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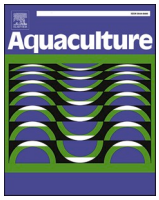

Mitigation of extreme winter stress in European seabass, *Dicentrarchus labrax* through dietary supplementation

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ABSTRACT

Climate change is driving the dangerous confluence of extreme weather events. Among others, aquatic ecosystems are the most affected one. This study sheds light on how dietary supplements affect the physiological responses of European seabass during extreme winter exposure. Fish were provided with three dietary supplements: vitamins C and E (diet-1), propolis (diet-2), and phycocyanin (diet-3) for 45 days. They were then exposed to a simulated extreme winter cold (7 °C) for 20 days. The results showed that dietary supplements do not have a significant impact on weight, growth rate, and protein efficiency ratio before cold exposure. Levels of Na + and Cl − in the fish remained stable across all diets during both control (20 °C) and 7 °C exposure. However, on the 20th day of cold exposure, fish fed diet-3 exhibited notably higher levels of K+ compared to the other two diets, whereas blood glucose was significantly lower in fish fed diet-1. On day 20, significantly higher GPT activities were observed in fish fed diet-1. No significant effects of dietary supplementation were observed in fish serum cholesterol and protein levels. Before cold stress, fish fed the control diet exhibited significantly lower triglyceride levels ($p < 0.05$). However, during cold exposure, triglyceride levels increased in fish fed diet-1 and the control diet. On day 20, fish fed diet-3 exhibited a comparatively higher lactate content. Conversely, cortisol levels were significantly lower ($p < 0.05$) in fish fed diets 2 and 3 on the same day. No dietary effects were observed on fish muscle HSP70 gene expression. On days 10 and 20, SREBP1 was significantly upregulated in fish fed all diets. On day 20 of cold exposure, FADS2 and GLUT2 genes were downregulated in the fish fed control diet compared to those fed diets 2 and 3. Before cold stress, fish on the control diet showed higher TNF-1α expression in the spleen. SREBP1 showed a significant upregulation on day 10 in fish fed diet-2. FADS2 gene expression remained unchanged during cold stress. GLUT2 expression showed no noticeable effects throughout the study. Overall, during extreme cold exposure, we observed decreased levels of blood cortisol, transaminase, dehydrogenase, and metabolites, along with a comparatively higher upregulation of growth, metabolic, and immune genes in fish fed diets supplemented with propolis, phycocyanin, and vitamins C&E. These results suggest that dietary supplements can help European seabass cope better with extreme cold, at least for short periods of up to 20 days.

1. Introduction

The frequency and intensity of extreme temperature events induced by climate change are increasingly evident (Smith et al., 2023). Temperature is the master abiotic factor in aquaculture, controlling and constraining fish development and physiology at all stages (Reid et al., 2022; Sopinka et al., 2016). Fundamental physiological limitations at the individual level underlie alterations in fish growth, physiology, reproduction, metabolism, and immunity during extreme temperature events. The effects of extreme temperatures on fish vary depending on stress magnitude and associated factors (Islam et al., 2022). Extreme weather events are becoming more intense and frequent, imposing substantial constraints on both fish and aquaculture systems (Galappaththi et al., 2020). European seabass is one of the dominant aquaculture fish in southern Europe and the Mediterranean. The aquaculture industry for European seabass has experienced robust growth since the

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early 1990s and stands as one of the most significant activities in this sector. This fish is primarily cultivated intensively in the coastal waters of southern Europe, with production reaching 235,537 tons in 2001 (seafish.org, 2022). This sparid teleost inhabits the Mediterranean Sea, the eastern coast of the Atlantic, from southern Norway to Senegal, and the Baltic Sea. Estuaries, lagoons, coastal waters, and rivers are among the most common habitats of this fish (O'Neill, 2017; Vandeputte et al., 2019). European seabass aquaculture has benefited from a better understanding of the optimal requirements for reproduction and growth (reviewed in Kousoulaki et al., 2015). However, this fish is vulnerable to lower temperatures, which can cause so-called winter disease or winter syndrome and, in some cases, may lead to high mortalities (Islam et al., 2021b; Yilmaz et al., 2020). European seabass cannot acclimatize properly in ponds with low temperatures (<13 °C) (Nathanailides et al., 2010). When winter temperatures drop below 5 °C (Nathanailides et al., 2010) or even at 8 °C (Islam et al., 2020a), mass mortality occurs. Thus, farming this fish at (seasonally) low temperatures is problematic (Nathanailides et al., 2010).

A critical temperature of 12 °C is indicated for the European seabass, below which the fish cease feeding (Pastoureaud, 1991). In the wild, during the winter when surface water temperatures decrease, European seabass migrate to deeper, warmer waters (Kousoulaki et al., 2015), which is not possible for farmed fish. Thus, winter disease commonly affects cultured European sea bass (Islam et al., 2020a). The optimal temperature for juvenile European seabass growth is 20-24 °C. During the winter season, growth stops when the temperature goes down to 10 °C, and a further decline below 7 °C results in a cessation of food intake (Dülger et al., 2012). Winter represents a crucial phase, particularly for the young of the year (Pastoureaud, 1991), regardless of the aquaculture system employed. Water temperatures can drop to 0–5 °C in semi-intensive culture systems in salt marshes along the Atlantic coast of France. In the northern region of the Mediterranean, there has been a recent increase in the occurrence of low winter temperatures accompanied by sporadic frost events (Aranda et al., 2005; Larcher, 2000; Simon et al., 2022). For example, in the southern part of Turkey and the north-western part of Italy, there were occurrences where winter temperatures abruptly dropped from approximately 20 °C to 9.5–10.6 °C (Besson et al., 2016; Llorente and Luna, 2013). Moreover, the lowest average winter water temperatures were documented in some Mediterranean estuaries, e.g. Balgzand (10.6 °C ± 4.5) and Vilaine (12.6 °C ± 3.7) (Vaz et al., 2019). Several physiological, metabolic, and immunological problems have been described in European seabass during extremely cold winter farming episodes (Islam et al., 2022; Shahjahan et al., 2022; Yilmaz et al., 2020). These include ionic imbalances, impaired digestive systems, altered hematological and liver functioning, and immune suppression that renders the fish more vulnerable to infection and diseases (de Moraes et al., 2018; Dengiz Balta et al., 2017; Islam et al., 2020a, 2021b). During cold stress, these symptoms appear to be exacerbated not just by lower feed intake and fasting periods, but also by a reduced capacity to digest and absorb nutrients (Ciji and Akhtar, 2021). Therefore, specifically in farming conditions, it is essential to increase the ability of European seabass to cope better with extreme winter events.

In recent years, one of the most promising areas of research has been the development of strategies to mitigate the negative effects of stress in aquaculture fish. This can be done by employing water quality control, feeding and feed quality management, site shifting, and stocking density optimization, among others (Ciji and Akhtar, 2021; Engle et al., 2017). However, these options are not always feasible. For example, while fish translocation is increasingly being considered to address long-term environmental issues, it is not feasible to reduce the effects of rapid climate change (Hoffmann and Sgró, 2011; Sorby et al., 2018). In some recent studies, dietary management strategies such as supplementation of functional feed ingredients have been reported as a promising option to ameliorate winter cold stress in teleosts (Hassaan et al., 2019; Kumar et al., 2018; Turchini and Nie, 2021; Župan et al., 2015). Different feed additives or nutraceuticals (vitamins C, E, pyridoxine, lecithin, propolis, phycocyanin, and β-glucan) have been found effective in mitigating stress effects (Hajirezaee et al., 2020; Dawood et al., 2020; Fawole and Nazeemashahul, 2023). When their diets were supplemented with the mentioned ingredients, a range of fish species demonstrated improved growth and physiological responses, e.g., gilthead seabream, *Sparus aurata* (Ibarz et al., 2010), Indian carp such as mrigal, *Cirrhinus mrigala*, and rohu, *Labeo rohita* (Kumar et al., 2014, 2018; Tejpal et al., 2014), and tilapia, *Oreochromis niloticus* (Dawood et al., 2020; Hassaan et al., 2019). Dietary ingredients, for instance, propolis (reviewed by Farag et al., 2021), phycocyanin (Zhang et al., 2020), and vitamins C and E (Dawood and Koshio, 2018; Gao et al., 2014), have been getting increased attention to mitigate stress effects in fishes. In a prior study on European seabass, we observed that fish fed on diets enriched with vitamins C and E, pure propolis, and pure phycocyanin performed better during extreme heatwave events (Islam et al., 2021a). Across a range of studies, fish have shown beneficial outcomes with specific supplements: vitamin C at levels of 0.05–4.0 g kg^{-1}; vitamin E at 1.2–3.0 g kg^{-1} (Betancor et al., 2012; Ortuño et al., 2003); propolis ranging from 2 to 10 g kg^{-1} (Farag et al., 2021; Islam et al., 2021a); and phycocyanin as algae extract within the range of 0.2 to 0.50 g kg^{-1} (Elabd et al., 2020; Hassaan et al., 2021; Jin et al., 2020).

