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Haemato-biochemical changes in epizootic ulcerative syndrome (EUS) infected striped murrel (*Channa striata*) cultured in inland saline water

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Abstract

Epizootic ulcerative syndrome (EUS) causes infection in a wide range of fish species, often leading to significant mortality worldwide. Among the susceptible hosts, snakeheads are reported to be particularly vulnerable. A 120-day investigation was undertaken to examine the impact of salinity on *Channa striata* cultured in inland saline water. Twenty-four circular fibre-reinforced plastic (FRP) tanks (400 l capacity) were filled with water adjusted to eight salinity levels *viz.*, 0 ppt (control) and treatments T1-T7 (0, 2, 4, 6, 8, 10, 12 and 14 ppt) each in triplicates, following a completely randomised design. Each tank was stocked with 24 juveniles (mean weight: 8±2.09 g) and fed with Growel feed containing 42% crude protein twice daily (09.00 hrs and 17.00 hrs). Mortalities were noticed in fish reared at 0, 12 and 14 ppt salinity levels, around the 90th day after introduction into the experimental tanks. Affected fish were found lethargic, swimming at water surface, pale and isolated, having fungal growth and haemorrhages with ulcerated skin. Polymerase chain reaction (PCR) confirmed the presence of EUS in affected fish. Both biochemical and haematological parameters were altered in the affected fish. The findings suggest that moderate salinity levels in inland saline water may help prevent the occurrence of EUS and could be effectively employed for sustainable aquaculture of *C. striata*.

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Introduction

Aphanomyces a fungal-like oomycete, is the primary microbial genus responsible for epizootic ulcerative syndrome (EUS). Food and Agriculture Organization (FAO) formally acknowledged EUS as a significant concern during an Expert Consultation held in Bangkok, Thailand in 1985 (Lilley *et al*., 1998; Kamilya and Baruah, 2014). It is now understood to be synonymous with the conditions known as red spot disease (RSD) and mycotic granuloma, initially identified in *Plecoglossus altivelis* in Japan in 1971 (Egusa and Masuda, 1971; Kamilya and Baruah, 2014) and in grey mullet in Eastern Australia in 1972 (McKenzie and Hall, 1976). The disease typically manifests as seasonal epizootics in both wild and farmed aquatic organisms (OIE, 2003). By 1972, reports highlighted severe ulceration and high mortality associated with the disease across many countries in the Asian Pacific nations (Chinabut, 1994). Since 1980s, EUS has affected freshwater fish in culture systems and wild populations across South and South-east Asia (Lilley *et al*., 1992; Adinarayana *et al*., 2015). In India, the first EUS outbreak was noticed in Tripura in 1988. By 1991, the disease had spread to other parts of the country, resulting in heavy mortalities and substantial decline in aquaculture production (Lilley *et al*., 1992; Herbert *et al*., 2019). Throughout the 1980s, EUS outbreaks were recorded across several Asian countries, including Thailand, Malaysia, Lao PDR, Vietnam, China, Cambodia, Myanmar, Bangladesh,

Sri Lanka, Bhutan, Hong Kong, Philippines, Nepal, Singapore, India and Pakistan (Lilley *et al*., 1998; Issac *et al*., 2020). Notably, an EUS outbreak was reported in Canada in 2010, in a newly susceptible species, *Ameiurus nebulosus*. As of 2013, EUS has been documented in 26 countries across four continents (Kamilya and Baruah, 2014).

EUS remains prevalent in wild fish throughout the winter and monsoon periods in freshwater and brackishwater ecosystems (Lilley *et al*., 1992; Kamilya and Baruah, 2014). The condition is characterised by enormous haemorrhagic necrotising ulcer, spreading deep into the tissue and causing mass mortality in affected fish (Lilley *et al*., 1992; Roberts *et al*., 1993). Fish afflicted by EUS typically exhibit appetite loss, lethargy and listlessness. The afflicted fish initially displays pinhead-sized red patches on body surfaces, developing into tiny dermal ulcers at the intermediate stage. Advanced haemorrhages and necrotic ulcers on the body surface indicate advanced stage lesions (Kamilya and Baruah, 2014; Herbert *et al*., 2019). In addition to pathogenic factors, poor water quality, elevated ammonia levels, high stocking densities and ectoparasitic infestations are also considered contributing factors in the aetiology of EUS outbreaks, particularly in inland water species (Azad *et al*., 2001; Cai *et al*., 2004). EUS posed a significant socio-economic impact particularly on small-scale fisheries. Capture fisheries from lakes, rivers, reservoirs, irrigation tanks, estuaries and other backwater resources have been severely impacted (Chinabut, 1994; Kamilya and Baruah, 2014). The disease resulted in heavy economic losses, as visibly infected wild-caught fish were unmarketable. Simultaneously, consumer fear and misconceptions surrounding EUS, resulted in reduced demand even for healthy fish species, further aggravating the economic burden (Mohan and Bhatta, 2002).

