

Digestive, Antioxidant and Muscle Metabolic Enzyme Activities in Female Hilsa (*Tenualosa ilisha*) During Monsoon Upstream Migration from the Bay of Bengal to the Rupnarayan River

Arindam Mondal*, Moumita Mahato, Shampa Mandal, Ashim Kumar Nath

Department of Zoology, Sidho-Kanho-Birsha University, Purulia-723104, West Bengal, India

*Corresponding Author. E-mail address: arindambio04@jkcprl.ac.in

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Abstract: Hilsa (*Tenualosa ilisha*), an anadromous migratory fish, demonstrates significant physiological adaptation when transitioning between marine and freshwater habitats. This study assessed the activity of key digestive and metabolic enzymes in Hilsa from both environments to evaluate biochemical changes during migration. Digestive enzymes— α -amylase, β -galactosidase, lipase, alkaline protease, and alkaline phosphatase—showed markedly higher activity in freshwater individuals ($p < 0.05$). Notably, α -amylase and β -galactosidase increased by 2.6-fold and 2.5-fold respectively ($p < 0.05$), indicating enhanced carbohydrate digestion. Lipase activity rose 1.6 times, while protease and phosphatase levels were also significantly upregulated ($p < 0.05$), reflecting dietary reactivation after the migratory fasting phase. In contrast, metabolic enzyme activities—citrate synthase (CS), lactate dehydrogenase (LDH), and catalase—varied by muscle type but not habitat ($p > 0.05$). CS and catalase were higher in red muscle ($p < 0.0001$), supporting sustained swimming, while LDH activity dominated in white muscle ($p < 0.001$), aiding burst movements. Digestive enzymes were strongly correlated with each other, whereas digestion showed a modest inverse relationship with red muscle metabolism, suggesting compartmentalized energy allocation. These findings underscore Hilsa's enzymatic plasticity as a critical adaptation for efficient resource use and physiological preparedness across changing salinities during migration.

Key words: *Tenualosa ilisha*, digestive enzymes, metabolic enzymes, anadromous migration.

1 Introduction

Hilsa (*Tenualosa ilisha*) is the prized fish of Bengal, celebrated for its delicacy, pungent aroma, and shimmering silver colour. Every monsoon, millions of fishermen participate in Hilsa harvesting [1]. It is an anadromous fish, migrating into freshwater during the monsoon season, spanning from August to October. Hilsa's upstream migration may be influenced by elevated freshwater discharge, increased nutrient availability, and other environmental cues. Although reproduction is the primary reason for anadromous fish migration, feeding might also play an important role in driving this behaviour. Adult Hilsa, ranging from 20 to 25cm, including both males and females, enter the

Hooghly-Bhagirathi estuarine region and migrate upstream, reaching as far as the Farakka Barrage [2]. Before the construction of the Farakka Barrage, Hilsa could migrate as far as Delhi, covering an astonishing distance of nearly 1200 km inland. Their swimming speed can reach up to 70 km per day during migration [3]. During their journey through the estuarine zone, a small fraction of Hilsa diverts into the Rupnarayan River, one of the major tributaries of the Hooghly River. However, their upstream movement within the Rupnarayan is quite restricted, with limited migratory distance. Before the development of the thermal power plant at Kollaghat, Hilsa landings in the Rupnarayan were considerably higher. Increased siltation, reduced freshwater discharge, and

other forms of habitat degradation [4,5] have now made the river less favourable for migration. Notably, at the same latitude, more fishes land at Godakhali, situated along the Hooghly River, possibly due to better environmental conditions. For our study, we selected Kolaghat for several reasons: (i) very limited studies have been conducted on Hilsa physiology in the Rupnarayan River, (ii) fishes at Kolaghat rarely migrate further upstream, thus representing a distinct physiological state, (iii) Hilsa caught in Godakhali might already be undergoing active migration, complicating interpretations, and (iv) logistical ease of sample transport to our laboratory.

Hilsa captures also occur outside the monsoon season, particularly during the winter spawning period, but the catch volumes are significantly lower. Some studies suggest the possibility of different stocks or genetic divergence among migratory populations based on their spawning periods [6]. Migration in Hilsa, as in other fishes, remains a complex interplay of external environmental cues and internal physiological changes [7]. There is considerable debate among connoisseurs about whether Hilsa from the Rupnarayan, Hooghly, or Padma rivers tastes better. Much of this difference is believed to be influenced by the type of food the fish consume during their upstream journey. To understand the feeding behaviour of migrating Hilsa, we analysed the activity of five key digestive enzymes: amylase, beta-galactosidase, lipase, alkaline protease, and alkaline phosphatase. Each enzyme corresponds to a particular type of dietary intake, and their levels provide insights into the types of food particles encountered and ingested [8].

