Effects of Salinity on Gills' Chloride Cells, Stress Indices and Gene Expression of Asian Seabass (*Lates Calcarifer*, Bloch, 1790)

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Abstract

A two-week research was carried-out to assess water salinity (WS) effects including 0, 15, 35 and 50‰ on osmoregulatory mechanisms and stress indices in Asian sea bass (34.4 g) juveniles. Except for fish reared at 50‰, in the other treatments gradually decreased to the prescribed WS during a 10-day period (-5‰ a day). After 10-day of acclimation period, fish reared at the prescribed WS for two weeks. Fish reared at 15 and 35‰ had higher chloride cell (CC) counts in the interlamellar region. The number of CC in the interlamellar region elevated with increment of WS up to 35‰, but they were pronouncedly reduced in 50‰ group . The diameter of CC in the interlamellar region was not affected by WS. The least nucleus diameter of CC in the interlamellar region was observed in fish reared at 15‰ ($P < 0.05$). The greatest and the least amounts of serum aspartate aminotransferase content were observed in fish reared at freshwater and 15‰, respectively. Fish reared at 35‰ had the highest serum sodium and potassium contents. Serum chloride content and total osmolality increased with increment of WS ($P < 0.05$). Serum cortisol and glucose contents gradually increased with elevation of WS up to 35‰, then their contents remarkably decreased. The relative expression of insulin like growth factor-1 in the liver of fish reared at 35‰ was strikingly higher than the other groups. The relative expression of *HSP70* gene in fresh water group was pronouncedly elevated compared to other treatments. The relative expression of interleukin-1β in 15 and 35‰ groups was higher than the other groups; however, the relative expression of lysozyme gene in the liver of fish reared at fresh water was pronouncedly lower than the other treatments. The results of this study suggested rearing *L. calcarifer* at 15‰ closer to the isosmotic point and better provide its welfare.

1. Introduction

Asian seabass (*Lates calcarifer*) is a protandrous fish and tolerate a wide range of salinities (Mozanzadeh et al., 2021). Because of several favorable characteristics such as high tolerance to sudden environmental changes, great growth and feed utilisation, ease of reproduction in captive condition and high fecundity it has been considered as a worldwide candidate for marine cage culture (Mathew, 2009). Due to its euryhaline characteristics, this species is commercially cultured in different aquaculture systems such as earthen ponds or recirculating systems supplied with fresh or brackish water (0–20‰) and in marine cages.

Euryhaline fish species have a great capacity to maintain their homoeostasis in hypo- and hypersaline environments (Evans, 2008). Gills, kidneys and gut are the vital organs in euryhaline fish for the uptake and excretion of salt in hypoosmotic and hyperosmotic environments, respectively (Evans et al., 2005). Chlorides cells (CC), mainly developed in gills, particularly developed for osmoregulation (Hirose et al., 2003). These mitochondrial rich cells have high energy requirement for osmoregulation and they contain specific enzymes (*e.g.* Na^+/K^+-ATPase pump among the others) by which they can actively secrete NaCl (Evans, 2008). It has been confirmed that hyperosmotic environments induce the proliferation of CC (Caberoy & Quinitio 2000; Carmona et al., 2004), but hypoosmotic condition reduce CC counts in the gills of fish (Fielder et al., 2007). Besides counts, it has been confirmed that size and location of CC in gills and

also the activity of Na^+/K^+-ATPase pump correlate with water salinity (WS) in euryhaline fish (Lin et al., 2003; Laiz-Carrión et al., 2005a; Fielder et al., 2007). It has been confirmed that the function and morphological properties of CC during WS challenges modified by endocrine system are mainly catecholamines and growth hormone (GH) (Sakamoto & Hirano, 1993; Sakamoto & McCormick, 2006; Jiang et al., 2008). In this sense, it has been confirmed that increment of plasma cortisol induced CC proliferation and enhanced Na^+, K^--ATPase activity in the gills of Mozambique tilapia (*Oreochromis mossambicus*, Jiang et al., 2008). Furthermore, GH and insulin-like growth factor I (*IGF-1*) seem to act synergistically with cortisol in the seawater acclimation process by affecting the activity of Na^+, K^+-ATPase pump in CC (Madsen, 1990; McCormick et al., 1991; Madsen & Bern, 1993; Sakamoto & Hirano, 1993; McCormick, 1996; Seale et al., 2002; Sakamoto & McCormick, 2006). In addition, the amounts of *IGF-1* mRNA transcription increased in the liver of coho salmon (*Oncorhynchus kisutch*) during smolting process (Duan et al., 1995).

Thus, physiological processes also may affect by WS in euryhaline fish such as immunocompetence (El-Leithy et al., 2019) and stress indices (Deane *et al.*, 2004). For example, Gu et al. (2018) reported that acute exposure to hypersaline condition pronouncedly reduced expression of the immune-related genes (*e.g.* interleukin 1-receptor type 2) in the gill cells of Japanese eels (*Anguilla japonica*). In addition, up-regulation of heat shock protein 70 (*HSP70*) gene transcript has been observed in Yellow Perch (*Perca flavescens*) under salinity treatment (Eissa et al., 2017). The above-mentioned authors also demonstrated a strong relationship between *IGF-1* and *HSP70* genes expression under salt treatment, which suggested them as valuable stress biomarkers in fish.