Propolis is rich in antiseptic, antimicrobial, antioxidant, and anti-inflammatory compounds. Animals fed propolis-supplemented diets have shown improved growth and immunity (Hassaan et al., 2019; Kaplan and Erdoğan, 2021). Phycocyanin-supplemented diets have been found effective in enhancing growth, immunity, oxidative stress, and other physiological performance in fishes (Hassaan et al., 2021; Jin et al., 2020; Pérez-Legaspi et al., 2020; Zhang et al., 2020). However, the direct utilization of pure phycocyanin as a feed supplement for mitigating winter stress in fish remains unknown. Furthermore, vitamins C and E are natural antioxidants that protect animals from oxidative stress. Due to their complementary effects, combined application of these two vitamins is advised for animal diet formulation (Betancor et al., 2012; Gao et al., 2014; Narra et al., 2015). However, for European seabass, there is a dearth of information about the potential of supplementing diets with propolis, vitamins C and E, and phycocyanin to alleviate extreme winter stress.

For fishes, parameters such as growth, individual fitness, physiological performances, metabolic and molecular stress responses are considered valuable indicators to assess thermal stress impacts (Islam et al., 2022; Shahjahan et al., 2022). Given the intricate relationship among growth, physiology, metabolism, and molecular networks, the multilayers of biomarkers are considered reliable for comprehending stress status in fish (Islam et al., 2022; Shahjahan et al., 2022; Zafalon-Silva et al., 2017). We hypothesize that dietary supplementation with propolis, vitamins C and E, and phycocyanin could help fish fare better when exposed to extreme winter events. A variety of parameters associated with growth, physiology, and metabolism were measured to evaluate this hypothesis.

2. Materials and methods

2.1. Diets formulation and dietary supplements

We used the same dietary formulation that was employed in our previous study on the effect of dietary supplements on heat stress (Islam et al., 2021a). Four diets were formulated that met the nutritional requirements of European seabass but differed in their supplemental components [vitamin C & E, propolis, phycocyanin, and no supplement (control diet)] (Table 1). All components were finely mixed, pelleted (2.0 mm) through cold extrusion, and stored at −4 °C before being fed to the fish. Hereafter, the diets formulated with vitamins C & E, propolis, and phycocyanin are referred to as 'Diet-1″, "Diet-2″, and "Diet-3″, respectively. The nutritional contents of the formulated diets were evaluated according to AOAC (1995). In this study, L-ascorbic acid, and

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

Diet composition (g kg^{-1})	Diets			
	Control	Diet-1	Diet-2	Diet-3
Fishmeal LT	280.00	280.00	280.00	280.00
Fishmeal 60	200.00	200.00	200.00	200.00
Fish hydrolysate	25.0	25.0	25.0	25.0
Soya concentrates	50.0	50.0	50.0	50.0
Gluten (wheat)	55.0	55.0	55.0	55.0
Gluten (corn)	50.0	50.0	50.0	50.0
Soybean meal	90.0	90.0	90.0	90.0
Wheatmeal	55.0	55.0	55.0	55.0
Peas (Whole)	50.0	50.0	50.0	50.0
Fish oil [h]	135.0	135.0	135.0	135.0
Vitamin and mineral	10.0	10.0	10.0	10.0
Vitamin-C	–	4.0	–	–
Vitamin-E	–	3.5	–	–
Propolis	–	–	4.5	–
Phycocyanin	–	–	–	0.03
Nutritional composition (g kg^{-1})				
Dry matters	951.10	964.10	932.20	974.90
Crude proteins	549.10	540.70	553.00	534.30
Crude lipids	189.20	183.40	170.10	160.50
Ash	108.60	105.60	109.30	107.50
Carbohydrate	153.10	170.30	144.00	197.70
Phosphorous	14.40	13.90	14.40	14.00
Gross energy (kJ g^{-1})	233.10	231.50	232.10	230.40

α-tocopherol (Sigma Aldrich, Germany) were used as vitamin C and E supplements. Pure propolis extracted from beehives (Bioland, Germany) was used. *Spirulina platensis* extracts were used as pure phycocyanin (99.99%) (Spirulysat®, MiAL, Germany).

2.2. Experimental fish, initial health check, and rearing conditions

Juvenile European seabass originated from the Poissons du Soleil hatchery, France. The fish were kept in a 1000 L tank for 14 days [20 °C and 30 PSU, 12:12 h light: dark regime] at the Alfred Wegener Institute for Polar and Marine Research (AWI), Germany. The initial health condition of the fish was checked by the supplier. Furthermore, fish feeding and behaviour were routinely monitored during the 14 days of initial acclimatization. Then fish were transferred to the experimental tanks located in the same facility, maintaining the same water quality parameters stated above and monitored for another 12 days. During the 12-day acclimatization period at the experimental site, we carefully monitored fish behaviour including normal movement, schooling pattern, shyness, boldness, and feeding behaviour. By the end of the first week of acclimatization in experimental tanks, the fish exhibited completely normal behaviour comparable to their pre-transfer state. The experiment was conducted at AWI in a temperature-controlled recirculatory water system outfitted with twelve fiberglass rectangular tanks (75 cm × 45 cm × 45 cm) with a constant supply of filtered seawater. Biofilters, UV-light, lava stone, continuous aeration, and thermostatically regulated heaters and coolers were used to maintain the desired water quality. After 12 days of acclimatization to the experimental conditions [20 °C, 30 PSU, 12:12 h light: dark regime], 180 juvenile European seabass, *Dicentrarchus labrax* [initial body weight: 12.7 ± 1.58 g, $n = 180$, Max =16.5 g, Min = 9.0 g] were randomly assigned into the tanks [15 fish $tank^{-1}$]. This particular size of fish was chosen because juveniles are less likely to have experienced winter stress in natural conditions before the experiment, because small fishes are generally more sensitive and responsive to stress (Feugere et al., 2021; Knight, 2022; Sloman and Mcneil, 2012; Vagner et al., 2019), and because small fishes recover faster from stress, which allowed to analyse multiple stress responses at three sampling points without compromising survival. After 45 days of feeding trial, fish were subjected to a simulated winter cold stress event (7 °C). To emulate cold stress, water temperature was ramped down (~3 °C day^{-1}) from 20 °C to 7 °C and maintained at this temperature for 20 days. Stress duration was counted since the temperature reached 7 °C. Throughout the experiment, fish were fed with the tested diets twice a day (9.00 and 16.00 h) to apparent visual satiety. Uneaten feed and faces were cleaned daily. To ensure optimum water quality, DO (>6.0 mg L^{-1}), NH_3 (<0.05 mg L^{-1}), NO_3^- and NO_2^- (<0.2 mg L^{-1}) were maintained throughout the experiment. Furthermore, approximately 50–60% of seawater (30 PSU, prior temperature-adjusted) was exchanged two times per week to prevent nitrogenous waste accumulation. The experiment was performed following the EU Directive (2010/63/EU) regarding the protection of animal rights for scientific research approved by the Bremen State Veterinary Authority (authorization code TVA 153).

2.3. Sample collections

Previous experience with the response European seabass to extreme winter stress (Islam et al., 2021) motivated us to explore potential remediation/mitigation measures to fare better during extreme winter stress. In the present study, we therefore followed a similar sampling scheme as the one in Islam et al. (2021). Before the onset of extreme winter stress, we conducted a 45-day feeding trial following common practice in the literature (Jovanović et al., 2018; Koch et al., 2021; Kumar et al., 2014; Radhakrishnan et al., 2024; Yar Ahmadi et al., 2014). After the 45-day feeding trial, three fish from each tank (a total of nine fish per treatment) were collected at three different sampling points. These sampling points included the day before the commencement of cold stress and days 10 and 20 of the extreme cold exposure, hereafter denoted as 'Day 0', 'Day 10', and 'Day 20', respectively. Before sampling, fish were fed-deprived for 24 h before being dissected in the post-absorptive state. Immediately after sampling, fish were anesthetized by immersing them in MS-222 (50 mg L^{-1}) (Topic Popovic et al., 2012). Following anesthesia, fish weight was recorded, and blood samples were taken through caudal vein puncture with non-heparinized syringes. Blood samples collected from fish in each replicate tank were pooled together to get enough volume for analysis ($n = 3$), which is a common procedure in fish and small animal (Alfano et al., 2015; Armesto et al., 2014; Corrêa et al., 2017; de Mattos et al., 2019; Fiess et al., 2007; Hossain et al., 2019; Pérez-Sánchez et al., 2017). Serum was collected from whole blood after centrifugation at 4000 ×*g* for 15 min. Furthermore, muscle and liver samples were taken from the same fish that was used to obtain blood. Tissue samples were flash-frozen in liquid nitrogen and stored at −80 °C until further analysis. It is to be noted that the number of samples per time point and treatment reported in the manuscript (n = 3) refers to the number of pooled samples, which is 3 times lower than the number of fish that were actually considered ($n = 9$ per treatment and time point). We also note that the initial health status of the fish on the different tested diets was assessed at Day 0 (20 °C) before the onset of extreme winter stress with all the parameters employed in the study at Day 0 (20 °C). This sampling point was used as a non-stress control.

