

EFFECT OF 17 $\beta$ -ESTRADIOL ON THE IMMUNOCOMPETENCE OF  
JAPANESE SEA BASS (*LATEOLABRAX JAPONICUS*)

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**Abstract**—Environmental contaminants can interfere with hormonal regulation in both vertebrates and invertebrates, and these contaminants may disrupt the endocrine system of human and other organisms. Evidence is growing that contaminants may be partly responsible for the observed increase of disease in marine organisms by adversely affecting their immunity. Fish are commonly used as sentinel organisms in vertebrate immunotoxicology; however, to date, studies have been undertaken only on a single size group of fish (juvenile/adult) and for acute exposure. In the present study, *Lateolabrax japonicus* fingerlings and juveniles were exposed to two sublethal concentrations (200 and 2,000 ng/L) of 17 $\beta$ -estradiol (E<sub>2</sub>) for 30 d under laboratory conditions, and alterations in immune parameters comprising differential leukocyte count, respiratory burst, myeloperoxidase, immunoglobulin levels, serum lysozyme, and bactericidal activity were investigated to establish whether estrogen produced immunomodulation and to understand the effects of long-term exposure on these immune parameters in fish fingerlings and juveniles. The results revealed a significant elevation of respiratory burst activity, myeloperoxidase, immunoglobulin levels, and differential leukocyte counts of the fish exposed to estrogen compared to the control. The remaining parameters were significantly reduced in the experimental groups when compared to the control. The results indicated that sublethal E<sub>2</sub> exposure induced immunomodulation in both fingerling and juvenile *L. japonicus*, and the changes caused by estrogen might affect the function of immune system in fish.

**Keywords**—*Lateolabrax japonicus* 17 $\beta$ -Estradiol Estrogen Immunotoxicity Immune parameters

## INTRODUCTION

Organic extrinsic compounds enter (and are widespread in) aquatic ecosystems along various routes, including direct discharge, land runoff, atmospheric deposition, in situ production, abiotic and biotic movement, and food-chain transfer. Public concern is increasing about the potential adverse effects on human health and ecological safety of various environmental contaminants designated as endocrine-disrupting chemicals (EDCs) [1]. 17 $\beta$ -Estradiol (E<sub>2</sub>) is increasingly reported as an environmental contaminant [2,3] and the most potent of all xenoestrogens that are able to affect aquatic organisms, namely fish. Furthermore, E<sub>2</sub> is the most potent EDC, having the potential to exert its effects at extremely low concentrations [4], and E<sub>2</sub> has been reported not only to affect the reproductive system but also to markedly influence the immune system [1]. The interaction of the endocrine and immune systems is an important regulatory process in vertebrates. The increasing regulatory trend toward assessing sublethal effects in aquatic ecosystems and the array of proven, rapid immunological assays has heightened the curiosity in the field of fish immunotoxicology [5,6].

The occurrence of hormonal steroids in the aquatic environment has risen markedly in recent decades because of their increasing use in human medicine and livestock farming, becoming a matter of concern and an interesting research topic in the field of ecotoxicology. Johnson et al. [7] reported that females were excreting E<sub>2</sub> through urine at 2.3 to 259  $\mu$ g/person/d and males at 1.6  $\mu$ g/person/d. The other important sources of E<sub>2</sub> are livestock waste [1] and agriculture runoff

[8]. Poultry dry waste has been reported to contain from 14 to 533 ng/g of E<sub>2</sub> [9].

Fish living in the aquatic environment are frequently in contact with various pathogenic and nonpathogenic microorganisms and, thus, may develop complex defense mechanisms that contribute to their survival. As indicated in previous studies concerning the pathogenesis of diseases, exposure to chemical contaminants may impair the immune system and increase host susceptibility to infectious diseases [10,11]. The immune system of aquatic organisms, such as fish, is continuously affected by periodic or unexpected changes in their environment. Adverse environmental conditions may acutely or chronically affect the health status of fish, either by altering some biochemical parameters or by modulating their innate and adaptive immune responses [12]. Immune defense mechanisms are of obvious strategic importance to fish when faced with environmental challenges. Thus, fish and their biological parameters associated with immunity may represent important scientific indicators in biomonitoring of environmental quality, particularly immunotoxic environmental pollution.

Endocrine-disrupting chemicals are of high environmental relevance not only for their role in fish production but also in terms of growth, digestion and food utilization, gut transport, shifts in body composition, intermediary metabolism, osmoregulation, and immunity [13]. Therefore, research into the effect of EDCs on immune systems is of great importance. The presence of EDCs in the form of estrogens or estrogen-mimicking compounds in sewage effluents has been well documented in recent years [14,15]. As in other vertebrates, the endocrine and immune systems are interrelated in fish, and changes in endocrine functions may be reflected in functional immune defense.

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Fish are susceptible to endocrine disruption by estrogens and xenoestrogens [16], and estrogens inhibit various aspects of the immune response in fish, including phagocytosis [17], antibody production [18], reduction in circulating lymphocytes and granulocytes [19], and immune-related gene transcription [20]. 17 $\beta$ -Estradiol suppressed the immunoglobulin M (IgM)-producing cells in a dose-dependent manner [21], and E<sub>2</sub> treatment suppressed the production of IgM and decreased disease resistance in fish [13,18,22]. These reported effects, however, have not always been consistent between studies and with different estrogens. These discrepancies in the immunomodulatory effects of estrogen on fish demand further evaluation to understand the neuroendocrine immune system cross talk in teleosts.

The effects of E<sub>2</sub> exposure on different aspects of the immune function in fish are well documented [13,17,19,23–25]. The available literature regarding the alteration of immunity in fish because of E<sub>2</sub> show that earlier studies were focused on one or more specific immune parameters, such as respiratory burst, total immunoglobulin, and myeloperoxidase (MPO) activity [13,17,26]. Most of these studies suggest a certain degree of immune system impairment. Moreover, the information available concerning long-term exposure to E<sub>2</sub> during in vivo studies was restricted to certain immune parameters (differential leukocyte count and respiratory burst activity). To our knowledge, no work has involved a long-term study of the alteration of immune parameters in fish because of E<sub>2</sub> in comparison to different fish size groups. Therefore, the present study investigated the effect of sublethal concentrations of E<sub>2</sub> on the nonspecific immune-associated parameters, including respiratory burst, MPO activity, serum lysozyme activity, bactericidal activity, and circulating IgM, and also on cellular components (leukocytes) in two different sizes of *Lateolabrax japonicus*. 17 $\beta$ -Estradiol was tested at sublethal concentrations, and the long-term effect was investigated to determine the extent of the immunomodulation produced.

