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Evaluate salinity stress in *Mystus gulio* to explore potential farming in brackish water

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This study aims to assess the effects of salinity on the growth and survival of freshwater catfish *Mystus gulio* and to analyse the biochemical and hormonal alterations. The fish were exposed to increasing salt concentrations of upto 22.27 PSU in experimental tanks within a study period of 21 days with an aim to investigate into the effect of salinity stress on the total protein profile and, synthesis of the stress hormone cortisol and stress protein HSP70 as well as the sex steroid hormones estradiol and testosterone. This euryhaline fish showed tolerance with different salinities upto approximately 9 PSU beyond which, most fishes succumbed to higher salinities. 50% of mortality was noticed at salt concentrations between 8.14-8.19 PSU. There was a gradual decrease in the protein concentration with increasing salt concentrations. SDS-PAGE revealed the disappearance of a 91.5 kDa, 77.8 kDa and an 18.2 kDa protein band and the appearance of a 60.7 kDa, 21.4 kDa and a 15.5 kDa protein band at higher salt concentrations. The Cortisol and HSP70 concentration showed a gradual increase in muscle tissue with increasing salinities but in case of gills there was a gradual decline after an initial sharp rise. Low salinity stress upregulated estradiol synthesis but level of testosterone showed a decrease with increasing salt concentrations.

Keywords*:* Protein, SDS-PAGE, Cortisol, HSP70, Testosterone, Estradiol

Mystus gulio[1] belonging to the family Bagridae, is a small to medium sized species of catfish. It is a euryhaline teleost fish that occurs mostly in freshwater but has also been reported to thrive in backwaters of low salinity[2,3]. Reported to be primarily a brackish-water fish that enters and lives in fresh water[4], this small indigenous fish species is well suited for both fresh- and brackish-water aquaculture[5] and is a commercially important food fish. It fetches high market price and is in increasing demand, making it a desirable candidate species for aquaculture in Southeast Asia[6]. Based upon its economic value, easy availability, and wide consumption by the people, *Mystus gulio* was selected for this study. Being able to survive both in freshwater and brackish water with low salinities, it can serve as an appropriate model to study the responses to salinity changes, stress resistance and range of tolerance.

Euryhaline fishes adapt to salinity perturbations mainly through physiological functions namely osmotic adjustment, transport of ions, and energy metabolism. Any adverse environmental condition poses a significant challenge to the fish's ability to maintain homeostasis[7]. Being a key factor affecting the survival of aquatic organisms, changes in salinity greatly affect physiological activities in organisms including fishes[8]. *Mystus gulio* while in freshwater is generally hyperosmotic to its environment as in case of most fresh water organisms[9-10]. When in brackish water, it must maintain elevated body fluid levels to acclimate and survive across a range of salinities.

For a successful selective fishery, it is important that released fish survive and maintain normal physiological functions. Their survival and growth are greatly influenced by environmental variables, particularly salinity, as about 20–50% of the total energy budget of fish is used for osmoregulation alone[11]. Hence, fish growth may increase if appropriate salinity levels are maintained, which may consequently decrease the energy expended to maintain homeostasis[12]. Fish culture at low salinity levels also reduces the occurrence of diseases[13].

Cortisol, synthesised by the interrenal cells of the head kidney, is one of the most predominant glucocorticoids in fishes, regulating osmolarity, metabolism, immune and stress related responses[14-16]. In teleosts, cortisol plays a very important role in osmoregulation under hyperosmotic or hypoosmotic conditions[17,18], and is a major stress hormone and a reliable indicator of stress in fish[19]. Besides this, cortisol has also been found to regulate the activity and expression of gill Na^+/K^+-ATPase and proliferation of gill chloride cells in several fishes thus establishing its role in hydromineral regulation apart from its role in carbohydrate metabolism[20-24].

Heat shock proteins are also natural biomarkers that play a vital role in the adaptive survival of fish under environmental stress. The functions of HSPs include the regulation of various aspects of fish

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physiology, including development and aging, immunology, stress tolerance and acclimation[25]. The HSP response has not only been found to vary with different tissues but also with the type of stressor the fish is exposed to[26-29]. Negative impact on the distribution and reproductive process of fish is also expected when there is escalated variation in the amplitude of salinity and temperature gradients.

The present study aims to understand the effects of salinity stress on the growth and biochemical constituents of *Mystus gulio* reared under varying salinity conditions for 21 days. The primary objective of this study was to investigate how salinity influences metabolism in *Mystus gulio,* providing a foundation for understanding the regulatory mechanisms in freshwater and brackish waters. Based on the comprehensive comparative data obtained across various salinities, strategies can be recommended for the development of fisheries in brackish waters and for assessing the possibility of farming this freshwater species in low salinity brackish water areas.