The objective of this study was to compare the activities of digestive enzymes, muscle metabolic enzymes, and the oxidative stress enzyme catalase in female hilsa (*Tenualosa ilisha*) collected from marine and freshwater habitats during monsoon upstream migration to understand its digestive and metabolic adaptability. we studied the activity of three key metabolic

enzymes in both red and white muscles. Citrate synthase (CS) serves as a marker for aerobic respiration, predominantly occurring in red muscle and supporting prolonged endurance swimming [9]. Lactate dehydrogenase (LDH) acts as a marker for anaerobic metabolism, abundant in white muscle, and supports burst movements [10]. To monitor oxidative stress associated with different metabolic modes, we measured catalase activity, a crucial antioxidant enzyme that detoxifies harmful reactive oxygen species (ROS). The physiological state of Hilsa during migration is heavily influenced by the changing riverine conditions. Indian rivers, including the Hooghly and its tributaries, are facing immense environmental challenges due to chemical pollution, illegal sand mining, and rampant overfishing, including fishing during breeding bans. These disruptions not only alter river ecology but also significantly impact migratory fish species. Our study aims to bridge the gap by analysing changes in digestive and metabolic enzymes during upstream migration, providing a glimpse into the physiological adaptations that allow Hilsa to survive and successfully migrate under changing and often harsh riverine conditions.

2 Materials and methods

2.0.1 Sample Collection, Transportation and Tissue Preparation

Freshwater specimens of *Tenualosa ilisha* were collected from the Rupnarayan River, specifically near the Kolaghat town (Station A), using small fishing boats operated by local fishermen. Marine specimens were collected from the Bay of Bengal (Station B), approximately 20 km offshore from the Mandarmani coast, using a mechanized trawler equipped with specialized fishing nets (gill net). Immediately after capture, all fish were transferred into containers filled with water from their respective sampling locations to minimize handling stress. All collection takes place

between 1st to 5th July, 2025. Within a few minutes of capture, fish were anesthetized using MS-222 (Merck) solution (20 mg/l) to ensure minimal suffering and ethical handling. Specimens were then euthanized by decapitation. For consistency in experimental design and to minimize physiological variability, only female individuals weighing between 190-217 g were selected from both habitats. This selection ensured uniformity and eliminated potential influences of reproductive physiology on metabolic and digestive enzyme activity. The fish samples were transported to the laboratory under cold conditions using crushed ice in a 1:2 (W/W) ratio. Ice was replenished as needed throughout transport. Upon arrival, the anterior intestine ($1/3^{rd}$ of total intestinal length) was dissected, homogenized in five volumes of ice-

cold 10 mM Tris-HCl buffer (pH 7.4) using a chilled glass mortar and pestle, and centrifuged at 10,000g for 20 minutes at 4°C. The supernatants were aliquoted and stored at -20°C until further analysis.

For tissue-specific enzyme analysis, red and white muscle tissues were excised from the mid-body region of each fish. Tissues were rinsed in 10 mM phosphate buffer (pH 7.4) and homogenized in four volumes of the same ice-cold buffer. Homogenates were centrifuged at $500 \times g$ for 10 minutes to remove tissue debris and lipids. The resulting supernatants were collected and stored at -20°C. Enzyme assays for lactate dehydrogenase (LDH) were conducted on 10-fold diluted samples. All metabolic enzyme analyses were performed within one week of sample preparation.

2.1 Determination of Enzyme Activity and Protein Concentration

All enzymatic assays were conducted at a constant room temperature of 25°C. Spectrophotometric measurements were carried out using either a microplate reader (Biorad imark) or UV-VIS spectrometer (Genesys 10s UV-VIS), depending on the assay requirements. Alkaline protease activity was assessed following the protocol of Prihanto [11], with minor modifications for microplate adaptation. The reaction mixture included 50 μ L of 1.5% Hammarsten casein (SRL chemicals) and 50 μ L of 10 mM Tris-HCl buffer (pH 8). The reaction was initiated by the addition of 50 μ L enzyme extract and incubated for 15 minutes. The reaction was stopped using 150 μ L of 10% trichloroacetic acid (TCA), followed by centrifugation at 5,000 RPM for 10 minutes. Absorbance was measured at 750 nm. Enzyme activity was quantified using a standard curve prepared with tyrosine. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μ mol of tyrosine per minute per mg of protein. Alkaline Phosphatase

