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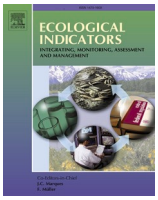

Influence of extreme ambient cold stress on growth, hematological, antioxidants, and immune responses in European seabass, *Dicentrarchus labrax* acclimatized at different salinities

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ABSTRACT

Climate change-driven extreme climatic events are expected to challenge ectotherms' physiological tolerance. The hemato-physiological modulation potentials of fish during ambient extreme-cold events at various salinities are poorly studied. In this study, we evaluated the growth, hemato-physiological, antioxidants, and immunological responses of European seabass, *Dicentrarchus labrax* acclimatized at 30, 12, 6, and 3 PSU followed by an extreme ambient cold (8 °C) exposure for 20 days. Juveniles acclimatized at 30 and 3 PSU showed significantly low growth performance ($p < 0.05$). Red blood cells (RBC) count, hematocrit, hemoglobin, and serum protein content were decreased in 3 and 30 PSU fish. In contrast, significantly higher white blood cells (WBC) count, skin mucus cortisol, different types of erythrocytic cellular abnormalities (ECA), and erythrocytic nuclear abnormalities (ENA) were observed in 30 and 3 PSU fish. Also, higher activities of serum antioxidants [superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), glutathione reductase (GR)], and upregulation of tumor necrosis factor α (TNF-α) in the spleen were observed in 3 and 30 PSU fish. In addition, on day 20, phagocytic respiratory burst (RB) and serum lysozyme activities (LSZ) were significantly higher in 3 and 30 PSU fish during extreme cold exposure. None of the repeatedly evaluated parameters indicated acclimation capacity to cope with tested salinities during cold exposure. However, taken together, our results indicate that *Dicentrarchus labrax* acclimatized at intermediary salinities (6 and 12 PSU) can perform comparatively better during ambient extreme-cold exposure (8 °C).

1. Introduction

Global climate change is a grievous and growing threat to natural systems and their species. Specifically, aquatic environments are harshly affected by the alterations in temperature and precipitation patterns, which affect water quality parameters including temperature and salinity (Cline et al., 2020; O'Gorman et al., 2016; Poff, 2002; Sharma et al., 2015; van Vliet et al., 2013). In recent decades, temperature and precipitation patterns have changed noticeably at global and regional scales (Jensen et al., 2015; Tsuzuki et al., 2007) and are predicted to occur more intensely and frequently than ever before (Fischer et al., 2013; García-Herrera et al., 2010; Pfahl et al., 2017). These predicted trends are consistent with observed extreme climate events (Donat et al., 2016; Fischer et al., 2013; Fischer and Knutti, 2014; Galappaththi et al., 2020; Webster, 2020). Southern Europe and the Mediterranean region have witnessed extreme temperatures and precipitation events (Diodato et al., 2019; López-Moreno et al., 2017; Philandras et al., 2011; Seager et al., 2019). The Mediterranean region, located in the transition zone between the dry subtropical and wet European mid-latitude climate, is very sensitive to deviations from the global mean climate state (Barcikowska et al., 2018; Gao et al., 2006). Over the Mediterranean Basin, decreased frequency of low-medium-intensity precipitation is projected to intensify extreme precipitation (Polade et al., 2017). Despite a decrease in mean precipitation during winter, drastic heavy rainfall is predicted to further increase across the Mediterranean (Barcikowska et al., 2018; Sen et al., 2019). In addition, extreme winter precipitation events are predicted to intensify (Coppens et al., 2020; Kotsias et al., 2020) with more intense cold events (abrupt temperature drops,

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snowfall, rain and sleet events) (Bech et al., 2013; Collados-Lara et al., 2018; Diodato et al., 2019; Fayad et al., 2017; Pérez-Palazón et al., 2018).

Climate change is expected to challenge ectotherms' ability to function optimally outside of their scope of tolerance (Kyprianou et al., 2010; Pörtner and Peck, 2010) by modifying water quality and hydrological dynamics (Klein et al., 2017; Ruaro et al., 2019; Zafalon-Silva et al., 2017). Climate change-induced temperature and salinity fluctuations are anticipated to affect teleost's physiological functions in tropics and temperate regions (Carney Almroth et al., 2019; Rosa et al., 2014; Watson et al., 2018; Webster, 2020). This is specifically true for coastal environments including aquaculture systems (Carney Almroth et al., 2019; Rosa and Marques, 2012; Vargas-Chacoff et al., 2020). Changes in water temperature impair growth, physiological fitness (Besson et al., 2016; Carney Almroth et al., 2019; Donaldson et al., 2008; Islam et al., 2020a; Yilmaz et al., 2020), and metabolism (Mateus et al., 2017; Nadermann et al., 2019; Richard et al., 2016). Thermal stress also impairs hydromineral balance (Christensen et al., 2017; Downie et al., 2018; Islam et al., 2020c; Vargas-Chacoff et al., 2020), hormonal regulation, stress, and immune responses (Bly and Clem, 1992; Burgerhout et al., 2019; Phuc et al., 2017), and antioxidant responses (Feidantsis et al., 2020; Madeira et al., 2016; Martínez-Álvarez et al., 2005) in fish. Salinity changes also cause impairment in fish osmoregulation, endocrine responses, physiological fitness (Islam et al., 2020b; Ma et al., 2020; Saillant et al., 2003; Vargas-Chacoff et al., 2019), development, growth (Bœuf and Payan, 2001; Han et al., 1995; Servili et al., 2020; Yilmaz et al., 2020), antioxidants (Birnie-Gauvin et al., 2017; Chowdhury and Saikia, 2020; Islam et al., 2020b; Lushchak, 2011) and immune responses (Bowden, 2008; Fries, 1986; Makrinos and Bowden, 2016). Combined stress from temperature and salinity impairs growth, physiological, metabolic, hormonal, and immune responses (Bento et al., 2016; Masroor et al., 2019, 2018; Qiang et al., 2013) and beyond the (species-specific) preferred window are likely to result in excessive fish suffering (Gamperl et al., 2020; Islam et al., 2020b, 2020c; Musa et al., 2017; Phuc et al., 2017). Fish are less likely to obtain relief from temperature and osmotic stress (Antonopoulou et al., 2020; Campos et al., 2019; Evans and Kültz, 2020; Portner and Farrell, 2008), or combinations thereof (Evans and Kültz, 2020; Islam et al., 2020b; Masroor et al., 2019; Phuc et al., 2017) through physiological and behavioral adaptations (Feidantsis et al., 2020, 2018; Portner and Farrell, 2008).

Hematological parameters, antioxidant, immune responses, and cytokines genes are considered significant stress biomarkers for organisms exposed to thermal and osmotic stress, pollutants, and genotoxic agents (Gomes et al., 2015; Islam et al., 2019, 2020b; Roche and Bogé, 1996; Val et al., 2015; Witeska, 2013). Blood cells count, erythrocytic cellular-nuclear abnormalities, hematocrit, and hemoglobin, among others, are considered significant (Ashaf-Ud-Doulah et al., 2019; Islam et al., 2020c; Jahan et al., 2019; Sulikowski et al., 2003). Serum glucose, lactate, and antioxidants [glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione reductase (GR), catalase (CAT)] are also reported as important thermal and osmotic stress indicators in fish (Cossins, 1977; Islam et al., 2020c; Madeira et al., 2016). Different blood cell types such as ECA (echinocytic shape, elongated, teardrop, fusion, and twin); and ENA (micronucleus, binuclei, blebbed, notched, bud, and bridge) have been reported as thermal and osmotic stress biomarkers for fish (Avrova, 1999; Cossins, 1977; Gracey et al., 2004; Islam et al., 2020c; Jahan et al., 2019). Tumor necrosis factor (TNF-α) an important pro-inflammatory cytokine (He et al., 2017; Shen et al., 2018; Yada and Tort, 2016) acts as an important indicator of inflammation, immune responses (He et al., 2017; Rymuszka and Adaszek, 2012; Shen et al., 2018; Yada and Tort, 2016) and blood cell peripheral abnormalities for fish during osmotic and thermal stress (Islam et al., 2020b; Pettersen et al., 2005; Uribe et al., 2011). Cellular-metabolic viability and turnover, enzymatic antioxidants, and cytokine biomarkers can reveal the impacts of climate change-induced cold events in fish (Islam et al., 2020c; Val et al., 2015).

European seabass, *Dicentrarchus labrax* is a popular marine aquaculture fish that spend their first year in shallow coastal water (Crespel et al., 2017; Claridge and Potter, 1983). In nature, European seabass hatch in the open sea (30 to 38 PSU) during winter, and young-of-the-year seabass are found close to the coast (Varsamos et al., 2001). Despite euryhaline nature and having well-developed osmoregulatory systems, for aquaculture practices, the ideal salinity of this fish at their juvenile stage ranges from 12 PSU to 25 PSU (Kokou et al., 2019; Saillant et al., 2003). Beyond this salinity range, the growth and physiological fitness of this teleost are impaired (Hwang et al., 2018; Islam et al., 2020b; Masroor et al., 2019; Yilmaz et al., 2020). Although this fish can survive in a wide range of temperatures from 8 °C to 32 °C (Islam et al., 2020b, 2020a; Islam et al.2020c;; Kousoulaki et al., 2015; Maulvault et al., 2017), but is sensitive to temperatures below 16 °C and over 25 °C (Islam et al., 2020a, 2020c; Claridge and Potter, 1983). The critical thermal minimum (CTmin) temperature of European seabass is 6.7 °C (Dülger et al., 2012). During extreme winter events, some parts of the Mediterranean coastal water temperature can decrease to 5.4–10 °C (Aranda et al., 2005; Besson et al., 2016; Dufour et al., 2009; Giorgi and Lionello, 2008; Llorente and Luna, 2013; Sen et al., 2019), which can result in lower growth, survival rate, and physiological impairment (Dülger et al., 2012; Islam et al., 2020a; Yilmaz et al., 2020). Alongside cold events increased winter heavy precipitation, predicted to intensify more in the future at the Mediterranean coast, could drop water salinity below 12 PSU (Bech et al., 2013; Coppens et al., 2020; Diodato et al., 2019; Pérez-Palazón et al., 2018).