2.4. Growth performance

After the 45-day feeding trial (Day 0), fish were sampled to assess the following growth performance parameters. The fish average weight of each replicate tank was determined by dividing the bulk weight by the number of individuals ($n = 15$). The following equations were used to calculate growth performance:

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

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

$$\text{Protein efficiency ratio(PER)} = \frac{\text{Live weight gain (g)}}{\text{Dry protein intake (g)}} \text{X } 100$$

2.5. Measurement of metabolic and cellular stress responses

Serum metabolites (glucose, triglycerides, cholesterol, protein, urea, creatine, and urea), serum osmolytes (Na+, K+, Cl-), and cellular enzymes [glutamic-pyruvic transaminase (GPT), glutamic-oxaloacetic transaminase (GOT), and lactate dehydrogenase (LDH)] were measured using an automated blood analyzer (Fuji Dri-CHEM NX500i). Serum cortisol was measured with a commercial kit (Cortisol Saliva ELISA, IBL International, Germany) following the manufacturer's instructions. Blood lactate was measured from the whole blood of each fish (n = 9) using Lactate Scout (EKF Diagnostics, Germany).

2.6. Gene expression analysis

Muscle and spleen tissues were analyzed for mRNA expression levels. Total RNA was extracted using RNA Miniprep (Monarch, USA) following the manufacturer's instructions. The quality and quantity of RNA were evaluated in a random subset of samples by gel electrophoresis alongside a 1.0 kb DNA ladder. Synthesis of cDNA was performed with 1 μg of the extracted total RNA with the RevertAid cDNA synthesis kit (Thermo Fisher Scientific). Gene expression levels were determined by q-PCR (CFX Manager™, Bio-Rad, USA). The analyses were conducted using a mixture consisting of 1 μL of diluted cDNA, 0.5 μL of each primer (at a concentration of 10 μM), 12.5 μL of SYBR Green (Roboklon, Germany), and 5.5 μL of DEPC treated water (Thermo Fisher Scientific), resulting in a total volume of 20 μL. Thermal cycling was initiated with incubation at 95 °C for 3 min, followed by 40 cycles of 95 °C for 15 s (denaturing), 54–60 °C for 30 s (annealing), and 72 °C for 20 s (extension). Previously used and validated gene primers used for the same species were employed in the present study (Table 2). The quantification of target mRNA expression levels was performed using the housekeeping gene elongation factor 1α (El 1α). The mRNA expression was quantified with the $2^{(-\Delta\Delta CT)}$ method, following the principles of normalized relative quantification (Rao et al., 2013; Steibel et al., 2009).

2.7. Statistical analysis

The data were checked for normality and homoscedasticity using the Kolmogorov-Smirnov and Levene tests, respectively. In instances where the assumption of homoscedasticity was violated and the distribution was non-normal, the data were log-transformed to better align with the normality assumption. To assess the impact of diets, the duration of cold stress, and their potential interactions, statistical comparisons were performed using a two-way MANOVA. In this analysis, we followed a 2 × 2 factorial design, with diet and duration considered as fixed factors. In the case of growth data, the one-way MANOVA was applied to compare growth performance among dietary groups. Both types of MANOVA used in this analysis were followed by a Bonferroni post hoc correction to account for multiple comparisons. Greenhouse-Geisser adjustment was employed when the Mauchly sphericity assumption was not met. The statistical significance level of 0.05 was used as the threshold for null hypothesis rejection.

3. Results

3.1. Growth performance

For all tested diets, the final weight of the fish almost tripled during the 45 days of trial (initial weight ~ 12.5 g, final weight ≥ 32 g, $n = 3$ per treatment). There were no significant effects of vitamin C&E (Diet-1), propolis (Diet-2), and phycocyanin (Diet-3) inclusion on final weight (FW), weight gain (WG), specific growth rate (SGR, %), and protein efficiency ratio (PER, %) (Fig. 1).

3.2. Serum ions

The mean [Na+] and [Cl−] concentrations remained stable in the serum of fish fed on all four tested diets (control, diet-1, diet-2, and diet-3) during exposure to both 20 °C and 7 °C (Fig. 2A and Fig. 2C). Surprisingly, on day 10, the concentration of [K+] was significantly and drastically lower (almost zero) in all tested dietary groups. However, by day 20 of cold exposure, fish fed diet-3 exhibited significantly higher levels of [K+] (Fig. 2 C).

3.3. Serum metabolites

3.3.1. Glucose, cholesterol, triglycerides, and protein concentrations

Throughout the 45-day feeding period at 20 °C and up to day 10 of extreme cold (7 °C) stress, no discernible diet-related effects were observed in serum glucose levels. However, on the 20th day of cold stress, fish that were fed diet-1 displayed significantly ($p < 0.05$) lower glucose levels in comparison to those fed the control diet (Fig. 3A). No significant dietary effects were observed for total cholesterol and protein levels in fish fed all four experimental diets (Fig. 3B and Fig. 3C). Before the commencement of cold stress (Day 0), the triglycerides level was significantly ($p < 0.05$) lower in fish fed the control diet compared to those fed diets 1, 2, and 3. During the extreme cold exposure, fish fed diets 2 and 3 exhibited significantly higher levels of triglycerides than fish fed diet-1 and the control diet (Fig. 3D).

3.3.2. Lactate and creatinine concentrations

On day 0 (before starting cold stress), lactate concentration was significantly decreased in fish fed on diet-3 than those fed the control

Table 2
Realtime RT-PCR primers details.

Genes	Primer (5′-3′)		Accession No.	References
HSP70	Forward:	GTCTGGACAAAGGCAAGAGC	MG711592.1	(Enes et al., 2006)
	Reverse:	TTGTGAGAGGGCCAAGAGAA		
TNF-α	Forward:	GCCAAGCAAACAGCAGGAC	DQ200910	(Azeredo et al., 2015)
	Reverse:	ACAGCGGATATGGACGGTG		
Igf1	Forward:	ATGTACTGTGCACCTGCCAA	GQ924783.1	(Islam et al., 2020a)
	Reverse:	CTTTGTGCCCTGCGGTACTA		
SREBP1	Forward:	CTGGAGCCAAAACAGAGGAG	FN677951	(Geay et al., 2010)
	Reverse:	GACAGGAAGGAGGGAGGAAG		
GLUT2	Forward:	GAGCCCACGGTACCTTTACA	EF014277	(Castro et al., 2015)
	Reverse:	CGGATCAAAGAAAGGATGGA		
FADS2	Forward:	CCTTCACTGCTTTCATCCCAA	EU439924	(Geay et al., 2010)
	Reverse:	CCCAGGTGGAGGCAGAAGAA		
El-1α	Forward:	AGATGACCACGAGTCTCTGC	FM019753	(Mitter et al., 2009)
	Reverse:	CTTGGGTGGGTCGTTCTTG		

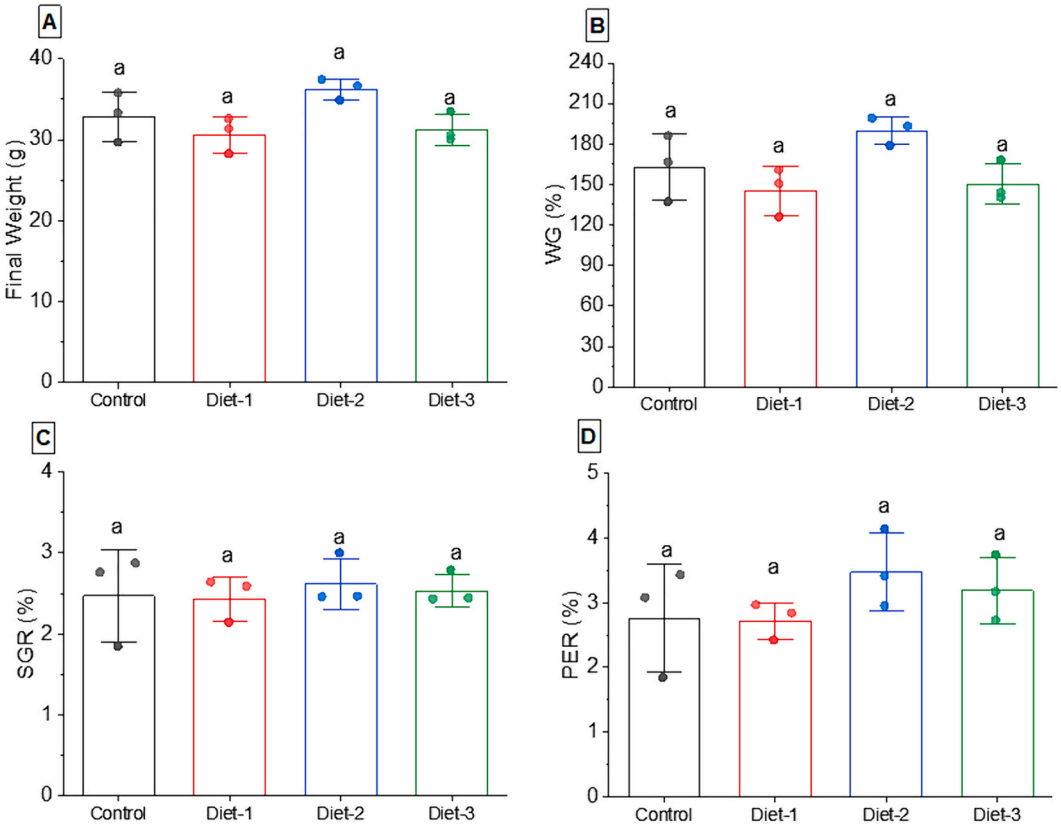

Fig. 1. Growth response parameters of European seabass juveniles reared for 45 days at 20 °C. (A) Final weight (g), (B) Weight gain (WG, %), (C) Specific growth rate (SGR, %), and Protein efficiency ratio (PER.%). Values are means ±SD, $n = 3$. Different letters displayed on the bars indicate statistically significant differences ($p < 0.05$).

diet. No dietary effects were measured in fish fed diets 1 and 2 compared to fish fed the control diet. On day 10 of cold exposure, no significant dietary supplementation effects were observed for lactate levels in fish fed on any of the tested diets. However, by day 20 of cold exposure, fish fed diets 2 and 3 exhibited comparatively higher lactate levels than fish fed diet-1 and the control diet (Fig. 4A). During the 45-day feeding period at 20 °C, creatinine levels were significantly lower in fish fed the control diet than those in fish fed diets 1, 2, and 3 ($p < 0.05$). However, during extreme cold exposure, no discernible diet-related effects were observed for serum creatinine levels (Fig. 4B).

