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Research Article

Cellular, Physiological, and Biochemical Basis of Adaptive Response to Variable Osmotic Environments by the River Shad, *Tenualosa ilisha*

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The river shad, hilsa (*Tenualosa ilisha*), is an anadromous fish that migrates from marine to freshwater for spawning. This transition/migration poses severe osmotic stress that hilsa needs to successfully minimize. The present study was conducted to evaluate the cellular (ultrastructure of gill and kidney), physiological (hemocyte counts, blood, and water osmolality), and biochemical (free amino acids, free fatty acids, blood glucose, and cortisol levels) parameters of hilsa collected from four different environmental salinity levels (0‰, 10‰, 20‰, and 30‰ salinity levels; using 10 fish samples from each salinity). Results indicate that increased amount of fatty/mucus cells in the gill and lower number of glomerular capsules in kidney were observed at low (0‰ and 10‰) salinities compared to high (20‰ and 30‰) salinities. Water and blood osmolality (total ionic content) showed declining trends with lowering salinities. Total number of blood cells was also found to vary significantly ($P < 0.05$) among salinities. Salinity-specific blood cortisol and glucose levels of hilsa were observed as 0‰ > 10‰ > 30‰ > 20‰. Total essential and free amino acids of hilsa blood showed significantly declining trends ($P < 0.05$) with salinity reductions. No significant differences were observed between 10‰, 20‰, and 30‰ salinities for different types of fatty acids, while hilsa collected from 0‰ showed significantly higher ($P < 0.05$) levels of fatty acids compared to the remaining three salinities. Findings indicate that hilsa rapidly alters cellular, physiological, and biochemical traits for successful transition between different salinity habitats.

1. Introduction

The migratory river shad (*Tenualosa ilisha*) is the national fish of Bangladesh (locally known as the hilsa), which is the most dominant, commercially important, and a major fishery in Bangladesh. It is an anadromous and important transboundary fish that lives in the Bay of Bengal and migrates to the upstream rivers of Bangladesh (86% share), India (8% share), and Myanmar (4% share) for breeding, and the nursing of offspring [1, 2]. Since 2016, the annual capture of hilsa has increased to 0.5 million tones and this single species alone contributes ≈12% (0.53 million MT) of the total fish production in Bangladesh [3]. Besides, hilsa contributes approximately 1.15% to the total gross domestic product (GDP) of Bangladesh. While the wild capture of hilsa shad has been increasing for the last few years, it faced severe production decline in the mid-1990s [4]. Due to implementation of strict conservation act (essentially banning the catch of gravid females during the spawning season), capture of hilsa has been increasing yearly. At present, Bangladesh alone contributes 80% of the world's total hilsa harvest. As a migratory species, ~0.3 million ton hilsa is harvested in the freshwater river systems after spawning (postmonsoon season) while majority of the total hilsa is harvested across the coastal rivers (brackish waters) and sea throughout the year [5]. However, the production of the upstream migratory rivers remained stable or even decreased due to the disruption of migratory routes by heavy siltation, loss of spawning, feeding, and nursing grounds, the indiscriminate catching of juveniles, and an increased fishing of adults [1, 6].

As a migratory (anadromous) fish, hilsa spends most of its life cycle in the sea water and migrate to the freshwater rivers for the purpose of spawning [7, 8]. Newly hatched larvae undergo many metamorphosis stages for two months in the rivers and start to migrate towards lower estuaries and coasts as "Jatka" [7], following which the juveniles move further down to the sea for growth where they continue regular feeding. Due to its migratory nature, hilsa regularly experiences broad spectrum changes in environmental salinity throughout their life cycle. Therefore, this species is specialized with the adaptation abilities between hyperosmotic seawater and hypo-osmotic freshwater environments. Changes in cellular, physiological, biochemical, and also some gene transcriptional alterations are known to provide appropriate adaptive response to large scale salinity change in different aquatic organisms [9–11]. So, it is of great interest to investigate the biological changes (cellular, physiological, and biochemical alterations) in hilsa (*T. ilisha*) for responding to the heterogeneous environmental salinities. This will help to understand the underlying mechanisms how hilsa manage large scale salinity change when they migrate from the marine waters to the freshwater river systems for breeding.

The main challenge for acclimation and survival in low salinity (freshwater) environments during migration is to maintain ionic regulation (osmoregulation) between body fluid and the surrounding medium [12, 13]. Broad spectrum changes in environmental salinity levels can impose severe osmotic stress on organisms that ultimately results in slower growth performance, increasing disease susceptibility and massive mortality [14, 15]. In order to cope with the osmotic stress, organisms must regulate internal biological mechanisms (cellular, physiological, biochemical, and transcriptional/gene expression) rapidly to deal with the adverse effects of salinity changes [16–18]. Gill and kidney are the primary organs/tissues in teleosts that help to adjust with any aspect of salinity fluctuations while intestine also plays an important role in this process particularly for water exchange. Therefore, the gill and kidney represent the most important target tissues/organs for studying various issues involved with osmoregulation or salinity change [15, 19].

The cellular basis of adaptive response to osmotic stress involves (i) changes in gill ultrastructure (increased mass of fatty acids in the gill region to restrict passive or diffusive ion loss in freshwater environments or maintaining leaky junctions in seawater to expel excessive ions) and (ii) extension (known as regulatory cell volume increase, RVI) or contraction (regulatory cell volume decrease, RVD) of gill lamellae [14, 20]. The main physiological alterations occur during the osmotic stress response due to salinity change involve (i) changes in blood osmolality or total ionic composition (active uptake or release of ions based on external environmental salinity) and (ii) increased rate of O_2 consumption (increase metabolic performance) to support extra energy for stress response [21, 24]. The biochemical changes are (i) reduced amount of free amino acids (FAAs) in the blood, (ii) increased mass of free fatty acids (FFAs) in the gill region, (iii) reduced number of blood cells due to cell lysis under stressful conditions, and (iv) glucose and hormonal changes (particularly the vertebrate stress hormone, cortisol) in the blood stream [23, 24]. Finally, the genetic alterations involve the changes in the expression levels of underlying candidate genes (osmotic stress response and osmo/ion-regulatory genes).

