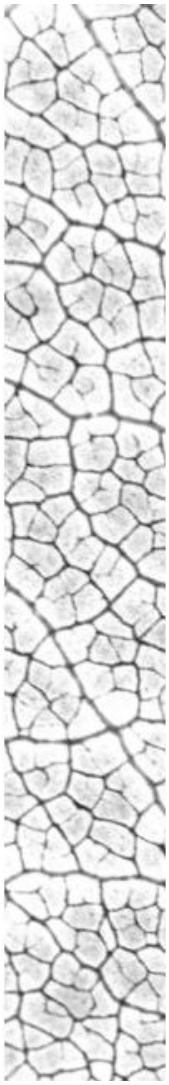

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Differential morphological responses to osmotic changes in euryhaline cichlid, *Etroplus suratensis* (Bloch, 1790)

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Abstract
Aquaculture of *Etroplus suratensis* is being practised at various water salinities. In this investigation, morphological responses of gill tissue of *E. suratensis* to various osmotic changes induced by freshwater (0%) and sea water (36%) were studied. Ultra-structural studies revealed significant decrease in the density of type I Mitochondrial rich cells in the apical surface of gills at 18% and complete disappearance of these cells at 36%. Type III subtype density increased consistently from 0% to 36%. Variations in type II cells density were comparatively less in any of the experimental groups. Light microscopic changes included reduction in mucous cell number with increasing salinity. Haemorrhages, lamellar fusion, lamellar curling, lifting of epithelium & hyperplasia are major histological responses to salinity shock. Observable clinical signs in fish exposed to increased salinity changes included agitated behaviour and respiratory distress.

Keywords: *Etroplus suratensis*, Gills, osmotic changes, mitochondrion-rich cells, scanning electron microscopy

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INTRODUCTION

Etroplus suratensis, commonly known as 'Pearlspot', is a euryhaline cichlid well known for its osmoregulatory ability. Pearlspot is the largest among the indigenous cichlids that is naturally acclimated to freshwater, but actually is a brackish water fish. This species of fish is a native of peninsular India and Srilanka. Pearl spot has a remarkable market value and its rate is high when compared with other local food fish in addition to the importance as an ornamental fish. *E.suratensis* grows fast than other species of this genus and are generally farmed in polyculture ponds and low volume cages. The osmoregulatory ability of *E.suratensis* has been studied extensively to understand its scope in aquaculture under varying salinity ranges (Chandrasekar et al. 2014, Islam and Tareq 2015). Ability to adapt to wide salinity regimens increases the profitability of pearl spot aquaculture.

For the inland saline soil, the seasonal fluctuations in salinity are the major concern for its culture. As the fish uses either brackish or freshwater to complete the life cycle, earlier studies states that brackish water is the main source of seeds for initiating its culture practiced in inland saline water & fresh water but the fry & fingerlings does not withstand stress of salinity when directly transferred from salt water to fresh water or vice versa. (Menon et al. 1959). Even though kidney, gills and intestine are considered to be the important organ that mediates osmoregulation, gills are the one responsible for ion movement and balancing (Hirose et al. 2003). Like any other teleost species, the gills of pearl spot is an organ that performs multiple functions and is responsible for gaseous exchange, osmoregulation, acid and base balance, ammonia excretion, hormone production, modification of circulating metabolites and immune defence. Mitochondrial rich cells also known as chloride cells are generally dispersed among the pavement cells in the inter-lamellar epithelium of gills (Evans et al. 1999). Ion secretion in seawater (SW) as well as freshwater (FW) fishes mostly takes place in mitochondrial rich cells (Evans et al. 2005).

Gill histology helps to explore the health impacts caused by environmental stressors including tissue responses to salinity fluctuations especially in estuarine fishes which are normally exposed to wide salinity variations (Thophon et al. 2003). Histopathological

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biomarkers can be used in environmental monitoring by examining the changes in targeted organs like kidney, gills and liver (Gernhofer et al. 2001) . Morphological changes in these osmoregulatory organs are easy to recognise than functional ones (Fanta et al. 2003; Tech, 2020). Further, in many fishes the morphological features of the bronchial epithelium depends on bronchial ion turnover (Van der Heijden et al. 1997). By considering wide saline adaptation ability of pearl spot, the present study was designed to provide an insight into the tissue level histological alterations inflicted by salinity changes. This study reports the histological and ultra-structural responses to salinity variations in *E.suratensis* which is one of the first study of its kind as far as our knowledge.