## MATERIALS AND METHODS

### Fish

Japanese sea bass of different sizes were obtained from Zhang Pu Fish Culture Farm in Fujian Province, China, and were acclimatized in laboratory conditions with a temperature of 24  $\pm$  1°C, salinity of 30  $\pm$  1 ppt, and pH 7.8  $\pm$  0.1. The fish were acclimatized in a 1,000-L capacity polyvinyl chloride tank for 10 d before being used in the experiments, and the fish were divided into two groups based on size: Fingerling and juvenile. Fish with a length of 7  $\pm$  1 cm were considered as fish fingerlings, and fish with a length of approximately 12  $\pm$  2 cm were considered as juveniles. Glass aquaria (90  $\times$  60  $\times$  60 inches) were used in the experiments. The fish were fed with fresh prawns at a rate of 3% of body weight per day, and the water (and test solutions) was replaced daily.

### Chemicals

Estrogen (E<sub>2</sub>; purity, 99%), *Micrococcus lysodeikticus*, nitro blue tetrazolium (NBT), 3,3',5,5'-tetra methyl benzidine, hen egg white lysozyme (HEWL), trypsin, and azocasein were obtained from Sigma Chemicals. All other chemicals used were of analytical grade.

### Toxicity test for immunotoxicological biomarkers

After acclimation, toxicity tests were performed following the standard guidelines of Environmental Protection Agency/

Republic of China [27]. The highest concentration (2,000 ng/L) employed in the present study was chosen to mimic endogenous levels of presumed accumulative estrogen in spawning female fish, as cited by Teles et al. [28], whereas the lowest concentration (200 ng/L) was chosen based on the highest concentrations found in the aquatic environment, as reported previously by Bowman et al. [29]. To assess changes in the biomarkers, both fingerling and juvenile fish were divided into four groups of 15 specimens each. Group I was reared in normal seawater, group II with a solvent control (95% ethanol), and groups III and IV exposed to seawater containing 200 and 2,000 ng/L, respectively, of estrogen. The test solution was replaced daily to provide a constant effect. Parallel duplicate tanks with identical experimental setup were used, and after 5, 15, and 30 d of exposure, three fish from each group were killed to assess their immune parameters.

### Sample collection

Blood samples were collected individually from the caudal vein of fish after days 5, 15, and 30 of exposure with a 24-gauge needle and a 2-ml syringe. Three fish in duplicate fingerlings or juveniles as one group were used at each exposure time. Blood samples were divided into two aliquots, one of which was heparinized (50 IU/ml blood) and the other allowed to clot at room temperature for 30 min and then kept at 4°C for 24 h. The clotted sample was centrifuged at 4,500 g for 5 min at 4°C to collect the serum, which was stored immediately at -80°C until use. The heparinized blood was immediately divided into two aliquots, one of which was used for the NBT assay and differential leukocyte counting and the other immediately centrifuged at 300 g for 5 min. Plasma was collected within half an hour of blood collection.

### Serum protein, albumin, and globulin contents

Serum protein was determined using the method described by Bradford [30]. Twenty microliters of diluted serum were added to each well in 96-well microtiter plates, and 200  $\mu$ l/well of Bradford solution (Sigma) was added. The absorbance was read at 595 nm. The albumin was determined using a diagnostic kit supplied by Nanning Jiancheng Bioengineering Institute following the method described by Weichselbaum [31]. Serum and standard were diluted with distilled water. First, 4.0 ml of reagent (bromocresol green solution) were placed in a sterile test tube, and then 0.02 ml of diluted serum was added. Similarly, a standard (4 ml of reagent and 0.02 ml of standard protein) and a blank (4 ml of reagent and 0.02 ml of distilled water) were taken in separate test tubes. Then, all test tubes were mixed well and kept for 5 min at room temperature. The optical density (OD) was measured at 640 nm against the blank. The serum globulin value was determined by subtracting the serum albumin value from the total serum protein value, and the albumin to globulin ratio was determined by dividing the albumin values by the globulin values.

### Plasma protein and total immunoglobulin assays

Plasma protein was determined using the method described by Bradford [30]. Twenty microliters of diluted plasma were added to each well in a 96-well microtiter plate, and 200  $\mu$ l/well of Bradford solution (Sigma) was added. The absorbance was read at 595 nm. The immunoglobulin concentration was measured using the method described by Klein and Siminovich [32] modified as follows: Immunoglobulins were precipitated with polyethylene glycol (10,000 kDa). Plasma (25

$\mu\text{l}$ ) was mixed with an equal volume of 12% polyethylene glycol solution for 2 h at room temperature under constant shaking. After centrifugation at 5,000 g for 15 min, the supernatant was collected and the concentration of protein determined as described above. The total immunoglobulin concentration was calculated by subtracting this value from the total plasma protein concentration.

#### *Differential leukocyte count*

A blood smear was prepared on a slide by using 20  $\mu\text{l}$  of heparinized blood and fixed immediately in 95% methanol for 5 min before staining according to the method described by Horton and Okamura [33]. Methanol-fixed blood smears were stained with Giemsa for 5 min. The slides were washed in tap water and allowed to dry before microscopic examination. Leukocytes were counted under the microscope in random fields until the count reached 100 cells/slide to calculate the percentage of monocytes, lymphocytes, neutrophils, and thrombocytes in the blood.

#### *Respiratory burst activity*

For the detection of intracellular superoxide anion production, the method described by Stasiack and Baumann [34] was followed. Briefly, 50  $\mu\text{l}$  of heparinized blood were placed into a U-bottomed Eppendorf tube and incubated at 37°C for 1 h to facilitate adhesion of cells. Then, the supernatant was removed, and the adhered cells were washed three times with phosphate buffer solution (PBS; pH 7.2). After washing, 50  $\mu\text{l}$  of 0.2% NBT were added, and the cells were incubated for a further 1 h. The tubes were shaken for 5 min and centrifuged at 1,500 rpm for 10 min, after which the supernatant was removed. The cells were fixed with 100% methanol for 2 to 3 min and washed twice in 30% methanol. After the final washing, the methanol was removed. The tubes were then air-dried, and 2 ml of extraction fluid (6 ml of KOH and 7 ml of dimethyl sulfoxide) were added into each tube to dissolve the deposits. Tubes were then centrifuged at 8,000 rpm for 15 min. The OD of the supernatant was read at 630 nm against the reagent blank in a spectrophotometer.

