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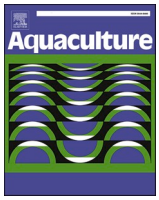

Salinity and dietary manipulation can ameliorate extreme summer heatwave stress in European seabass, *Dicentrarchus labrax*

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ABSTRACT

Climate change-induced extreme heatwaves are rapidly becoming a common environmental stressor, having a significant impact on fish. There is a growing emphasis on researching mitigation options for fish to fare better during heatwave events. To explore potential mitigation strategies, European seabass, *Dicentrarchus labrax* (36.19 ± 2.68 g) was raised under four experimental conditions: Control [30 practical salinity unit (PSU)], Treatment-1 (12 PSU), Treatment-2 (12 PSU while fed the diet supplemented with 4.5 % propolis), and Treatment-3 (30 PSU while fed the diet supplemented with propolis) for 60 days followed by 20 days of heatwave (30 °C) exposure under controlled laboratory conditions. To understand fish response, this study focused on the assessment of growth, feeding performances, and a range of biochemical, metabolic, and molecular parameters. These findings indicate that fish reared in 12 PSU while fed propolis supplemented diet (Treatment-2) exhibited significantly higher final body weight (FWG), percent weight gain (PWG), and specific growth rate (SGR) compared to the control group ($p < 0.05$). During heatwave exposure, fish in the 12 PSU group, whether fed propolis (Treatment-2) or not (Treatment-1), exhibited lower cortisol levels than the fish in control condition. On the 20th day of heatwave exposure, compared to fish in control condition, fish in the 12 PSU group, whether fed propolis or not, exhibited significantly higher superoxide dismutase assay (SOD) activity in gill tissue, whereas a contrasting trend was observed in muscle tissue. Glutathione peroxidase (GPx) activity was found significantly lower in fish in the 12 PSU group, whether fed propolis or not. On day 20 of heatwave exposure, the heat shock protein 70 (HSP70) and immunoglobulin growth factor 1 (Igf1), fatty acid desaturase 2 (FADS2), and tumor necrosis alpha (TNF-1α) genes were significantly ($p < 0.05$) upregulated in the liver tissue of fish raised at 30 PSU group when fed propolis (Treatment-3). Most of the tested parameters exhibited variable patterns across tissues suggesting that the fish stress response is influenced by both propolis supplemented diet and varying salinities during heatwave exposure. During the heatwave exposure European seabass reared in 12 PSU while feeding on propolis-supplemented diet or not, demonstrated higher growth and physiological responses than fish in control condition. Moreover, there were positive physiological benefits observed in fish raised in 30 PSU (Treatment-3), while fed propolis supplemented diet. The study proposes that adjusting salinity level and incorporating propolis into the diet can be effective strategies to mitigate the negative impacts of heatwave in European seabass.

1. Introduction

The ongoing repercussions of climate change are increasingly evident, manifesting in increased and prolonged extreme weather events that pose severe threats to the stability of global ecosystems (Hai and Perlman, 2022; van Gevelt et al., 2023). In marine environments, these events represent a significant challenge to the physiological and ecological dynamics of numerous species (Smith et al., 2023; Vieira et al., 2018). Extreme heatwaves driven by climate change are rapidly becoming a ubiquitous environmental stressor, profoundly impacting ecosystems and associated species (Ozbayram et al., 2022; Woolway et al., 2021) by disrupting critical biological processes. Rising temperatures in the aquatic environment have far-reaching implications for the physiological, behavioral, and ecological aspects of fish, potentially jeopardizing their well-being and survival. Thus, extreme heatwaves, characterized by prolonged periods of excessively high temperatures, present intense challenges to fish (Smale et al., 2019; Smith et al., 2023).

European seabass, *Dicentrarchus labrax*, an important aquaculture

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species having both ecological and economic values (seafish.org, 2022; Vandeputte et al., 2019), is confronting challenges due to the increased frequency and intensity of heatwaves (Islam et al., 2021b; Islam et al., 2020a; Islam et al., 2020c). This fish is a prominent inhabitant of coastal waters in the Mediterranean and Eastern Atlantic regions, playing a crucial role in both marine ecosystems and the aquaculture industry. Its ecological importance stems from its position as a key predator, influencing prey populations and maintaining ecological balance (De Pontual et al., 2023; Kelley, 1988; Oikonomidou et al., 2019). However, the vulnerability of this species to extreme heatwaves, intensified by climate change demands attention and comprehensive mitigation strategies (Ciji and Akhtar, 2021; Islam et al., 2022). As a temperature-sensitive species, this fish experiences increased stress during heatwave events, affecting its growth, reproductive performance, and overall fitness (Masroor et al., 2019; Nathanailides et al., 2010). Thermal preferendum of European seabass ranges from 20 to 24 °C, the fish cannot thrive best beyond 24 °C. Moreover, fish struggle to survive if exposed to >29 °C for extended periods (Claridge and Potter, 1983; Person-Le Ruyet et al., 2004; Vinagre et al., 2012). In the southern part of the Mediterranean, especially during summer, the temperature of shallow waterbodies may occasionally exceed 33–34 °C. These extreme heatwaves usually last from 3 to 15 days and are projected to extend to over 20 days by the end of the 21st century (Dülger et al., 2012; Molina et al., 2020; Simon et al., 2022). Thus, in this region, fish in both wild and farming systems have to cope with intense summer heatwave events. As climate change continues its relentless progression by means of frequency, duration, and intensity (Galappaththi et al., 2020; Simon et al., 2022), necessitating effective strategies to mitigate their impacts on European seabass and other fish (Islam et al., 2022; Rosa et al., 2012; Sarà et al., 2018).

Stress management in aquaculture animals has become a focal point of research. Strategies to mitigate stress in farmed fish encompass diverse approaches such as water quality control, feeding optimization, site shifting, and stocking density management. However, some options, like fish translocation, may not be viable for rapidly addressing climate change impacts (Ciji and Akhtar, 2021; Herrera et al., 2019; Islam et al., 2022; Farag et al., 2021). Dietary manipulation strategies have arisen as a viable approach to alleviate stress in fish (Islam et al., 2022). For fish, some feed nutraceuticals and additives have shown efficacy in counteracting stress effects, growth improvement, and physiological functions (Hassaan et al., 2019; Herrera et al., 2019; Kumar et al., 2018; Kumar et al., 2015; Zhang et al., 2020). Among dietary ingredients, propolis has garnered increased attention for its potential to alleviate stress in fish (reviewed by Farag et al., 2021). In a previous study, European seabass fed on propolis-supplemented (4.5 g kg^{-1}) diets showed significant benefits (growth performance, hematological parameters, oxidative stress, and immunity) during heatwave extremes (Islam et al., 2021a). For fish, several studies have also highlighted the thermal stress-alleviating properties of propolis (2 to 10 g kg^{-1}) supplemented diets by measuring and interpreting a range of parameters including hematocrit, hemoglobin, blood cell count, GOT, GPT, LDH, triglycerides, and cholesterol content in Nile tilapia and European seabass (Hassaan et al., 2019; Kelestemur et al., 2012; Šegvić-Bubić et al., 2013). In addition, salinity control could be another avenue to mitigate thermal stress in European seabass. This fish reared between 6 and 12 PSU showed better growth and physiological performance during temperature extremes (Islam et al., 2021b; Islam et al., 2021a). Juvenile European seabass inhabit near-shore areas where salinity levels are typically lower compared to the deep sea (Kelley, 1988). Despite being euryhaline and having an effective osmoregulatory capacity, juvenile European seabass exhibit a salinity preferendum ranging between 15 and 25 PSU (Saillant et al., 2003a). During extreme weather events, European seabass fared better at salinities ranging from 6 to 12 PSU (Islam et al., 2021b, Islam et al., 2021a). The question remains unanswered as to whether suboptimal salinities and dietary supplementation can modify the temperature tolerance, growth, and physiological performances of this fish, thereby enhancing its ability to withstand extreme heatwave events.

This research intends to identify effective strategies to mitigate the detrimental impacts of extreme heatwaves on European seabass. The study focuses on evaluating three primary mitigation approaches: environmental management (salinity control), dietary supplementation (propolis), and a synergistic integration of both strategies. Each approach is poised to offer unique benefits, addressing various physiological and behavioral aspects of fish to enhance resilience against heatwave stress. Through a comprehensive analysis and comparison of these three strategies, this study seeks to discern the most effective approach to mitigating the impacts of extreme heatwaves on fish. By understanding and implementing efficient strategies, we aim to sustain the health and productivity of European seabass in the face of a changing climate, thereby ensuring the resilience and stability of coastal ecosystems and aquaculture enterprises.

2. Materials and methods

2.1. Experimental fish and rearing conditions

The experiment took place at the Centre for Aquaculture Research (ZAF), Alfred Wegner Institute for Polar and Marine Research (AWI), Bremerhaven, Germany. European seabass *Dicentrarchus labrax* was procured from Les Poissons du Soleil, Balaruc-les-Bains, France. The research was conducted in thermostatically controlled four separate recirculatory systems, featuring twelve rectangular (4 Treatments × 3 Replicates) fiberglass tanks [75 cm × 45 cm × 45 cm; 60 L (effective water volume) continuously supplied with filtered seawater (30 PSU, 20 °C). A total of 144 individuals, with an average weight of 36.19 ± 2.68 g (ranging from a minimum of 29.40 g to a maximum of 42.36 g), were randomly distributed with 12 fish allocated to each tank. The recirculation system was connected to essential components, including biofilters, protein skimmers, UV light, continuous aeration, and thermostatic sensors. Prior to commencing the experiment, a 14-day acclimation period was provided to allow the fish to adapt to experimental conditions. During the study, fish were fed twice daily, at 9:00 and 16:00 h, with 2 mm pellets containing 54 % protein (Alltech Coppens, Netherlands) until visual satiety. Following the acclimation period, fish were subjected to four experimental conditions: reared at 30 PSU and 12 PSU hereafter referred to as Control and Treatment-1, respectively. Whereas fish reared at 12 PSU and 30 PSU while fed on propolis supplemented diet, were hereafter referred to as Treatment-2 and Treatment-3. Six tanks (3 tanks per treatment in separate systems) were maintained with the acclimation salinity (30 PSU) to achieve the conditions for two treatments: [Control (30 PSU) and Treatment-3 (30 PSU+ Propolis)]. To attain the desired salinity for another two treatments, the water of targeted six replicate tanks (3 tanks per treatment in separate systems) was gradually reduced (~2–4 PSU per day) to establish the conditions for another two 12 PSU treatments [Treatment-1 (12 PSU) and Treatment-2 (12 PSU+ Propolis)]. Under these conditions, fish were fed for 60 days [twice a day (at 9:00 and 16:00 h)] with the designated diets up to visual satiety. The photo regime was set at 10 h of light and 14 h of darkness. After 60 days of feeding, subsequently, fish were subjected to an extreme summer heatwave event (30 °C). To simulate the extreme heatwave stress, the water temperature increased at a rate of approximately 2 °C per day to reach 30 °C and maintained for 20 days. The count of extreme heatwave stress duration commenced when the temperature reached 30 °C (Fig. 1). To maintain optimal water quality, parameters such as dissolved O_2 (>6.0 ppm), NH_3 (<0.05 mg L-1), NO_3^-, and NO_2^- (<0.2 mg L-1) were monitored and maintained consistently. Daily cleaning ensured the removal of uneaten feed and waste. Uneaten feed was collected, dried, and quantified to get data and calculate fish growth performance. Moreover, approximately 50–70 % of temperature-adjusted seawater (30 PSU) was exchanged two times per week to minimize nitrogenous excretion. The experiment adhered to the guidelines outlined in the EU directive for animal experiments [EU Directive 2010/63/EU].