(ALP) activity was measured based on the method described by Chinnadurai [12], optimized for microplate readings. A total of 10 μ L of enzyme extract was mixed with 190 μ L of substrate solution consisting of 10 mM glycine NaOH buffer (pH 8.5), 2 mM $MgCl_2$, and 1.25 mM para-nitrophenyl phosphate (SRL chemicals) as substrate. The mixture was incubated for 15 minutes, and absorbance was recorded at 415 nm. Para-nitrophenol (SRL Chemicals) standard curves were used to calculate enzyme activity, expressed as μ mol of para-nitrophenol released per minute per mg of protein. Lipase activity was evaluated following the protocols as described by Vo, C. V. T. [13] with some modifications. We used para-nitrophenol palmitate (long chain fatty acid) (pNPP) (Thermo Fisher Chemicals) as substrate. The reaction buffer included 4% acetonitrile-dissolved pNPP (20 mM) in 10 mM Tris-HCl (pH 7.4) with 1 mM NaCl, 1 mM $CaCl_2$, and 0.4% (w/v) gum arabic. Triton X-100 (0.2%, v/v) was added to facilitate substrate solubilization. After incubating the enzyme with the reaction mixture for 15 minutes, absorbance was mea-

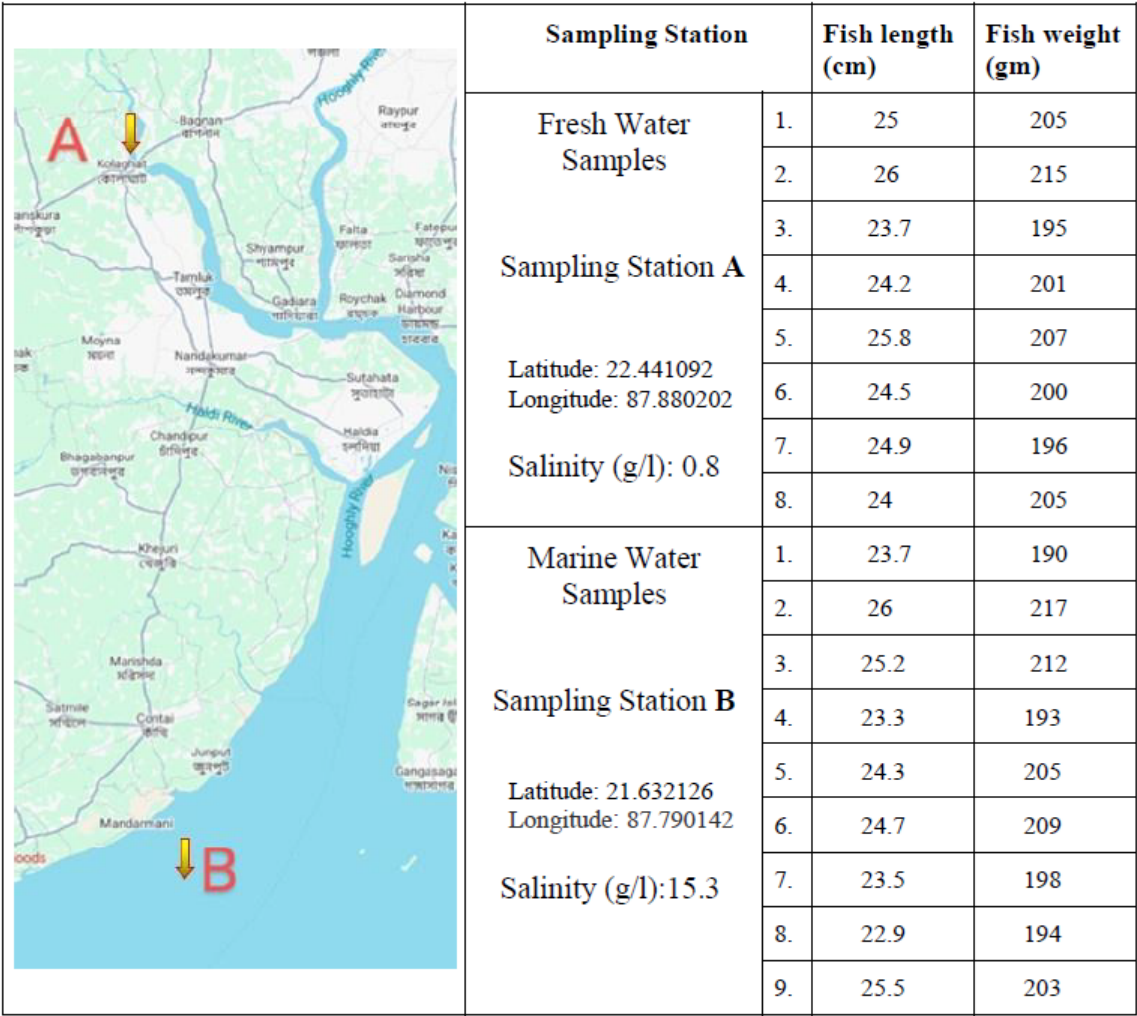


Figure 1: Map showing the two sampling sites: Station A and Station B. Station A is situated along the bank of the Rupnarayan River (Freshwater sampling site), while Station B lies within the estuarine zone of the Bay of Bengal. The map was generated using Google Maps. Coordinates are taken from portable gps tracker.