MATERIALS AND METHODS

Source of Fish

E. Suratensis (75 numbers, 12±2 cm length, 75-100 gm weight) required for the study were obtained from pearl spot farms with 16‰ salinity around Ernakulam, Kerala, India and acclimated in the laboratory for 15 days in one ton capacity fibre reinforced plastic tanks containing 500 L water with continuous aeration. (Salinity: 16‰, temperature: 28±1 and pH: 7.8±.4).

Species Identification with COI Amplification

Total DNA was extracted from fresh muscle tissue with the standard DNA barcoding methods for fish (Ward et al. 2005). The COI gene was amplified using the primers specified by Ward et al and Sanger sequenced. Sequences were manually edited using SeqMan program (DNASTAR) combined with manual proofreading before submitting to GenBank.

Salinity Tolerance Experiment

After acclimatization, the fishes were divided into 3 groups of 18 fishes in each group. Each group was maintained in triplicates with 6 fish in each replicate. The fishes were maintained in 250 L experimental glass tanks. First group was exposed to 0% water (fresh water, FW), second group to 36% (sea water, SW) and the third group to was exposed to 18% (brackish water, BW) for 21 days. The required salinity was attained by steadily increasing/decreasing the salinity by 2-3‰ every day.

Sampling and Microtomy

After attaining the respective salinity, the fishes were maintained in that salinity for four weeks. At the end of the experiment, fishes were anaesthetised with 2-phenoxy ethanol and representative samples of gills were excised, fixed in 10 % NBF (neutral buffered formalin) for 24 to 48 h, then acid decalcified. The decalcified gill tissues were processed following routine histological procedures, cleared in xylene and embedded in paraffin. Sections cut at 5µ thickness using a Microtome (Leica, Germany) were stained with Harris hematoxylin and Eosin and Periodic Acid Schiff (PAS). The sections were viewed under Leica microscope and micro-photographed.

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to examine the excised gill filaments surface as described by Lee et al. (1996). Briefly, tissue were fixed in a solution containing 0.2M sodium coccodylate buffer and 2.5% Glutraldehyde at 4ºC for 12 h followed by three washes in sodium coccodylate buffer at 10 minutes interval. The washed gill filaments were then treated with 4% osmium tetroxide dissolved in sodium coccodylate buffer (0.2M) for 4h, and washed in sodium coccodylate buffer thrice at 10 min interval. The tissues were then dehydrated in acetone series (30-100%) for 10 minutes. Finally, the tissues were washed with amyl acetate and dehydrated with critical point drier (Hitachi HCP-2). After sputter coating with gold particles using Quorum SC7620 sputter coater, samples were examined and images captured with a TESCAN VEGA 3 SEM. Various sub types of mitochondrial rich cells were identified based on the morphology and size of apical surfaces . Two areas (10000 μm^2) on trailing edge were chosen randomly and counted at 2800X on each of two gill filaments from a fish.

Analysis of Date

The data were expressed as mean ± SD. The results of time course experiments were analysed with one-way ANOVA accompanied by posteriori comparisons using Dunnet's test where $p<0.05$ was fixed as the significance level.

RESULTS

Species Identification

COI consensus length (660bp) of barcode sequence was edited and submitted to the GenBank database holding an accession number MN626365. Species identification was based on the BLAST results, which substantially confirmed that the organism is *E.suratensis.*

Morphological Description

In case of fish acclimatized to FW and BW, chloride cells (CC) were found less frequently in inter-lamellar regions of filaments and at the base of lamella, whereas, in the SW acclimatized fishes, the CC (chloride cells) were higher in numbers and larger in size, these cells are frequently observed in inter-lamellar regions (**Fig. 1C**). Along the filament mucous cells were distributed almost in all the groups (**Fig. 3**). The PAS staining revealed that mucus cells were PAS positive. Increased numbers of PAS + ve mucus cells were seen along filament of gills of fish acclimatized to FW (26 to 29/Lamella/Filament (LPF); whereas, the mucus cells were least in SW acclimatized fish (11 to 14/LPF) and moderate in BW exposed fish (18 to 21/ LPF) (**Fig. 2**). Partial lamellar fusion, mild congestion and dilation of

