



Original Research Paper

Field-Based Assessment of Hematological, Antioxidant, and Innate Immune Responses in Cultured *Mugil cephalus* from Ponneri, Tamil Nadu, India

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Abstract

Aquaculture systems are frequently exposed to variations in water quality that can alter the physiological condition, oxidative balance, and immune competence of cultured fish. The present study evaluated hematological, antioxidant, and innate immune responses in juvenile grey mullet (*Mugil cephalus*) collected from three culture ponds at Pioneer Aquaculture Consultants, Ponneri, Tamil Nadu, India. Fourteen fish were sampled on 5 May 2026, comprising four fish from Pond 1, four from Pond 2, and six from Pond 3. Differential leukocyte profiles, plasma total protein concentration, erythrocyte superoxide dismutase (SOD) and catalase (CAT) activities, and plasma lysozyme activity were determined to assess the integrated physiological response of the fish. Lymphocytes were the predominant leukocyte population in all ponds; however, their proportion decreased from $68.4 \pm 4.2\%$ in Pond 1 to $52.6 \pm 6.3\%$ in Pond 3, whereas neutrophils increased from $18.2 \pm 3.1\%$ to $32.8 \pm 4.7\%$. Total protein concentration declined from 42.8 ± 3.6 to 33.9 ± 3.8 mg/mL. In contrast, antioxidant and innate immune responses increased progressively across the ponds. SOD activity increased from 18.6 ± 2.1 to 31.7 ± 3.4 U/mg protein, CAT activity increased from 12.4 ± 1.5 to 21.6 ± 2.7 U/mg protein, and lysozyme activity increased from 15.8 ± 1.9 to 27.9 ± 3.1 U/mL. Pond 3 therefore exhibited the most pronounced integrated biomarker response, characterized by reduced lymphocyte percentage and total protein concentration and increased neutrophil percentage, SOD, CAT, and lysozyme activities. These findings indicate that the combined assessment of hematological, antioxidant, and innate immune biomarkers can provide a useful early-warning approach for monitoring physiological stress in cultured fish. However, because of the relatively small and unequal sample sizes and the absence of matched pond-specific water-quality measurements for all sampled ponds, the observed relationships should be interpreted as exploratory associations rather than definitive causal effects.

Keywords: Aquaculture; cultured fish; water-quality variation; environmental stress; hematological biomarkers; oxidative stress; antioxidant enzymes; innate immunity; lysozyme activity; complement activity; respiratory burst activity; fish health; biomonitoring; Pioneer Aquaculture Consultants; Ponneri; Tamil Nadu.

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1. Introduction

Aquaculture plays an important role in meeting the increasing demand for fish protein and supporting rural livelihoods. However, intensive and semi-intensive culture systems are frequently affected by fluctuations in water quality associated with stocking density, feed inputs, organic-matter accumulation, metabolic waste, seasonal variation, and inadequate water exchange. Changes in temperature, pH, dissolved oxygen, ammonia, nitrite, salinity, and other physicochemical parameters can disturb physiological homeostasis in cultured fish and may reduce growth, disease resistance, survival, and production efficiency.

Pioneer Aquaculture Consultants operates the J.K. Centre for Sustainable Aquaculture Research and Development at Manopuram Village, Ponneri, Tamil Nadu, India. The centre is involved in aquaculture consultancy, project development, input supply, and research activities related to fish and other cultured aquatic organisms. Ponneri is also an important location for fisheries education, research, and aquaculture development. These facilities provide a relevant setting for evaluating the biological responses of cultured fish under variable farm-based conditions.

Fish respond to environmental stress through coordinated hematological, biochemical, antioxidant, and immune changes. Hematological parameters such as erythrocyte count, hemoglobin concentration, hematocrit, total leukocyte count, and differential leukocyte count provide useful information about oxygen transport, metabolic condition, tissue function, and immune status. Alterations in these parameters may occur before visible signs of disease or mortality and may therefore serve as early indicators of physiological disturbance.