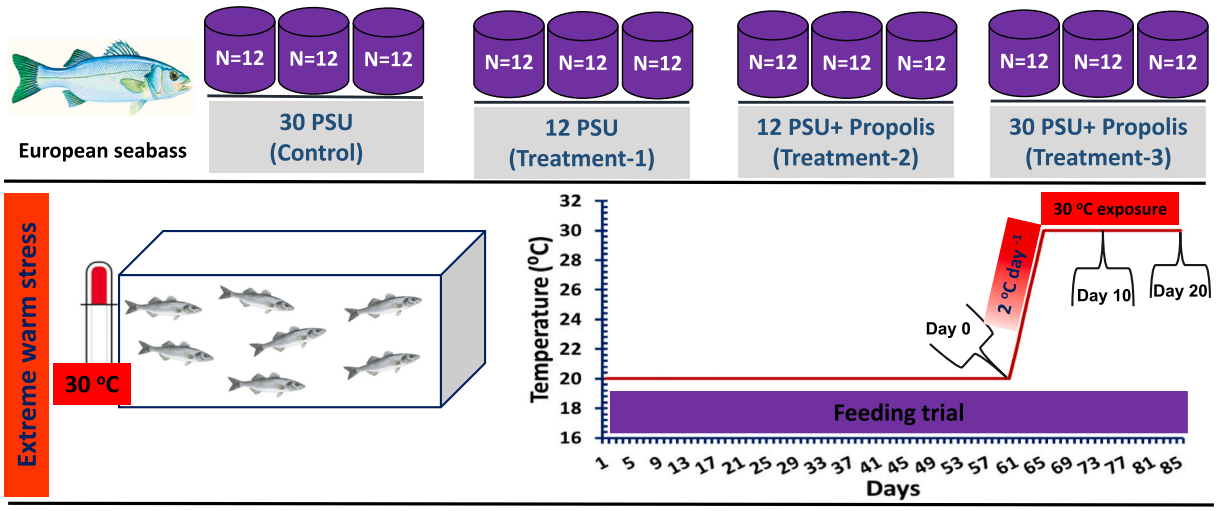

Fig. 1. Experimental design and sampling protocol. Fish were reared under four experimental conditions: 30 PSU; 20 °C (Control), 12 PSU (Treatment-1), 12 PSU+ Propolis supplemented diet (Treatment-2), and 30 PSU + Propolis supplemented diet (Treatment-3). With these treatments fish were reared for 60 days followed by a 30 °C heatwave for 20 days. Prior starting heatwave fish were sampled (No Stress), on the mid of heatwave exposure (Stress Day 10), and at the end of heatwave exposure (Stress Day 20).

2.2. Diet formulation

Pure propolis from honeybee hives (Bioland, Germany) was used to formulate the diet. Two diets were prepared to fulfill the dietary needs of experimental fish, differing only in their supplementary components [one containing propolis and the other without any supplement] (Table 1). All ingredients were thoroughly mixed and pelletized (size: 2.0 mm) through the twin-screw cold extrusion method (SPAROS, Portugal), and subsequently stored at −4 °C until use. The nutritional composition of the diets was assessed following the AOAC (1995) guidelines.

2.3. Sampling

After 60 days of feeding trial, three fish from each tank (nine fish per treatment) were sampled on the day before the onset of the heatwave, and on days 10 and 20 of extreme heatwave (30 °C) stress exposure; hereafter mentioned as 'No Stress', 'Stress Day 10' and 'Stress Day 20', respectively. Prior to sampling, the fish were subjected to feed deprivation for 24 h before being dissected in their post-absorptive stage. Fish were sedated immediately before sampling by immersion in an overdose of tricaine methanesulfonate (MS-222, 50 mg L^{-1}). After anesthesia, the weight of the fish was measured, and blood samples were obtained by puncturing the caudal vein using nonheparinized syringes. To achieve sufficient volume for analysis, blood samples from fish in each replication tank were pooled ($n = 3$). Following centrifugation at 4000 $\times g$ for 15 min, the serum was separated from the blood. Additionally, samples of muscle, liver, and kidney were collected from each fish used for blood collection. Tissue samples were immediately frozen in liquid N_2 and maintained at −80 °C for further analysis.

2.4. Growth performance

At the end of the 60-day feeding trial and before simulating heatwave stress, fish were sampled to assess weight gain, and feed utilization efficiency [specific growth rate (SGR), protein efficiency ratio (PER), and feed intake (FI)]. The mean individual length and weight of fish from each replicate tank were calculated by dividing the total weight by the number of individuals ($n = 12$). Growth performance was then determined using the following equations:

Table 1
Formulation of experiential diets and nutritional composition (g kg^{-1}).

Diet composition (g kg^{-1})	Diets	
	Control	Propolis
FishmealLT70[a]	280.00	280.00
Fishmeal 60[b]	200.00	200.00
Fish soluble concentrate [c]	25.0	25.0
Soy protein concentrate [d]	50.0	50.0
Wheat gluten [e]	55.0	55.0
Corn gluten [f]	50.0	50.0
Soybean meal 48 [g]	90.0	90.0
Wheat meal	55.0	55.0
Whole peas	50.0	50.0
Fish oil [h]	135.0	130.5
Vitamin and mineral premix [i]	10.0	10.0
Propolis	–	4.5
Nutritional composition (g kg^{-1})		
Dry matters	951.10	932.20
Crude proteins	549.10	553.00
Crude lipids	189.20	170.10
Ash	108.60	109.30
Carbohydrate	153.10	144.00
Phosphorous	14.40	14.40
Gross energy (kJ g^{-1})	233.10	232.10

[a] Peruvian fishmeal LT, 71 % crude protein (CP), 11 % crude fat (CF), EXALMAR, Peru.
[b] Fair Average Quality (FAQ) fishmeal, 62 % CP, 12 % CF, COFACO, Portugal.
[c] CPSP90, 84 %CP, 12 %CF, Sopropeche, France.
[d] Soycomil P, 65 % Cp, 0.8 % CF, ADM, The Netherlands.
[e] VITEN, 85 %CP, 1.3 %CF, ROQUETTE, France.
[f] Corn gluten feed, 61 %CP, 6 %CF, COPAM, Portugal.
[g] Solvent extracted dehulled soybean.
[h] COPPENS International, The Netherlands.
[i] Premix for marine fish, PREMIX Lda, Portugal. Vitamins (IU or mg kg − 1 diet), DL-alpha tocopherol acetate, 100 mg; sodium menadione bi-sulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 2000 IU; thiamin, 30 mg; riboflavin, 30 mg; pyridoxine, 20 mg; cyanocobalamin, 0.1 mg; nicotinic acid, 200 mg; folic acid, 15 mg; ascorbic acid, 1000 mg; inositol, 500 mg; biotin, 3 mg; calcium pantothenate, 100 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg kg − 1 diet), cobalt carbonate, 0.65 mg; copper sulphate, 9 mg; ferric sulphate, 6 mg; potassium iodide, 0.5 mg;manganese oxide, 9.6 mg; sodium selenite, 0.01 mg; zinc sulphate, 7.5 mg; sodium chloride, 400 mg; calcium carbonate, 1.86 g; excipient wheat middling.

$$\text{Percent Weight gain (WG\%)} = \frac{\text{Final weight (FW)} - \text{Initial weight (IW)}}{\text{Initial weight (IW)}} \text{X} 100$$

$$\text{Feed intake (FI) (g/Fish/day)} = \frac{\text{Given dry diet weight} - \text{Recovered remaining dry diet weight}}{\text{Number of fish}}$$

$$\text{Specific growth rate (SGR, \%/day)} = \frac{\{ln(\text{Final weight}) - (\text{Initial weight})\}}{\text{Duration}} \text{X } 100$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Dry feed intake (g)}}{\text{Live weight gain (g)}}$$

$$\text{Protein efficiency ratio(PER)} = \frac{\text{Live weight gain (g)}}{\text{Dry protein intake (g)}}$$

2.5. Metabolic and cellular stress response measurement

Cellular enzymes in serum [glutamate pyruvate transaminase (GPT), glutamic oxaloacetic transaminase (GOT), and lactate dehydrogenase (LDH)] were quantified using a blood analyzer (Fuji Dri-CHEM NX500i, Fujifilm, Japan). Blood lactate levels were determined from whole blood samples collected from each fish ($n = 9$) with Lactate Scout (EKF Diagnostics, Germany). Serum cortisol levels were assessed using a commercial kit (Cortisol Saliva ELISA, IBL International, Germany).

2.6. Enzymatic analysis

Glutathione peroxidase (GPx) and glutathione reductase (GR) activities were assessed with modified methods outlined by Flohé and Günzler (1984) and Carlberg and Mannervik (1975), respectively. Superoxide dismutase (SOD) activity was determined using the method outlined by McCord and Fridovich (1969). Protein concentrations in the enzyme extracts were quantified following the Bradford method using the commercial dye reagent (BioRad 600–0006). Bovine γ-globulin (Sigma Aldrich, Germany) at concentrations of 1 mg ml^{-1}, was employed as standard (Lima et al., 2007).

2.7. Gene expression analysis

Analyses of mRNA expression levels were performed for muscle, liver, and kidney tissue of European seabass (Table 2). Tissue samples from each replicate tank were pooled and used for gene expression analysis. Total RNA extraction was performed using an RNA Miniprep kit (Monarch, USA). The quality and quantity of extracted RNA were assessed using gel electrophoresis (with a 1.0 kb DNA ladder) and spectrophotometry (Tecan, Switzerland) respectively. Gene expression levels were assessed using q-PCR (CFX Manager™, Bio-Rad, USA). The cDNA synthesis and gene expression analyses were followed according to our previously published paper (Islam et al., 2024b).

