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Chronic hyperosmotic stress interferes with immune homeostasis in striped catfish (*Pangasianodon hypophthalmus*, S.) and leads to excessive inflammatory response during bacterial infection

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ABSTRACT

Hyperosmotic stress has often been investigated from osmoregulation perspectives while the effects of such stress on the immune capacity remain largely unexplored. In this study, striped catfish were submitted to three salinity profiles (freshwater, low saline water, saline water) during 20 days, followed by infection with a virulent bacteria, *Edwardsiella ictaluri*, responsible for the enteric septicaemia of catfish. Osmoregulatory (plasma osmolality, gill Na^+K^+ATPase), immune (blood cells, lysozyme activity, complement activity, respiratory burst) parameters and mortality rate were investigated. In addition, abundances of heat shock protein 70 and high mobility group box 1 were explored. With elevated salinity, plasma osmolality severely increased while gill Na^+K^+ATPase slightly increased. Salinity alone stimulated the number of granulocytes, lysozyme activity and respiratory burst but depleted the number of thrombocytes. Salinity in combination with infection stimulated the number of monocytes and ACH50. On the contrary, erythrocytes, hematocrit, heat shock protein 70 and high mobility group box 1 did not significantly vary with salinity profiles. Then, salinity induced earlier onset on mortalities after *E. ictaluri* inoculation whereas cumulative mortality reach 79.2%, 67.0% and 91.7% respectively in freshwater, low saline water and saline water. In conclusion, salinity stimulates several immune functions in striped catfish but prolonged exposure to excessive hyperosmotic condition may lead to excessive inflammatory response and death.

1. Introduction

The striped catfish (*Pangasianodon hypophthalmus*, Sauvage) is a potamodromous catfish endemic of the Mekong River Basin and the Chao Praya River in Thailand. Nowadays, striped catfish farms are the major inland aquaculture production in Southeast Asia. In 2014, worldwide striped catfish production reached 1.2 million of tons, which represent almost 1.7 billion US$ in the international trade [1]. Vietnam is by far the largest striped catfish producer, exporting striped catfish to more than 80 countries, mainly in the European Union and United States [1]. In Vietnam, the Mekong Delta accounts for more than 75% of the Vietnamese production with a total farming area of 5509 ha in 2011 [2]. Nevertheless, striped catfish industry in the Mekong Delta is currently facing many climatic challenges, particularly extensive salinity intrusion induced by the global climate changes. According to 4 RCP (representative concentrations pathways), ocean thermal expansion (30–55%) and glaciers melting (15–35%) will induce a global sea level rise comprised between 0.26 and 0.98 m by 2100. In the Mekong Delta, in 2016, saline water intrusions have already been observed up to 90 km far from the River Mouth, thereby rising salinity level up to 12 ppt in many aquacultural provinces [3]. The latter study also suggested that such salinity conditions may affect catfish hematological and immune status [unpublished data].

Environmental salinity is an important parameter for aquatic organisms. Modification of salinity may be responsible for

Abbreviations: HSP, Heat shock proteins; HMGB1, High-mobility group protein B1; DAMPS, Damage-associated molecular patterns; LD5096, Lethal dose 50% 96 h; HBSS, Hank's Balanced Salt Solution; RRBC, Rabbit red blood cells; ACH50, Alternative complement hydrolysis 50%; ESC, Enteric septicaemia of catfish.

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important biochemical and physiological troubles [4]. Studies evaluating fish acclimation from hyposmotic to hyperosmotic environment (relative to plasma) have been mainly looking at changes in the osmoregulatory system while the effects of such stressors on the immune capacity remain largely unexplored. Gilthead seabream (*Sparus aurata*) acclimated to low saline water (6 ppt) showed lower peroxidase and alternative complement activity in plasma compared to fish acclimated to brackish (12 ppt) and saline water (38 ppt) [4]. In Mozambique tilapia (*Oreochromis mossambicus*), renal and plasma lysozyme activities increased 1 h and 24 h after transfer from freshwater to saline water (25 ppt) while respiratory burst increased in spleen and kidney as early as 8 h post transfer [5]. In rainbow trout (*Oncorhynchus mykiss*), 3-days acclimation to hyperosmotic water (12 and 29 ppt) resulted in elevated plasma lysozyme activity [6]. Hypo- or hyperosmotic shocks in grouper fry (*Epinephelus* sp.) induced higher susceptibility to infectious pancreatic necrosis virus [7]. Regarding adaptative immune function, a study on fish vaccination revealed that barramundi (*Lates calcarifer*) acclimated to seawater produced a higher adaptative mucosal antibody response than barramundi acclimated to freshwater [8].

In eukaryotes, Heat Shock Proteins (HSP) and High-Mobility Group B1 (HMGB1) are constitutive and highly conserved molecular chaperones for DNA and proteins [9–11]. During cellular stress, HSPs and HMGB1 may be produced and released either actively or passively in the extracellular environment, in order to inhibit protein aggregation and to repair denatured proteins [12–14]. For immune defence, HMGB1 and HSPs (particularly HSP70 and 90) have key role in inflammation as well as in innate and adaptative immune function during bacterial or viral infection [10,15]. In the extracellular environment, they act as damage-associated molecular patterns (DAMPs) and affect many aspects of the immune response, particularly through activation of cell surface innate immune receptor, typically Toll like Receptor [16]. In fish, upregulation of HSP70 has been documented during biotic (i.e. infectious disease) and abiotic (e.g. osmotic, acidosis, heat, anoxia, toxins, protein degradation) stressors [10]. On the other hand, reports on HMGB1 are still limited and regulation of HMGB1 level by abiotic factors remains largely unknown. In red drum (*Sciaenops ocellatus*) and goldfish (*Carassius auratus*), bacterial infection elevated the abundance of HMGB1 transcript and protein from 12 to 48 h post challenge [17,18].

Following sterile or microbial injury, inflammatory processes are essential for tissue and wound repair. However, prolonged exposure to the detrimental agents and continuous release of DAMPs (e.g. typically HSPs, HMGB1, S100 calcium-binding proteins and purine metabolites) may lead to excessive inflammation and severe immunopathological conditions that may end up with tissue damage and death [19].