#### *MPO activity*

The total MPO content in the serum was measured according to the method described by Quade and Roth [35] with slight modification. Serum (15  $\mu\text{l}$ ) was diluted in 135  $\mu\text{l}$  of Hank's balanced salt solution ( $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  free) in a 96-well plate. To each well, 50  $\mu\text{l}$  of 20 mM 3,3',5,5'-tetra methyl benzidine and 5 mM  $\text{H}_2\text{O}_2$  were added. The reaction was stopped after 2 min by adding 50  $\mu\text{l}$  of 4 M sulfuric acid. The OD was read at 450 nm in a microplate reader.

#### *Antiprotease activity*

For assay of antiprotease activity, a modification of the method described by Ellis [36] was used. Briefly, 10  $\mu\text{l}$  of undiluted serum were incubated with the same volume of standard trypsin solution (5 mg/ml) for 30 min at 22°C. To this, 1 ml of azocasein dissolved in phosphate buffer (2.5 mg/ml; pH 7.0) was added, and the samples were incubated for 1 h at 22°C. The reaction was stopped by adding 500  $\mu\text{l}$  of 10% trichloroacetic acid, and to remove the precipitate, all tubes were centrifuged at 10,000 g for 5 min. Then, 100  $\mu\text{l}$  of the supernatant were transferred to a 96-well plate containing 100  $\mu\text{l}$  of 1 N NaOH per well. The OD was read at 450 nm. For the positive control, buffer replaced the serum, and for the

negative control, buffer replaced both serum and trypsin. The percentage inhibition of trypsin activity by each sample was calculated by comparing it to the 100% control sample.

#### *Serum bactericidal activity*

The serum was assayed for bactericidal activity following the method described by Rainger and Rowley [37]. For this assay, an *Aeromonas hydrophila* culture was centrifuged at 1,000 g for 15 min, and the pellet was washed and suspended in PBS. The OD of the suspension was adjusted to 0.509 at 600 nm. This bacterial suspension was serially diluted (1:10) with PBS. Serum bactericidal activity was determined by incubating 2  $\mu\text{l}$  of the diluted *A. hydrophila* suspension with 20  $\mu\text{l}$  of serum in a microvial for 1 h at 37°C. In the control group, PBS was used in place of the serum, and in the positive control, nutrient broth was used in place of the serum. After incubation, the number of viable bacteria was determined by counting the colonies that grew on a nutrient agar plate after 24 h at 37°C.

#### *Serum lysozyme assay*

Serum lysozyme assay was determined using a modified turbidimetric assay developed by Hutchinson and Manning [38]. Briefly, a 0.3 mg/ml suspension of freeze-dried *M. lysodeikticus* was prepared in 0.05 M  $\text{Na}_2\text{HPO}_4$  buffer immediately before use, and the pH was adjusted to 6.0 using a few drops of 1.0 M NaOH. Ten microliters of serum were added to 250  $\mu\text{l}$  of the bacterial suspension and allowed to equilibrate at 28°C. As an external standard, HEWL with a specified activity of 46,200 U/mg was used. After dissolving the HEWL in buffer at a concentration of 25,000 U/ml, this HEWL solution was added to each well (10  $\mu\text{l}$ /well). The reduction in absorbance at 450 nm was determined over a 10-min period at 28°C in a microplate reader. The standard curve was constructed using HEWL. The amount of lysozyme present in the serum was calculated from this standard curve.

#### *Statistical analyses*

Statistical comparisons were performed using a two-way analysis of variance (ANOVA) and SPSS software (Ver 10.0; SPSS). Briefly, duplicate experimental chambers were maintained for both control and experimental groups, with each containing 15 fish. Results are reported as the mean  $\pm$  standard deviation of six individuals per group per time point ( $n = 3$  fish/replication). The data were first tested for normality and homogeneity using Bartlett's test. Because all data were normal, we then determined, using ANOVA, whether the groups differed and if the ANOVA-calculated  $p$  value was significant ( $p \leq 0.05$ ). Tukey's multiple-comparison post hoc test was performed to identify statistical differences among individual treatment groups [39]. Spearman's correlation analysis was used to test for any interaction between respiratory burst and MPO activity.

## RESULTS

For all analyses,  $\text{E}_2$  was used at sublethal concentrations of 200 and 2,000 ng/L. Both fingerling and juvenile groups were exposed to these concentrations for 30 d, and assays were performed periodically. The behavior of the fish was observed to be normal. No mortality was recorded for either concentration tested for 30 d. The data presented in Figure 1 show the changes in serum protein, albumin, globulin, and albumin to globulin ratio. The serum protein significantly decreased in the fish exposed to the  $\text{E}_2$  concentration in all the exposure