In this regard, in this study we aimed to assess the influences of different WS on osmoregulatory and physiological responses in *L. calcarifer* by considering microscopic changes of CC in gills, serum electrolytes, stress indices and transcription of some immune and stress related genes.

2. Materials And Methods

2.1 Research design

The current study was run in the aquatic research laboratory of Persian Gulf University, Bushehr, Iran. The juveniles of Asian seabass were purchased from Ramooz company (Delvar, Bushehr) and transferred into the lab. They were stocked into a 1000 L fiberglass tank. One hundred and eighty *L. calcarifer* (initial weight = 34.4 ± 0.4 g) juveniles were randomly distributed into twelve 250-L cylindrical polyethylene tanks (15 specimens in each tank) that supplied with hypersaline (50‰) that pumped from Persian Gulf. The selected WS treatments were fresh water (0‰), 15, 35 and 50‰. Except for the 50‰ group, WS in the other treatments gradually decreased to the prescribed WS during a 10-day period (-5‰ per a day). After 10-day of acclimation period, fish were reared at the prescribed WS for two weeks. The husbandry system was supplied with sand-filtered and disinfected water with the prescribed salinities. The prescribed WS were adjusted by diluting hypersaline water (50‰) of Persian Gulf with fresh water in 1000-L polyethylene tanks and pumped into the system. The mean values (mean ± standard deviation) for

temperature, pH and dissolved oxygen were 25.0 ± 2.0°C, 7.9 ± 0.2 and 80% saturation level, respectively and photoperiod was 12L: 12D (Light: Darkness). About 70% of water of system was exchanged with new water daily. Fish were fed twice daily (0800 and 1600) with a commercial diet (size 3.0 mm, 500 g kg^{-1} protein, 160 g kg^{-1} lipid, Beyza Feed Mill 21, Shiraz, Iran) to visual satiation for two weeks.

2.2 Sampling

After finishing two-weeks of the husbandry trial, six specimens of each tank were anesthetized with 2-phenoxyethanol and blood samples was collected from their caudal vein with syringes. Five h after blood coagulation, blood samples were centrifuged (4000 *g*, 10 min, at room temperature) and serum was separated and was kept at − 80°C until their analysis. After blood collection, the same fish were sacrificed with an overdose (1000 mg L^{-1}) of the anesthetic to dissect gills and the liver. The dissected gill was fixed in formalin 10% for 24 h and then transferred to 70% ethanol until examination and the liver samples were snap-frozen by using liquid nitrogen then kept in -80°C until their analysis.

2.3 Histological studies and serum biochemical assessments

A classic histology along with hematoxylin and eosin staining methods were done for evaluating the histoarchitecture of CC in the gills samples using an Olympus BH-2 photomicroscope. A computerized microscopic image analyzer (Digimizer 4.1.1) was used to determine histomorphometric parameters of gills including number of chloride cells (CC) in the interlamellar region, diameter of CC and their nucleus in the interlamellar region of 10 fish per treatment were evaluated.

Serum glucose (GLU), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and electrolytes (sodium, potassium and chloride) were measured by means of an automated analyzer (Technicon RA-1000, Technicon Instruments) using diagnostic kits (Pars Azmoon Kit, Iran). Serum cortisol concentration was measured by radioimmunoassay using a commercial kit (#IM1841, Beckman Coulter, Immunotech) (Ellis et al., 2004). Serum total osmolality (mOsmol kg^{-1}) were determined by a freezing point osmometer (Knauer, K-7400, Germany). Plasma Cl^{-}, Na^{+} and K^{+} levels were measured was determined by atomic absorption spectrophotometry (Radiometer mod. EML-100).

2.4 Evaluation of relative expression of growth and immune-related genes in the liver

Total RNA of the liver samples was extracted by a commercial kit (Roche, Manheim, Germany) as described by the manufacturer instructions and then were treated with DNase I to remove contaminating genomic DNA. RNA quantity and purity were assessed by measuring the A260/A280 ratio using Spectrophotometer (Biotech photometer WPA), and the RNA quality was assessed through electrophoresis on a 1% agarose gel. Elongation factor 1α was applied for internal housekeeping gene as used a suitable reference gene by Mohd-Shaharuddin et al. (2015) and Paria et al. (2015) in previous studies on Asian seabass. A cDNA synthesis Kit (Cinna gene, Tehran, Iran) and primers (oligo(dT),

Random Hexamer (Metabion)) following the manufacturer's instructions was applied for synthesizing cDNA. The expression of insulin-like growth factor I (*IGF-1*), interleukin 1β (*IL-1β*), heat shock protein 70 (*HSP70*) and lysozyme in the liver of *L. calcarifer* juveniles were assessed by quantitative real-time PCR assays in triplicate (Table 1). A real-time PCR machine (Rotor Gene-3000, Sydney, Australia) was applied to assess the expressions of these genes as described by Zeynali et al. (2020). The Ct of Ef1a under the influence of salinity did not change significantly between different treatments ($P < 0.01$). Data analysis of the real-time PCR was performed in triplicate with Rotor-Gene, RG-3000 (Australia) software. The relative expression levels of candidate genes were calculated using the 2-ΔΔCt method (Livak & Schmittgen, 2001).