Poor or fluctuating environmental conditions may also increase the production of reactive oxygen species. When reactive oxygen species production exceeds the capacity of the antioxidant defense system, oxidative stress may develop, leading to cellular dysfunction and tissue damage. Antioxidant biomarkers, including superoxide dismutase and catalase, are useful indicators of antioxidant defense capacity. Malondialdehyde and other lipid-peroxidation products may provide additional evidence of oxidative damage.

The innate immune system is another important component of fish health. Lysozyme activity and leukocyte profiles are commonly used to evaluate nonspecific immune competence in fish. Changes in these biomarkers may indicate immune activation, suppression, or physiological stress. Therefore, innate immune biomarkers can complement hematological and antioxidant measurements.

Keywords

Aquaculture; *Mugil cephalus*; water-quality variation; environmental stress; hematological

biomarkers; oxidative stress; antioxidant enzymes; innate immunity; lysozyme activity; fish health; biomonitoring; Ponneri; Tamil Nadu.

2. Materials and Methods

2.1 Study site and sampling design

The study was conducted at Pioneer Aquaculture Consultants, Ponneri, Tiruvallur District, Tamil Nadu, India. Juvenile grey mullet, *Mugil cephalus*, were collected from three culture ponds on 5 May 2026.

Four fish were collected from Pond 1, four from Pond 2, and six from Pond 3, giving a total of 14 fish. The fish weighed approximately 8–12 g and measured 5–9 cm in total length. The study was designed as a field-based assessment of hematological, antioxidant, and innate immune biomarkers in cultured fish.

2.2 Fish collection and transport

Fish were collected using a clean cast net and immediately transferred into clean, aerated containers containing water from the respective culture ponds. The fish were transported to the laboratory under aerated conditions and processed immediately after arrival.

Each fish was examined for visible external abnormalities, lesions, fin damage, abnormal pigmentation, and other apparent signs of disease. Apparently healthy fish were selected for analysis. The body weight and total length of each fish were recorded individually.

2.3 Water sampling and analysis

Water samples were collected from approximately 20–30 cm below the water surface in each of the three ponds on 5 May 2026. Clean, labelled sampling bottles were used, and care was taken to avoid disturbing the bottom sediment.

The water-quality parameters assessed were:

- Temperature.
- pH.
- Dissolved oxygen.
- Salinity.

The measurements were performed using calibrated instruments and applicable procedures described in *Standard Methods for the Examination of Water and Wastewater*, 24th edition.

Only water-quality measurements corresponding to Ponds 1–3 and collected on the same sampling date should be used for comparison with the fish-health biomarkers. Data from separately labelled experimental groups or different culture periods should not be presented as pond-specific data.

2.4 Fish sacrifice and blood collection

Fish were sacrificed according to the approved laboratory procedure. Blood was collected immediately after sacrifice by caudal-vein puncture using a sterile syringe and needle. Approximately 0.5–1.2 mL of blood was obtained from each fish, resulting in a total blood volume of approximately 8 mL. The blood was transferred into sterile tubes containing EDTA as an anticoagulant. Samples

were maintained under chilled conditions and processed within 20 min of collection. Blood smears were prepared immediately, while the remaining blood was used for plasma separation and erythrocyte preparation.

2.5 Blood-smear preparation and Giemsa staining

Peripheral blood smears were prepared from freshly collected EDTA-treated blood. A small drop of blood was placed on a clean glass slide and spread using a second slide to produce a thin smear.

The smears were air-dried and stained with 10% Giemsa stain prepared in phosphate buffer at pH 7.2. The smears were stained for 10–15 min, gently rinsed with phosphate buffer, air-dried, and examined under a light microscope. General cell morphology was initially observed under lower magnification. Detailed examination of erythrocytes and leukocytes was performed at 1000× magnification using an oil-immersion objective. For differential leukocyte analysis, at least 100 leukocytes were counted per smear and classified according to their morphological characteristics. The relative proportions of the leukocyte types were calculated and expressed as percentages.