Osmoregulatory activities enable aquatic animals to adjust with external environmental salinity levels [25, 26]. Ability to rapidly alter the internal biological mechanisms to counterbalance the adverse environmental stressors due to salinity change determines organismal migration success (e.g., migration success for spawning of hilsa). Freshwater environments are hypotonic in nature (contains less ions) compared to the fish blood and thus, hilsa tend to loss ions from their body and also face the challenge of water influx. The reverse pattern is the common biological event in case of sea water environments where hilsa tend to gain ion but lose water. Therefore, migratory fish (hilsa) must deploy an appropriate adaptation mechanism to counteract these challenges in hypertonic to hypotonic medium [27]. The hyperosmoregulatory mechanisms compensate with a low water intake, active absorption of ions by the gills and production of hypotonic urine by kidney [3, 20, 25]. In sea water, hypo-osmoregulatory mechanisms compensate for water loss and ionic invasion. To avoid the dehydration, *Tenualosa ilisha* makes up the extra water loss through drinking higher rates of sea water. The water is absorbed by intestine, and gills restrict the entry of excess ions inside their body [12, 28, 29].

As a migratory species, hilsa (*Tenualosa ilisha*) must have developed specialized internal biological mechanisms to rapidly adapt to the large spectrum salinity change during their spawning migration. Although hilsa is commercially the most important and national fish of Bangladesh, no studies have been conducted to investigate the underlying cellular, physiological, genetic, and biochemical mechanisms to date. Therefore, this study aims to investigate the changes in selected cellular, physiological, and biochemical changes in hilsa collected from different salinity habitats.

2. Materials and Methods

2.1. Sample Collection. Live hilsa fish were collected from four different salinity habitats (Figure 1), ranging between 0 and 30‰ with 10‰ interval (from freshwater to full strength sea water environments). Live fish were collected from the wild by using specific nets with the help of local fishermen. Salinity level of each of the sampling site was recorded by using a salinity meter (model no: GR-100, Temecula, CA, USA). The recorded salinity levels at each of the sampling sites were 0‰, 10‰, 20‰, and 30‰. In total, 10 live individuals of hilsa (5 males and 5 females) were collected from each location. Adult fish (body weight ranging between 600 and 850 g) were collected from each location for this study. This study was approved by the Animal Ethics Committee of the Khulna University (Ref. No.: KUAEC-2021/09/20).

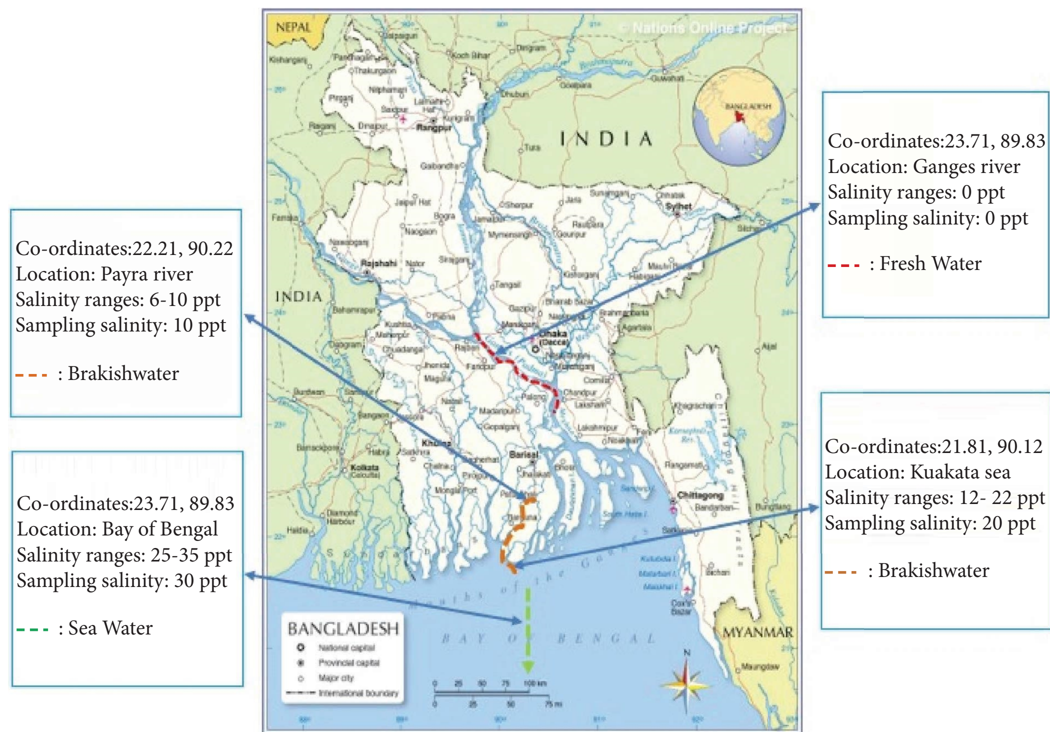

Figure 1: Sampling sites for collecting hilsa samples from different salinity habitats. "Coordinates" indicate longitude and latitude of the sampling sites; "location" indicates name of the sampling site/place; "salinity range" depends on tidal action and season (the highest range of salinity is found during high tide of dry season); "sampling salinity" indicates salinity measured at the sampling site during sample collection. Sampling was conducted during the high tide of dry season (February, 2022).

2.2. Dissection of Hilsa and Tissue Preservation. Live hilsa fish collected from different salinity habitats (rivers and sea) were euthanized in ice at the sampling sites (in the field) and dissected immediately to obtain fresh tissue samples. At first, blood samples were collected from each fish and immediately preserved in anticoagulant solution (200 IU/ml of heparin). Initially, the concentration of heparin was 5000 IU/ml which was diluted to 200 IU/ml by using Hayme's solution [30]. Blood was collected from each of the fish using a 23 gauge heparinized syringe by inserting the needle through caudal puncture. 500 μl of blood was collected from each fish and immediately transferred in tubes containing an equal volume of anticoagulant solution (heparin). The total volume of the blood samples was 1000 μl (500 μl of blood + 500 μl of heparin) for each fish. Blood samples were then kept in ice until transportation to the laboratory. Following blood collection, gill and kidney tissues were dissected out. Dissected gill and kidney tissues were sliced into smaller pieces and preserved in appropriate buffer (2.5% glutaraldehyde). Prior to sampling, 2.5% glutaraldehyde solution was prepared by mixing with appropriate amount (97.5%) of phosphate-buffered saline (PBS). Preserved gill and kidney tissues were first maintained at ambient temperature for 4 hours and then kept in ice for transportation to the laboratory. All the samples (blood, gill, and kidney) were maintained at 4°C in the laboratory for subsequent analysis.

