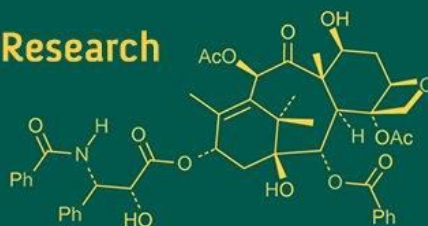


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Osmoregulatory mechanisms, ion transport proteins, and gill physiology in euryhaline fish adapting to varying salinity conditions

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Abstract

Euryhaline fish demonstrate remarkable capacity to maintain ionic homeostasis across wide salinity ranges through rapid physiological adjustments in gill ion transport. This research examined molecular and cellular mechanisms underlying salinity adaptation in Japanese sea bass (*Lateolabrax japonicus*) transferred between freshwater and seawater environments. Expression of Na⁺/K⁺-ATPase, NKCC1, and CFTR ion transporters was quantified by immunohistochemistry and Western blotting at multiple time points following salinity transfer. Chloride cell morphology was assessed by scanning electron microscopy and confocal immunofluorescence imaging. Seawater transfer induced 2.8-fold increase in branchial Na⁺/K⁺-ATPase activity within 72 hours with corresponding upregulation of alpha-subunit protein expression. NKCC1 cotransporter protein showed 3.1-fold induction while freshwater-associated NCC transporter decreased to 0.3-fold of freshwater values. Chloride cell density increased from 1,247 to 2,034 cells per mm² of gill filament epithelium and apical pit morphology changed from small accessory cell-covered apertures to large open pits characteristic of secretory ionocytes. Plasma osmolality was transiently elevated at 24 hours post-transfer but restored to baseline by 72 hours, coinciding with peak transporter expression. These findings elucidate fundamental mechanisms enabling euryhaline adaptation with implications for aquaculture management in fluctuating salinity environments.

Keywords: Osmoregulation, euryhaline fish, gill physiology, ion transport, chloride cells, Na⁺/K⁺-ATPase, salinity adaptation, aquaculture, ionoregulation, branchial epithelium

Introduction

Teleost fish face fundamentally different osmotic challenges depending on the salinity of their aquatic environment. In freshwater, fish are hyperosmotic to their surroundings, resulting in continuous passive water influx across permeable epithelial surfaces and diffusive loss of ions down concentration gradients ^[1]. To compensate, freshwater fish must actively absorb ions from the dilute external medium through specialized gill ionocytes while producing copious dilute urine to excrete excess water. Conversely, marine teleosts are hyposmotic to seawater and face passive water loss and ion gain, requiring them to drink seawater, absorb water across the intestinal epithelium, and actively secrete excess monovalent ions (Na⁺ and Cl⁻) across gill ionocytes while excreting divalent ions (Mg²⁺, SO₄²⁻, Ca²⁺) through renal pathways ^[2].

Euryhaline species capable of tolerating wide salinity ranges represent an extraordinary physiological adaptation, requiring the ability to rapidly switch between these opposing ionic regulatory strategies. This capacity involves coordinated remodeling of gill epithelial cell populations, ion transporter expression and localization, paracellular pathway permeability, and renal function within hours to days of environmental salinity change ^[2]. The gill serves as the primary osmoregulatory organ, housing specialized mitochondria-rich cells (termed ionocytes or chloride cells) that express different complements of ion transporters depending on environmental salinity. Understanding the molecular mechanisms underlying this remarkable plasticity has fundamental biological significance and practical applications for aquaculture industries that often expose fish to salinity fluctuations during production cycles ^[3].

