# DECIPHERING SALINITY-INDUCED TRANSCRIPTOMIC VARIATIONS IN OSMOREGULATORY TISSUES GILLS AND KIDNEY OF HILSA SHAD (*TENUALOSA ILISHA*)

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**ABSTRACT**

The hilsa shad (*Tenualosa ilisha*), the national fish of Bangladesh, is an anadromous species that migrates across environments with varying salinities, placing selective pressure on gene expression to support osmoregulation. This study examined gene expression in the gill and kidney tissues of hilsa, assessing salinity acclimation across freshwater (<0.5 ppt), brackish water (12.0 ppt), and seawater (26.0 ppt). Illumina NovaSeq 6000 sequencing generated over 1.67 billion high-quality reads, which were assembled into a *de novo* reference transcriptome of 218,867 unigenes with an average contig length of 620 bases. Sequence alignment was performed against NCBI-nr, Swiss-Prot, GO, KEGG, and COG databases, with 6,070 unigenes annotated in all databases. Using freshwater samples as a control, 502 and 257 DEGs were identified in the gills of brackish and seawater hilsa, respectively, while 632 and 89 DEGs were observed in the kidney. GO analysis highlighted osmoregulatory functions such as ion transport, transmembrane transporter activity, and metal ion binding. KEGG pathway analysis revealed involvement in pathways essential to osmoregulation, including MAPK and cAMP signaling, lipid metabolism, and adherens junctions. Differentially expressed genes related to osmoregulation included solute carrier proteins (NKCC, NHE3), channel-mediated proteins (AQP), and tight junction proteins (Claudin, Cadherin). These findings enhance understanding of *T. ilisha*'s osmophysiology and the genetic basis of its adaptive mechanisms.

**KEYWORDS:** Hilsa, Anadromous fish, RNA-seq, Transcriptome, Osmoregulation, Differential Gene Ex-pression.

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## Introduction

The hilsa shad (*Tenualosa ilisha*), Bangladesh's national fish, holds cultural and economic significance in South Asia. This anadromous species moves between marine and freshwater environments during its life cycle to spawn, feed, and grow (Sahoo et al., 2018; Hossain et al., 2019). These migrations expose the fish to highly variable salinity conditions, necessitating precise osmoregulatory control (Margolis et al., 1982). Euryhaline fish like hilsa can tolerate a wide range of salinities, from freshwater (<0.5 ppt) to seawater (30–40 ppt) (Kültz, 2015). They achieve this by adjusting physiological processes to manage salt and water balance. These adjustments are controlled by molecular mechanisms that detect environmental changes and activate specific transport systems to maintain internal stability (Marshall, 2005; Beyenbach, 2004). This remarkable adaptability supports their migratory behavior and underpins their ecological and economic importance.

Key osmoregulatory organs in fish include the gills and kidneys, both of which play essential roles in maintaining ionic and osmotic homeostasis across varying salinities. Gills are multifunctional organs that not only facilitate gas exchange but also regulate ion transport, acid-base balance, and nitrogenous waste excretion. Specialized cells such as mitochondrion-rich cells (MRCs) and pavement cells actively mediate ion absorption and secretion, enabling fish like hilsa to adapt to changing environments (Seo et al., 2013; Lai et al., 2015). The kidney plays an equally critical role in osmoregulation, particularly in regulating internal water and salt balance through dynamic control of glomerular filtration and tubular reabsorption. In freshwater, fish kidneys produce large volumes of dilute urine to eliminate excess water, whereas in saltwater, they reduce urine output and reabsorb ions to conserve fluids (Teranishi et al., 2013; Cliff and Beyenbach, 1988). In euryhaline species, the kidneys' ability to switch between these contrasting functions is vital for long-term salinity acclimation. Despite its importance, the kidney has been underrepresented in molecular studies of osmoregulation in hilsa. Previous studies have mainly focused on gill tissues (Das et al., 2018; et

al., 2019; Vindhya Mohindra et al., 2023), leaving a critical gap in understanding how the kidney contributes to salinity adaptation. As a single species, hilsa contributes significantly to national fish production in Bangladesh, comprising over 12% of the total annual yield and nearly 1% of the national GDP (FRSS, 2019). Traditional conservation efforts, like breeding programs, have had limited success in sustainably managing hilsa population (Sahoo et al., 2018). As hilsa face growing threats from pollution, habitat loss, and climate change (Rahman et al., 2020), it is increasingly important to understand the molecular mechanisms behind their salinity adaptation. Transcriptomic studies in other euryhaline fish have identified genes and pathways involved in ion transport, osmosensing, and hormone regulation, including MAPK and cAMP signaling (Fiol & Kültz, 2007; Qian et al., 2014; Heras, 2021). Such insights have important applications in conservation biology and aquaculture.

This study aims to perform a comparative transcriptomic analysis of gill and kidney tissues of *T. ilisha* across freshwater, brackish water, and seawater conditions to identify differentially expressed genes and molecular pathways involved in osmoregulation.

## Methods and Materials

### Sample Collection and Maintenance

Hilsa (*Tenualosa ilisha*) samples were collected from diverse habitats: freshwater (FW) sites on the Padma river at Munshiganj (23°25'17.6" N, 90°19'11.0" E) and the Meghna river at Barishal (22°50'03.5" N, 90°20'06" E), a brackish water (BW) site on the Payra river at Khepupara (21°57'29.0" N, 90°15'35.7" E), and from deep seawater (DS) in the Bay of Bengal near Kuakata (21°33'33.8" N, 90°15'41.4" E). Fish were measured for weight and size before being dissected under aseptic conditions to obtain gill and kidney tissues. The collected tissues were preserved in RNAlater™ stabilization solution from Thermo Fisher Scientific (Gorokhova, 2005) to prevent RNA degradation, then stored at -80°C until RNA extraction. The necessary documentation for securing animal ethics clearance and conducting fieldwork received approval from the appropriate governing body before the commencement of this study (Reference Number: KUAEC-2021/09/20). Sample collection and ethical clearance details are also mentioned in our previously published paper (Chowdhury at el, 2023).

