Comparative Biochemistry and Physiology, Part A 309 (2025) 111923

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Research Article

Low temperature-driven physiological responses in ballan wrasse (*Labrus bergylta*): Stress, inflammation, and liver health

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ARTICLE INFO

Editor: Michael Hedrick

Keywords:
Cleaner fish
Ballan wrasse
Temperature
Stress
Inflammation
Immune
Liver

Abstract

This study investigated the impact of winter conditions on the physiology of ballan wrasse (*Labrus bergylta*), a widely used biological control agent in salmon farming. Fish were subjected to temperatures encompassing winter and summer conditions for short-term (8 days at 6, 10, or 14 °C) or long-term (2.5 months at 7 or 15 °C) period. Their stress, inflammatory, and hepatic responses were then examined. After short-term exposure, no significant differences were found in terms of morphology (bod weight, total length, condition factor), levels of stress markers (cortisol, glucose, and head kidney expression of stress-related genes), and inflammatory markers (circulating lipid inflammatory mediators, liver expression of *cox* and *lox* genes). However, at 6 °C, fish exhibited lower lactate levels and increased head kidney *cyp11a1* expression, indicating some metabolic and physiological changes during thermal acclimation. In comparison, long-term exposure to 7 °C resulted in stunted growth, along with elevated levels of stress markers, indicating tertiary stress response. Additionally, long-term exposure to 7 °C significantly lowered the levels of lipid anti-inflammatory mediators, particularly those synthesized by COX and derived from DHA and EPA, while increasing the pro-inflammatory cytokine *il-1β* expression in the head kidney, suggesting a heightened inflammatory capacity at low temperatures. Moreover, ballan wrasse at 7 °C displayed a larger hepatosomatic index and enlarged hepatocytes, indicative of energy substrate accumulation and storage in the liver. Findings highlight the physiological challenges and adaptive responses of ballan wrasse to winter temperatures, providing crucial insights for optimising farming practices and enhancing species' health, welfare and performance during deployment.

1. Introduction

Optimal thermal conditions are essential for sustaining normal physiological processes in fish, such as growth and immunity. Temperatures beyond the optimal range can be perceived as stress and trigger stress response (Donaldson et al., 2008). As in other vertebrates, stress response in fish consists of three levels, starting with the primary response which involves the release of corticosteroids (mainly cortisol) and catecholamines into the circulation followed by the secondary response which involves changes in blood chemistry (e.g., glucose and lactate levels), immune functions, and metabolic activity (Schreck and Tort, 2016; Urbinati et al., 2020; Wendelaar Bonga, 2011). Tertiary stress response occurs during chronic exposure to a stressor and involves reallocation of some energy from immune, growth and reproductive functions into vital survival functions such as breathing, swimming, and tissue repair (Urbinati et al., 2020). It should be noted that exposure of fish to sub-optimal thermal conditions regularly occur in the natural environment (e.g., diurnal, seasonal). Thermal navigation is a common adaptation in fish wherein they seek areas with optimal thermal conditions (Schulte, 2011; Soyano and Mushirobira, 2018). Another form of adaptation observed in some species of fish is dormancy which involves changes in physiology (e.g., metabolic rate depression) and behaviour (e.g., inactivity) as observed in Chinese sleeper (*Perccottus glenii*), crucian carp (*Carassius carassius*), cunner (*Tautogolabrus adspersus*), and

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https://doi.org/10.1016/j.cbpa.2025.111923
Received 23 June 2025; Received in revised form 30 July 2025; Accepted 13 August 2025
Available online 18 August 2025
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mummichog (*Fundulus heteroclitus*) (Haverinen et al., 2024; Niu et al., 2024; Reeve et al., 2022; Varis et al., 2016).