Over the past 50 years, *A. invadans* has spread to major continents, with the exception of South and Central America. To date, over 160 fish species susceptible to EUS, spanning 54 families and 16 orders have been documented (Kamilya and Baruah, 2014). While nearly all brackishwater and freshwater fish species are considered susceptible, eels, milkfish, catfishes, loaches, Indian major carps (IMCs), common carp, striped mullet and Nile tilapia, have shown high levels of tolerance to EUS as they have either not been found naturally infected or have failed to develop EUS symptoms under experimental conditions (Lilley *et al*., 1998; Oidtmann *et al*., 2008). Previous studies have indicated that most fish impacted by natural outbreaks are bottom-dwelling fishes (Chondar and Rao, 1996) or fish possessing accessory respiratory organs (Roberts *et al*., 1994). However, this is not always the case, as species from various ecological niches including those with accessory respiratory organs may also become infected. Age or size can also influence susceptibility to EUS. Snakeheads are vulnerable to EUS regardless of their size (Cruz-Lacierda and Shariff, 1995), while IMCs are more susceptible during fingerling stage (Roberts *et al*., 1989), but typically develop resistance when they reach 1 year age class (Pradhan *et al*., 2007). Several snakehead species have been identified as susceptible to EUS including Formosan snakehead (Miyazaki, 1994), walking snakehead (Khan *et al*., 2002), ocellated snakehead (Hanjavanit *et al*., 1997), spotted snakehead (Kanchanakhan, 1996; Podeti and Benarjee, 2017; Deshmukh *et al*., 2020), striped snakehead (Mohan and Shankar, 1995) and both the giant and red snakeheads (Lilley *et al*., 1998).

The striped murrel, *C. striata,* is known for its high tolerance to environmental stress, including low dissolved oxygen levels and relatively poor water quality during prolonged culture periods (Vidthayanon, 2002). Recently, the species has attracted significant interest from aquaculture entrepreneurs due to its excellent flesh quality, distinctive flavour and notable nutritional, restorative and medicinal properties (Kumari *et al*., 2018). According to FAO (2019), global production of *C. striata* reached 92,523 t. This study aims to evaluate the effect of salinity on the occurrence of EUS in *C. striata.* with a focus on polymerase chain reaction (PCR)-based diagnosis and haemato-biochemical responses of fish reared in inland saline water (ISW).

Materials and methods

Experimental design and husbandry

The experiments were conducted following the standards established by the CPCSEA (Committee for Control and Supervision of Experiments on Animals), Animal Welfare Division, Ministry of Environment and Forests, Government of India, for conducting scientific research on animals.

The incidence of EUS was noticed while conducting the salinity tolerance studies in *C. striata* from March to June 2023 at the Rohtak Centre of ICAR-Central Institute of Fisheries Education (ICAR-CIFE), Haryana, India. The rearing system consisted of 7 treatments with differing salinity levels, including T1 (2 ppt), T2 (4 ppt), T3 (6 ppt), T4 (8 ppt), T5 (10 ppt), T6 (12 ppt), T7 (14 ppt) and control (0 ppt), all administered with three replicates following a completely randomised design (CRD). The culture unit contained twenty-four circular fibre-reinforced plastic (FRP) tanks with a capacity of 400 l (1 m dia, 0.5 m depth) holding 250 l of water of respective salinity. The culture tanks were thoroughly cleaned, treated with a disinfectant (potassium permanganate, $KMnO_4$) at 4 ppm) Inland ground saline water of 15.3 ppt salinity was pumped from a borewell, followed by filtration using a 100 µ filter bag and transferred to rectangular cement tanks of 9000 l (3x2x1.5 m) capacity.

Water at salinities of 2, 4, 6, 8, 10, 12, and 14 ppt was prepared by mixing borewell water with freshwater and stored in 7 circular tanks (1000 l capacity, 1.08 m dia, 0.93 m depth). These pre-mixed saline waters were then used to fill the respective experimental tanks. *C. striata* (6±2.73 g) were procured from an aqua consultant in Andhra Pradesh and were acclimatised in freshwater for 25 days in a 9000 l (3x2x1.5 m) tank. After acclimatisation, the salinity levels were gradually increased to 2, 4, 6, 8, 10, 12 and 14 ppt, respectively, by adding 1 ppt per day over a twenty day period. Healthy *C. striata* juveniles with a mean weight of 8±2.09 g were stocked at a density of 24 fish per tank. Fish were randomly assigned to each tank and continuous aeration was provided using an air blower (A1 Blowers India Pvt. Ltd.) with a capacity of 2 HP, delivering air at a a flow rate of 160 $m^3 h^{-1}$ at 1200 rpm. The experimental tanks were routinely siphoned to remove waste and uneaten feed to maintain water quality parameters in the acceptable range. For a period of 120 days, *C. striata* were fed with Growel feed consisting of 42% crude protein, 3% crude fibre, 8% crude fat and 12% moisture content daily at 09.00 and 17.00 hrs, at the rate of 5% of the biomass. The health status of the animals in each treatment group was monitored

regularly throughout the experimental period. After observing the clinical changes during the rearing phase, representative fish were sampled from each treatment group for PCR-based detection of pathogen as well as for analysis of haemato-biochemical parameters.