2.6 Plasma preparation and storage

Following blood collection, EDTA-treated samples were processed under chilled conditions. The blood samples were centrifuged at 12,000 rpm for 10 min at 4 °C using a Remi CM-12 Plus centrifuge (Remi Elektrotechnik Ltd., India). After centrifugation, the plasma fraction was carefully separated without disturbing the cellular layer and transferred into sterile, labelled microcentrifuge tubes. Plasma samples were stored at –80 °C until biochemical and innate immune analyses. Repeated freeze–thaw cycles were avoided.

2.7 Preparation of the erythrocyte fraction

After plasma separation, the erythrocyte fraction was collected for antioxidant enzyme analysis. The erythrocytes were washed three times with chilled physiological saline. Following each washing step, the erythrocyte fraction was centrifuged under chilled conditions to remove residual plasma and other contaminants. The washed erythrocyte fraction was used to determine superoxide dismutase and catalase activities.

2.8 Determination of total protein

Total protein concentration was determined using the Bradford method described by Bradford (1976). An appropriate volume of sample was mixed with Coomassie Brilliant Blue G-250 reagent and incubated for 10 min at room temperature. Absorbance was measured at 595 nm using a spectrophotometer. Bovine serum albumin was used as the protein standard. Total protein concentration was calculated from the corresponding standard curve and expressed as

mg/mL. Protein concentration was used to normalize SOD and CAT activities.

2.9 Superoxide dismutase activity

Superoxide dismutase activity was determined in the washed erythrocyte fraction following the method of Misra and Fridovich (1972). The assay was based on the inhibition of epinephrine autooxidation by SOD under alkaline conditions.

The reaction mixture was prepared using an appropriate carbonate buffer and epinephrine substrate according to the validated laboratory protocol. The change in absorbance was monitored spectrophotometrically at 480 nm.

SOD activity was calculated based on the inhibition of epinephrine autooxidation and expressed as units per milligram of protein.

2.10 Catalase activity

Catalase activity was determined in the washed erythrocyte fraction according to the method described by Aebi (1984). The assay was based on the decomposition of hydrogen peroxide by catalase.

The reaction mixture contained an appropriate concentration of hydrogen peroxide prepared in a suitable buffer according to the validated laboratory protocol. The decrease in absorbance resulting from hydrogen peroxide decomposition was monitored spectrophotometrically at 240 nm.

Catalase activity was calculated from the rate of hydrogen peroxide degradation and expressed as units per milligram of protein.

2.11 Lysozyme activity

Innate immune analysis was performed using the stored plasma samples. Lysozyme activity was determined using a validated turbidimetric method with *Micrococcus lysodeikticus* as the substrate.

The decrease in absorbance resulting from bacterial-cell lysis was monitored spectrophotometrically. Lysozyme activity was expressed as units per millilitre of plasma.

Complement activity and respiratory burst activity were not included because these assays were not documented as having been performed.

2.12 Assay replication

Total protein, SOD, CAT, and lysozyme assays were performed in triplicate for each individual fish. The mean of the technical replicates was used for subsequent statistical analysis.

Technical replicates were not treated as independent biological replicates. Individual fish were considered biological replicates, while pond identity was retained as a sampling factor.

2.13 Statistical analysis

All data were expressed as mean $\pm$ standard deviation (SD). The biological replicate numbers were $n = 4$ for Pond 1, $n = 4$ for Pond 2, and $n = 6$ for Pond 3.

Statistical analysis was performed using GraphPad Prism version 10.0. Data normality was assessed before statistical comparisons. Differences among

the three pond groups were evaluated using one-way analysis of variance followed by an appropriate post hoc multiple-comparison test.

When parametric assumptions were not satisfied, an appropriate non-parametric test was applied. A probability level of $p < 0.05$ was considered statistically significant.

Exact p -values should be reported wherever they are available from the original statistical output. Exact p -values cannot be calculated from the reported means, standard deviations, and sample sizes alone.