sured at 415 nm. Activity was determined based on para-nitrophenol standard curves. Activity was expressed as μmol of para-nitrophenol released per minute per mg protein. Beta-galactosidase activity was measured using *o*-Nitrophenyl- β -D-Galactopyranoside (ONPG) as the substrate, following He et al [14]. The assay mixture contained 20 μL of enzyme extract, 280 μL of 100 mM sodium phosphate buffer (pH 7.5), 1 mM MgCl_2 , and ONPG (280 μg). After 30 minutes of incubation, absorbance was recorded at 415 nm. Enzyme activity was calculated using an ortho-nitrophenol standard curve. Activity was expressed as μmol of ortho-nitrophenol released per minute per mg protein. Amylase activity was assessed according to Nolasco-Soria [15]. The enzyme was incubated with the starch substrate for 15 minutes, and the released maltose was quantified at 550 nm using a maltose standard curve. Activity was expressed as μmol of maltose released per minute per mg protein.

LDH activity was measured spectrophotometrically at 340 nm, based on the reduction of NADH. The assay mixture included 20 μL of enzyme sample added to 1980 μL of substrate buffer containing 0.4 mM NADH and 3 mM sodium pyruvate. Absorbance was recorded every 15 seconds for 2 minutes. The rate of NADH oxidation was used to calculate LDH activity. CS activity was determined using the

method described by Spinazzi [16]. The reaction mixture contained 100 μM DTNB and 115 μM acetyl-CoA (SRL Chemicals) in 50 mM potassium phosphate buffer. A 20 μL enzyme sample was added to 380 μL of substrate mixture. Absorbance was recorded at 412nm, at 15-second intervals over 2 minutes. Activity was expressed in nmol/min/mg protein. Catalase activity was measured following Weydert et al [17]. The enzyme's ability to decompose hydrogen peroxide (H_2O_2) was monitored using a UV-VIS spectrophotometer. Activity was expressed in U/mg protein. Total protein content of the samples was determined using the Himedia Bradford kit, following the manufacturer protocol.

2.1.1 Statistical Analysis

Unpaired *t*-tests were conducted using GraphPad Prism (version 8.0.1) to determine statistical significance; all enzymes activity related graphs were also generated using the same software. Pearson correlation matrices were visualized using the 'Performance Analytics' package in R. Statistical significance is indicated as: ns not significant ($p > 0.05$), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Standard curves were created in Excel spreadsheet. Data of particular groups are presented as Mean $\pm$ SD.

3 Results and discussions

Hilsa collected from freshwater habitats exhibited significantly elevated ($p < 0.05$) digestive enzyme activities compared to marine individuals, reflecting key physiological adjustments during migration. Amylase activity, essential for carbohydrate digestion, ranged from 0.45 to 0.81 Umg^{-1} (mean: $0.7116 \pm 0.1753 \text{ Umg}^{-1}$) in freshwater, markedly higher than the 0.18-0.34 U/mg (mean: $0.275 \pm 0.068 \text{ Umg}^{-1}$) observed in marine fish; a 2.5-fold increase. This suggests enhanced carbohydrate digestion aligned with resumed foraging and

access to phytoplankton-rich diets post-migration [18]. Given hilsa migrate in a semi-starved state to conserve energy, the broader amylase range in freshwater may also reflect variability in gut content and arrival timing. Beta-galactosidase, involved in hydrolysing non-starch polysaccharides [19], averaged $0.0485 \pm 0.0149 \text{ Umg}^{-1}$ in freshwater versus $0.0187 \pm 0.004 \text{ Umg}^{-1}$ in marine samples. This increase may also indicate microbial activity in digestion [20], suggesting a more complex carbohydrate diet in freshwater. Co-