Table 1
Primers sequences and amplification efficiencies

Gene name	Sequences of primers	Accession number	Efficiency	Product size
Ef1a	F: AAATTGGCGGTATTGGAAC	GQ507427.1	97%	83
	R: GGGAGCAAAGGTGACGAC			
IGF-1	F: ACGCTGCAGTTTGTATGTGG	XM_018697285.1	98%	157
	R: CCTTAGTCTTGGGAGGTGCA			
HSP70	F: AAGGCAGAGGATGATGTC	HQ646108.1	97%	186
	R: TGCAGTCTGGTTCTTGTC			
IL-1β	F: CCTGTCGCATTTCAGTACGG	XM_018669006.1	95%	147
	R: ATTTCCACCGGCTTGTTGTC			
Lysozyme	F: GGTGTTTCTGCTCTTGGTGG	XM_018667849.1	99%	196
	R: GCCGTAGTCAGTGGATCCAT			

2.5 Statistical assessments

A SPSS ver. 16.0 (Chicago, IL, USA) software was used for data analyses. Kolmogorov-Smirnov and Leven tests were used for confirmation of normality and homogeneity of data, respectively. A one-way ANOVA and Duncan's multiple-range as post-hoc test were performed for comparisons between groups. The $P < 0.05$ was considered as significant for all statistical tests.

3. Results

The survival rate in all groups was 100%. The histological study of the gill demonstrated that fish reared at 15 and 35‰ had more CC counts in the interlamellar region ($P < 0.05$, Table 2, Fig. 1, 2). The number

of CC in the interlamellar region increased with increasing WS up to 35‰, but they decreased in fish reared at 50‰. The diameter of CC in the interlamellar region was not affected by WS. The nucleus diameter of CC in the interlamellar region in fish reared at 15‰ was lower than the other groups ($P < 0.05$).

Table 2

Gills' chloride cells (CC) morphology of *Lates calcarifer* juveniles reared in water salinities (0, 15, 35 and 50 ‰) at the end of the trial. A different superscript in the same row denotes statistically significant differences ($P < 0.05$).

	Water salinities (‰)			
Treatments	0	15	35	50
CC counts in the interlamellar region	6.4 ± 0.5^{b}	9.6 ± 0.5^{a}	9.0 ± 0.6^{a}	7.5 ± 0.4^{b}
Diameter of CC in the interlamellar region	7.9 ± 0.2^{a}	7.1 ± 0.3^{a}	7.0 ± 0.3^{a}	7.6 ± 0.2^{a}
Diameter of CC nucleus in the interlamellar region	5.3 ± 0.1^{a}	4.8 ± 0.2^{b}	5.4 ± 0.3^{a}	5.7 ± 0.2^{a}

Serum ALP and ALT contents did not change in fish reared at different WS (Table 3). Fish reared at freshwater and 15‰ had the highest while lowest serum AST content, respectively ($P < 0.05$).

Table 3

Serum enzymes contents ($U\ L^{-1}$) (mean ± SE, n = 3 tank) of *Lates calcarifer* juveniles reared in water salinities (0, 15, 35 and 50‰) at the end of the trial. A different superscript in the same row denotes statistically significant differences ($P < 0.05$).

	Water salinities (‰)			
Treatments	0	15	35	50
CC counts in the interlamellar region	6.4 ± 0.5^{b}	9.6 ± 0.5^{a}	9.0 ± 0.6^{a}	7.5 ± 0.4^{b}
Diameter of CC in the interlamellar region	7.9 ± 0.2^{a}	7.1 ± 0.3^{a}	7.0 ± 0.3^{a}	7.6 ± 0.2^{a}
Diameter of CC nucleus in the interlamellar region	5.3 ± 0.1^{a}	4.8 ± 0.2^{b}	5.4 ± 0.3^{a}	5.7 ± 0.2^{a}
Abbreviations: ALP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase.				

Fish reared at 35‰ had the highest serum sodium and potassium contents and fish reared at 15 and 50‰ showed the lowest values (Table 4). Serum chloride content and total osmolality increased with increment of WS ($P < 0.05$).

Table 4

Serum electrolytes (mmol L^{-1}) and total osmolality (mOsmol kg^{-1}) (mean ± SE, n = 3 tank) of *Lates calcarifer* juveniles reared in water salinities (0, 15, 35 and 50 ‰) at the end of the trial. A different superscript in the same row denotes statistically significant differences ($P < 0.05$).

	Water salinities (‰)			
Treatments	0	15	35	50
Sodium	114.0 ± 1.0^{b}	102.7 ± 3.8^{c}	157.7 ± 2.1^{a}	95.0 ± 2.1^{c}
Potassium	3.2 ± 0.2^{b}	2.4 ± 0.2^{c}	3.9 ± 0.1^{a}	2.3 ± 0.1^{c}
Chloride	135.3 ± 1.8^{b}	146.3 ± 7.8^{b}	161.7 ± 1.5^{a}	170.0 ± 1.0^{a}
Total Osmolality	391.7 ± 2.9^{c}	413.7 ± 1.8^{b}	438.7 ± 5.2^{a}	439.3 ± 2.7^{a}

Serum cortisol (Fig. 3a) and glucose (Fig. 3b) levels gradually increased with elevation of WS up to 35‰, then their contents remarkably decreased in serum of fish reared at 50‰.