3.3.3. Serum enzymatic activities

Throughout the 45-day feeding period at 20 °C and up until 10 days of extreme cold exposure (7 °C), no discernible dietary supplement-related effects were observed regarding serum GPT and GOT activities. However, by day 20 of cold exposure, fish fed diet-1 displayed higher GPT activities, and fish fed diets 1 and 2 exhibited increased GPT activities, although these values are not statistically significant (Fig. 5A, Fig. 5B). Before the initiation of extreme cold stress, fish fed diets 2 and 3 exhibited higher LDH activities than those fed control diet. On day 0, LDH activities were lowest in fish fed the control diet, followed by those fed diets 1, 2, and 3. On day 10 of cold exposure, no dietary supplementation effect was evident for LDH activity. On day 20, however, LDH activity was significantly ($p < 0.05$) higher in fish fed diet-3 (Fig. 5C).

3.3.4. Serum cortisol

During the 45-day feeding period at 20 °C, no discernible dietary supplement-related effects were observed for serum cortisol levels. However, by day 10 of the cold exposure, fish fed diet-3 displayed significantly higher ($p < 0.05$) cortisol content. Before the initiation of extreme cold stress, fish fed diets 2 and 3 exhibited higher lactate dehydrogenase (LDH) activities than those fed control diet. Cortisol content was significantly lower ($p < 0.05$) in fish fed on diets 2, and 3 compared to fish fed on diet-1 and the control diet on day 20 (Fig. 6).

3.4. Gene expression

3.4.1. Muscle gene expression

Throughout the 45-day feeding period at 20 °C, and during days 10 and 20 of cold exposure, no noticeable effects on HSP70 in fish muscle were observed due to the dietary supplements (Fig. 7A). On Day 0, fish fed diets 1 and 3 exhibited higher upregulation of Igf1 compared to those fed the control diet. On day 10, Igf1 was upregulated in fish muscle for both the control diet and diet-2. However, on day 20, fish fed diet-1 showed a notably higher upregulation of Igf1 in their muscle tissue (Fig. 7B). On Day 0, the expression level of TNF-1α was significantly lower in fish fed diet-2 compared to those fed diet 1. By day 10 of cold exposure, fish fed diet-2 continued to exhibit significantly lower expression of TNF-1α in their muscle tissue. Whereas, on day 20, fish fed control diet showed a higher level of TNF-1α expression (Fig. 7C). During the 45-day feeding period at 20 °C and 20 days of extreme cold exposure, no discernible effects on SREBP1 in fish muscle were observed. However, during cold stress exposure (days 10 and 20), fish fed four tested diets exhibited a notable upregulation of SREBP1 compared to day 0 (Fig. 7D). For the FADS2 and GLUT2 genes, during the 45-day feeding trial at 20 °C and until day 10 of cold exposure, there were no noticeable effects in fish muscle attributable to the diets. But on day 20 of cold exposure, both FADS2 and GLUT2 genes were down-regulated in the muscles of fish fed on the control diet compared to those fed on diets 2 and 3 (Fig. 7E, F). In the muscle tissue of fish fed all four tested diets, except Igf1, five other reported genes exhibited significantly different expressions during the study period (Table 3).

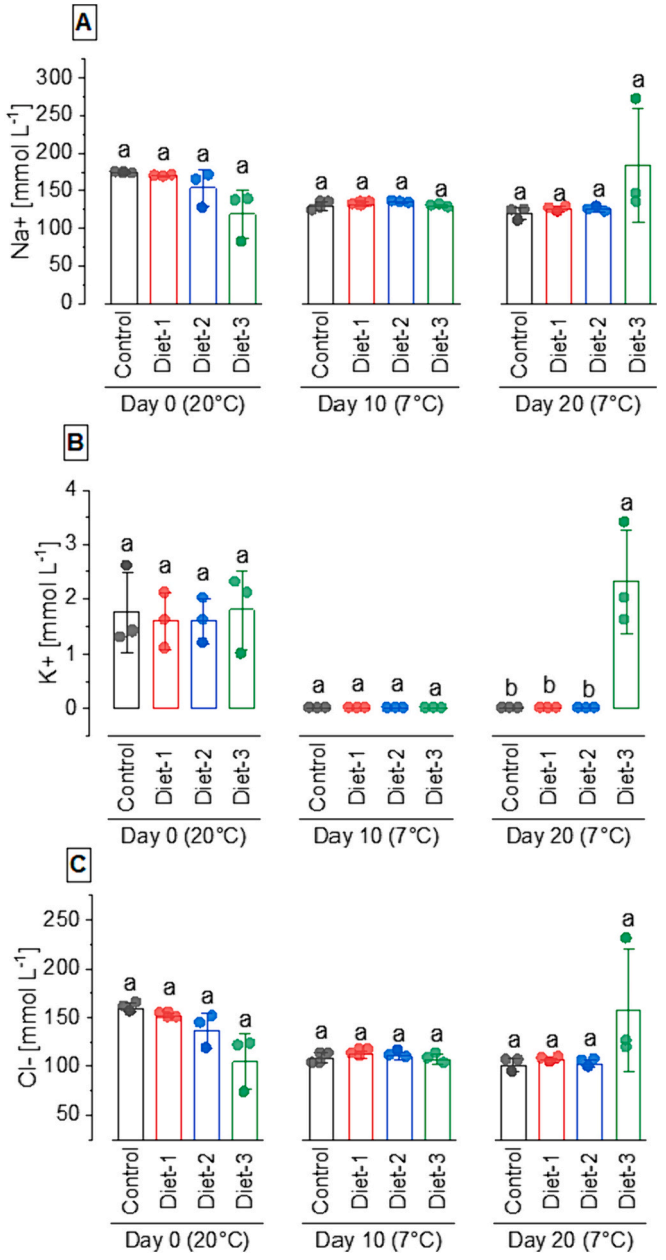

Fig. 2. Serum ions concentration: A. [Na+], B. [Cl−], and C. [K+] concentrations in European seabass juveniles reared at 20 °C while fed on tested diets for 45 days followed by a 20-day cold (7 °C) exposure. The reported values represent means ± SD, n = 3. On each sampling day, different letters displayed on the bars indicate statistically significant differences ($p < 0.05$).

3.4.2. Spleen gene expression

In the spleen, throughout the 45-day feeding period at 20 °C, fish diets 1, 2, and 3 exhibited significantly higher ($p < 0.05$) upregulation of HSP70 compared to those fed on the control diet. But during cold exposure (days 10 to 20), there were no noticeable effects on HSP70 attributable to the diets (Fig. 8A). For the Igf1 gene, during the 45-day feeding trial at 20 °C (on day 0), fish fed on diets 2 and 3 showed significant ($p < 0.05$) downregulation compared to those fed on the control diet. Like HSP70, during cold exposure, no noticeable effects on Igf1 expression in the spleen were attributable to the diets (Fig. 8B). TNF-1 was significantly increased in fish fed on the control diet before initiating cold stress compared to fish fed on diet 3 (Fig. 8C). There were no noticeable effects on SREBP1 gene expression in the spleen of fish fed on all four tested diets during the 45-day feeding period at 20 °C. However, on day 10 of cold stress exposure, fish fed diet-2 exhibited a significant upregulation ($p < 0.05$) of SREBP1 compared to fish fed the control diet (Fig. 8D). On day 0, fish fed diet-2 had higher FADS2 expression than those fed the control diet. FADS2 gene expression was unchanged with cold stress (days 10 and 20) exposure (Fig. 8E). Throughout the entire study period, no noticeable effects on GLUT2 gene expression in fish spleen were observed due to the diets (Fig. 8E). During the study period, notably in spleen tissue, significantly different expression was observed for HSP70, Igf1, and SREBP1 genes in fish fed all four tested diets (Table 3).