Physico-chemical parameter of rearing water

Water quality parameters like temperature, salinity, pH and dissolved oxygen were monitored daily. Other factors such as alkalinity, hardness, ammonia, nitrate nitrogen, nitrite nitrogen, calcium (Ca^{2+}), potassium (K^{+}) and sodium (Na^{+}) were measured at the time of infection. A thermometer (MERCK KGaA, Darmstadt, Germany) was used to record the water temperature of the experimental tanks. Water quality analyzer (type WQC-24, DKK-TOA CORPORATION, Japan) was used to measure dissolved oxygen and salinity. pH-Scan (Eutech instruments, Singapore) was used to measure pH. The total alkalinity and hardness were estimated by titration method as per APHA (2005). Ammonia (Phenol hypochlorite method), nitrite, and nitrate nitrogen were analysed spectrophotometrically. K^{+} and Na^{+} ions were recorded by flame photometry (Essico Instruments, India), while Ca^{2+} ions were estimated by titration method (APHA, 2005).

Gross observations

The EUS-infected *C. striata* were sampled to examine the gross morphology and progression of skin lesions and developmental stages of the lesion. The most distinctive EUS lesions were documented and representative samples were collected for further analysis.

Sampling, collection of tissue and serum preparation

EUS-infected (n=3) and healthy (n=3) fish were collected from the same tanks and anaesthetised using 50 µl l^{-1} of clove oil. Blood was drawn by piercing the caudal vein using a 3 ml hypodermal tuberculin syringe (Dispovan India Ltd.) and immediately transferred to an anti-coagulant ethylenediaminetetraacetic acid (EDTA) coated vial and stored at 4°C for subsequent examination of various haematological parameters. Muscle tissue samples for PCR diagnosis and serum for enzymatic assays were collected from healthy (n=3) as well as infected fish (n=3) Skin, gills and fins of healthy as well as infected fish were evaluated for presence of lesions. Fishes were randomly selected for PCR diagnosis, based on gross examination from 0, 12 and 14 ppt treatments. The collected tissues were preserved in absolute alcohol for PCR detection. The blood drawn from EUS-infected and healthy fish from each treatment was allowed to clot at room temperature and then centrifuged at 3000 *g* for 10 min. to collect serum in sterile vials for assaying various parameters (Nuwansi *et al*., 2021). Serum samples was preserved at -20°C for further analyses.

PCR for diagnosis of *A. invadans*

The primer pair BO73 (5'-CTTGTGCTGAGCTCACACTC-3') and BO639 (5'-ACACCAGATTACACTATCTC-3'), targeting the Internal Transcribed Spacer (ITS) region was used for PCR amplification. The PCR master mix (50 µl reaction volume) comprised 0.6 µM of each primer, 0.2 mM of each deoxynucleoside triphosphates (dNTPs), 1.5 mM $MgCl_2$, 0.625 U of Taq DNA polymerase and approximately 5 ng of genomic DNA (2.5 µl DNA template) extracted from 25 mg of tissue from both EUS infected and healthy fish suspended in 100 µl of buffer solution. The thermal cycling conditions consisted an initial denaturation at 96°C for 5 min; 35 cycles of denaturation at 96°C for 1 min, annealing at 58°C for 1 min and extension at 72°C for 1 min; followed by a final extension at 72°C for 5 min (Oidtmann *et al*., 2008). European eel *Anguilla anguilla* and rainbow trout *Oncorhynchus mykiss* were challenged by intramuscular injection of zoospores of *Aphanomyces invadans*, the oomycete associated with epizootic ulcerative syndrome Agarose gel electrophoresis was used to analyse the PCR products, which yielded an amplicon 564 bp size. The amplified PCR products were separated on a 1% agarose gel and stained with 10 mg ml^{-1} ethidium bromide (M.P. Biomedicals, India). A 1000 bp DNA ladder (Thermo Scientific, India) was used as a molecular weight marker to confirm the product size. The gel was visualised and photographed using a gel documentation system (BioRad, Germany).