The relative expression of *IGF-1* in the liver of fish reared at 35‰ was significantly higher than the other groups, but fish reared at fresh water and 15‰ showed the lowest liver *IGF-1* gene expression (Fig. 4a). The relative expression of *IL-1β* in the liver of fish reared at 15 and 35‰ was higher than at fresh water and 50‰ (Fig. 4b). The relative expression of *HSP70* gene in fish reared at fresh water was pronouncedly higher than other treatments (Fig. 4c); however, the relative expression of lysozyme gene in the liver of fish reared at fresh water was remarkably lower than those reared at salt waters (Fig. 4d).

4. Discussion

In many teleosts, changes in counts and/or dimension of CC mainly correlated to acclimation to WS throughout ontogenic development (Hiroi & McCormick, 2007). The findings of the present research indicated that the CC morphology in the gills remarkably responded to different WS. The number of CC in the interlamellar region pronouncedly increased in fish with increment of WS especially in fish reared at 35‰, then decreased at 50‰. It was noticed that, the proliferation of CC particularly in fish reared at 35‰ was coincided with elevation of serum cortisol level and up-regulation of the liver *IGF-1* gene. These results suggesting the direct influence of these hormones on osmoregulation of *L. calcarifer* by modifying the morphology of CC in the gills. Furthermore, the augmentation of CC counts indicating the greater

requirement for ionoregulation through these cells for keeping homeostasis. Similarly, remarkable increment of CC counts in the gill filaments was reported in different hyperosmotic-acclimated fish such as European sea bass (*Dicentrarchus labrax*, Varsamos et al., 2002), killifish (Lima & Kültz, 2004), Adriatic sturgeon (*Acipenser naccarii*, Martínez-Álvares *et al*., 2005), Mozambique tilapia (Hiroi et al., 2005), fat snook (*Centropomus parallelus*, Sterzelecki et al., 2013). It should be mentioned that fish reared at 15‰ had the smallest nucleus diameter in CC suggesting these fish were at isosmotic condition and less osmotic stress pressure was on them; however, fish reared at other WS showed nucleus hypertrophy mas a result of hypo and/or hyperosmotic stress condition. Similarly, Laiz-Carrión et al., (2005a) revealed that the number and size of CC were remarkably enhanced in gilthead seabream reared at 5‰ and 60‰, whereas exposure of fish to intermediate WS (15‰ and 25‰) reduced their CC size as a result of lower need for ion pumps required in fish reared at isosmotic environments. Increment of CC size in the hypo- and/or hyperosmotic environments indicates the increasing the permeability of cells' junctions and CC complexes for augmentation of Na^+ and Cl^- turnover (Miyazaki et al., 1998).

The amount of liver enzymes in body's fluids is valuable biomarkers of fish welfare and health in response to stressful condition (Wagner & Congleton, 2004). In the present study, the amount of serum ALP and ALT did not change in fish reared at different WS, but fish reared at fresh water or 15‰ had the highest and least amount of plasma AST. These results indicated that fish reared at freshwater may be metabolized amino acids derived from proteolysis or used exogenous amino acids pool as a fuel source for gluconeogenic activity to cope with stressful condition; meanwhile fish reared at isosmotic condition (15‰) underwent the least stress. In accordance with these findings, Farshadian et al., (2018) revealed that the value of ALP was not affected in yellowfine seabream reared at 5‰ and 35‰.

In this study, serum cortisol increased in fish reared at 35‰ that was in concomitant with hyperglycemia and the up-regulation of the liver *IGF-1* gene that may be correlated with increase of CC in the interlamellar region. It should be mentioned that the concentrations of serum Na^+ and K^+ also increased in fish reared at 35‰ that may as consequence of increase in CC counts in this group. These findings indicated that cortisol and *IGF-1* can synergically enhance the salinity resistance in *L. calcarifer*. In this sense, it has been reported that cortisol can act directly on gills to augment Na^+, K^+-ATPase activity and CC density (Madsen & Bern, 1993). The increment of serum glucose in fish reared at 35‰ may be related to the increasing transfer of metabolites as a stored fuel source to deal with stress and to satisfy the energy demand for higher Na^+, K^+-ATPase activity in CC under the regulation of adrenalin and cortisol hormones (Wendelaar-Bonga, 2011).

The determination of the amounts of blood electrolytes, especially Na^+, K^+ and Cl^-, and osmolality and ion levels after changes in WS can provide information regarding the ionoregulatory ability and also successful acclimation of fish in a saline environment (Stewart et al., 2016). In the current research, the amounts of serum Na^+ and K^+ pronouncedly enhanced in fish reared at 35‰ that was associated with enhancing serum cortisol in this group indicating the increment tightening the junction between polygonal pavement cells in order to limit passive salt gain or loss during SW or FW acclimation,

respectively (Chasiotis et al., 2012). However, the levels of these ions in fish reared at 35‰ were higher than those reared at 15 and 50‰. In this context, because of any correlation among result of Na with other ions and osmolality, the significant difference in the metioned parameter could probably be attributed to an error in Na^+ evaluation in the lab. In our study, the serum osmolality and Cl^- linearly enhanced with elevation of WS suggesting strong osmoregulatory ability of this species as also reported in other euryhaline fish (Laiz-Carrion et al., 2005b; Saud et al., 2007; Herrera et al., 2009; Vargas-Chacoff et al., 2011). It has been suggested that these increased ions levels are due to elevated Na^+/K^+-ATPase activities mainly in gut and kidney for uptaking ions from gut fluids and urine.