4. Discussion

Acute temperature fluctuations pose a significant threat to aquaculture species, particularly as the intensity of extreme weather events has worsened recently. Fish can suffer severe growth inhibition and mortality as a result of low-temperature stress (Reid et al., 2022). However, nutritional supplementation could play a crucial role in mitigating these risks. In this study, we explored the potential of dietary manipulation to enhance fish tolerance to low temperatures. The results of our research demonstrated the effectiveness of propolis, vitamins C & E, and phycocyanin-supplemented diets in improving fish performance during extreme winter events. The present study shows that before cold exposure, the final weight, and weight gain slightly improved in fish fed propolis supplemented diet. This finding is in line with several previous reports and suggests that feeding propolis-supplemented diets promotes higher growth rates in various fish species (de la Cruz-Cervantes et al., 2018). The observed growth rates and feed efficiency ratios in this study align with previous trials conducted on European seabass (Islam et al., 2021a; Šegvić-Bubić et al., 2013), indicating favorable growth in fish fed on propolis supplemented diet. Specific growth rate and protein efficiency ratio did not differ significantly among dietary treatments. Some other studies also reported that propolis inclusion did not improve growth performance in common carp, *Cyprinus carpio* (Alishahi et al., 2018), and rainbow trout, *Oncorhynchus mykiss* (Choobkar et al., 2017). In the present study, the inclusion of tested ingredients did not adversely affect growth performance, indicating that fish feed characteristics and growth were not hindered by dietary manipulation and can be safely implemented.

Temperature fluctuations outside the optimum range have been documented to lead to poor hydro-mineral balance in various studies (Islam et al., 2020b; Mackay, 1974; Nakamura et al., 2020; Phuc et al., 2017; Stewart et al., 2019). In our study, European seabass exhibited a significant impairment in K+ levels during days of cold stress, accompanied by decreases in Na + and Cl- contents, when compared to serum ion levels before the onset of cold stress (Day 0). Fish osmolality adjustments are primarily influenced by temperature rather than salinity variations (Vargas-Chacoff et al., 2019, 2020). Both lower and higher thermal stress have been demonstrated to have a negative influence on the osmoregulatory capacity of fish (reviewed in Islam et al., 2022), including European seabass (Islam et al., 2020b; Stewart et al., 2019); brook trout, *Salvelinus fontinalis* (Chadwick and McCormick, 2017); and salmon, *Salmo salar* (Vargas-Chacoff et al., 2018). On days 10 and 20 of simulated winter stress, a significant decrease in K+ ion was measured in fish fed all four tested diets, indicating that none of the diets were effective in alleviating osmotic stress during cold exposure. However, by day 20, fish fed on the phycocyanin-supplemented diet (diet-3) showed a notable recovery of osmotic ions (Na+, K+, and Cl-). Elevated cortisol content has been associated with ionic imbalances (Koakoski et al., 2014; Laiz et al., 2002; Lin et al., 2011). On day 20, the significantly increased cortisol levels found in fish fed diet-1 and control diet indicate dysfunction of ion pumps. From this, we assume that phycocyanin may support fish in coping with prolonged cold exposure. However, further studies are needed to confirm this hypothesis.

On day 10, the decreased levels of lactate and LDH content in fish fed all tested diets suggest a reduction in aerobic activity. It is well

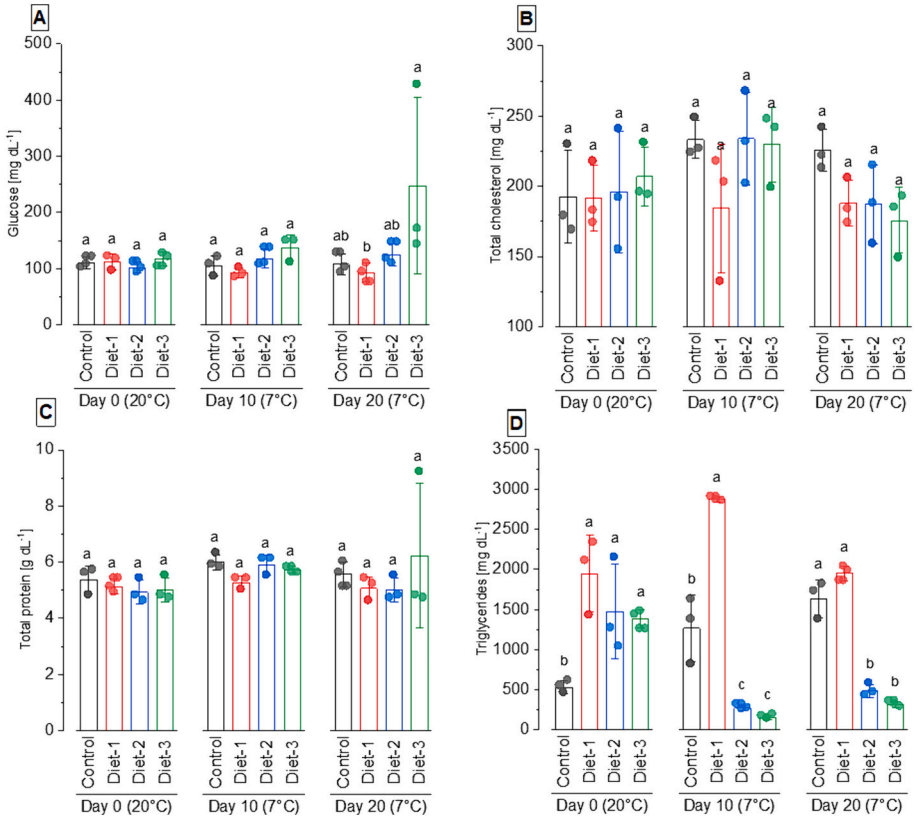

Fig. 3. Serum metabolites: A. Glucose, B. Total cholesterol, C. Total protein, and D. Triglycerides concentrations in European seabass juveniles reared at 20oC while fed on tested diets for 45 days followed by a 20-day cold (7 °C) exposure. The reported values represent means ± SD, n = 3. On each sampling day, different letters displayed on the bars indicate statistically significant differences ($p < 0.05$).

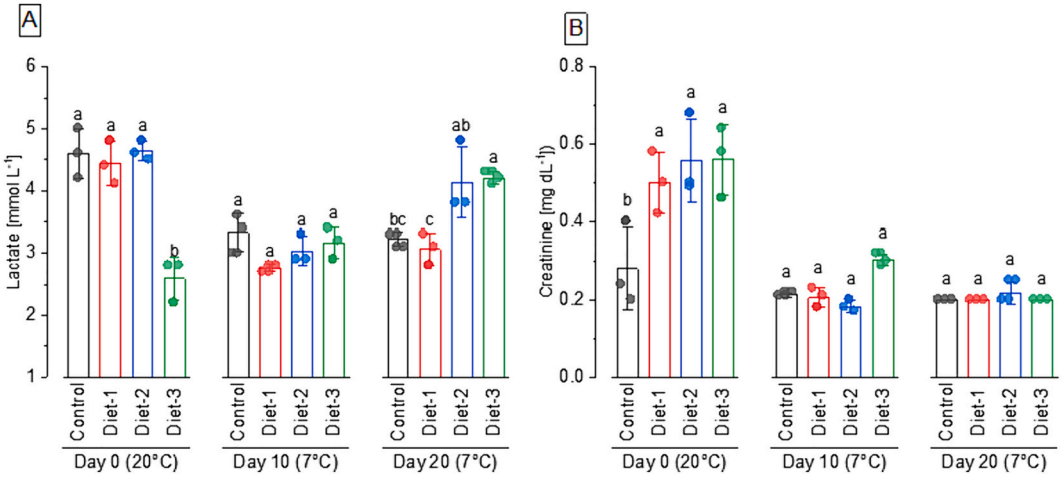

Fig. 4. Blood metabolites: A. Blood lactate and B. Creatinine concentrations in European seabass juveniles reared at 20oC while fed on tested diets for 45 days followed by a 20-day cold (7 °C) exposure. The reported values represent means ± SD, n = 3. On each sampling day, different letters displayed on the bars indicate statistically significant differences ($p < 0.05$).

documented that during cold stress, fish experience weakening due to decreased aerobic activity at the lower thermal tolerance limits (Dai et al., 2022; Pörtner and Farrell, 2008; Zhou et al., 2019). Conversely, increased levels of lactate and LDH activity indicate activation of anaerobic metabolic activity during cold exposure. By day 20 of cold stress, fish fed diets 2 and 3 exhibited higher accumulation of lactate and LDH content. As temperatures approach the lower thermal boundaries of the aerobic scope, inadequate oxygen supply results in anaerobic metabolism and the buildup of anaerobic byproducts (Pörtner et al., 2009; Pörtner and Farrell, 2008). Throughout the study, the energy content (glucose, total cholesterol, and total protein) remained stable. However, serum triglyceride levels exhibited significant changes across diets and during cold exposure. On day 0, prior to cold exposure, serum triglyceride content was notably lower in fish fed control diet than in those fed all three tested diets. This indicated that fish fed diets 1, 2, and 3 had a higher stored energy content. During cold exposure, however, fish fed diets 2 and 3 had much lower triglyceride levels than fish fed the control diet. These findings imply that fish might have used triglycerides