Analysis of haematological and serum parameters

Serum glucose levels were estimated using ERBA-Manheim glucose kit with (Catalogue No. BLT00027), with optical density (OD) measured at wavelength range of 490-550 nm. Serum cortisol levels in healthy and EUS-infected *C. striata* was assessed using a cortisol enzyme-linked immunosorbent assay (ELISA) assay kit (Catalogue No. 500360; Cayman Chemical), following the manufacturer's instructions. Total serum protein was determined by the Biuret method (Reinhold, 1953). Albumin concentration was quantified based on its binding to bromocresol green (Doumas *et al*., 1971). Both standard and test values were measured spectrophotometrically at 630 nm, with readings taken relative to a reagent blank. Globulin (g dl^{-1}) concentration was calculated by subtracting the albumin value from the total serum protein. The Albumin to Globulin ratio (A/G ratio) was subsequently computed using the formula: A/G ratio = Albumin (g%)/Globulin (g%).

Haemoglobulin (Hb) concentration in blood was estimated using the cyan-methemoglobin method (Qualigens Diagnostics), employing Drabkin's solution and spectrophotometric analysis. A 20 µl blood sample was mixed with 5 ml of Drabkin's working solution and the OD was measured at 540 nm using a spectrophotometer (MERCK-Thermo Electron, WI, USA). Total erythrocytes count (TEC) and total leucocytes count (TLC) were determined using a Neubauer counting chamber The cell count per mm^3 was calculated using the formula: Number of cells per mm^3 = (No of cells counted × Dilution)/(Area counted × Fluid depth). The haematocrit (HCT)/packed cell volume (PCV) was measured by capillary centrifugation. Blood samples were drawn into microhaematocrit tubes *via* capillary action, sealed at one end with a synthetic sealant and centrifuged. PCV was then calculated as percentage using a microhaematocrit reader (Thomas Scientific).

Statistical analysis

The statistical program SPSS version 22 was used to analyse the data. One-way Analysis of the variance (ANOVA) followed by Duncan's multiple-range tests (DMRT) was performed to determine significant differences among treatments, with a significance level set at $p<0.05$.

Results and discussion

Physico-chemical parameters of water

The physico-chemical parameters of each experimental group at the time of infection are presented in Table 1. Significant differences ($p<0.05$) were observed among treatment groups for temperature, pH, dissolved oxygen; ammonia-N, nitrite-N, nitrate-N, total alkalinity, total hardness as well as for concentrations of Na^+, Ca^{2+} and K^+. Water quality is a critical factor influencing the survivability and health of aquatic organisms. During the experimental period, key parameters such as pH, temperature, dissolved oxygen, ammonia-N, nitrite-N and nitrate-N, remained within acceptable range for *C. striata* culture in inland saline groundwater (ISGW). However, other parameters such as total hardness, alkalinity, potassium, calcium and sodium, varied significantly with salinity levels and reflected changes in ionic composition consistent with findings by Sarma *et al*. (2020) and Patel *et al*. (2022). It is also well documented that the ionic concentration of inland saline water (ISW) can vary by location, even at identical salinity levels. Compared to freshwater, ISE has a higher mineral content, with elevated total hardness (Prangnell and Fotedar, 2006). Previous studies by Choongo *et al*. (2009) and Issac *et al*. (2020) have reported that various physico-chemical parameters of the rearing environment can act as risk factors for EUS outbreak. Baldock *et al*. (2005) and Podeti and Benarjee (2017) opined that, EUS infections are often triggered by drops in water temperature. In the present study, *C. striata* reared at 0, 12 and 14 ppt salinities exhibited EUS symptoms when water temperature dropped to 28°C, indicating a possible link between thermal stress and disease manifestation.

Gross examination

The observed changes during the experimental period are presented in Fig. 1. Gross examination of healthy fish showed no clinical signs (Fig. 2a). In contrast, infected fish exhibited distinct symptoms including fungal growth in the head region (Fig. 2b), ulcerated skin (Fig. 2c), skin haemorrhages (Fig. 2d) and red spots on the body surface (Fig. 2e). Fungal hyphae were also observed microscopically in the infected specimens (Fig. 2f). Infected fish displayed a pronounced inflammatory response, evident from localised skin swelling and reddening. Internal examination revealed granulomatous inflammation in multiple organs (Fig. 2g). These gross pathological features are consistent with previous reports by Viswanath *et al*. (1997) and Kamilya and Baruah (2014). The localised skin inflammation and swelling reflect a strong host immune response in *C. striata*, consistent with earlier studies. Deep ulceration caused by fungal hyphae led to granulomatous lesions in the internal organs, which were totally absent in healthy fish. Similar findings have also been reported by Baldock *et al*. (2005) and Herbert *et al*. (2019).