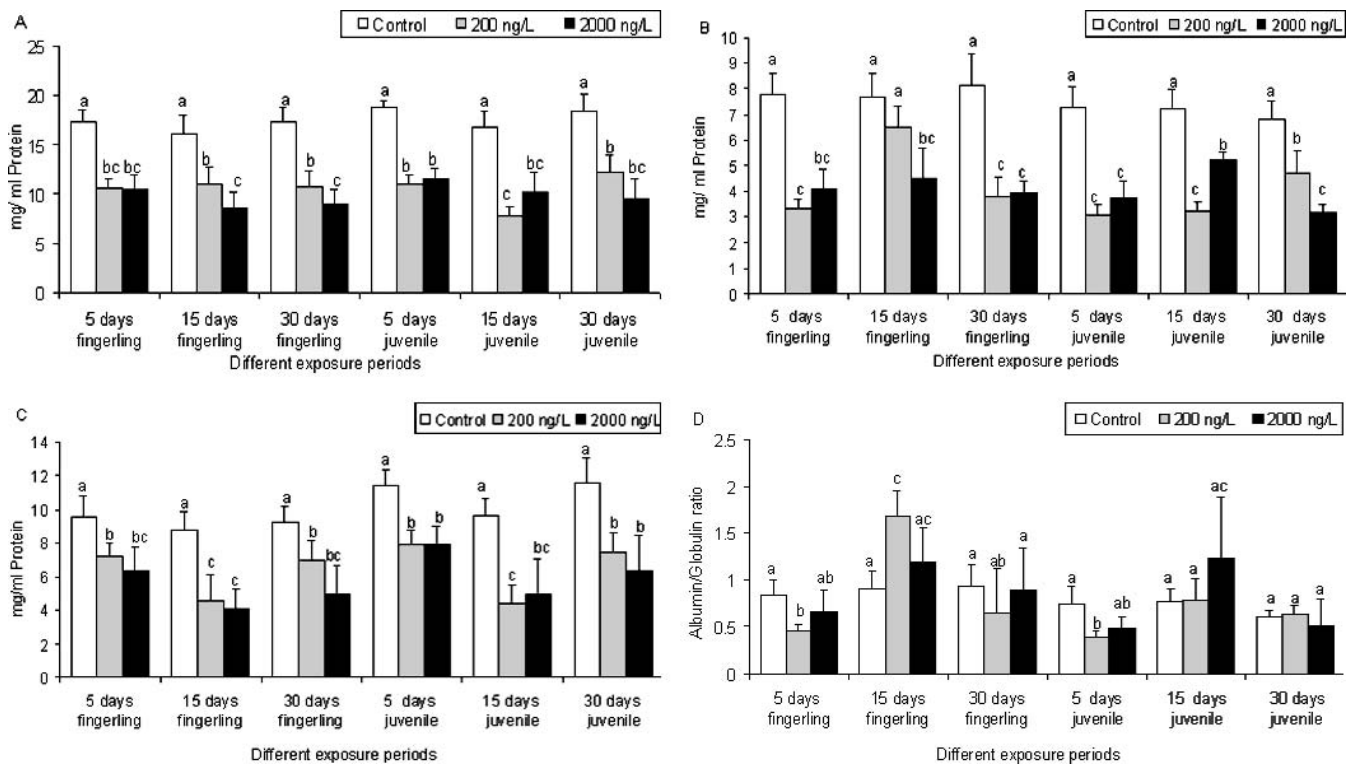


Fig. 1. Effect of 17 $\beta$ -estradiol on (A) protein content, (B) albumin content, (C) globulin content, and (D) albumin and globulin ratio in blood serum of *Lateolabrax japonicus*. Data, representing the mean  $\pm$  standard deviation of six determinations using samples from different preparations, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b, c) indicate no significant difference between exposure groups at different exposure periods, whereas different letters indicate statistically significant differences ( $p \leq 0.05$ ) between different exposure periods and groups.

periods, and the trend was similar for both fingerlings and juveniles (Fig. 1A).

The fingerling serum albumin level decreased nearly 48, 15, and 44% after 5, 15, and 30 d, respectively, of exposure at 200 ng/L, whereas it decreased nearly 50% in all the exposure periods when fingerlings were exposed to 2,000 ng/L (Fig. 1B). In juvenile fish, the serum albumin level decreased nearly 47, 45, and 30% at 2,000 ng/L after 5, 15, and 30 d, respectively, of exposure. At 200 ng/L, however, the albumin level decreased by 48, 28, and 53% after 5, 15, and 30 d, respectively, of exposure (Fig. 1B). A similarly significant decrease was observed for serum globulin level when both groups were exposed to E<sub>2</sub> concentrations for 5, 15, and 30 d (Fig. 1C). The albumin to globulin ratio showed a significant increase in fish fingerlings exposed to 200 ng/L after 15 d. Both groups exposed to 200 ng/L, however, showed a significant decrease in albumin to globulin ratio after 5 d of exposure (Fig. 1D).

The levels of plasma protein rose moderately in both groups exposed to E<sub>2</sub>, but the increase was statistically significant when fingerlings were exposed to 200 ng/L after 5 d of exposure and to 2,000 ng/L after 15 and 30 d of exposure (Fig. 2A). For the juvenile group, a significant change was observed after 5 d of exposure at 200 ng/L and after 15 d of exposure at 2,000 ng/L (Fig. 2A). Total immunoglobulin levels increased when both groups were exposed to E<sub>2</sub>, but the increase was statistically significant only for fingerlings exposed to 2,000 ng/L after 15 d when compared to the control. The juveniles, however, showed significant change at both E<sub>2</sub> concentrations after 5 d of exposure and at 2,000 ng/L after 15 d of exposure (Fig. 2B). The effects of E<sub>2</sub> on IgM and IgM-secreting cells

in different animal models as compared with the present study are presented in Table 1.

The differential leukocyte counts of *L. japonicus* control and experimental groups are shown in Figure 3. The number of thrombocytes increased significantly in fish fingerlings exposed to both E<sub>2</sub> concentrations when compared to the control after 5 d of exposure. This decreased significantly, however, when juveniles were exposed to both E<sub>2</sub> concentrations, except at 200 ng/L for 5 d (Fig. 3A). In fingerlings, lymphocyte counts decreased significantly after 5 d of exposure to both concentrations of E<sub>2</sub>; however, no significant change was observed after 15 and 30 d of exposure. The juvenile group exposed to E<sub>2</sub> concentrations showed no significant change in lymphocyte count when compared to the control (Fig. 3B). The neutrophil count decreased significantly in fingerlings exposed to 2,000 ng/L after 5 d, whereas it increased significantly when the juveniles were exposed to 2,000 ng/L for 5 d and to 200 ng/L for 15 d (Fig. 3C). The monocyte count decreased significantly in fingerlings exposed to 2,000 ng/L after 5 d. The monocyte count increased in the juvenile group exposed to E<sub>2</sub>, but this increase was not statistically significant (Fig. 3D).