Understanding how salinity changes impact fish health and disease resistance is of critical importance, particularly for high commercial species such as the striped catfish. In the present study, we aimed to characterize the effects of hyperosmotic stressor on passive and induced innate immune defences of striped catfish. We hypothesized that prolonged exposure to hyperosmotic conditions (10 and 20 ppt) might unbalance the immune homeostasis of healthy fish by inducing chronic inflammation. Key factors of the osmoregulatory, inflammatory and immune components have been analysed at different time points during salinity exposure and compared to values obtained under freshwater conditions. Then, we further hypothesized that chronic inflammation may result in an excessive inflammatory response following microbial challenge, leading to serious diseases status and death. To verify this hypothesis, fish were challenged with a virulent strain of *Edwardsiella ictaluri*, responsible for ESC (enteric septicaemia of catfish) disease [20]. Key parameters of the innate immune response, molecular chaperones (i.e. HSP 70 and HMGB1 protein) and cumulative mortality were compared between freshwater-held fish and salinity-challenged fish prior and after inoculation of the bacteria.

2. Methods

2.1. Fish and in vivo stress experiment

Investigations have been conducted according to the guidelines for animal use and care in compliance with Belgian and European regulation on animal welfare (ethical protocol n°KE 12/189). One week-old striped catfish (*Pangasianodon hypophthalmus*, Sauvage) were provided by the Nam Sai catfish farm (Ban Sang, Thailand). Juveniles were maintained in fish facilities in the University of Namur (Belgium) at 28 °C under constant aeration and photoperiod (12L:12D) in recirculating aquaculture systems. Fish were daily fed *ad libitum* with commercial dry pellets (Troco Supreme 4.5 mm, Coppens, The Netherlands). After 3 months, fish (40–50 g) were equally divided into 3 experimental groups each including four 100 L tanks (30 fish/tank). Fish were acclimated to their new housing conditions during 10 days. The first group of fish was maintained in freshwater (0.4 ppt) during all the experiment. The second group of fish was exposed to low salinity water stressor namely to a gradual water salinity increase of 0.5 ppt per day during 20 days (0.4–10 ppt). The third group of fish was exposed to a higher saline stressor namely to a gradual salinity increase of 1 ppt per day during 20 days (0.4–20 ppt). Salinity was increased by adding marine salt (Ocean Fish, Prodac, Italy) mixed with tap water until day 20 and then remained stable during the following bacterial challenge. On day 20, fish were anaesthetized in tricaine methanesulfonate MS-222 (150 mg L^{-1}) and intraperitoneally injected with 0.025 ml g^{-1} fish of a bacterial solution (10^6 bacteria ml^{-1} ($LD50_{96h}$) of Hank's Balanced Salt Solution (HBSS, Sigma-Aldrich)). Mortality was daily recorded during 10 days. Fish (6 fish/tank) were sampled at the following time points: days 0, 10, 20 and 23. On the sampling days, fish were randomly collected in the tanks with nets and anaesthetized in MS-222 (Sigma-Aldrich) (150 mg L^{-1}). Blood was collected by caudal vein puncture using a sterile 1 ml heparinized syringe within 5 min and euthanized by cervical dislocation. Blood was kept on ice until plasma was separated by centrifugation at 4 °C (7000 g, 10 min) and frozen at −80 °C pending analyses. The whole kidney was rapidly collected by gentle scratching with tweezers along the vertebral column and immediately frozen in liquid nitrogen. Gill filaments from left arches 1–2 were taken out and immediately frozen in liquid nitrogen. Spleen was stored on ice in L-15 media until respiratory burst assay was carried out the same day. Physicochemical data were measured daily in the outlet pipe using a multiparameter probe (WTW, Multi 350i): O_2:5.7 ± 0.5 mg L^{-1}; pH: 8.4 ± 0.24; temperature: 28.2 ± 0.1 °C, $N\text{-}NO_3^-$: 3.55 ± 2.27 mg L^{-1}; $N\text{-}NO_2^-$: 0.019 ± 0.005 mg L^{-1}; $N\text{-}NH_3^+$: 0.19 ± 0.25 mg L^{-1}. Measured salinities were closed to the expected values (±0.3 ppt) (Fig. 1). The feed intake was measured daily.

2.2. Bacterial challenge

2.2.1. Culture of the bacterial strain

Virulent strain of *E. ictaluri* (TNA 015) was cultured on BHI (Brain Heart Infusion) agar (Sigma-Aldrich) at pH 7.4 and 28 °C. Small round and transparent colonies appeared after 48 h of incubation. Colonies were checked for specific shape and Gram staining under light microscopy.

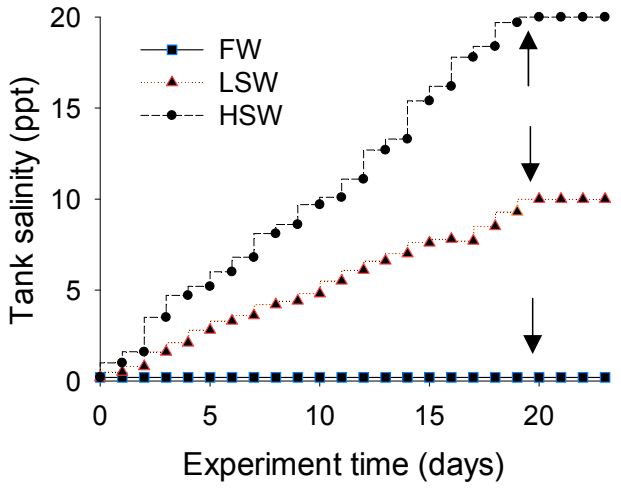

Fig. 1. Striped catfish salinity experiment regimens. Striped catfish were exposed (or not) to increasing salinity during 20 days in three independent recirculating systems. FW: Freshwater; LSW: Low saline water (0–10 ppt); SW: saline water (0–20 ppt). The arrows represent bacteria inoculation at day 20.

2.2.2. Bacteria count

Bacteria were first numbered in a reference bacteria solution with optical density of 0.1 at 590 nm. Serial exponential dilutions (100 to 10^{10}) of 100 µl of this reference bacteria solution were cultured in BHI agar during 48 h at 28 °C and the colonies were counted. A method of confirmation using DNA fluorochrome 4′-6′-diamidino-2-phenylindole (DAPI) was used. Serial exponential dilutions (10^4 à 10^8) were incubated 1 h in DAPI, filtered and counted on black filters under fluorescent microscopy. Based on these bacteria counts, it has been estimated that a reference *E. ictaluri* solution of optical density 0.1 cultured in BHI contained approximately 10^9 bacteria ml^{-1} solution.

2.2.3. Lethal dose 50% (LD 50) 96 h

In a preliminary experiment, exponential doses of bacteria suspended in HBSS were injected to fish using an inoculation volume of 0.025 ml g^{-1} fish (4 tanks, n = 6 per tank) in order to estimate the LD 50_{96h}. The results indicated that an injection of 0.025 ml g^{-1} fish of a bacterial solution containing 10^6 CFU ml^{-1} induced 50% mortality after 96 h.