To date, the literature contains research on European seabass related to temperature, salinity preferences (Dülger et al., 2012; Masroor et al., 2019, 2018; Saillant et al., 2003), growth and nutritional management (reviewed in Oliva-Teles, 2000; Kousoulaki et al., 2015), physiological, immune responses (reviewed by Chistiakov et al., 2007). Metabolic, physiological, and molecular responses at altered salinities and temperatures have also been documented (Enes et al., 2006; Islam et al., 2020c; Saillant et al., 2003). European seabass exposed to 8 °C and 32 PSU salinity for 30 days adjusted its physiology to cold exposure within the first 20 days of exposure and remained almost the same afterward (Islam et al., 2020a, 2020c). Still, there is little information on the responses when these fish are exposed to ambient extreme-cold events in winter months preceded by heavy precipitations-driven hyposaline water (Dülger et al., 2012; Rosa et al., 2012; Sarà et al., 2018). Information is lacking on fish response when temperatures and salinities fall below 10 °C and 15 PSU, respectively during intense precipitation and cold events in winter (Bento et al., 2016; Dülger et al., 2012; Person-Le Ruyet et al., 2004; Yilmaz et al., 2020). The combination of hemato-physiological, cellular, immunological, and molecular responses of this fish facing extreme ambient cold and hyposaline events have not yet been studied comprehensively. Thus, the current study intended to compare growth performance, selected fundamental hemato-physiological, immunological, and molecular biomarkers responses of European seabass before being exposed to a range of environmentally realistic hyposaline water regimes following an ambient extreme cold thermal (8 °C) stress exposure. This study hypothesized that European seabass is fully functional within the optimal salinities and thermal window, but could encounter varying physiological challenges outside of their preferred salinities during ambient extreme cold events. The current study experimentally tested this hypothesis by evaluating a range of hemato-physiological parameters. Growth performance was evaluated at different salinities to reveal individual fitness (Angilletta, 2009; Jin et al., 2015; Li et al., 2015) while cellular, immunological, and molecular responses were observed to examine physiological fitness (Islam et al., 2020b; Madeira et al., 2013; Vinagre et al., 2012) during ambient extreme-ambient cold exposure.

2. Materials and methods

2.1. Experimental animals and system design

The experiment took place at Alfred Wegener Institute for Polar and Marine Research (AWI), Bremerhaven, Germany. The full experimental design has been depicted in the supplementary file-1 (Fig. S1). Juveniles European seabass (24.8 ± 1.69 g) were obtained from Les Poissons du Soleil marine hatchery, France. Before starting the experiment, fish were acclimatized to laboratory conditions for three weeks in a 2000 L tank at 22 °C and 30 PSU. Then, a total of 192 fish of similar sizes were randomly distributed into 12 (4 treatments × 3 replicates) fiberglass tanks (100 L, 22 °C, and 30 PSU) with 16 individuals per tank. After 21 days, fish were gradually exposed to four experimental salinities (30, 12, 6, and 3 PSU) at 22 °C. The required water salinity was achieved by a reduction of 5 PSU every 3 days until the chosen salinity was reached (Islam et al., 2020c). All experimental salinity groups had been acclimatized to the experimentally preferred salinity for 45 days. Following this, fish of all salinities were exposed to 8 °C for 20 days. To mimic an extreme ambient cold event, the temperature was decreased at the rate of 4.5 °C day^{-1} to reach 8 °C rapidly from 22 °C (acclimation temperature). Three consecutive days were required to reach the desired temperature. Intended temperature was reduced in the morning (9.00) of each day of the required three days. The 20 days of exposure to the extreme cold event was counted once the water temperature reached at 8 °C. Throughout the experiment, the fish were reared to a 10:14 h light–dark photoperiod regime. The fish were fed twice a day (at 09.00 and 16.00) with a commercial pellet (Alltech Coppens, Netherlands) containing 54% protein (please see Supplementary file-2, Table S 1 for nutritional composition). Each replicate tank was connected separately to a recirculatory water system fitted with a biofilter, UV light sterilization facilities, protein skimmers to ensure required water quality parameters were maintained. The desired temperature was maintained with a temperature sensor (Inkbird, Germany) mounted with thermostatic cooling (Titan, Aqua Medic, Germany) and heating systems (Titanium, Aqua Medic, Germany). Also, the whole experiment system was placed in a climate-controlled room to minimize errors. Any uneaten feed and fish feces were removed daily, and 50–60% water (with appropriate salinity and temperature) was exchanged to minimize ammonia nitrates and nitrites accumulation. This experiment was approved by the AWI laboratory animal ethics committee and based on EU Directive 2010/63/EU for animal experiments.

2.2. Sampling

During the ambient extreme-cold temperature stress period, on days 1, 10, and 20, three fish were sampled randomly from each of the replicate tank (9 individuals from each saline water treatment; n = 9); hereafter mentioned as Day 1, Day 10 and Day 20 group, respectively (Supplementary file 1, Fig. S1). Prior to sampling, fish were starved for 24 h. Immediately before the sampling fish were compassionately euthanized with MS 222 (50 mg L^{-1}) on collection from the rearing tank. After recording length and weight, blood samples were withdrawn through caudal puncture with sterile plastic syringes rinsed with EDTA anticoagulant (20 mM). Full blood sampling was completed within 4 min of anesthesia in order to minimize and avoid stress impacts in the blood (Islam et al., 2020b). Approximately, 100 μL of fresh blood was stored in EDTA rinsed microfuge tubes for blood cell counting. Besides, additional blood was withdrawn from the replicate tank's fish; pooled and centrifuged (15 min, 4000*g* at 4 °C) to get the adequate amount of serum and stored at −80 °C until analyses. Skin mucus was collected for cortisol analysis following the method described by Guardiola et al. (2014). Briefly, skin mucus was collected by gently scraping the dorsolateral surface of fish. Collected mucus samples were homogenized in 500 μL sterile water and vigorously mixed. Then the supernatant was collected after centrifugation at 2000*g*, 10 min, 4 °C, and preserved at −80 °C until use (Guardiola et al., 2016). Fish used to collect blood were dissected to collect liver, spleen, and viscera and weighed to calculate somatic performance. Spleen samples were stored at −80 °C for analysis later.

2.3. Evaluation of growth and somatic outcomes

At the experimental beginning and onset of the ambient extreme-cold exposure, all fish were counted, measured, and weighed. The mean individual weight of each experimental unit (tank) was calculated by dividing the bulk weight by the total number of fish. The following equations were used to evaluate growth and somatic performance, and nutrient utilization efficiency.

$$\text{Feed intake (FI) (g fish}^{-1}\text{ days}^{-1}) = \frac{\text{(dry feed applied)}}{\text{number of individuals}}$$

$$\text{Weight gain(WG, \%)} = \frac{\text{mean final body weight} - \text{mean initial body weight}}{\text{mean initial body weight}} \times 100$$

$$\text{Specific growth rate (SGR, \%day}^{-1}) = \frac{\{\ln(\text{mean final body weight}) - \ln(\text{mean initial body weight})\}}{\text{duration of the trial}} \times 100$$

$$\text{Protein efficiency ratio (PER, \%)} = \frac{\text{weight gain(g)}}{\text{total protein intake(g)}} \times 100$$

$$\text{Conditionfactor(CF, \%)} = \frac{\text{weight gain (g)}}{(\text{fish body length})^3}$$

$$\text{Survival (\%)} = \frac{\text{number of fish survived}}{\text{the initial number of fish stocked}} \times 100$$

$$\text{Hepatosomatic index(HSI, \%)} = \frac{\text{weight of liver}}{\text{weightoffish}} \times 100$$

$$\text{Viscerasomatic index}(\text{VSI},\%) = \frac{\text{fish viscera weight}}{\text{weight of fish}} \times 100$$

$$\text{Intestine somatic index (ISI},\%) = \frac{\text{weight of intestine}}{\text{weight of fish}} \times 100$$

$$\text{Spleen somatic index (SSI},\%) = \frac{\text{fish spleen weight}}{\text{weight of fish}} \times 100$$

2.4. Hemato-physiological parameters

2.4.1. Hematological and skin mucus cortisol analysis

RBC and WBC were counted with a flow cytometer (BD Accuri™ C6, UK) following the direct volumetric method. Before measuring experimental samples, instrument performance validation and fluidics calibration were performed. With the original samples, validation checks and optimization trials were conducted to choose the exact dilution. Finally, dilution of whole blood was carried out at a 1:4 ratio with PBS as the stock solution. For whole blood cell counts, 10 μL of diluted blood, and 990 μL of PBS was used during flow cytometry. Besides, RBC and WBC dilution media (Himedia, India) were used to dilute samples. Briefly, 5 μL of whole fresh blood was mixed in 990 μL of RBC diluent and 195 μL of WBC diluent to acquire 200x and 40x dilution respectively. The stained diluted sample was inverted six times before analysis to ensure homogeneity. Samples were run at a medium flow rate (<3000 events s^{-1}) until 100 μL samples suctioned; with the SC-H threshold of 50,000 to exclude debris and electronic noise (Supplementary file-1, Fig. S 2). Blood cell counting (10^6 mL^{-1}) was performed by the direct volumetric method. A traditional blood cell count was also performed using a Neubauer Hemocytometer to validate the results from flow cytometry. Blood hematocrit was estimated in triplicate through centrifugation of blood in microhematocrit capillaries at 7500 rpm for 5 min. Hemoglobin content was measured (in triplicate) spectrophotometrically at 540 nm, with an extinction coefficient of 11.01 $mmol^{-1}$ cm^{-1} (Islam et al., 2020b; Steucke and Schoettger, 1967). Serum protein was assessed spectrophotometrically using a blood analyzer machine (Fuji Dri Chem NX500i, Japan). Mucus cortisol was measured with a cortisol analysis kit (IBL International, Germany) following the manufacturer's instructions used in previous studies (Islam et al., 2020c, 2020d).

2.4.2. Estimation of erythrocytic nuclear abnormalities (ENA) and erythrocytic cellular abnormalities (ECA)

Immediately after collection, a blood drop was smeared on a glass slide and left for 10 min to dry. Afterward, slides were cleaned and fixed with 99% ethanol. After 20 min, ethanol cleaned slides were stained by dipping in Giemsa stain solution (5%) for 12 min, then washed through running distilled water and left overnight to dry. Slides were mounted with DPX medium and stored at ambient temperature until analysis. For each fish, three slides were prepared; 2000 cells for each slide were counted at ×40 magnification. Thus, for each tested salinity group, around 18,000 cells were examined to check ENA and ECA. The blind scoring method was applied to code slides randomly to avoid biases and technical variation (Islam et al., 2020b). ENA and ECA were classified and scored according to Carrasco et al. (1990). Briefly, ENA were classified as follows: (i) binucleated: cell with two nuclei, (ii) micronuclei: small chromatin like object at the peripheral part of the cell, (iii) notch nuclei: looks like nucleus but do not have nuclear materials, (iv) nuclear bud: evagination of bud-like structure from the nucleus, and (v) blebbed nuclei: small euchromatin evagination of the nuclear membrane. Whereas, ECA were categorized as (i) echinocytic: cell surface consists of serrated boundaries despite uniform cell size and shape, (ii) elongated: noticeable longer than the surrounding cells, (iii) tear-drop: deformed erythrocyte and narrowed to nipple shape at one end, (iv) fusion: two (more) cells fused and, (v) twin: two cells surface-attached and fused (Ashaf-Ud-Doulah et al., 2019; Islam et al., 2020b).

