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Environmental modulation of innate immune gene expression in freshwater and coastal farmed Nile tilapia (*Oreochromis niloticus*)

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Abstract

The expression of immune genes in fish can serve as a biomarker for monitoring disease outbreaks and assessing the impact of environmental stressors. This study investigated the expression of nine innate immune genes in cultured Nile tilapia from freshwater and coastal water aquaculture to understand immune responses to environmental variations. The expression of immune genes was monitored using quantitative RT-PCR. Results showed that IL-1β and viperin were significantly higher in the muscle of freshwater tilapia, while GP1 was higher in coastal tilapia muscle. Conversely, TNF-α, TP4, and Hep2 were significantly lower in coastal tilapia muscle. Metallothionine (MT) expression was significantly higher in the gills of both freshwater and coastal tilapia compared to RAS controls. Between the two culture systems, significant variations occurred in the muscle for IL-1β, TNF-α, GP1, viperin, TP4, Hep1 and Hep2, whereas no significant differences were observed in gills, indicating tissue-specific regulation. Pearson correlation revealed strong positive associations between MT and IL-1β, TNF-α, Hep1 and Hep2 in muscle, as well as between several immune genes in gills. Most immune gene expressions were negatively correlated with water quality parameters, except GP1, which correlated positively with pH. Stocking density showed a positive effect on IL-1β, TNF-α, MT, Hep1 and Hep2. Overall, the findings highlight the influence of environmental factors on tilapia immune responses, emphasizing distinct patterns of inflammatory, antimicrobial, and stress-related gene expression in different aquaculture conditions.

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Introduction

Tilapia is one of the fastest growing farmed trade fish species and has gained great popularity as a commercial fish in Africa, South America, and Southeast Asia(1). Bangladesh is one of the top tilapia producing countries where tilapia has been 3rd highest aquaculture producing species contributes 8% of the total farmed fish production as a single species in Bangladesh(2). However, tilapia in aquaculture settings is frequently susceptible to various infectious diseases and environmental stressors. To safeguard tilapia from the burden of infectious diseases, the innate immune system plays an important role to protect them from pathogens and other harmful microorganisms(3). The immune system serves as the primary means of protection against infections, and promptly responds to invaders. Head, kidney, gills, liver and gut-associated lymphoid tissue are key components of this system. These organs and tissues contain a variety of immune cells and produce antimicrobial substances that help protect fish from infections in their aquatic environments. Immune cells, such as macrophages and dendritic cells, produce and release proinflammatory cytokines in response to pathogens or other harmful stimuli encountered by fish(4). Proinflammatory cytokines are important players in the innate immune response of fish. Interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) are among the important proinflammatory cytokines in fish produced and released by immune cells in response to harmful stimuli or pathogens. IL-1β is produced by immune cells such as macrophages and dendritic cells in response to infection or injury(5). TNF-α is another proinflammatory cytokine that is produced by immune cells in response to infection or injury(6). It helps to activate other immune cells and promote inflammation, and it has also a role in apoptosis, or programmed cell death(7). Researchers in aquaculture have explored the possibility of utilizing proinflammatory cytokines as biomarkers to monitor the health of fish and detect the existence of infections. Metals induced protein, metallothionein (MT) is a family of low-molecular-weight cysteine-rich protein with cysteinyl residues that are highly conserved(8). Glutathione peroxidase 1 (GP1) gene expression in fish can be triggered by various factors that can cause stress in fish such as environmental stressors, dietary alterations, disease status, and other stress-inducing factors(9). Viperin is an interferon-inducible protein which appears to work in a variety of methods to prevent viruses from replicating and able to control various circumstances within the cell(10).

Antimicrobial peptides (AMPs) are small, cationic peptides that are produced by various cells in the body, including immune cells, in response to infection or other types of stress(11). Fish hepcidin gene expression is generally linked to bacterial infection or inflammation. The two hepcidin peptides found in teleost fish have been discovered to have some sub-functionalization of their roles, with hepcidin 1 (Hep1) being more involved in iron metabolism regulation and hepcidin 2 (Hep2) primarily serving as an antibacterial peptide(12). Tilapia piscidin 2 (TP2) and tilapia piscidin 4 (TP4) are two members of the piscidin family of antimicrobial peptides produced by tilapia. Both TP2 and TP4 exhibit broad-spectrum antimicrobial activity against bacteria, fungi, and viruses, and are important components of the innate immune system in fish. In addition to their antimicrobial activity, TP2 and TP4 have immunomodulatory effects, meaning that they can regulate the activity of other immune cells(13).