### RNA Isolation, Library Preparation, and Sequencing

Preserved tissues were ground into a fine powder using liquid nitrogen. RNA extraction was performed using a TRIzol/chloroform method, followed by purification with the RNeasy Mini Kit (Qiagen) as per the manufacturer's instructions (Simms, Cizdziel, and Chomczynski, 1993). RNA concentration and purity were assessed using a Thermo Scientific™ NanoDrop™ One UV-Vis spectrophotometer, and RNA integrity was confirmed by 1% agarose gel electrophoresis. Before shipping, RNA samples were precipitated in a mixture of sodium acetate (3M) and ethanol (100%) for stabilization. All RNA samples were then sent to Macrogen Inc. (Seoul, Korea) for sequencing. RNA quality was re-evaluated at Macrogen using an Agilent 2100 Bioanalyzer, with samples meeting a RIN $\geq$ 5.5 and an rRNA ratio $\geq$ 0.7 selected for sequencing. While a RIN threshold $\geq$ 5.5 is considered moderate, it was deemed acceptable given the challenges of working with wild-caught, non-model fish tissues. This threshold has also been used in other transcriptomic studies (Chowdhury at el, 2023) where high-quality RNA is difficult to obtain, and it still supports reliable expression profiling. mRNA was isolated, and libraries were constructed using poly-A selection from RNA of gill and kidney tissues across three salinity conditions, with the following replicates: FW (n = 4 gill, n = 3 kidney), BW (n = 3 gill, n = 3 kidney), and DS (n = 2 gill, n = 2 kidney). Libraries were sequenced on the Illumina NovaSeq-6000 platform. The overall workflow of the study was demonstrated in **Figure 1.**

### *De novo* Transcriptome Assembly and Gene Annotation

Raw reads underwent quality assessment using FastQC, with MultiQC used for combined sample visualization (Ewels et al., 2016). Adapters and low-quality bases (Q<20) were trimmed with FastP (Chen et al., 2018). The cleaned reads were then re-evaluated using FastQC. *De novo* transcriptome assembly was conducted with Trinity (v2.13.2), utilizing the Inchworm, Chrysalis, and Butterfly modules for efficient processing of high-throughput RNA-seq reads (Haas et al., 2013). Approximately 1.59 billion paired-end reads from 17 samples were normalized *in silico* and assembled. The longest isoform per gene was selected, and non-redundant transcripts were further clustered with CD-HIT-EST (v4.8.1) at 95% similarity to reduce redundancy while retaining closely related transcript variants, following standard practice for de novo transcriptome assembly in non-model organisms (Li and Godzik, 2006). Assembly quality was assessed using BUSCO (v5.2.2) with Actinopterygii odb10 and Vertebrata odb10 lineage datasets, and TransRate (Smith-Unna et al., 2016).

Functional annotation was conducted by aligning transcripts against multiple databases (NCBI non-redundant (Nr), Swiss-Prot, COG, KEGG, and GO) with an E-value cutoff of 1e-5. TransDecoder (http://transdecoder.github.io) was employed to predict coding regions. Protein alignment was performed with DIAMOND (v2.0.15) against the Swiss-Prot database (Buchfink et al., 2015). BLASTx and BLASTp were utilized with a maximum E-value of 1e-5 and one target sequence.

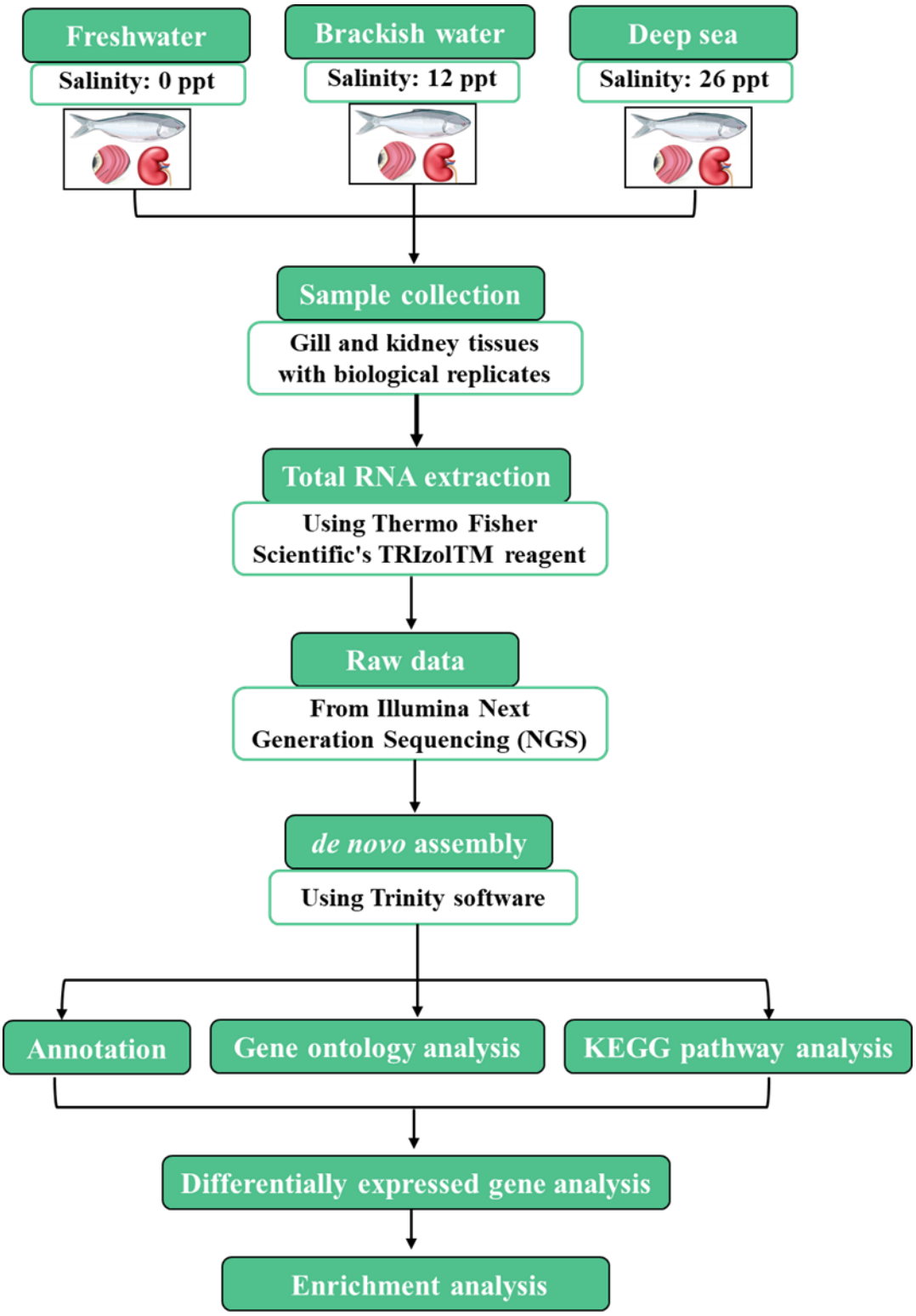


**Figure 1.** Overall workflow of this study, including sample collection, RNA extraction, sequencing, de novo transcriptome assembly, functional annotation, and differential gene expression analysis.

**Differential Gene Expression and Enrichment Analysis**

Transcript quantification was performed using RSEM (Li, 2011), and differential expression analysis was conducted with DESeq2 (Love et al., 2014). DEGs were identified by comparing FW vs. BW and FW vs. DS groups, with log2 fold change > 2.00 or < -2.00 and a p-value cutoff of 0.05. Data visualization, including hierarchical clustering and Pearson correlation analysis, was performed in RStudio with ggplot2 (Wickham, 2016).