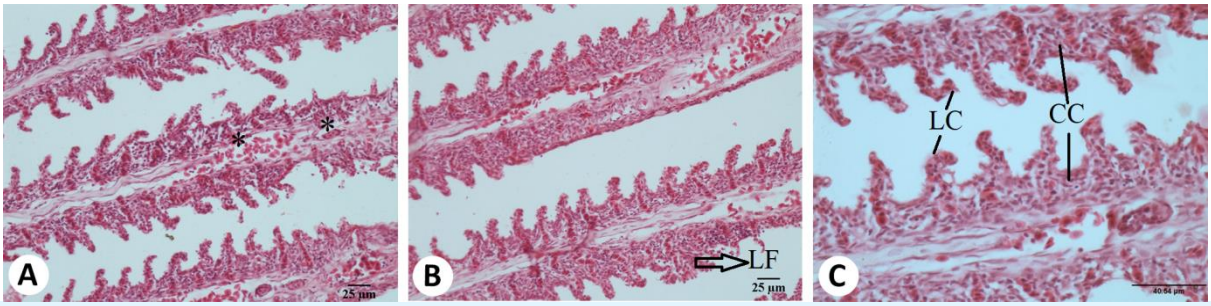

Fig. 1. Gill structure of *E.Suratensis*, exposed to 36‰ salinity, 36‰ showed severe A) Lamellar hyperplasia & necrosis (*), Lamellar fusion (LF), Lamellar lifting (black arrow heads) and Lamellar curling (LC) (H & E stain, 20x objective) (A and B scale bar: 25µm, C scale bar: 40.54µm)

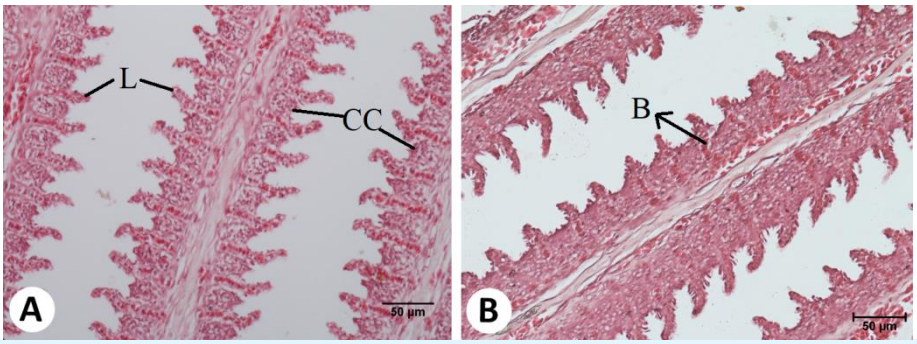

Fig. 2. Gill structure exposed to 0‰ and 18‰ salinity (control) without any major /severe physiological changes. L- Lamella, CC- Chloride cells and B- Blood channels (Scale bar: 50µm)

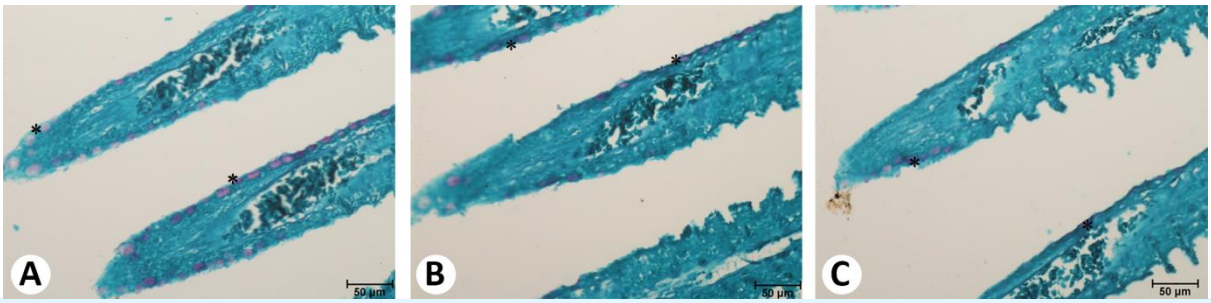

Fig. 3. Mucous cells (*) number variation in 0‰, 18‰ and 36‰ acclimatised *E.suratensis.* (Scale bar: 50µm)