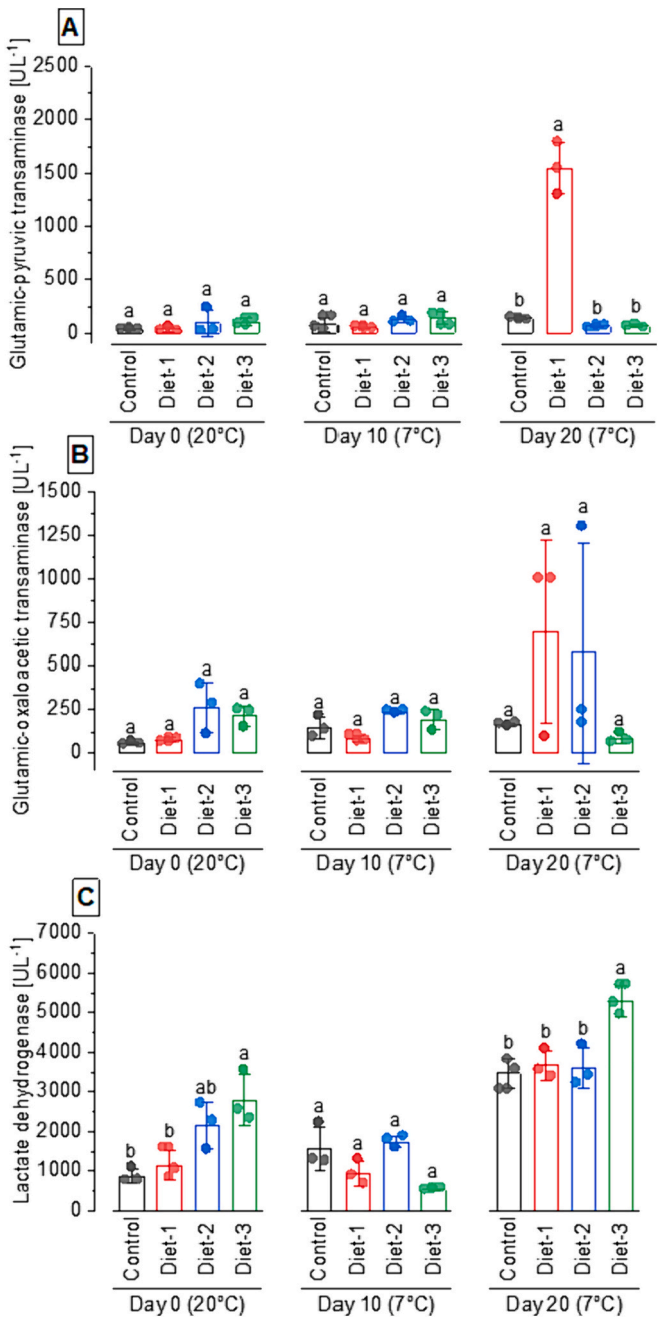

Fig. 5. Serum metabolic enzymes: A. GPT, B. GOT, and C. LDH concentrations in European seabass juveniles reared at 20 °C while fed on tested diets for 45 days followed by a 20-day cold (7 °C) exposure. The reported values represent means ± SD, n = 3. On each sampling day, different letters displayed on the bars indicate statistically significant differences ($p < 0.05$).

as an energy source to deal with the stress exerted by cold exposure. The significantly lower cortisol levels observed in fish fed diets 2 and 3 also support this assumption, as reduced cortisol levels are often associated with reduced stress responses and energy expenditure.

On day 20, total cholesterol levels also started reducing in fish fed on diets 1, 2, and 3. This may be due to the catabolism of cholesterol in fish after triglycerides. We observed that fish consumed little food during cold exposure, primarily due to the reduced metabolism induced by the low temperatures. However, during stressful situations, it is crucial to have an abundant energy supply in the bloodstream, often in glucose form, to satisfy the increased energy demand (Krumschnabel et al., 1994; Rich et al., 2022; Soengas, 2014). Apart from glucose,

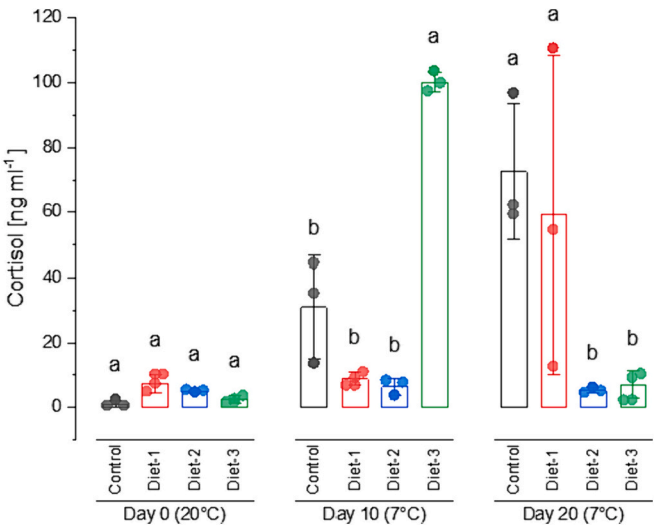

Fig. 6. Serum cortisol concentrations in European seabass juveniles reared at 20 °C while fed on tested diets for 45 days followed by a 20-day cold (7 °C) exposure. The reported values represent means ± SD, n=3. In each sampling day, different letters displayed on the bars indicate statistically significant differences ($p < 0.05$).

triglycerides and cholesterol also contribute significantly to energy metabolism. The energy expenditure in fish during extreme winter cannot be completely countered by metabolic processes alone (Alam et al., 2020; Schleger et al., 2022; Soengas, 2014; Suarez, 2012). On days 10 and 20, we found that fish fed diets 2 and 3 had significantly lower triglyceride levels than fish fed the control diet. This suggests that in response to stress, fish used additional triglycerides to meet the increased metabolic energy demand, a phenomenon often linked to disruptions in lipid metabolism in response to stress (Dai et al., 2022; Jia et al., 2021; Suarez, 2012). The findings from this study reveal a temporary decline in serum triglyceride levels during winter cold stress, suggesting its utilization as a source of energy. During cold acclimation, changes in lipid metabolism have been seen in several fish species, including European seabass (Bouaziz et al., 2017; Schleger et al., 2022). European seabass, when subjected to a 30-day cold stress period characterized by a decrease in temperature from 16 °C to 8 °C, displayed reduced feeding, decreased body weight (Islam et al., 2022, 2020a), and an increased reliance on the aerobic metabolism of fatty acids (Laiz et al., 2002; Reid et al., 2022). These alterations in lipid storage play a crucial role in meeting the heightened energy demands resulting from the activities of fish (physiological and swimming) during the acclimation to cold temperatures (Dai et al., 2022; Schleger et al., 2022).

In animals, specific cellular enzymes are released into the bloodstream due to metabolic activities (Chen, 2020; Roychowdhury et al., 2020). During cold exposure, a significant accumulation of GPT was observed in fish fed diet-1 and GOT in fish fed on diets 1 and 2, indicating heightened metabolic activity and improved liver function. While several studies have explored the effects of vitamin C, E, propolis, and phycocyanin incorporation in fish nutrition, the influence of these ingredients on European seabass in terms of cellular enzyme activity to cope with low temperatures has not been previously reported. Therefore, direct comparisons with our results were not always possible. It is worth noting that, in contrast to our findings, previous studies have reported an increase in GPT, GOT, and LDH levels in Rohu (*Labeo rohita*) during exposure to cold stress (Roychowdhury et al., 2020). However, this study focused solely on varying cold stress exposure with the same basal diet. When transaminases are activated, they can impair fish immune function and lead to skeletal muscle breakdown and decreased growth (Nemova et al., 2016; Rosenthal et al., 2017). In our study, on day 10, the increased cortisol in fish fed diet 3 and the control diets were