Materials & Methods

Experimental setup

Fish (*Mystus gulio*) were transported from the local fish farm. Almost equal sized fishes measuring approximately 9-10 cm were collected. Fish were set randomly in 3 glass aquaria with 36 litres of dechlorinated tap water in each tank and acclimatized to captive conditions for two weeks by maintaining them in normal conditions (pH 7.7, temperature 27.24°C, dissolved oxygen 4.72 mg/mL and a salinity of 1.13 PSU approximately). One tank (Tank 1) was set as control. The other two tanks (Tank 2 & 3) were set as experimental tanks. At the outset of the trial, there were 10 fishes in each tank. These were fed with formulated diet at 4% of fish total body weight. Each aquarium was equipped with an aerator for proper oxygen supply and a filtration system to remove faecal matter and other debris, keeping the water clean. A variable amount of NaCl was added to the water of the experimental tanks per day to increase the salinity of the tanks by 1 PSU per day. The salinity of tank 2 was increased to approximately 15.94 PSU on the 15th day and the salinity in tank 3 was increased up to 22.27 PSU within a span of 21 days. The control aquarium was maintained at the salt concentration of normal tap water without adding any salt. The value of temperature, pH, dissolved oxygen and salinity were measured in each tank on a regular basis using a Multi-parameter Probe (Make: Hanna Instruments, Model no.: H198194).

Sampling of fish and tissue collection

Sampling was performed on the1st day (2.25 PSU), 8th day (9.13 PSU), 15th day (15.94 PSU) and 21st day (22.27 PSU) from the 3 tanks for hormone (cortisol, estradiol and testosterone) and HSP70 protein assay and for protein estimation and SDS-PAGE analysis, fish were sacrificed on 1st, 7th, 8th, 9th, 12th, 13th, 14th, 15th, 16th, 17th and 21st day. All operations on the fish in this study followed the standard practices of animal care and use. The fish were sacrificed and tissue collected for the different assays. Muscle tissue was collected from euthanized fish by making an incision along the lateral line and a piece was carefully cut and transferred to labelled cryovials. Gill tissue was collected by opening the gill operculum and cutting a section of the gill filament. For the reproductive organs, the abdominal cavity was cut open with a longitudinal incision and the reproductive organs were located and carefully excised. The sex of each specimen was identified by visual examination and confirmed after dissection and examination of the gonads following standard protocol. Blood sample was collected from each fish by cardiac puncture according to the method of Lawrence[30] with the help of a small gauge needle as the fish were very small and transferred to eppendorf tubes. The blood sample was immediately centrifuged for 10 minutes at 0.5 g to collect the serum. All tissue samples were placed in labelled cryovials and immediately stored on ice if immediately used or stored at -20°C for later studies.

Morphometric studies

Before collection of tissues, each fish was weighed with the help of a digital electric balance. Morphometric measurements were done with the help of a steel scale. This included total length (cm) of the fish and the standard length (cm) of the fish.

Statistical analysis

The datasets for water quality and histological measurements of gonads were small in nature; hence the Shapiro-Wilk Normality tests were conducted using CRAN R software (Version: 4.1.1) for all water variables and gonadal tissues, followed by measurements of their central tendencies.

Protein extraction and estimation

Protein was extracted from fish muscle tissue by homogenizing in 0.2 M Tris HCl buffer (pH 7.4) at

4°C. The extract was clarified by centrifugation at 12000×g for 20 minutes at 4°C. The supernatant was collected and stored at -20°C. The protein concentration in the extract was estimated by the modified method of Lowry[31]. A calibrated solution of bovine serum albumin was used as a standard.

SDS-PAGE

SDS-PAGE was performed to study the total protein profile of muscles. Gel Documentation System (Make: Biotech, Model No.13-01-03) has been utilised for the determination of molecular weights. The protein sample was heated with an equal amount of sample buffer [0.06M Tris HCl (pH 6.8), 1% SDS, 10% sucrose, 0.5% β-mercaptoethanol, 0.01% Bromophenol blue] at 100°C for 3 minutes. The sample was loaded in the well of a 10% T mini-gel (8×7 cm gel) and the gel was run using Laemmli buffer system [0.05 M Tris, 0.192 M Glycine, 0.1% SDS, pH 8.4] at room temperature for 2 hours 30 minutes at 70V. The gel was calibrated using a marker mixture [Protein Molecular Weight Marker (Broad Range), Make: Genei, Cat. No. 3110475001730] consisting of Myosin, Rabbit Muscle (205 kDa), Phosphorylase b (97.4 kDa), Bovine Serum Albumin (66 kDa), Ovalbumin (43 kDa), Carbonic Anhydrase (29 kDa), Soyabean Trypsin Inhibitor (20.1 kDa), Lysozyme (14.3 kDa), Aprotinin (6.5 kDa) and Insulin and chains (3.5 kDa). After electrophoresis, the gel was stained with 0.1% Coomassie Brilliant Blue R 250 and destained with methanol: acetic acid: water (4:1:5) mixture.