2.3. Cellular Observations. Salinity specific changes of hilsa at the cellular level were evaluated by investigating changes in the ultrastructure of specialized osmoregulatory tissues (gill and kidney). Preserved gill and kidney tissues (in 2.5% glutaraldehyde at 4°C) were taken out from the fridge and kept at room temperature for 30 minutes. Gill and kidney tissues were used for washing with increasing concentrations of ethanol and hexamethyldisilazane (HMDS) for imaging using scan electron microscope (SEM). These washing steps make the tissue samples completely dry (dehydrate) without rupturing/damaging the cells. The following procedure (Figure 2) was followed to prepare the samples.

Following the two consecutive washing steps, samples were incubated at ambient temperature overnight to completely remove the residual chemicals. Tissue samples were then gently wrapped with aluminum foil and carefully placed in a plastic box. Samples were sent to the Genome Research Center of Jashore Science and Technology University, Bangladesh, for SEM.

2.4. Total Blood Cell Counts. 100 μl of the preserved blood from each sample was used for counting the number of blood cells according to the methods outlined in the studies of De et al. [31], Islam et al. [32], and Rahi et al. [24]. In brief, 100 μl of 10% formalin was added to the 100 μl anticoagulated blood samples to fix for 30 min at room

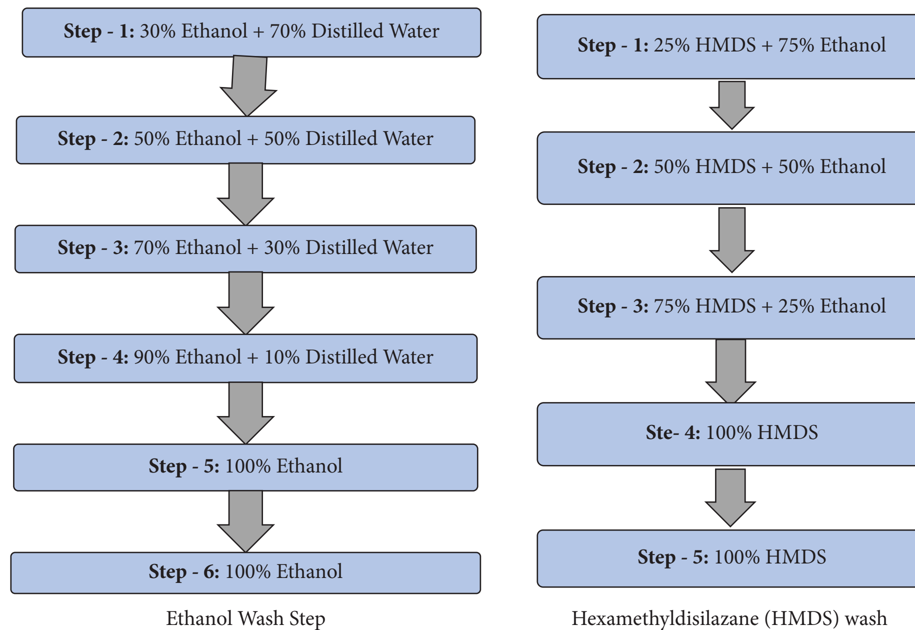

FIGURE 2: Flowchart for serial washing steps in ethanol and hexamethyldisilisane (HMDS) for gill and kidney tissues of hilsa collected from different salinity habitats.

temperature. Samples were then centrifuged at 16,000 g for 2 minutes at 4°C. Ice-cold phosphate-buffered saline (PBS, 20 mM, pH 7.2) was then added to the fixed blood samples (200 μl) with serial dilutions (at 2, 4, 8, 16, and 32 times). Finally, total numbers of blood cells were counted (no. of cells/ml of blood) by using a hemocytometer (Boeco, Germany) fitted with a microscope (SOLARIS-T-LED, Italy) at 40× magnification.

2.5. Assaying Blood and Water Osmolality. In total, 100 μl of preserved blood samples (50 μl blood + 50 μl anticoagulant) were used for measuring blood osmolality. Plasma was separated from the preserved samples by centrifuging at 6000 g for 10 minutes. Plasma osmolality was then measured using an osmometer (Osmomat 030, Gonotec, Germany). Water osmolality of the sampling sites was also measured following the same procedures.

2.6. Determining Blood Glucose and Cortisol Levels. Firstly, preserved blood samples were centrifuged at 1000 rpm for 10 minutes, then 10 μl of the supernatant was mixed well with 1 ml glucose mono reagent (Johnson & Johnson, USA) and incubated at room temperature for 10 minutes, following which blood glucose levels were analyzed using an automatic chemistry analyzer (Vitros DT60II Chemistry System, Johnson & Johnson Clinical Diagnostics Inc., New York, USA).

For determining cortisol levels in the blood plasma, 400 μl anticoagulated samples were centrifuged for 2 minutes at 16,000 g (at 4°C) to obtain adequate quantities of plasma. Approximately, 80 μl plasma was separated and preserved at −80°C for subsequent analysis. Preserved plasma samples were denatured by heating at 80°C for 1 hour. All of the heat-denatured samples were vortexed for 20 seconds, then diluted in 0.05 M phosphate-buffered saline (PBS) and vortexed again for 20 seconds. The sample mixtures were centrifuged at 13,000 g for 20 minutes to collect the supernatant. Following which, 50 μl of the supernatant was used from each sample for the competitive enzyme-linked immunosorbent assay (ELISA) in triplicate in a microtiter plate coated with anti-cortisol antibodies of rabbit. Plasma cortisol levels were then determined by using an ELISA system (IBL International, Germany), as described previously [33, 34]. Plasma cortisol levels were finally quantified by calculating the standard curve and sample value with the cubic spline method (SRS1 Cubic Spline for Excel, version 2.5.1.0) according to Bögner et al., [33].

2.7. Free Amino Acid (FAA) Levels in Blood. FAA levels in the blood of hilsa samples were determined according to the methods outlined in Long et al. [35] and Cheng et al. [36]. In brief, 100 μl of the anticoagulated blood samples were deproteinized by adding an equal volume (100 μl) of 12% trichloroacetic acid and mixed well by using a vortex. Samples were then maintained at 4°C for 20 minutes; following this step, the mixtures were centrifuged at 10,000 g for 10 minutes (at 4°C). The supernatants (100 μl from each sample) were collected and appropriate amount of 6M NaOH solution was added to the supernatant to adjust the pH level to 2.2. Finally, FAA analysis (including characterization of different amino acids) was performed using a Hitachi L-8900 amino acid analyzer (Hitachi, Tokyo, Japan) with a Li column.