The respiratory burst activity of *L. japonicus* leukocytes increased significantly when the fish fingerlings were exposed to 2,000 ng/L after 5 d, and in both E<sub>2</sub> concentrations after 15 d, when compared to the control (Table 2). Interestingly, this decreased significantly in fingerlings exposed to 2,000 ng/L after 30 d of exposure when compared to the control. The respiratory burst activity in juveniles showed a significant increase after all the exposure periods, however, in both E<sub>2</sub> concentrations except at 200 ng/L after 30 d. The MPO activity increased when the fish fingerlings were exposed to both con-

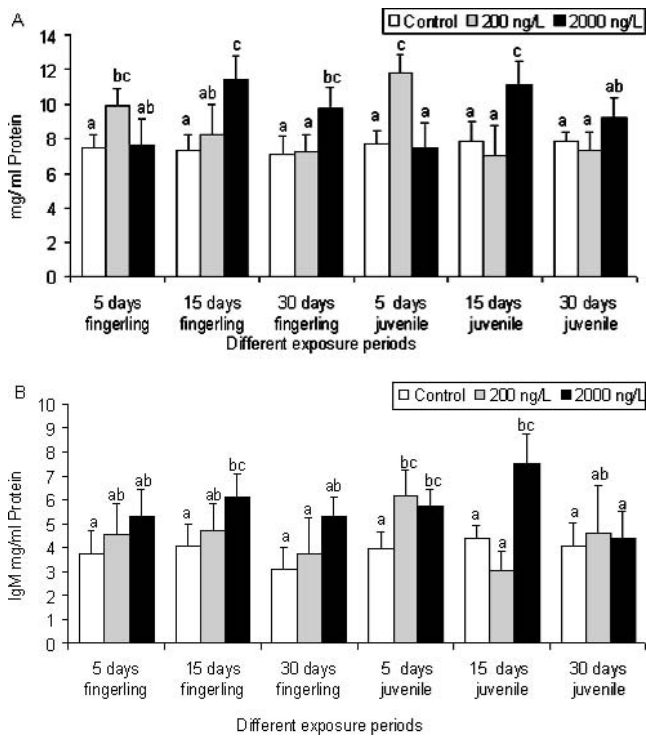


Fig. 2. Effect of 17 $\beta$ -estradiol on (A) protein content and (B) total immunoglobulin in blood plasma of *Lateolabrax japonicus*. Data, representing the mean  $\pm$  standard deviation of six determinations using samples from different preparations, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b, c) indicate no significant difference between exposure groups at different exposure periods, whereas different letters indicate statistically significant differences ( $p \leq 0.05$ ) between different exposure periods and groups.

concentrations of E<sub>2</sub> up to 15 d, and the increase in activity was statistically significant when compared to the control (Table 2). After 30 d of exposure, however, the MPO activity decreased significantly in fingerlings exposed to 2,000 ng/L when compared to the control. The juveniles exposed to E<sub>2</sub> concentrations did not show any significant increase in MPO activity after 5 or 30 d of exposure but did show a significant increase after 15 d of exposure.

The effect of sublethal concentrations of E<sub>2</sub> on the anti-protease activity of *L. japonicus* is shown in Figure 4. The results revealed that E<sub>2</sub> had a significant effect on anti-protease activity in fingerlings after 15 d of exposure in both concentrations when compared to the control. Juveniles exposed to E<sub>2</sub> at sublethal concentrations revealed similar results for anti-protease activity as seen after 15 and 30 d of exposure.

The alteration in serum bactericidal activity observed in both the fingerling and juvenile groups exposed to E<sub>2</sub> concentration is shown in Figure 5. The serum bactericidal activity

decreased significantly after 5 d of exposure when the fingerlings were exposed to both E<sub>2</sub> concentrations. Similarly, for juveniles, serum bactericidal activity decreased at 2,000 ng/L after 5 d and at both concentrations after 30 d of exposure. The changes in the level of the serum lysozyme activity are shown in Figure 6. The lysozyme activity decreased significantly in fingerlings exposed to both concentrations of E<sub>2</sub> after 5, 15, and 30 d of exposure. A similar trend was observed for juveniles exposed to E<sub>2</sub> concentrations. After 30 d of exposure, however, juvenile fish exposed to 200 ng/L showed no significant decrease in activity.

## DISCUSSION

Our results showed that sublethal concentrations of E<sub>2</sub> significantly modulated the immune response of the Japanese sea bass (*L. japonicus*), because the majority of the parameters reflecting cellular and humoral responses were affected. To our knowledge, the present study is the first to examine the effect of E<sub>2</sub> over long-term (30-d) exposure on the immune competence of this fish.

Total protein and lysozyme levels were thought to be measurable humoral components of the nonspecific defense mechanism [40]. The decrease in levels of total protein, globulin, and lysozyme as well as the change in the albumin to globulin ratio was observed in our present study, showing the degree of immunomodulation. Changes in blood protein levels because of pollutant exposure have been reported [41,42]; the pollutant is suggested to react with the cell nucleoproteins and nucleic acids, resulting in altered protein synthesis and cell integrity. In the present study, the immunoglobulin level was higher in estrogen-exposed fish compared to the control, with the exception of day 15 for juveniles and day 30 for fingerlings in the lower concentration (200 ng/L). The levels increased as the concentration of estrogen increased, and long-term exposure to estrogen resulted in a significant increase in the production of total immunoglobulin in exposed fish compared to that in the control. Conversely, in earlier reports, E<sub>2</sub> suppressed the IgM-producing cell in a dose-dependent manner [22], and E<sub>2</sub> treatment suppressed production of IgM and decreased resistance to disease in fish [13,18,22]. The contradiction in the results among studies (Table 1) may be caused by the concentrations of E<sub>2</sub> used, the mode of exposure, and the sizes or different species of fish tested.

Interestingly, modulation of differential leukocyte counts differed between fingerlings and juveniles when the fish were exposed to E<sub>2</sub> concentrations. The lymphocytes decreased significantly only in fingerlings after 5 d of exposure, but no significant changes were observed in either group during the exposure period. As described by Filby et al. [19], reductions in the number of circulating lymphocytes were indicative that immunosuppression occurred in fish exposed to the potent estrogenic effluent. In addition, both neutrophils and monocytes

Table 1. Effect of 17 $\beta$ -estradiol on immunoglobulin M (IgM) and IgM-secreting cells in different animal models

Parameters involved	Organisms tested	Size groups	Changes	References
Total IgM	Fish gilthead seabream ( <i>Sparus aurata</i> )	Sexually immature male	Suppression	Cuesta et al. [13]
IgM	Fish ( <i>Oncorhynchus mykiss</i> )	Juvenile	Suppression	Hou et al. [18]
IgM-producing cells	Fish ( <i>O. mykiss</i> )	Juvenile	Suppression	Yi and Dong [21]
IgM	Fish ( <i>O. mykiss</i> )	Juvenile	Suppression	Hou et al. [22]
IgM-secreting cells	Fish ( <i>Cyprinus carpio</i> )	Sexually immature (sex not mentioned)	No effect	Saha et al. [60]
IgM level	Mice and hamster	Adult	Increase	Grossman [61]
Total IgM	Fish ( <i>Lateolabrax japonicus</i> )	Fingerling/juvenile	Increase	Present study