Cortisol hormone and HSP70 assay

Competitive ELISA was performed for both Cortisol and HSP70 assay. Tissue samples (0.1gm) were suspended in 1mL of 1M Phosphate buffer (pH 7) overnight, subjected to two freeze-thaw cycles, vortexed for 30 minutes and centrifuged for 10 minutes at 12000 x g. The supernatant (50μL) was then used directly in the assay without dilution. Each of these samples was analysed in duplicate, across 96-well plates.

Cortisol hormone assay was done using ELISA kit [Fish cortisol ELISA Kit Catalogue Number: CSB-E08487f, Make – Cusabio] according to the manufacturer′s recommendations. The microtiter plate of the assay was pre-coated with an antigen. The standards and test samples were added to the appropriate microtiter plate wells with antibodies specific for Cortisol and Horseradish Peroxidase (HRP) conjugated goat-anti-rabbit antibodies. The competitive inhibition reaction was launched between pre-coated Cortisol and Cortisol in samples by incubating the plate at 37°C for 30 minutes. After washing, tetramethylbenzidine (TMB) substrate solution was added to the wells for detection of HRP activity and the plate was incubated for 20 minutes at 37°C. The blue colour that developed was in amount opposite to the amount of Cortisol in the sample. A stop solution was added and mixed well by gentle shaking. The colour changed into yellow after adding acidic stop solution. The cortisol level was determined by reading the optical density at 450 nm wavelength using a microplate ELISA Reader.

Heat Shock Protein 70 was assayed using ELISA kit [Fish Heat Shock Protein 70 (HSP-70) ELISA Kit Catalogue Number: CSB-E16327Fh, Make – Cusabio] according to the manufacturer′s recommendations. A competitive inhibition reaction was launched between HSP70 (Standards and samples) and biotin-conjugated HSP70 with the pre-coated antibody specific for HSP70. The more the amount of Hsp70 in the samples, the less the antibody was bound by biotin-conjugated HSP70. After washing, avidin conjugated Horseradish Peroxidase (HRP) was added to the wells. Substrate solution (TMB) was then added to the wells and the colour developed was in opposite to the amount of HSP70 in the sample. The colour development reaction was stopped and the intensity of the colour measured at 450 nm.

Cortisol and HSP70 concentrations were calculated based on a standard curve run on each plate using the software Cusabio Curve Expert 1.4. For cortisol a standard curve was calculated using eight standards, and the cortisol concentration in the tissue was extrapolated from this curve with r^2=0.97726 whereas for HSP70, a standard curve was calculated using six standards, and the HSP70 concentration in the tissue was extrapolated from this curve with r^2=0.96350.

Estradiol and testosterone assay

Estradiol and testosterone hormone assay from the samples was done using ELISA kit (Fish Estradiol E2 Elisa Kit, Make: Biospes, Chongqing Biospes Co. Ltd., Catalogue No. BYEK1437 for Estradiol and Fish Testosterone T Elisa Kit, Make: Biospes, Chongqing Biospes Co. Ltd., Catalogue No. BYEK1437 for testosterone) following the manufacturer′s recommendations. Each of the samples was analysed in duplicate, across 96-well plates. Hormone concentrations were calculated based on a standard curve.

The assay was based on standard sandwich ELISA technique. For both the hormones (estradiol and testosterone), 50 µL of undiluted samples were added to the well of the assay plate coated with purified anti-E2 antibody and anti-T antibody respectively and incubated at 37°C for 30 minutes. The solution in each well were then discarded and washed 5 times with diluted wash buffer. 50µL of HRP conjugated anti-E2 antibody (for estradiol) and HRP conjugated anti-T antibody (for testosterone) was then added to each well and incubated at 37°C for 30 minutes. This was followed by the washing procedure. 50 µL of TMB substrate A was then added to each well followed by 50 µL of TMB substrate B, vortexed and incubated in the dark at 37°C for 15 minutes. A blue color developed in the wells. The reaction was stopped by adding 50 µL of Stop solution into each well. The plate was read at 450 nm using a microplate ELISA Reader. The estradiol and testosterone concentration of the samples were then interpolated from the standard curve. The final reading of the hormone level was averaged according to respective treatments.

Results and Discussion

Water quality variables

A summary of the statistical results of the observed water quality parameters in each of the experimental tanks is presented in Fig. 1. Temperature, pH and dissolved oxygen levels were relatively stable during the course of the experiment. The water quality variables were monitored every 24 hours using a handheld Multi-parameter Probe in the experimental tanks after each addition of salt, as well as in the control tank, during the 21 day period of study. Variables like water temperature (°C), pH, salinity (in practical salinity unit), % saturation and amount (in mg/L) of dissolved oxygen were measured in triplicate from the subsurface for each tank. Shapiro-Wilk Normality tests were performed for each variable and each tank and subsequent data distribution and analysis were ascertained. The water temperature was normally distributed in all tanks and ranged between 22.22 – 29.08°C. The pH levels in control tank (8.02 ± 0.19), experimental tank 2 [8.06 ± 0.04 (SE)] and tank 3 (8.01 ± 0.19) showed an overall range of 7.58 - 8.56. The salinity remained between 1.06 - 1.39 PSU in the control tank with tap water while in the experimental tanks it was gradually raised from 1.06 PSU to 15.94 PSU in experimental tank 2 and to 22.27 PSU in experimental tank 3 by 24 hourly salinity treatments during the study period. The saturation of dissolved oxygen showed normal distribution (75.67 ± 12.68%) and ranged from 48.10 - 96.20 % in the control tank while it was not-normally distributed in experimental tank 2 [80 ± 4.81(SE)%] and tank 3 [77.85 ± 2.88(SE)%] with an overall range of 25.50-90.40 %. The