2.8. Statistical analysis

The data were assessed for normality by employing the Kolmogorov test and homoscedasticity using the Levene test. In cases when the assumption of normality and homoscedasticity was violated, log transformations were performed to enhance conformity with the normality assumption. To analyze the impacts of diet, heatwave stress, salinity, and their interplay, a three-way MANOVA was performed with diets, salinity, and stress duration as fixed variables. Growth data were subjected to a two-way MANOVA to assess growth performance within the dietary and salinity groups. Bonferroni post hoc correction was applied to both types of MANOVA. A probability value of $p < 0.05$ was considered statistically significant.

Table 3
Fish growth performance at the end of the 60-day feeding trial and before starting summer heatwave (30 °C) stress.

Parameters	Treatments			
	Control	Treatment-1	Treatment-2	Treatment-3
IBW[1]	36.38 ± 0.66[a]	36.51 ± 1.22[a]	36.17 ± 0.64[a]	36.19 ± 0.67[a]
FBW[2]	110.08 ± 2.92[b]	119.44 ± 2.50[ab]	130.78 ± 5.05[a]	116.83 ± 5.48[b]
WG[3]	202.74 ± 12.96[b]	227.28 ± 7.73[ab]	261.72 ± 18.90[a]	223.01 ± 19.58[ab]
SGR[4]	2.46 ± 0.06[b]	2.65 ± 0.05[ab]	2.85 ± 0.09[a]	2.60 ± 0.10[b]
PER[5]	3.80 ± 0.39[a]	4.84 ± 0.49[a]	5.03 ± 0.60[a]	4.63 ± 0.46[a]
FI[6]	35.67 ± 4.20[a]	30.98 ± 3.01[a]	36.54 ± 1.71[a]	32.47 ± 0.90[a]
FCR[7]	2.11 ± 0.19[a]	1.64 ± 0.16[a]	1.69 ± 0.21[a]	1.76 ± 0.20[a]

*Values are means of triplicate groups ±SD. N = 3. Values followed by different letters within the same row are significantly different values from two-way ANOVA results ($P < 0.05$). [1] IBW = Initial body weight (g); [2] FBW=Final body weight (g); [3] WG = Percent weight gain (%); [4] SGR = Specific growth rate (% day^{-1}); [5] PER = Protein efficiency ratio; [6]FI=Feed Intake; [7]FCR = Food Conversion Ratio.

Table 2
Realtime RT-PCR primers details.

Gene	Primer sequence (5′-3′)		BP	Annealing Temp.	Primer efficiency	Accession No.	References
HSP70	Forward:	GTCTGGACAAAGGCAAGAGC	181	59 °C	104.0 %	MG711592.1	(Enes et al., 2006)
	Reverse:	TTGTGAGAGGGCCAAGAGAA					
TNF-α	Forward:	GCCAAGCAAACAGCAGGAC	77	60 °C	105.0 %	DQ200910	(Azeredo et al., 2015)
	Reverse:	ACAGCGGATATGGACGGTG					
Igf1	Forward:	ATGTACTGTGCACCTGCCAA	106	59 °C	90.0 %	GQ924783.1	(Islam et al., 2020a)
	Reverse:	CTTTGTGCCCTGCGGTACTA					
FADS2	Forward:	CCTTCACTGCTTTCATCCCAA	202	60 °C	104.0 %	EU439924	(Geay et al., 2010)
	Reverse	CCCAGGTGGAGGCAGAAGAA					
El-1α	Forward:	AGATGACCACGAGTCTCTGC	127	60 °C	105.0 %	FM019753	(Mitter et al., 2009)
	Reverse:	CTTGGGTGGGTCGTTCTTG					

Table 4
Two-way MANOVA test results of measured growth parameters in European seabass reared under different salinities and dietary conditions. Probability values (p) <0.05 were considered significant.

	Measured parameters	Salinity					Diets					Salinity*Diets				
		DF	DF (Error)	F-statistic	*p*-value	Π^2 (Partial)	DF	DF (Error)	F-statistic	p-value	Π^2 (Partial)	DF	DF (Error)	F-statistic	p-value	Π^2 (Partial)
Growth performances	IBW[1]	1	8	0.324	0.585	0.039	1	8	0.024	0.880	0.003	1	12	0.013	0.913	0.002
	FBW[2]	1	8	13.943	**0.006**	0.635	1	8	0.897	0.371	0.101	1	12	23.156	**0.001**	0.743
	WG[3]	1	8	9.235	**0.016**	0.536	1	8	0.619	0.454	0.072	1	12	12.343	**0.008**	0.607
	SGR[4]	1	8	13.774	**0.006**	0.633	1	8	0.793	0.399	0.090	1	12	23.278	**0.001**	0.744
	PER[5]	1	8	0.554	0.478	0.065	1	8	7.557	**0.025**	0.486	1	12	0.038	0.850	0.005
	FI[6]	1	8	1.677	0.231	0.173	1	8	2.894	0.127	0.266	1	12	6.066	**0.039**	0.431
	FCR[7]	1	8	1.993	0.196	0.199	1	8	3.374	0.104	0.297	1	12	6.274	**0.037**	0.440
MANOVA results (Wilks' lambda, λ) Effects		7.0	2.0	3.041	0.27	0.914	7.0	2.0	2.28	0.338	0.889	7.0	2.0	3.29	0.25	0.920

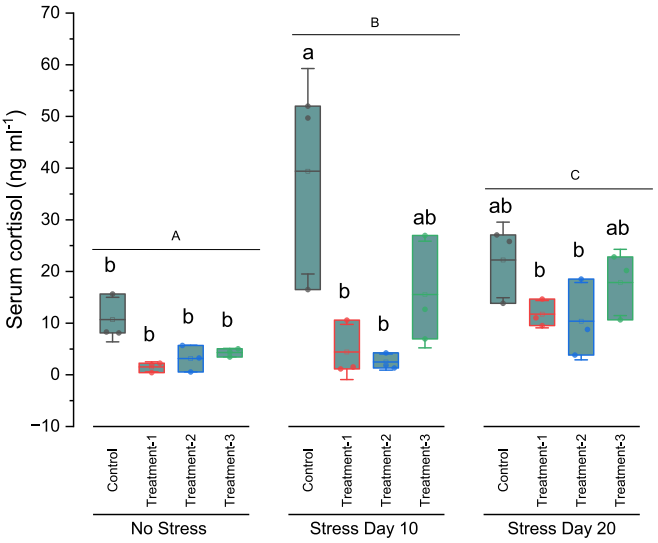

Fig. 2. Serum cortisol levels in European seabass reared under different salinities and dietary conditions. Values are represented as mean ± SD, $n = 3$. On each bar different letters refer to significance differences and each sampling point labeled with different block letters refer significance difference ($p < 0.05$, Bonferroni).

3. Results

To enhance readability, here the treatment explanation has been clarified once again: 30 PSU; 20 °C (Control), 12 PSU (Treatment-1), 12 PSU+ Propolis supplemented diet (Treatment-2), and 30 PSU + Propolis supplemented diet (Treatment-3).

3.1. Growth performance

Fish reared under the Treatment-2 (30 PSU+ Propolis) condition exhibited significantly higher FBW, PWG, and SGR compared to those in the control group ($p < 0.05$). Furthermore, FCR and PER were observed to be higher with fish reared under Treatment-2; however, these high values did not reach statistical significance (Table 3). Growth performances have been found to be affected by both salinity, diets, and their interactions (Table 4).

3.2. Serum cortisol and blood lactate levels

Following 60 days of exposure to different salinities and dietary conditions, preceding the onset of heatwave stress, serum cortisol content was higher (though not significant) in fish reared under control conditions compared to the other three treatments. But on day 10 of heatwave stress, fish reared under treatments 1 and 2 showed significantly lower cortisol levels compared to those in the control ($p < 0.05$). Whereas, on stress day 20, a significantly different cortisol level was not observed among the fish reared at four treatments (Fig. 2). It can be observed from Fig. 2 and Table 5 that cortisol content varied significantly ($p < 0.05$) with stress duration, salinity, and the interaction between warm stress duration and salinity. Whereas, diets and interaction between diet, stress duration, and salinity were nonsignificantly different ($p > 0.05$). Blood lactate levels did not vary among fish reared under four treatments (Fig. 3). Both serum cortisol and lactate levels demonstrated significant differences before and after heatwave stress while feeding at different salinities. The interaction among diet, stress duration, and salinity has been found no significant impact on lactate levels (Fig. 3, Table 5).

Table 5
Three-way MANOVA test results of measured parameters in European seabass reared under different salinities and dietary conditions. Probability values (p) <0.05 were considered significant.