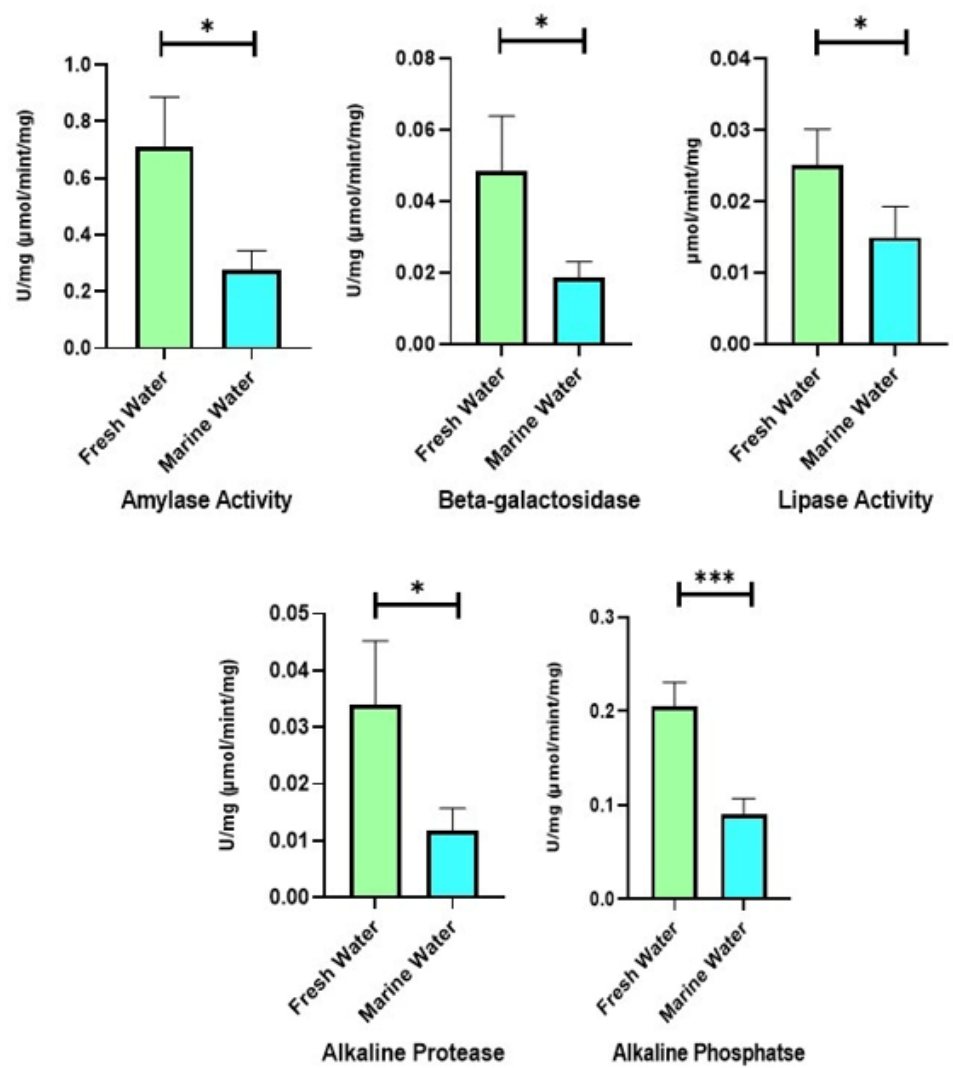


Figure 2: Comparative digestive enzyme activity in the intestinal extracts of Hilsa (*Tenuklosa ilisha*) collected from marine and freshwater habitats. The chart illustrates significant differences in the activities of key enzymes, including amylase, lipase, alkaline protease, β -galactosidase, and alkaline phosphatase, reflecting the physiological adaptations associated with habitat transition during migration. Data are presented as mean $\pm$ standard deviation.

activation of amylase and β -galactosidase supports efficient utilization of diverse plant polysaccharides [21], crucial for energy production and gonadal maturation. Lipase activity rose 1.5-fold in freshwater ($0.025 \pm 0.00499 \text{ Umg}^{-1}$) compared to marine ($0.0149 \pm 0.004 \text{ Umg}^{-1}$). These enzymes, active at the intestinal brush border [22], hydrolyses triglycerides into fatty acids, replenishing lipid reserves needed for reproduction [23]. Alkaline protease levels were significantly higher in freshwater ($0.03384 \pm 0.011 \text{ Umg}^{-1}$) than in marine samples ($0.0116 \pm 0.004 \text{ Umg}^{-1}$), sup-

porting protein digestion for tissue repair and enzyme/gonad development. Alkaline phosphatase (ALP) also increased over twofold ($p < 0.05$), suggesting elevated phosphate metabolism and cellular turnover. Strong positive correlations among all digestive enzymes indicate an integrated response typical of omnivorous teleost like hilsa, allowing rapid adaptation to diverse dietary resources-phytoplankton, zooplankton, and crustaceans-encountered during migration.

