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Insights into gill histology and erythro-morphological alterations in juvenile *Oreochromis niloticus* under acute salt exposure

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Abstract
Aquaculture success hinges on managing stressors that impact aquatic organisms' welfare, health, growth and survival. While salt addition is known for stress reduction and antiseptic properties, it poses osmotic challenges for freshwater fish. Our study assessed salt exposure's impact on healthy monosex *Oreochromis niloticus* juveniles. A one-hour exposure to 10 and 30 g/L induced notable behavior, histology and erythro-morphological changes. Tissue histology revealed hyperplasia in epithelial and lamellar regions, with swelling, burst secondary lamellar tips, epithelial damage and pillar cell vacuolation. Epithelial hyperplasia seemed protective against excessive exosmosis. While behavior, gill histology and erythrocyte morphology showed recovery signs in 10 g/L, the short-term excessive 30 g/L osmotic shock could be fatally consequential. Prudent application of this method is crucial. However, careful consideration of concentration and duration is vital for aquatic organisms' well-being and survival. Further research should explore optimal salt exposure strategies and their long-term impact on fish health.

Keywords: Sodium chloride, aquaculture, nile tilapia, erythrocyte, gill

Introduction
Aquaculture meets surging protein demand, with growing salt use for disease control (FAO, 2020) [3]. Sodium chloride's safety, low accumulation and transport benefits have made it popular (Souza-Bastos *et al*., 2016; Tavares Dias, 2021) [9, 10], but its empirical use neglects fish tolerance and salinity stress effects. *Oreochromis niloticus*, despite being a freshwater species, is recognized for its resilience and adaptability to varying salinity levels (FAO, 2020) [3]. We address the dearth of data on *O. niloticus* response to salt exposure (10 g/L and 30 g/L), evaluating erythrocyte morphology and gill histology. Being the first of its kind, our study aims to establish effects which occur following salt treatment.

Aquaculture stands out as a rapidly expanding global farming venture, playing a vital role in meeting the escalating demand for animal protein amid a burgeoning human populace (FAO, 2020) [3]. Notably, the utilization of salt (sodium chloride, NaCl) in the cultivation of freshwater fish has gained momentum as a recently explored and investigated protocol. This practice has gained widespread popularity due to its affordability, accessibility, and inherent safety – it neither accumulates within fish tissues nor poses a threat to human health upon consumption (Tavares Dias, 2021) [10]. This application holds particular relevance during stressful events such as transportation, where it has been observed to be beneficial (Souza-Bastos *et al*., 2016) [9]. Furthermore, sodium chloride exhibits prophylactic attributes, functioning as an antiseptic agent with notable efficacy against specific parasitic infections (Tavares Dias, 2021) [10]. Nevertheless, the implementation of salt in aquaculture frequently occurs empirically, often overlooking the fish's tolerance levels and euryhaline nature (Tavares Dias, 2021) [10]. Variations in water salinity, contingent on concentration and species-specific responses, can potentially induce stress in fish. Salt presence may disrupt the osmotic equilibrium in freshwater fish, thereby influencing their overall health, growth, and even survival (Tavares Dias, 2021) [10]. Of note, *Oreochromis niloticus* has emerged as a key contributor to freshwater aquaculture, boasting a global production of 4407.2 thousand tonnes in 2021, amounting to a noteworthy 9% of the total aquaculture output (FAO, 2020) [3]. Despite being a freshwater species, *O. niloticus* is recognized for its resilience and adaptability to varying salinity levels. Particularly in the context of West Bengal, it ranks among the most cultivated fish after carps, spurring the prevalent utilization of salt exposure.

However, it is imperative to exercise caution, as the administration of salt in excessively high concentrations may verge on toxicity, potentially encroaching upon therapeutic thresholds. While earlier studies have highlighted the detrimental effects of elevated doses of salt on freshwater fish (Tavares Dias, 2021) [10], scant literature exists pertaining to its impact on erythrocyte morphology and *O. niloticus* gill histology. Souza-Bastos *et al*. (2016) [9] proposed 10 g/L salt acute exposure was safe for freshwater fish. However, no such safety data are available for *O. niloticus.* This lacuna underscores the present study's significance, aimed at assessing and contrasting the extent of erythro-morphological changes and gill histological alterations induced by salt administration (10 g/L and 30 g/L) in *O. niloticus* juveniles.