Fish are usually used as good bioindicators of environmental pollution, as changes in their innate immune gene expression can signal exposure to toxins and other environmental stressors. Environmental stressors, such as salinity changes, pollutants, and temperature variations, can significantly influence immune function. However, the specific gene expression changes in response to these stressors in natural habitats remain underexplored. Moreover, the different immune organs of fish such as gills, anterior kidney, spleen and skin are usually considered for gene expression study. The fish organs particularly gills play a key function in immune response of tilapia(14). In addition to gills, fish skeletal muscle is also considered for the immune gene expression study in response to pathogenic infection(15). However, a limited number of studies has been reported on gene expression from fish skeletal muscle. Previous studies in Nile tilapia have mainly examined immune gene expression following immune stimulation or dietary interventions, such as lipopolysaccharide/peptidoglycan exposure or nanoparticle supplementation, focusing on cytokines and Toll-like receptor pathways under experimental challenge conditions(16,17). In contrast, the present study investigates innate immune gene expression in naturally farmed tilapia reared under two different environmental water conditions without artificial stimulation. To address this gap, this study was conducted on Nile tilapia cultured in two different water environments such as freshwater and coastal water targeting two organs (gills and skeletal muscle) for gene expression. Therefore, the objectives of the study were to analyze nine innate immune genes expression in gills and skeletal muscle of Nile tilapia to observe the immune responses in relation to different environmental stressors and to understand their overall health status.

Materials and Methods

Sampling site and sample collection: Nile tilapia, *Oreochromis niloticus* from freshwater and coastal water sources was used as an experimental organism. The samples were collected from Mymensingh, Khulna, and Satkhira districts between October and November in 2021. Freshwater farmed Nile tilapia (n = 8, average weight: 271.39 g and length: 22.21 cm) samples were collected from Trishal of Mymensingh district. On the other hand, coastal water cultured Nile tilapia (n = 6, average weight: 124.39 g and length: 19.12cm) samples were collected from Dumuria of Khulna and Kaliganj of Satkhira districts (Fig. 1). Nile tilapia collected from re-circulating aquaculture system (RAS) was used as control fish (n = 3, average weight: 865.07 g and length: 33.6 cm) to determine the relative expression of target genes in tilapia of freshwater and coastal water aquaculture. The freshwater tilapia was collected from Mymensingh region as this region is one of the most freshwater tilapia producing regions while Khulna and Satkhira are coastal water tilapia producing regions. Tilapia fish samples were collected in live condition and we examined the fish externally and did not find any external parasites or any infections. The fishes were harvested from the ponds by netting and fish from the RAS was harvested by scoop net. All handling procedures were conducted carefully to minimize stress. After collection fish were anesthetized with 2-phenoxyethanol at a concentration of 400 mg/L before dissecting to collect muscle and gill samples. The collected tissues were immersed in RNA protector (TaKaRa, Japan) until further processing.

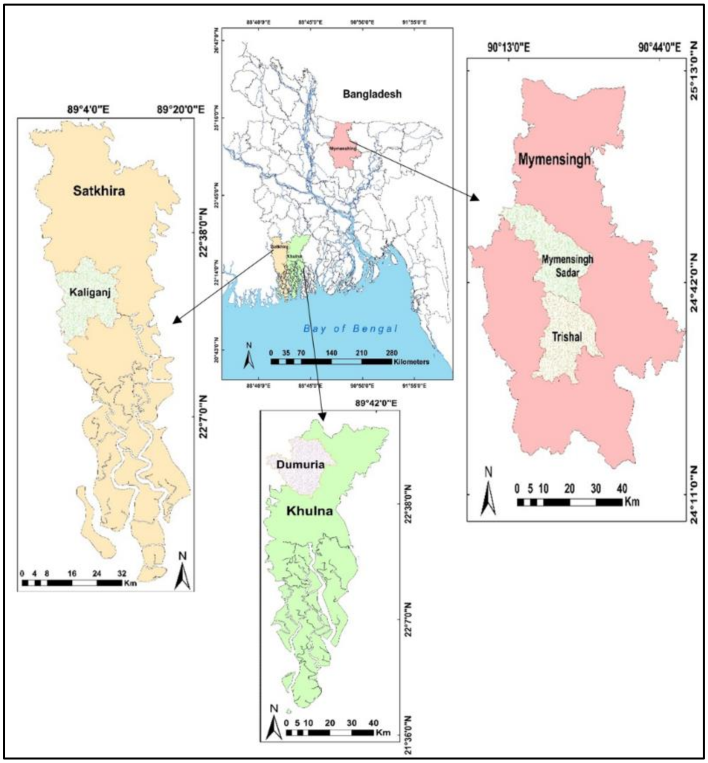

Fig. 1. Map showing the sampling locations of tilapia culture ponds situated in freshwater and coastal water environments.