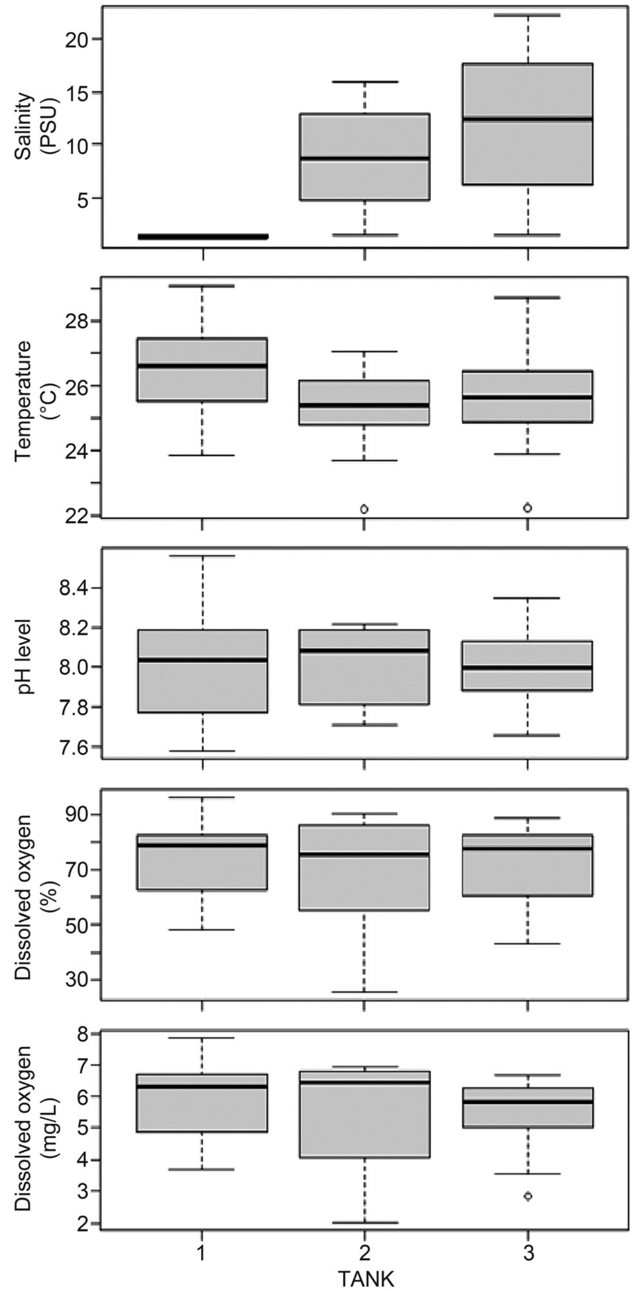

Fig. 1 — Tank wise water quality variables during the experimental duration of the 21 days.

DO concentration (mg/L) were not normally distributed in experimental tank 2 [6.58 ± 0.38(SE)] and tank 3 [5.81 ± 0.23(SE)] with a lowest record of 2.03 mg/L in tank 2. The overall DO ranged from 2.03 - 6.68 for the experimental tanks and from 3.67-7.85 for the normally distributed DO levels (6.044 ± 1.14) in the control tank.

Effect of salinity on fish growth

Fishes with an average total length of approximately 9-10 cm and weighing 11-12 g were introduced randomly into the three experimental tanks. In case of the control tank, the average weight of male fish increased up to 14 g on an average and in case of females up to 13.2 g within the span of 21 days. But in the experimental tanks, in case of both the male and the female fish, although there was an increase in the average total length, but the average standard length and average weight of fishes did not increase proportionately and showed a retarded growth (Table 1). These findings suggest that *Mystus gulio* being a fresh water fish may require more metabolic energy for osmotic pressure adjustment at high salt concentrations, thus not allowing more energy allocation towards growth. Hence manipulation of the external environment can minimize energy consumption associated with ion and osmotic pressure regulation[32]. The cumulative survival (%) at different time intervals and exposure to different salinity concentrations in experimental tank 2 and 3 are presented in Fig. 2. 50% of mortality was noticed at salt concentration 8.19 PSU in tank 2 and at 8.14 PSU in tank 3, both within 7 days of exposure.