In euryhaline teleosts, *GH* and *IGF-1* have osmoregulatory effects and act on the gill is through changes in tissue responsiveness to cortisol through elevation the numbers of gill cortisol receptors (Shrimpton et al., 1995; Sakamoto & McCormick, 2006). Thus, *GH* and *IGF-1* synergically along with cortisol appears to control gills' osmoregulatory function by affecting the activity of Na^+/K^+-ATPase, distribution and density of CC (Sakamoto & McCormick, 2006; Deane & Woo, 2009). In the current research, the expression of *IGF-1* remarkably enhanced in the liver of fish reared at 35 and 50‰ indicating the key role of this hormone for maintaining homeostasis at hyperosmotic environments. In addition, up-regulation of liver *IGF-1* gene in 35‰ group was associated with the increment of serum cortisol, which consequently enhanced CC in the interlamellar region in this group. Similarly, it has been found that liver *IGF-1* expression increased by seawater exposure in black sea bream and Atlantic salmon (Deane & Woo 2005; Breves et al., 2017).

The *HSP* family mainly functions as molecular chaperones in cells to prohibit protein disruption, regulate protein homeostasis and contribute in refolding of misfolded proteins. They are also implicated in the general protection of stressed cells (Basu et al., 2002). Our findings demonstrated that fish reared at freshwater has higher liver *HSP70* gene expression that was coincided with the highest liver AST content compared to other groups suggesting this treatment was under stressful condition. These results indicate a direct role of the stress protein in salinity tolerance by *L. calcarifer*. The key role of *HSP70* in the adaptation of fish to changes of WS has been well documented (Smith et al., 1999). For example, hypo- or hyperosmotic shock enhanced the branchial expression of *HSP70* in the silver sea bream (*Sparus sarba*, Deane *et al.*, 2004). The authors of the above-mentioned study revealed that the activity and mRNA levels of *HSP70* were lower around isosmotic WS that was attributed to the best growth performance in silver sea bream.

It has been confirmed that there is a direct relationship between immune-related genes and environmental salinity in fish (Gu et al., 2018). Inflammatory-related genes (pro-inflammatory cytokines) such as *IL-1β* enable the organisms in responding to stress condition by inducing neutrophil chemo-attractant ability and their migration toward inflammatory sites (Uribe et al., 2011). In the present study, rearing fish at 15 and 35‰ induced liver *IL-1β* up-regulation suggesting changes in WS can modify immune responses in this species. Similar to our findings, proliferation of leucocytes and their activities after acute salinity change were found in pipefish (*Syngnathus typhle*) (Birrer et al., 2012). Furthermore, El-Leithy et al. (2019) reported that levels of IL-1β, IL-8, and cc-chemokine were higher in the liver of Nile tilapia (*Oreochromis niloticus*) reared at 16‰ compared to groups reared at 20‰ suggesting pro-inflammatory respond in

fish reared at 16‰. In contrast, Choi et al. (2012) reported that rapid decreases in salinity, did not affect splenic leucocytes IL-1β transcription in Nile tilapia. In addition, chronic hyperosmotic stress in striped catfish (*Pangasianodon hypophthalmus*, Sauvage), inhibited kidneys' toll-like receptors expression suggesting immune-suppressive effects of salinity stress (Schmitz et al. 2017).

Lysozyme possesses a direct antibacterial effect by splitting peptidoglycan layers of Gram-positive bacteria and act as an opsonin that trigger phagocytes to destroy Gram-negative bacteria (Yano, 1996). In the present study, *Lysozyme* gene expression down-regulated in the liver of fish reared at freshwater that was associated with up-regulation of the liver *HSP70* in this group suggesting immunosuppressive effects of hypoosmotic stress. In this sense, Yada *et al.* (2012) reported that hyperosmotic condition increased LZ gene expression in the gills of Atlantic salmon (*Salmo salar*). Furthermore, it has been reported that increasing WS enhanced serum/plasma lysozyme activity in brown trout (Marc et al., 1995), rainbow trout (Yada *et al.*, 2001; Fast et al., 2002), Nile tilapia (Dominguez *et al.*, 2005), sablefish (Kim et al., 2017), yellowfin seabream and Asian seabass (Mozanzadeh et al., 2021). These results indicate that WS can directly affect fish immunocompetence by affecting immune-related genes, chaperones as well as endocrine system especially catecholamines and GH.

In conclusion, the findings of this study indicated that changes in WS pronouncedly alter the histoarchitecture of CC of gills maybe through stress response pathway (*e.g.* cortisol) and *IGF-1* also synergically modified these responses. Immune-related genes also triggered by intermediate WS (15 and 35‰), suggesting mediatory role of WS in fish immunity. Finally, rearing *L. calcarifer* at intermediate salinities (*e.g.* 15‰) is suggested because of lower concentration of AST in the liver and this salinity is closer to its isotonic point compared to the other salinities.

5. Declarations

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Authors' contributions

All persons listed as authors have read, contributed to preparing the manuscript as given below:

Maryam Azodi, Sakineh Avizhgan and Ahmad Ghasemi carried out fish maintenance and sample collection

Mahmoud Nafisi Bahabadi carried out experimental design and statistical analyses

Vahid Morshedi carried out digestive enzymes analyses

Raheleh Shahraki, Omid Khademzadeh and Shirin Hamedi carried out antioxidant enzymes analyses

Mansour Torfi Mozanzadeh carried out data interpretation

Ethics approval

This study was carried out in accordance with the principle of the Basel Declaration and recommendations of the Faculty of Veterinary Medicine at University of Tabriz, the FVM.REC.1396.939. The protocol was approved by the FVM.REC.1396.939.