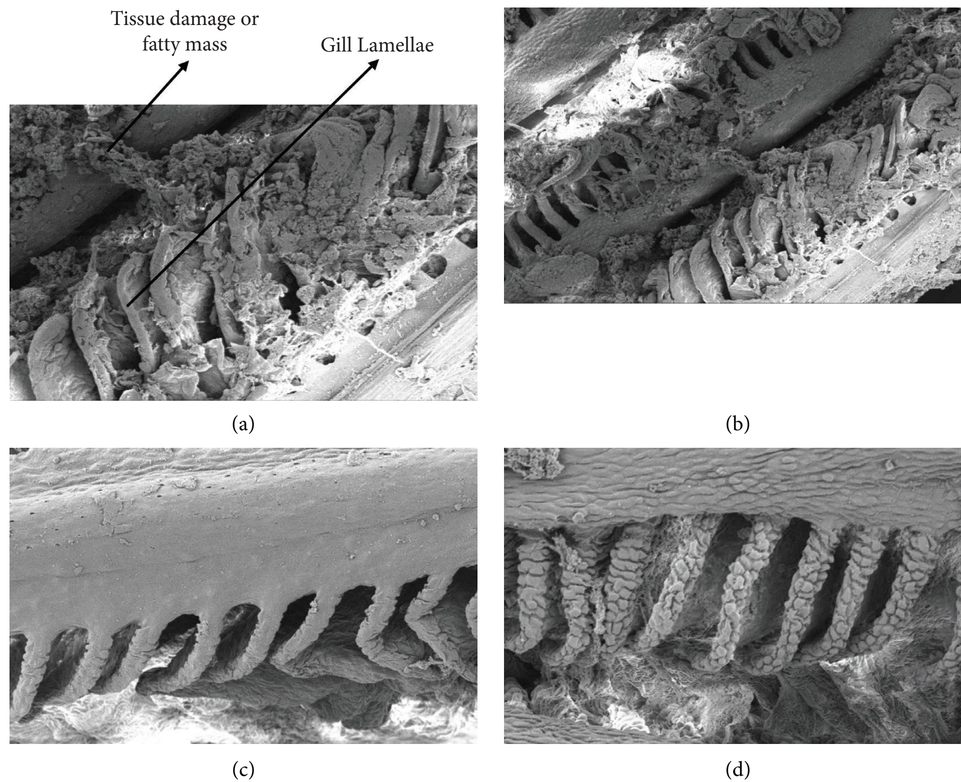

Figure 3: Changes in gill ultrastructure (SEM imaging) of hilsa collected from four different environmental salinity levels (0–30‰): (a) 0‰, (b) 10‰, (c) 20‰, and (d) 30‰. Images were taken at 1000× magnification measuring 20 μm surface area.

2.8. Measuring Free Fatty Acid (FFA) Levels. Gill tissues were dissected from the experimental fishes for FFA analysis. Gill tissues were preserved in 2.5% glutaraldehyde solution (as mentioned previously in 2.2) for this analysis. Preserved gills were taken out from the refrigerator and kept at room temperature for 30 minutes, then homogenized and freeze-dried, individually [24]. Total lipids were extracted from the gill tissues using chloroform-methanol (2 : 1, v/v) method according to Folch et al. [37]. Extracted total lipids were subsequently esterified with the 14% of boiling boron trifluoride/methanol (w/w) which was then used for fatty acid methyl ester (FAME) extraction by using hexane. FAME samples were injected to a Thermo Trace GC Ultra gas chromatograph (100 m × 0.25 mm ID, 0.2 μm film thickness) fitted Supelco SP-2560 capillary column (Supelco, Bellefonte, PA, USA) for further analysis using flame ionization detection (FID) according to Long et al. [35]. Following this step, the peaks for different fatty acids were identified by comparing retention times with a known standard (Sigma-Aldrich Co., St. Louis, MO, USA). Finally, fatty acid profiles were expressed as percentage of each fatty acid to the total fatty acids (% total fatty acids).

2.9. Statistical Analysis. Levels of significance ($\alpha = 0.05$) for different experimental results were tested for one-way ANOVA (data were also tested for Duncan's multiple range and post hoc tests, homoscedasticity, and normality tests) using the software package SPSS (version 23). These statistical analyses were performed to assess the effects of different environmental salinity levels on the selected physiological (blood osmolality and blood cell counts) and biochemical (blood glucose, cortisol, FAA, and gill FFA levels) responses of *Tenualosa ilisha*. Regression analyses were also performed between different biological markers to investigate the pattern of relatedness among the experimental data.

3. Results

The present study revealed a significant effect of different environmental salinity levels on the cellular (ultrastructure of gill and kidney), physiological (hemocyte counts, blood, and water osmolality), and biochemical (free amino acids, free fatty acids, blood glucose, and cortisol levels) parameters of hilsa (*Tenualosa ilisha*).

3.1. Changes in Gill and Kidney Ultrastructure. Scan electron microscopic (SEM) view of gill (Figure 3) and kidney (Figure 4) tissues of hilsa clearly showed salinity-specific differential view in cellular structure. Increased amount of mucus aggregations (could also be loads or mass of fat) in the gill tissues were observed at 0‰ and 10‰ salinities while lower amount of fatty mass or mucus aggregations was observed at 20‰ and 30‰ salinities for both the gill and kidney tissues. Therefore, lower numbers of cellular structures (likely be the glomerular capsules) were observed in the kidney tissue at 0‰ and 10‰ salinities (probably due to