2.5. Antioxidant enzyme analyses

2.5.1. Glutathione peroxidase (GPx) and glutathione reductase (GR) assays

GPx and GR activities were measured following Flohé and Günzler (1984) and Carlberg and Mannervik (1975). Briefly, serum samples were 10 times diluted with 100 mM PBS [2 mM Na_2-EDTA: pH 7.5]. Finally, GPx reaction mixture was: 100 mM PBS with [2 mM Na_2-EDTA (pH 7.5)], 2.4 UL^{-1} GR, 1 mM GSH, 0.15 mM NADPH (Sigma Aldrich, Germany) in 0.1% $NaHCO_3$ and 1.2 mM H_2O_2 (Sigma Aldrich, Germany). The GR test mixture finally contained: 100 mM PBS in 2 mM Na_2-EDTA (pH 7.5), 0.1 mM NADPH and 1.0 mM GSSG (Islam et al., 2020b). Sample protein content was determined according to Bradford (1976). Bovine γ-globulin (1.0 mg mL^1) commercial dye reagent (BioRad 600–0006) was used during protein content determination. Standard glutathione reductase (Sigma Aldrich, Germany) was used to define unit activity. Activities were expressed as nmol. min^{-1}.mg^{-1} of total protein.

2.5.2. Catalase (CAT)

The principle of the CAT assay method has been described in Claiborne (1985), adapted to the microplate. CAT was measured by following the consumption of the substrate (H_2O_2) measured at 240 nm with a 1 min 30 s time interval after adding H_2O_2. Briefly, serum samples were 10 times diluted in 50 mM PBS (pH 7.0). The final assay concentration was: 50 mM PBS and 10 mM H_2O_2 (pH 7.0) (Sigma Aldrich, Germany). Finally, CAT activity was calculated using the molar extinction coefficient of 40 $M^{-1}cm^{-1}$ (240 nm). Catalase standard (Sigma Aldrich, Germany) was used to measure unit activity. The activity has been expressed as nmol. min^{-1}.mg^{-1} of total protein.

2.5.3. Superoxide dismutase (SOD)

The SOD activity assay was carried out after following the protocol described by McCord and Fridovich (1969). SOD standard (Sigma Aldrich, Germany) was used to calculate unit activity. Activities were expressed as %inhibition.mg^{-1}.of total protein. Briefly, serum samples were diluted (10 times) in PBS [0.05 M, pH: 7.4]. The final reaction concentrations for SOD assay were: 100 μL PBS [0.05 M, pH: 7.4], cytochrome *C* [0.06 M], xanthine [0.14 mM], and xanthine oxidase [0.01 U mL^{-1}]. Finally, SOD activity was assessed at 550 nm for 10 min using a second-degree polynomial equation.

[(Abs_{550} min^{-1} negative control − Abs_{550} min^{-1} sample)/Abs_{550} min^{-1} negative control] × 100

2.6. Immunological analysis

2.6.1. Respiratory burst (RB)

Phagocytic respiratory burst (RB) was measured using bitrotetrazolium blue chloride (NBT) (Anderson and Siwicki, 1995; Hasan et al., 2019). Briefly, an equal volume of 0.2% NBT reagent (Sigma Aldrich, Germany) and a blood sample was mixed and incubated for 30 min at room temperature. Then 50 μL of the mixture was added to 1 mL N-N-dimethylformamide and centrifuged at 2000 g for 5 min. Optical density (OD) of the collected supernatant (800 μL) was measured spectrophotometrically at 540 nm (Tecan, Switzerland), where N-N-dimethylformamide was used as a blank.

2.6.2. Serum lysozyme activity (LSZ)

Serum lysozyme (LSZ) activity was measured using a turbidometric assay (Hultmark et al., 1980) adopted for a 96 well plate. Briefly, lyophilized *Micrococcus lysodeikticus* was dissolved (0.2 mg mL^{-1}) in PBS (pH 5.52). Then 180 μL *Micrococcus lysodeikticus* solution and 20 μL serum (10 times diluted with PBS, pH = 5.52) were added to each well. The absorbance was then measured spectrophotometrically (450 nm) at 0 and 30 min (Infinite M200, Tecan, Switzerland). A 0.001 min^{-1} reduction in absorbance was considered 1 unit of LSZ activity (Hasan et al., 2019).

Table 1
qPCR primer sequences, length, melting temperature, and calculated efficiency.

Gene	Primer sequence (5′-3′)		Base pair	Melting temperature	Efficiency	Accession No.	References
TNF-α	Forward:	GCCAAGCAAACAGCAGGAC	106	60 °C	103%	DQ 200,910	El Aamri et al. (2015)
	Reverse:	ACAGCGGATATGGACGGTG					
El-1α	Forward:	AGATGACCACGAGTCTCTGC	127	57 °C	98.6%	FM 019,753	Mitter et al. (2009)
	Reverse:	CTTGGGTGGGTCGTTCTTG					

2.7. Gene expression study

Spleen RNA extraction was carried out following the manufacturer's instructions (Monarch RNA Kit, USA). Briefly, spleen tissues were placed in a 0.5 mL RNA protection buffer. DNA-ase I was used to eliminating potential contamination of the gDNA. Total RNA extract was eluted in 50 μL of nuclease-free water preserved at −80 °C until analysis. RNA integrity was checked and confirmed on 1% TAE-agarose gels alongside a DNA Ladder. A 3 μg of total RNA extracted sample was reverse-transcribed into cDNA following the manufacturer's instructions (Reverse Transcription Kits, ThermoFisher Scientific). The qRT-PCR was performed according to the reported protocol for Elongation factor 1α (El 1α) and Tumor Necrosis Factor (TNF-α) (Islam et al., 2020b). For each sample, RT-PCR reactions were performed in triplicate, where sterile DEPC treated water was used as a negative control, and a confirmed RNA sample was used as the positive control. RT-PCR quantification was performed in a 96-well plate with a 25 μL master mix for each well after following the manufacturer's instruction (Roboklon, Germany). The real-time cycling protocol was as follows: initial denaturation at 95 °C; 3 min followed by 39 cycles of 15 s at 95 °C, 15 s at 60 °C, and 20 s 72 °C (Table 1). Melting curves (65–95 °C, 0.5 °C s^{-1}) were checked carefully to ensure PCR reaction without primer-dimers and a single peak dissociation curve. The relative mRNA expression was calculated by using the 2–ΔΔCt normalized relative expression (NRE) method, $NRE = 2^{(Ct\ Ref.\ gene - Ct\ Target\ gene)}$ (Kokou et al., 2019; Livak and Schmittgen, 2001; Yuan et al., 2006).

Table 2
Growth performance of European seabass acclimated at four salinities for 45 days followed by 20 days of cold temperature (8 °C) stress. *

Parameter	Salinities (PSU)				P-value
	30 PSU	12 PSU	6 PSU	3 PSU	
Initial body weight [IBW] (g)	24.53 ± 1.88[a]	24.03 ± 1.96[a]	24.28 ± 1.85[a]	24.24 ± 1.06[a]	0.68
Final body weight [FBW] (g)	40.93 ± 3.67[b]	43.37 ± 1.38[a]	49.64 ± 1.92[a]	36.62 ± 0.90[b]	<0.05
Feed intake [FI] (g $fish^{-1}$ 45 $days^{-1}$)	21.42 ± 1.13[b]	24.23 ± 0.38[a]	24.24 ± 0.17[a]	21.57 ± 1.69[b]	<0.05
Percent weight gain [WG] (%)	72.22 ± 5.63[b]	125.96 ± 5.34[a]	120.95 ± 6.08[a]	80.73 ± 5.22[b]	<0.05
Specific growth rate [SGR] (% day-1)	1.71 ± 0.36[b]	2.15 ± 0.26[a]	2.36 ± 0.14[a]	1.47 ± 0.11[b]	<0.05
Protein efficiency ratio [PER] (%)	1.55 ± 0.38[b]	1.63 ± 0.10[b]	2.03 ± 0.17[a]	1.13 ± 0.06[b]	<0.05
Condition factor [CF] (%)	0.51 ± 0.05[b]	0.60 ± 0.09[a]	0.65 ± 0.02[a]	0.53 ± 0.06[b]	<0.05
Survival rate [SUR] (%)	97.22 ± 0.98[b]	100.00 ± 0.00[a]	100.00 + 0.00[a]	97.93 ± 1.96[ab]	<0.05
Viscera somatic index [VSI][1]	11.93 ± 0.88[b]	13.68 ± 1.57[a]	13.19 ± 1.08[a]	11.59 ± 0.85[b]	<0.05
Intestine somatic index [ISI][1]	2.89 ± 0.47[b]	3.86 ± 0.63[a]	3.44 ± 0.15[a]	2.74 ± 0.76[b]	<0.05
Hepatosomatic index [HSI][1]	2.19 ± 0.44[b]	3.03 ± 0.12[a]	2.71 ± 0.11[a]	2.51 ± 0.22[b]	<0.05
Spleen somatic index [SSI][1]	0.07 ± 0.01[a]	0.09 ± 0.02[a]	0.08 ± 0.01[a]	0.08 ± 0.02[a]	<0.05

*Values are means of triplicate groups ± SD. Values followed by different superscripts in the same row are significantly different after Bonferroni's post-hoc test ($p < 0.05$). [1]Nine individuals were sampled for each salinity treatment and statistical analyses were performed based on the mean value of each replicate tank (n = 3 per treatment).

2.8. Statistical analysis

Data normality and homoscedasticity were confirmed by Kolmogorov-Smirnov and Levene's tests, respectively. Data were log-transformed when normality and homoscedasticity assumptions were violated. For all but growth data, one-way repeated measure mixed-model ANOVA followed by Bonferroni's post-hoc correction was applied to evaluate salinity impacts during ambient extreme-cold exposure. Growth performance was compared among treatments with a one-way ANOVA. Moreover, principal component analysis (PCA) and discriminant function analysis (DCA) were performed to obtain tested parameters' overall responses during ambient extreme-cold exposure. The first two components, PCA and DCA scores, were plotted as biplots. Differences in treatment means were considered when the probability value (p) was < 0.05.