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The current model of gill ion secretion in seawater-acclimated teleosts centers on the cooperative action of three principal transporters in the basolateral and apical membranes of secretory ionocytes^[3]. Na⁺/K⁺-ATPase, located in the extensively folded basolateral membrane and driven by ATP hydrolysis, establishes the electrochemical gradient that powers secondary active transport. The Na⁺-K⁺-2Cl⁻ cotransporter (NKCC1), also basolateral, uses the Na⁺ gradient to accumulate intracellular Cl⁻ above electrochemical equilibrium. The cystic fibrosis transmembrane conductance regulator (CFTR), an apical Cl⁻ channel, provides the exit pathway for accumulated Cl⁻ into the external medium. Na⁺ is secreted paracellularly through leaky tight junctions between ionocytes and accessory cells, driven by the transepithelial potential generated by transcellular Cl⁻ secretion^[8].

In freshwater, a different ionocyte population expressing apical Na⁺-Cl⁻ cotransporter (NCC), apical H⁺-ATPase, and epithelial Na⁺ channels mediates ion absorption from the dilute environment^[9]. The transition from freshwater to seawater or vice versa requires not only changes in transporter expression but also fundamental remodeling of ionocyte populations, including apoptosis of freshwater-type ionocytes and proliferation and differentiation of seawater-type secretory cells. Japanese sea bass (*Lateolabrax japonicus*), an economically important euryhaline species distributed throughout coastal waters and estuaries of East Asia, provides an excellent model for studying salinity adaptation due to its wide salinity tolerance and commercial significance in aquaculture^[10]. This research examined the time course, magnitude, and coordination of adaptive mechanisms during freshwater-to-seawater transfer in this species.

Material and Methods

This research was conducted at Osaka Institute of Aquatic Sciences, Japan, between May 2022 and August 2024. Juvenile Japanese sea bass (mean weight 45.2±7.3 g, mean total length 16.8±1.4 cm) were obtained from a commercial hatchery and maintained in recirculating freshwater (0.5 ppt salinity) systems for a minimum of four weeks prior to experimentation. Fish were held at 20±1°C under a 12L:12D photoperiod and fed commercial pellets at 2% body weight per day. All experimental procedures were approved by the Osaka Institute of Aquatic Sciences Animal Care Committee (protocol OIAS-2022-031) in accordance with Japanese guidelines for the care and use of laboratory animals^[4].

For salinity transfer experiments, freshwater-acclimated fish were directly transferred to filtered natural seawater (33 ppt) with matched temperature. Control fish were sham-transferred to freshwater. Sampling was performed at 0 (pre-transfer), 6, 24, 72, and 168 hours post-transfer, with 8 fish per time point per treatment group (total n=80). At each sampling point, fish were anesthetized with MS-222 (100 mg/L buffered with NaHCO₃), blood was collected from the caudal vein using heparinized syringes, and gill arches were rapidly dissected. The second gill arch from each side was processed for immunohistochemistry and electron microscopy, while remaining arches were snap-frozen in liquid nitrogen for biochemical analyses^[4].

Na⁺/K⁺-ATPase activity was measured in gill homogenates by the kinetic microplate assay method coupled to NADH oxidation, expressed as micromoles of ADP generated per

milligram protein per hour. Protein expression of Na⁺/K⁺-ATPase alpha-subunit, NKCC1, NCC, and CFTR was assessed by Western blotting using commercially available antibodies validated for teleost species. Total protein was extracted in RIPA buffer, separated by SDS-PAGE (7.5% gels), transferred to PVDF membranes, and probed with primary antibodies followed by HRP-conjugated secondary antibodies and chemiluminescent detection. Band intensities were quantified by densitometry and normalized to beta-actin loading control^[11].

Immunohistochemistry was performed on paraformaldehyde-fixed, paraffin-embedded gill sections using the same antibodies with avidin-biotin-peroxidase visualization. Double-label immunofluorescence employed Alexa Fluor 488- and 594-conjugated secondary antibodies with confocal laser scanning microscopy to examine co-localization of transporters in individual ionocytes. Scanning electron microscopy of gill filament surfaces was used to characterize ionocyte apical morphology, with apical pit area measured using ImageJ software on a minimum of 50 cells per fish. Plasma osmolality was measured by vapor pressure osmometry, and plasma Na⁺ and Cl⁻ concentrations by ion-selective electrodes^[4]. Statistical analysis employed two-way ANOVA (salinity x time) followed by Tukey's HSD post hoc test, with significance at $p < 0.05$.