Tissues	Measured parameters	Stress duration				Diets				Salinity				Stress duration*diets				Salinity*Diets				Stress duration*Salinity				Stress duratin*Salinity*Diets			
		DF	DF (Er)	F	P	DF	DF (Er))	F	P	DF	DF (Er)	F	P	DF	DF (Er))	F	P	DF	DF (Er)	F	P	DF	DF (Er)	F	P	DF	DF (Er)	F	P
Enzymes	GPT	2	24	10.583	**0.001**	1	24	18.380	**0.000**	1	24	0.496	0.488	2	24	8.986	**0.001**	2	24	4.944	**0.036**	2	24	1.628	0.217	2	24	15.010	**0.000**
	LDH	2	24	14,090	**0.000**	1	24	1093.70	**0.000**	1	24	1047.3	**0.000**	2	24	880.880	**0.000**	2	24	1547.01	**0.000**	2	24	899.94	**0.000**	2	24	756.36	**0.000**
	GOT	2	24	5.050	**0.015**	1	24	1.847	0.187	1	24	13.343	**0.001**	2	24	19.051	**0.000**	2	24	4.757	**0.039**	2	24	16.280	**0.000**	2	24	7.320	**0.003**
Metabolites	Cortisol	2	24	11.983	**0.000**	1	24	2.188	0.152	1	24	34.821	**0.000**	2	24	0.824	0.451	2	24	1.933	0.177	2	24	3.472	**0.047**	2	24	0.277	0.761
	lactate	2	24	3.299	**0.054**	1	24	0.038	0.847	1	24	1.437	0.242	2	24	2.515	0.102	2	24	1.961	0.174	2	24	0.140	0.870	2	24	1.849	0.179
Gill	GPx	2	24	2.149	0.138	1	24	0.073	0.790	1	24	0.900	0.352	2	24	0.038	0.963	2	24	1.691	0.206	2	24	0.173	0.842	2	24	0.148	0.863
	SOD	2	24	5.452	**0.011**	1	24	0.098	0.757	1	24	1.555	0.224	2	24	0.190	0.828	2	24	1.181	0.288	2	24	9.089	**0.001**	2	24	0.585	0.565
	GR	2	24	55.844	**0.000**	1	24	2.961	0.098	1	24	0.899	0.353	2	24	0.354	0.705	2	24	0.268	0.610	2	24	3.669	**0.041**	2	24	1.130	0.340
Liver	GPx	2	24	6.499	**0.006**	1	24	3.032	0.094	1	24	17.350	**0.000**	2	24	0.281	0.757	2	24	1.618	0.216	2	24	2.576	0.097	2	24	1.502	0.243
	SOD	2	24	35.228	**0.000**	1	24	2.712	0.113	1	24	22.975	**0.000**	2	24	2.140	0.140	2	24	2.054	0.165	2	24	7.481	**0.003**	2	24	5.691	**0.009**
	GR	2	24	29.187	**0.000**	1	24	0.638	0.432	1	24	6.416	**0.018**	2	24	1.898	0.172	2	24	5.962	**0.022**	2	24	11.667	**0.000**	2	24	2.911	0.074
Muscle	GPx	2	24	21.189	**0.000**	1	24	0.861	0.363	1	24	21.815	**0.000**	2	24	0.050	0.951	2	24	0.066	0.799	2	24	20.990	**0.000**	2	24	0.388	0.682
	SOD	2	24	2.661	0.090	1	24	0.836	0.370	1	24	3.718	0.066	2	24	0.652	0.530	2	24	14.113	**0.001**	2	24	6.620	**0.005**	2	24	4.095	**0.029**
	GR	2	24	19.39	**0.000**	1	24	3.504	0.073	1	24	2.500	0.127	2	24	2.637	**0.092**	2	24	4.469	**0.045**	2	24	36.769	**0.000**	2	24	3.386	**0.051**
Muscle gene	HSP70	2	24	0.413	0.666	1	24	0.799	0.380	1	24	0.098	0.757	2	24	3.124	0.062	2	24	3.570	0.071	2	24	17.795	**0.000**	2	24	11.126	**0.000**
	TNF-1α	2	24	11.215	**0.000**	1	24	0.813	0.376	1	24	55.564	**0.000**	2	24	1.223	0.312	2	24	0.477	0.626	2	24	0.477	0.626	2	24	3.079	0.065
	Igf1	2	24	5.056	**0.015**	1	24	1.365	0.254	1	24	1.105	0.304	2	24	0.050	0.951	2	24	7.354	**0.003**	2	24	7.354	0.003	2	24	0.754	0.481
	FADS2	2	24	0.794	0.463	1	24	0.115	0.738	1	24	3.332	0.080	2	24	0.999	0.383	2	24	0.623	0.545	2	24	0.623	0.545	2	24	0.924	0.411
Liver genes	HSP70	2	24	2.332	0.119	1	24	0.984	0.331	1	24	5.457	**0.028**	2	24	14.285	**0.000**	2	24	5.464	**0.011**	2	24	5.464	**0.011**	2	24	13.563	**0.000**
	TNF-1α	2	24	3.338	**0.053**	1	24	2.480	0.128	1	24	33.400	**0.000**	2	24	11.264	**0.000**	2	24	6.676	**0.005**	2	24	6.676	**0.005**	2	24	14.021	**0.000**
	Igf1	2	24	9.472	**0.001**	1	24	23.521	**0.000**	1	24	8.652	**0.007**	2	24	8.949	**0.001**	2	24	12.751	**0.000**	2	24	12.751	**0.000**	2	24	5.371	**0.012**
	FADS2	2	24	17.607	**0.000**	1	24	20.689	**0.000**	1	24	3.511	0.073	2	24	13.463	**0.000**	2	24	18.297	**0.000**	2	24	18.297	**0.000**	2	24	20.401	**0.000**
Kidney genes	HSP70	2	24	1.424	0.260	1	24	0.456	0.506	1	24	97.294	**0.000**	2	24	0.920	0.412	2	24	1.840	0.180	2	24	1.840	0.180	2	24	0.608	0.553
	TNF-1α	2	24	96.38	**0.000**	1	24	4.571	**0.043**	1	24	73.825	**0.000**	2	24	4.589	**0.021**	2	24	101.99	**0.000**	2	24	101.99	**0.000**	2	24	3.770	**0.038**
	Igf1	2	24	0.860	0.436	1	24	0.009	0.926	1	24	39.771	**0.000**	2	24	0.768	0.475	2	24	1.805	0.186	2	24	1.805	0.186	2	24	0.049	0.953
	FADS2	2	24	1.523	0.238	1	24	1.526	0.229	1	24	7.077	**0.014**	2	24	1.152	0.333	2	24	0.461	0.636	2	24	0.461	0.636	2	24	0.681	0.516
MANOVA results (Wilks' lambda, λ) Effects		2	48	50.55	0.019	1	24	55.85	0.15	1	24	36.27	**0.13**	2	48	3.96	0.221	1	24	54.38	0.106	2	48	12.85	0.074	2	48	7.634	0.122

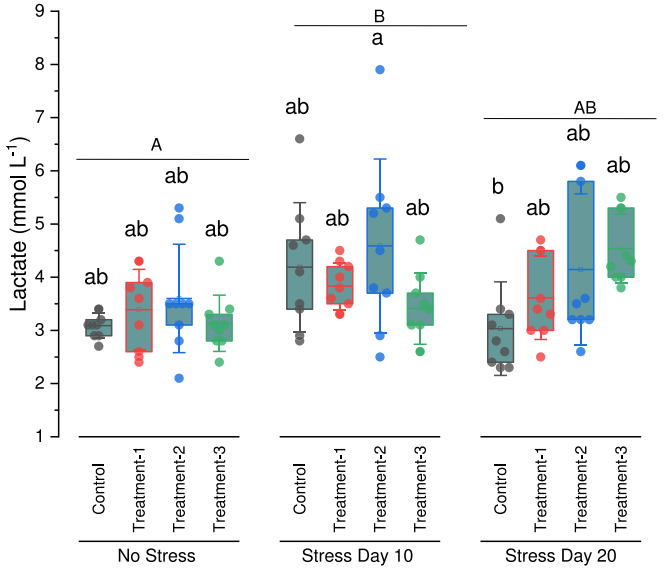

Fig. 3. Blood lactate levels in European seabass reared under different salinities and dietary conditions. Values are represented as mean ± SD, $n = 9$. On each bar different letters refer to significance differences and each sampling point labeled with different block letters refer significance difference ($p < 0.05$, Bonferroni).

3.3. Oxidative stress measurement

3.3.1. Superoxide dismutase (SOD) assays

Before initiating heatwave stress, fish reared under Treatment-1 showed higher SOD activity in gill tissue compared to fish in the other three treatment conditions and remained almost the same until stress day 10. However, on stress day 20, fish reared under treatments 1 and 2 exhibited higher SOD activity in gill tissue than in fish reared under the control condition and Treatment-3 (Fig. 4 A). In the case of SOD activity in muscle, at no stress stage, the value was higher in fish reared at Treatment-1 than in the other three treatments. While, on day 20 of heatwave stress, SOD activity was significantly lower in fish reared under treatments 1 and 2 than in fish reared under control and Treatment-3 conditions (Fig. 4 B). For the liver, on stress day 10, SOD activity was significantly lower in fish reared under treatments 1 and 2 compared to the control condition (Fig. 4 C). SOD activities in gill and liver tissues were significantly impacted by stress duration, salinity, and interaction between these two variables. Whereas for muscle, the activity was impacted by only the interactions among salinity and diet as well as stress duration and salinity (Fig. 4, Table 5).

3.3.2. Glutathione peroxidase (GPx) assays

During stress days 10 and 20, GPx activity was measured higher (although not significant) in the gill tissue of fish reared under the control condition, followed by fish reared under treatments 1, 2, and 3 (Fig. 5 A). In muscle tissue, on stress day 10, GPx activity was observed significantly higher in fish reared in treatments 1 and 2 compared to those in Treatment 3. GPx activity was found to decrease as the heatwave stress duration progressed (Fig. 5 B). For liver tissue, until day stress 10, both non-stressed and stressed fish exhibited almost similar GPx activity (no significant difference was observed). However, on stress day 20, fish reared under treatments 1 and 2 exhibited significantly lower GPx activity compared to fish reared at the control and Treatment-3 conditions (Fig. 5 C). GPx activities in muscle and liver tissues were significantly influenced by stress duration, salinity, and the interaction between these two factors (Fig. 5, Table 5).

3.3.3. Glutathione reductase (GR) assays

In gill tissue, at the "no stress" sampling point, GR activity was

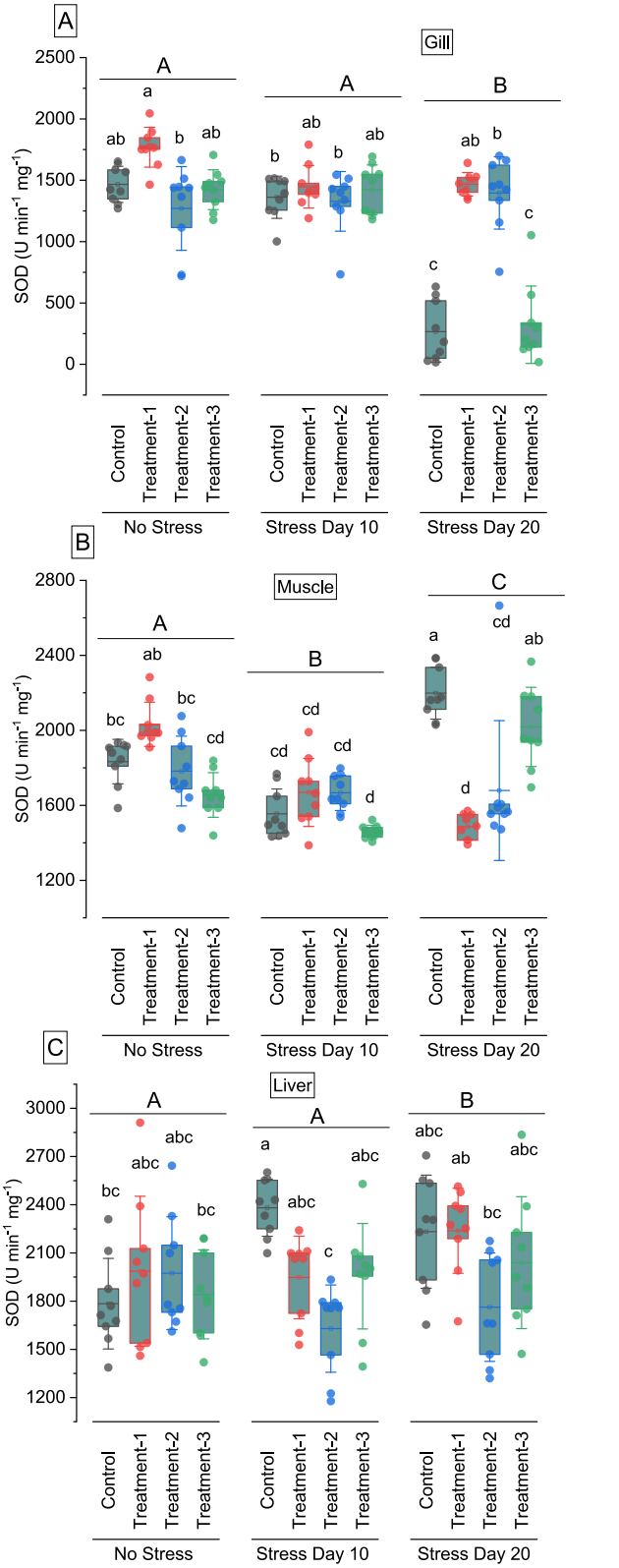

Fig. 4. Superoxide dismutase (SOD) assays conducted for European seabass reared under different salinities and dietary conditions: A. SOD activity in gill, B. SOD activity in muscle, and C. SOD activity in liver. Values are represented as mean ± SD, n = 9. On each bar different letters refer to significance differences and each sampling point labeled with different block letters refer significance difference ($p < 0.05$, Bonferroni).