Conflicts of interest/Competing interests

The authors declare that they have no conflict of interest.

Availability of data and material (data transparency)

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Consent to participate

The authors declare that they have every consent to participate.

Consent for publication

The authors declare that they have every consent for publication.

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Figures

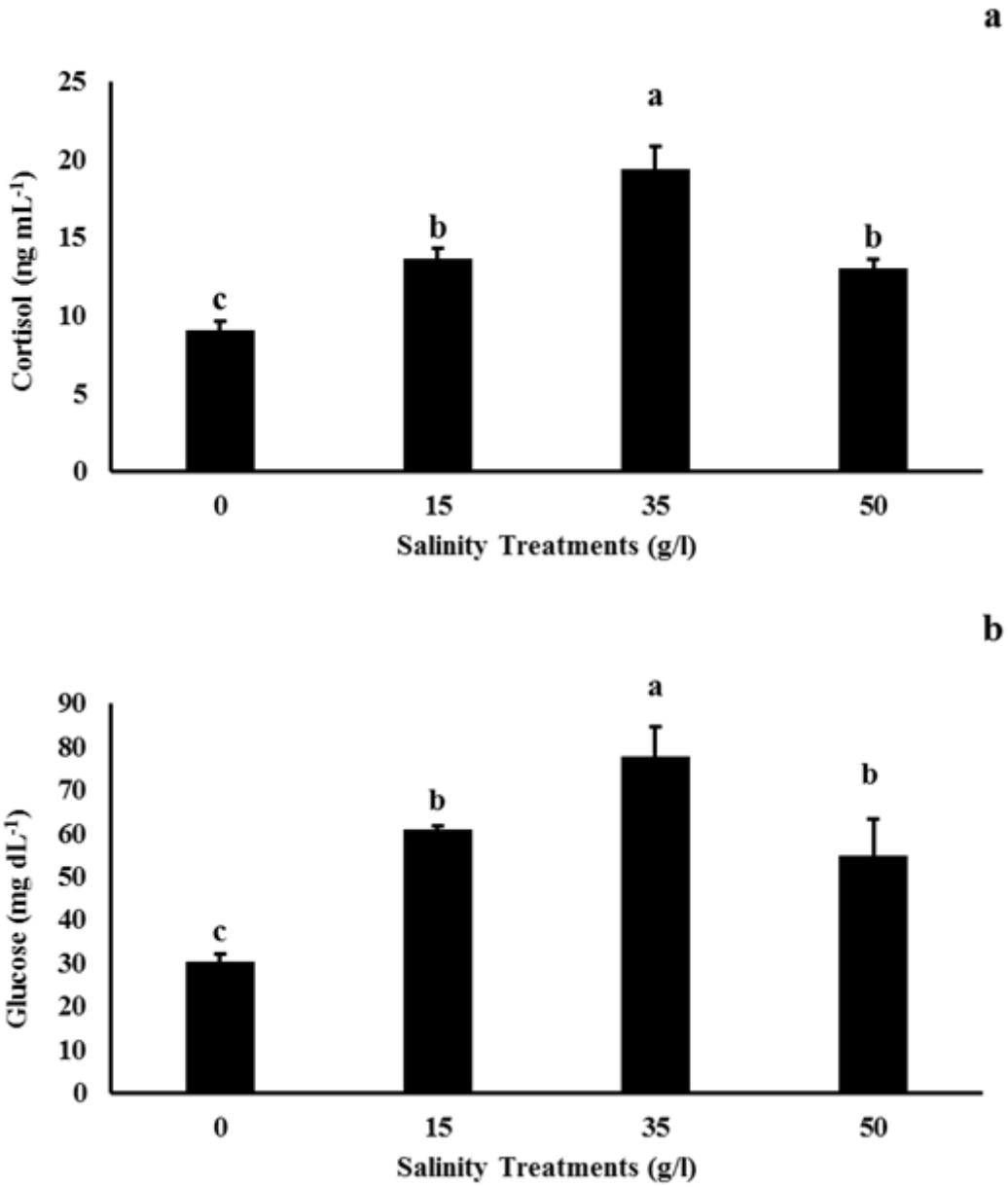

Figure 1

The survival rate in all groups was 100%. The histological study of the gill demonstrated that fish reared at 15 and 35‰ had more CC counts in the interlamellar region ($P < 0.05$, Table 2, Fig. 1, 2). The number of CC in the interlamellar region increased with increasing WS up to 35‰, but they decreased in fish reared at 50‰. The diameter of CC in the interlamellar region was not affected by WS. The nucleus diameter of CC in the interlamellar region in fish reared at 15‰ was lower than the other groups ($P < 0.05$).