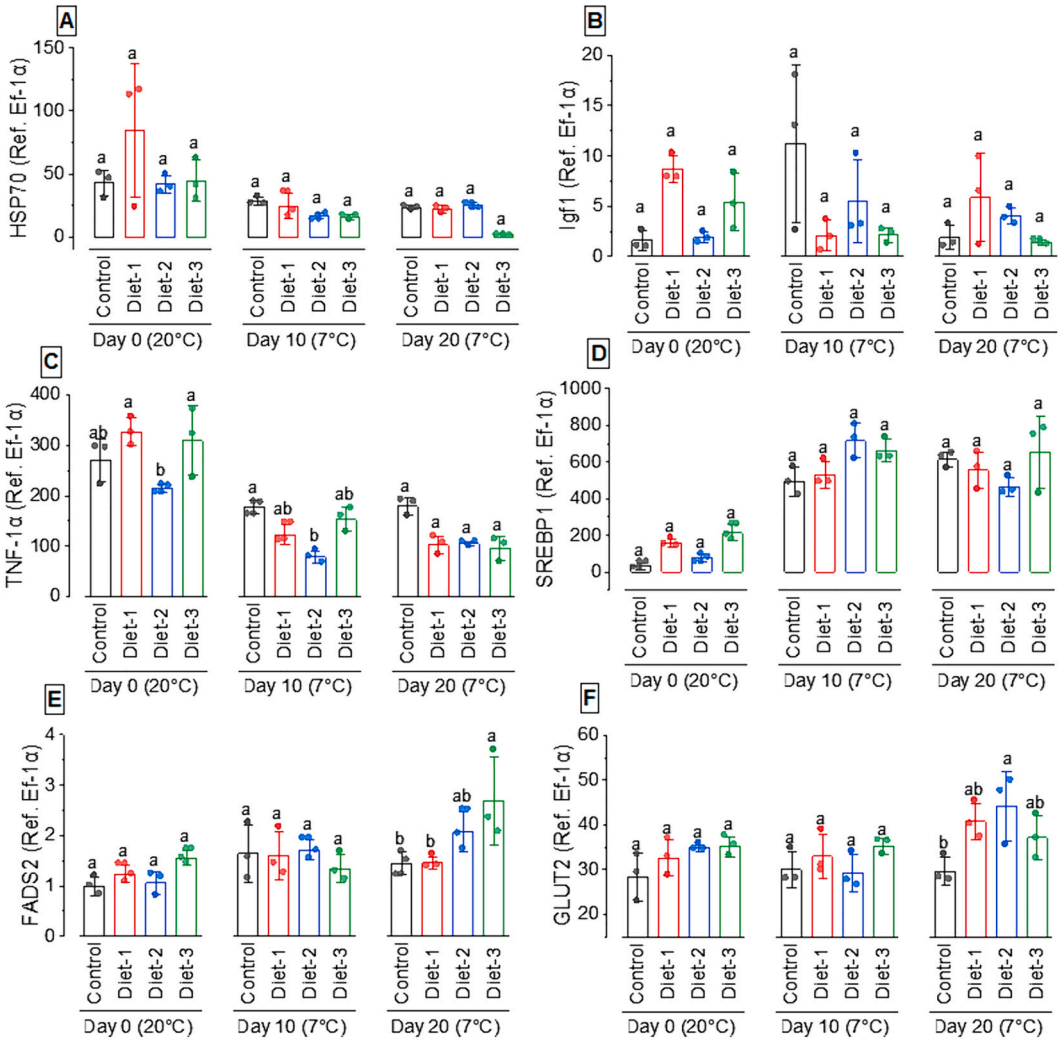

Fig. 7. Gene expression in muscle of European seabass juveniles reared at 20oC while fed on tested diets for 45 days followed by a 20-day cold (7 °C) exposure. A. HSP70, B. Igf1, C. TNF-1α, D. SREBP1, E. FADS2, and E. GLUT2. The reported values represent means ± SD, n = 3. On each sampling day, different letters displayed on the bars indicate statistically significant differences ($p < 0.05$).

consistent with higher triglyceride levels in fish. During cold exposure, relatively lower expression of TNF-1α in the muscles of fish fed on diets 1, 2, and 3 compared to fish fed on the control diet further justifies this assumption. The drop in serum triglyceride levels was seen in previous studies focusing on the effects of cold stress (Mukherjee et al., 2022; Sun et al., 2019). On days 10 and 20, lower serum triglyceride levels and higher GPT and GOT activity in fish given diets 1, 2, and 3 imply improved performance in terms of liver metabolic functions and energy sources. On day 20 of cold exposure, a rise in cortisol levels was seen in fish fed Diet-1 and the control, passively indicating higher stress levels. Cortisol is a key stress response hormone in fish, and its plasma concentration fluctuates rapidly under stress (Schreck and Tort, 2016; Tort, 2011; Wendelaar Bonga, 1997). Cold shock has been known to modulate cortisol concentrations in various fish, including European seabass (Chen et al., 2002; Fan et al., 2019; Islam et al., 2020a, 2021b).

HSP70 protein is highly conserved and serves a universal protective role in animals, including fish. It is essential for maintaining cellular and protein homeostasis, preventing protein aggregation, and assisting in protein requirements (Antonopoulou et al., 2020; Eid et al., 2016; Yamashita et al., 2010). The expression of HSP70 in the muscles of fish fed the tested diets remained consistently higher, suggesting a lower molecular response to cold stress exposure. However, in spleen tissue, HSP70 exhibited a different expression pattern compared to muscle tissue. Before the onset of cold stress, HSP70 was significantly upregulated in the spleen of fish fed the control diet and diet-3, as opposed to diets 1 and 2. Similar findings have been documented for different tissues in fish during cold stress, notably in (Fiocchi et al., 2020; Islam et al., 2020a, 2020b; Kokou et al., 2019), *Oreochromis niloticus* (Waheed et al., 2020), *Lateolabrax maculatus* (Shin et al., 2018) and *Larimichthys crocea* (Xu et al., 2018). Lower temperatures have been associated with the downregulation of Igf1 in *Oncorhynchus tshawytscha* (Beckman, 2011) and European seabass (Islam et al., 2021b). However, some studies have found HSP70 upregulation in the muscles of fish exposed to low temperatures, which contradicts our findings. To date, there are no published studies on HSP70 in the tissues of fish exposed to cold exposure while being fed diets supplemented with the ingredients used in the present study. Therefore, a direct comparison with this study may not always be feasible. Further studies employing proteomics and metabolomics would offer a more comprehensive understanding of the dietary influence in mitigating cold stress.

On day 20 of cold exposure, this study observed upregulation of Igf1 in the muscles of fish fed on diets 1 and 2. Temperature plays a role in regulating Igf1 expression in fish. Changes in nutrition and temperature can impact Igf1 regulation in fish, as documented in various studies (Gabillard et al., 2003; Nakano et al., 2015; Nipkow et al., 2018). Furthermore, temperature has been demonstrated to alter the expression

Table 3
Two-way MANOVA test results of measured parameters on three sampling days for European seabass juveniles reared at 20 °C while fed on four experimental diets for 45 days followed by a 20-day cold (7 °C) exposure. The significance level was set at 0.05 for probability values.

	Measured parameters	Days					Diets					Days*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)
Serum ions	Na+	2	24	2.985	0.070	0.199	3	24	0.066	0.977	0.008	6	24	4.300	0.004	0.518
	K+	2	24	12.506	0.000	0.510	3	24	3.260	0.039	0.289	6	24	2.646	0.041	0.398
	Cl+	2	24	7.118	0.004	0.372	3	24	0.224	0.879	0.027	6	24	5.246	0.001	0.567
Serum metabolites	Glucose	2	24	1.809	0.185	0.131	3	24	6.842	0.002	0.461	6	24	2.167	0.082	0.351
	Total protein	2	24	2.360	0.116	0.164	3	24	0.961	0.427	0.107	6	24	0.540	0.773	0.119
	Triglycerides	2	24	31.795	0.000	0.726	3	24	125.243	0.000	0.940	6	24	48.585	0.000	0.924
	GPT	2	24	16.602	0.000	0.580	3	24	2.954	0.053	0.270	6	24	12.749	0.000	0.761
	LDH	2	24	119.999	0.000	0.909	3	24	7.091	0.001	0.470	6	24	17.227	0.000	0.812
	GOT	2	24	3.314	0.054	0.216	3	24	4.185	0.016	0.343	6	24	4.011	0.006	0.501
	CHO	2	24	2.403	0.112	0.167	3	24	1.494	0.241	0.157	6	24	1.245	0.319	0.237
	CRE	2	24	14.028	0.000	0.539	3	24	4.455	0.013	0.358	6	24	1.611	0.187	0.287
	Cortisol	2	24	41.812	0.000	0.777	3	24	4.411	0.013	0.355	6	24	17.277	0.000	0.812
	Lactate	2	24	29.066	0.000	0.708	3	24	8.452	0.001	0.514	6	24	20.351	0.000	0.836
Muscle tissue	HSP70	2	24	53.806	0.000	0.818	3	24	24.666	0.000	0.755	6	24	15.663	0.000	0.797
	TNF1	2	24	111.677	0.000	0.903	3	24	17.026	0.000	0.680	6	24	6.756	0.000	0.628
	Igf1	2	24	0.791	0.465	0.062	3	24	0.982	0.418	0.109	6	24	5.470	0.001	0.578
	FADS2	2	24	12.927	0.000	0.519	3	24	3.237	0.040	0.288	6	24	2.993	0.025	0.428
	GLUT2	2	24	5.966	0.008	0.332	3	24	5.472	0.005	0.406	6	24	1.828	0.136	0.314
	SREBP1	2	24	255.985	0.000	0.955	3	24	18.125	0.000	0.694	6	24	14.418	0.000	0.783
Spleen tissue	HSP70	2	24	95.885	0.000	0.889	3	24	20.766	0.000	0.722	6	24	8.603	0.000	0.683
	TNF1	2	24	68.746	0.000	0.851	3	24	5.045	0.008	0.387	6	24	2.077	0.094	0.342
	Igf1	2	24	98.291	0.000	0.891	3	24	10.330	0.000	0.564	6	24	2.414	0.057	0.376
	FADS2	2	24	4.963	0.016	0.293	3	24	1.535	0.231	0.161	6	24	1.450	0.237	0.266
	GLUT2	2	24	0.001	0.999	0.000	3	24	0.639	0.597	0.074	6	24	1.680	0.169	0.296
	SREBP1	2	24	288.573	0.000	0.960	3	24	10.723	0.000	0.573	6	24	3.850	0.008	0.490
MANOVA results (Wilks' lambda, λ)																
Effects		48	2	42.209	0.01	0.999	72	3.85	12.259	**0.014**	0.996	144	13.741	8.891	0.000	0.988