Because the study included only 14 fish and unequal numbers of fish from the three ponds, the findings were interpreted as exploratory. Effect sizes, mean differences, variability, and biological response patterns were considered in addition to p -values. Formal correlations between water-quality parameters and fish-health biomarkers were not performed because matched pond-specific water-quality measurements were not available.

3. Results

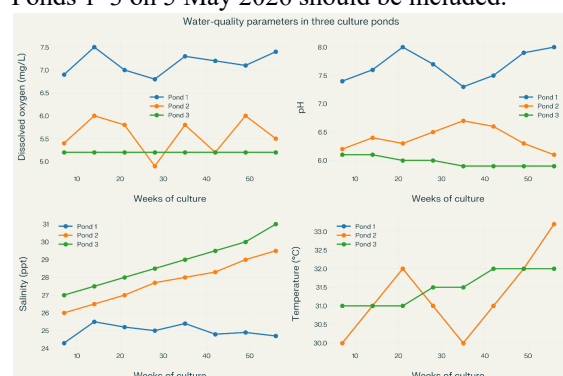
3.1 Fish sampling and sample collection

Fourteen juvenile *Mugil cephalus* were collected from three culture ponds on 5 May 2026. Four fish were collected from Pond 1, four from Pond 2, and six from Pond 3.

The fish weighed 8–12 g and measured 5–9 cm in total length. Approximately 0.5–1.2 mL of blood was collected from each fish, giving a total blood volume of approximately 8 mL.

3.2 Water-quality parameters

The water-quality measurements should be presented in graph 1. Only values recorded from Ponds 1–3 on 5 May 2026 should be included.



3.3 Differential leukocyte profile

Lymphocytes were the predominant leukocyte population in all three ponds. The lymphocyte percentage decreased progressively from Pond 1 to Pond 3, whereas the neutrophil percentage increased.

The lymphocyte percentage decreased from $68.4 \pm 4.2\%$ in Pond 1 to $52.6 \pm 6.3\%$ in Pond 3, representing an approximate 23.1% reduction. The neutrophil percentage increased from $18.2 \pm 3.1\%$ in Pond 1 to $32.8 \pm 4.7\%$ in Pond 3, representing an approximate 80.2% increase.

The monocyte percentage increased modestly from $8.6 \pm 1.8\%$ in Pond 1 to $10.1 \pm 2.4\%$ in Pond 3. Eosinophils remained relatively stable, ranging from $2.8 \pm 0.7\%$ to $3.1 \pm 0.9\%$. Other cells ranged from $1.6 \pm 0.4\%$ to $1.7 \pm 0.5\%$.

These findings indicate differences in the differential leukocyte profile among the sampled ponds. They do not establish that a particular environmental factor produced these changes.

Table 2. Differential leukocyte profile of juvenile *Mugil cephalus*.

Leukocyte type (%)	Pond 1 (n = 4)	Pond 2 (n = 4)	Pond 3 (n = 6)
Lymphocytes	68.4 ± 4.2	61.8 ± 5.1	52.6 ± 6.3
Neutrophils	18.2 ± 3.1	24.6 ± 3.8	32.8 ± 4.7
Monocytes	8.6 ± 1.8	9.2 ± 2.0	10.1 ± 2.4
Eosinophils	3.1 ± 0.9	2.8 ± 0.7	2.9 ± 0.8
Other cells	1.7 ± 0.5	1.6 ± 0.4	1.6 ± 0.5

Values are presented as mean $\pm$ SD. n represents the number of individual fish and biological replicates in each pond.

3.4 Plasma total protein concentration

Total protein concentration decreased progressively across the ponds. The highest concentration was recorded in Pond 1, followed by Pond 2, whereas the lowest concentration was recorded in Pond 3. The concentrations were 42.8 ± 3.6 , 38.6 ± 4.1 , and 33.9 ± 3.8 mg/mL in Ponds 1, 2, and 3, respectively. The difference between Pond 1 and Pond 3 represented an approximate 20.8% reduction. The exact statistical result should be reported as follows after checking the original analysis. Total protein concentration differed among the ponds ($p =$ insert exact value). Total protein concentration differed among the ponds ($p < 0.05$).