2.2.4. Confirmation of infection

Using API and Biolog biochemical identification systems, infection was confirmed (100% probability for *E. ictaluri*) by the CER group (Belgian reference laboratory for animal health, Centre d'Economie Rurale-CER, Aye, Belgium), a laboratory specialized in screening and diagnosis of fish viral, bacterial and parasitic pathologies.

2.3. Osmoregulatory parameters

2.3.1. Gill Na^+K^+ ATPase activity

Gill lysates were obtained by homogenizing (1:5) tissue in ice cold SEI buffer (Sucrose 0.25 M, EDTA 1 mM, Imidazole 50 mM - pH 7.4) containing a protease inhibitor cocktail (Sigma) for 2 × 30 s using a sterile potter homogenizer. Main debris were removed by 2 successive centrifugations at 10 000 g during 5 min at 4 °C. An aliquot (50 µl) was used to measure the Na^+K^+ ATPase activity according to the method of Mc Cormick (1995) [21]. One unit of Na^+K^+ ATPase activity represents the consumption of 1 µmole NADH min^{-1} ml^{-1}. Analyses were performed in duplicates.

2.3.2. Plasma osmolality

Plasma osmolality (100 µl) was measured with a micro-osmometer (Type 6, Löser Messtechnik, Germany) in duplicates according to the depression of freezing point compared to pure water.

2.4. Hematology

2.4.1. Blood cell populations

Blood cells populations were analysed by flow cytometry (Flow Activated Cell Sorter Calibur, Flow Cytometry System) according to Inoue et al. (2002) [22a] (Fig. 2). Briefly, 10 µl of fresh heparinized blood were mixed with 1950 µl of HBSS and 40 µl of fluorochrome $DiOC_6$ (3,3-dihexyloxacarbocyanine, Sigma-Aldrich, Germany) diluted 1:10 in ethanol. The tube was mixed gently and incubated at RT (room temperature) during 10 min. The FACS was calibrated with TrueCount Beads diluted in HBSS. Each blood cell population was identified by its typical location in a FL-1 v. SSC and FSC v. SSC according to Inoue et al. (2002) [22a] and Pierrard et al. (2012) [22b] (Fig. 2). Five clusters were identified including erythrocytes, thrombocytes and lymphocytes, monocytes, eosinophils and heterophils. In order to validate this method, differential blood counts were previously done in 20 fish in light microscopy. Regarding to erythrocytes, blood was diluted 200× in HBSS and erythrocytes were counted on Neubauer hemocytometer. Regarding to leukocytes, subpopulations were differentially counted on blood smear staining with May Grunwald Giemsa according to Vazquez et al. (2007) [23]. Fig. 3 shows blood cell populations identified in light microscopy (Fig. 3). Flow cytometer analyses were in agreement with microscopic counts.

2.4.2. Hematocrit

Fresh heparinized blood was centrifuged in microhematocrit tubes at 10 000 g during 5 min at RT.

2.5. Immune parameters

2.5.1. Plasma lysozyme assay

The lysozyme activity protocol was adapted from Ellis et al. (1990) [24]. Briefly, lysozyme activity assay was initiated by mixing 10 µl of kidney homogenate or 10 µl of plasma with 250 µl of lyophilized *Micrococcus lysodeikticus* (Sigma) suspension (0.6 mg ml^{-1} in phosphate buffer at pH 6.2). The difference in absorbance at 450 nm was monitored between 0 min and 15 min (linearity range). One unit of lysozyme activity represents the amount of lysozyme causing a 0.001 decrease in absorbance per minute. Samples were measured in duplicates.

2.5.2. Plasma alternative complement pathway

The alternative complement pathway was assayed in duplicates according to Sunyer and Tort (1995) [25]. Briefly, 10 µl of rabbit red blood cells suspension (RRBC, Biomerieux) suspended at 3% in veronal buffer (Biomerieux) were mixed with serial dilutions of plasma. After incubation for 2 h at 28 °C, the samples were centrifuged (2000 g, 5 min, 4 °C). A positive control sample (100% haemolysis) was obtained by adding 60 µl of distillate water to 10 µl of RRBC. A negative control sample was obtained by adding 60 µl of veronal buffer to 10 µl of RRBC. The absorbance of supernatant was measured at 405 nm. The ACH 50 value is defined as the reciprocal of the plasma dilution which induces 50% haemolysis of RRBC.

2.5.3. Spleen respiratory burst

Spleen respiratory burst was adapted from Rook et al. (1985) [26]. Using the back of a syringe piston, spleen tissues were gently mashed with 1 ml of L-15 medium through a 100 µm nylon mesh

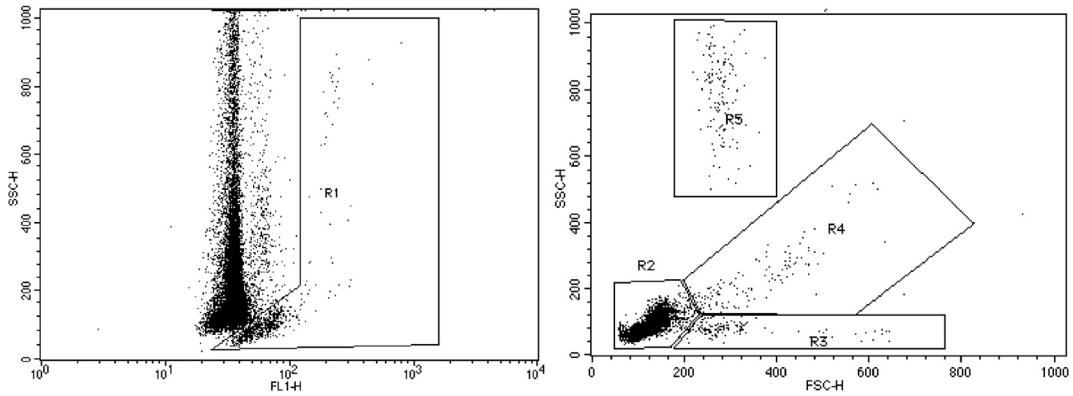

Fig. 2. FACS analyses of blood cells of striped catfish stained with DiOC$_6$. (A) FL1 as a function of SSC, erythrocytes and isolation of leukocytes population (R1) (B) FSC as a function of SSC (R2: thrombocytes and lymphocytes; R3: monocytes; R4: eosinophils; R5: heterophils).