Targeted genes: The expression of nine innate immune genes including Interleukin 1 beta (IL-1β), Tumor Necrosis Factor alpha (TNF-α), Metallothionein (MT), Glutathione peroxidase 1 (GP1), Viperin, Tilapia Piscidin 2 (TP2), Tilapia Piscidin 4 (TP4), Hepcidin 1 (Hep1) and Hepcidin 2 (Hep2) in the muscle and gill of freshwater and coastal water tilapia was investigated using quantitative real-time RT-PCR (qRT-PCR). Beta actin gene was used as an internal control for relative quantification of the expression of each target gene. The list of primers of the genes is given in table 1.

RNA extraction, cDNA synthesis and qPCR analysis: Total RNA extraction was performed using TRIzol™ reagent (ThermoFisher Scientific). The concentration and purity of extracted RNA were measured using NanoDrop spectrophotometer (NanoDrop™ 2000, Thermo Fisher Scientific Inc., Wilmington, DE, USA). RNA was reverse-transcribed into First-strand complementary DNA (cDNA) using the Precision nanoScript2 Reverse Transcription kit (Primerdesign, UK) using 1000 ng of total RNA for each sample. The following reagents were added to a sterile 200 μL PCR tube for 25 μL of each RT-PCR reaction. GoTeq Mastermix (2X) 12.5 μL, forward and reverse primers (10 pM) 1 μL each, cDNA template 1 μL and nuclease free water up to the volume of 25 μL. Reaction condition for RT-PCR was as follow: initial denaturation at 95°C for 1 minute for 1 cycle followed by 35 cycles of denaturation at 95°C for 10 s, annealing at 56-60°C (varied with primers) for 30 s and extension at 72°C for 15 s. One cycle of final extension at 72°C for 2 minutes was used. A thermocycler (Biometra, T3000) was used to conduct RT-PCR. Conventional PCR was conducted to confirm the single product for each of the primer pair of each of the target gene. Two percent of agarose gel dyed with ethidium bromide was used to run the PCR products. Gel was visualized in gel documentation system (UVstar 312, Biometra, analytikJena).

Quantitative RT-PCR was performed in this study to analyze the mRNA expression of the target immune genes in the muscle and gill tissues of Nile tilapia. Real-Time PCR (qTower^3 G, Analyticjena) was used to amplify the cDNA to analyze amplification curve, melting curve and to quantify the relative expression of the target genes. The following temperature profile was used for qPCR reaction: Pre-treatment at 50°C for 10 minutes, initial denaturation for 2 minutes at 95°C and 40 cycles of denaturation of the cDNA at 95°C for 15 s, annealing at 59°C for 30 s and extension at 72°C for 30 s followed by melt curve analysis (50°C for 5s, 62°C for 10 s and 95°C for 30 s). The PCR was carried out in a reaction volume of 20 μl which included 10 μl qPCR master mix (Luminous Color HiGreen, ThermoFisher Scientific), 0.5 μl of forward primer (10 pM), 0.5 μl of reverse primer (10 pM), 2.0 μl template cDNA (diluted as 1:10), 7.0 μl nuclease free water.

Water quality parameters and stocking densities of Nile tilapia: Water quality parameters including temperature, pH, salinity, dissolved oxygen, total dissolved solids, turbidity, conductivity and oxidation reduction potential (RedoX) of the sampling pond were measured by a portable multi-parameter water quality analyzer (HORIBA-50, Japan).

Data analysis: The relative expression of target genes was measured using Delta-Delta CT method (2^{-DDCT}) where beta actin was used as an internal control. The expression of the target genes was normalized with the expression of beta actin. The fold change of expression was calculated compared to control sample where fish sample from RAS was used as control sample as the system maintains optimal water conditions for the fish rearing and is free from contaminants and environmental as well as biological stressors.

One-way ANOVA was performed by using GraphPad Prism version 9 (San Diego, CA, USA). To assess the normality assumption, we conducted the Shapiro-Wilk test before conducting ANOVA and the data passed the normality test at alpha = 0.05. Significant differences were identified based on the adjusted p value. Pearson correlation was used to check correlation among the expression of target genes and the level of expression of different genes as well as water quality parameters and stocking density of the sampling ponds by using GraphPad Prism version 9 (San Diego, CA, USA). Before performing correlation analysis, we identified and excluded the outliers and then we performed the normality test and the variables which passed the normality test were used for correlation analysis.