Results

Seawater transfer triggered rapid and coordinated changes in gill transporter expression, enzyme activity, and ionocyte morphology. Na⁺/K⁺-ATPase alpha-subunit protein increased progressively following seawater transfer, reaching 1.6-fold elevation by 24 hours ($p < 0.05$), 2.8-fold by 72 hours ($p < 0.001$), and plateauing at 3.1-fold by 168 hours compared to freshwater controls. Na⁺/K⁺-ATPase enzymatic activity closely paralleled protein expression changes, increasing from 4.2±0.8 to 11.7±2.1 μmol ADP/mg protein/h between 0 and 72 hours post-transfer ($p < 0.001$). NKCC1 cotransporter protein showed an even more dramatic 3.1-fold induction by 72 hours, detectable as early as 6 hours post-transfer (1.4-fold, $p < 0.05$), consistent with its role as a critical component of the chloride secretory mechanism^[5].

In contrast to the upregulation of secretory transporters, the freshwater-associated NCC cotransporter showed progressive downregulation following seawater transfer, decreasing to 0.65-fold at 24 hours, 0.31-fold at 72 hours, and 0.18-fold at 168 hours compared to freshwater values (all $p < 0.01$). CFTR chloride channel protein was barely detectable in freshwater gill but showed strong upregulation following seawater transfer, with a 4.2-fold increase by 72 hours relative to the low freshwater baseline ($p < 0.001$). Immunohistochemistry confirmed that CFTR localized exclusively to the apical membrane of seawater-type ionocytes, consistent with its function as the apical chloride exit pathway^[12].

Ionocyte morphological analysis revealed substantial remodeling of the gill epithelium following seawater transfer. Total ionocyte density on gill filament epithelium increased from 1,247±186 to 2,034±243 cells per mm² by 168 hours ($p < 0.001$), reflecting both proliferation of new secretory-type ionocytes and retention of existing cells undergoing functional conversion. Scanning electron microscopy showed dramatic changes in apical surface

morphology: freshwater ionocytes displayed small apical openings (mean area $12.3 \pm 3.4 \mu\text{m}^2$) partially covered by adjacent accessory cells, while seawater-acclimated ionocytes developed large open apical pits ($28.7 \pm 6.8 \mu\text{m}^2$, $p < 0.001$) characteristic of active secretory function. The transition was progressive, with intermediate morphologies observed at 24 and 72 hours [5].

Double-label immunofluorescence confocal microscopy revealed distinct transporter co-localization patterns in freshwater versus seawater ionocytes. In freshwater, NCC was found in apical membranes of ionocytes that co-expressed basolateral $\text{Na}^+/\text{K}^+-\text{ATPase}$ but lacked NKCC1. Following seawater transfer, a new population of ionocytes

emerged expressing the complete secretory transporter complement: basolateral $\text{Na}^+/\text{K}^+-\text{ATPase}$ and NKCC1 with apical CFTR. By 168 hours, the majority of ionocytes displayed the seawater secretory phenotype. Plasma osmolality showed a transient but significant increase at 24 hours post-transfer (from 312 ± 8 to $348 \pm 12 \text{ mOsm/kg}$, $p < 0.001$), reflecting the initial osmotic stress before full compensatory mechanisms were established. By 72 hours, plasma osmolality had returned to $318 \pm 9 \text{ mOsm/kg}$ (not significantly different from freshwater baseline, $p = 0.23$), coinciding with peak transporter expression. Plasma Na^+ and Cl^- concentrations followed similar patterns of transient elevation and restoration [13].