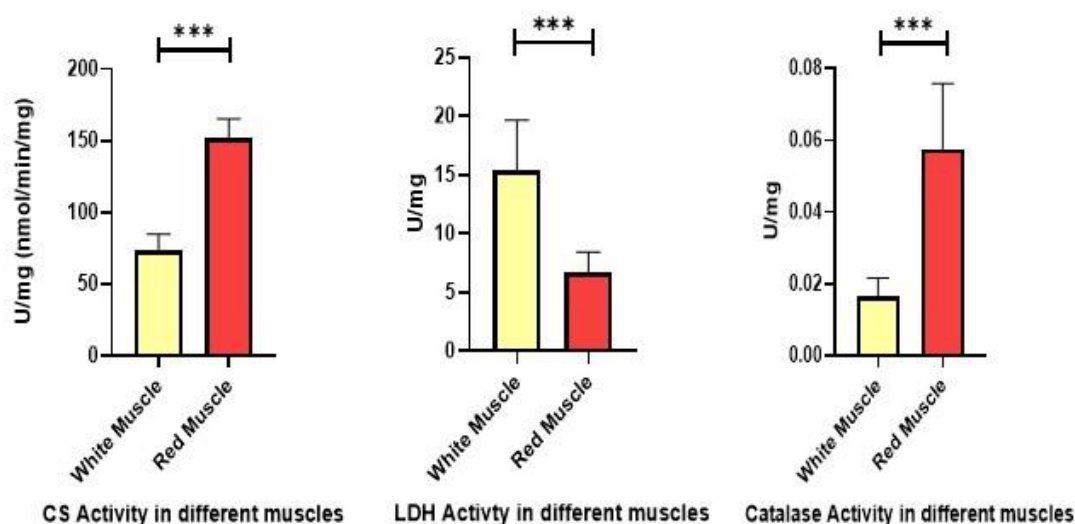


Figure 3: Activity of metabolic enzymes (mean $\pm$ standard deviation) (LDH, CS, and catalase) in red and white muscle tissues of Hilsa ($n=17$). Comparison is made between tissue types. Statistically significant differences were observed between tissues ($*** < 0.001$).

Contrasting with the digestive response, muscle-based metabolic enzymes displayed variation based on muscle type but not significantly between marine and freshwater habitats. Citrate synthase (CS), a mitochondrial enzyme indicative of aerobic capacity, was nearly three times higher ($p < 0.0001$) in red muscle ($151.80 \pm 13.577 \text{ nmolmin}^{-1}\text{mg}^{-1}$) than in white muscle ($73.484 \pm 11.50 \text{ nmolmin}^{-1}\text{mg}^{-1}$). CS is a mitochondrial matrix enzyme that forms a connecting link between aerobic respiration and the glycolytic pathway. Red muscle contains a higher number of mitochondria, making it the prime region for aerobic

respiration. It is situated along the lateral side of the fish's body, approximately at mid-length, and is essential for sustained endurance swimming. Since aerobic respiration occurs in red muscle, it also serves as the main site for beta-oxidation during migration [24,25]. Conversely, lactate dehydrogenase (LDH), essential for anaerobic metabolism, showed higher activity in white muscle ($15.43 \pm 4.24 \text{ Umg}^{-1}$) than in red muscle ($6.656 \pm 1.785 \text{ Umg}^{-1}$; $p < 0.001$), facilitating short bursts of movement for prey capture or predator evasion. LDH catalyses the reversible conversion between L-lactate and pyruvate. Al-