3.5 Antioxidant biomarkers

3.5.1 Superoxide dismutase activity

SOD activity increased progressively from Pond 1 to Pond 3. The activity was 18.6 ± 2.1 , 24.3 ± 2.8 , and 31.7 ± 3.4 U/mg protein in Ponds 1, 2, and 3, respectively. Compared with Pond 1, SOD activity increased by approximately 70.4% in Pond 3. This pattern indicates a difference in erythrocyte SOD activity among the sampled ponds.

3.5.2 Catalase activity

CAT activity showed a pattern similar to that of SOD. The lowest CAT activity was recorded in Pond 1, followed by Pond 2, whereas the highest activity was recorded in Pond 3. The activity was 12.4 ± 1.5 , 16.8 ± 2.0 , and 21.6 ± 2.7 U/mg protein in Ponds 1, 2, and 3, respectively.

CAT activity increased by approximately 74.2% from Pond 1 to Pond 3. The results indicate differences in erythrocyte CAT activity among the sampled ponds.

3.6 Lysozyme activity

Lysozyme activity increased progressively among the ponds. The activity was 15.8 ± 1.9 , 21.4 ± 2.5 , and 27.9 ± 3.1 U/mL in Ponds 1, 2, and 3, respectively. Lysozyme activity increased by approximately 76.6% from Pond 1 to Pond 3. This pattern may indicate differences in innate immune activity among the sampled fish. However, elevated lysozyme activity should not automatically be interpreted as improved health because immune biomarkers may increase during both protective activation and physiological stress.

3.7 Integrated biomarker pattern

Fish from Pond 3 showed the most pronounced overall biomarker differences compared with fish from Pond 1.

Table 3. Relative changes in biomarkers between Pond 1 and Pond 3.

Biomarker	Change from Pond 1 to Pond 3
Lymphocyte percentage	Decreased by < 23.1
Neutrophil percentage	Increased by < 80.2%
Total protein concentration	Decreased by < 20.8%
SOD activity	Increased by < 70.4%
CAT activity	Increased by < 74.2%
Lysozyme activity	Increased by < 76.6%

The strongest relative increase was observed in neutrophil percentage, followed by lysozyme activity, CAT activity, and SOD activity. The combined decrease in lymphocyte percentage and total protein concentration, together with increases in neutrophil percentage, antioxidant enzyme activities, and lysozyme activity, indicates a distinct integrated physiological response in fish from Pond 3.

Because the study was observational and field-based, these findings should be described as associations rather than causal effects.

4. Discussion

The reduction in lymphocyte percentage and increase in neutrophil percentage in Pond 3 indicate an alteration of the differential leukocyte profile. Similar changes may occur during physiological stress or immune activation. However, the present study does not establish that a specific water-quality parameter produced these changes.

The reduction in plasma total protein concentration may reflect differences in nutritional status, metabolic condition, or physiological state among the sampled fish. The increases in SOD and CAT activities indicate differences in erythrocyte antioxidant responses among the ponds.

The increase in lysozyme activity indicates variation in innate immune activity among the sampled fish. Nevertheless, this result should be interpreted cautiously because increased lysozyme activity may reflect immune activation, physiological stress, or other biological differences. The integrated biomarker pattern observed in Pond 3 is consistent with a distinct stress-associated physiological profile. However, causal interpretation is not justified because the study was conducted under field conditions, included a small number of fish, used a single sampling date, and lacked matched pond-specific water-quality measurements for formal analysis.