Effect of salinity on protein concentration and SDS-PAGE protein profile

In fish farming and processing, taste and nutrition are two important indexes for evaluating the quality of fish flesh[33]. Although these qualities are regulated

Table 1 — Morphometric data of male and female of *Mystus gulio* fish exposed to different salt concentration

Sex	Salt concentration in the experimental tank	Day of sampling	Average total length (cm)	Average standard length (cm)	Average body weight (g)
Male	Control (1.12 PSU)	21st	11.59	9.26	14.00
	9.14 PSU	8th	11.00	9.30	13.28
	15.94 PSU	15th	11.60	8.80	13.31
	22.27 PSU	21st	11.70	8.60	10.94
Female	Control (1.12 PSU)	21st	11.19	9.16	13.20
	9.14 PSU	8th	10.30	8.00	11.01
	15.94 PSU	15th	10.20	8.50	9.90
	22.27 PSU	21st	10.50	8.50	8.66

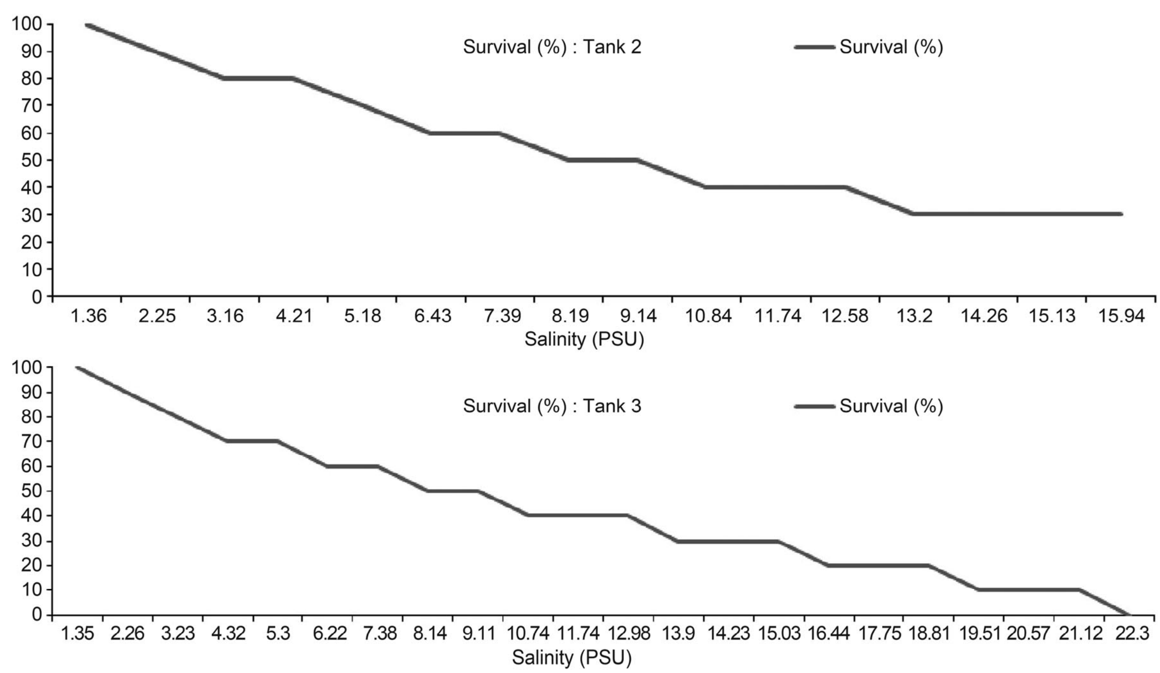

Fig. 2 — Survivorship curve of *Mystus gulio* at different salinities (PSU) in experimental tank 2 and tank 3.

by the genetic characteristics of the species, they are also influenced by other factors such as feed composition and rearing environment[34]. Ambient salinity is one of the crucial factors affecting fish flesh quality and there are specific mechanisms by which salinity influences changes in fish muscle nutrition. Consequently in this study, the crude protein content in the muscle of *Mystus gulio* was found to vary with different salinity conditions. There was a gradual decrease in the protein concentration with increasing salt concentrations. In the experimental tank 2, the amount of protein per gram of muscle tissue decreased from an average of 20.36 µg at 2.25 PSU to 17.71 µg in fishes exposed up to 15.94 PSU of salt concentration. In the experimental tank 3, the average protein concentration also decreased from 27.57 µg at 2.26 PSU to 11.11 µg at 22.27 PSU. Several other studies in fishes have revealed significant differences in the nutritive value of fish muscle between seawater and freshwater aquaculture environments[35,36]. Water-holding capacity, is another indicator for evaluating muscle quality as demonstrated by Li *et al.*[37] - as aquaculture salinity increases, the water-holding capacity decreases.