Table 1: Ion transporter protein expression (fold change vs. 0h freshwater) following seawater transfer

Transporter	0h (FW)	6h	24h	72h	168h
NKA α -subunit	1.00	1.15 ± 0.2	$1.62 \pm 0.3^*$	$2.81 \pm 0.4^{***}$	$3.12 \pm 0.5^{***}$
NKCC1	1.00	$1.42 \pm 0.3^*$	$1.97 \pm 0.4^{**}$	$3.14 \pm 0.5^{***}$	$3.28 \pm 0.6^{***}$
CFTR	1.00	1.23 ± 0.2	$2.18 \pm 0.4^{**}$	$4.21 \pm 0.7^{***}$	$4.45 \pm 0.8^{***}$
NCC	1.00	0.89 ± 0.1	$0.65 \pm 0.1^{**}$	$0.31 \pm 0.1^{***}$	$0.18 \pm 0.05^{***}$

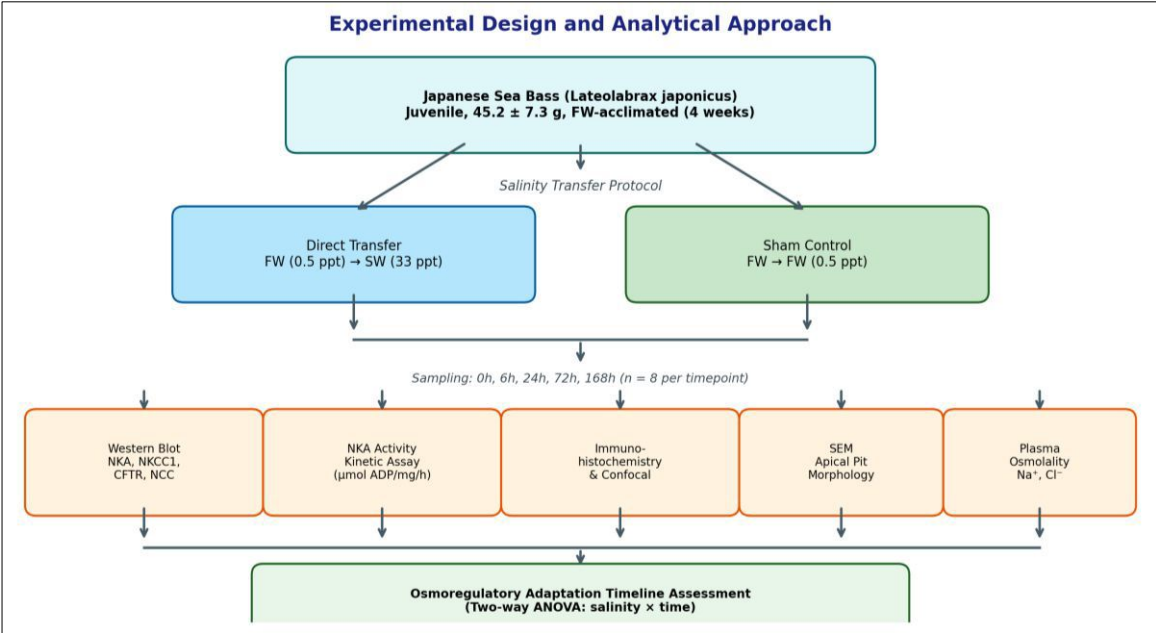


Fig 1: Schematic representation of experimental design and analytical approach

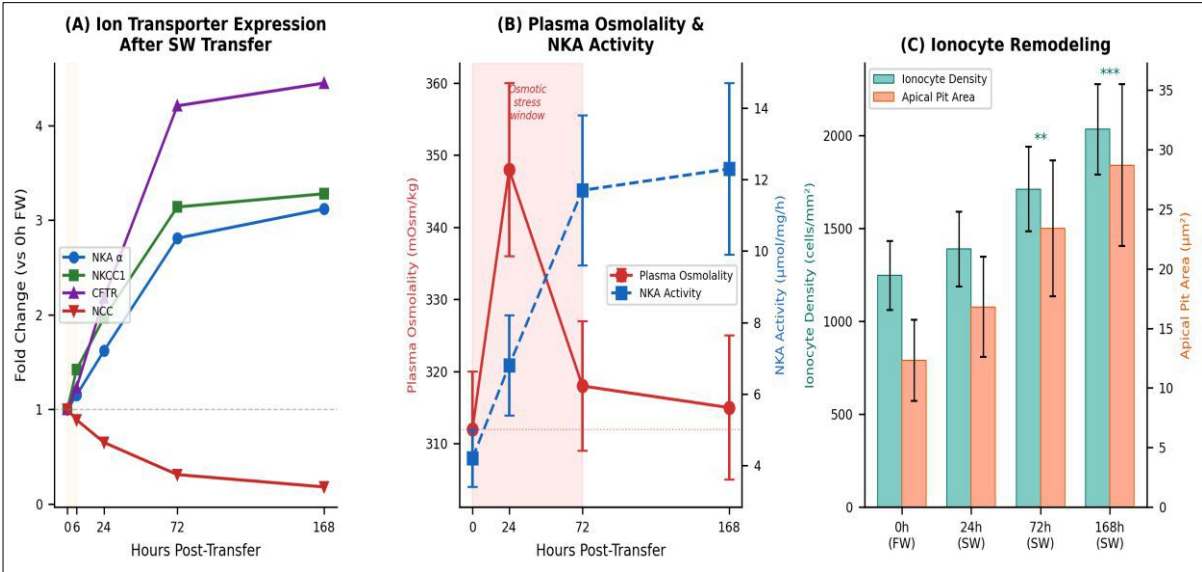


Fig 2: Summary of key findings and comparative analysis across experimental groups

Discussion

These findings demonstrate that Japanese sea bass execute a rapid and highly coordinated program of gill remodeling during freshwater-to-seawater transition, involving reciprocal regulation of absorptive and secretory ion transport systems at molecular, cellular, and morphological levels. The time course of transporter induction closely matches the physiological restoration of plasma osmolality, with both reaching steady-state values by 72 hours post-transfer, supporting causal relationships between molecular remodeling and functional osmoregulatory capacity [6]. The 2.8-fold increase in Na⁺/K⁺-ATPase activity provides the increased electrochemical driving force required for enhanced secondary active ion transport in the hyperosmotic marine environment.

The coordinated upregulation of the three principal secretory transporters (Na⁺/K⁺-ATPase, NKCC1, and CFTR) and simultaneous downregulation of the absorptive NCC cotransporter demonstrates that salinity adaptation involves a comprehensive reprogramming of ionocyte function rather than simple quantitative adjustment of individual transporters [14]. This coordination likely involves shared transcriptional