GO and KEGG enrichment analyses of DEGs were conducted to identify enriched biological functions and pathways. KEGG pathway annotation was performed with KAAS (Moriya et al.,

2007), and enrichment was determined with ClusterProfiler in R (Yu et al., 2012), using a corrected p-value threshold of 0.05 for significant terms.

## Results

### Transcriptome Sequencing and *de novo* Assembly

Using paired-end Illumina NovaSeq 6000 sequencing, transcriptomic data from the gill and kidney tissues of *Tenualosa ilisha* across different salinity environments yielded over 1.67 billion high-quality reads. Quality control metrics indicated high precision, with Q20 and Q30 values reaching 97.4% and 93.23%, respectively, for the reads. **Supplementary Table S1** provides a summary of the raw sequence read counts from gill and kidney tissue samples of hilsa with replicates. Following quality filtering to remove adapter sequences, ambiguous 'N' bases, and low-quality sequences, a total of 1.59 billion clean reads were retained. This final dataset enabled the generation of 305,601 assembled transcripts from approximately 240 million bases, with CD-HIT-EST clustering at 95% similarity further reducing the transcript count to 218,867. Transcriptome assembly statistics is stated in **Table 1**. BUSCO analysis indicated 97.4% completeness for the Metazoa_odb10 and 82.3% for Actinopterygii_odb10 lineages, demonstrating high assembly quality (**Figure 2**).

**Table 1.** Transcriptome Assembly Statistics Based on Trinitystats.Pl and Transrate

| Parameter | Before CD-HIT-EST | After CD-HIT-EST |
|---|---|---|
| **Smallest contig length** | 181 | 201 |
| **Largest contig length** | 16238 | 16238 |
| **Average contig** | 787.34 | 620.23 |
| **Contig N50** | 1392 | 903 |
| **Contig N90** | 298 | 261 |
| **Contig over 10kb length** | 80 | 34 |
| **Contig over 1kb length** | 68642 | 31190 |
| **GC (%)** | 45.01 | 44.16 |
| **Median contig length (base)** | 409 | 349 |
| **Total assembled bases** | 240612417 | 135746953 |
| **Total trinity genes** | 226764 | 218867 |
| **Total trinity transcripts** | 305601 | 218867 |

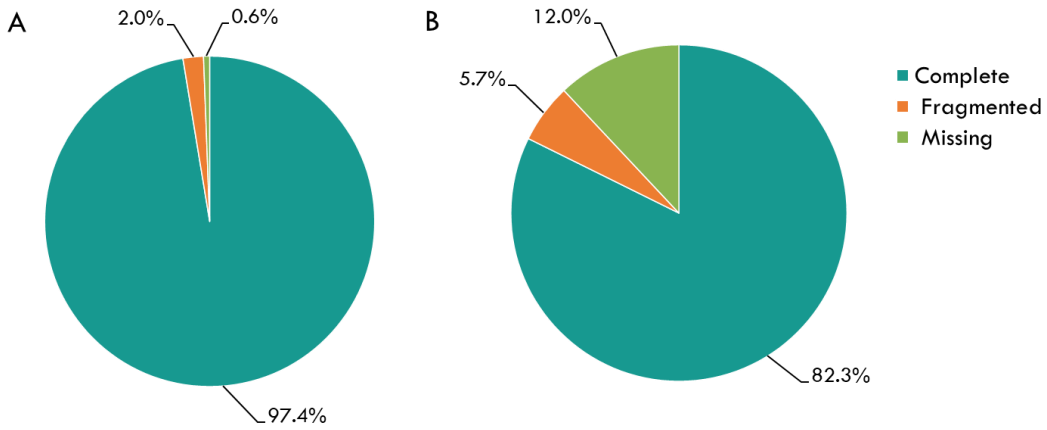


**Figure 2. BUSCO analysis of the assembled transcriptome using two ortholog databases:** (A) Metazoa_odb10 and (B) Actinopterygii_odb10. The pie charts represent the percentage of complete, fragmented, and missing orthologs, providing a measure of transcriptome completeness.

**Annotation of Assembled Transcriptome**

All assembled unigenes were annotated through BLASTX alignment against the NCBI-nr, Swiss-Prot, GO, KEGG, and COG databases. Annotation results identified 65,612 (29.98%) unigenes in NCBI-nr, 47,939 (21.90%) in Swiss-Prot, 16,918 (7.72%) in GO, 45,737 (20.90%) in KEGG, and 11,368 (5.20%) in COG (**Figure 3**). GO functional analysis classified 16,918 unigenes into 45 subcategories across three primary categories: biological process, cellular component, and molecular function. Dominant biological processes included cellular processes, metabolic processes, and biological regulation, while the major molecular functions were binding and catalytic activity. For cellular components, key categories included cell parts and protein complexes (**Figure 4**). COG analysis categorized 11,368 unigenes into 20 major functional classes, with the largest groups being related to translation, ribosomal structure, and biogenesis (J); posttranslational modification, protein turnover, chaperones (O); and signal transduction mechanisms (T) (**Figure 5**).

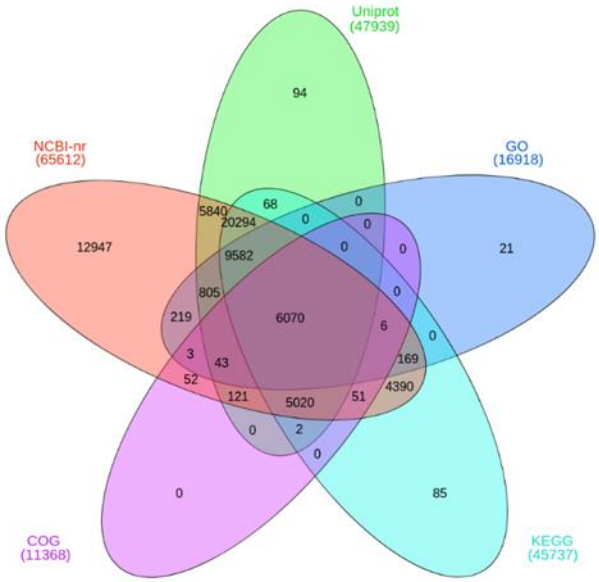


**Figure 3. Venn diagram showing BLASTX-based functional annotation of transcripts** against five public databases: NCBI-nr, Swiss-Prot, KEGG, GO, and COG. Overlapping areas indicate transcripts annotated in multiple databases.