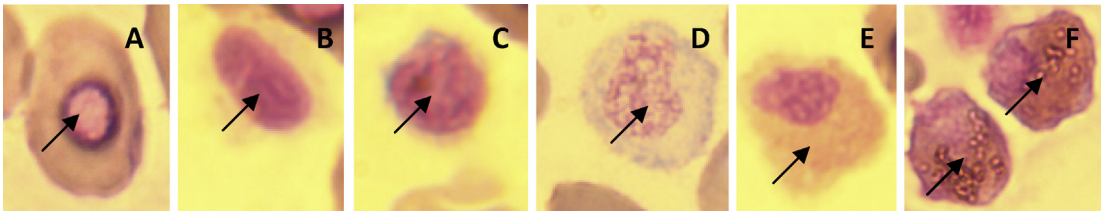

Fig. 3. Blood smear of striped catfish stained by May Grunwald Giemsa. (A) erythrocytes; (B) thrombocyte; (C) lymphocyte; (D): monocyte; (E) eosinophil; (F) heterophils. Arrow indicates the corresponding cell.

grid settled at the bottom of a Petri dish. The cell suspension was washed twice and 100 µl of the final cell suspension were incubated in duplicates with 100 µl of nitroblue tetrazolium (1 mg ml^{-1} in PBS, pH 7.4) during 1 h at RT. Then, samples were washed in PBS, methanol and finally air-dried at RT. The blue formazan in each tube was dissolved in 240 µl of KOH 2 M and 280 µl of N-dimethylformamide and absorbance was measured at 550 nm. Negative control samples were not incubated but directly brought to methanol fixation step. A standard curve was performed using serial dilutions of nitrobluetetrazolium directly dissolved in KOH 2M and N-dimethylformamide.

2.5.4. Kidney HSP70 and HMGB-1

2.5.4.1. Kidney lysates. Using Speed Mill Vac Bound Homogenizer, kidney lysates were obtained by homogenizing kidney tissue for 2 × 30 s in the following buffer (1:3): Tris-HCl 50 mM, NaCl 150 mM, SDS 0.1%, Triton X-100 0.1%, apopritin 0.001 mg ml^{-1}, pH 8. Lysates were then sonicated 3 × 10 s at 45 kHz and 5 × 1 s at 65 kHz on ice and centrifuged at 10 000 g for 10 min to remove main debris. Total protein abundance in the samples was measured by Pierce method.

2.5.4.2. Blotting. Western blot analyses were performed to validate the specificity of the antibodies. So, 20 µg of kidney lysate proteins were mixed with dithiothreitol 0.5 M 0.1% and NuPage Lithium Dodecyl Sulfate (1:3) and heated at 70 °C for 7 min. Samples were then centrifuged at 13 000 g for 5 min to remove debris. Chemiluminescent ladder (ECL DualVue Marker, Amersham) and 20 µg of kidney proteins were separated on a 4–12% NuPage Novex Bis Tris gel during 1h30 at 100 V and transferred onto a PVDF (polyvinylidene difluoride) membrane using an electrophoretic transfer system during 2h15 at 0.8 mA cm^{-2} (BioRad). Then the membrane was blocked overnight with PBST (PBS pH 7.4, 0.1% Tween-20) containing 5% skimmed milk at 4 °C. The next day, the membrane was rinsed 2 × 5 min in PBST and probed with anti-HSP70 3A3 (ThermoScientific) 1:5000 diluted in 2% blocking buffer for 1 h at RT under constant agitation. After washing 2 × 5 min with PBST, membrane was incubated with horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody (GE Healthcare) 1:10 000 diluted in 2% blocking buffer for 1 h at RT. Membrane was rinsed 2 × 10 min in PBST, 20 min in PBS and revealed with ECL Plus Western Blotting substrate (Thermo Scientific) in ChemiDoc MP Imaging System (BioRad). The same procedure was applied for HMGB-1 western blotting except that the PVDF membrane was probed with anti-HMGB-1 19N15F4 (ThermoScientific) 1:500 diluted in 2% blocking buffer for 1 h at RT as a primary antibody. Quantification was performed by dot blotting. Proteins extract (1 µl) was directly spotted to the wetted PVDF membrane and allowed to dry out during 30 min at RT. PVDF membrane was then transferred in a blocking solution (PBST, 5% skimmed milk) during 1 h at RT and probed in the same manner than for western blotting. Dot quantification was done using Image J software.

2.6. Statistical analyses

Heterogeneity of variances was tested by Levene test and normality was checked by Shapiro-Wilk test. The changes in physiological, osmoregulatory and immune parameters were analysed by two-way analysis of variance ANOVA followed with pairwise multiple comparisons procedures by Scheffe test ($p < 0.05$) in SigmaStat. Data are represented as the mean ± SD and tanks were used as the statistical unit ($n = 4$).

3. Results

3.1. Growth performance

The feed intake was significantly higher in fish held in

freshwater (23.6 ± 2.2 g fish^{-1}) compared to fish held in low saline (17.3 ± 1.3 g fish^{-1}) and saline (18.2 ± 2.5 g fish^{-1}) water. The weight gain ((final weight − initial weight)/initial weight × 100) and the specific growth rate (ln (final weight) − ln (initial weight)/time × 100) did not significantly vary between groups and averaged respectively 25.7 ± 1.2% and 1.1 ± 0.03%. Fulton's coefficient (K) (100 × weight/length$^{2.88}$) was calculated on each sampling day (days 0, 10, 20 and 23) and averaged 1.04 ± 0.05. K was significantly lower on day 23 compared with day 0, 10 and 20 ($p < 0.001$).

3.2. Autopsy

Autopsies were performed on day 20 (n = 6 fish per treatment) in collaboration with the CER laboratory. In the group exposed to saline water (0–20 ppt), 100% of the fish were suffering from high congestions on fins and tail as well as on the membranous flap of skin of the opercula (Table 1). Only 15% of the fish were suffering from congestion of barbels and abdomen. No symptoms were observed on the skin mucosal fluidity neither on gill integrity (fluidity, lamellar integrity and vascularisation). Dissection of mouth and ocular structures revealed no macroscopic damages or lesions. No ulcers or lesions were observed on the organs. During infection, all fish showed typical clinical signs of ESC (Enteric Septicaemia of Catfish) including decrease in gill vascularisation, abdominal septicaemia, high congestion of eyes, fins and tail, white gills and nodular round lesions (1–3 mm) on kidney, spleen and, to a smaller extent, on liver.