5. Study limitations

The study has the following limitations:

1. The total sample size was small, with only 14 fish.
2. The sample sizes were unequal among the three ponds.
3. Sampling was conducted on only one date.
4. The study did not include repeated seasonal sampling.
5. The available water-quality data were not clearly matched to Ponds 1–3 on 5 May 2026.
6. Formal correlations between water-quality parameters and biomarkers were therefore not performed.
7. The study used field-collected fish and did not include a controlled exposure experiment.
8. Exact *p*-values were not available in the supplied manuscript text.

6. Conclusion

The present study demonstrated measurable differences in the hematological, antioxidant, and innate immune biomarkers of cultured juvenile *Mugil cephalus* collected from three farm ponds.

Fish from Pond 3 showed a lower lymphocyte percentage and plasma total protein concentration, together with higher neutrophil percentage, SOD activity, CAT activity, and lysozyme activity. These findings support the potential use of an integrated

biomarker panel for the early assessment of sublethal physiological responses in cultured fish. However, the findings should be interpreted as exploratory associations rather than definitive causal effects because of the small sample size, unequal pond-level sampling, single sampling date, and absence of matched pond-specific water-quality data.

Future studies should include larger and balanced sample sizes, repeated seasonal sampling, pond-specific water-quality measurements collected on the same dates as biological samples, and controlled exposure experiments.

7. Acknowledgements

The authors sincerely thank Pioneer Aquaculture Consultants, Ponneri, Tiruvallur District, Tamil Nadu, India, for providing access to the aquaculture facility and assisting with the collection of *Mugil cephalus* samples.

The authors also acknowledge the laboratory staff and technical personnel who supported fish handling, blood collection, sample processing, microscopic examination, biochemical assays, and water-quality analysis.

8. Funding

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9. Conflict of interest

The authors declare that they have no competing financial or non-financial interests.

10. Ethics approval

Fish collection, handling, sacrifice, blood sampling, and laboratory analyses were performed in accordance with applicable institutional guidelines for the ethical use of animals in research

11 Research gap

Although hematological, antioxidant, and innate immune biomarkers have been widely investigated individually, their integrated responses under practical farm-based conditions remain insufficiently characterized. Limited information is available on the simultaneous variation of leukocyte profiles, plasma total protein concentration, erythrocyte antioxidant enzymes, and plasma lysozyme activity in cultured juvenile grey mullet, *Mugil cephalus*, from the Ponneri region of Tamil Nadu.

Many previous studies have focused on individual biomarkers or controlled laboratory exposure conditions. Consequently, the relationship among hematological status, antioxidant defense, and innate immune activity in farm-reared juvenile *M. cephalus* remains incompletely understood.

12 Novelty of the study

The novelty of the present study lies in the field-based integration of:

- Differential leukocyte profiling.
- Plasma total protein estimation.
- Erythrocyte superoxide dismutase activity.
- Erythrocyte catalase activity.
- Plasma lysozyme activity.

These biomarkers were evaluated in juvenile *M. cephalus* collected from three culture ponds. This integrated approach provides a broader assessment of fish physiological status than the use of a single biomarker.

13 Aim and objectives

The present study aimed to evaluate the hematological, antioxidant, and innate immune responses of cultured juvenile *Mugil cephalus* under farm-based conditions in Ponneri, Tamil Nadu.

The specific objectives were:

1. To examine the differential leukocyte profile of juvenile *M. cephalus* collected from three culture ponds.
2. To determine plasma total protein concentration.
3. To evaluate erythrocyte superoxide dismutase and catalase activities.
4. To determine plasma lysozyme activity.
5. To compare biomarker responses among the three culture ponds.
6. To assess the potential usefulness of an integrated biomarker panel for fish-health monitoring.

14. Consent to participate

Not applicable.

15. Consent for publication

Not applicable.

16. Data availability

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

17. Author contributions

Praveena Durairaj conducted the data analysis and contributed to the interpretation of the results. Kokila Sumathi assisted with manuscript writing. Eesa Elayavanan contributed to manuscript preparation. Deivasigamani Balaraman supervised the study and provided critical guidance during manuscript development. All authors reviewed and approved the final version of the manuscript.

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