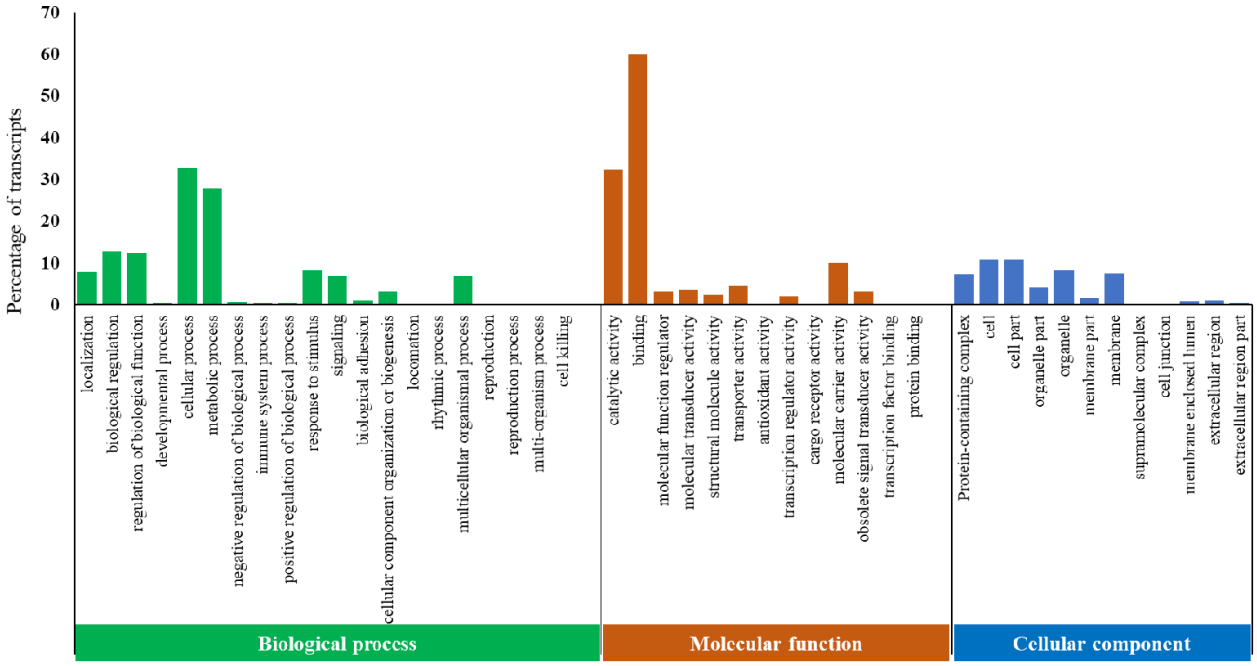


**Figure 4. GO classification of assembled transcripts.** Categories are divided into Biological Process, Molecular Function, and Cellular Component, based on sequence homology.

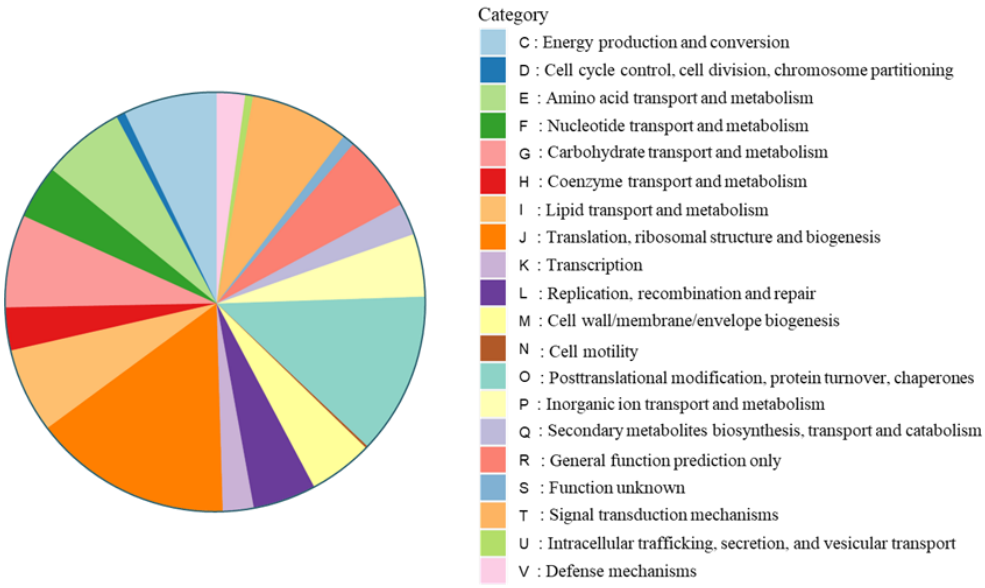


**Figure 5. COG functional classification of assembled transcripts.** The pie chart represents the distribution of annotated transcripts across major COG functional categories.

**Differential Gene Expression Analysis for Salinity Tolerance**

To investigate gene expression changes associated with salinity tolerance ,17 transcriptome samples were categorized into six groups based on tissue type and salinity: freshwater gill (FWG), brackish water gill (BWG), deep-sea gill (DSG), freshwater kidney (FWK), brackish water kidney (BWK), and deep-sea kidney (DSK). Freshwater tissue samples served as controls in each analysis. Genes were identified as differentially expressed (DEGs) if they showed a log2 fold change ≥ 2 for upregulation or < -2 for downregulation, with a p-value cutoff of 0.05. For gill tissue, 502 DEGs were identified in BWG (327 upregulated, 175 downregulated) and 257 in DSG (110 upregulated, 147 downregulated) compared to FWG. In kidney tissue, 632 DEGs were detected in BWK (479 upregulated, 153 downregulated) and 89 in DSK (51 upregulated, 38 downregulated) when compared to FWK (**Figure 6A**). A subset of DEGs was shared across environments, with 11 overlapping DEGs in gill tissues and 12 in kidney tissues between salinity conditions (**Figures 6B and 6C**). All DEGs for gill and kidney with cutoff values are shown in **Figure 7**.

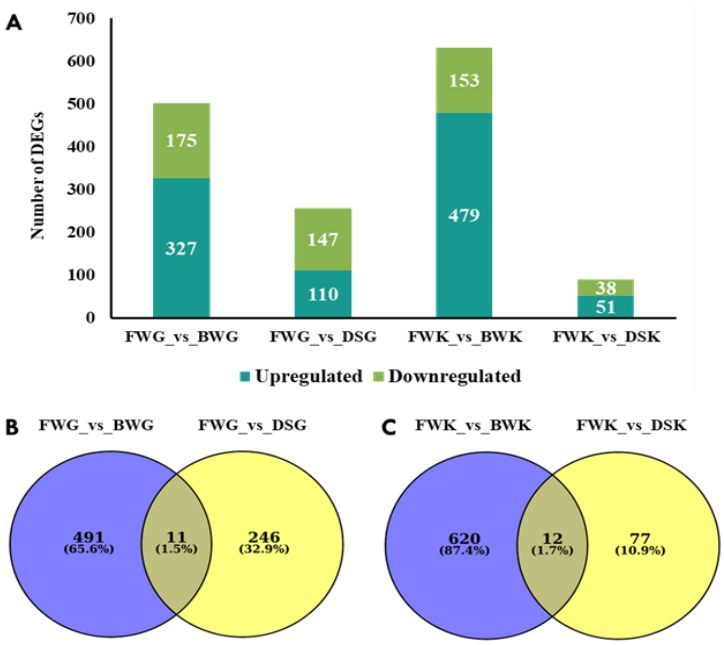


**Figure 6. Summary of differentially expressed genes (DEGs) across salinity conditions.** (A) Bar graph showing the number of upregulated and downregulated genes in gill and kidney tissues under brackish and deep-sea conditions compared to freshwater (control). (B) Venn diagram of DEGs identified in gill tissue under different salinity conditions. (C) Venn diagram of DEGs identified in kidney tissue under different salinity conditions.