3.3. Osmoregulatory response

The osmoregulatory response of striped catfish to increasing salinity was investigated through plasma osmolality (Fig. 4, A) and gill Na^+K^+ ATPase (Fig. 4, B). In freshwater, plasma osmolality was comprised between 254 and 271 mosm. With increasing salinities, plasma osmolality gradually increased to reach 288 ± 6 mosm at 10 ppt and 370 ± 3 mosm at 20 ppt on day 20 ($p < 0.001$). The bacterial infection did not induce significant changes in plasma osmolality. Prior to infection, gill Na^+K^+ ATPase activity was comprised between 0.27 and 0.75 U mg^{-1} gill min^{-1} in freshwater and low saline water respectively. At 20 ppt on day 20, activity increased up to 1.68 ± 0.32 U mg^{-1} gill min^{-1} ($p < 0.05$). During infection, gill Na^+K^+ ATPase activity significantly decreased from 0.69 U mg^{-1} gill min^{-1} in freshwater to 0.38 at 20 ppt ($p < 0.001$).

3.4. Hematology

Salinity did not induce any significant effect on the number of erythrocytes and hematocrit (Table 2). Before infection, the abundance of heterophils increased with salinity, peaking at 29.3 million cells µl^{-1} blood at 20 ppt, which corresponds to a level 4 fold higher than in freshwater and low saline water ($p < 0.01$) (Table 2). During infection, fish also exhibited significant changes in their blood cell populations (Table 2). The number of thrombocytes/lymphocytes significantly decreased by one third on day 23 in all groups compared with day 0, 10 and 20 ($p < 0.001$). Moreover, increase in salinity also significantly lowered the number of thrombocytes/lymphocytes from 124.8 million cells µl^{-1} blood in freshwater to 82.53 million cells µl^{-1} blood in saline water ($p < 0.01$). As the decrease is higher than the proportion of lymphocytes in the cluster, as suggested by microscopic stain, thrombocytes may be, at least partly, responsible for the drop of the cluster in cytometry. The number of eosinophils and heterophils significantly increased during infection (day 23) compared with day 0, 10 and 20 but was not significantly affected by salinity ($p < 0.01$). During infection, the number of monocytes was enhanced with salinity by two fold to reach 30.7 million cells µl^{-1} blood.

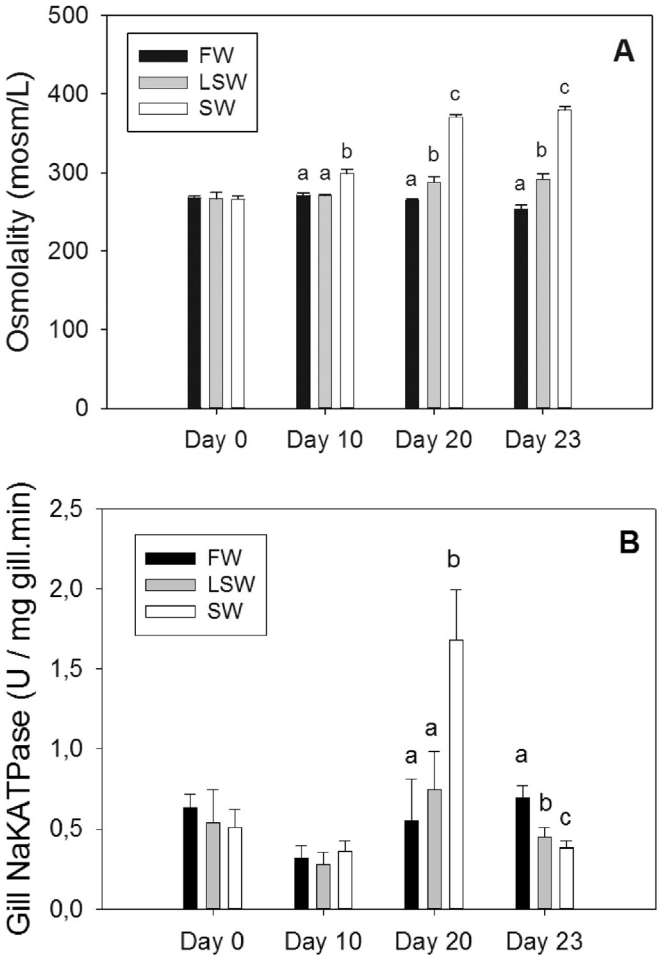

Fig. 4. Osmoregulatory responses of striped catfish exposed (or not) to elevated salinity and experimentally infected with *Edwardsiella ictaluri*. Plasma osmolality (A) and gill Na^+K^+ ATPase activity (B) of striped catfish exposed to freshwater (FW, black), low saline water (LSW, grey) and saline water gradient (SW, white) during 20 days and 3 days post-infection (day 23). The values were presented as the mean ± SD with n = 4 (4 tanks, 6 fish/tank). The statistical letters (a,b,c) indicate a significant change ($p < 0.05$) between salinity treatments on the same sampling day.

Table 1
Autopsy of striped catfish exposed to increasing salinity and macroscopic symptoms of ESC disease after 20 days of exposure to salinity increase (n = 6 fish/treatment).

	FW	LSW	SW	Infected
Congestions of fins and tail	−	−	+	++
Congestion of opercula	−	−	+	++
Congestion of barbels and eyes	−	−	+	++
Diffuse abdominal congestion	−	−	+	++
Mucosal fluidity (skin)	+	+	+	+
Gill integrity (mucus, vascularisation)	+	+	+	−
Cutaneous petechial haemorrhages	−	−	−	+
Lesion or ulceration in organs	−	−	−	+
Abdominal septicaemia	−	−	−	+

Freshwater (FW), low saline water (LSW, 0–10 ppt) and saline water (SW, 0–20 ppt). −/+: absence/presence; +/++: comparative gravity of the lesions.

3.5. Immune parameters

Salinity significantly enhanced plasma lysozyme activity, alternative complement pathway and spleen respiratory burst. The

Table 2
Hematology of striped catfish exposed (or not) to elevated salinity and experimentally infected with *Edwardsiella ictaluri*. The letters (a, b) indicate a significant change ($p < 0.05$) between salinity treatment on a same sampling day.