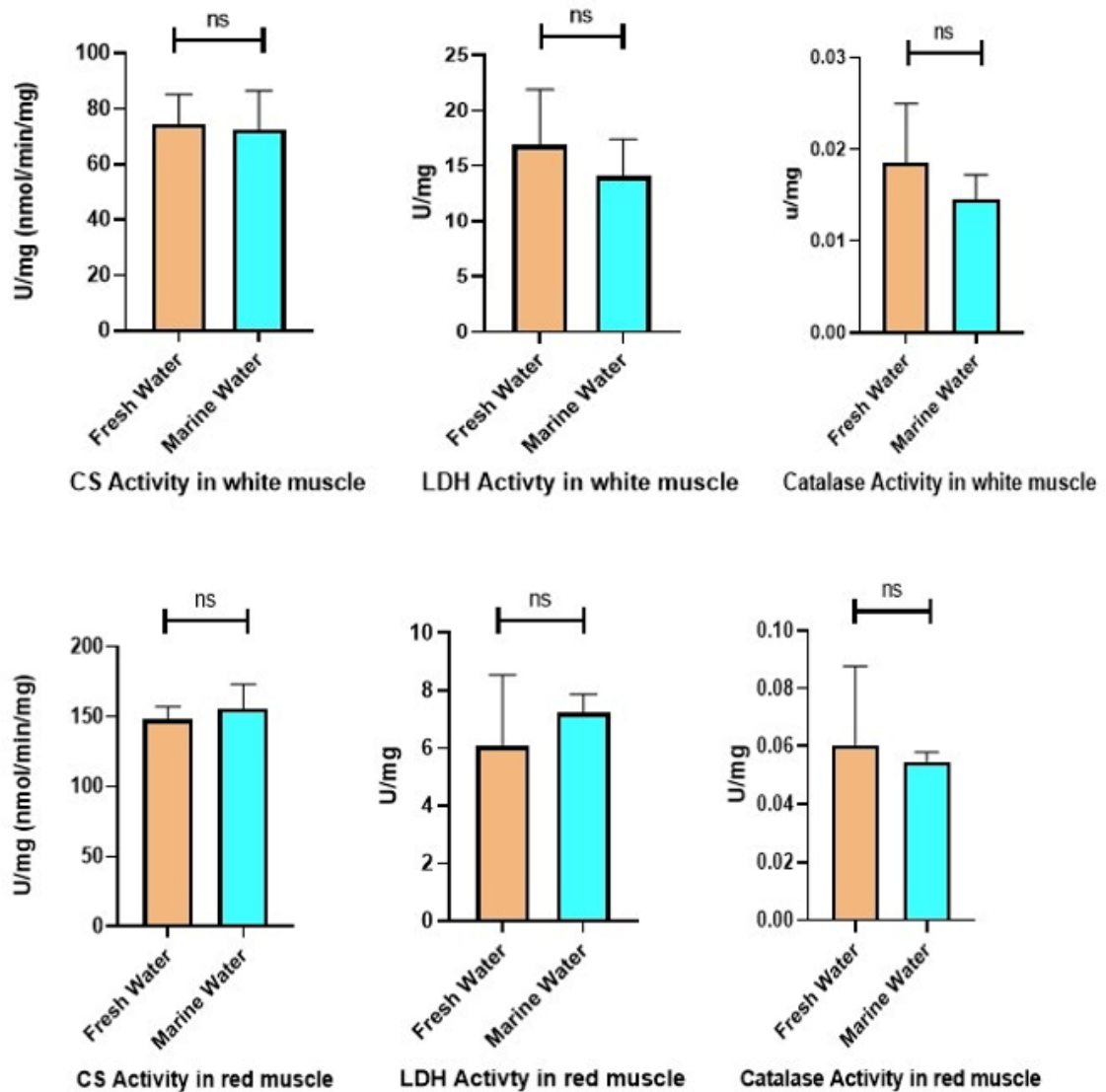


Figure 4: Activity of metabolic enzymes (mean $\pm$ standard deviation) lactate dehydrogenase (LDH), citrate synthase (CS), and catalase (CAT) in red and white muscles of hilsa collected from marine (n=9) and freshwater habitats (n=8). Comparisons were made separately for red and white muscles across the two habitats. No statistically significant differences were observed between the habitats for any of the enzymes (ns, $p > 0.05$).

though ATP yield from anaerobic respiration is lower than that of aerobic respiration, it is crucial for generating energy rapidly during sudden, intense activity without relying on mitochondrial pathways. This highlights a trade-off between the immediacy and efficiency of ATP production [26]. Catalase, an antioxidant enzyme that mitigates oxidative stress, was also significantly higher ($p < 0.0001$) in red muscle ($0.0575 \pm 0.0182 \text{ U mg}^{-1}$) compared to white muscle ($0.01658 \pm 0.004 \text{ U mg}^{-1}$), consistent with the elevated oxidative load in mitochondria-rich tissues [27]. This well-known enzyme detoxifies cells by neu-

tralizing reactive oxygen species. Its higher expression in red muscle can be attributed to the greater metabolic activity and higher rate of beta-oxidation in this tissue, requiring enhanced antioxidant defence.

These enzymes did not show significant differences between habitats. As the fish were captured within a week of arrival at the upstream location (Station A), it is possible that they may require more time to fully acclimate to the new environment, which could later be reflected in changes in metabolic enzyme activities.