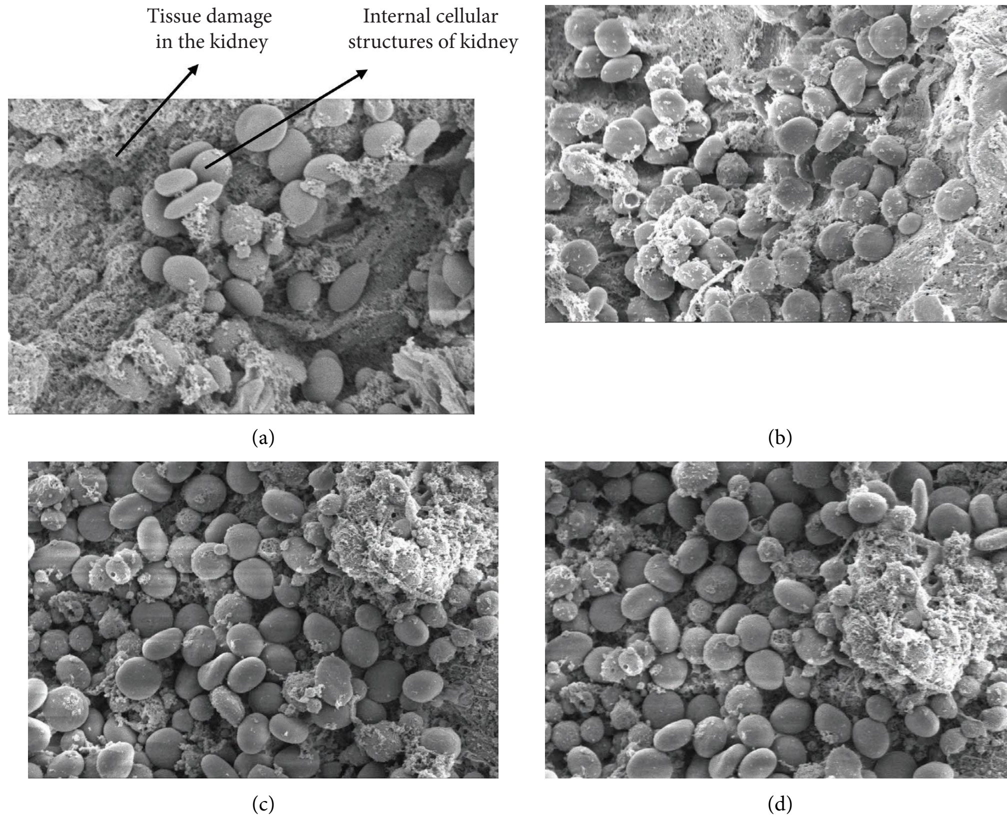

Figure 4: SEM of the kidney tissue of hilsa collected from four different environmental salinity levels (0–30‰): (a) 0‰, (b) 10‰, (c) 20‰, and (d) 30‰. Images were taken at 1000× magnification measuring 5 μm surface area.

higher amount of fatty/mucus mass) compared to the 20‰ and 30‰ (higher numbers of cellular structures in the form of glomerular capsules were observed at higher salinities).

3.2. Changes in Water and Blood Osmolality of Hilsa. A significant interaction was observed between salinity with blood and water osmolality (amount of total ionic content in a solution) in this study. A generalized declining trend in blood osmolality of hilsa (significantly lower: $P < 0.05$) was observed with lowering environmental salinity levels (from 30‰ to 0‰ salinity levels). Water osmolality was also found in a declining trend with reduced environmental salinity levels. Gradual change was observed for the blood osmolality levels while water osmolality change was rapid (Figure 5). Blood osmolality of hilsa was found to be higher than the water osmolality up to 10‰ salinity but water osmolality was significantly higher than the blood osmolality at 20‰ and 30‰ salinity. Significant differences were observed for the blood and water osmolality levels among all the environment salinity levels.

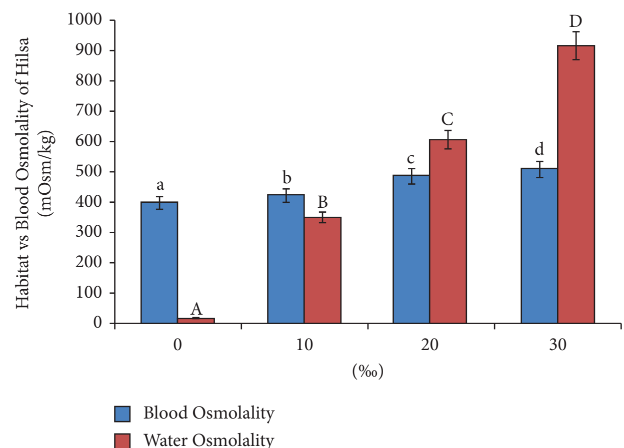

Figure 5: Habitat (salinity) specific changes in water and blood osmolality of hilsa (*Tenualosa ilisha*). Different letters above the bar indicate significant differences at $P < 0.05$. $N = 10$ fish samples per salinity. Capital letters indicate comparison among water osmolality while small letters are among blood osmolality.

3.3. Environmental Salinity-Specific Changes in the Number of Total Blood Cells. Lower levels of environmental salinity (0–10‰) significantly reduced ($P < 0.05$) total number of blood cell counts (Figure 6). No significant differences were observed between the higher salinities (20–30‰). Higher salinity levels (20‰ and 30‰) showed significantly higher number of total blood cell counts ($P < 0.05$) over the lower salinities (0‰ and 10‰). Significantly higher blood cell count was also observed for the 10‰ salinity over 0‰.

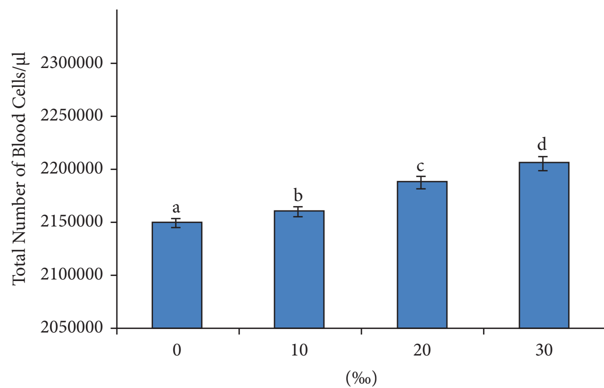

Figure 6: Total number of blood cell counts of hilsa (*T. ilisha*) with environmental salinity changes. Different letters above the bar indicate significant differences at $P < 0.05$. $N = 10$ fish samples per salinity.

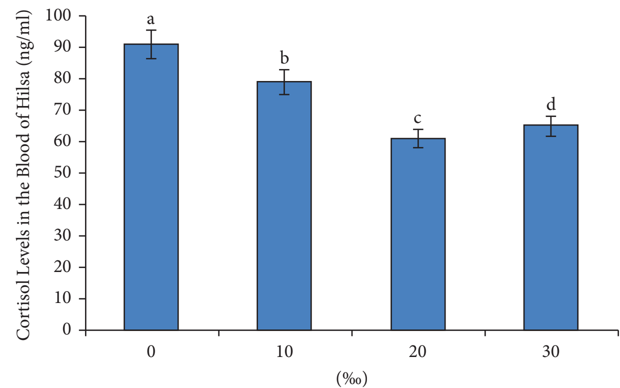

Figure 7: Environmental salinity-specific changes in blood cortisol (stress hormone) levels of hilsa (*Tenualosa ilisha*). $N = 10$ fish samples per salinity.