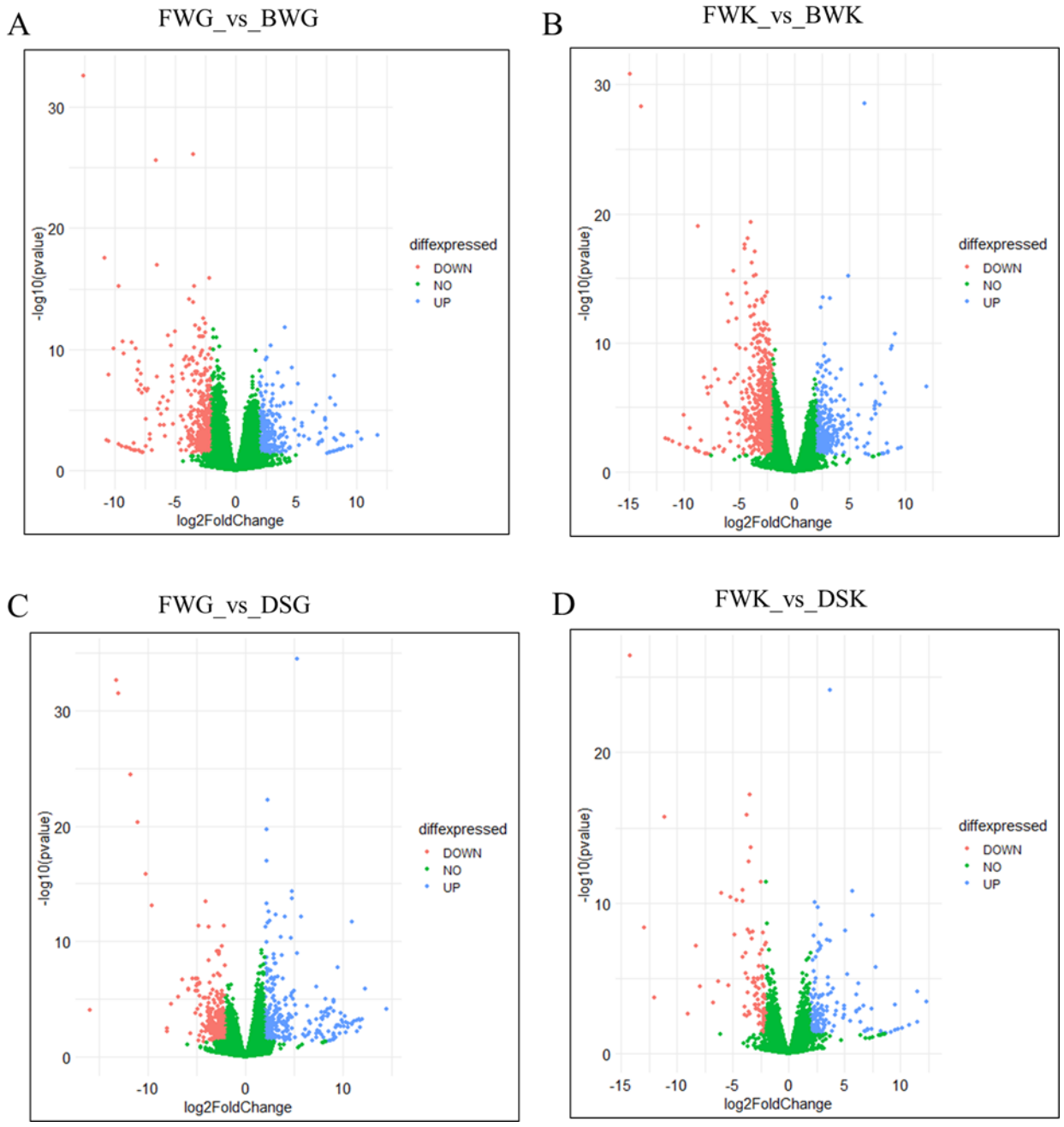


**Figure 7. Expression profiles of DEGs** in gill and kidney tissues under salinity stress. (A, B) Volcano plots of DEGs in the gill and kidney between control (freshwater) and low salinity (brackish water). (C, D) Volcano plots of DEGs between control and high salinity (deep-sea).

**Functional Enrichment and Pathway Analysis**

GO enrichment and KEGG pathway analyses were performed on the identified DEGs to elucidate the biological functions and pathways associated with salinity adaptation. Enrichment analysis revealed key GO terms among DEGs, such as cellular process, regulation of biological processes, and metabolic processes in the biological process category; binding and catalytic activity in molecular functions; and cell part and organelle in cellular components (**Figure 8**).

KEGG pathway analysis indicated that the DEGs were involved in several critical pathways linked to salinity adaptation. In FWG vs. BWG, pathways such as MAPK signaling, protein processing in the endoplasmic reticulum, and cAMP signaling were significantly enriched (**Figure 9A**). For FWG vs. DSG, enriched pathways included cell adhesion molecules, arachidonic acid metabolism, and ether lipid metabolism (**Figure 9C**). In FWK vs. BWK, enriched pathways included the cAMP signaling pathway, purine metabolism, renin secretion, and arachidonic acid metabolism (**Figure 9B**). For FWK vs. DSK, significant pathways included arachidonic acid metabolism, cortisol synthesis and secretion, and aldosterone synthesis and secretion (**Figure 9D**). These pathways are implicated in osmoregulation and salinity acclimation with specific DEGs listed in **Tables 2-5**.