Table 1. List of targeted primers applied in gene expression in Nile tilapia, *Oreochromis niloticus*

Gene	Sequence (5′---3′)	Accession number
TNF-α	F- CAGGATCTGGCGCTACTCAG R- TAGCTGGTTGGTTTCCGTCC	NM_001279533.1
IL-1 β	F-TGAGAGCCTACTTTAGGATTCTGC R-GCGGCTATTACAACCAATGCT	XM_005457887.2
Metallothionein (MT)	F- CTGCAACTGCGGAGGA R- GGTGTCGCATGTCTTTCCTT	XM_003447045.5
GP1	F- CCAAGAGAACTGCAAGAACGA R- CAGGACACGTCATTCCTACAC	XM_003444086.3
Viperin	F- ATCAACTTCTCTGGCGGA R- AGATAGACACCATATTTCTGGAAC	XM_003453237.3
TP2	F- ATGAAGTGTGCTGCAGTATTTCTTATGCTGTCC R- CTAGTCAAAATTAAGTCGACGAGGGGT	JX006071
TP4	F- GGTCGTCCTCATGGCTGA R- GTCGTATGAGGCGATGGATAG	JX006073
Hep1	F- CAG CAG CAC ATG AGG AGG CA R- ACC TGC AGC AAA CTC CAC AGA	MH651353.1
Hep2	F- GA GCA ATG ACA CTC CAG TTG R- AGA TGG CTC TGA CGC TTT TG	MH651359.1
Beta actin	F- GCTACTCCTTCACCACCACAG R- CGTCAGGCAGCTCGTAACTC	KJ126772.1

Results and Discussion

Expression of cytokine IL-1β and TNF-α: The expression of pro-inflammatory cytokine IL-1β in muscle sample of freshwater tilapia was significantly higher than that of control fish collected from RAS ($p < 0.05$) while in coastal water farmed tilapia the level of expression was similar to control sample ($p > 0.05$; Fig. 2A). However, the expression of IL-1β in the muscle of freshwater farmed tilapia was significantly higher than that of coastal water farmed tilapia ($p < 0.05$). In the gill samples from both freshwater and coastal water systems the expression of IL-1β was similar to control sample.

In the case of TNF-α, an inflammatory cytokine, the expression in the muscle of coastal water tilapia was significantly lower than control sample ($p < 0.05$, Fig. 2B). Moreover, the expression of TNF-α in the muscle of coastal water tilapia was significantly lower than that of freshwater farmed tilapia ($p < 0.05$). However, the expression remained similar in the gill of tilapia from all sources ($p > 0.05$).

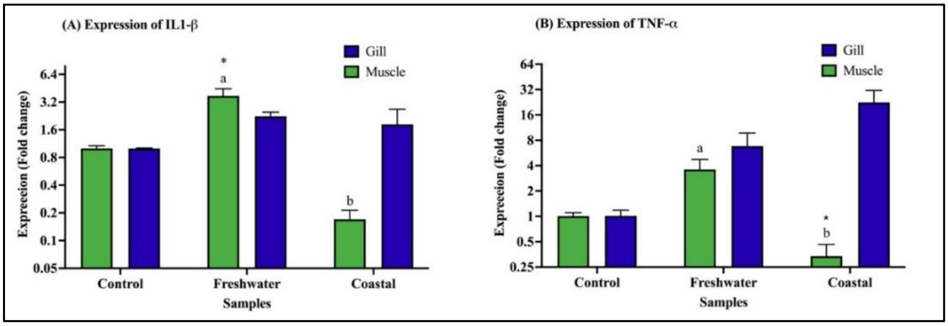

Fig. 2. Relative expression of IL-1β (A) and TNF-α (B) in the muscle and gill of Nile tilapia. Asterisk * represents significantly different at $p < 0.05$ between control and freshwater or control and coastal water samples while bars with same color different letters are significantly different at $p < 0.05$ between freshwater and coastal water samples.

The non-specific immune system can be impacted by disease-causing agents and environmental pollutants, which may alter the function of immune system modulators such as pro-inflammatory cytokines IL-1β and TNF-α. The higher expression of IL-1β in freshwater tilapia suggests increased inflammation due to cytokine production(18). Tilapia may develop inflammation from stimuli such as bacterial, viral, parasitic, or fungal infections, physical injuries (e.g., wounds), or exposure to environmental stressors like changes in temperature, salinity, or dissolved oxygen(19). Parasites also induce inflammation in fish, as external protozoans and monogeneans, and internal cestodes and nematodes, trigger immune responses that elevate IL-1β in *Oreochromis niloticus*(20,21). As the studied fish lacked external parasites or wounds, the observed inflammation might be linked to internal parasitic infection. Conversely, the lower TNF-α expression in the muscle of coastal-water tilapia may reflect reduced inflammation, impaired cell proliferation, or chronic stress. Chronic stress has been associated with decreased TNF-α expression in fish, and pollutants like heavy metals or pesticides can similarly downregulate this cytokine(21). Studies show significant TNF-α downregulation in *Oreochromis niloticus* from lakes with high zinc, manganese, nickel, and lead levels(22). The lower expression of TNF-α in coastal-water tilapia may therefore relate to heavy-metal exposure, consistent with reported moderate to high metal pollution in Bangladesh's coastal regions(23). Environmental fluctuations in salinity or oxygen may further induce chronic stress, influencing TNF-α expression.