There have been extensive studies on the temperature-induced stress in fish especially among farmed species, constantly subjected to water temperature fluctuation in lakes and seas but constrained from thermal navigation. The subject of recent studies have notably focused on heat-induced stress aimed at understanding and addressing risks associated with global warming in aquaculture (Alfonso et al., 2021). Exposure to elevated temperatures (24–30 °C from ambient 18 °C) for 20 days caused apoptosis in muscle tissues and prompted inflammatory response in gilthead seabream (*Sparus aurata*) (Feidantsis et al., 2020). In pike-perch (*Sander lucioperca*), liver injury (i.e., apoptosis) and inflammation were observed in response to acute heat stress (Liu et al., 2022). In terms of primary and secondary responses, acute heat stress has been shown to prompt significant increase in the circulating levels of cortisol and lactate in Atlantic cod (*Gadus morhua*), Atlantic sturgeon (*Acipenser oxyrinchus oxyrinchus*), and shortnose sturgeon (*A. brevirostrum*) (Hori et al., 2010; Penny et al., 2023). Cold-induced stress also triggers increase in circulating levels of glucose and lactic dehydrogenase as reported in orange-spotted grouper (*Epinephelus coioides*) (Sun et al., 2019). In some aquaculture fish species such as gilthead seabream and Atlantic salmon (*Salmo salar*), cold stress during winter can cause multi-organ dysfunction in a disease referred to as winter syndrome (Ibarz et al., 2010; Rojas et al., 2024). The impact of cold stress on the physiology of fish remains poorly studied, even more so in species that exhibit adaptation to winter through dormancy (Soyano and Mushirobira, 2018).

Ballan wrasse (*Labrus bergylta*) is a cleaner fish species widely used in the UK and in Europe to control sea lice outbreak during salmon farming (Brooker et al., 2018; Overton et al., 2020). At about 20 g body weight, ballan wrasse are typically deployed in salmon cages where, as natural predators of small crustaceans, they feed on sea lice attached on salmon skin, effectively preventing outbreaks (Leclercq et al., 2014a; Treasurer, 2018). Due to long period of salmon on-growing (at least 2 years) however, ballan wrasse are exposed to seasonal temperature fluctuations. In Scottish lochs, where the majority of UK salmon farms are located, water temperatures reach 14–15 °C in the summer while they drop to ~6 °C in winter which falls below the intrinsic thermal tolerance range of ballan wrasse (7.9 to 16.8 °C; Palma et al., 2025). During winter when temperature falls below ~8 °C, delousing performance is minimal due to ballan wrasse being inactive, mainly staying at the bottom or in substrates, which is consistent with their behaviour in the wild during winter dormancy (Dipper et al., 1977; Morel et al., 2013). In a previous study wherein ballan wrasse were reared at 6 °C for 4.5 months, fish displayed weight loss, reduction in epidermal thickness and skin mucous cells (Kottmann et al., 2023). These observations suggest that this cold-temperature adaptation comes with health and welfare trade-off. Despite the importance of thermal physiology to the performance and welfare of ballan wrasse during deployment, current knowledge remains fragmentary.

Exposure to winter thermal conditions where ballan wrasse exhibits dormancy likely stimulates stress response that subsequently affect other aspects of physiology such as inflammation and liver health. Thus, in this study, we aimed to examine the response of ballan wrasse to short-term and long-term exposure to temperatures encompassing winter and summer conditions. The circulating levels of stress markers as well as selected stress-related genes in head kidney were analysed. Plasma lipid inflammatory mediators and selected inflammation-related genes in head kidney and liver were also quantified. Finally, changes in liver size and histology were examined. Novel findings from this study advance our understanding of how ballan wrasse response to cold or winter conditions, which is essential for supporting effective health and welfare management during deployment in salmon cages.

2. Materials and methods

2.1. Animal ethics

Fish trials were carried out based on the guidelines of the Animals Scientific Procedures Act 1986 (Home Office Code of Practice. HMSO: London January 1997) and under the Project Licence number PBBB474D5 (Environmental and genetic regulation of fish physiology) and Personal Licence number I76131148 (P.A. Palma). Details of the experiment were reviewed and granted approval by the Animal Welfare and Ethical Review Body of the University of Stirling (AWERB 2022 7597 7975; AWERB 2023 14,299 11,177) prior to implementation. During tissue and blood sample collection, fish were killed by overdose (200 ppm) MS-222 bath (Pharmaq, UK) followed by decapitation.

2.2. Experimental animals

The fish used in this study, which involves two trials (Section 2.3), were hatchery-bred ballan wrasse obtained from Otter Ferry Seafish Ltd. (OFS; Tighnabruaich, Scotland). In both trials, fish were sourced from a single population (i.e., one rearing tank) to minimize potential effects related to family or genetic variation. Ballan wrasse are protogynous hermaphrodites, meaning all individuals mature first as females and later undergo sex reversal to become males (Leclercq et al., 2014b; Palma et al., 2023). The sizes used in this study have been previously shown to represent a very early stage of gonad development (i.e., differentiation of oogonia into chromatin-nucleolar oocyte stage) (Palma et al., 2023). Hence, this precludes potential effects due to sexual dimorphism or reproductive maturity.

