use in preventing infectious diseases in cultured finfish. pp. 303-324. *In* Lavilla-Pitogo, C.R. and Cruz-Lacierda, E.R. (eds.). Diseases in Asian Aquaculture IV, Fish Health Section, Asian Fisheries Society, Manila.
- Findlay, V.L and Munday, B.L. 2000. The immunomodulatory effects of levamisole on the nonspecific immune system of Atlantic salmon, *Salmo salar* L. *J. Fish Dis.* 23:369-378.
- Hesser, E.F. 1960. Methods for Routine Fish Haematology. *Progressive Fish Culturist* 22:164-171.
- Hibiya, T. 1982. An Atlas of Fish Histology-Normal and Pathological Features. Kodansha, Tokyo, Japan. 147 p.
- Kabata, Z. 1985. Parasites and Diseases of Fish Cultured in the Tropics. Taylor and Francis, London. 318 p.

- Mulero, V., Esteban, M.A., Munoz, J. and Meseguer, J. 1998. Dietary intake of levamisole enhances the immune responses and disease resistance of the marine teleost gilthead seabream (*Sparus aurata* L). *Fish and Shellfish Immunology* 8:49-62.
- Pillay, T.V.R. 1990. Aquaculture, Principles and Practices. Blackwell Science, Ltd. London, UK. 575 p.
- Sakai, M. 1999. Current research status of fish immunostimulants. *Aquaculture* 172: 63-92.
- Secombes, C. J. 1996. The non specific immune system cellular defenses, pp 63-94. In Iwama, G. and Nakanishi, T. (eds.). The Fish Immune System. Organism, Pathogen and Environment. Academic Press, California.
- Siwicki, A. K. 1987. Immunomodulating activity of levamisole in carp spawners, *Cyprinus carpio* L. *J. Fish Biol.* 31:245-246.
- Siwicki, A.K. 1989. Immunostimulating influence of levamisole on nonspecific immunity in carp (*Cyprinus carpio*). *Dev. Comp. Immunol.* 13:87-91.
- Subasinghe, R. P. 1992. Hatchery diseases of freshwater fish in Sri Lanka, pp 209-214. In Shariff, M., Subasinghe, R.P. and Arthur, J.R. (eds.). Fish Health Section, Asian Fisheries Society, Manila.
- Treves-Brown, K.M. 2000. Applied Fish Pharmacology. Kluwer Academic Publishers, Dordrecht, the Netherlands. 309 p.
- Wijendra, G.D.N.P. and Pathiratne, A. 2004. Assessment of the effects of adistration of levamisole through feed on the immune system of an Indian carp, *Labeo rohita*, a culturable food fish in Sri Lanka. Proceedings of the Sri Lanka Association for Fisheries and Aquatic Resources 5.
- Wimalawickrama, N.D. and Pathiratne, A. 2005. Evaluation of the effects of infestation by trematodes and copepodes on *Catla catla* an Indian carp cultured in Sri Lanka. *Sri Lanka J. Aquat. Sci.* 10:85-96.
- Zar, J. H. 1999. Biostatistical Analysis. Prentice Hall, Englewood Cliffs, NJ, USA. 663 p.