Expression of metallothionein (MT), glutathione peroxidase 1 (GP1) and viperin: Similar expression of metallothionein (MT) was observed in the muscle of control, freshwater and coastal water farmed samples while the expression was significantly higher in the gill of Nile tilapia from both the culture systems compared to that of control samples ($p < 0.01$; Fig. 3A). In case of glutathionase peroxidase 1 (GP1), the expression in the muscle of Nile tilapia from coastal water aquaculture was significantly higher than that of control fish ($p < 0.001$) and freshwater farmed tilapia ($p < 0.05$). However, the expression of GP1 was similar in the gills of tilapia from all sources (Fig. 3B). The expression of viperin was significantly higher in the muscle of tilapia compared to control fish ($p < 0.0001$) and coastal water tilapia ($p < 0.05$; Fig. 3C). However, the expression of viperin in the muscle of tilapia from coastal water aquaculture remained similar to that of control fish. In the gill of Nile tilapia from all sources, similar expression of viperin was observed.

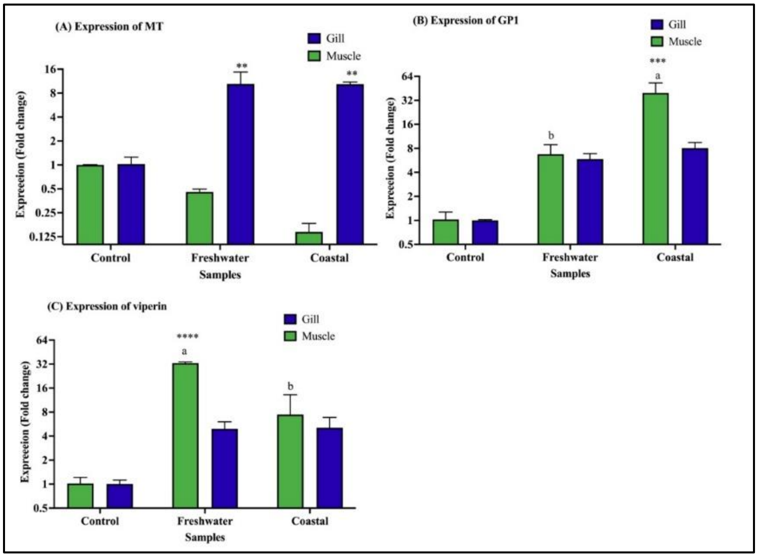

Fig. 3. Relative expression of MT (A) GP1 (B) and Viperin (C) in the muscle and gill of Nile tilapia. Asterisk **, ***, **** represents significantly different at $p < 0.01$, $p < 0.001$ and $p < 0.0001$, respectively between control and freshwater or control and coastal water samples while bars with same color different letters are significantly different at $p < 0.05$ between freshwater and coastal water samples.

MT serves as a biomarker for heavy-metal pollution and detoxification of metals, pesticides, and other toxic materials. The combined influence of aquaculture drug use and heavy-metal contamination may explain the elevated MT levels in both systems(24). MTs limit toxicant availability at undesired sites(25) and their upregulation in *Cyprinus carpio* has been linked to abiotic stress(26). Seasonal increases of MT expression in *Oreochromis niloticus* liver were also reported(27). The greater MT expression in gills compared to muscle in the present study aligns with the gill's direct contact with the aquatic environment and its key role in detoxification. A significantly higher GP1 expression in coastal-water tilapia muscle suggests an enhanced oxidative-stress response under saline or pollutant-rich conditions. GP1 protects cells from reactive oxygen species damage(28) and is often upregulated in

Oreochromis niloticus under oxidative stress. This may be triggered by heavy-metal exposure, salinity fluctuations, or other environmental factors(29). Coastal ponds may accumulate more pollutants from agricultural runoff, industrial discharge, or sewage(11) and elevated conductivity and TDS observed in the coastal ponds may also induce stress. Additional oxidative stressors include pathogens or poor diet. Viperin, an interferon-inducible antiviral protein, was significantly upregulated in freshwater tilapia muscle compared to control and coastal fish. This may reflect activation of antiviral immunity, possibly due to viral exposure or environmental stressors that stimulate innate defenses. Elevated viperin expression indicates an immune response to potential viral challenges common in aquaculture settings.