		FW	LSW	SW
Erythrocytes (million cells μl^{-1} blood)	Day 0	3.84 ± 0.20	4.10 ± 0.20	4.09 ± 0.40
	Day 10	3.85 ± 0.09	3.81 ± 0.12	4.15 ± 0.28
	Day 20	4.08 ± 0.18	3.87 ± 0.36	4.36 ± 0.15
	Day 23	4.37 ± 0.13	4.13 ± 0.27	4.6 ± 0.22
Hematocrit (%)	Day 0	37.6 ± 1.59	33.67 ± 1.44	33.60 ± 2.51
	Day 10	33.67 ± 1.28	31.54 ± 1.56	33.20 ± 2.12
	Day 20	36.93 ± 1.94	35.78 ± 1.60	36.62 ± 1.77
	Day 23	32.29 ± 1.17	32.17 ± 1.05	31.90 ± 2.38
Thrombocytes and lymphocytes (thousand cells μl^{-1} blood)	Day 0	171.3 ± 23	170.9 ± 38.6	152.8 ± 17.5
	Day 10	167.7 ± 32.9	167.5 ± 7.3	164.8 ± 11.6
	Day 20	153.3 ± 8.3	171.0 ± 16.1	149.9 ± 29.4
	Day 23	**124.8 ± 15.2**^{a}	**109.4 ± 24.8** ^{ab}	**82.53 ± 11.9** ^{b}
Monocytes (thousand cells μl^{-1} blood)	Day 0	18.1 ± 13	7.3 ± 1.9	10.2 ± 8.2
	Day 10	7.3 ± 5.4	13.1 ± 9.1	6.8 ± 4.6
	Day 20	39.3 ± 19.2	16.8 ± 11.1	14.6 ± 6.0
	Day 23	**17.4 ± 2.3**^{ab}	**13.4 ± 4.9**^{a}	**30.7 ± 8.9** ^{b}
Eosinophils (thousand cells μl^{-1} blood)	Day 0	7.6 ± 2.6	9.0 ± 1.9	8.9 ± 8.4
	Day 10	7.8 ± 1.9	7.5 ± 1.4	5.2 ± 0.7
	Day 20	7.6 ± 3.4	5.4 ± 1.4	6.9 ± 0.7
	Day 23	8.7 ± 0.9	11.0 ± 7.2	12.9 ± 1.6
Heterophils (thousand cells μl^{-1} blood)	Day 0	5.4 ± 2.4	6.2 ± 4.5	4.1 ± 2.1
	Day 10	3.3 ± 3.0	1.8 ± 1.1	3.2 ± 1.0
	Day 20	**7.2 ± 3.7**^{a}	**4.6 ± 1.7**^{a}	**29.3 ± 6.3** ^{b}
	Day 23	17.8 ± 4.4	11.3 ± 9.0	11.9 ± 5.1

Freshwater (FW), low saline water (LSW) and saline water (SW) during 20 days and 3 days post-infection (day 23). Values presented as the mean ± SD with n = 4 (4 tanks, 6 fish/tank). Significant results in bold. The statistical letters (a,b) indicate a significant change ($p < 0.05$) between salinity treatments.

strongest significant effect was observed on plasma lysozyme activity which gradually but significantly increased with elevating salinity ($p < 0.001$) (Fig. 5 A). On day 0, lysozyme activity varied between 194 and 219 U ml^{-1} plasma. On day 10, lysozyme activity peaked at 264 U ml^{-1} plasma in fish held in saline water compared to 217 U ml^{-1} plasma in fish held in low saline water and 199 U ml^{-1} plasma in fish kept in freshwater. On day 20, it reached 519 and 346 U ml^{-1} plasma respectively in fish of high and low saline water groups while it averaged 268 U ml^{-1} plasma in freshwater fish. During the bacterial challenge, lysozyme activity significantly increased by 3-fold compared to values observed in fish prior to infection ($p < 0.001$) and the stimulating effect of

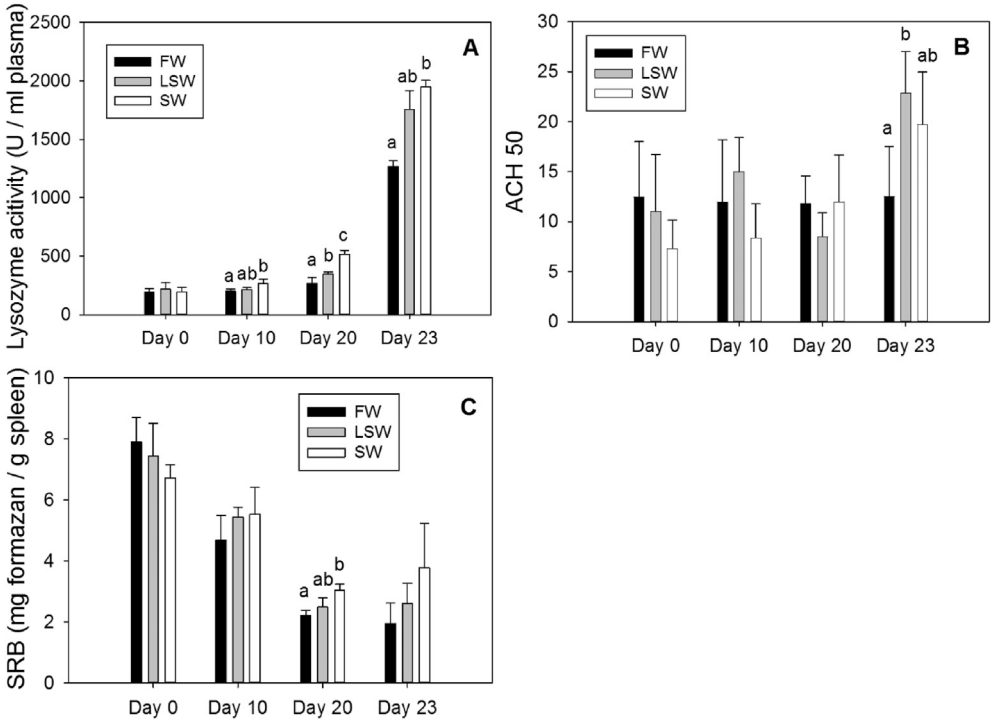

Fig. 5. Immune response of striped catfish exposed (or not) to elevated salinity and experimentally infected with *Edwardsiella ictaluri*. Plasma lysozyme activity (A), alternative complement activity (B) and spleen respiratory burst (C) of striped catfish exposed to freshwater (FW, black), low saline water (LSW, grey) and saline water (SW, white) during 20 days and 3 days post-infection (day 23). Values were presented as the mean ± SD with n = 4 (4 tanks, 6 fish/tank). The statistical letters (a,b,c) indicate a significant change ($p < 0.05$) between salinity treatments on a same sampling day.