PCR for diagnosis of *A. invadans*

PCR amplification using the primer pair BO73 and BO639,yielded a product of 564 bp, confirming the presence of *A. invadans* (Fig. 3). Diagnostic confirmation of *A. invadans* infection in *C. striata* exhibiting clinical symptoms can be achieved through histopathological examination, oomycete isolation, or PCR amplification. The extensive geographic distribution of *A. invadans* has been demonstrated using these methods. PCR-based diagnostics have previously been employed to confirm infections in both naturally and experimentally infected fish species. Lilley *et al*. (1998) developed species-specific PCR protocols to detect *A. invadans* isolates associated with EUS. In a related study, Phadee *et al*. (2004) employed PCR technique to detect *A. invadans* in goldfish exposed to an EUS challenge study. Later, these PCR methods were widely used for identifying and screening *A. invadans* in various sample types (Vandersea *et al*., 2006; Oidtmann *et al*., 2008) of ulcerative skin disease caused by a fungus-like agent of wild and cultured fish. We developed sensitive PCR and fluorescent peptide nucleic acid *in situ* hybridisation (FISH). In the current investigation, a similar species-specific PCR technique successfully confirmed the presence of *A. invadans* in EUS affected *C. striata* across various salinity treatments.

Serum biochemical parameters

The serum biochemical indices, such as Alanine transaminase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), cortisol and glucose levels showed significant differences ($p<0.05$) between healthy and EUS-infected fish. Infected fish exhibited significantly ($p<0.05$) elevated ALT, ALP, AST, cortisol and glucose levels; compared to healthy fish (Table 2). Albumin, globulin, total protein, ALT, AST and ALP levels were markedly higher in

Table 1. Physio-chemical parameters of the rearing water in the experimental tanks during infection studies of *C. striata* juveniles

Parameter	0 ppt	2 ppt	4 ppt	6 ppt	8 ppt	10 ppt	12 ppt	14 ppt
Temperature (°C)	$28.67^{b} \pm 0.14$	$28.71^{b} \pm 0.01$	$28.69^{b} \pm 0.01$	$28.78^{b} \pm 0.01$	$28.33^{a} \pm 0.01$	$28.3^{a} \pm 0.01$	$28.53^{ab} \pm 0.01$	$28.66^{b} \pm 0.16$
pH	$7.94^{a} \pm 0.01$	$7.95^{ab} \pm 0.01$	$7.95^{ab} \pm 0.01$	$7.96^{bc} \pm 0.01$	$7.96^{bc} \pm 0.01$	$7.94^{a} \pm 0.01$	$7.95^{ab} \pm 0.01$	$7.97^{c} \pm 0.01$
Dissolved oxygen (mg l^{-1})	$6.04^{bc} \pm 0.01$	$6.05^{cd} \pm 0.01$	$6.01^{a} \pm 0.01$	$6.05^{cd} \pm 0.01$	$6.05^{cd} \pm 0.01$	$6.06^{cd} \pm 0.01$	$6.04^{bc} \pm 0.01$	$6.03^{ab} \pm 0.01$
Alkalinity (mg l^{-1})	$115.54^{a} \pm 0.28$	$126.34^{b} \pm 0.46$	$142.42^{c} \pm 0.43$	$156.51^{d} \pm 0.23$	$175.67^{e} \pm 0.19$	$195.42^{f} \pm 0.08$	$214.81^{g} \pm 0.20$	$235.13^{h} \pm 0.41$
Hardness (mg l^{-1})	$156.03^{a} \pm 0.14$	$629.71^{b} \pm 0.13$	$1158.28^{c} \pm 0.77$	$1664.66^{d} \pm 0.36$	$1829.13^{e} \pm 0.39$	$2396.16^{f} \pm 0.43$	$2866.04^{g} \pm 0.69$	$3217.75^{h} \pm 1.17$
Calcium (mg l^{-1})	$55.14^{a} \pm 0.45$	$84.82^{b} \pm 0.34$	$136.05^{c} \pm 0.45$	$177.29^{d} \pm 0.37$	$207.59^{e} \pm 0.37$	$244.95^{f} \pm 0.50$	$278.75^{g} \pm 0.22$	$321.08^{h} \pm 0.50$
Potassium (mg l^{-1})	$2.52^{a} \pm 0.03$	$5.62^{b} \pm 0.18$	$7.23^{c} \pm 0.05$	$10.61^{d} \pm 0.12$	$11.70^{e} \pm 0.03$	$12.71^{f} \pm 0.08$	$14.87^{g} \pm 0.02$	$15.10^{g} \pm 0.10$
Sodium (mg l^{-1})	$15.45^{a} \pm 0.23$	$633.22^{b} \pm 0.88$	$1159.53^{c} \pm 0.45$	$1567.74^{d} \pm 0.32$	$2437.54^{e} \pm 0.56$	$2985.21^{f} \pm 0.14$	$3365.19^{g} \pm 0.19$	$3780.04^{h} \pm 9.23$
Nitrite (mg l^{-1})	$0.001^{a} \pm 0.01$	$0.001^{a} \pm 0.01$	$0.001^{a} \pm 0.01$	$0.001^{a} \pm 0.01$	$0.001^{a} \pm 0.01$	$0.002^{a} \pm 0.01$	$0.003^{b} \pm 0.01$	$0.004^{c} \pm 0.01$
Nitrate (mg l^{-1})	$0.11^{a} \pm 0.01$	$0.11^{a} \pm 0.01$	$0.14^{b} \pm 0.01$	$0.15^{c} \pm 0.01$	$0.16^{c} \pm 0.01$	$0.18^{d} \pm 0.01$	$0.20^{e} \pm 0.01$	$0.28^{f} \pm 0.01$
Ammonia (mg l^{-1})	$0.013^{a} \pm 0.01$	$0.013^{a} \pm 0.01$	$0.026^{b} \pm 0.01$	$0.036^{c} \pm 0.01$	$0.053^{d} \pm 0.01$	$0.063^{e} \pm 0.01$	$0.066^{e} \pm 0.01$	$0.086^{f} \pm 0.01$