3. Results

3.1. Fish growth and performance parameters

Significantly ($p < 0.05$) lower final body weight (FBW), feed intake (FI), weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), condition factor (CF), and survival rate (SUR) was observed in fish maintained at 3 PSU and 30 PSU water when compared to 12 and 6 PSU. Similarly, viscera somatic index (VSI), intestine somatic index (ISI), and hepatosomatic index (HSI) values were also significantly ($p < 0.05$) lower in 3 PSU and 30 PSU fish (Table 2).

3.2. Hemato-physiological and biochemical markers

3.2.1. Blood cells count

RBC counts ($\times 10^6$ mm^{-3}) decreased overall during cold stress exposure, but significantly more so ($p < 0.05$) in 30 and 3 PSU compared to fish in 12 and 6 PSU on days 1, 10, and 20 (Fig. 1 A). In contrast, WBC number increased significantly ($p < 0.05$) in 30 PSU and 3 PSU fish on days 1, 10, and 20. With the progression of cold exposure, increasing trends have been observed for all four salinities (Fig. 1*B*). Mean values, standard deviation (SD), and repeated measure ANOVA results of RBC and WBC are presented in full in the Supplementary file-2 (Table S 2).

3.2.2. Hematocrit, mucus cortisol, hemoglobin, and serum protein contents

On day 1, mucus cortisol was significantly ($p < 0.05$) higher in 12 PSU and 6 PSU fish. In contrast, on day 20, the values were significantly ($p < 0.05$) higher in 30 PSU and 3 PSU fish (Fig. 2 A). Hematocrit value was significantly ($p < 0.05$) reduced in 30 PSU and 3 PSU fish compared to 12 PSU and 6 PSU fish on days 1, 10, and 20 (Fig. 2*B*). Hemoglobin contents have been significantly ($p < 0.05$) lower in 30 PSU and 3 PSU fish during the ambient extreme-cold exposure (Fig. 2*C*). For serum protein content, the value was significantly ($p < 0.05$) lower in 30 PSU and 3 PSU fish than 12 PSU and 6 PSU fish on days 1 and 10, whereas on day 20, serum protein content was found almost similar in all four saline water groups (Fig. 2 D). Decreased trends have been found for hematocrit, cortisol, hemoglobin, and protein content with the duration of ambient extreme-cold exposure (Fig. 2). Mean values, standard deviation (SD), and repeated measure ANOVA results of these measure

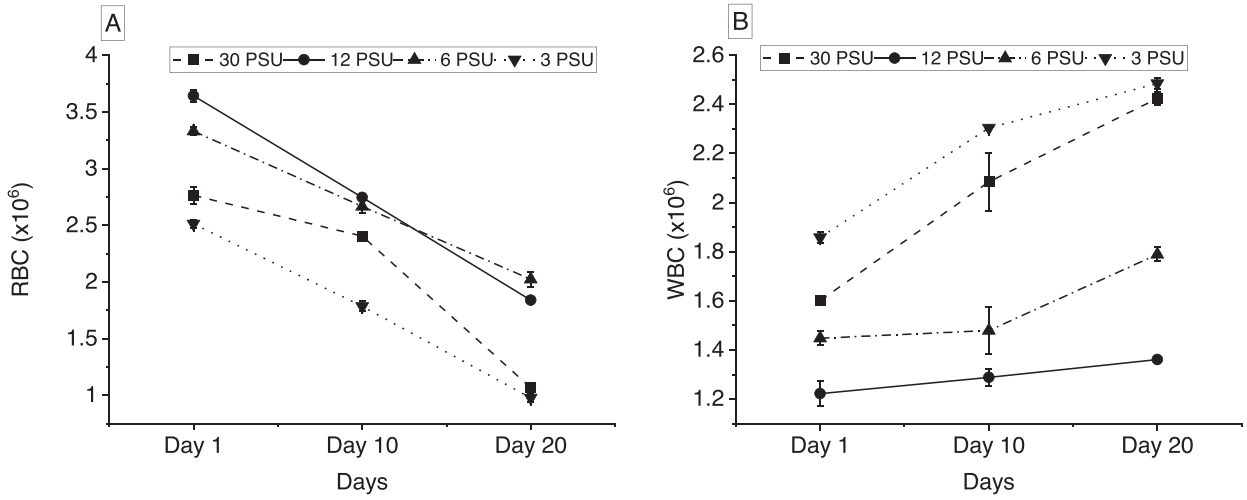

Fig. 1. Changes in A) red blood cell counts (RBC), and B) white blood cell counts (WBC) in juvenile European seabass on days 1, 10, and 20 of extreme ambient cold temperature (8 °C) prior to being acclimatized at four different salinities. On each sampling day, nine individuals were sampled for each salinity group. Values represent mean ± SD.

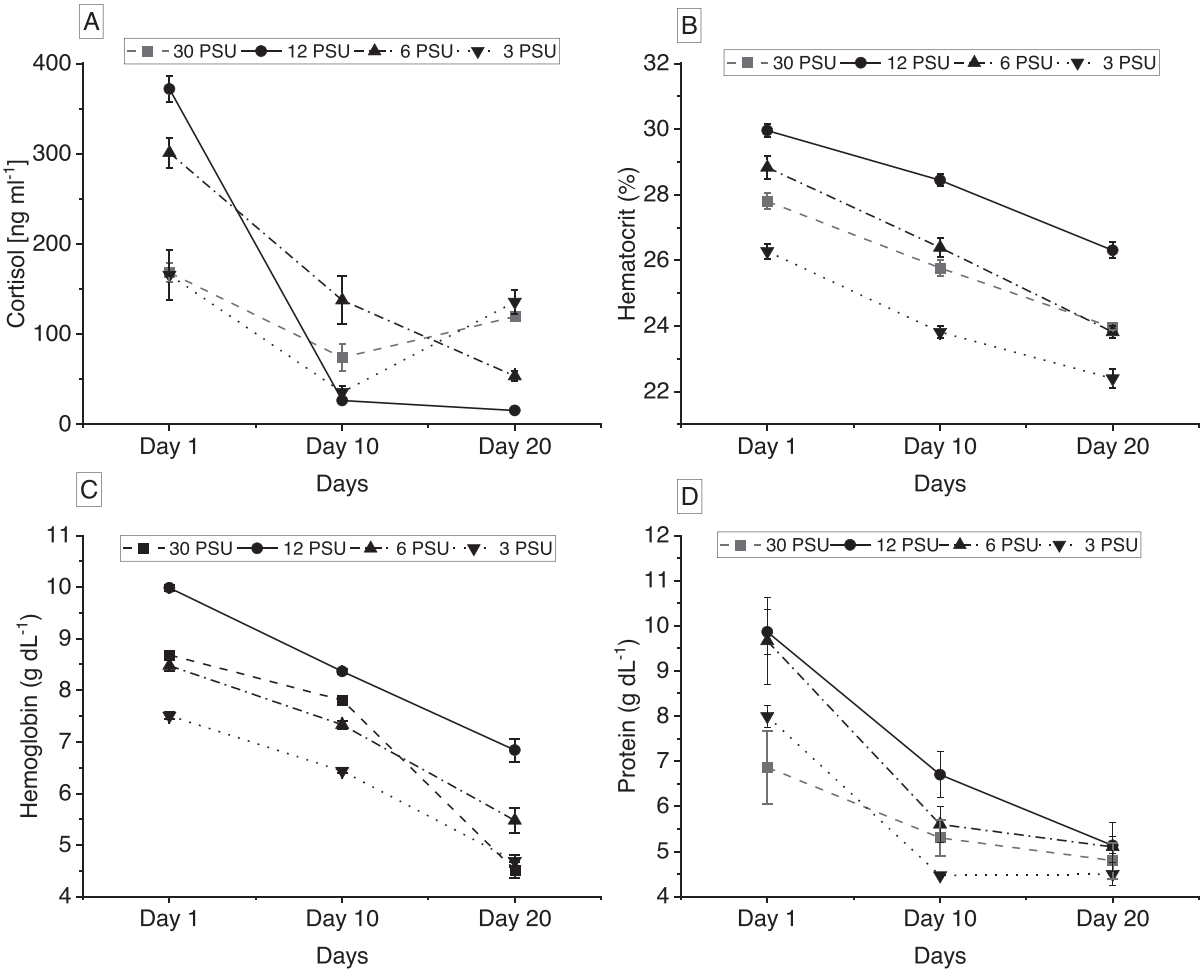

Fig. 2. Changes in A) Mucus cortisol, B) Hematocrit, C) Hemoglobin and D) Plasma protein in juvenile European seabass on days 1, 10, and 20 of extreme ambient cold temperature (8 °C) prior being acclimatized at four different salinities. On each sampling day, nine individuals were sampled for each salinity group. Values represent mean ± SD.