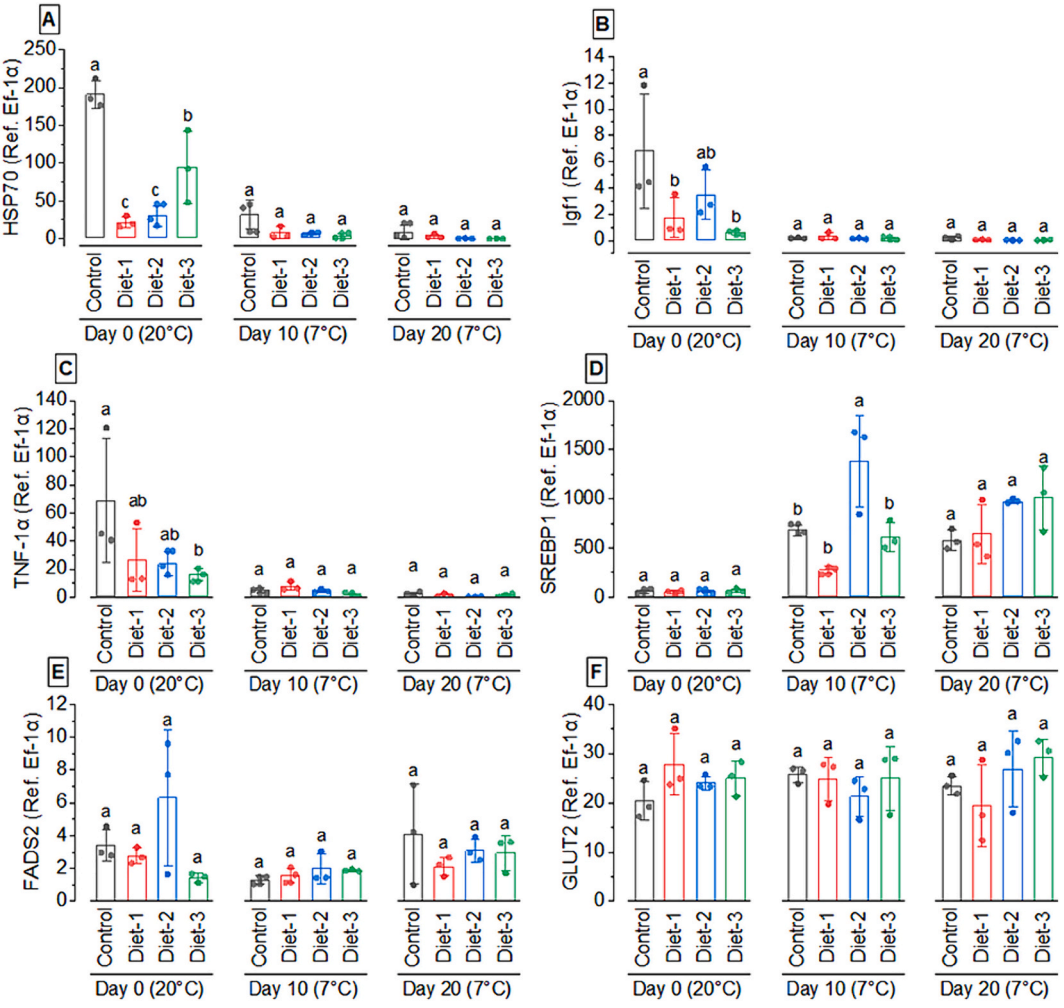

Fig. 8. Gene expression in spleen of European seabass juveniles reared at 20 °C while fed on tested diets for 45 days followed by a 20-day cold (7 °C) exposure. A. HSP70, B. Igf1, C. TNF-1α, D. SREBP1, E. FADS2, and E. GLUT2. The reported values represent means ± SD, n = 3. On each sampling day, different letters displayed on the bars indicate statistically significant differences ($p < 0.05$).

of growth-related genes such as Igf1 in fish. Decreased temperatures have been found to downregulate the Igf1 gene in European seabass (Islam et al., 2020a, 2021b). Furthermore, the nutritional status of rainbow trout was also found to affect the expression of Igf1, with levels being related to the growth rate and inversely proportional to the rearing temperature (Nakano et al., 2015). During cold stress exposure, the present study evidenced upregulation of the pro-inflammatory innate immune cytokine gene TNF-α in the muscle of fish fed the control diet. TNF-α expression is often associated with damage to tissues and pro-inflammatory responses to stress and disease (Al-Rashed et al., 2020; Roher et al., 2008). In fish, qualitative changes in gene expression, including genes linked to inflammation such as TNF-α, are associated with cold stress (Healy and Schulte, 2019). TNF-α upregulation has been observed in marine teleosts when exposed to cold temperatures (Islam et al., 2021b; Mladineo and Block, 2009). Lower TNF-α expression observed in fish fed on diets 1, 2, and 3 in the current study may be connected to robust humoral innate immune responses after cold exposure. Thus, the higher expression of Igf1 and downregulation of TNF-1α in fish fed diets 1, 2, and 3 indicate the potential of these ingredients to support fish and could be integrated into winter feed formulations.

FADS2 plays a crucial role in the biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFA) in fish. This gene is extensively expressed during acclimation to temperature stress, facilitating the introduction of double bonds (fatty acid saturation) within biomembranes. In teleosts, it is solely responsible for desaturation reactions in the LC-PUFA pathway (Bláhová et al., 2020; Martos-Sitcha et al., 2019; Xi et al., 2023). In this study, the inclusion of propolis and phycocyanin exerted an influence on FADS2. Fish fed on diets 2 and 3 exhibited upregulation of FADS2, whereas those on the control diet had the lowest expression. The upregulation of the FADS2 gene in fish could be attributed to the abundance of short-chain fatty acids present in propolis and phycocyanin extract. This finding aligns with Kabeya et al. (2015), who showed that the FADS2 expression is influenced by dietary composition and dietary fatty acids. Moreover, the results of the present study are in line with Tocher et al. (1996), who emphasized that FADS2 expression is responsive to various factors such as diets and temperature. In the context of cold exposure, a study on zebrafish revealed that long-chain saturated fatty acids synthesized by the evolving gene, which is a component of the LC-PUFA pathway, have a beneficial impact on increasing cold stress resilience in fish (Chung and Zulkharnain, 2016). Changes in membrane fluidity through hepatic lipid unsaturation in rainbow trout due to alterations of dietary formulation enhance cold protection, aiding fish to tolerate and adapt to colder conditions during the winter season (Polley et al., 2003). These findings support the idea that changes in lipid saturation play a substantial role in cold tolerance and adaptation. Further research is needed to fully understand the involvement of FADS2 in the response of fish to cold stress.

In addition to various functions, fish activate FADS2, SREBP1, and GLUT2 genes to maintain energy metabolism, antioxidant levels, tissue

repair, and immunity (Bouaziz et al., 2017; Geay et al., 2010; Shi et al., 2018). These genes play a crucial role in protecting cells from heat stress and facilitating the removal of damaged proteins through ubiquitination and proteolysis pathways within the cell (Esmaeili et al., 2021). The current study evidenced upregulation of FADS2, SREBP1, and GLUT2 genes in fish muscle tissue from all four experimental diets. This suggests an increased demand for energy to synthesize unsaturated fatty acids, which is crucial for maintaining membrane fluidity during extreme cold exposure. SREBP1 plays a role in fish during cold stress exposure (Flik et al., 2006). SREBP1 is a master regulator of lipid metabolism (Hu et al., 2020). SREBP1 is involved in regulating lipid metabolism in fish during cold stress, which is important for their survival and adaptation to low temperatures (Sun et al., 2021). The expression of SREBP1 and GLUT2 genes in fish changes with cold stress exposure, depending on the fish species and stress duration. SREBP1 expression was significantly reduced in the liver of zebrafish when GLUT2 was knocked off, which blocked glucose uptake (Xi et al., 2023). This indicates that in fish, SREBP1 regulates fatty acid production and glucose metabolism. Cold stress (4 °C) did not affect SREBP1 expression in rainbow trout liver after 24 h or 7 days of exposure (Yamashita et al., 1998). This suggests that SREBP1 is not involved in the cold response of rainbow trout. Another study reported that after 24 h of acute cold stress (13 °C), GLUT2 expression was dramatically elevated in the gill of tiger barb (Liu et al., 2020). This shows that GLUT2 engages in glucose absorption and transport in cold-stressed fish. Therefore, it seems that SREBP1 and GLUT2 expressions in fish may change with cold stress exposure, but the changes may vary depending on the fish species and the duration of the stress. However, the spleen did not exhibit the same shifting pattern of gene expression, nor did it demonstrate any synergistic effects. This may be due to the limited function of this organ to mitigate cold stress. This is consistent with the fact that the spleen is primarily involved in immunity not metabolic regulation. More studies are needed to compare the expression of these genes in different fish species and organs under different cold stress conditions.

5. Conclusions

Overall, during extreme cold exposure, we observed decreased levels of blood cortisol, transaminase, dehydrogenase, and metabolites, as well as an upregulation of growth and immune genes in fish fed diets enriched with propolis, phycocyanin, and vitamins C&E. These contrasting trends suggest that European seabass fed on diets enriched with these ingredients perform better during extreme cold exposure. This approach may offer a potential strategy for formulating climate-smart aquafeeds to mitigate the impact of extreme winter events in aquaculture. Nevertheless, considering the experimental design and dietary supplements tested, along with the parameters assessed, it is important to acknowledge that the present study constitutes an initial investigation. Further research is warranted to gain a thorough comprehension of the growth, immunostimulatory, and antioxidant attributes of the tested dietary supplements, along with their optimal incorporation into winter diets for European seabass and other fish species. Moreover, conducting additional research on proteomic and metabolomic responses across different tissues would provide a more holistic understanding of fish physiology and nutritional status when subjected to extreme winter conditions.

CRediT authorship contribution statement

Md Jakiul Islam: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Oscar Puebla:** Writing – review & editing, Supervision, Conceptualization. **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.

Data availability

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

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