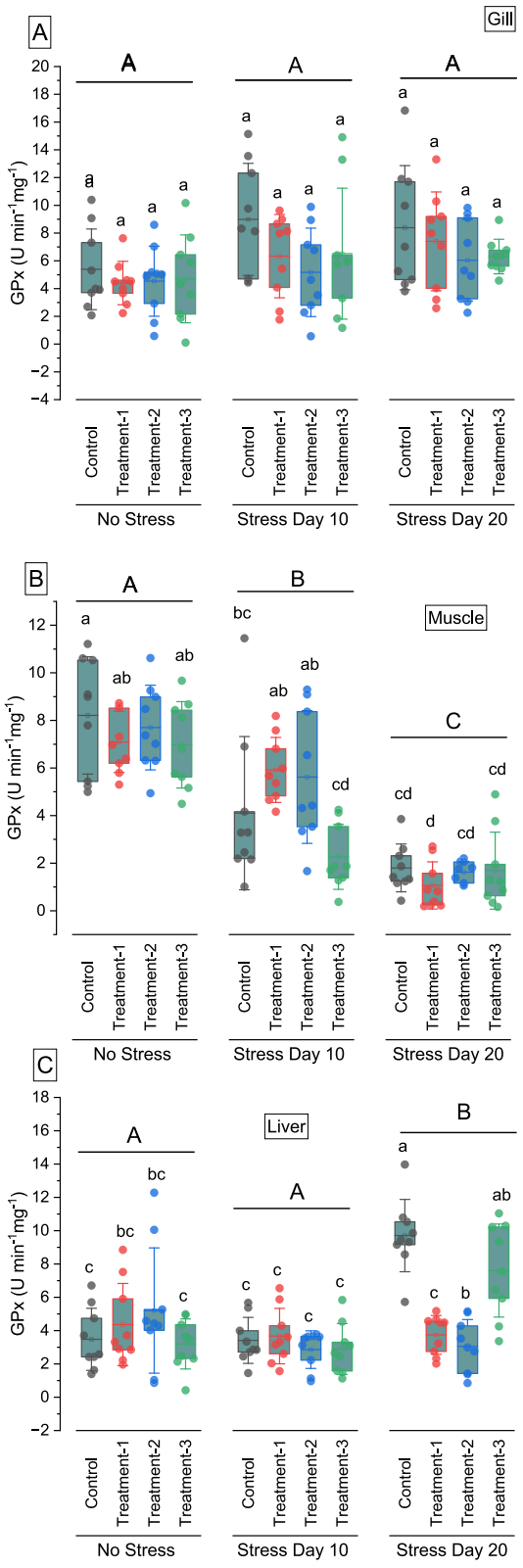

Fig. 5. Glutathione peroxidase (GPx) assays conducted for European seabass reared under different salinities and dietary conditions: A. GPx activity in gill, B. GPx activity in muscle, and C. GPx activity in liver. Values are represented as mean ± SD, n = 9. On each bar different letters refer to significance differences and each sampling point labeled with different block letters refer significance difference ($p < 0.05$, Bonferroni).

significantly lower in fish reared under Treatment-1 than those in control and Treatment-3 conditions. Whereas, on days 10 and 20, no significant influence on GR activity was observed among treatments (Fig. 6 A). Similarly, in the muscle and liver tissues, prior to starting heatwave stress no significant influence on GR activity was observed among treatments. However, on days 10 and 20, GR activity did not vary significantly among the treatments' conditions (Fig. 6 B, C). GPx activities in gill, liver, and muscle tissues were significantly ($p < 0.05$) influenced by stress duration and the interaction between duration and salinity. No significant effect of diet was observed on this antioxidant activity (Fig. 6, Table 5).

3.4. Cellular enzymatic stress measurement

Without heatwave stress, GPT levels were found to be significantly higher in fish reared under Treatment-3 compared to the control and Treatment-2 conditions. On stress day 10 of heatwave stress, fish reared in Treatment-1 exhibited significantly higher GPT levels compared to those in treatments 2 and 3. However, on stress day 20, the values showed no significant difference among the treatments (Fig. 7 A). Regarding LDH, the value was lowest in fish reared under control conditions, followed by treatments 1, 2, and 3. On stress day 10, the LDH value was measured significantly higher in fish reared under control conditions compared to fish under treatments 1 and 3. In contrast, on stress day 20, the LDH value did not vary significantly among treatments (Fig. 7 B). GOT did not vary significantly in fish among tested treatments until stress day 10 of heatwave stress. However, by stress day 20, fish subjected to Treatment 1 exhibited significantly higher GOT levels compared to fish at control and treatments 2 and 3 conditions (Fig. 7 C). GPT, LDH, and GOT activities showed significant variations ($p < 0.05$) and were notably influenced by stress duration, diet, salinity, and the interactions among these factors (Fig. 7, Table 5).

3.5. Gene expression

3.5.1. Muscle genes

Before the onset of heatwave simulation, HSP 70 gene did not vary significantly among treatments. On stress day 10, HSP70 gene was significantly upregulated in fish reared at Treatment-2 compared to the control and the other two treatments. On stress day 20, HSP70 expression was found to be upregulated but did not reach statistically significant differences in fish reared under treatments 1 and 2 (Fig. 8 A). For the Igf1 gene, until day 10 of heatwave stress, fish reared under treatments 1 and 2 showed lower expression (although not significant) compared to those in control and Treatment-3. However, on stress day 20, Igf1 was found upregulated (not significant) in fish reared under treatments 1 and 2 compared to the control condition (Fig. 8 B). On heatwave stress days 10 and 20, fish reared in treatments 1 and 2 exhibited significant FADS2 upregulation ($p < 0.05$) compared to those in control (Fig. 7 C). On stress days 10 and 20, fish subjected to treatments 1 and 2 had significantly higher TNF-1α levels ($p < 0.05$) compared to the control group (Fig. 7 D). In fish muscle, HSP70 expression was significantly ($p < 0.05$) influenced by the interaction among stress duration, salinity, and diet. In contrast, TNF-1α expression was significantly ($p < 0.05$) affected by stress duration and salinity, with no interaction effects observed for this gene. For Igf1, expression was significantly ($p < 0.05$) altered by stress duration, as well as by the interactions between stress duration and salinity, and salinity and diet (Fig. 8, Table 5).

3.5.2. Liver genes

Prior to starting heatwave stress, HSP70, Igf1, and FADS2 expressions were non-significantly higher in the liver tissue of fish reared under treatments 1 and 2 than in the control condition. On day 10, HSP70 and FADS2 expressions were almost similar in fish reared under all treatment conditions. Igf1 was downregulated (although not

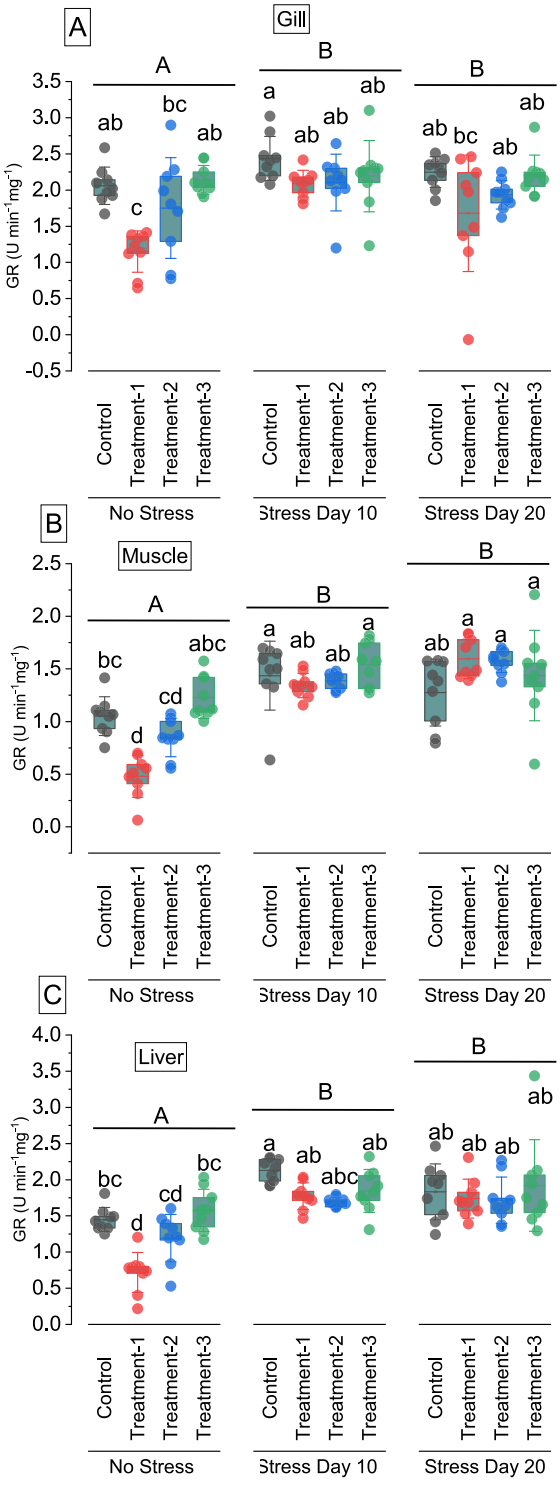

Fig. 6. Glutathione reductase assays conducted for European seabass reared under different salinities and dietary conditions: A. GR activity in gill, B. GR activity in muscle, and C. GR activity in liver. Values are represented as mean ± SD, n = 9. On each bar different letters refer to significance differences and each sampling point labeled with different block letters refer significance difference ($p < 0.05$, Bonferroni).