Materials and Methods

Monosex *Oreochromis niloticus* specimens (mean weight: 18.4 ± 0.2 g; mean length: 12.8 ± 0.2 cm; n = 38) were procured from a fish farm located in Sarisha, South 24 Parganas district (Lat 23.84° E Long 91.27° N). The fish were transported in oxygen-enriched plastic bags preventing any mortality during transportation. Upon arrival, the fish were introduced to cemented tanks encompassing 250 liters of dechlorinated freshwater. These tanks were equipped with continuous aeration and maintained under optimal physiochemical conditions (temperature: 22.00 °C–30.00 °C, pH: 7.80–8.60, dissolved oxygen: 4.90–5.20 mg/L, ammonia: 0.002–0.008 mg/L, nitrite: 0.14–0.53 mg/L and nitrate: 0.13–0.55 mg/L). They were acclimatized for 7 days, while being fed commercial feed (AquaExcel, 32% protein) and any uneaten food was promptly removed from the tanks after 20 to 30 minutes to ensure optimal water quality. Notably, the fish were withheld from feeding for a period of 24 hours preceding the commencement of the experiments.

Analytical-grade pure salt (Nice®) was introduced into glass aquaria (50 L) with dechlorinated freshwater. This addition aimed to achieve final concentrations of 10 (T_1) and 30 (T_2) g/L, a process conducted 24 to 48 hours before subjecting the fish to these experimental conditions. Prior to transferring the prepared water to the aquaria, the salinity of the water was verified using a refractometer (Chem Fine, Vadodara), ensuring the desired experimental salinities of approximately 9.84 and 29.26 practical salinity units (psu) or g/L of seawater salt. Individual fish were exposed for a duration of 1 h to either the control or one of the experimental salinities (T_1 or T_2). This exposure took place in aquaria with a 20 L capacity, containing 4 L of water. Following the exposure period, the fish were anesthetized using clove oil (eugenol; 20 µL/L), which was diluted in the same experimental water. Once complete anesthesia was achieved (approximately 2 mins), blood samples were collected through caudal vein puncture, utilizing insulin syringes (2 mL) filled with a 1.5 parts ethylenediaminetetraacetic acid (EDTA). Subsequently, the fish were euthanized with a severe blow to the cranium and promptly dissected, to facilitate the removal of the gill tissues, stored in Bouins fluid for histological analyses. It's noteworthy that the experimental methodology adhered to the guidelines set forth by The Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA, 2021) [1].

The collected blood from control, T_1 and T_2 was smeared (Roberts, 2012) [7] and stained with 0.2% safranin and observed under the microscope at 100× magnification for erythro-morphological alterations. Gill histological sections from the 3 experimental groups were outsourced from a lab (Pathowynd Clinical Laboratories, Kolkata) followed by photo-micrographic observations under 10× and 40× magnifications.

Results and Discussion

While no mortalities were recorded, the observed behavior of the fish in the treatment tank exhibited a distinct pattern. Upon introduction to the treatment tank, fish initially struggled with controlled swimming, experiencing erratic escape responses and periods of lethargy. The T_2 group showed more pronounced abnormal behavior than T_1. After around 2 hours, both groups transitioned to intense but uncoordinated swimming, interspersed with balance issues. Over time, equilibrium improved, leading to a gradual return to normal swimming patterns in both T_1 and T_2.

A comprehensive assessment of erythrocyte morphology revealed a notable prevalence of irregularly shaped erythrocytes characterized by membrane deformities in T_1 (Fig. 1B). Concurrently, there was a discernible increase in the nucleus-to-cytoplasm ratio when compared to the control group. The T_2 fish showed similar erythro-morphological alterations but in higher vehemence. These morphological alterations are likely attributable to an osmolarity imbalance, potentially stemming from a substantial influx of salt via the gills (Jahan *et al*., 2019) [6]. A substantial presence of smudge cells was observed. These smudge cells, essentially delicate leukocytes on the precipice of apoptosis, exhibited compromised structural integrity, rendering them unclassifiable from a morphological perspective. Traditionally, peripheral blood smears featuring smudge cells have been associated with degenerated lymphocytes (Groff and Zinkl, 1999) [4].