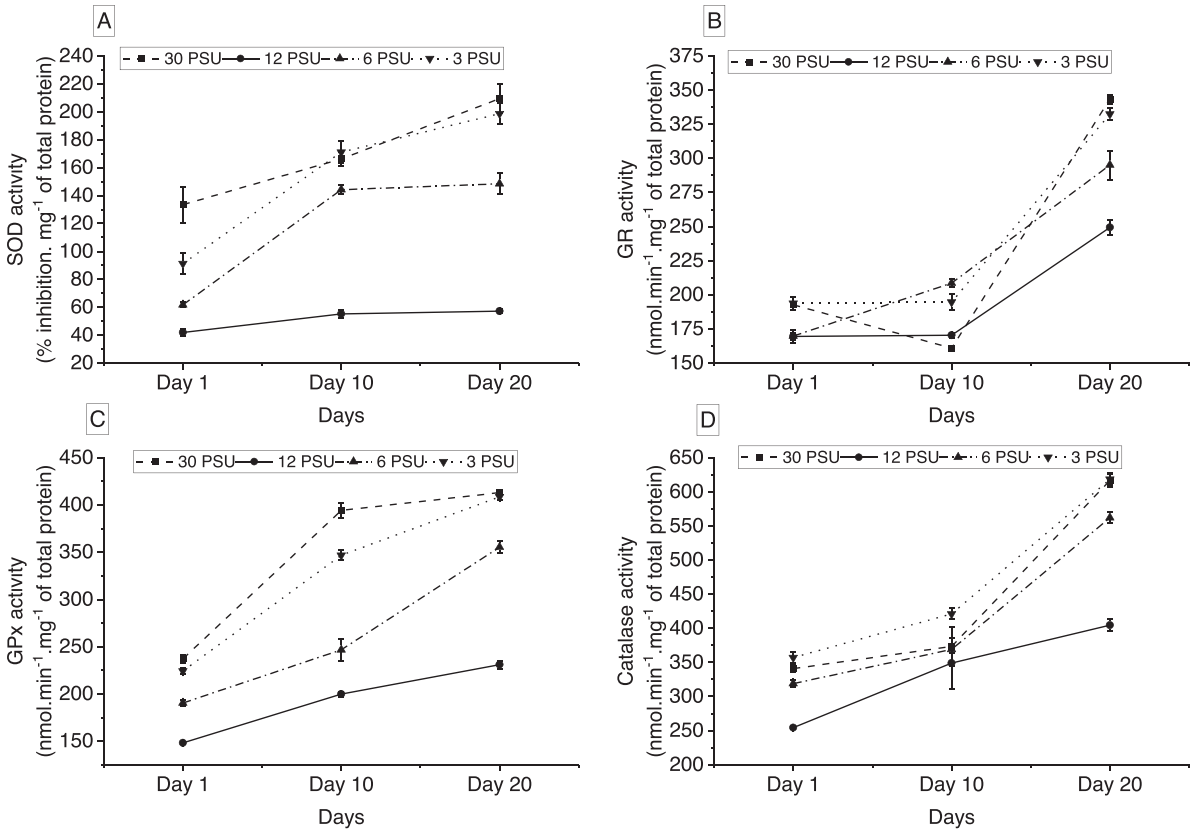

Fig. 3. Changes in serum antioxidants A) Superoxide dismutase (SOD), B) Glutathione reductase (GR), C) Glutathione peroxidase (GPx), and D) Catalase activity in juvenile European seabass on days 1, 10, and 20 of extreme ambient cold temperature (8 °C) prior being acclimatized at four different salinities. On each sampling day, nine individuals were sampled for each salinity group. Values represent mean ± SD.

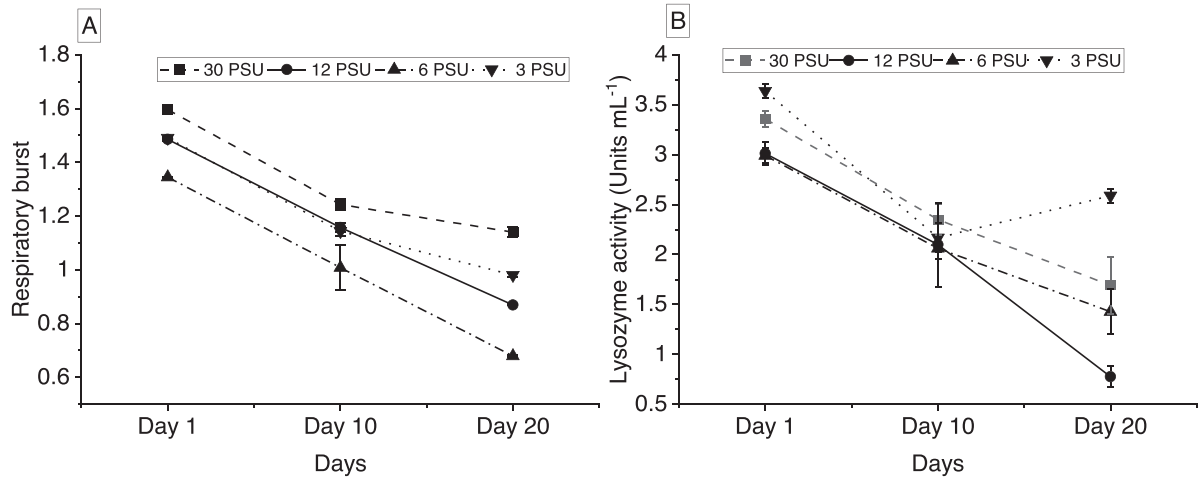

Fig. 4. Changes in A) Respiratory burst (RB) and B) Lysozyme activity in juvenile European seabass on days 1, 10, and 20 of extreme ambient cold temperature (8 °C) prior to being acclimatized at four different salinities. On each sampling day, nine individuals were sampled for each salinity group. Values represent mean ± SD.

parameters are reported in the Supplementary file-2 (Table S 2).

3.2.3. *Serum antioxidant activities*

During tested cold exposure, on days 1, 10, and 20, SOD activity in serum were significantly ($p < 0.05$) higher in fish acclimatized at 30 PSU and 3 PSU fish than 12 PSU and 6 PSU fish (Fig. 3 A). In the case of GR activity, significantly ($p < 0.05$) lower values were measured in fish acclimatized at 30 PSU and 3 PSU on days 1 and 20 (Fig. 3*B*). Whereas for GPx, significantly higher activity was observed in 30 PSU and 3 PSU fish on days 1, 10, and 20 of ambient extreme-cold exposure (Fig. 3*C*). For serum catalase, on days 1, 10, and 20 significantly ($p < 0.05$) higher activity was found in 30 PSU and 3 PSU fish (Fig. 3 D). During the extreme ambient cold exposure period, increased trends were observed for SOD, GR GPx, and catalase activities (Fig. 3 A, B, C, D). Mean values,

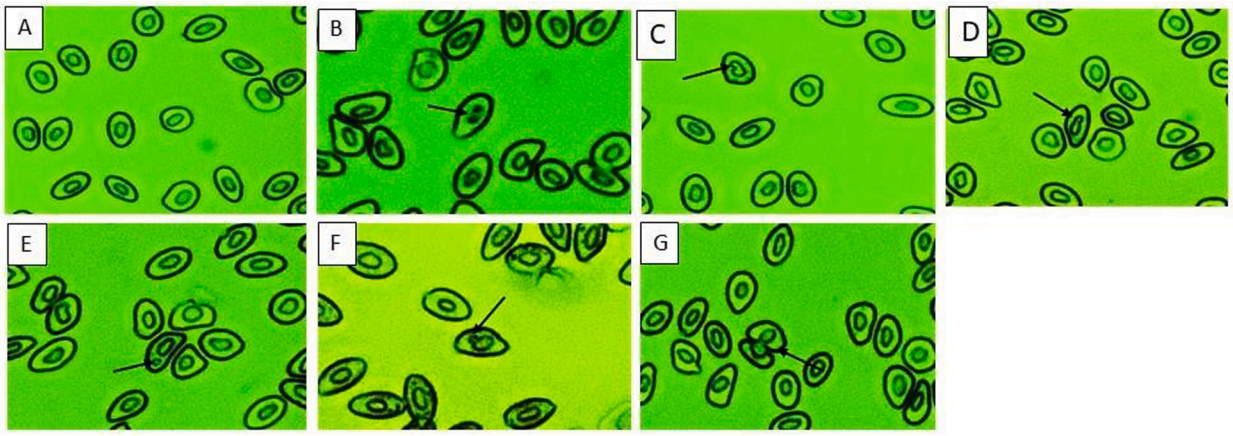

Fig. 5. Erythrocyte nuclear abnormalities (ENA) observed with Giemsa stained blood smears in juvenile European seabass during extreme ambient cold temperature (8 °C) prior to being acclimatized at four different salinities. A) Regular cell, B) Binuclei (%), C) Notch nuclei (%), and D) Micronuclei (%), E) Blebbed (%), F) Nuclear bud (%), and G) Nuclear-bridge (%).

standard deviation (SD), and repeated measure ANOVA results of SOD, GR, GPx, and catalase activities are presented in the supplementary file-2 (Table S 3).

3.2.4. Respiratory burst and serum lysozyme activities

On days 1 and 10, phagocytic respiratory burst (RB) activity was found to increase significantly in 30 PSU and 3 PSU fish, while on day 20, the activity was significantly higher ($p < 0.05$) in 30 PSU fish (Fig. 4 A). In the case of lysozyme activity (LSZ), the values were low in 12 PSU and 6 PSU fish on day 1, whereas, on day 20, the RB was significantly higher in 3 PSU fish (Fig. 4B). For both RB and LSZ activities, decreasing trends were observed during ambient extreme-cold exposure (Fig. 4 A, B). Mean values, SD, and repeated measure ANOVA results of these immunological parameters are presented in the Supplementary file-2 (Table S 3).

3.2.5. Erythrocytic nuclear abnormalities (ENA)

Several types of ENA, such as binuclei (Fig. 5B), blebbed (Fig. 5C), notched-nuclei (Fig. 5 D), micronucleus (Fig. 5 E), nuclear bud (Fig. 5F), nuclear bridge (Fig. 5 G) were observed in 30 PSU, 12 PSU, 6 PSU, and 3 PSU acclimatized fish during ambient extreme-cold exposure (8 °C). ENA types significantly ($p < 0.05$) increased in 30 PSU and 3 PSU fish on days 1, 10, and 20 (Fig. 6 A–F). An increasing trend was found for the mentioned ENA types during cold exposure (Fig. 6). ENA frequencies were significantly ($p < 0.05$) higher on day 20 compared to days 1 and 10. ENA types were significantly affected by salinity and extreme ambient cold exposure. Mean values, SD, and one-way mixed model repeated measure ANOVA results of observed ENA are reported in supplementary file-2 (Table S 4).

3.2.6. Erythrocytic cellular abnormalities (ECA)

ECA types found in the different saline water acclimatized fish during ambient extreme-cold acclimation were echinocytic (Fig. 7B), tear-drop shaped (Fig. 7C), fusion (Fig. 7D), elongated (Fig. 7E), and twin (Fig. 7F). Similar to ENA, statistically significant increases ($p < 0.05$) in ECAs were found in fish during ambient extreme-cold (8 °C) exposure (Fig. 8A-E). On day 20, ECA counts were significantly ($p < 0.05$) higher than on days 1 and 10. ECA frequencies tended to increase during the tested cold exposure (Fig. 8). Mean values, SD, and one-way mixed model repeated measure ANOVA results of observed ECAs are shown in the Supplementary file-2 (Table S 4).

3.3. Expression of Tumor necrosis factor (TNF-α) gene in spleen tissues

The TNF-α mRNA relative expression in the spleen was significantly ($p < 0.05$) highest in 3 PSU fish compared to 30 PSU, 12 PSU, and 6 PSU fish on days 1, 10, and 20 (Fig. 9). Whereas on day 1, significantly lower expression was observed in 12 PSU. Spleen TNF-α mRNA regulation was significantly impaired by both salinities and extreme cold exposure duration (Supplementary file-2, Table S 3).