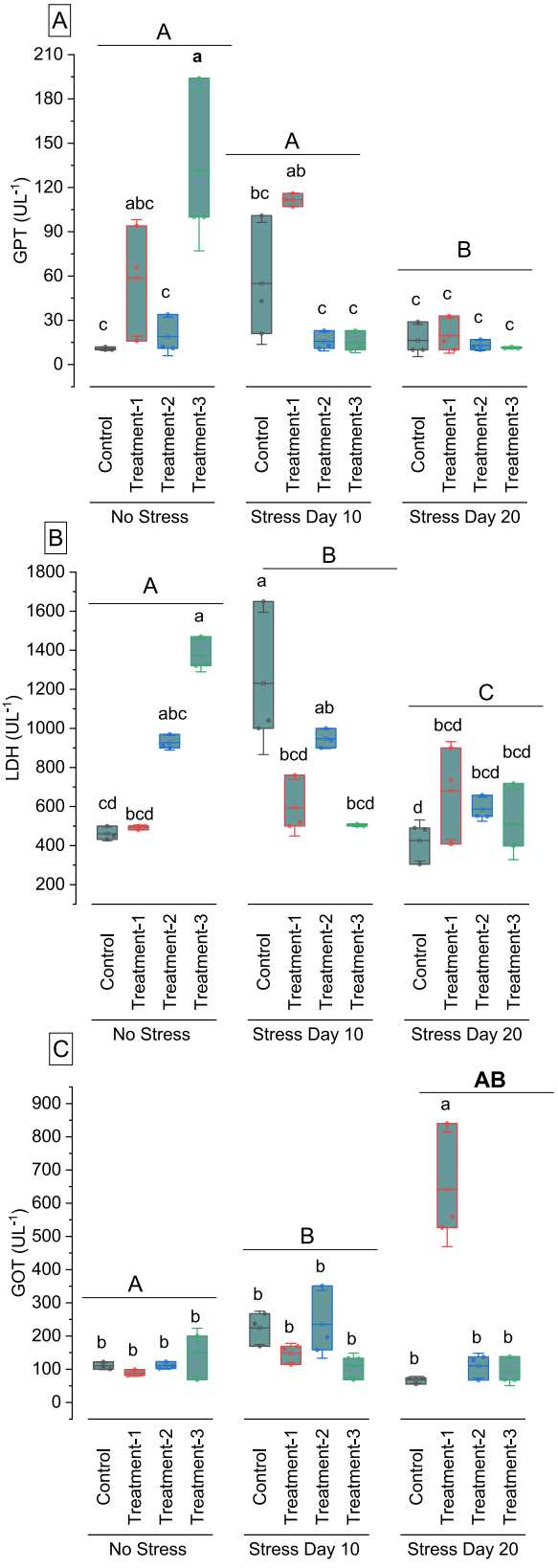

Fig. 7. Cellular enzymatic activity assays conducted for European seabass reared under different salinities and dietary conditions: A. GPT, B. LDH, and C. GOT. Values are represented as mean ± SD, n = 3. On each bar different letters refer to significance differences and each sampling point labeled with different block letters refer significance difference ($p < 0.05$, Bonferroni).

significant) in fish subjected to treatments 1 and 2 than the control condition. In contrast, on stress day 20, fish reared in Treatment-3 showed significant upregulation ($p < 0.05$) of HSP70, Igf1, and FADS2 compared to those in the control (Fig. 9 A, B, C). During the no-stress period and on stress day 10, TNF-1α expression was significantly

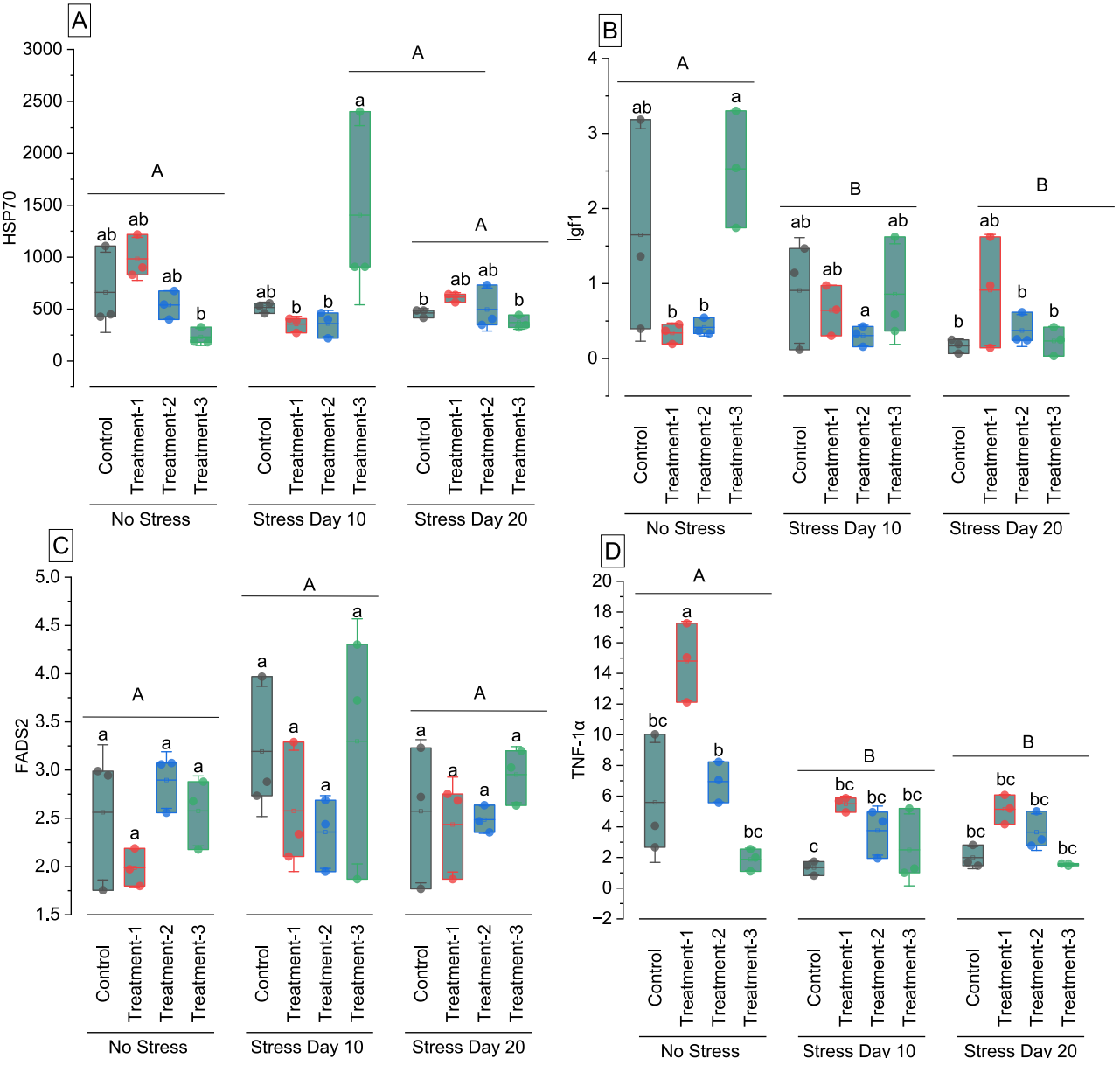

Fig. 8. Gene expression in muscle of European seabass reared under different salinities and dietary conditions: A. HSP70, B. Igf1, C. FADS2, and D. TNF-1α. Values are represented as mean ± SD, n = 3. On each bar different letters refer to significance differences and each sampling point labeled with different block letters refer significance difference ($p < 0.05$, Bonferroni).

higher ($p < 0.05$) in fish reared under Treatment-1 compared to the control condition ($p < 0.05$). On stress day 20, TNF-1α expression was observed significantly higher ($p < 0.05$) in fish reared under Treatment-3 compared to fish under the control condition (Fig. 9 D). In fish liver, HSP70 and TNF-1α expressions were significantly ($p < 0.05$) influenced by the interaction among stress duration, salinity, and diet. In contrast, TNF-1α expression was significantly ($p < 0.05$) affected by salinity, with interaction effects among stress duration, diet, and salinity. For both FADS2 genes, expression was significantly ($p < 0.05$) altered by stress duration, diet, and salinity as well as by the interactions among them (Fig. 9, Table 5).

3.5.3. *Kidney genes*

Prior to starting heatwave stress and during heatwave stress, both HSP70 and Igf1 genes were non-significantly upregulated in the kidney tissue of fish reared under treatments 1 and 2 compared to the control condition (Fig. 10 A, B). Whereas, before heatwave exposure and until stress day 10, FADS2 expression in the kidney was observed higher (but did not reach a significant difference) in fish reared under Treatment-3 followed by treatments 2, 1, and control conditions (Fig. 10 C). On stress day 10, fish reared under treatments 1 and 2 had significantly lower ($p < 0.05$) TNF-1α expression than the control condition. In contrast, on stress day 20, fish reared under treatments 1 and 2 exhibited significant upregulation ($p < 0.05$) compared to fish reared under the control and Treatment-3 conditions (Fig. 10 D). In kidney tissue, most of the studied genes were significantly influenced ($p < 0.05$) by salinity. Specifically, the TNF-1α gene was significantly affected by stress duration, diet, salinity, and the interactions among these factors (Fig. 10, Table 5).

4. Discussion

The occurrences of extreme weather events are unpredictable phenomena that can have serious consequences on fish and their habitats (Donat et al., 2016; Smith et al., 2023). Heatwaves are extreme climatic