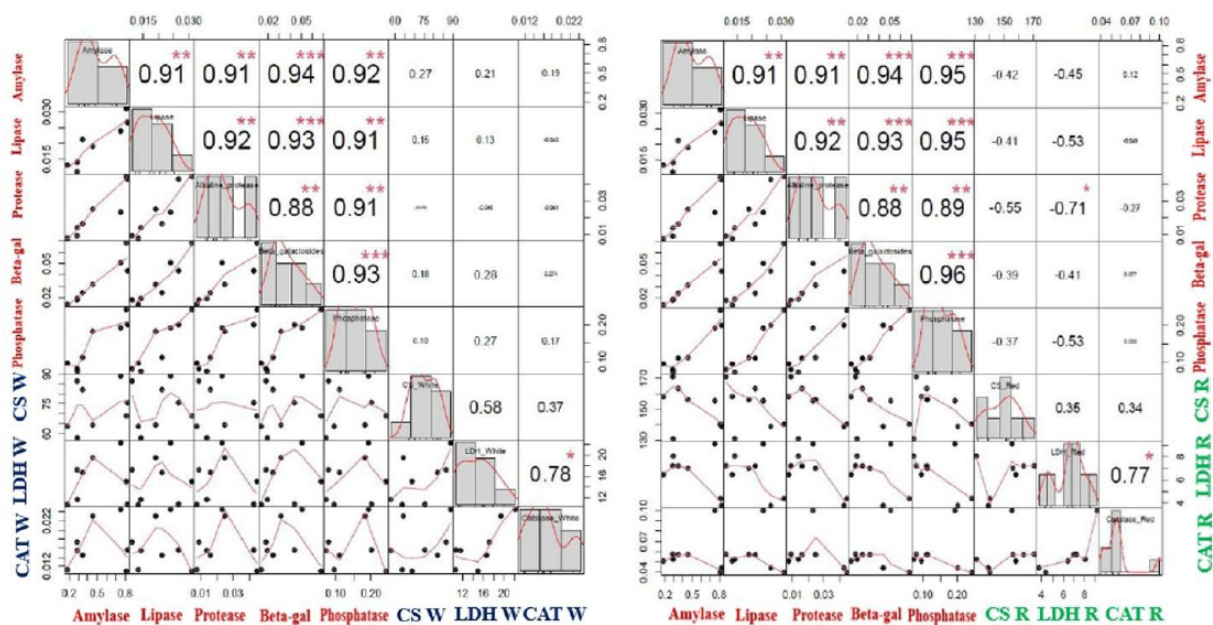


Figure 5: Pearson correlation analysis between various muscle metabolic enzymes and intestinal digestive enzymes in female Hilsa (*Tenulosa ilisha*), visualized using the “Performance Analytics” R package. The left correlation matrix depicts correlations between white muscle metabolic enzymes and digestive enzymes, while the right one illustrates the corresponding relationships for red muscle.

We also explored potential relationships between digestive and metabolic enzymes. A strong positive correlation was observed among the various digestive enzymes, suggesting a coordinated digestive response. This pattern supports the omnivorous nature of Hilsa, which consumes both carbohydrate- and protein-rich resources during migration, possibly explain-

ing the simultaneous presence of multiple digestive enzymes in the intestine. However, no consistent correlation was found between digestive enzymes and metabolic enzymes. In red muscle, the metabolic enzymes showed negligible or even negative correlations with digestive enzymes, while a similar pattern was observed in white muscle. Although catalase activity

did not vary significantly across habitats, it showed a strong positive correlation with LDH activity in white ($r^2 = 0.78$) and red muscle ($r^2=0.77$). Minor fluctuations in both LDH and catalase activity may account for these tissue-specific patterns. A slight non-significant negative trend in red muscle and positive trend in white muscle metabolic enzymes was observed, possibly indicating reduced red muscle activity with a corresponding increase in white muscle involvement during foraging. The absence of significant difference in muscle metabolic enzymes activities between the two sites suggests that the locomotory metabolic capacity of hilsa remains relatively stable during upstream migration. This may reflect the continuous swimming activity required both in the marine feeding grounds and during migration to freshwater spawning habitats, thereby maintaining comparable metabolic demands in skeletal muscle. It should be noted that the residence time of hilsa in freshwater during migration is relatively short, which may not be sufficient to induce substantial changes in muscle metabolic enzyme activities. These findings suggest that, unlike digestive enzymes, which rapidly respond to changes in feeding conditions, muscle metabolic enzymes are more conserved and maintain the metabolic capacity required for sustained locomotion throughout the migration.

4 Conclusion

Altogether, these findings shed some light on the physiological characteristics of Hilsa in response to its anadromous life cycle. The upregulation of digestive enzymes in freshwater is not merely indicative of resumed feeding but also reflects key traits such as dietary plasticity and coordinated enzyme expression. These adaptations are crucial for survival, reproductive preparedness, and ecological success in the dynamic estuarine and riverine environments. Understanding these physiological shifts helps

clarify how migratory fish like Hilsa balance nutrient uptake with energy demands throughout their journey, and can support the development of sustainable management strategies that align with their complex ecological physiology while minimizing anthropogenic impacts on their natural behaviour.

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Ethics Approval and Consent to Participate

The authors declare that ethics approval and consent to participate were not required for this work.

Conflict of Interest

The authors declare no conflicts of interest.

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