3.4. Changes in Blood Cortisol and Glucose Levels of Hilsa (Tenualosa ilisha). Both the blood cortisol (stress hormone) and glucose levels showed reverse pattern compared to the blood cell counts. Cortisol and glucose levels of the hilsa blood showed increasing trends with decreasing salinity levels (Figures 7 and 8). The levels of blood cortisol and glucose levels were observed as 0‰ > 10‰ > 30‰ > 20‰; the highest levels at 0‰ while the lowest levels at 20‰. Significant differences were observed for the cortisol and glucose levels ($P < 0.05$) of hilsa collected from four different environmental salinities except for 20‰ and 30‰ (no significant differences were observed between these two salinities).

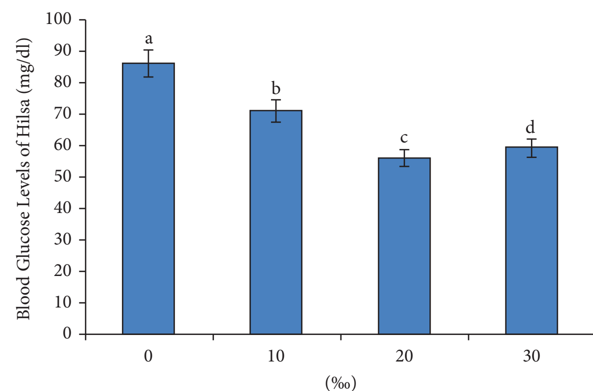

Figure 8: Salinity-specific blood glucose levels (mg/dl) of hilsa (*Tenualosa ilisha*). $N = 10$ fish samples per salinity.

3.5. Changes in the Amount of Free Amino Acids (FAAs) in the Blood of Hilsa. Amino acid profiling of the blood samples of hilsa collected from four different salinity habitats revealed 27 distinct amino acids (including essential amino acids) and amino acid derivatives (Table 1). Total essential amino acids (∑EAAs) and total free amino acids (TFAAs) showed general declining trends with reductions in environmental salinity levels. The highest levels of EAAs were observed in the blood samples of hilsa collected from 30‰ salinity (759.5 nmol·mL^{-1}); significantly higher EAAs were observed ($P < 0.05$) at higher salinities (20‰ and 30‰) compared to the lower salinities (0‰ and 10‰), but no significant differences were observed between the 20‰ and 30‰ salinities. Similarly, total free amino acids (TFAAs) were found to be in a similar trend, with the highest levels observed at 30‰ (4593.7 nmol·mL^{-1}), but no significant differences were observed between the 20‰ and 30‰ salinities. Three particular amino acids (alanine, glycine, and proline) were found to be at the highest levels at 0‰ and at the lowest levels at 20‰ salinity (Table 1).

3.6. Changes in the Amount of Free Fatty Acids (FFAs) in Gill Region. Fatty acid profiling of the gill tissue showed general increasing trends with decreasing environmental salinity levels (Table 2). No significant differences were observed between 10‰, 20‰, and 30‰ salinities for different types of fatty acids, while hilsa collected from 0‰ salinity showed significantly higher ($P < 0.05$) levels of different types of fatty acids compared to the remaining three environmental salinity levels.

4. Discussion

Variable osmotic environments significantly altered physiological (blood and water osmolality), biochemical (FAAs, FFAs, number of total blood cells, glucose, and cortisol levels), and cellular (gill and kidney ultrastructures) parameters of the migratory river shad (*Tenualosa ilisha*).

Maintaining blood osmolality (amount of ionic content) is the main challenge for any migratory aquatic species [21, 38]. Ability to rapidly adjust blood ionic content (osmolality) with changing environmental salinity determines organismal migration success and subsequent spawning activities [12, 14, 24]. Large spectrum change in environmental salinity imposes severe osmotic stress on migratory fish (e.g., hilsa). Fish typically act as hyperosmoregulators (maintaining blood ionic content below the surrounding medium) in marine environments while act as hypo-osmoregulators (maintain blood osmolality above water)

Table 1: Characterization of different amino acids (FAAs) in the blood (nmol·ml^{-1}) of hilsa (*Tenualosa ilisha*) collected from four different salinity habitats.

Amino acids	Different salinities			
	0‰	10‰	20‰	30‰
Alanine*	686.3^a ± 41.2	551.5^b ± 38.4	406.7^c ± 43.8	453.6^d ± 33.4
β-Alanine	27.8^a ± 1.1	24.6^b ± 0.8	8.76^c ± 0.7	9.66^c ± 0.9
Arginine**	407.5^a ± 3.5	443.3^b ± 4.9	419.8^c ± 5.8	428.6^d ± 6.2
Asparagine	55.9^a ± 2.8	63.5^b ± 4.1	56.2^{ab} ± 4.3	54.6^{ab} ± 3.9
Aspartic acid	40.4^a ± 3.8	38.6^a ± 3.1	23.6^b ± 3.6	24.1^b ± 2.15
α-AAA	29.1^a ± 2.4	34.7^b ± 2.1	22.6^c ± 1.9	21.9^c ± 1.6
α-ABA	29.4^a ± 1.1	20.6^b ± 0.8	4.9^c ± 0.4	5.3^c ± 0.6
Citrulline	24.4^a ± 0.6	27.9^b ± 0.7	15.4^c ± 0.3	14.8^c ± 0.4
Cystathionine	25.5^a ± 2.1	19.28^b ± 0.6	3.97^c ± 0.4	4.57^c ± 0.5
Cystine	18.73^a ± 0.4	20.11^b ± 0.5	6.71^c ± 0.4	6.02^c ± 0.7
Glutamic acid	76.9^a ± 4.4	70.7^b ± 3.3	53.6^c ± 3.4	55.4^c ± 3.6
Glutamine	246.8^a ± 10.4	276.4^b ± 33.4	242.8^c ± 39.1	230.4^d ± 38.4
Glycine*	446.5^a ± 7.6	406.7^b ± 9.8	379.6^c ± 6.9	391.5^d ± 9.9
Histidine**	58.5^a ± 2.9	51.3^b ± 2.1	33.6^c ± 1.9	34.8^c ± 1.7
Isoleucine**	40.4^a ± 1.1	36.3^a ± 1.7	20.3^b ± 1.4	21.2^b ± 1.3
Leucine**	52.4^a ± 1.9	45.8^b ± 1.4	29.7^c ± 2.2	30.6^c ± 1.6
Lysine**	55.5^a ± 2.7	70.2^b ± 3.9	63.9^c ± 4.3	63.4^c ± 3.8
Methionine**	17.11^a ± 0.5	19.01^a ± 0.6	5.91^b ± 0.2	6.06^b ± 0.7
Ornithine	53.1^a ± 3.4	45.7^b ± 3.6	26.38^c ± 4.4	22.41^c ± 2.9
Phenylalanine**	33.9^a ± 1.2	39.7^b ± 1.4	30.6^c ± 1.5	29.9^c ± 1.8
Proline*	1186^a ± 56.7	995.5^b ± 50.9	923.5^c ± 60.8	930.6^c ± 43.8
Serine	69.3^a ± 4.1	93.6^b ± 6.2	87.8^c ± 3.9	86.1^c ± 5.4
Taurine	606.8^a ± 23.7	461.5^b ± 41.7	384.1^c ± 10.4	390.4^c ± 39.4
Threonine**	61.2^a ± 3.9	80.4^b ± 4.1	86.3^b ± 6.5	87.1^b ± 5.2
Tryptophan**	17.92^a ± 0.8	19.0^a ± 0.9	6.13^b ± 0.8	6.27^b ± 1.1
Tyrosine	17.98^a ± 0.6	19.1^a ± 0.6	6.2^b ± 0.7	5.6^b ± 0.5
Valine**	45.2^a ± 4.26	61.8^b ± 4.38	52.3^c ± 4.18	51.6^c ± 4.39
∑EAAs	563.63^a ± 5.3	685.9^b ± 9.3	748.5^c ± 9.6	759.5^c ± 6.9
TFAAs	3960.9^a ± 57	4225.3^b ± 69	4503.6^c ± 71	4593.7^c ± 66