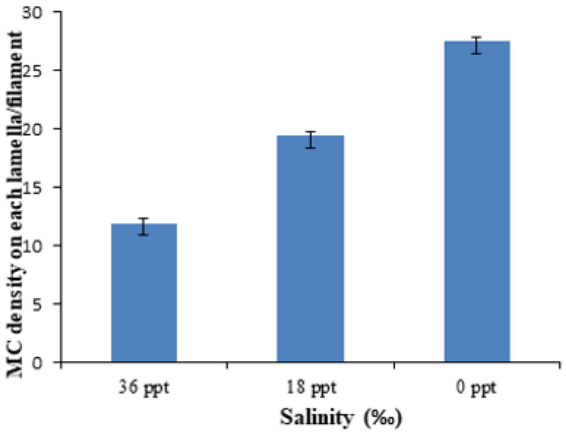

Fig. 4. Mucous cell density in SW, BW and FW acclimatised *E.suratensis* (n=6 for each salinity). The experiment indicates the significance increase in the number of mucous cell from SW to FW acclimated ($p<0.05$, one way ANOVA)

marginal channels were also observed in gills of FW acclimated fish. Histological changes observed in the gills of fish acclimatized to SW included extensive lamellar epithelial hyperplasia, lifting and hypertrophy with epithelial fusion in many places, curling of lamella and rupture of epithelia with haemorrhages (**Fig. 1**).

Ultrastructure Description

SEM revealed different subtypes of mitochondrial rich cells which varied with respect to apical surface morphology and size. The overall apical opening of gill mitochondrial rich cell densities remained almost the same throughout, but the distribution of each subtypes varied according to the acclimation. In *E.suratensis* acclimated from BW to FW, there was an increase in wavy-convex (subtype I) and shallow basin (subtype II) but deep hole (subtype III) were reduced. In contrast, those acclimated from BW to SW revealed reduction in wavy-convex subtype significantly ($p<0.05$), decrease in

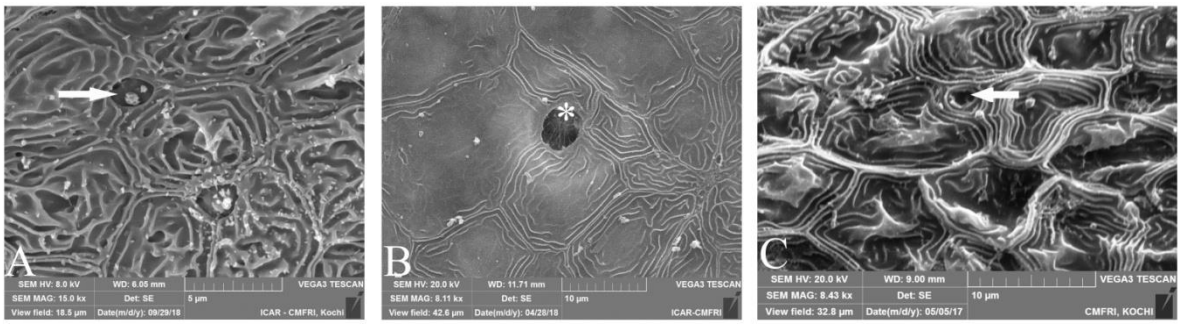

Fig. 5. The apical openings of Mitochondrial Rich cells including A) Subtype I-Wavy convex (white arrow), B) Subtype II-Shallow basin (*) and C) Subtype III-Deep hole (white arrow) (Scale-5µm (A), 10µm (B & C))