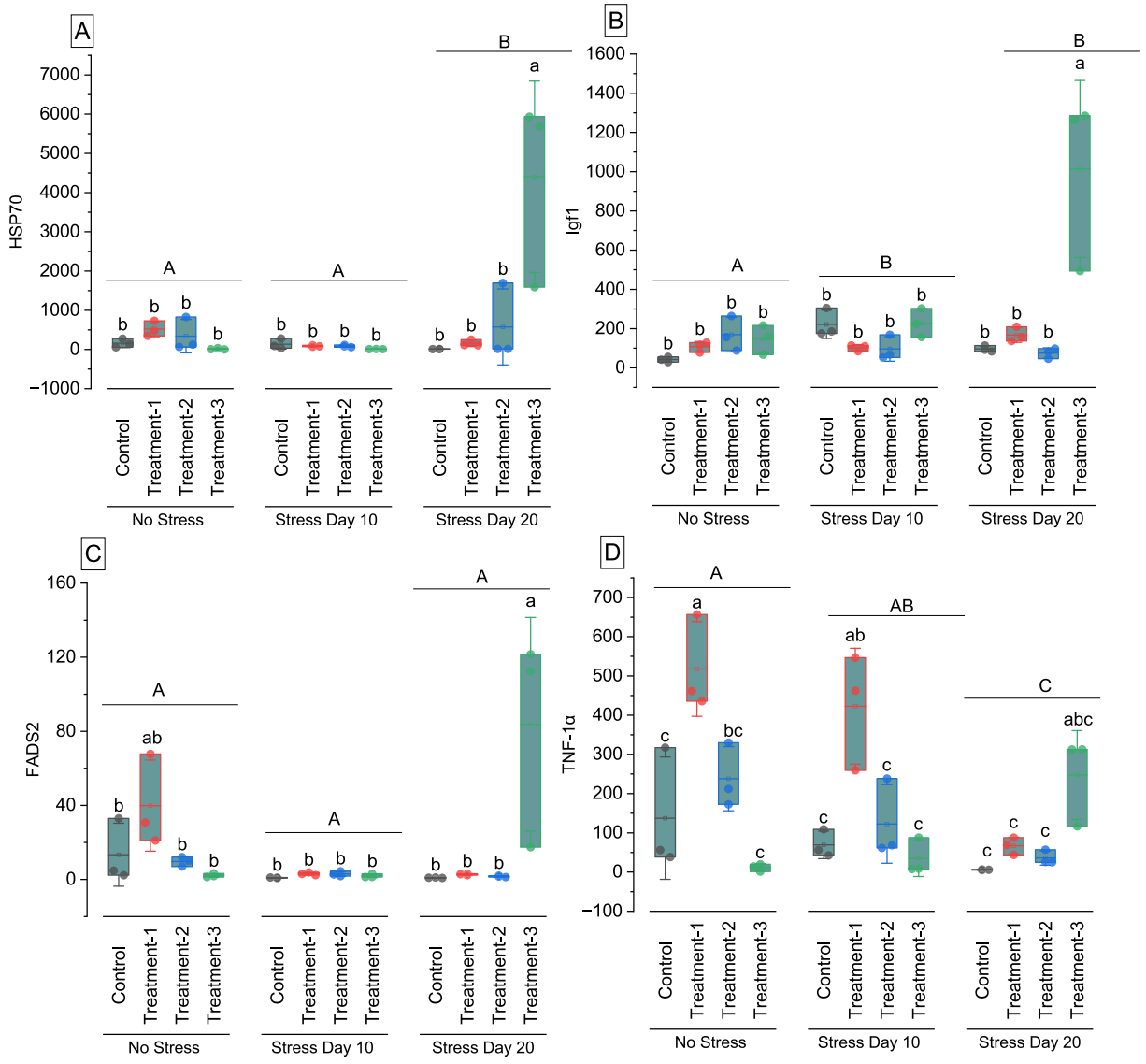

Fig. 9. Gene expression in liver of European seabass reared under different salinities and dietary conditions: A. HSP70, B. Igf1, C. FADS2, and D. TNF-1α. Values are represented as mean ± SD, n = 3. On each bar different letters refer to significance differences and each sampling point labeled with different block letters refer significance difference ($p < 0.05$, Bonferroni).

events that can cause severe stress and mortality to fish by impacting water quality, and thus fish growth, immune system, and metabolism. Therefore, it is critical to identify techniques to alleviate the detrimental impacts of heatwaves on fish (Islam et al., 2022; Naz et al., 2023). Among others, improving water quality and providing adequate nutrition and functional feed ingredients are found to be the most suitable to enhance the immunity and stress response of fish (Fawole and Nazeemashahul, 2023; Herrera et al., 2019). To find out possible mitigation options, we studied growth, feeding performance, and a range of biochemical and physiological parameters for fish while rearing at two salinities and while either feeding on propolis supplemented diet or not, followed by 20 days of heatwave (30 °C) exposure under laboratory condition. This research is intended to identify effective strategies to mitigate the detrimental effects of extreme heatwaves on European seabass. We focused our evaluation on three primary mitigation approaches: salinity management, propolis supplementation, and synergistic integration of both strategies. Each approach is poised to offer benefits, addressing growth, and various metabolic, physiological, and molecular aspects of the European seabass to enhance their resilience against heatwave stress. Through comprehensive analysis and comparison of these three strategies, this study tries to identify the most effective approach to mitigate the extreme heatwave impacts on European seabass and sustain them better in the face of a changing climate. The study indicates that both salinity and dietary manipulation play a major role in helping fish mitigate heatwave stress.

Growth and feeding performance were significantly higher in European seabass reared under 12 PSU while feeding on propolis supplemented diet (Treatment 2) compared to fish reared under 30 PSU (resembling the natural environment). The salinity preferendum of juvenile European seabass is around 15 PSU (Saillant et al., 2003b). The salinity of 12 to 15 PSU closely aligns with the blood osmotic pressure of juvenile European seabass (Hwang et al., 2018; Islam et al., 2020c). This salinity offers juvenile fish the lower energy expenditure required for osmoregulation, because of the most suitable osmotic environment and other salinity-dependent physiological functions (Islam et al., 2020c; Maulvault et al., 2017; Saillant et al., 2003a). Researchers have reported that European seabass tends to grow faster at 20 PSU and 25 °C (Yilmaz et al., 2020), or 15 PSU and 20 °C (Goda et al., 2019; Saillant et al., 2003a) along with lower feed conversion ratios. For the same fish, growth and physiological performance were better between 6 and 12

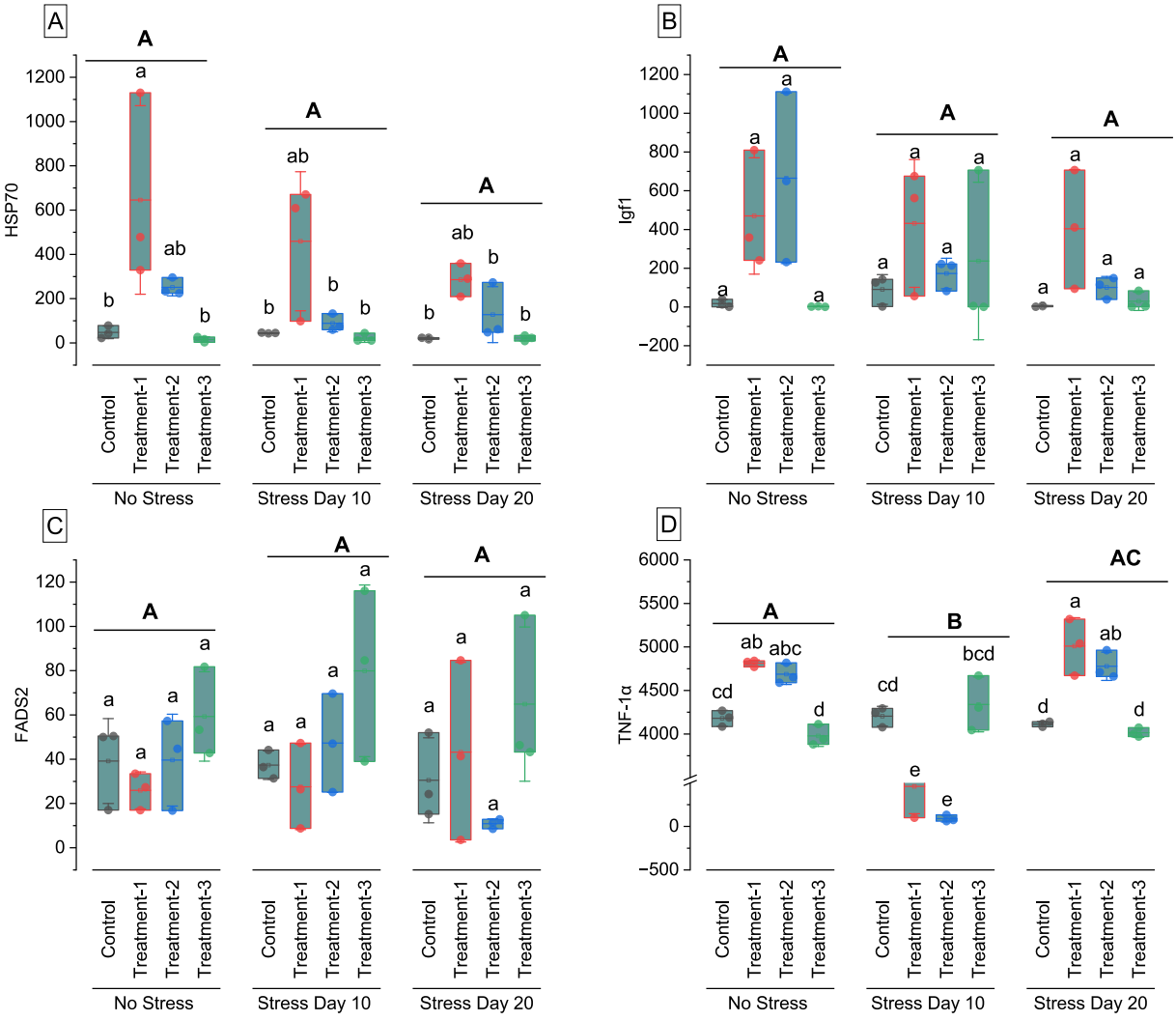

Fig. 10. Gene expression in kidney of European seabass reared under different salinities and dietary conditions: A. HSP70, B. Igf1, C. FADS2, and D. TNF-1α. Values are represented as mean ± SD, n = 3. On each bar different letters refer to significance differences and each sampling point labeled with different block letters refer significance difference ($p < 0.05$, Bonferroni).

PSU while exposed to extreme heatwave events (Islam et al., 2020c). Besides, propolis contains a range of bioactive compounds (flavonoids, phenolic acids, terpenes, and steroids) that have antioxidant, anti-inflammatory, antimicrobial, and immunomodulatory properties. It has been used as a dietary additive in fish to mitigate temperature stress and enhance the immune system (Herrera et al., 2019). Propolis can modulate cortisol, glucose, lactate, and energy metabolism. It can increase immunity and heat shock protein (HSP) expression, which help the fish from diseases, protect cells from damage, and restore their normal functions (Acar, 2018; Hassaan et al., 2019; Herrera et al., 2019; Kaplan and Erdoğan, 2021). Thus, for our study, both 12 PSU and propolis may have contributed to better fish growth performances. Lower serum cortisol content measured in fish reared at 12 PSU either feeding propolis or not (treatments 1 and 2) further justifies the better growth performance.

Increased temperature induces oxidative stress and heat shock response in different tissues of fish, which leads to an increase in reactive oxygen species (ROS) production (Roychowdhury et al., 2021). This has a subsequent impact on antioxidant defense responses through changes in SOD, GPx, and GR activities, among others (Madeira et al., 2016; Schleger et al., 2022). Fish metabolic rate increases during heatwave stress, which leads to increased oxygen intake and thus contributes to generating ROS (Forgati et al., 2017; Madeira et al., 2016; Nakano et al., 2014). To tackle the excess ROS, fish (animals) activate antioxidant defense to cope with stress adversity. In fish gill tissue, liver, and muscle tissues play a significant role in tackling excess ROS production (Chinnadurai et al., 2022; Pan et al., 2022; Xu et al., 2022). In this study, on the 20th day of heatwave stress, SOD activities were significantly higher in the gill tissue of fish reared under treatment 1 (12 PSU) and treatment 2 (12 PSU + propolis supplemented diet). This phenomenon may be attributed to the direct exposure of the gill tissue to the physico-chemical conditions of water (Bhanot and Hundal, 2021; Jiang et al., 2018). While muscle and liver tissues exhibited opposite trends. The synergistic effect of antioxidant response in fish reared under varying salinities and fed on propolis while exposed to heatwave stress was not reported before. This made it difficult to compare our results with others. Heatwave stress increases the oxygen demand, and for fish, gills act as the first port for oxygen entry. Thus, gill tissue generates a higher amount of ROS. To prevent oxidative tissue damage, gills tissue activates antioxidant machinery. We assume this is the possible cause of the observed higher SOD in gill tissue during heatwave stress. In contrast, SOD in the muscle and liver of fish reared under treatments 1 and 2 exhibited lower activities compared to those fish reared in the control condition. Lower SOD activities indicate lower oxidative damage (Bainy et al., 1996; Shen et al., 2015). On stress day 20, lower GPx activities in muscle and liver further justify this.