"*" denotes specific amino acids with particular roles in osmoregulation, "**" represents essential amino acids. EAA = essential amino acid; TFAA = total free amino acid. Different superscripts indicate significant difference at $P < 0.05$.

Table 2: Characterization of free fatty acid (FFA) (% of total fatty acids) in the gill tissue of hilsa (*Tenualosa ilisha*).

Fatty acids	Different salinities			
	0‰	10‰	20‰	30‰
C14:0	0.46^a ± 0.05	0.37^b ± 0.03	0.30^c ± 0.02	0.35^d ± 0.03
C15:0	0.42^a ± 0.06	0.29^b ± 0.04	0.27^b ± 0.03	0.28^b ± 0.02
C16:0	15.54^a ± 0.79	14.34^b ± 0.61	13.96^c ± 0.62	14.26^b ± 0.59
C17:0	0.53^a ± 0.04	0.46^b ± 0.04	0.44^b ± 0.03	0.45^b ± 0.03
C18:0	7.95^a ± 0.08	7.56^b ± 0.08	6.86^c ± 0.06	7.66^d ± 0.07
C20:0	0.98^a ± 0.04	0.88^b ± 0.04	0.76^c ± 0.03	0.86^b ± 0.02
C22:0	0.96^a ± 0.06	0.77^b ± 0.03	0.74^b ± 0.05	0.71^c ± 0.04
∑SFAs	26.86^a ± 0.66	24.67^b ± 0.61	23.33^b ± 0.56	24.57^b ± 0.65
C16:1n7	3.46^a ± 0.05	2.93^b ± 0.07	2.76^c ± 0.06	2.89^{bc} ± 0.08
C18:1n9	19.83^a ± 0.18	19.06^b ± 0.16	18.61^c ± 0.11	18.99^b ± 0.13
C18:1n7	3.78^a ± 0.09	3.36^b ± 0.05	2.98^c ± 0.02	3.18^d ± 0.06
C20:1n7	1.04^a ± 0.04	0.93^b ± 0.04	0.83^c ± 0.01	0.89^{bc} ± 0.02
∑MUFAs	28.11^a ± 0.32	26.06^b ± 0.29	25.18^b ± 0.22	24.97^b ± 0.28
C18:2n6	7.86^a ± 0.14	7.44^b ± 0.11	6.96^c ± 0.06	7.33^{bc} ± 0.09
C18:3n3	0.87^a ± 0.05	0.71^b ± 0.04	0.59^c ± 0.03	0.64^{bc} ± 0.01
C20:2n6	2.45^a ± 0.05	2.34^b ± 0.06	2.26^c ± 0.02	2.29^{bc} ± 0.08
C20:3n6	0.28^a ± 0.05	0.22^b ± 0.02	0.19^c ± 0.01	0.20^{bc} ± 0.03
C20:4n6	17.03^a ± 0.21	16.50^b ± 0.09	16.02^c ± 0.15	16.46^b ± 0.17
C20:5n3	10.42^a ± 0.23	9.97^b ± 0.16	9.68^c ± 0.18	9.86^b ± 0.12
C22:6n3	5.41^a ± 0.08	5.76^b ± 0.08	5.96^b ± 0.04	6.35^c ± 0.22

Table 2: Continued.

Fatty acids	Different salinities			
	0‰	10‰	20‰	30‰
∑PUFAs	$46.32^{a} \pm 0.17$	$42.94^{b} \pm 0.26$	$41.66^{b} \pm 0.12$	$43.12^{b} \pm 0.24$
∑n-3PUFAs	$15.56^{a} \pm 0.12$	$15.06^{ab} \pm 0.10$	$14.35^{b} \pm 0.11$	$14.98^{ab} \pm 0.09$
∑n-6PUFAs	$24.52^{a} \pm 0.10$	$23.96^{ab} \pm 0.09$	$23.32^{b} \pm 0.07$	$23.76^{ab} \pm 0.08$
n-3/n-6	$0.64^{a} \pm 0.03$	$0.62^{b} \pm 0.04$	$0.61^{c} \pm 0.01$	$0.62^{b} \pm 0.03$
∑HUFAs	$35.06^{a} \pm 0.25$	$34.11^{b} \pm 0.27$	$33.60^{b} \pm 0.32$	$34.06^{b} \pm 0.29$

Data represent mean ± SE. Different superscripts indicate significant differences among treatments at $P < 0.05$. ∑SFAs = total saturated fatty acids, ∑MUFAs = total monounsaturated fatty acids, ∑PUFAs = total polyunsaturated fatty acids, and ∑HUFAs = total highly unsaturated fatty acids.

in low-salinity environments [39–41]. Large-scale osmotic stress, in turn, can affect overall biological activities of hilsa (*T. ilisha*): alterations in cellular (ultrastructure of major osmoregulatory tissues), physiological (blood osmolality, growth, metabolic activity, and immunity status), biochemical (hormonal imbalance and blood hematological changes as well as FAAs and FFAs), and gene expression traits. Inability to rapidly regulate or adjust these traits will retard reproductive performance and increase mortality and susceptibility to infections/diseases that adversely affect overall wellbeing or existence of a species [20, 26].