3.4. Principle component analysis (PCA) and discriminant function analysis (DCA)

Both PCA and DCA biplots of measured parameters depicted a well-separated grouping based on salinities and sampling days (Fig. 10, Fig. 11). Component 1 and 2 (first two PCA components), together explaining 87.3% of data variability. Component 1 (70.3% of variance) assembled different ECA and ENA, RBC, WBC, serum protein, respiratory burst, LSZ, whereas component 2 (17.0% of variance) assembled GPx, GR, SOD, and catalase (Fig. 10). The DCA biplot showed well-separated grouping with sampling days referring to varying stress responses during ambient extreme-cold stress (Fig. 11).

4. Discussion

Extreme climatic events, such as temperature extremes and heavy precipitation, are predicted to increase in frequency and magnitude as a consequence of global warming. These events result in significant challenges to the water environment and create hydrological challenges (Donat et al., 2016; Islam et al., 2020b; Reverter et al., 2020; Wernberg et al., 2013). Nonetheless, their eco-hydrological impacts are poorly understood, particularly in relation to fish in the wild and in aquaculture systems. In the face of climate change, it is necessary to understand the impacts of such events on the physiological fitness, hematological, antioxidant, and immune responses of commercially significant fish to ensure future aquaculture productivity, as well as fish welfare. Our study findings indicate increased stress response trends in terms of growth performance, hemato-physiological, antioxidants, and immune parameters during ambient extreme-cold exposure.

Differences in experimental design, parameters tested, and techniques used, make comparing our findings with others was challenging. Overall, the lowest growth and survival rates were observed in 30 PSU and 3 PSU fish. Salinity affects many aspects of physiology in ectotherms such as metabolic rate, food intake, and hormonal stimulation (Bœuf and Payan, 2001). Fish spend more energy to maintain physiological balance in both hypo- and hypersaline environments, impairing growth, development, and survival (Herrera et al., 2009; Hwang et al., 2018; Thompson, 2019). For marine teleosts, 20 to 50% of the energy budget is dedicated to osmoregulation, and better growth has been reported for

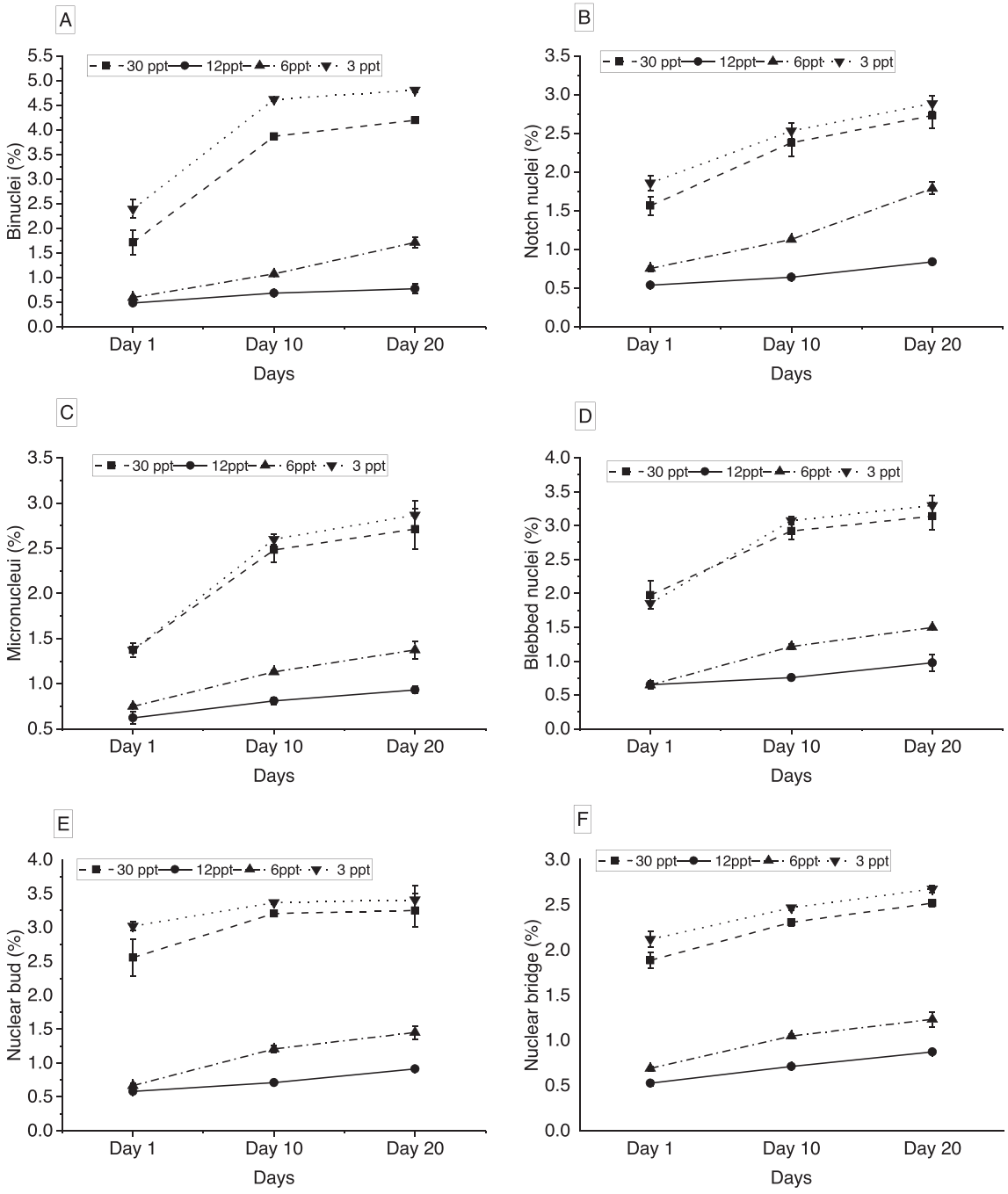

Fig. 6. Erythrocytic nuclear abnormalities (ENA) observed in juvenile European seabass on days 1, 10 and 20 of extreme ambient cold temperature (8 °C) prior to being acclimatized at four different salinities. A) Binuclei (%), B) Notch nuclei (%), and C) Micronuclei (%), D) Blebbed (%), E) Nuclear bud (%), and F) Nuclear-bridge (%). Nine fish were sampled in each sampling day for each salinity group. Three slides were prepared from each fish, 2000 cells were scored from each slide. Values represent mean ± SD.

intermediate salinities (8–20 PSU) at their juvenile stages (Bœuf and Payan, 2001; Evans and Kültz, 2020). Growth and survival rate were maximal at 15 PSU than 37 PSU in 22 °C and European seabass showed a low salinity preference (Masroor et al., 2018; Saillant et al., 2003). Blood osmolarity of European seabass is near to 15 PSU (Hwang et al., 2018). Higher survival rate, growth, and low FCR have been found at 20–25 PSU, 25 °C (Yilmaz et al., 2020), 6 PSU to 12 PSU, 24 °C (Islam et al., 2020b), and 12–15 PSU, 20 °C (Goda et al., 2019; Saillant et al., 2003). These can be attributed to low energy used to maintain osmotic balance in medium to low saline water environments (Hwang et al., 2018; Islam et al., 2020b; Maulvault et al., 2017; Saillant et al., 2003). It is important to note that, for this study, mortality occurred only during extreme cold (8 °C) exposure and no mortality was observed during salinity acclimation. For euryhaline fish, the physiological and osmotic modulations are dominated more by temperature than salinity (Vargas-Chacoff et al., 2020). Temperatures below 16 °C led to inadequate ion regulation and lower acclimatization capability in the European seabass (Dülger et al., 2012; Islam et al., 2020a), which conforms with our study.

In our study, a significant reduction of RBC, hematocrit, hemoglobin, respiratory burst (RB), serum lysozyme activities (LSZ), and survival rate were observed in 30 PSU and 3 PSU fish during the ambient extreme-cold exposure. A significant increase in WBC, antioxidant

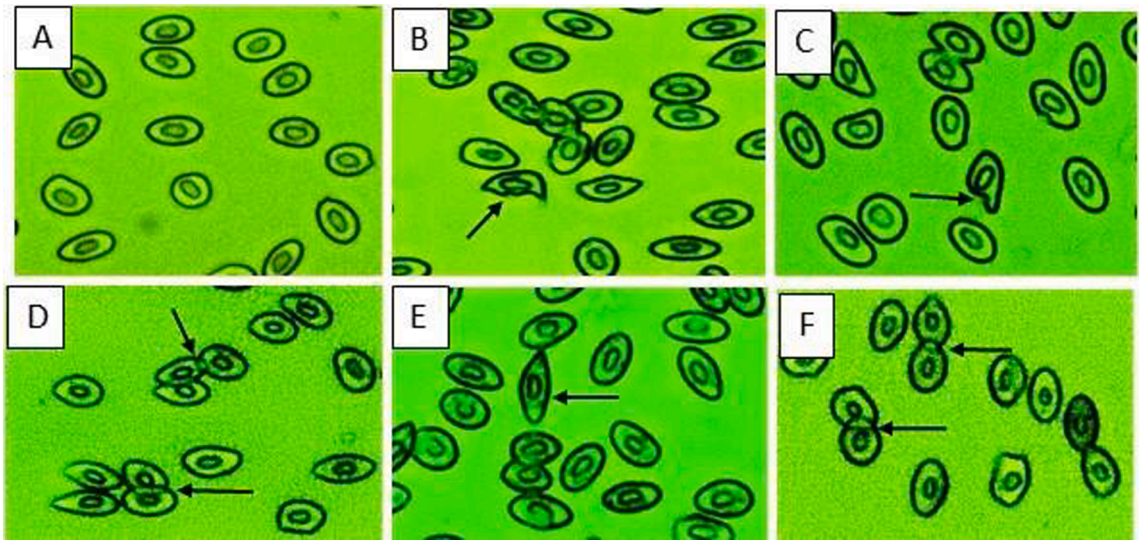

Fig. 7. Erythrocyte cellular abnormalities (ECA) observed with Giemsa stained blood smears in juvenile European seabass during extreme ambient cold temperature (8 °C) prior to being acclimatized at four different salinities. A) regular cell; B) echinocytic; C) tear-drop shaped; D) fusion; E) elongated; and F) twin.