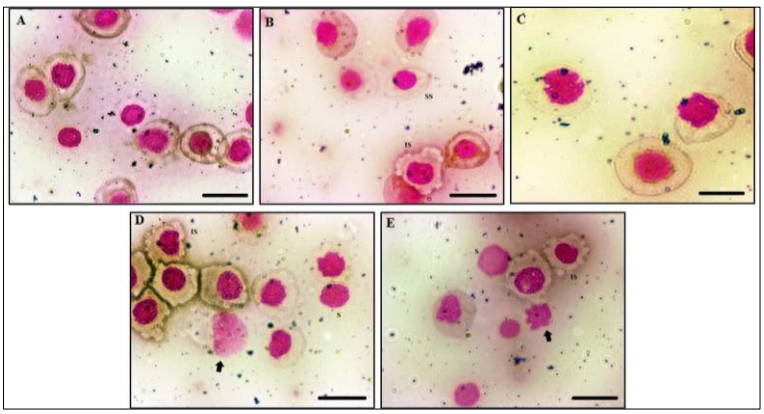

Fig 1: Effect of acute salt exposure on the erythrocyte morphological characteristics of *Oreochromis niloticus* juveniles at 10 g/L (B) and 30 g/L (D) for 1 hour and compared with the control group (A). Black arrow demarcates nuclear changes in dead erythrocyte and eryptosis. Blood smears from 6 hour post exposure in 10 g/L (C) and 30 g/L (E) showed lingering nuclear membrane deformities (red arrow). S: Smudge cell; IS: Irregular shaped erythrocyte. ×1000 Safranin staining

The heightened occurrence of smudge cells strongly indicates leukocyte death due to osmotic stress. Analogously, the blood smears of the treated fish prominently displayed deceased erythrocytes. Evidently, this phenomenon is aligned with eryptosis, a process facilitated by osmotic shock and stress, subsequently prompting an excessive efflux of cellular fluid (Igbokwe, 2018; Farag and Alagawany, 2018) [5, 2]. Further evidence is provided by the identification of cellular remnants wherein only the nucleus remains intact. The initiation of osmotic shock is a plausible contributor to these observed phenomena. Although recovery was attained in both T_1 and T_2 groups with many erythrocytes returning to normalcy (Fig 1C), nuclear anomalies seemed to persist hinting at induced cytotoxicity.

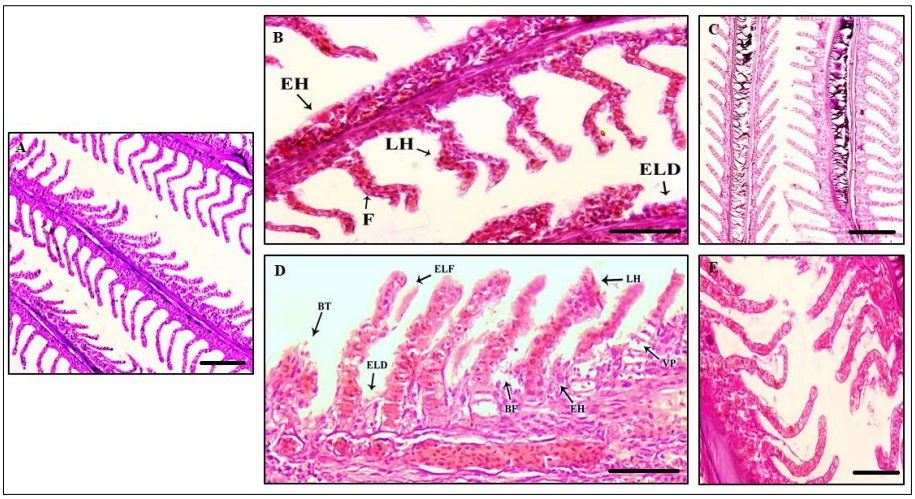

Fig 2: Effect of acute salt exposure of gill tissues of *Oreochromis niloticus* juveniles at 10 g/L (B) and 30 g/L (D) for 1 hour and compared with the control (A). Gill tissues from 6 hour post exposure in 10 g/L (C) and 30 g/L (E) showed incomplete recovery. EH: epithelial hyperplasia; LH: lamellar hyperplasia; F: fusion of secondary lamella; ELD: epithelial layer degeneration; BT: burst secondary lamellar tips; ELF: epithelial layer lifting; BF: breaking of secondary lamellar filaments; VP: Vacuolation of pillar cells. ×200 (A, C, E) and ×400 (B, D) magnification.

Gills are widely recognized as key target organs in fish, often exhibiting prompt sensitivity to unfavorable environmental conditions (Santos *et al*., 2014) [8]. As such, gills frequently bear the brunt of toxicant exposure, with histological analysis of gill tissue being particularly illuminating in revealing sublethal effects, including structural alterations induced by toxicants (Santos *et al*., 2014) [8].