Before simulating heatwave stress, except for GR, both SOD and GPx activities remained the same in the gill tissue and muscles of fish reared under all experimental treatments. Whereas GR activity was observed lower in muscle, gill tissue, and livers of fish reared under treatments 1 and 2. Moreover, GR activity was found to have increased as the heatwave duration progressed. GR plays a crucial role in safeguarding hemoglobin, red cell enzymes, and biological cell membranes against oxidative damage. The increased concentration of reduced glutathione aids in neutralizing and counteracting oxidative stress, thereby protecting essential cellular components and structures from potential harm induced by oxidative processes (Chowdhury and Saikia, 2020; Sukhovskaya et al., 2017). Fish achieve this by elevating the level of reduced glutathione to maintain aerobic glycolysis during stress (Lushchak and Bagnyukova, 2006; Ming et al., 2019). Relatively stable amounts of lactate levels in fish reared under all experimental conditions indicate that during the heatwave stress, GR played an important role in mitigating heatwave stress. From this, we can assume that without heatwave stress, SOD and GPx activities in fish were not affected by the salinities and diet we employed. For the same fish, another study reported that, as a defensive strategy, fish employed SOD to detoxify ROS at a later stage (Islam et al., 2020b). However, during heatwave stress (stress days 10 and 20), SOD and GPx activities varied across tissues in fish reared under the conditions of treatments 1 and 2 compared to fish under control. During heatwave exposure, in all three measured tissues (gill, livers, and muscle), when decreased SOD activity was observed, GPx activity was found to have increased. From this observation, we can assume that during heatwave stress, SOD activity is the first ROS defense system in fish. During the heatwave simulation, fish reared under control conditions (30 PSU) exhibited higher stress by generating higher SOD, GPx, and GR activities. The increased SOD activity, while beneficial in reducing superoxide radicals, can also lead to the production of hydrogen peroxide (H_2O_2) (Cadenas et al., 2022; Hassan and Fridovich, 2022). On stress days 10 and 20, the increased H_2O_2-scavenging enzymes (GP_X and GR) activity in fish reared under treatments 1 and 2, suggests their relatively better ability to counteract oxidative damage to tissues (Sopinka et al., 2016; Tort, 2011). European seabass exposed to 32 PSU while exposed to heatwave events showed a higher degree of antioxidant responses (Islam et al., 2020b). During heatwave simulation, European seabass reared under 12 PSU while feeding on propolis supplemented diet provided the best antioxidant response.

In animals, damage to functional tissues and organs can lead to the release of certain (cellular) enzymes into the blood circulatory system (Salamat and Zarie, 2016; Sweet, 2004). Before heatwave exposure, the higher amount of GPT, GOT, and LDH in fish reared under Treatment-3 (30 PSU while fed on the propolis-incorporated diet) indicates that fish in this combination experienced impairments in metabolic and liver function (Islam et al., 2024a). This passively indicates hepatic and cellular damage and other cellular injuries. Higher levels of GPT, GOT, and LDH in fish showed damaged hepatocytes and other cellular injuries reported in several studies, for examples, silver carp, *Hypophthalmichthys molitrix* (Li et al., 2007), tilapia, *Oreochromis mossambicus* (Chinnadurai et al., 2022), and olive flounder, *Paralichthys olivaceus* (Najib et al., 2016). Further and detailed studies are required to understand hepatic functions and cellular damage in fish at lower salinities while feeding on propolis.

On stress day 10, the increased GPT and LDH activity in the serum suggests cellular and tissue damage, which might result from disturbances in normal physiological processes, such tricarboxylic acid (TCA) cycle, and the subsequent enzyme leakage into the blood circulation system through the hepatic cytosol (Baysal, 2005; Samanta et al., 2014). On stress day 20, fish reared under Treatment-1 exhibited higher LDH and GOT activities compared to fish reared under Treatment-2. This suggests that propolis might have provided some sort of metabolic and cellular enzymatic protection to fish during heatwave stress. Similar to the current investigation results, elevated activities of GPT, GOT, and LDH have been documented in yellow croaker, *Pseudosciaena crocea*, during periods of temperature stress (Ji et al., 2009). The activation of transaminases and protein catabolism can impair fish immunity and lead to skeletal muscle degradation (Nemova et al., 2021; Nemova et al., 2016).

In our study, on stress days 10 and 20, cortisol levels increased in fish reared under Treatment-3 and control conditions. Heatwave stress modulates cortisol concentrations in fish. Elevated cortisol levels trigger protein breakdown, weaken fish immunity, degrade skeletal muscle, and induce HSP70 and TNF1α gene upregulation (Basu et al., 2001; Dagoudo et al., 2023; Islam et al., 2020b; LeBlanc et al., 2012). The comparatively higher HSP70 expression in fish reared under Treatment-3 corroborates the higher cortisol level. As a molecular chaperone and due to its highly conserved nature, HSP70 protein plays a vital role in cellular and protein homeostasis (Dammark et al., 2018; Oksala et al., 2014). Upregulation of HSP70 observed in the muscle, kidney, and liver of fish reared under the conditions of Treatment-3 indicates a relatively higher stressful condition compared to those fish in treatments 1 and 2. Thermal stress has been linked to the induction of HSP70 in a variety of fish species, including European seabass (reviewed in Islam et al., 2022). Igf1 is considered an important gene involved in growth regulation (Duran et al., 2022; Liu et al., 2022). Higher muscle growth is linked with higher growth. Igf1 expression in muscle provides more accurate information about growth status than in metabolic organs e.g., liver and kidney (Fuentes et al., 2013; Pfaffl et al., 1998; Reichel et al., 2000). Stress and growth genes are physiologically linked in animals, including fish. The expression of Igf1 in fish is influenced by factors such as nutritional status, environmental temperatures, and salinity levels (Nakano et al., 2015; Nakano et al., 2013; Reichel et al., 2000). Our study observed significantly higher expression of Igf1 in the muscle of fish reared under Treatment-3 than in the control, which contrasts with HSP70 regulation. However, in the present study, an inverse relationship between HSP70 and Igf1 has been observed, which contrasts with previous studies (Eissa et al., 2017; Fiocchi et al., 2020). This could potentially be attributed to the study design, fish size, and feed employed in this research. Further comprehensive studies are warranted to thoroughly understand the intricate relationship between HSP70 and Igf1 expression in fish during heatwave exposure, particularly rearing under varying salinities and dietary intake. In this study, TNF-α gene was significantly upregulated in fish reared under treatments 1 and 2 conditions. This upregulation of the TNF-1α gene is indicative of an increased immune response and a more robust immune performance (Burke et al., 2014; Yamada et al., 2009). The upregulation of TNF-α in fish under treatments 1 and 2 may be linked to a stronger humoral innate immune response during heatwave exposure. In marine teleosts, including European seabass, during high-temperature stress, TNF-α upregulation has been documented (Islam et al., 2020b; López-Castejón et al., 2007).

FADS2 plays a critical function in the production of long-chain polyunsaturated fatty acids (LC-PUFA) (Dong et al., 2023). During acclimation to temperature stress, FADS2 expression becomes widespread, enabling the insertion of double bonds to saturated fatty acids. In fish, FADS2 functions as the only enzyme for desaturation processes in the LC-PUFA pathway (Kabeya and Tocher, 2017; Zhao et al., 2020). In our study, fish reared under Treatment-3 and the control conditions exhibited FADS2 upregulation, while those in treatments 1 and 2 exhibited downregulation. The higher expression of FADS2 may be related to higher metabolic activities and increased energy demand during heatwave stress in the control condition [full-strength saline water, 30 PSU]. Dietary components impact the expression of FADS2 (Koletzko et al., 2019; Lankinen et al., 2018). Furthermore, the findings of this study align with those of Koletzko et al. (2019), who highlighted that FADS2 is regulated by various factors, including dietary components and thermal conditions. During heatwave stress exposure, research conducted on zebrafish demonstrated that very long-chain saturated fatty acids, synthesized by FADS2 involved in the LC-PUFA pathway, contribute positively to heatwave mitigation in fish. Furthermore, modifications in membrane fluidity due to lipid unsaturation,

triggered by changes in diet composition, enhance thermal protection, aiding rainbow trout in tolerating and adjusting to summer conditions (Almaida-Pagán et al., 2015; Lankinen et al., 2018). These findings indicate that lipid saturation plays an important role in temperature tolerance and acclimation. However, more research is necessary to fully comprehend the FADS2 role in fish during heatwave events.

5. Conclusions

The findings of this study suggest that, during heatwave exposure European seabass responded differently to heatwave stress duration, propolis supplementation, salinity and the interactions among them. Fish raised in 12 PSU regardless of propolis supplementation exhibited higher growth performance and lower levels of stress, immunity, metabolic, cellular, and oxidative responses. Despite the fish being eurythermic and euryhaline, the interplay among diet, salinity, and heatwaves significantly influences growth, stress, metabolic and physiological activities. The interactions observed among the examined tissues demonstrated varying responses during exposure to the 30 °C heatwave. For measured parameters, varying patterns observed across tissues suggest that the stress response in fish is influenced not only by the propolis-supplemented diet but also by salinity during heatwave exposure. Fish reared at higher salinities (30 PSU) while feeding on propolis supplemented diet also provide some sort of physiological benefits during heatwave stress. Overall results indicate that during heatwave exposure, European seabass can fare best at 12 PSU while fed on propolis supplemented diet. Thus, manipulating salinity and incorporating propolis into the diet could be effective in mitigating the adverse effects of extreme heatwaves in farmed European seabass. These strategies could be beneficial to improve growth, mitigate oxidative stress, and enhance the overall fitness of fish during heatwave events.

CRediT authorship contribution statement

Md Jakiul Islam: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Amir Hossain:** Writing – review & editing, Validation, Methodology, Formal analysis. **Andreas Kunzmann:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Conceptualization.

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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Data availability

The data produced during this study are available on request from the corresponding author.

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