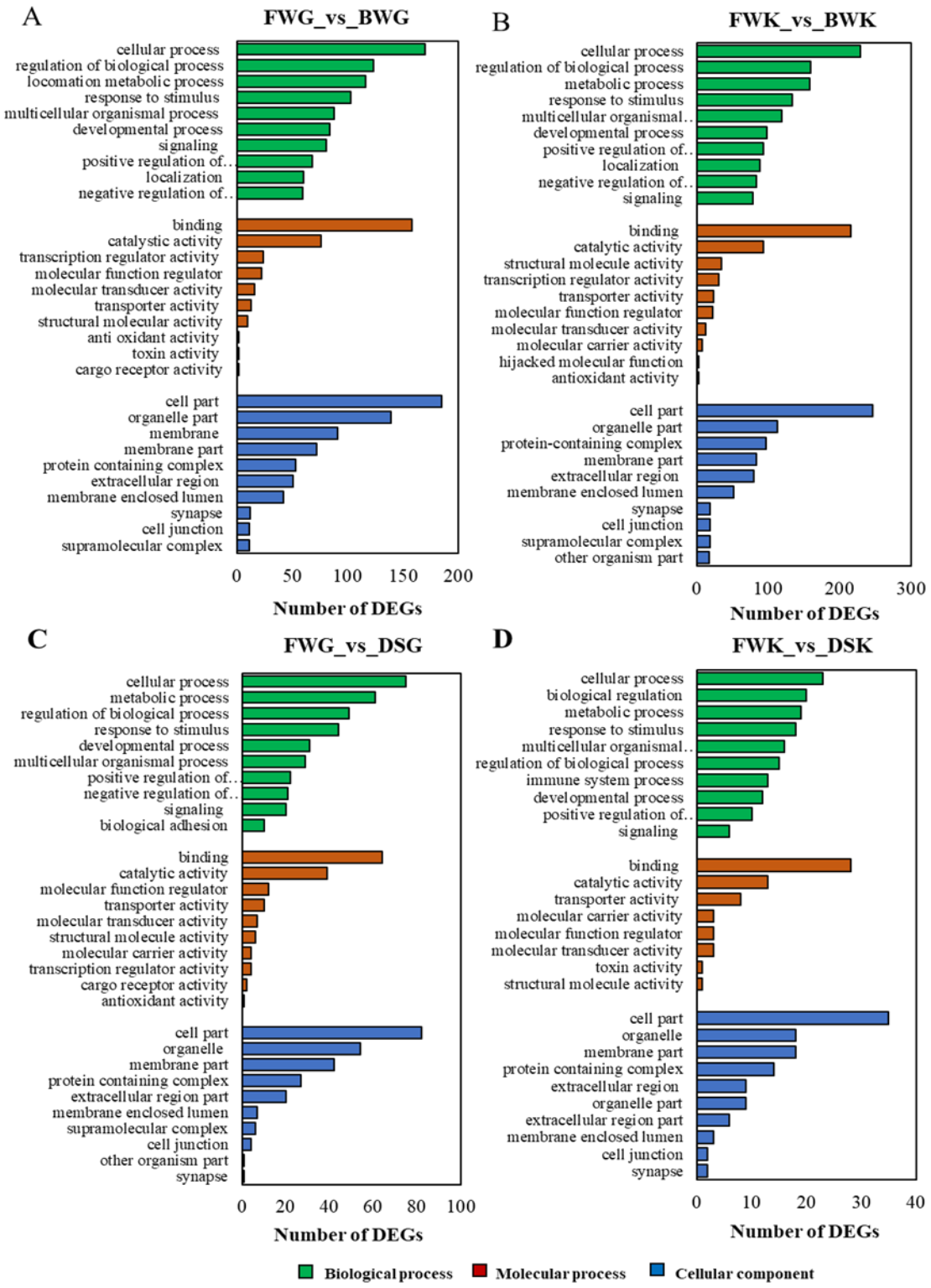


**Figure 8. Gene Ontology enrichment of DEGs** in response to salinity changes. (A, B) GO terms enriched in gill and kidney DEGs from freshwater vs brackish water. (C, D) GO terms from freshwater vs deep-sea comparisons. Categories include Biological Process, Molecular Function, and Cellular Component.

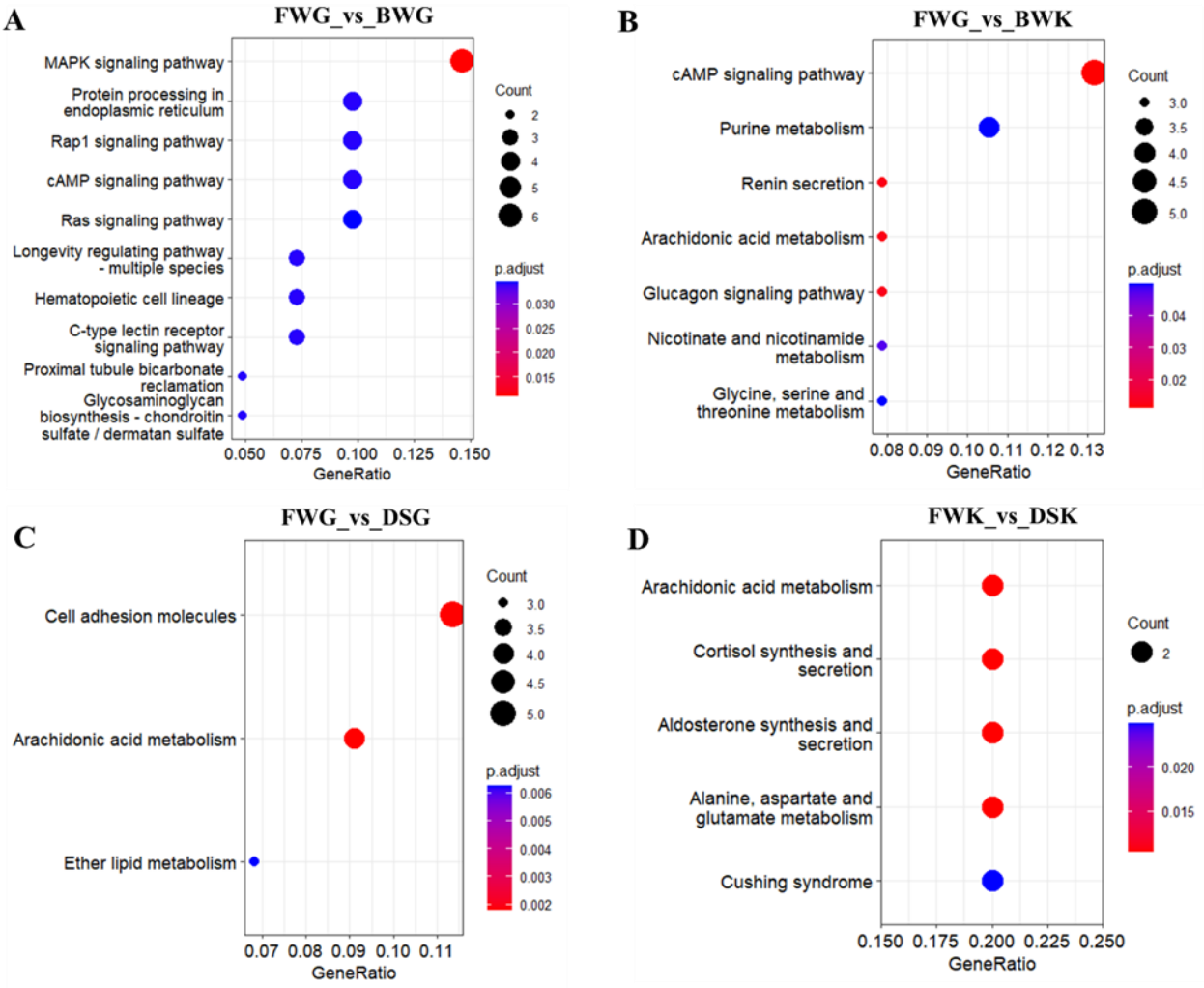


**Figure 9. KEGG pathway enrichment of DEGs** under different salinity conditions. (A, B) Significant KEGG pathways in gill and kidney between freshwater and brackish water. (C, D) Pathways in freshwater vs deep-sea comparisons.