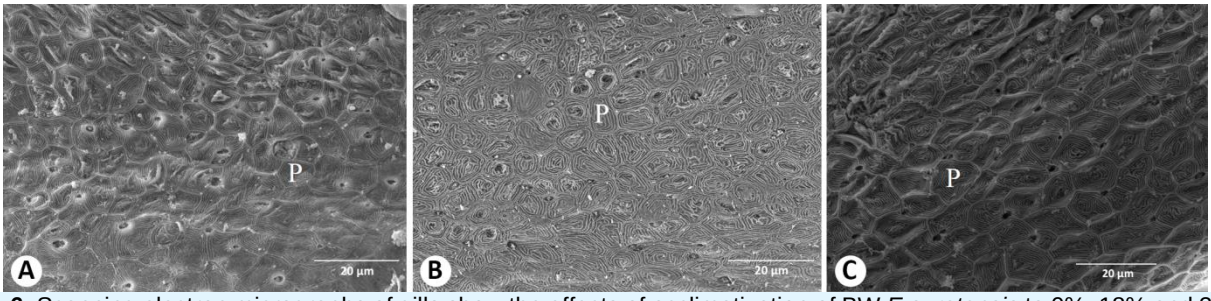

Fig. 6. Scanning electron micrographs of gills show the effects of acclimatization of BW *E.suratensis* to 0%, 18% and 36% and the densities of various subtypes. (P- Pavement cells) (Scale-20µm)

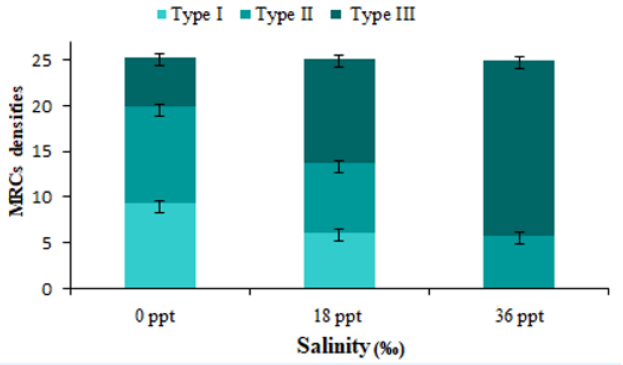

Fig. 7. The significant difference in densities (per 10000 µm^2) of total number of MITOCHONDRIAL RICH cells opening crypt between the acclimated experimental salinities in *E.suratensis* (n=6 for each salinity) ($p<0.05$, one way ANOVA)

basin density; whereas, the deep hole subtype showed significant increase in its density (**Fig. 7**).

DISCUSSION

Present investigation describes the morphological changes that facilitate *E. Suratensis* to adapt to a wide salinity range. The capacity to adapt with varying salinities in teleost largely depends on integrated osmoregulatory functions of organs such as kidney, digestive tract and gills (Cioni et al. 1991). Increase in number and size of chloride cells in gills is known to occur in euryhaline teleost *Oreochromis sp* transferred to SW from FW (Avella et al. 1993). In FW fishes chloride cells located in lamella may be responsible for ion regulation (Avella et al., 1987: Perry and Wood, 1985); whereas, in SW fishes elevated chloride cell number in filaments reflects the adaptation of teleost to increased external salinity (Laurent and Dunel 1980) and may be the reason for salt expulsion in hypertonic medium (Avella et al. 1993). This interpretation was strongly supported by studies conducted on opercular epithelium of Tilapia and Fundulus (Foskett et al. 1981) which is rich in CC (chloride cells). Proliferation and activation of immature CC (chloride cells) followed by its differentiation is involved in ion transport mechanism of euryhaline teleost (Fosket et al. 1983). Cells of these kind may have role in ion transport in both fresh and saltwater-adapted teleost. The morphological variations in the gill tissue observed in fish exposed to 36‰ included hyperplasia, lamellar fusion and lamellar curling. Most common morphological response of gills induced by toxic substances and other chemical as well as environmental stressors are necrosis, hyperplasia and lamellar fusion (Mallat 1985). The increasing salinity concentrations can interrupt the respiratory system in fish which can be one of the reason for lamellar hyperplasia (Ried et al. 2006). One of the earliest injuries found in SW acclimated fish were respiratory epithelium lifting. Displacement of lining epithelium of secondary lamella is the characteristic feature of lamellar lifting which becomes oedematous risking gas exchange process due to gill surface reduction (Winkaler et al. 2001). Proliferation of lamellar cell occurs during hyperplasia lead to the reduction of inter-lamellar space which can be the reason for lamellar fusion (Fracário et