salinity was maintained ($p < 0.001$). Indeed, in freshwater condition lysozyme activity averaged 1268 U ml^{-1} plasma in freshwater fish, 1756 U ml^{-1} plasma in fish from low saline water and 1948 U ml^{-1} plasma those from saline water. No significant effects of salinity on the alternative complement activity, which varied between 7.3 and 12.5 U ml^{-1} plasma, were observed in fish prior to infection (Fig. 5 B). However, ACH50 (Alternative Complement Hydrolysis 50%) significantly increased in infected fish (day 23), particularly in low saline water and saline water ($p < 0.001$). ACH50 response was significantly higher during bacterial infection in fish from low saline water (12.5 ± 5.0 U ml^{-1} plasma) compared to freshwater fish (22.8 ± 4.2 U ml^{-1} plasma) ($p < 0.01$). Spleen respiratory burst significantly decreased up to 4 fold during the experiment in all groups ($p < 0.001$) (Fig. 5 C). On day 20, respiratory burst significantly increased up to 3.0 ± 0.2 mg formazan g^{-1} spleen in fish held in saline water compared to those held in freshwater (2.2 ± 0.2 mg formazan g^{-1} spleen) ($p < 0.001$).

On the opposite, salinity did not induce any significant changes in the abundance of HSP70 and HMGB-1 in kidney. Relative to its abundance in freshwater fish from day 0, production of HSP70 varied from 0.46 to 1.63 fold on days 10, 20 and 23 (Fig. 6 A). However, production of HMGB-1 was significantly higher in infected fish (day 23) compared to days 0, 10 and 20 ($p < 0.001$). Relative production of HMGB-1 varied from 0.60 to 1.96 fold in fish prior to injection and increased 2.80–5.94 fold in infected fish, compared with the abundance of HMGB-1 in freshwater on day 0 (Fig. 6 B).

3.6. Sensitivity to Edwardsiella ictaluri

Fig. 7 shows cumulative mortalities during 10 days after bacteria inoculation in the three salinity groups. At 72 h post inoculation, mortalities were significantly higher in saline water (67 ± 14%) compared to freshwater (0 ± 0%) and saline water (13 ± 16%) ($p < 0.05$). At 96 h post inoculation, mortalities begun in fish held in freshwater (42 ± 10%) but were significantly lower than those recorded in saline water (75 ± 10%) ($p < 0.05$). On day 5, cumulative mortality was significantly higher in saline water (92 ± 10%) compared to low saline water (67 ± 14%). No additional mortalities were recorded from day 5 and up to day 10 post inoculation in the three salinity groups.

4. Discussion

Plasma osmolality values measured in this study were in the same range than those reported by other authors in striped catfish and other siluridae, differing by less than 10% [27,28]. Increase in salinity was responsible for elevated blood plasma osmolality while gill Na^+K^+ ATPase activity only increased at 20 ppt. When striped catfish were submitted to low saline stress up to 10 ppt (270 mosm), plasma osmolality equilibrated with external salinity. However, higher salinity up to 20 ppt (540 mosm) induced a significant increase in plasma osmolality although to a lower level than that of the environment (370 mosm ± 3 mosm). This indicated that gill electrolyte clearance is insufficient to cope with the increased salt load. Similarly, seawater survival of channel catfish is limited due to the absence of efficient electrolyte excretion. Transfer of channel catfish from freshwater to seawater induced an elevation in plasma osmolality while the activity of functional renal glomeruli decreased [27,29]. In the current study, infection subsequent to salinity exposure lowered gill Na^+K^+ ATPase activity whereas plasma osmolarities remained unchanged. As live *E. ictaluri* gain access to blood circulation system across the gills, decrease in gill vascularisation is frequent in infected fish (including striped catfish in this study) and may be responsible for gill ion transport collapse [30,31].

In saline water (20 ppt), striped catfish juveniles suffered from high congestions throughout the body. Prolonged high osmotic pressure in blood vessels may damage the endothelium and induce

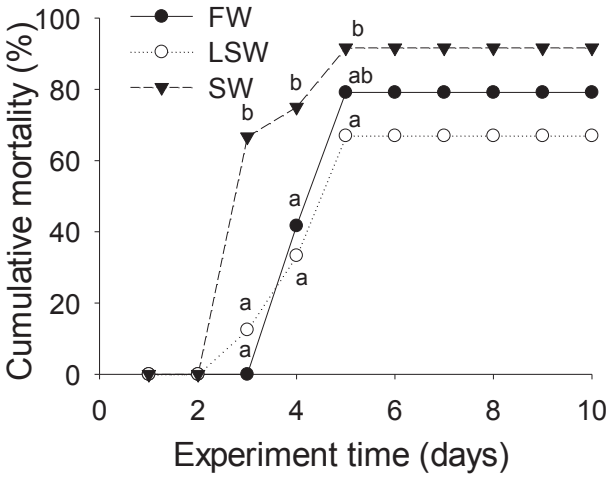

Fig. 7. Cumulative mortality of striped catfish exposed (or not) to elevated salinity during 10 days after inoculation of *Edwardsiella ictaluri*. Mean cumulative mortality of striped catfish exposed to freshwater (FW), low saline water (LSW) and saline water (SW) during 10 days. The values were represented as the mean cumulative mortality (4 tanks, 6 fish/tank).

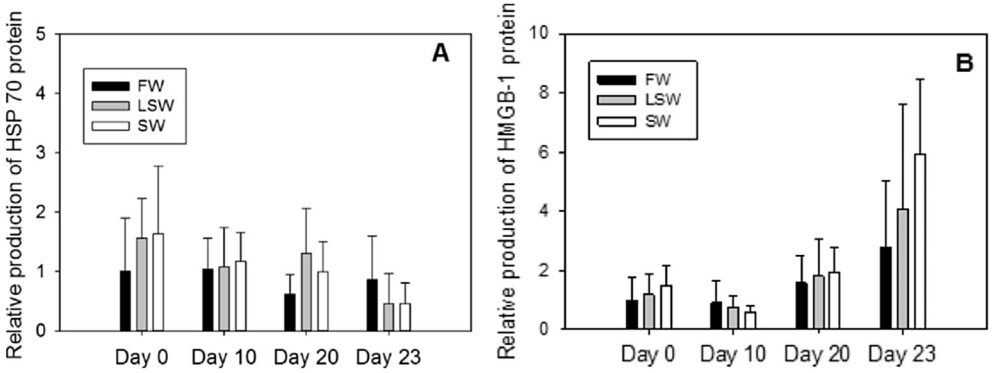

Fig. 6. Chaperone response of striped catfish exposed (or not) to elevated salinity and experimentally infected with *Edwardsiella ictaluri*. Heat Shock Protein 70 (A) and High-Mobility Group protein B1 (B) of striped catfish exposed to freshwater (FW, black), low saline water (LSW, grey) and saline water (SW, white) during 20 days and 3 days post-infection (day 23). Values were presented as the mean ± SD with n = 4 (4 tanks, 6 fish/tank).

sterile inflammation. Particularly, high congestions have been observed on the opercular membrane, a membranous flap of skin located along the edge of the opercula that might play key role in resistance to hypoxia [32]. Whether increased osmotic pressure in the opercula may impair the resistance of striped catfish to hypoxia is unknown but should be investigated, as oxygen levels in typical Vietnamese aquacultures are inferior to 5 kPa [33].