**Table 2.** Osmoregulation-related Differentially Expressed Genes (DEGs) in Brackish Water Gill (BWG) Compared to Freshwater Gill (FWG).

| FWG_vs_BWG | | |
|---|---|---|
| **Gene name** | **Pathway ID** | **Log2FoldChange** |
| Heat shock protein 30 (HSP 30) | K09542 | 3.82 |
| Heat shock 70 kDa protein (HSP70) | K03283 | 3.01 |
| Solute carrier family 25 member 33 (SLC25A33) | K15116 | 2.87 |
| Solute carrier family 16 member 3 (Monocarboxylate transporter 4) | K08180 | 2.82 |
| Hydroperoxy icosatetraenoate dehydratase (ALOXE-3) | K18684 | 3.66 |
| GTPase HRas | K02833 | 2.57 |

| | | |
|---|---|---|
| Calcium/calmodulin-dependent protein kinase type II delta 1 chain (CAMK2D1) | K04515 | 2.51 |
| Solute carrier family 4 member 4 (Sodium bicarbonate cotransporter) (SLC4A4) | K13575 | 2.43 |
| Polyunsaturated fatty acid lipoxygenase (ALOX15B) | K00460 | 2.37 |
| Solute carrier family 2 member 1 (Glucose transporter type 1 GTR1) | K07299 | 2.37 |
| Sodium-dependent lysophosphatidylcholine symporter 1-B (NLS1B) | K23894 | 2.23 |
| Solute carrier family 38 member 2 (Sodium-coupled neutral amino acid symporter 2) | K14207 | 2.15 |
| Long-chain-fatty-acid--CoA ligase ACSBG2 (Acetyl-CoA synthetase) | K15013 | 2.14 |
| E3 ubiquitin/ISG15 ligase GRAIL | K10652 | 2.12 |
| Claudin-1 | K06087 | 2.10 |
| Actin-related protein 2-A (ACTR2A) | K17260 | 2.07 |

**Table 3.** Osmoregulation-related Differentially Expressed Genes (DEGs) in Deep-sea Gill (DSG) Compared to Freshwater Gill (FWG).

| **FWG_vs_DSG** | | |
|---|---|---|
| **Gene name** | **Pathway ID** | **Log2FoldChange** |
| Arachidonate 12-lipoxygenase | K18684 | 3.74 |
| Solute carrier family 12 member 9 (potassium/chloride transporters) | K06087 | 2.27 |
| ABC-type oligopeptide transporter ABCB9 | K05654 | -9.71 |
| Claudin-4 | K06087 | -3.19 |
| Heat shock protein beta-11 | K09542 | -2.51 |

**Table 4.** Osmoregulation-related Differentially Expressed Genes (DEGs) in Brackish Water Kidney (BWK) Compared to Freshwater Kidney (FWK).

| **FWK_vs_BWK** | | |
|---|---|---|
| **Gene name** | **Pathway ID** | **Log2FoldChange** |
| Heat shock protein beta-1 | K04455 | 3.96 |
| Solute carrier family 41 member 1 | K15122 | 3.74 |
| Serine/threonine-protein kinase WNK4 | K04456 | 2.75 |

| | | |
|---|---|---|
| Solute carrier family 9 family 2 (Na+/H+-exchanger) | K12040 | 2.71 |
| Chloride intracellular channel protein 2 | K16501 | 2.61 |
| Calcium-activated potassium channel subunit alpha-1 | K04936 | 2.53 |
| Actin, alpha skeletal muscle | K12314 | 2.53 |
| Solute carrier family 13 member 1 (Renal sodium/sulfate cotransporter) | K14444 | 2.33 |
| Claudin-1 | K06087 | 2.41 |
| Calcium-dependent secretion activator 1 | K19933 | 2.30 |
| Aquaporin-7 (AQP-7) | K08771 | 2.19 |
| Heat shock protein HSP 90-alpha | K04079 | 2.09 |
| Solute carrier family 16 member 7 (Monocarboxylate transporter 2) | K08184 | 2.05 |
| Hydroperoxide isomerase ALOXE3 | K18684 | -2.07 |
| Cystic fibrosis transmembrane conductance regulator Claudin-1 | K05031 | -3.12 |
| cadherin-related family member 1 | K16501 | -7.95 |

**Table 5.** Osmoregulation-related Differentially Expressed Genes (DEGs) in Deep-sea Kidney (DSK) Compared to Freshwater Kidney (FWK).

| **FWK_vs_DSK** | | |
|---|---|---|
| **Gene name** | **Pathway ID** | **Log2FoldChange** |
| Solute carrier family 26 member 6 | K14704 | 4.66 |
| Solute carrier family 41 membrane 1 | K15122 | 3.57 |
| Carbonic anhydrase | K18245 | 2.13 |
| Hydroperoxide isomerase ALOXE3 | K08022 | -2.61 |

**Osmoregulatory Gene Expression in Gill and Kidney Tissues**

Gill Tissue Osmoregulatory Genes: In gill tissue, several genes were found upregulated, likely contributing to osmoregulation in response to salinity change. Claudin-1, associated with cell adhesion and tight junction assembly, and polyunsaturated fatty acid lipoxygenase (alox15b), involved in cell junction and calcium binding, were upregulated. Prostasin, a gene linked to sodium transport, and several solute carrier (SLC) genes (e.g., SLC2A1 for glucose transport and SLC12 for cation-chloride symport) were also upregulated, suggesting roles in ionic balance and osmoregulation. In contrast, claudin-4 and heat shock proteins, associated with cellular junctions and ATP binding, were downregulated, suggesting modulation of specific junctional proteins in response to high salinity.