activities, and different ECA types, ENA was also found in 30 PSU and 3 PSU fish during cold exposure. These may have resulted from the failure of the hematopoietic system, exacerbated by salinity (Elarabany, 2017; Masroor et al., 2019, 2018) and cold temperature (Islam et al., 2020c; Zarejabad et al., 2010b). Cold thermal stress beyond the optimum thermal range resulted in the decrease of RBC, hematocrit, hemoglobin values, and, at the same time, increased WBC, ENA, ECA, and mortality reported for European seabass (Islam et al., 2020c). The effects of salinity, temperature, and combinations thereof on the hematological and physio-immunological responses depend on the ranges of salinities and temperatures, stress duration, fish age, and associated biotic-abiotic factors (reviewed in Ahmed et al., 2020; Birrer et al., 2012; Elahee and Bhagwant, 2007). Low RBC counts and higher WBC, ECA, ENA have also been reported for pangas catfish, *Pangasianodon hypophthalmus* (Jahan et al., 2019), Indian carp, *Labeo rohita* (Ashaf-Ud-Doulah et al., 2019). Reduced hematocrit and hemoglobin values were observed in 30 PSU and 3 PSU fish during cold exposure. These conform with other studies, that report a significant reduction in RBC, hematocrit, hemoglobin, and increased WBC counts in sturgeon, *Huso huso* (Zarejabad et al., 2010a), tilapia, *Oreochromis niloticus* (Elarabany, 2017; Soegianto et al., 2017), *Cyprinus carpio* (Salati et al., 2010), mullet, *Mugil cephalus* (Fazio et al., 2013), and *Dicentrarchus labrax* (Islam et al., 2020c; Pascoli et al., 2011) during cold stress exposure. Reduction of hematocrit and hemoglobin could be related to decreased RBC counts (Ashaf-Ud-Doulah et al., 2019; Islam et al., 2020b) and volume caused by osmotic changes and ion leakage from the serum (Ahmed et al., 2020; Pascoli et al., 2011; Soegianto et al., 2017).

WBC counts in fish are a good indicator of physiological stress (Grzelak et al., 2017; Sopinka et al., 2016). This study shows increased leucocytes count in fish maintained at 30 PSU and 3 PSU water. Similar results were reported for rainbow trout, *Oncorhynchus mykiss* reared in high salinity compared to freshwater fish (Sahafi et al., 2013), and in *Dicentrarchus labrax* acclimatized to near freshwater (Islam et al., 2020b). Increased WBC counts indicate leukocytosis, which is considered essential for the adaptive measures during variable stress responses (Begg and Pankhurst, 2004; Christensen et al., 2017; Kang et al., 2015). This increase may result from the interaction of increased cortisol and antioxidants activities and decreased protein as non-specific immune responses (Blier, 2014; Chowdhury and Saikia, 2020; Hossain et al., 2019), linked with augmented antioxidants production; essential to cope and recover from stressors (Evans and Kültz, 2020; Joshi and Ghose, 2003; Martínez-Álvarez et al., 2005; Puerto et al., 2009). Decreased growth performance increases hepatic aminotransferase activity (Chowdhury et al., 2020) and cortisol release (Barton et al., 1985), which results in protein catabolism at the expense of physiological fitness and growth (Islam et al., 2020a; Jentoft et al., 2005; Van der Vyver et al., 2013). Increased cortisol, antioxidant activities, and decreased protein content in the current study are in good agreement with the results. The decreased trends of RB and LSZ during ambient extreme-cold exposure indicate immune dysfunction. On day 20, significantly higher RB and LSZ activities in 30 PSU and 3 PSU fish indicated a higher degree of stress than 12 PSU and 6 PSU fish. Significant upregulation of TNF-α observed in 30 PSU and 3 PSU fish in the present study corroborates this. Thus, during the cold stress period, depleted serum protein and increased RB and LSZ in 30 PSU and 3 PSU fish could explain to some extent changes in stored energy mobilization and poor growth performance (Hossain et al., 2018; McEwen and Wingfield, 2003).

In this study, significantly higher ECA and ENA were observed in 30 PSU and 3 PSU fish blood smears during cold stress. These findings corroborate other studies, where fish experienced stressful temperatures and salinities (Islam et al., 2020c, 2020b; Jahan et al., 2019). These abnormal cellular and nuclear structures might have resulted from changes in lipid layer viscosity, protein-lipid phase distribution-interaction, and increased lipid peroxidation in erythrocytes (Avrova, 1999; Bhanu and Divya, 2014; Islam et al., 2020b, 2020c; Kreps, 1981). Among others, extreme abiotic factors impair cell metabolism and damage cell membranes (Islam et al., 2019; Jahan et al., 2019). Temperature and salinity influence the erythrocytic cellular-nuclear membrane through homeo-viscous adaptation, change protein-lipid interaction phase, and fatty acids composition (Cossins, 1977; Cossins and Prosser, 1978; Gracey et al., 2004). Thus, ECA and ENA could result from increased lipid peroxidation in erythrocytes exposed to low temperatures (De et al., 2019; Ghaffar et al., 2015) and stressful salinities (Jahan et al., 2019; Klein et al., 2017; Martínez-Álvarez et al., 2005, 2002; Roche and Bogé, 1996). Changes in water temperatures and salinities affect cell membrane's biophysical properties, cellular enzymatic activities, and membrane transportation processes. To cope with this situation, fish accelerate fatty acids synthesis processes to maintain cell membrane lipid bilayer biophysical properties (Guderley and St-Pierre, 2002; Hazel, 1984; O'Brien, 2011). In the current study, increased WBC counts, TNF-α expression, and serum antioxidants activities in fish reared in 30 PSU and 3 PSU saline water during extreme ambient cold exposure were observed. These results corroborate other reports with

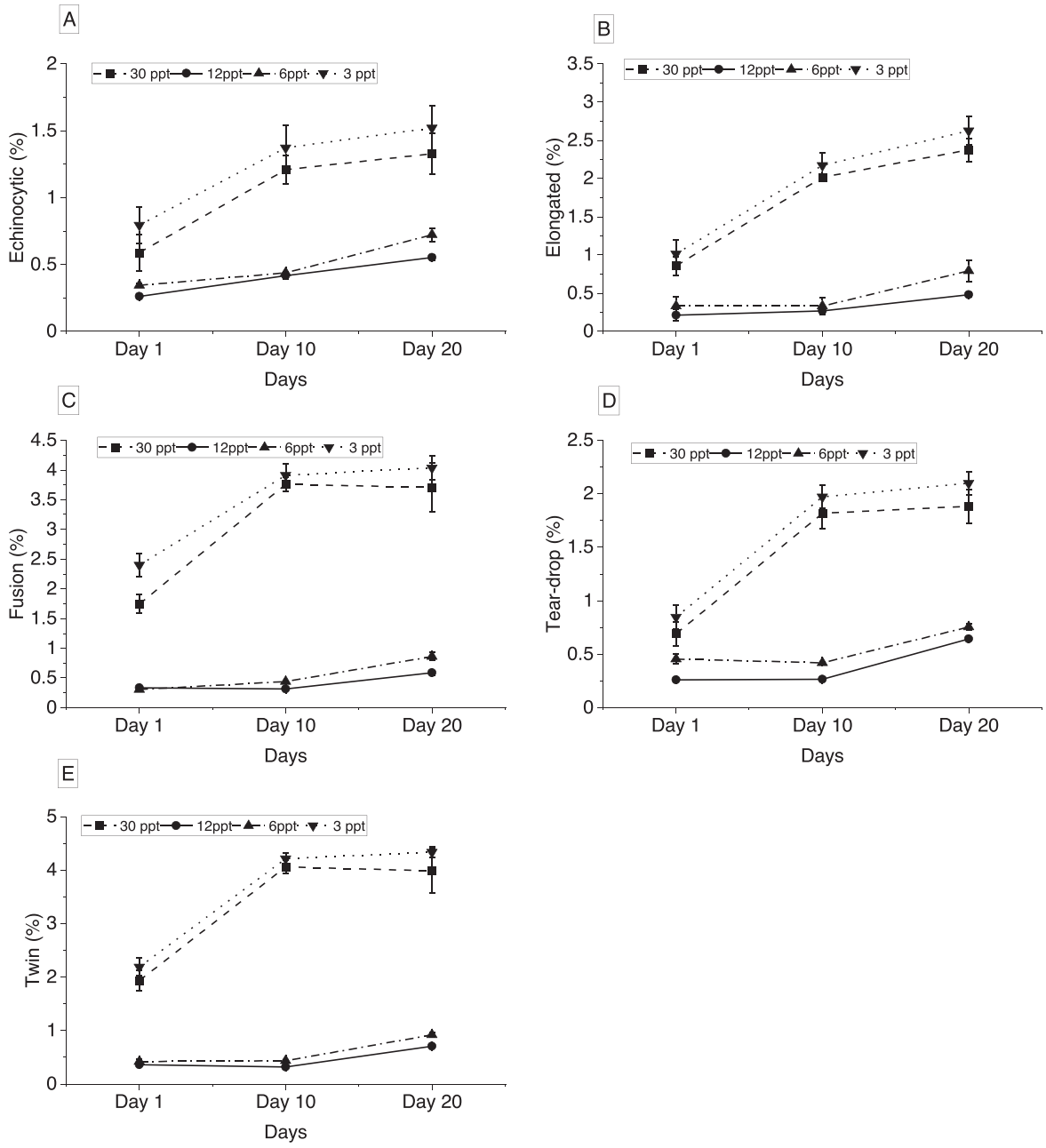

Fig. 8. Erythrocytic nuclear abnormalities (ENA) observed in juvenile European seabass on days 1, 10 and 20 of extreme ambient cold temperature (8 °C) prior to being acclimatized at four different salinities. A) Echinocytic, B) Elongated, C) Fusion, D) Tear-drop, and E) Twin. Nine fish were sampled at each sampling day for each salinity group. Three slides were prepared from each fish, 2000 cells were scored from each slide. Values represent mean ± SD.

pangas catfish, *Pangasius hypophthalmus* (Jahan et al., 2019), *Oreochromis niloticus* (Elarabany, 2017), *Oncorhynchus mykiss* (Sahafi et al., 2013), and *Dicentrarchus labrax* (Islam et al., 2020b, 2020c) exposed to different stressful temperatures and salinities.