Values bearing differentuperscripts varied significantly ($p<0.05$) for each water quality parameter. Values are stated as mean±SE, n=3.
One-way ANOVA was used for analysing each parameter, followed by Duncan's multiple range tests in statistical software SPSS version 22.0.

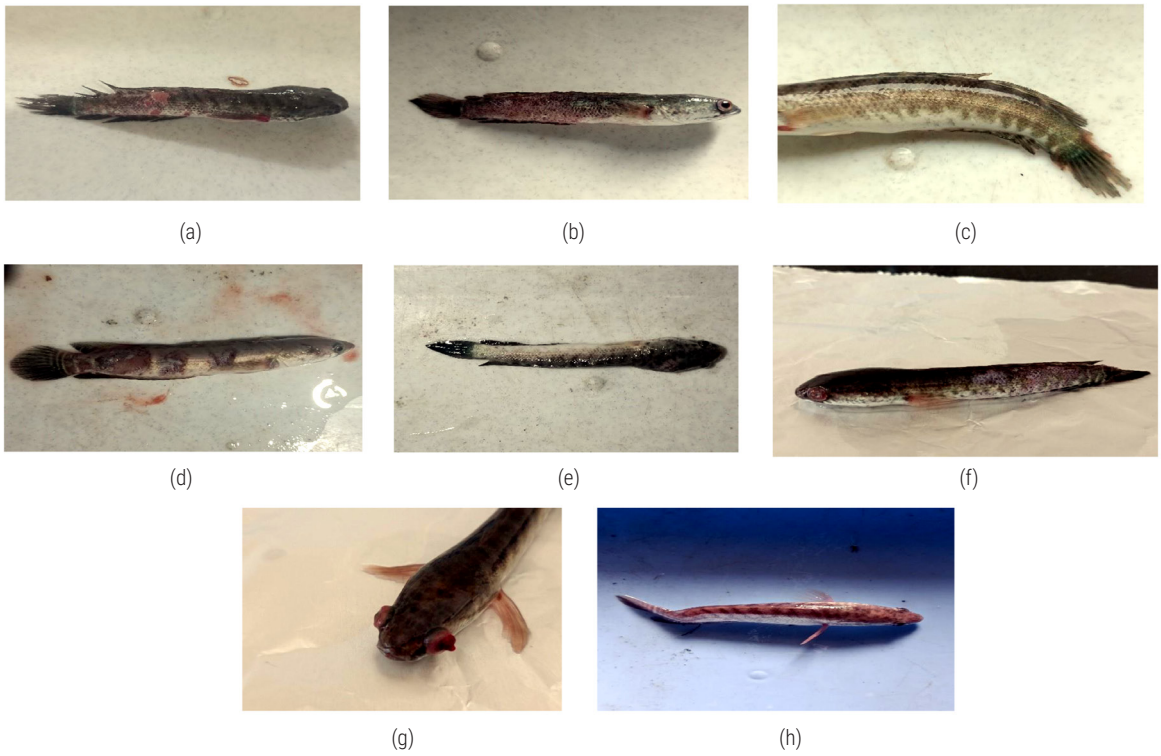

Fig. 1. Impact of varying salinity levels on morphological and behavioural changes in *C. striata*. (a) Skin ulceration, (b) Scale loss, (c) Tail rot, (d). Haemorrhages, (e) Emaciation, (f) Sunken eye, (g) Swollen eye and (h) Vertebral deformity