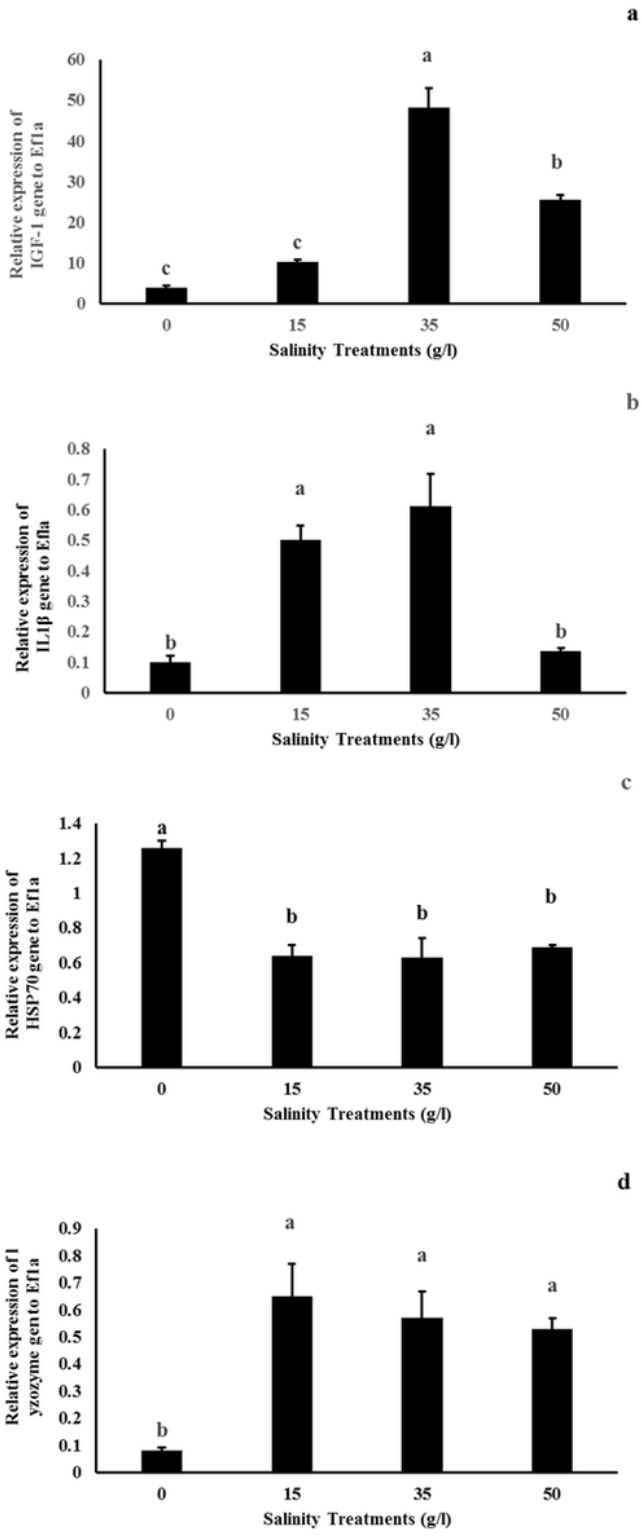

Figure 2

The survival rate in all groups was 100%. The histological study of the gill demonstrated that fish reared at 15 and 35‰ had more CC counts in the interlamellar region ($P < 0.05$, Table 2, Fig. 1, 2). The number of CC in the interlamellar region increased with increasing WS up to 35‰, but they decreased in fish reared at 50‰. The diameter of CC in the interlamellar region was not affected by WS. The nucleus

diameter of CC in the interlamellar region in fish reared at 15‰ was lower than the other groups ($P < 0.05$).