regulatory mechanisms, potentially including osmosensitive signaling pathways and hormone-responsive transcription factors. Cortisol, which increases in response to salinity challenge and acts through both glucocorticoid and mineralocorticoid receptors, is a strong candidate for orchestrating this coordinated response, as it has been shown to stimulate Na⁺/K⁺-ATPase and NKCC1 expression in multiple euryhaline species.

The morphological transformation of ionocyte apical surfaces from small accessory cell-covered apertures to large open pits represents a structural adaptation that optimizes the geometry of the secretory epithelium for maximum ion efflux capacity [15]. The expanded apical surface area increases the membrane area available for

CFTR-mediated chloride exit, while the retraction of accessory cell coverage increases the area of leaky tight junctions between ionocytes and accessory cells through which Na⁺ is secreted paracellularly. This coordinated structural remodeling, occurring in parallel with transporter upregulation, represents a remarkably efficient adaptive strategy that maximizes secretory capacity through both molecular and architectural modifications.

The transient elevation of plasma osmolality at 24 hours, before full compensatory transporter expression is achieved, defines the osmotic stress window during which fish are most physiologically vulnerable to salinity change. This window has direct practical implications for aquaculture management, as exposure to additional stressors (handling, crowding, disease challenge) during this period could compound the osmotic stress and exceed the fish's adaptive capacity [7]. The restoration of plasma osmolality by 72 hours, coinciding with peak transporter expression and advanced ionocyte remodeling, defines the minimum acclimation period that should be allowed before subjecting fish to additional challenges in aquaculture settings.