Expression of antimicrobial peptides (AMPs): In the present study, the expression of tilapia piscidin 2 (TP2) in the muscle and gill of freshwater and coastal water farmed Nile tilapia was similar to that of control tilapia ($p > 0.05$; Fig. 4A). On the other hand, the expression of tilapia piscidin 4 (TP4) in the muscle of Nile tilapia from both freshwater and coastal water aquaculture was significantly lower than that of control fish ($p < 0.05$; Fig. 4B). Moreover, the TP4 expression in the muscle of tilapia from freshwater aquaculture was significantly higher than that of coastal water tilapia ($p < 0.05$). However, TP4 expression in the gill of tilapia from all the sources remained similar ($p > 0.05$; Fig. 4B). The expression of hepcidin 1 (Hep1) in the muscle and gill of the tilapia from both aquacultures remained similar when compared to control fish ($p > 0.05$; Fig. 4C). However, Hep1 expression in the muscle of freshwater tilapia was significantly higher than that of coastal water tilapia ($p < 0.05$). In case of hepcidin 2 (Hep2), the expression in the muscle of freshwater tilapia was similar to the control fish ($p > 0.05$). However, the expression was significantly higher than that of coastal water fish ($p < 0.05$). Moreover, the expression of Hep2 in the muscle of coastal water tilapia was significantly lower than that of control fish ($p < 0.0001$; Fig. 4D). However, similar expression of Hep2 in the gill of tilapia from all the sources was observed.

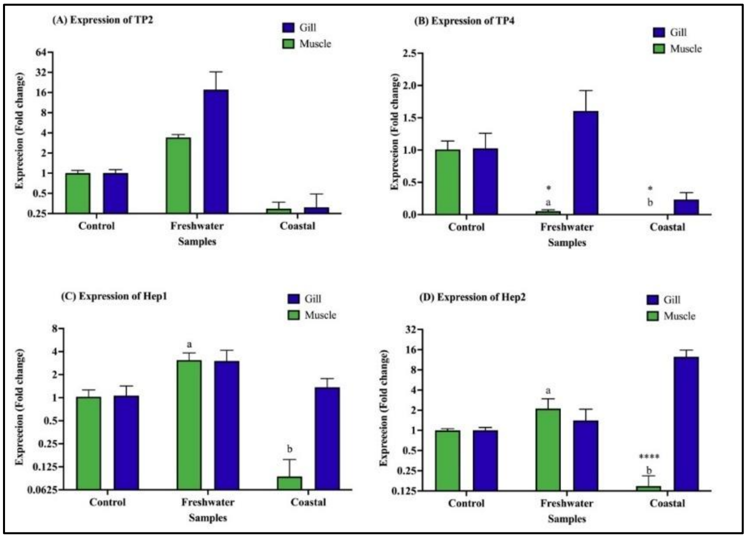

Fig. 4. Relative expression of TP2 (A) TP4 (B) Hep1 (C) and Hep2 (D) in the muscle and gill of Nile tilapia. Asterisk * and **** represent significantly different at $p < 0.05$ and $p < 0.0001$, respectively between control and freshwater or control and coastal water samples while bars with same color different letters are significantly different at $p < 0.05$.

AMPs are multifunctional defense peptides that provide the first line of protection against bacteria, fungi, and viruses and act as immune modulators(30). In this study, the significantly lower expression of TP4 and Hep2 in coastal-water tilapia compared to control and freshwater fish suggests compromised innate defense. Reduced AMP expression may be influenced by genetic variation, environmental conditions, pollution, and fish health. Poor water quality or nutrient imbalance in coastal systems may downregulate AMP expression and weaken antimicrobial defenses.

Correlation between the level of expression of different genes: The Pearson correlation was performed between the fold change of expression of different genes in the muscle of Nile tilapia where a significantly positive correlation was found between the expression of IL-1β vs MT ($p < 0.01$), TNF-α vs MT ($p < 0.05$), Hep1 vs MT($p < 0.05$), Hep2 vs MT ($p < 0.01$), Hep1 vs IL-1β ($p < 0.001$), Hep2 vs IL-1β ($p < 0.01$) and Hep1 vs Hep2 ($p < 0.001$) (Fig. 5A). On the other hand, in gill, a significantly positive correlation was observed between the expression of TP2 vs TP4 ($p < 0.05$), GP1 vs Hep2 ($p < 0.001$), viperin vs TNF-α ($p < 0.001$), viperin vs GP1 ($p < 0.05$) while a significantly negative correlation was observed only between the expression of MT vs IL-1β ($p < 0.05$; Fig. 5B).