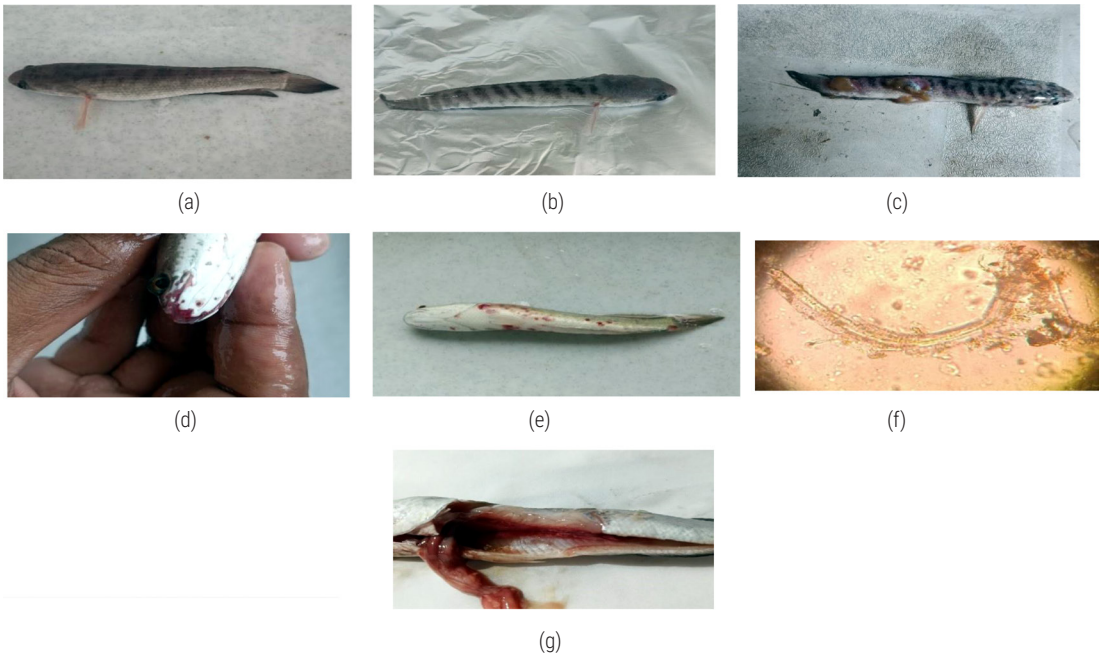

Fig. 2. Photographs showing healthy and EUS-infected *C. striata* reared in inland saline groundwater. (a) Unaffected *C. striata* (b) Infected *C. striata* with *A. invadans* (Preliminary infection of EUS with fungal hyphae in head region), (c) Infected *C. striata* with *A. invadans* in 0 ppt saline water (Ulceration due to severe inflammatory response), (d) Infected *C. striata* with *A. invadans* in 14 ppt saline water (Infection in mouth region with haemorrhages), (e) Infected *C. striata* with *A. invadans* in 14 ppt saline water showing red spots across whole body, (f) Microscopic image (40x) showing infected *C. striata* with *A. invadans* in 0 ppt saline water, (g) Organs of infected *C. striata* with *A. invadans* in 14 ppt saline water

EUS-infected groups compared to uninfected control fish. Only few studies have investigated the biochemical profile of fish affected with EUS. The elevated total protein concentration in infected fish may be attributed to the production of anti-*A. Invadans* antibodies in the serum as reported by Miles *et al*. (2001) and Thompson *et al*. (1999). Supporting the present findings, Ali and Ansari (2023), also reported elevated levels of glucose, ALT and AST values in EUS-infected fish. These elevated biochemical parameters in the infected fish could be attributed to damage in hepatocytes caused by invasive *A. invadans* hyphae. Baldock *et al*. (2005) documented that EUS infection can suppress immune responses, , which aligns with the observation of the current study.