Histological examination of gill sections unveiled pronounced modifications within both the epithelial and lamellar regions. Acute salt exposure elicited conspicuous instances of excessive epithelial hyperplasia, lamellar hyperplasia, lamellar fusion and epithelial layer degeneration in T_1 (Fig. 2B). Similar changes were documented in T_2 with increased intensities (Fig. 2D) and also encompassed instances of ruptured secondary lamellar tips, lifting of the epithelial layer, fractured secondary lamellar filaments and vacuolation within pillar cells (Fig. 2D). The rupture of secondary lamellar tips could likely be attributed to osmotic shock, given their direct contact with the hypertonic environment. Notably, the heightened epithelial and secondary lamellar hyperplasia appeared to function as a protective response of gill tissues, aiming to counteract excessive exosmosis and the eventual collapse of gill structures (Zhang *et al*., 2022) [12]. Furthermore, the inflicted damage upon gill tissues displayed a heterogeneous distribution, with areas exhibiting severe damage often juxtaposed with healthier regions. Notably affected were the tips of secondary lamellae and the epithelial segment of the gill arch. Vacuolation in pillar cells could likely be attributed to cellular demise prompted by osmotic stress. Particularly during acute exposure, the hyperplastic response was more pronounced, culminating in the fusion of certain secondary lamellae (Zhang *et al*., 2022) [12].

The histopathological harm inflicted upon gill surfaces following salt exposure is ascribed to elevated accumulations within the gills, instigating heightened mucous secretion, increased ventilation volume and a subsequent reduction in gill oxygen uptake efficiency. The reduced oxygen uptake consolidates the fish's lethargic response during exposure. Observations encompassing alterations in epithelial lifting, hyperplasia, hypertrophy of epithelial cells, and the partial fusion of some secondary lamellae can be attributed to defense mechanisms (Velasco-Santamaria and Cruz-Casallas, 2008) [11]. These mechanisms primarily contribute to an augmented separation between the external milieu and the bloodstream, effectively acting as a barrier against the ingress of contaminants (Velasco-Santamaria and Cruz-Casallas, 2008) [11].

Epithelial hyperplasia, intended to augment the epithelial area for diffusion, thereby diminishing the uptake of salt into the bloodstream (Zhang *et al*., 2022) [12]. In the context of this study, the observed desquamation of gill epithelium are direct consequences precipitated by salt action. The noted defensive responses include the elevation of the epithelium which elongates the distance traversed by toxicants to reach the bloodstream, and lamellar fusion which potentially provides protection by diminishing the extent of susceptible gill surface area. Gill hyperplasia likely functions as a defensive mechanism that leads to a reduction in respiratory surface and an increase in the diffusion distance for toxicants within the bloodstream. Nevertheless within 6 hours of post-salt exposure, the T_1 depicted substantial recovery (Fig. 2C) but the fish in T_2 failed to return to normalcy (Fig. 2E) proclaiming the tolerability of *O. niloticus* juveniles to 10 g/L salt exposure.

Conclusion

The triumph of aquaculture hinges fundamentally on the judicious mitigation of stress factors capable of compromising the well-being, health, growth, and even survival of the aquatic organisms. The incorporation of salt into aquaculture

practices has emerged as one such successful strategy, endowed with the capacity to serve as an effective stress mitigator and an agent with antiseptic attributes. Nevertheless, the introduction of salt to freshwater environments inevitably precipitates a perturbation to the delicate balance of osmotic homeostasis within freshwater fish. Consequently, prudent measures must be adopted to avert pronounced escalations in blood osmolality, along with the attendant implications on tissue hydration. The magnitude of this challenge is intricately linked to the quantum of salt introduced and the duration of exposure. Remarkably, our investigation into *Oreochromis niloticus* juveniles revealed discernible perturbations in behavior, tissue histology and erythrocyte characteristics subsequent to a 1-hour exposure to a salt solution. Notably, this concentration engendered extensive modifications within both tissue architecture and erythrocyte morphology. In light of these findings, it can be cautiously inferred that subjecting juvenile specimens of this species to a 1-hour exposure at 10 g/L, in pursuit of prophylactic objectives, appears to elicit relatively minor adverse effects. It is important, however, to underscore that while recuperation in terms of behavior, gill histology and blood profiles was perceptible, the transient osmotic shock endured during this short-term exposure has the potential to elicit fatal consequences. Consequently, the judicious application of this approach warrants careful consideration and thorough evaluation prior to implementation.

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