SDS-PAGE analysis of the total protein extracted from the muscle tissue revealed the disappearance of some protein bands, and induction of new protein bands with increasing salt concentrations. A 91.5 kDa protein was found to be most sensitive which disappeared after exposure to low salt concentration of above 9 PSU while a 77.8 kDa and a smaller 18.2 kDa protein band disappeared at higher salt concentrations of 17.75 PSU and above 15 PSU respectively (Table 2, Fig. 3). At the same time there

Table 2 — Comparative protein profile of the fish (*Mystus gulio*) exposed to different salt concentration

		Tank 1 (Control)			Tank 2 (Treated with salt)							Tank 3 (Treated with salt)									
	DAY →	1	15	21	1	8	9	12	13	14	15	1	8	9	12	13	14	15	16	17	21
Protein in kDa	Marker Protein	1.36 PSU	1.14 PSU	1.12 PSU	2.25 PSU	9.14 PSU	10.84 PSU	13.2 PSU	14.26 PSU	15.13 PSU	15.94 PSU	2.26 PSU	9.11 PSU	10.74 PSU	13.99 PSU	14.23 PSU	15.03 PSU	16.44 PSU	17.75 PSU	18.81 PSU	22.27 PSU
205	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
157.2	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
121.3	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
97.4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
95.4	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
91.5	-	+	+	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-
77.8	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
70	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
66	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
63.4	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
60.7	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	+	+
56.1	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
43	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
40.7	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27.4	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21.4	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
20.1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18.2	-	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-
15.5	-	-	-	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+
14.3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6.5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3.9	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
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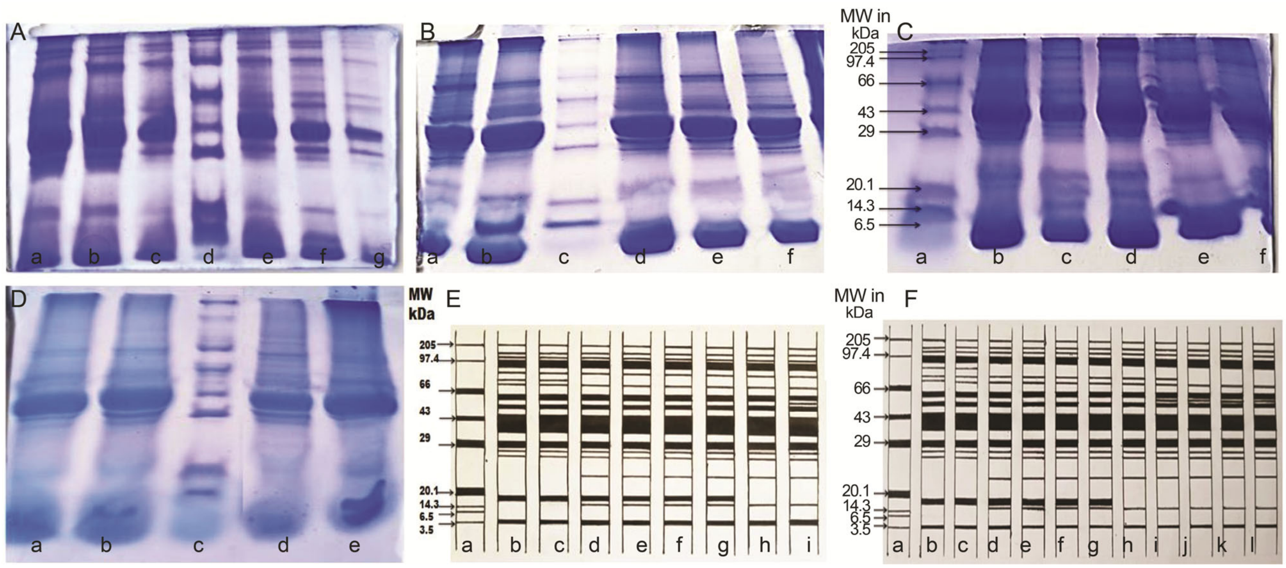

Fig. 3 — SDS-PAGE protein profile of the muscle tissue of *Mystus gulio* after exposure to different salt concentrations in the experimental tanks (A)- (a,b&c) 15µL, 10µL and 5µL of protein respectively in ET2 (2.25 PSU); (d) Marker protein; (e,f &g) 15µL, 10µL and 5µL of protein respectively in ET3 (2.26 PSU); (B)- (a) 10µL (ET3, 18.81 PSU); (b) 10µL (ET2, 15.13 PSU); (c) Marker protein; (d) 10µL (ET1, 1.14 PSU); (e) 10µL (ET2, 15.13 PSU); f) (10µL (ET3, 15.44 PSU); (C)- (a) Marker protein; (b) 10µL (ET2, 9.14 PSU); (c) 10µL (ET3, 9.11 PSU); (d) 10µL (ET2, 10.84 PSU); (e) 10µL (ET2, 13.2 PSU); (f) 10µL (ET3, 14.23 PSU); (D)- (a&b) 10µL (ET3, 17.71 PSU); (c) Marker protein; (d) 10µL (ET3, 22.27 PSU); (e) 10µL (ET1, 1.12 PSU); (E)- Experimental tank 2; (a) Marker protein, (b) Control fish (1.14 PSU), (c) 2.25 PSU, (d) 9.14 PSU, (e) 10.84 PSU, (f) 13.2 PSU, (g) 14.26 PSU, (h) 15.13 PSU, (i) 15.94 PSU, (F)- Experimental tank 3; (a) Marker protein, (b) Control fish (1.14 PSU), (c) 2.26 PSU, (d) 9.11 PSU, (e) 10.74 PSU, (f) 13.99 PSU, (g) 14.23 PSU, (h) 15.03 PSU, (i) 15.44 PSU, (j) 17.71 PSU, (k) 18.81 PSU, (l) 22.27 PSU.