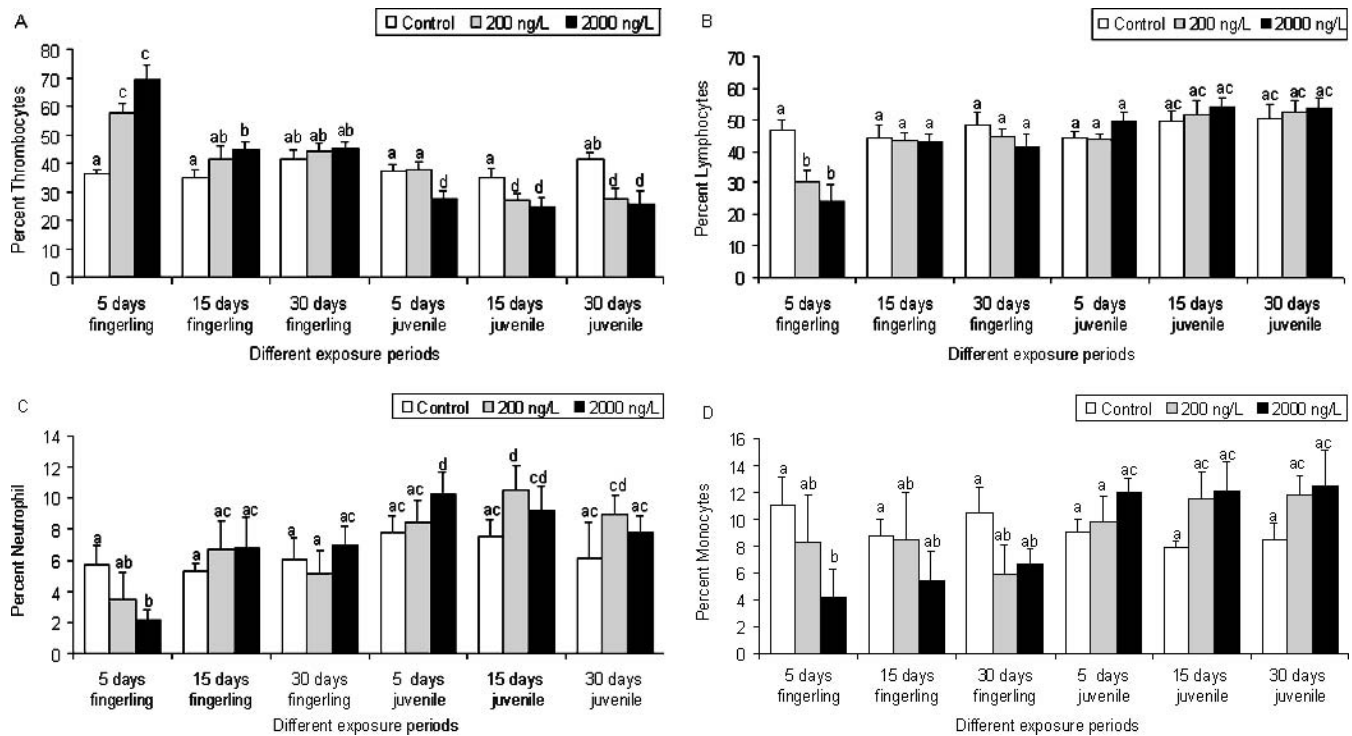


Fig. 3. Immunotoxic responses in fish exposed to a estrogenic concentration quantified by counts of (A) thrombocytes, (B) lymphocytes, (C) neutrophils, and (D) monocytes shown as percentages of total leukocytes. Data, representing the mean  $\pm$  standard deviation of six determinations using samples from different preparations, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b, c, d) indicate no significant difference between exposure groups at different exposure periods, whereas different letters indicate statistically significant differences ( $p \leq 0.05$ ) between different exposure periods and groups.

significantly decreased in fingerlings after 5 d of exposure, whereas an increasing trend was observed in juveniles through the exposure period. The increases in the number of circulating granulocytes and thrombocytes were indicative of nonspecific immune activation [19].

Respiratory burst assay has been used recently to investigate the effects of exposure to environmental toxicants on the immune function of fish [43–45]. The increase in respiratory burst activity in fish is a measure of oxidative radical production because of estrogen exposure, and the reactive oxygen species produced during the respiratory burst are thought of

as potent microbicidal agents [46], which indicates the modulation of nonspecific immunity. Enhanced respiratory burst activity in both groups exposed to estrogen was observed in the present study, and the increase was dependent on concentration and exposure period. The result was consistent with the report of Yamaguchi et al. [47]; in their study, carp macrophage respiratory burst was elicited gradually by increased in vitro estradiol from 0.1 to 1,000 nmol/L. After 30 d of exposure, however, the respiratory burst activity decreased in fingerlings compared to the respective control, whereas the juvenile group showed almost the same level as the control in the lower con-

Table 2. The effect of 17 $\beta$ -estradiol on respiratory burst and myeloperoxidase activity of *Lateolabrax japonicus*<sup>a</sup>

Concentration of 17 $\beta$ -estradiol	Respiratory burst		Myeloperoxidase	
	Fingerling	Juvenile	Fingerling	Juvenile
5 d				
Control	0.103 $\pm$ 0.006 A	0.112 $\pm$ 0.003 A	1.54 $\pm$ 0.05 A	1.69 $\pm$ 0.07 A
200 ng/L	0.122 $\pm$ 0.007 AB	0.142 $\pm$ 0.007 B	1.86 $\pm$ 0.08 B	1.70 $\pm$ 0.08 A
2,000 ng/L	0.132 $\pm$ 0.005 B	0.153 $\pm$ 0.012 B	1.92 $\pm$ 0.06 B	1.80 $\pm$ 0.13 AB
15 d				
Control	0.106 $\pm$ 0.009 A	0.117 $\pm$ 0.005 A	1.46 $\pm$ 0.19 A	1.40 $\pm$ 0.18 AC
200 ng/L	0.150 $\pm$ 0.006 C	0.166 $\pm$ 0.020 BC	1.95 $\pm$ 0.12 B	1.90 $\pm$ 0.12 B
2,000 ng/L	0.154 $\pm$ 0.013 C	0.194 $\pm$ 0.013 C	1.98 $\pm$ 0.10 B	1.98 $\pm$ 0.10 B
30 d				
Control	0.117 $\pm$ 0.006 A	0.111 $\pm$ 0.005 A	1.61 $\pm$ 0.06 A	1.68 $\pm$ 0.07 A
200 ng/L	0.093 $\pm$ 0.008 A	0.114 $\pm$ 0.013 A	1.19 $\pm$ 0.12 AC	1.12 $\pm$ 0.20 AC
2,000 ng/L	0.070 $\pm$ 0.013 D	0.140 $\pm$ 0.017 B	1.04 $\pm$ 0.02 C	1.35 $\pm$ 0.18 AC