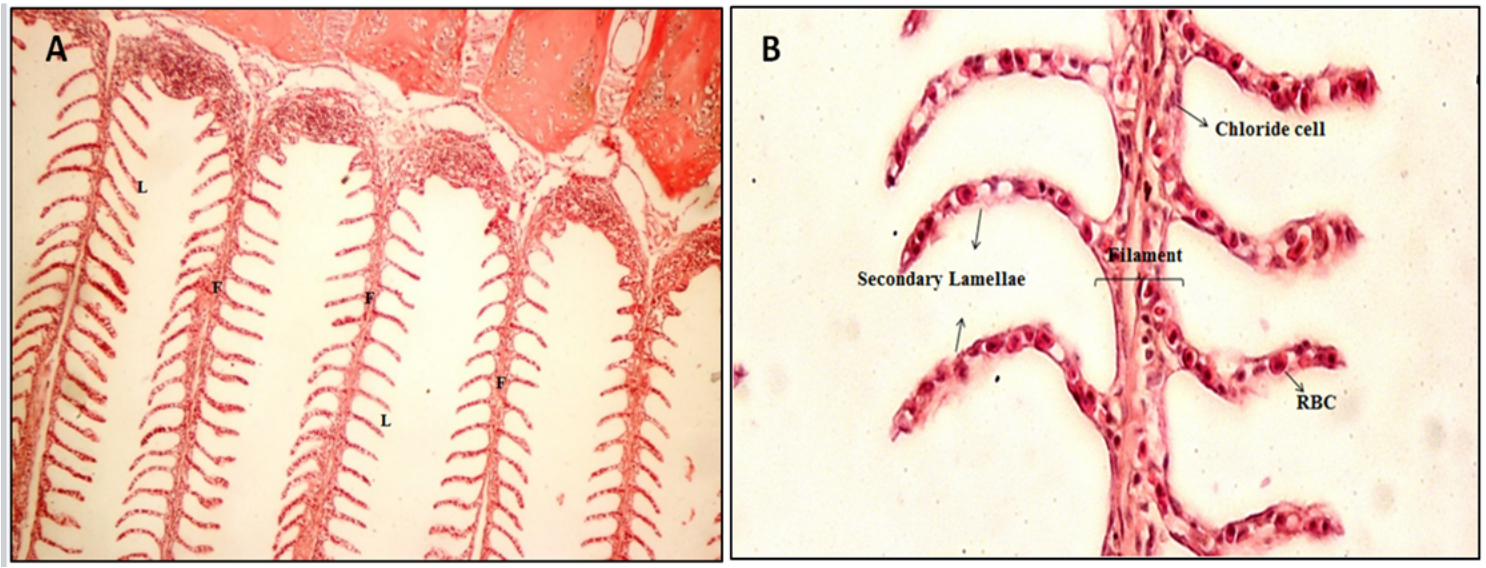

Figure 3

Serum cortisol (Fig. 3a) and glucose (Fig. 3b) levels gradually increased with elevation of WS up to 35‰, then their contents remarkably decreased in serum of fish reared at 50‰.

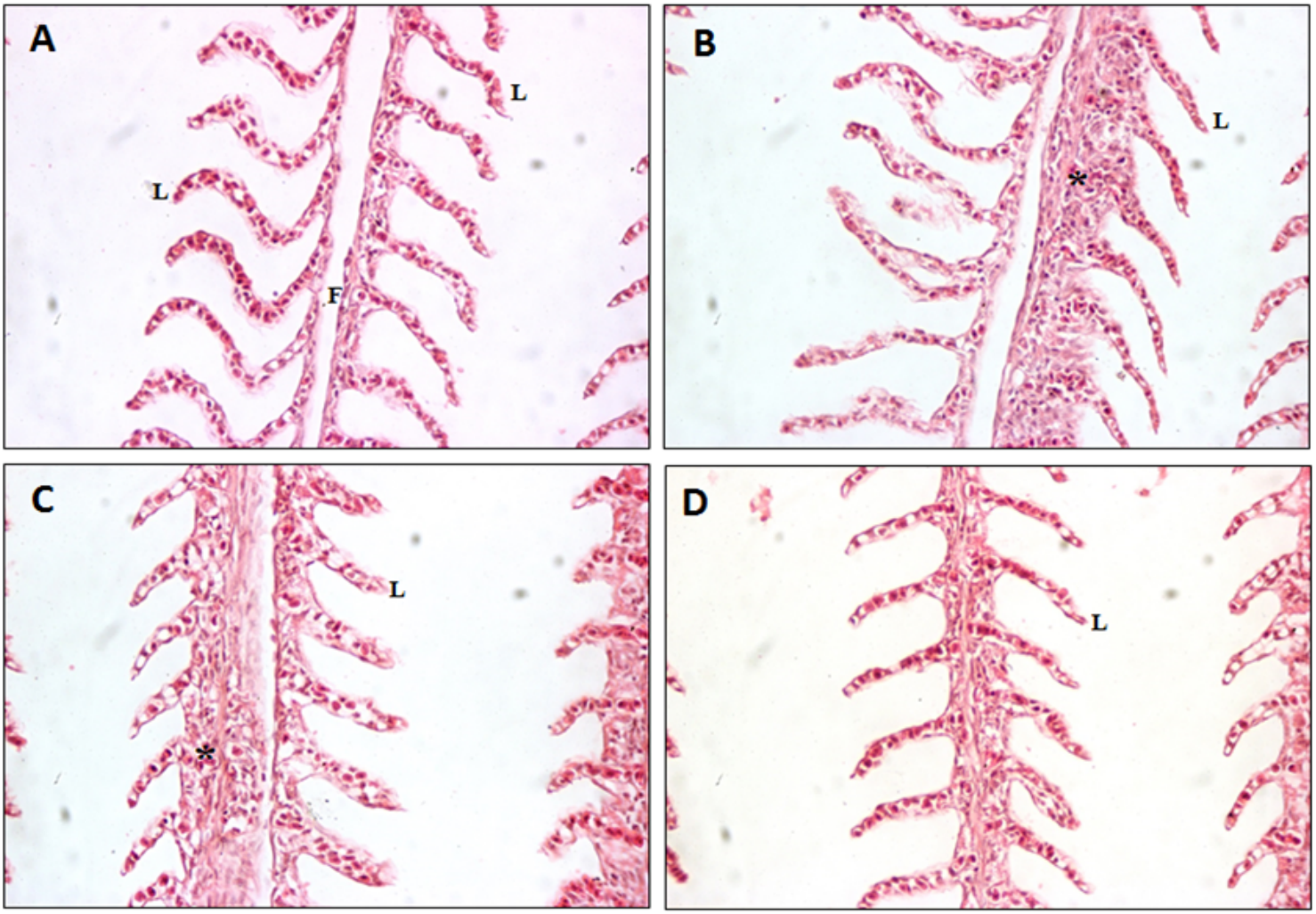

Figure 4

The relative expression of IGF-1 in the liver of fish reared at 35‰ was significantly higher than the other groups, but fish reared at fresh water and 15‰ showed the lowest liver IGF-1 gene expression (Fig. 4a). The relative expression of IL-1β in the liver of fish reared at 15 and 35‰ was higher than at fresh water and 50‰ (Fig. 4b). The relative expression of HSP70 gene in fish reared at fresh water was pronouncedly higher than other treatments (Fig. 4c); however, the relative expression of lysozyme gene in the liver of fish reared at fresh water was remarkably lower than those reared at salt waters (Fig. 4d).