was the appearance of two low molecular weight proteins of 21.4 kDa and 15.5 kDa at a salt concentration of above 9 PSU and a 60.7 kDa protein after exposure to a salt concentration of 15.94 PSU and above. As high salt concentration in the surrounding water causes ion imbalance and hyperosmotic stress, it may have toxic effects on biochemical processes including protein synthesis[38]. Salt stress can induce proteins which may be either salt stress proteins that accumulate solely under salt stress or they may be stress associated proteins, which accumulate in response to various environmental stresses[39]. These proteins maybe either synthesised de novo in response to salt stress or be constitutively expressed at low basal levels, which get upregulated upon exposure to elevated salinity.

Effect of salinity on Cortisol and HSP70

There was a significant difference in the mean cortisol and HSP70 protein concentration in the fish exposed to increasing salt concentrations compared to the control level.

Cortisol level was elevated after the fish was subjected to gradually increasing salinity as cortisol plays a crucial role in adaptation to hypertonic or hypotonic environments in teleosts[40] and is implicated in the osmoregulatory process[41]. In the muscle tissue, the cortisol level increased from, 0.263 ng/ml at 2.25 PSU to 0.45 ng/mL at 22.27 PSU (Fig. 4). With the addition of salt for the increase of salinity by 1 PSU every day, the fish in the experimental tanks expressed signs of agitation for 5-10 minutes, displaying erratic swimming approaches. They tended to settle at the bottom and crowd under the aerator. This behaviour can be explained by the fact that as salinity increases, dissolved oxygen exponentially decreases[42]. Such abnormal behaviours, including swimming upside down has, been reported earlier at different salt dilutions[43,44]. However in case of gills, although there was an initial sharp increase in cortisol level which peaked to 0.349 ng/mL at 2.25 PSU salinity as compared to the 0.297 ng/mL in the control (1.12 PSU), there was a subsequent decrease in cortisol level to 0.018 ng/mL at 22.27 PSU. The main target tissue of the cortisol hormone in the fish is the chloride cell in the gills and it activates Na^{+}–K^{+} ATPase activity that helps in the ion regulation[20]. This in turn reduces cortisol concentration in gills, following adaptation to the changes. The gill cortisol falls back to the resting levels, even though the fish may still be responding to the stressor.