al. 2003). Salinity changes may impact the physiological condition of the fish, causing stress and leading to reduced immune response. Dysfunction of gas exchange ability of fishes is a result of the gill degeneration which causes an anoxic internal behaviour (Ajani et al. 2007). The number of mucous cells in current study decreased as salinity changed from 0 to 36%. The mucous cells of *E. suratensis* are located mainly in the afferent and efferent gill filament borders. The migration and proliferation of the specialised mucous cells on the inter-lamellar tissue between secondary gill lamellae appears to be a protective response to induced ionic variations at gill surface (Shoman and Gabera 2003). The glycocalyx that lines the microridges can contribute to retention of mucus by reducing the abrasive action of particles suspended in water. The number of mucus cells further decreased when *E.suratensis* were adapted to BW and SW condition, which was similar to the observations made by Virabadrachari (1961) in *E.maculatus*.

Present investigations revealed gradual decrease in the density of subtype I and progressive increase in subtype III mitochondrial rich cells when salinity was changed from 0‰ to 18‰ and then to 36‰. Major role of each subtypes of mitochondrial rich cells as implicated by the different components of ion transporters were different. Studies on Cl^- influx in Tilapia gill (*O.mossambicus*) gives an insight on the main role of sub type I cells was chloride ion (Cl^-) uptake while sub type III cells involved in Cl^- secretion (Chang et al. 2003, Lin and Hwang 2001) . Subtype II mitochondrial rich cells having apical surface in the form of shallow basin were thought to be involved in Ca^{2+} uptake due to evident connection between cell density and ion influx (Tsai and Hwang 1998). The mitochondrial rich cells (branchial) in SW tilapia were found to be deep holes (subtype III) by Hirori et al in 2005.

E.suratensis mitochondrial rich cells of gills in this research work actively responded to SW, initially by reduction of wavy convex (Subtype I) in BW and complete disappearance in SW which can be due to apical membrane internalization (Lin and Hwang 2004) and also because of 'close' reaction as reported in adjacent pavement cells of mudskipper (*Periophthalmus modestus*) and mummichog (*Fundulus heteroclitus*) gills by Sakamoto et al in the year 2000 and Katoh & Kaneko in 2003 which in turn paved way for reduction in apical surface density of mitochondrial rich cells. These findings were similar to the observations of Pei-Jen Wang et al in 2009 who demonstrated that deep-hole (subtype III) ion secretory mitochondrial rich cells in *O.mossambicus* showed a significant hike in number when acclimatised to SW. No remarkable differences occurred in total cell density of mitochondrial rich cells apical-surfaces in FW, BW or SW acclimatised animals and at the same time density & constitution of mitochondrial rich cell subtypes varied. In SW and BW adapted fish, densities of functional mitochondrial rich cell reduced significantly. It can be referred as reversible & rapid modification of branchial mitochondrial rich cell types with varying function of ion-regulation which must be the strategy adopted by certain euryhaline teleost while FW-BW-SW adaptation.

CONCLUSION

To conclude, gradual increase in salinity condition causes respiratory distress in fishes. This has been proved by the histopathological observations, where the fish exposed to 36‰ exhibited tissue responses such as hyperplasia, lamellar fusion, curling and minor necrosis. This indicated that the animal was approaching their tolerance limit which can further results in osmoregulatory failures. The observation in the variation of mucous cells indicated the response of gills to salinity variations. The ultra-structural observation detailed the morphology of subtypes of Mitochondrial Rich cells and the relation between salinity and Mitochondrial Rich cell subtypes, where subtype I density reduced as the salinity increased and when salinity reached 36‰, subtype I was almost absent; whereas, subtype III increased and subtype II showed a minor reduction. The mechanism behind the replacement of subtype I with subtype III with the increase of salinity needs to be studied in future work.

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