Salinity habitat specific differences in cellular ultrastructure (SEM imaging) of hilsa in the current study (Figures 3 and 4) clearly indicate an appropriate and immediate adaptive response to successfully perform spawning migration. In low-salinity habitats (0‰ and 10‰), increased amount of mucus aggregations (fatty mass) were observed in the gill tissue (Figure 3). These cellular modifications in the gill ultrastructural view of hilsa could be due to migration from ion-rich marine to ion-deficient freshwater environments. In addition, the higher loads of fat or mucus might help to absorb or hold internal ions (and also from food) due to lower ionic content of the surrounding medium [15, 42–44]. In high-salinity environments (20‰ and 30‰), relatively lower levels of fatty mass (lower amount or even no mucus aggregations) in the gill and kidney likely help to release excessive loads of ions in high-ionic environments. Higher numbers of internal cellular structures (glomerular capsules) in the kidney (Figure 4) likely help to reduce water loss from body in high-salinity environments [45–49]. All of these findings clearly demonstrate an appropriate adaptive response by hilsa from cellular perspective during their migration pathway.

Migratory of the fish species are well known for the ability to regulate internal ionic content (blood osmolality) with environmental salinity changes, enabling them to rapidly acclimate with the surrounding environment [13, 47, 50, 51]. In low salinities (0‰ and 10‰), hilsa was found to maintained blood osmolality levels between 400 and 425 mOsm/kg H_2O, which is above the surrounding water (water osmolality levels at these salinities were 0‰ = 20 and 10‰ = 350 mOsm/kg H_2O), reflecting very strong osmotic gradients between hilsa blood and surrounding water (Figure 5). These higher levels of osmotic gradients between water and blood can potentially impose severe stress. At the highest salinity (30‰), the blood osmolality of hilsa was 510 mOsm/kg H_2O while the water osmolality was 920 mOsm/kg H_2O, which is below the medium and likely established a stronger osmotic gradient between blood and water. These differences in osmotic gradients between hilsa blood and surrounding water probably imposed significant osmotic stress on hilsa at higher salinities. Difference in osmolality level between blood and water was lowest at 20‰ (nearly an iso-osmotic condition) that probably imposed no osmotic stress on hilsa. Therefore, salinities slightly above 20‰ could be the iso-osmotic point (the point at which organisms do not face any osmotic stress) for hilsa. Adult marine fishes normally maintain blood osmolality levels at ≈280–360 mOsm/kg while some species at early developmental stages maintain ≈300–550 mOsm/kg [30, 52, 53]. Blood osmolality of hilsa was found to be considerably higher than the normal range (≈280–360 mOsm/kg) which is most likely be due to handling stress (hilsa was euthanized on ice which could initiate some degree of blood clot) and sample preservation at anticoagulant for approximately 72 hours (blood clotting likely increased the osmolality values).

Counting blood cells of fish indicate immunity status where higher number indicates better immunity [30, 54, 55]. Blood cell lysis (lower blood cell counts) can occur under stressful condition for stress mitigation that makes fish susceptible to diseases [18, 31, 56]. Although slightly lower number of total blood cell counts were obtained at low salinities (0‰ and 10‰), no significant difference in total blood cell counts between salinities (Figure 6) indicate no blood cell lysis for tackling osmotic stress. These two facts (regulating osmolality and blood cell counts rapidly) clearly indicate appropriate physiological adjustments within a short time to regularly disperse (for spawning migrations) from marine to freshwater environments for hilsa.

Measuring blood glucose and cortisol (vertebrate stress hormone) levels represents reliable biochemical method to determine the magnitude of stress on different fish species [53, 57, 58]. In the current study, significantly higher levels ($P < 0.05$) of cortisol hormone and blood glucose (Figures 7 and 8) were observed at low-salinity environments (0‰ and 10‰) indicating higher levels of stress at these two low salinities compared to the higher salinities. The lowest levels of blood glucose and cortisol at 20‰ indicate minimum stress on hilsa at this salinity, probably due to be nearly an iso-osmotic point. Free amino acids (FAAs) and free fatty acids (FFAs) are two additional biochemical components (act as intracellular osmolytes) playing important roles in ionic regulation (osmoregulation) in migratory aquatic

species [11, 59]. A gradual declining trend was observed for FAAs with decreasing salinity while a reverse pattern was observed for the FFAs (Tables 1 and 2). FAA breakdown occurs to fulfill adequate energy requirements for osmotic stress mitigation while increased amount of FFAs in the low-salinity conditions helps to hold internal ions by creating an impermeable membrane through gill [15, 60]. Three particular amino acids (alanine, glycine, and proline) are known to play vital roles in osmoregulation [35, 59]. These three amino acids showed significant differences ($P < 0.05$) in hilsa samples collected from different salinity habitats (Table 1), clearly indicating important roles of these amino acids for ionic regulation (osmoregulation) in the study species. Salinity habitat-specific changes in FFAs, FAAs, and specific amino acids (associated with osmoregulation) of hilsa (in the current study) show similar trend with those of other aquatic species [15, 35, 59]. Like the cellular and physiological changes related to osmoregulation, biochemical traits of hilsa also showed rapid alterations to cope with variable salinity environments. Although *T. ilisha* is a marine species, individuals regularly migrate between different habitats including the deep sea, inshore coastal waters, and freshwater environments (inland river systems) at different developmental stages of life. This regular transition between different salinity habitats undoubtedly imposes osmotic stress at different orders of magnitude depending on salinity change, having potential to hamper normal biological activities. Results of this study imply that the ability of hilsa to rapidly alter different cellular, physiological, and biochemical traits enable them to tolerate wide ranging salinity variations without adverse effects on them.

5. Conclusion

Osmoregulatory performance is considered as the principal mechanism for maintaining ionic balance for every aquatic organism when environmental salinity levels vary. The current study represents the effects of different osmotic gradients on cellular, physiological, and biochemical parameters of hilsa shad *Tenualosa ilisha.* Results of this study clearly indicate that the cortisol hormone, FFA, and FAA have an important role in adaptive response to salinity stress during migration in hilsa shad. Blood glucose and number of blood cells also exhibited remarkable differences among the four environmental salinity habitats. Outcomes of this study provide fundamental information on the osmoregulatory functions, migration biology, and adaptive response of *T. ilisha*, which are prerequisites for effective management of this species and other similar migratory species in Bangladesh and other countries.

Data Availability

The data used in this study are analyzed and presented in the results section in the form of tables and figures/graphs.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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