<sup>a</sup> Values are expressed as optical density (respiratory burst at 630 nm and myeloperoxidase at 450 nm). Data, representing the mean  $\pm$  standard deviation of six determinations using samples from different preparations, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (A, B, C, D) indicate no significant difference between exposure groups at different exposure periods, whereas different letters indicate statistically significant differences ( $p \leq 0.05$ ) between different exposure periods and groups.

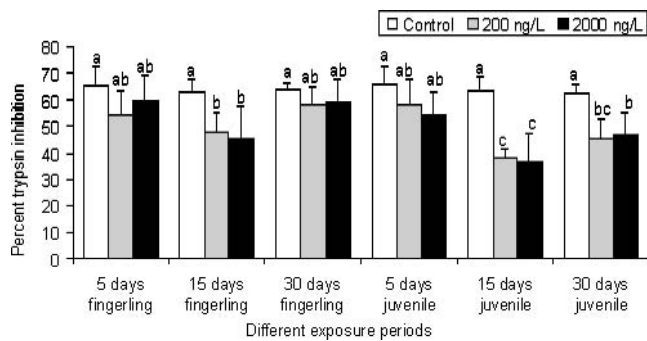


Fig. 4. Effect of  $17\beta$ -estradiol on antiprotease activity in blood serum of *Lateolabrax japonicus*. Data, representing the mean  $\pm$  standard deviation of six determinations using samples from different preparations, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b, c) indicate no significant difference between exposure groups at different exposure periods, whereas different letters indicate statistically significant differences ( $p \leq 0.05$ ) between different exposure periods and groups.

centration. The observation in the present study demonstrates that fingerlings were more sensitive than juveniles to  $E_2$  exposure.

Respiratory burst with the formation of superoxide anions, hydrogen peroxide, singlet oxygen, and hydroxyl radicals released by phagocytes is an important mechanism for the protection of an animal against many pathogens and for repairing its own damaged tissue [45]. The present study revealed that long-term exposure to  $E_2$  concentration decreased intracellular production of reactive oxygen species. When exposed to a toxicant, a decrement in production of reactive oxygen species may reduce an animal's ability to effectively kill ingested pathogens, thereby increasing the host's susceptibility to infectious agents [48,49]. Modulation of intracellular  $O_2^-$  generation after 5- and 15-d exposures possibly showed that intracellular generation was induced by  $E_2$ . Levels were initially low (5-d exposure period) but increased significantly compared to those of the control fish, and the levels were highest after 15 d of exposure. Interestingly, when juveniles were exposed to lower concentrations of  $E_2$ , the respiratory levels returned to almost the same level as the control after 30 d of exposure, indicating the possibility of juvenile fish adaptation to this concentration. All these results implied toxicity of  $E_2$  over a period of time

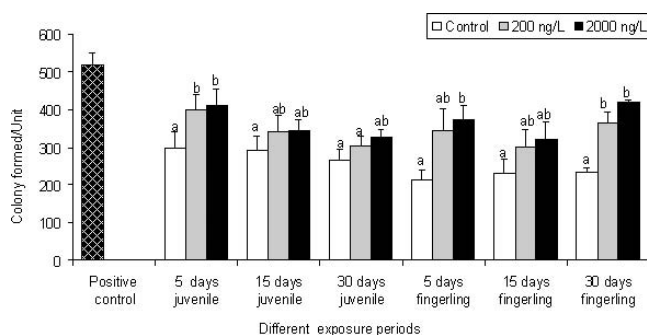


Fig. 5. Effect of  $17\beta$ -estradiol on serum bactericidal activity of *Lateolabrax japonicus*. Data, representing the mean  $\pm$  standard deviation of six determinations using samples from different preparations, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b) indicate no significant difference between exposure groups at different exposure periods, whereas different letters indicate statistically significant differences ( $p \leq 0.05$ ) between different exposure periods and groups.

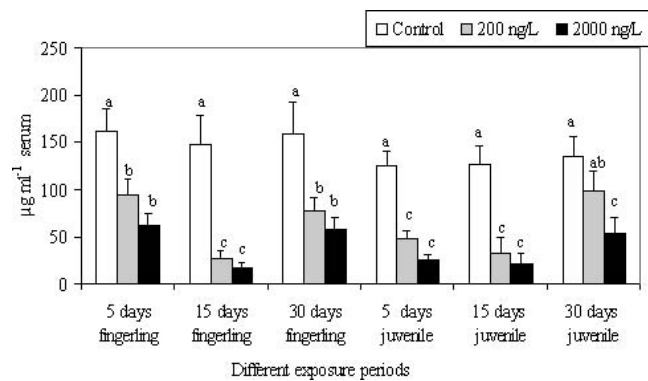


Fig. 6. Effect of  $17\beta$ -estradiol on lysozyme activity in blood serum of *Lateolabrax japonicus*. Data, representing the mean  $\pm$  standard deviation of six determinations using samples from different preparations, were analyzed using two-way analysis of variance followed by Tukey's post hoc test. The same letters (a, b, c) indicate no significant difference between exposure groups at different exposure periods, whereas different letters indicate statistically significant differences ( $p \leq 0.05$ ) between different exposure periods and groups.

caused by the increase in the number of days of  $E_2$  exposure, and the results also implied that prolonged exposure could cause the animal to adapt to the conditions, as evidenced by the fact that most parameters, such as MPO, lysozyme, bactericidal activity, albumin to globulin ratio, plasma protein, immunoglobulin, and circulating leukocytes, showed no significant changes (these results are summarized in Table 3). This finding is similar to that of Thiagarajan et al. [50], who report that mussels adapt to metal concentration after 25 d of exposure.