Extreme temperatures result in noticeable changes in enzymatic antioxidant machinery and heat shock response, essential for cellular defense mechanisms against the formation of reactive oxygen species (ROS) (Madeira et al., 2016, 2013; Madeira et al.2012;; Vinagre et al., 2012). In the current research, increased serum GPx, GR, SOD, and CAT activities were found in 30 PSU and 3 PSU fish compared to 12 PSU and 6 PSU fish during cold exposure (8 °C). Results are in line with other studies, which report on oxidative stress in fish due to stressful temperatures and salinities (Bagnyukova et al., 2007; Chowdhury and Saikia, 2020; Madeira et al., 2016; Martínez-Álvarez et al., 2005). The combined impact of stressful salinities and temperatures on antioxidant activities has not been investigated before (Islam et al., 2020b). The SOD, GPx, GR, and CAT activities, thus antioxidants stress responses, significantly increased with the progression of cold stress exposure. Increased SOD activity has been reported for *Pampus argenteus* (Yin et al., 2011), *Dicentrarchus labrax* (Islam et al., 2020b), *Anoplopoma fimbria* (Kim et al., 2017), and *Takifugu obscurus* (Cheng et al., 2017) during low salinity exposure. Increased SOD activity results in more H_2O_2 production, which triggers the release of H_2O_2 scavenging enzymes such as CAT, GPX, and GR. In our study, the increased SOD, GPx, GR, and CAT activities indicate incremental stress with the progression of ambient extreme-cold exposure. During cold exposure, the significant

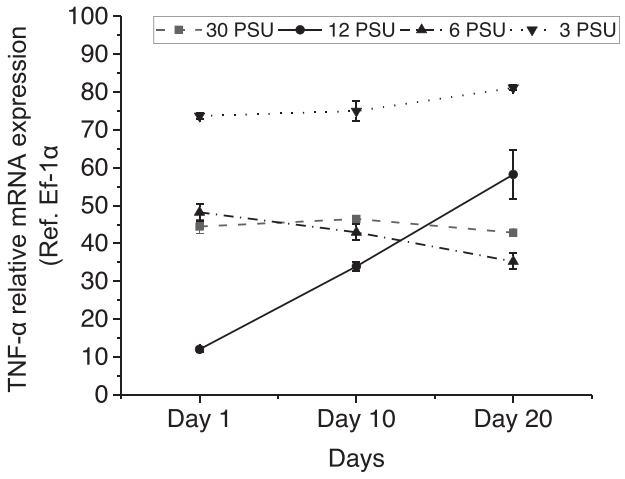

Fig. 9. Spleen Tumor necrosis factor (TNF-α) gene expression profile of juvenile European seabass acclimatized at 30 PSU, 12 PSU, 6 PSU and 3PSU on day 1, 10, and 20 of extreme ambient cold exposure (8 °C). On each sampling day, nine individuals were sampled for each salinity group. Values represent mean ± SD.

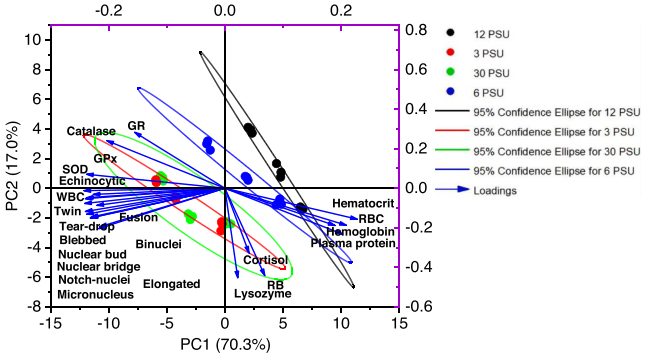

Fig. 10. Principal Component Analysis (PCA) representing the contribution of blood cellular abnormalities, serum antioxidants and immunological biomarkers measured in fish acclimatized at 30 PSU, 12 PSU, 6 PSU and 3 PSU water. The variable coordination is presented by the complementary cases analysis showing distribution of four salinity groups. Legend: RB, Respiratory burst; GRx, glutathione reductase; GPx, glutathione peroxidase; SOD, superoxide dismutase; CAT, catalase.

increase of GPX, GR, SOD, and CAT activities in 30 PSU and 3 PSU fish indicated their poor compensation ability, compared to 12 PSU and 6 PSU reared fish. RB is the rapid release of the reactive oxygen species (ROS), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2), from vertebrate phagocytic cells to minimize ROS species during stress responses (Bonilla and Menell, 2016; Kumar et al., 2005). The dropping RB values, juxtaposed to SOD, GPx, GR, and CAT activities, justify the increased antioxidant activities in our study. Moreover, LSZ has been found to decrease with the duration of cold exposure, indicating decreased immune capabilities. Lysozyme is part of the innate immune system (Revenis and Kaliner, 1992). On day 20, significantly lower RB and LSZ were observed than on day 1. Moreover, on day 20, increased RB, LSZ has been observed in 30 PSU and 3 PSU fish; this is maybe due to increased WBC counts and leukocytosis. Earlier studies have reported decreased RB activities in tilapia, *Oncorhynchus mykiss* (Nikoskelainen et al., 2004), tilapia, *Oreochromis mossambicus* (Dominguez et al., 2005), and stickleback, *Gasterosteus aculeatus* (Dittmar et al., 2014) during cold exposure. Reduced LSZ activities have been reported for *Oreochromis niloticus* (Dominguez et al., 2005) and *Oreochromis mossambicus* (Dominguez et al., 2005; Ndong et al., 2007).

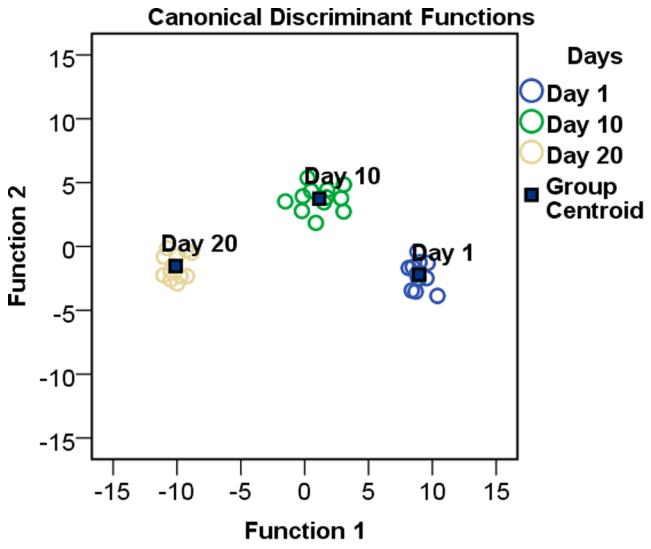

Fig. 11. Canonical discriminant function analysis (DCA) biplots. DCA representing the contribution of measured parameters during the extreme ambient cold exposure (8 °C). The variables coordination is presented by the complementary cases analysis showing distribution on days 1, 10, and 20.

TNF-α is a proinflammatory cytokine, considered an important innate immune-related gene. Fish accustomed at 3 PSU showed significantly higher inflammatory responses during cold exposure. The reason might be that 3 PSU fish could not keep immune function from the onset of cold stress; thus, salinity might have played a significant role in these immune disturbances (Bowden, 2008; Makrinos and Bowden, 2016). An increasing trend observed for 12 PSU fish indicated an incremental immune response with the time, and fish belonging to this salinity group enable to keep immune functions during the early cold stress period. Fish spleen plays a vital role in the hematopoiesis process, immune function, and hormonal production. Increased TNF-α mRNA expressions in the kidneys, blood erythrocytes, spleens, and livers have been reported for *Dicentrarchus labrax* during stress, infection, and disease (Islam et al., 2020b; Scapigliati et al., 2001). Higher expression of TNF-α during thermal stress has been stated for gilthead bream, *Sparus aurata* (López-Castejón et al., 2007), zebra fish, *Danio rerio* (Jia et al., 2017), and Atlantic cod, *Gadus morhua* (Seppola et al., 2008). Our study showed that significantly higher ECA, ENA, antioxidants, TNF-α, and decreased respiratory burst and lysozyme activities observed in 30 PSU and 3 PSU fish also support the TNF-α expression result.

Despite being a well-adapted thermal and osmotically tolerant fish, European seabass prefers intermediary salinities (~15 PSU) close to its blood osmolality (Islam et al., 2020d; Masroor et al., 2018; Saillant et al., 2003). This could be the reason that European seabass exhibited better osmoregulatory performance at low energy costs and thus increased growth (Hwang et al., 2018; Islam et al., 2020b; Vargas-Chacoff et al., 2018). Among other factors, temperature changes affect hydromineral balances in fish maintained at low and high osmotic stress reported for *Dicentrarchus labrax* (Islam et al., 2020b; Masroor et al., 2018; Thibaut et al., 2019) and in other marine fish (Gaumet et al., 1995; Islam et al., 2020c). The observed higher growth and survival rate in 12 PSU and 6 PSU than 30 PSU and 3 PSU fish conform to these reported studies. Both PCA and DCA graphs indicated a clear, well-separated cluster for four tested salinities and three sampling days. This segregated pattern refers to differential stress intensity during the extreme cold event at different salinities. Overall, PCA findings indicate that ECA, ENA, hematocrit, hemoglobin, SOD, GPx, GR, blood cells count, and serum protein could be used as potential indicators of cold stress responses in European seabass. Low growth performance, higher oxidative, and erythrocytic damages at 30 PSU and 3 PSU may indicate higher physiological demand, intensified by osmotic stress resulting from high ion pumping

activities. The current study's overall findings suggest that European seabass maintained between 6 PSU to 12 PSU could help cope better during ambient extreme-cold exposure (8 °C).

5. Conclusions

The current study examined the combined effect of different salinities and an extreme ambient cold temperature on European seabass, mimicking the environmental conditions potentially faced by this species in the future. Lower growth, survival rate, stress-linked immune-physiological responses, and a higher degree of oxidative stress, ECA, and ENA were found in fish acclimatized at 30 PSU and 3 PSU fish compared to 12 PSU and 6 PSU. Despite being euryhaline and eurythermic in nature, during ambient extreme-cold exposure, this fish encountered a higher degree of stress in terms of growth, immunity, and hemato-physiological responses. Besides, most of the measured parameters showed stressful trends during ambient extreme-cold exposure. These refer to continuous and incremental stress responses and no sign of adaptation during the 20 days of extreme ambient cold (8 °C) exposure. However, fish reared at 12 PSU, and 6 PSU water could fare better during the extreme cold spells, offering fish farmers a potential option to mitigate extreme-cold events. Based on our findings, further studies are recommended for this fish to understand the osmotic mechanisms and functions when exposing fish at environmentally realistic salinities and extreme cold temperatures.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ecolind.2020.107280.

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