Haematological and serum parameters

The blood and serum parameters of healthy and infected *C. striata* are summarised in Table 3. Significant differences ($p<0.05$) were observed among the infected and healthy fish. Healthy fish showed markedly higher ($p<0.05$) levels of albumin, globulin, total protein and A/G ratio as compared to infected fish. Haematological indices such as Hb, HCT and TEC levels were significantly ($p<0.05$) higher in healthy fish than in the infected group. Conversely, TLC was significantly ($p<0.05$) elevated in the EUS-infected group as compared to healthy fish. EUS infection in *C. striata* was found to have a significant effect on the haematological parameters, which corresponded with the study of Pathiratne and Rajapaksha (1998); Adinarayana *et al*. (2015) and Issac *et al*. (2020) in striped murrel. The present study reported a significant decline in TEC, Hb and HCT values in severely affected fish, suggesting anaemia. These observations are in agreement with the findings of Rao (2023) and Ali and Ansari (2023). In the present study, an increase in TLC was observed in EUS-infected *C. striata*, indicating leukocyte proliferation likely associated with inflammatory and immune responses. Similar findings were reported in walking catfish (Ali and Ansari, 2023); in striped murrel (Podeti and Benarjee, 2017; Rao, 2023) and in Atlantic menhaden infected with ulcerative syndrome (Faisal and Hargis, 1992). The elevated TLC levels in EUS-affected fish suggest an intensified immunological activity. Anaemia in the EUS-infected *C. striata* marked by reduced RBC count, PCV and Hb content, could be due to haemodilution caused by the loss of body fluid from necrotic lesions in infected fish. Similar results were observed in EUS-affected pearl spot and striped snakeheads (Cruz-Lacierda and Shariff, 1995; Pathiratne and Rajapaksha, 1998). Overall, healthy fish exhibited stable haematological and biochemical profiles, indicating better physiological status. The results of the present study suggest that moderate salinities (2-10 ppt) in inland saline groundwater (ISGW) support optimal health and growth performance of *C. striata*, In contrast, fish reared at 0, 12 and 14 ppt salinities exhibited signs of immune disruption and were more susceptible to EUS infection. The possible influence of leukocyte activation and proliferation in the development of granulomatous responses associated with the disease needs further investigation.

In the present study, incidence of EUS was confirmed in *C. striata* cultured at 0, 12 and 14 ppt ISGW, while no signs of infection were observed at moderate salinity levels (2, 4, 6, 8 and 10 ppt) highlighting the protective role of optimal salinity in mitigating disease outbreaks in aquaculture systems.

Table 2. Serum biochemical parameters of healthy and infected *C. striata* juveniles

Treatment	AST (IU l^{-1})	ALT (IU l^{-1})	ALP (IU l^{-1})	Glucose (mg dl^{-1})	Cortisol (mg dl^{-1})
Control	$21.91^{b} \pm 0.26$	$43.25^{b} \pm 0.80$	$31.45^{b} \pm 0.24$	$27.25^{b} \pm 0.55$	$19.82^{b} \pm 0.13$
EUS infected	$34.35^{a} \pm 0.49$	$72.92^{a} \pm 1.03$	$67.77^{a} \pm 0.32$	$50.39^{a} \pm 0.41$	$53.34^{a} \pm 0.76$

Values bearing different superscripts varied significantly ($p<0.05$) for each serum biochemical parameter. Data stated as mean ± SE, n=3.

Table 3. Serum biochemical and haematological levels in healthy and infected *C. striata* juveniles

Treatment	Total protein (g dL^{-1})	Albumin (g dL^{-1})	Globulin (g dL^{-1})	Albumin: Globulin ratio (g dL^{-1})	Haematocrit value (%)	Haemoglobin concentration (%)	Total erythrocyte count (10^{6} cells mm^{2})	Total leucocytes count (10^{6} cells mm^{2})
Control	$1.65^{a} \pm 0.12$	$0.38^{a} \pm 0.01$	$1.27^{a} \pm 0.01$	$0.30^{a} \pm 0.01$	$26.72^{a} \pm 0.10$	$9.37^{a} \pm 0.03$	$1.96^{a} \pm 0.01$	$7.31^{b} \pm 0.01$
EUS infected	$1.06^{b} \pm 0.14$	$0.18^{b} \pm 0.01$	$0.88^{b} \pm 0.01$	$0.20^{b} \pm 0.01$	$17.43^{b} \pm 0.10$	$3.78^{b} \pm 0.06$	$0.98^{b} \pm 0.01$	$9.37^{a} \pm 0.03$

All ranges in the same column bearing different superscripts varied significantly ($p<0.05$) for each parameter. Data is stated as mean±SE, n=3.

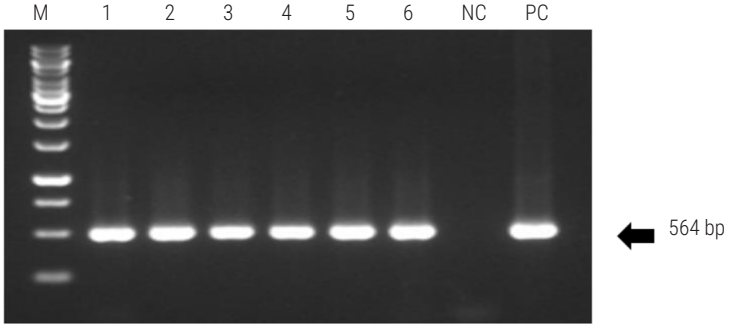

Fig. 3. Gel image of PCR products (564 bp) amplified with the primer BO73 and BO639. Lanes 1-6: Samples; Lanes 1 and 2 - Freshwater, Lanes 3 and 4 - 12 ppt; Lanes 5 and 6 - 14 ppt (in duplicates); M: 1000 bp ladder, NC - Negative control, and PC- Positive control

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