Myeloperoxidase activity showed a trend similar to that observed for respiratory burst (Table 2). A positive correlation (data not shown) was observed between the respiratory burst and MPO, suggesting that the majority of the  $O_2^-$  formed during this respiratory burst was converted to the bactericidal oxidant hypochlorous acid via a series of reactions involving superoxide dismutase and MPO. Myeloperoxidase levels can be correlated with neutrophil activation in a variety of conditions where neutrophils contribute to pathological conditions.

Although the serum of control fish exhibited increased antiprotease activity compared to the exposed fish throughout the study period, the difference was only significant after 15 d of exposure to 2,000 ng/L. This indicated that the serum of the exposed fish decreased the level of protease inhibitors. Lysozyme present in mucous secretions, blood, and other areas of virtually all eukaryotic organisms is a hydrolytic enzyme able to cleave the cell wall of gram-positive and some gram-negative bacteria. Lysozyme is found in most tissues and secretions of fish, and in immunotoxicological studies, lysozyme levels most frequently are examined in plasma or serum [51]. In the present study, exposure to estrogen caused a significant decrease in lysozyme activity (Fig. 6). The decrease was drastic after 5 and 15 d of exposure, but both groups eventually recovered after 30 d of exposure. The values differed significantly from the control group after 5 and 15 d of exposure, and the lysozyme value increased in both groups. O'Neill [52] and Snarski [53] report that toxicants affect lysozyme levels causing alterations in immunoregulatory functions in fish and that the lysozyme concentration in fish blood increases following infections or injections of foreign material or pathogens [54]. The decrease in resistance against *A. hydrophila* in fish exposed to estrogen can be explained by a decrease in the

Table 3. Summary of 17 $\beta$ -estradiol-induced immunomodulation<sup>a</sup>

Immune parameter	5 d		15 d		30 d	
	200 ng/L	2,000 ng/L	200 ng/L	2,000 ng/L	200 ng/L	2,000 ng/L
Serum protein	▼▼	▼▼	▼▼	▼▼	▼▼	▼▼
Albumin	▼▼	▼▼	▼	▼▼	▼▼	▼▼
Globulin	▼▼	▼▼	▼▼	▼▼	▼▼	▼▼
AGR	▼▼		▲			
Plasma protein	▲▲			▲▲		▲
Immunoglobulin	△	△		▲▲		
Thrombocytes	▲	▲▼	▼	▼	▼	▼
Lymphocytes	▼	▼				
Neutrophils		▼△	△			
Monocytes		▼				
Respiratory burst	△	▲▲	▲▲	▲▲		▼△
Myeloperoxidase	▲	▲	▲▲	▲▲		▼
Antiprotease			▼▼	▼▼	▼	▼
Lysozyme	▼▼	▼▼	▼▼	▼▼	▼	▼▼
Bactericidal	▼	▼▼			▼	▼

<sup>a</sup> Symbols represent statistically significant ( $p < 0.05$ ) increases (▲△), decreases (▼▼), and the cells without symbols represent no significant change when compared with the control. ▲ or ▼ = fingerling; △ or ▽ = juvenile; AGR = albumin:globulin ratio.

bactericidal activity of the serum. Serum bactericidal activity decreased during all exposure periods in both groups when compared to the control. The higher bactericidal activities possibly resulted from a higher lysosomal activity, as was evidenced in the control fish during the present study. Thus, sublethal concentrations of estrogen had effects on the immune system of *L. japonicus*, although it is imperative to note that such an alteration in immune parameters in the present study appeared to be more pronounced over short-term exposure.

Although the exact mechanisms involved in the toxicity of E<sub>2</sub> on the immune response of Japanese sea bass has not yet been established, our results showed that Japanese sea bass were sensitive to E<sub>2</sub> exposure. Moreover, long-term (30-d) exposure showed some level of adaptation by fish to estrogen-induced stress, possibly by altering their immune system to the duration and nature of exposure. In addition, the toxicant effect on one system clearly leads to subsequent effects on others, resulting in widespread disruption of normal physiological functions. The sublethal levels of the estrogen exerted an effect on immune competence, and the results revealed that E<sub>2</sub> effects were dose dependent. The higher concentration of E<sub>2</sub> caused significant effects (Table 3); conversely, the low concentration showed no significant effects in most of the parameters studied (Table 3). Although the concentration of 2,000 ng/L is relatively higher when compared to the existing environmental concentration, such levels in animals are quite possible as a result of bioaccumulation (a strong possibility in highly polluted natural environments), and these would have more drastic effects on the immune competence of the fish by suppressing the immune function at elevated estrogen concentration [55]. Environmental estrogens may affect the immune system by altering cytokine regulation. In fish, the natural estrogen E<sub>2</sub>,  $\beta$ -naphthoflavone, and 4-nonylphenol have been reported to cause genotoxic effects [28,56]. More studies show that exposure to treated sewage effluents containing various estrogenic compounds also has been associated with deleterious effects on gonad differentiation and development in various species of fish [57–59]. Therefore, environmental estrogens may affect the immune system directly or, more likely, indirectly through several tissues, and these estrogens may modulate the immune system by altering the patterns of cytokines and apoptosis [23].

In conclusion, this comparative short- and long-term sublethal exposure study of EDC-induced immunomodulation in both fingerlings and juveniles of *L. japonicus* showed changes caused by estrogen on the immune system. The results clearly reveal that E<sub>2</sub> significantly induced immunomodulation at higher concentration in all the exposure periods (Table 3). Fingerlings were more sensitive than juveniles when exposed to E<sub>2</sub>, but the response trend of all the parameters except for the leukocyte counts and respiratory burst activity for both sizes of fish were similar during the exposure period. The observed discrepancy in immune response between the fingerlings and juveniles may be caused by size variability and individual response of the fish exposed to E<sub>2</sub>, as mentioned previously [23]. Such a comparative study of fingerling and juvenile stages after long-term exposure throws more light on the long-term effects of toxicants on the parameters associated with immunity of animals. Nevertheless, this analysis is not definitive in terms of its conclusions, because the immune system is subject to seasonal variations among the groups, principally in the leukocyte population, the animal habitat, and other environmental factors, all of which together have a profound influence on the overall response to toxicants.

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