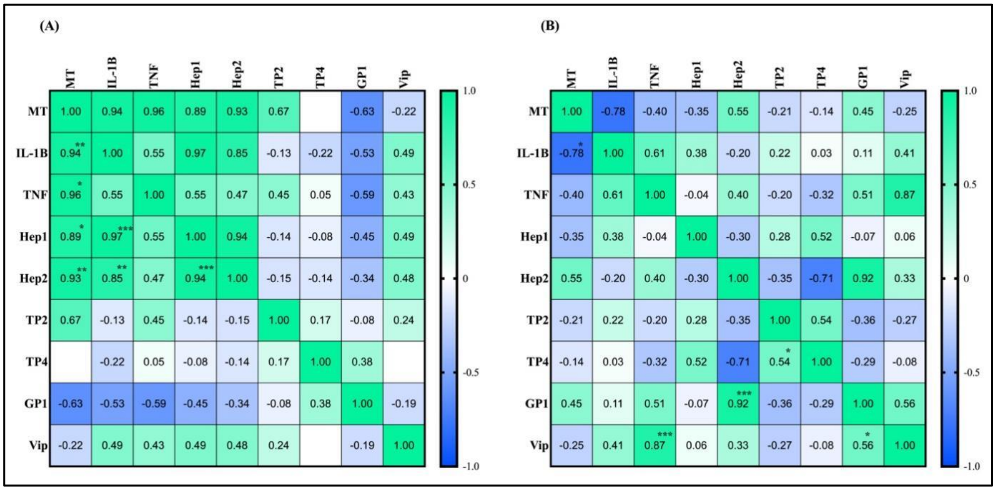

Fig. 5. Pearson correlation between the expression of different immune genes (fold change relative to housekeeping gene Beta actin) in the muscle (A) and gill (B) of Nile tilapia. Color code and the values in each box indicate the Pearson r value. The asterisk denotes the significant correlation between genes where * = $p < 0.05$. ** = $p < 0.01$ and *** = $p < 0.001$.

The strong positive correlation between MT expression and IL-1β, TNF-α, Hep1, and Hep2 implies a coordinated immune and detoxification response(22). MTs may regulate inflammation by sequestering metals and maintaining homeostasis, and their induction could reflect pollutant exposure linked to inflammation(22). The positive relationships between MT, IL-1β, Hep1, and Hep2 suggest that oxidative stress or microbial infection triggers a concerted immune activation, with IL-1β possibly inducing Hep1 and Hep2 expression.

Correlation between level of expression of different genes, water quality parameters and stocking density: The Pearson correlation was also conducted between gene expression and water quality parameters where significantly positive correlation was observed between GP1 and pH (Table 2). Significantly positive correlation was also observed between stocking density and expression of IL-1β, TNF-α, MT, TP4, Hep1 and Hep2 (Table 2). On the other hand, a significantly negative correlation was observed between pH and expression of IL-1β, MT, Hep1, Hep2; oxidation reduction potential (ReDox) and viperin; conductivity and expression of IL-1β, TNF-α, Hep1, Hep2; total dissolved solids and IL-1β, TNF-α, Hep1 and Hep2; salinity and expression of IL-1β, TNF-α, Hep1, Hep2 (Table 2). However, no significant correlation was observed between expression of any of the genes and DO and TDS ($p > 0.05$).

Table 2. Pearson correlation between level of expression of different genes and water quality parameters and stocking density of the sampling ponds. Asterisk (**) and (*) denote significant correlation at the p < 0.01 and p < 0.05, respectively (2-tailed)

Gene symbols	Test	Temp	pH	RedoX	Cond.	Turbidity	DO	TDS	Salinity	SD
IL-1 β	R	-0.4	-0.629*	-0.129	-0.693*	0.309	-0.513	-0.694*	-0.582*	0.718**
	p (2-tailed)	0.139	0.012	0.674	0.018	0.305	0.088	0.018	0.037	0.006
	N	15	15	13	11	13	12	11	13	13
TNF-α	R	-0.265	-0.513*	-0.456	-0.715*	0.552	-0.36	-0.721*	-0.604*	0.742**
	p (2-tailed)	0.381	0.073	0.158	0.02	0.079	0.307	0.019	0.038	0.009
	N	13	13	11	10	11	10	10	12	11
MT	R	-0.289	-0.838**	-0.415	-0.708	0.487	-0.63	-0.712	-0.702	0.72*
	p (2-tailed)	0.488	0.009	0.306	0.116	0.221	0.129	0.113	0.12	0.044
	N	8	8	8	6	8	7	6	6	8
GP1	R	-0.543	0.903**	-0.007	0.145	-0.536	0.613	0.194	0.463	-0.511
	p (2-tailed)	0.131	0.001	0.989	0.784	0.215	0.196	0.713	0.248	0.241
	N	9	9	7	6	7	6	6	8	7
Viperin	R	-0.598	0.514	-0.971*	-0.996	-0.423	-0.695	-0.996*	-0.27	0.892
	p (2-tailed)	6	6	4	3	4	3	3	5	4
	N	0.21	0.297	0.029	0.059	0.577	0.51	0.058	0.66	0.108
TP2	R	0.098	-0.015	-0.37	-0.373	0.464	0.054	-0.374	-0.286	0.227
	p (2-tailed)	0.728	0.957	0.213	0.258	0.11	0.867	0.258	0.344	0.456
	N	15	15	13	11	13	12	11	13	13
TP4	R	-0.59	-0.01	-0.02	0.15	-.44	-0.11	.19	0.24	0.712**
	p (2-tailed)	0.17	0.99	0.97	0.75	.32	.81	.68	0.61	0.001
	N	7	7	7	7	7	7	7	7	7
Hep1	R	-0.354	-0.658**	-0.116	-0.674*	0.331	-0.452	-0.676*	-0.605*	0.692**
	p (2-tailed)	0.195	0.008	0.707	0.023	0.27	0.141	0.023	0.028	0.009
	N	15	15	13	11	13	12	11	13	13
Hep2	R	-0.191	-0.729**	-0.111	-0.627*	0.42	-0.388	-0.631*	-0.641*	0.64*
	p (2-tailed)	0.495	0.002	0.718	0.039	0.153	0.212	0.038	0.018	0.018
	N	15	15	13	11	13	12	11	13	13