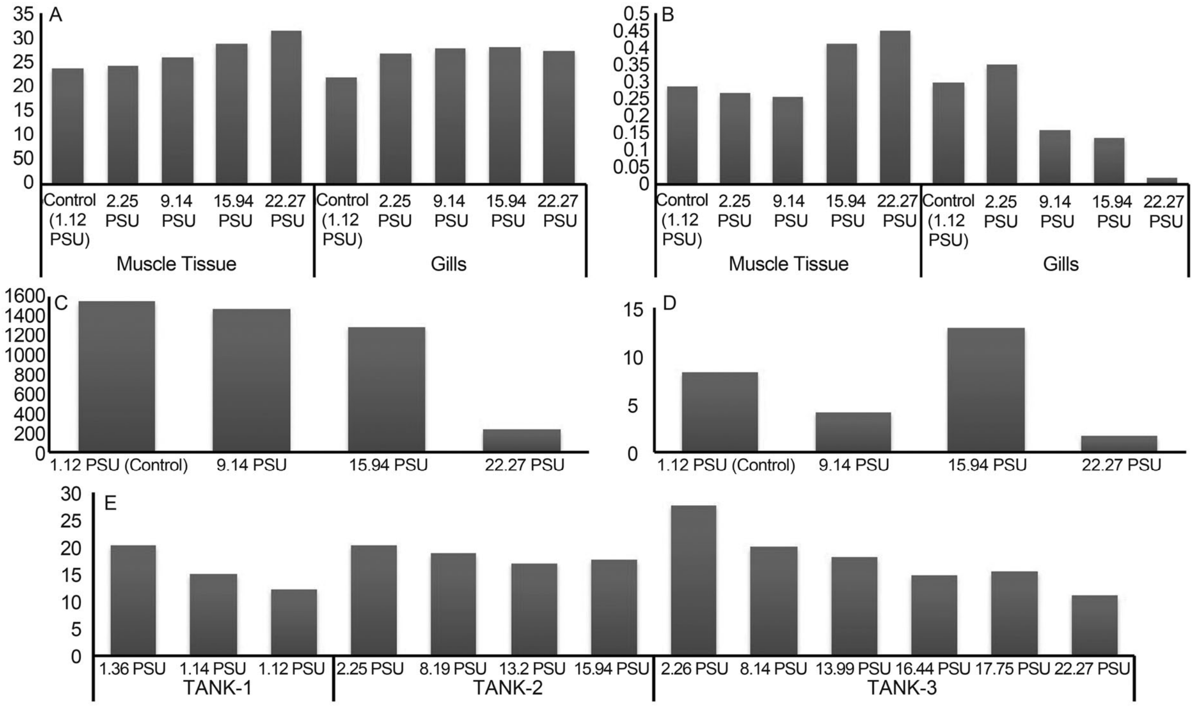

Fig. 4 — Comparative data of the effect of salt treatment on the concentration of cortisol, HSP70 protein, testosterone and estradiol in the muscle and gills of the experimental fish (*Mystus gulio*) and the total muscle protein concentration.

HSP70 also showed a difference in the secretion in the different organs and in each treatment. In case of the muscle, there was a gradual increase in HSP70 level, from 23.797 pg/mL in the control tank with a salinity of 1.12 PSU, to 31.404 pg/mL at a salinity level of 22.27 PSU (Fig. 4). On the other hand in gills, the HSP70 level was much higher than that of the muscle tissue even at a low salt concentration of 2.25 PSU. In gills the concentration of HSP70 was 26.808 pg/mL at 2.25 PSU compared to 24.211 pg/mL in the muscles at the same salt concentration. This may presumably be due to the fact that the gills being external respiratory organs are directly exposed to the environmental salinity and are the first organs to overcome salinity exposure[45]. Thus, HSP70 in the gills of the experimental fish reared at high salinity shows higher yields than in the muscles in order to protect the cells and maintain gill function. Salinity stress of the environment has also been found to disrupt protein synthesis and cause stress in aquatic animals[27]. As constitutive HSP (HSC70) synthesised in cells under normal conditions act as chaperons[46], it is possible that this stress induced HSP70 may help in protein refolding and bind abnormal proteins.

The cortisol and HSP70 level in the fish of the control tank although not exposed to salinity stress was considerably high after 21 days of the experimental tenure. This may be due to increased stocking density, competition for food and chasing, as none of the fishes in the control tank died, while in the other tanks there was gradual reduction in the number of fishes with increased salinities. Thus, basal and stressed cortisol and HSP70 levels not only differs between species but also affected by temperature, dissolved oxygen, gender, sexual maturity etc.[47].

Effect of salinity on Estradiol and testosterone

The levels of the sex steroid hormones in the control and salt treated fish have been presented in Fig. 4. There was a gradual decrease in the concentration of testosterone with increasing salt concentration. This may be due to the decreased testes development with the salinity stress as reported earlier[48]. Surprisingly on the other hand, there was an initial increase in the concentration of estradiol with the elevation levels of salinity from 9.13 to 15.94 PSU. Thereafter there was a decrease in estradiol level with a further increase in salinity up to 22.27 PSU. Thus, low salinity stress upregulated estradiol expression whereas testosterone synthesis was affected even at this salinity range. For a euryhaline fish like *Mystus gulio*, this increase might be advantageous as it may help in the adaptation of the female fish to both fresh water and brackish waters with low salinities. Low salinity stress has been earlier reported to induce estradiol synthesis which upregulates vitellogenin expression[49]. However, exposure to high levels of estrogen may reduce viability, induce gonadal malformations or feminization of genetic males[50], or sterilization[51] in fishes. All this might be responsible for the gonadal dysfunction at higher levels of salinity with subsequent decrease in estradiol synthesis.

Conclusion

The results of the present study showed that *Mystus gulio* is an efficient osmoregulator over a salt range of approximately 7-9 PSU, and alterations in protein, cortisol, and HSP70 levels are strategies to maintain ion and water homeostasis across different salinity regimes. Though the optimal salinity range for this species lies between freshwater and ~2.5 PSU, the fish demonstrated tolerance across a broader range up to ~9 PSU, beyond which most fish succumbed. Those that survived showed reduced growth. When exposed to salinity stress, the fish first showed an acute response, with an increase in cortisol levels in both muscle and gill tissue. This peak cortisol elevation was observed throughout the muscle tissue up to the maximum salinity of 22.27 PSU, but in the gills, this peak decreased with increasing salinity. The sex steroid hormone estradiol expressed significantly under low salinity stress with subsequent decrease at higher salt concentrations, but male testosterone synthesis was affected even at lower levels of salinity increase. Further investigations are necessary to understand the physiological restrictions pertaining to limited salinity tolerances in this species of fish. However, based on the comprehensive comparative data obtained under a varied salinity regime, it is recommended that fisheries may consider rearing *Mystus gulio* under low salinity conditions of ~2-7 PSU.

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Conflict of interest

The authors declare that there are no conflicts of interest.

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