Kidney Tissue Osmoregulatory Genes: In kidney tissue, upregulated genes included solute carrier family 13 member 1, which mediates sodium symport, and calcium-dependent secretion activator 1, involved in cell adhesion and ion binding. Additional upregulated genes involved in ion transport included chloride intracellular channel protein 2, aquaporin-7 for water channel activity, and carbonic anhydrase for salinity response. Downregulated genes included hydroperoxide isomerase ALOXE3 and cadherin-related family member 1, both involved in cell adhesion and ion transport (Tables 3.8 and 3.9). These gene expression profiles underscore kidney and gill roles in osmoregulatory adaptation, facilitating ionic and osmotic balance during salinity shifts.

## Discussion

Hilsa shad is an euryhaline species capable of surviving across a broad salinity gradient from freshwater (FW) to brackish water (BW) and marine environments such as the deep sea (DS). This study provides comparative transcriptomic insights into the molecular mechanisms of osmoregulation in both gill and kidney tissues, revealing tissue-specific and habitat-dependent gene expression patterns. Unlike previous studies, which focused solely on gills (e.g., Mohindra et al., 2019, 2023), our analysis offers a broader perspective by including kidney tissues, which are equally critical for salinity adaptation in fish.

### Gill-Specific DEGs and Pathways

Gill tissues exhibited extensive transcriptional changes in response to salinity, particularly in the BW samples compared to FW samples. Among the 502 differentially expressed genes (DEGs) identified in BW gills, genes such as claudin-1 and prostasin were notably upregulated. These genes are associated with tight junction maintenance and sodium transport, respectively—key functions in gill epithelium during osmotic stress (Evans et al., 2005). Additionally, SLC2A1, a glucose transporter, was elevated, suggesting enhanced metabolic support for ion regulation. Functional enrichment analyses showed activation of MAPK and cAMP signaling pathways in BW gills, which have been implicated in osmoregulatory and stress responses across fish species (Fiol & Kültz, 2007). In the DS condition, gill tissue also showed enrichment of pathways involved in cell adhesion and arachidonic acid metabolism. This latter pathway plays a known role in maintaining membrane fluidity and regulating inflammatory and stress-related signaling (Tocher, 2003). Notably, heat shock proteins (HSPs), commonly induced under osmotic or thermal stress (Feder & Hofmann, 1999), were downregulated in DS gills, potentially reflecting reduced acute stress and a more stable cellular environment under long-term saline exposure. Similarly, the downregulation of claudin-4 may indicate selective remodeling of tight junctions under high-salinity adaptation.

### Kidney-Specific DEGs and Pathways

In contrast to gills, kidney tissues exhibited fewer DEGs in DS conditions compared to BW. Genes such as SLC13A1, chloride intracellular channel protein 2, aquaporin-7, and carbonic anhydrase were upregulated in both BW and DS conditions, highlighting the kidney's role in solute transport and acid-base balance under osmotic challenge. The regulation of water channels and ion exchangers aligns with known renal adjustments in euryhaline fish during salinity shifts (Evans et al., 2005; Kültz, 2012). The enrichment of cAMP and renin secretion pathways underscores the kidney's roles in ion transport and blood pressure modulation. In DS conditions, the aldosterone synthesis and secretion pathway was also enriched, supporting the kidney's role in hormone-regulated ion homeostasis, as observed in marine teleosts (McCormick et al., 2013). Interestingly, cadherin-related family member 1, involved in cell-cell adhesion, was downregulated in DS kidney tissue. This could suggest a controlled reduction in epithelial permeability to renal filtration, as documented in other euryhaline species during high-salinity acclimation (Shaughnessy & McCormick, 2020).

### Shared Responses and Pathways

Several molecular responses were common across both tissues. Heat shock proteins (HSPs) were upregulated in BW and DS conditions, likely as part of a general protective response to salinity-induced protein denaturation. ALOX15B and ACSBG2, involved in fatty acid metabolism, were elevated, possibly contributing to membrane remodeling under osmotic pressure (Fonseca-Madrigal et al., 2012). Antioxidant enzymes such as superoxide dismutase and glutathione peroxidase were also upregulated, indicating oxidative stress mitigation.

Markers of cell turnover and immune regulation such as E3 ubiquitin ligase upregulation (GRAIL) may suggest a role for protein degradation and apoptosis in maintaining cellular integrity during salinity adaptation, a mechanism seen in other osmotically stressed fish (Fiol et al., 2011).

### Biological and Aquacultural Implications

These findings provide important molecular insights into the adaptive responses of *T. ilisha*, and highlight candidate genes that may serve as biomarkers for salinity tolerance. This knowledge has practical implications: as salinity regimes in riverine and estuarine systems shift due to climate change, sea-level rise, and upstream damming, identifying salinity-responsive genes can support selective breeding and conservation programs aimed at sustaining hilsa populations under future environmental pressures.

## Limitations

There are few limitations in the study. The number of biological replicates, particularly for deep-sea samples, was limited, which may reduce statistical power. Additionally, the absence of a reference genome necessitated *de novo* transcriptome assembly, which may impact annotation accuracy. The study also lacks physiological measurements such as ion concentration, osmolality, or hormonal levels, which would strengthen the functional interpretation of gene expression changes. Furthermore, differential gene expression results were not validated using quantitative PCR (qPCR), primarily due to resource constraints. Including qPCR validation in future studies would enhance the robustness of transcriptomic findings. Overall, future work incorporating physiological assays, qPCR-based validation, and broader sampling will help confirm and expand upon the molecular insights presented here.

## Conclusion

This study reveals key osmoregulatory genes and pathways in both gill and kidney tissues of *T. ilisha* across salinity gradients. Our findings contribute to the understanding of how euryhaline fish manage ionic, osmotic, and oxidative stress in changing environments. Future research should focus on validating these candidate genes through qPCR, functional assays, and correlating expression data with physiological traits. Such integrative studies could guide the development of salinity-resilient hilsa strains and inform climate-resilient aquaculture strategies.

## Supplementary information

**Supplementary Table S1**

**Availability of data and materials**

All data generated or analyzed during this study are included in this article and in supplementary information. Raw RNA-

Sequencing reads of muscle tissue of *Tenualosa ilisha* have been deposited in the NCBI Sequence Read Archive (SRA) database under the NCBI Bioproject (https://www.ncbi.nlm.nih.gov/bioproject/) with accession PRJNA850620 and Biosamples (https://www.ncbi.nlm.nih.gov/biosample/) with accessions SAMN29249928.

## Declaration of interests

The authors declare that they have no competing interests.

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## Author Contributions

Mohammad Riazul Islam, Haseena Khan, and Md. Ashraful Amin started and led the project. Mohammad Riazul Islam, Haseena Khan and Afsana Akter Rupa designed the overall project. Md. Lifat Rahi collected the samples. Md. Arko Ayon Chowdhury and Sadia Noor Mou processed the samples including extraction and QC. Afsana Akter Rupa, Mohammad Riazul Islam, Haseena Khan, and Md. Lifat Rahi processed, analyzed the data, and wrote the manuscript. Amin Ahsan Ali assisted in the analysis pipeline development. All authors revised the final manuscript.

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