The positive relationship between GP1 and pH suggests that oxidative-stress defense mechanisms respond sensitively to pH variation. Conversely, negative correlations between several environmental parameters (e.g., salinity, conductivity, TDS, ReDox) and immune-gene expression indicate that suboptimal water conditions may suppress immune responses in *Oreochromis niloticus*. As these environmental factors deviate from optimal ranges, immune-gene expression decreases, potentially compromising defense capacity. The positive correlations between stocking density and IL-1β, TNF-α, MT, TP4, Hep1 and Hep2 suggest that crowding stress induces immune activation, reflecting a physiological response to intensive culture conditions.

While this study provides new insight into innate immune gene expression in Nile tilapia under different environmental conditions, several limitations should be acknowledged. First, the sample size was relatively small, and the number of fish differed between the control group and the coastal and freshwater culture groups. These constraints may limit the statistical power of the findings. Second, fish size and age are known to influence immune function and gene expression profiles; however, these factors could not be fully standardized across sampling groups in the present study because of the availability of samples during the study period. Therefore, some of the observed differences in innate immune gene expression may partly reflect variation in physiological status rather than environmental conditions alone. Future work using larger, size-matched fish are required to confirm these patterns.

Conclusion

In conclusion, this research offers valuable insights into utilizing innate immune genes as indicators for the surveillance of fish well-being and the evaluation of environmental stressors within aquaculture systems. The higher expression of Interleukin-1 beta (IL-1β) and viperin in freshwater tilapia, as well as Glutathione peroxidase 1 (GP1) in coastal water tilapia, signifies distinct immune responses because of differing environments and potential encounters with pathogens. These genes are key components of inflammatory, antiviral and oxidative-stress pathways, and their altered expression may therefore reflect modulation of host-defence mechanisms in response to environmental change. Furthermore, the lower expression of Tumor necrosis factor-alpha (TNF-α), Tilapia piscidin 4 (TP4), and Hepcidin 2 (Hep2) in coastal water tilapia is probably a balance between adaptation to the coastal habitat and sustaining immune readiness. The increased expression of MT in both gill samples underscores its significance in responding to heavy metal and stressors in both aquatic habitats. Additionally, the relationships found between gene expression and water quality parameters, as well as stocking density, offer additional evidence of the impact of environmental conditions on fish health. The findings of the study indicate that the tested genes can be used as the molecular markers to determine the environmental suitability of the fish for their growth and defence against pathogens. The qRT-PCR technique can determine the physiological/immunological conditions of the fish in short time. Moreover, the correlation between gene expression and environmental parameters, and stocking density can give an idea about the optimum environment for aquaculture. However, there are some other suitable candidate genes related to stress and specific pathogens which can be used in future work such as additional interleukins (e.g., IL-6, IL-8, IL-10) and Toll-like receptors (e.g., TLR2, TLR4, TLR9), to further clarify mechanistic pathways. In addition, discovery-based approaches such as RNA-sequencing may identify additional immune-regulatory genes, while targeted platforms such as NanoString could support rapid routine monitoring in aquaculture systems. Other immune tissue, for instance anterior kidney, spleen and skin, might be considered for a complete understanding of the immune response.

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Conflict of interest

The authors declare that they do not have any financial or non-financial interests to disclose.

Ethical statement

The study was reviewed and approved by Faculty of Biological Sciences, University of Dhaka, Dhaka 1000, Bangladesh.

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