Blood composition and abundance of immune cells were modified by salinity in both healthy and infected fish. Prior to infection, the abundance of heterophils in the circulation of fish held in saline water increased by nearly 4 fold. During ESC, the abundance of macrophages increased by 2 fold in fish held in saline water while the abundance of thrombocytes was severely and gradually depleted. In this study, increase in monocytes and heterophils during hyperosmotic stress in healthy and infected fish respectively may indicate that the increased osmotic pressure is perceived as a threat to body integrity. In addition, the abundance of granulocytes (i.e. eosinophils and heterophils) increased in infected fish compared to the abundance before infection. Therefore, both stressors (infection and salinity) were responsible for accumulation of blood inflammatory cells including monocytes, eosinophils and heterophils. During prolonged cellular stress, it is known that persistence of such inflammatory cells may lead to excessive immune reaction and tissue damage [19]. On the contrary, salinity and infection had a synergic negative effect on the number of thrombocytes. In fish, decrease in the abundance of circulating thrombocytes is a frequent observation during pathogenic infection [34,35] while the effects of salinity on thrombocytes have not been investigated yet. In infected fish, the increased blood osmotic pressure may activate the coagulation system in response to blood vessel damage and decrease the number of free circulating thrombocytes. Although their implication in hemostasis and wound healing is well-studied, the role of thrombocytes in immune functions is often neglected. Due to their extensively high number in the circulation, thrombocytes may play a major role in inflammatory processes and immunity. In fish, thrombocytes express a high number of immune-relevant genes involved in inflammation and antigen presentation, have the capacity to phagocytose live bacteria and foreign particles and may have bactericidal activity [34–36].

In the present study, innate immune components were stimulated with increasing salinities or in combination with bacterial challenge. Plasma lysozyme activity of freshwater fish are in accordance with those of Sirimanapong et al. (2014) [37] and differ by less than 10%. Salinity and infection rapidly and gradually stimulated lysozyme activity. In fish, higher lysozyme activities during both acute and chronic hyperosmotic stress have also been described in euryhaline species [5,6,38,39]. In this study, ACH50 values of freshwater fish are within the same range of values than those measured by Hang et al. (2013) [40] on the same species. ACH 50 values increased during infection. In addition, a combination stimulatory effect of elevated salinity and infection was observed. In fish, variations of ACH50 during an osmotic stress have been poorly investigated. In gilthead seabream *Sparus aurata*, it has been shown that complement activities may increase or decrease when salinity increase, depending on acclimation time [4]. The respiratory burst was within the same range of values those already observed for other species [41,42]. A decrease in respiratory burst along the experiment have been observed and may be caused by insufficient acclimation time prior to the onset of the experiment. Our results suggest that salinity might stimulate spleen respiratory burst in striped catfish during hyperosmotic stress, similarly to the responses observed in several euryhaline species [5,43], but such modulation was not markedly affected by bacterial challenge. During bacterial infection, excessive respiratory burst induced by the persistence of immune cells such as activated granulocytes and macrophages may lead to oxidative stress and tissue injury if not adequately countered by antioxidant activities [19].

In our experiment, salinity stressor induced earlier onset of mortality in striped catfish during ESC. Moreover, cumulative mortalities after 10 days were significantly higher in fish held in saline water (92%) compared to fish held in low saline water (67%). In grouper fish, acute osmotic shock during 48 h (33 ppt–20 or 40 ppt) increased susceptibility to birnavirus from 10 to 90% [7]. Therefore, high salinity stressor may increase sensitivity to ESC. At the opposite, low salinity water may have a protective effect in striped catfish by preventing bacteria multiplication.

In this study, HSP70 level remained unchanged with saline gradient alone the experiment. During acute or chronic hyperosmotic stress, increase in branchial HSP70 expression has been documented in several euryhaline species [43–45] while HSP70 modulation in other tissues remained unclear. In silver seabream acclimated to a large range of salinities, expression of HSP70 multigene family remained unchanged in kidney [43]. Similarly to HSP70, no significant changes were observed in HMGB1 level during salinity exposure whereas HMGB1 level increased in infected fish compared to no infected fish. In addition, following infection, a non-significant increase was observed with salinity. In mammals, active secretion of HMGB1 mainly occurs when immunologically competent cells are exposed to pathogen or microbial associated molecular patterns [46,47]. On the contrary, sterile injury did not induce HMGB1 production but only leaded to passive release of inactive HMGB1 [46,47]. In the present study, it might be possible that sterile tissue damages induced by the hyperosmotic pressure did not induce active overproduction of HSP70 and HMGB1 but that passive extracellular release of HSP70 and HMGB1 occurred, making available some inflammatory mediators. Further, high standard deviation may reflect difference in HSP70 and HMGB1 response depending on individual fish susceptibility to the pathogen.

5. Conclusion

The present study demonstrated that salinity alone or salinity associated to infection increased some immune functions (i.e. lysozyme activity, complement activity, respiratory burst, abundance of monocytes and heterophils). Nevertheless, increase in immune factors does not necessarily indicate higher resistance to microbial disease, as suggested by the higher mortality rate in saline water. Chronic increase in the osmolality of body fluids may result in the persistence of sterile inflammatory processes such as granulocytes and macrophages accumulations, respiratory burst, release of inflammatory mediators and proteases. Therefore, it can be expected that such excessive inflammatory response may lead to immune exhaustion and that the resulting tissue damages may disrupt basal immune homeostasis, thereby creating an unfavourable environment for efficient immune defence in case of pathogen contamination.

In conclusion, these novel results show the importance of the multi-stress approach in fish. To a larger extent, salt is commonly used as an antiseptic to prevent and treat microbial diseases in fish farms. In striped catfish ponds, production under moderate salinities has already been suggested in order to prevent microbial contamination. However, salt sterilization process targeting halophile pathogens should be managed carefully in order to avoid prolonged exposure of fish to hyperosmotic conditions which might be responsible for immune defence impairment.

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