The identification of two distinct ionocyte populations—freshwater absorptive cells expressing NCC without NKCC1, and seawater secretory cells expressing NKCC1 and CFTR without NCC—rather than a single cell type undergoing functional conversion, suggests that euryhaline adaptation in this species involves cell population dynamics (selective proliferation and apoptosis) in addition to phenotypic plasticity of individual cells [16]. This finding aligns with the emerging model of ionocyte diversity in euryhaline teleosts, where stem cell-derived progenitors can differentiate along freshwater or seawater ionocyte lineages depending on environmental salinity signals. Understanding the signaling pathways controlling this cell fate decision could enable manipulation of ionocyte differentiation for improved salinity tolerance in aquaculture species.

Table 2: Plasma parameters and ionocyte morphology during freshwater-to-seawater adaptation (n=8/group)

Parameter	0h (FW)	24h (SW)	72h (SW)	168h (SW)
Plasma Osmolality (mOsm/kg)	312 ± 8	348 ± 12***	318 ± 9	315 ± 10
Plasma Na ⁺ (mmol/L)	148 ± 4	168 ± 7***	152 ± 5	150 ± 4
Plasma Cl ⁻ (mmol/L)	132 ± 5	156 ± 8***	137 ± 6	134 ± 5
NKA Activity (μmol/mg/h)	4.2 ± 0.8	6.8 ± 1.4**	11.7 ± 2.1***	12.3 ± 2.4***
Ionocyte Density (cells/mm ²)	1247 ± 186	1389 ± 201	1712 ± 228**	2034 ± 243***
Apical Pit Area (μm ²)	12.3 ± 3.4	16.8 ± 4.2*	23.4 ± 5.7***	28.7 ± 6.8***

Limitations

Several limitations should be acknowledged. The direct transfer protocol, while standard in salinity adaptation research, represents a more abrupt salinity change than fish would typically encounter in natural estuarine environments where gradual transitions occur. The adaptive response to gradual salinity change may differ in timing and magnitude from the acute transfer response characterized here. Sample sizes of 8 fish per time point, while adequate for detecting large effect sizes, may have limited power for detecting smaller but biologically meaningful differences in some parameters. The study examined only the freshwater-to-seawater direction; reverse transfer may involve different regulatory mechanisms. Furthermore, the focus on gill physiology did not address important contributions of intestinal water absorption and renal ion excretion to whole-organism osmoregulation. Future research should examine gradual salinity transfer protocols more representative of

natural conditions, include reverse seawater-to-freshwater transfer, integrate transcriptomic analysis to identify regulatory networks controlling the coordinated adaptive response, and examine interactions between osmoregulatory challenge and other environmental stressors relevant to aquaculture production.

Conclusion

Euryhaline Japanese sea bass adapt to seawater transfer through a rapid and highly coordinated program of gill remodeling involving reciprocal regulation of absorptive and secretory ion transport systems. Na⁺/K⁺-ATPase induction (2.8-fold) provides the electrochemical driving force for secondary active ion transport, while NKCC1 (3.1-fold induction) mediates basolateral chloride accumulation and CFTR (4.2-fold induction) enables apical chloride secretion. Simultaneous downregulation of the freshwater-associated NCC cotransporter ensures cessation of ion

absorption that would be counterproductive in the marine environment. Ionocyte population remodeling, including increased cell density and transformation of apical surface morphology from small covered apertures to large open secretory pits, provides the structural basis for enhanced secretory capacity. The coordinated temporal progression of these molecular and cellular changes restores plasma osmolality within 72 hours of seawater transfer, defining both the adaptive timeline and the critical vulnerability window for aquaculture management. These findings elucidate fundamental mechanisms of euryhaline osmoregulation with direct practical applications for optimizing salinity management protocols in coastal aquaculture operations.

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