2.3. Thermal exposure trials

2.3.1. Short-term trial

Fish (~20 g) were stocked in three 100 l tanks (60 fish/tank) and allowed to acclimate in the experimental set up for about two weeks at ambient temperature (~13 °C). During this period, fish were fed artificial fish pellets at 1.5 % biomass/day (Symbio Plus 1.5; BioMar, Denmark). The tanks were also provided with artificial hides, aeration, and flow-through water system. Each tank was equipped with an aquarium chiller for temperature control (TR30; TECO Srl, Italy). Photoperiod was maintained at 12:12 light:dark cycle and D.O. levels >90 % throughout the trial. Waste materials that settled at the bottom of the tank were removed (siphoned) every 2–3 days.

Following acclimation in the experimental set up, 15 fish (i.e., 5 fish/tank) were randomly sampled for initial body measurement (body weight; total length) and tissue collection. Body condition (K) was also calculated ([body weight in g ÷ total length in cm]^3 × 100). The water temperature in each tank was then gradually adjusted (~1 °C/day; Kelly et al., 2014; Underwood et al., 2012) to reach either 6, 10, or 14 °C, representing the range of seasonal temperatures that ballan wrasse typically experience during deployment in salmon cages in Scotland. Fish were then reared in these temperatures for 8 days. This period was chosen based on our previous study that showed one week was sufficient to induce physiological shift (i.e., thermal tolerance limits) in ballan wrasse (Palma et al., 2025).

The actual mean temperatures during the trial were 6.2 ± 0.1, 10.1 ± 0.1, and 13.8 ± 0.3 °C, respectively. Feeding was continued at 1.5 % biomass/day except on the last day (i.e., 24 h before final sampling) wherein feeds were not provided. Tanks were checked multiple times in the morning and afternoon to observe general fish behaviour and monitor for any mortalities. After 8 days, 12 fish from each tank were sampled.

2.3.2. Long-term trial

Fish (~8 g) were stocked in two 500 l tanks (~390 fish/tank) where they were allowed to acclimate for two weeks at ambient temperature

(~13 °C). During acclimation, fish were fed with fish pellets at 1.5 % biomass/day (Symbio Plus 1.5; BioMar, Denmark). Tanks were provided with hides, aeration, and flow-through water system. Photoperiod was maintained at 12:12 light:dark cycle and D.O. levels >90 % throughout the trial. Each tank was equipped with temperature control. Waste materials that settled at the bottom of the tank were removed (siphoned) every 2–3 days.

After acclimation, 20 fish were sampled (i.e., 10 fish per tank) for initial data and tissue sample collection. The water temperature in each tank was then gradually manipulated (~1 °C/day) to reach the experimental temperatures which were intended to be 6 °C or 14 °C. However, the actual mean temperatures in each tank during the trial were 7.1 ± 0.2 °C and 14.9 ± 0.4 °C, respectively. Hence, for accuracy, the temperature groups are referred to as 7 °C and 15 °C in this paper. Fish were then reared in the tanks for about 2.5 months wherein feeding was continued at 1.5 % biomass/day. Tanks were checked multiple times in the morning and afternoon to observe general fish behaviour. Any mortalities were immediately removed from the tanks. At the end of the rearing period, 15 fish from each tank were sampled for morphometric measurements and tissue sample collection.

2.4. Sample collection

During sampling, fish were anaesthetized with MS-222 bath (Pharmaq, UK). Body weight and total length were measured, and blood (~1 ml) was collected from the caudal vasculature using heparinized syringe (25 G needle). Plasma was separated by centrifugation of whole blood at 4000 rpm for 15 mins and then stored at −80 °C until analysis. Fish were then dissected to collect tissue samples including liver and head kidney. Total liver weight was measured for calculation of hepatosomatic index (HSI = [liver weight ÷ body weight] x 100). Samples of liver and head kidney tissues were placed in RNA preservation buffer (3.62 M ammonium sulphate, 20 mM sodium citrate, 10 mM EDTA di sodium salt; pH 5.2) and stored at −80 °C until RNA extraction. A portion of the liver (consistently taken from the same area across all samples) was fixed in 10 % Neutral Buffered Formalin (NBF) for histological analysis.

2.5. Plasma stress markers

Circulating levels of glucose were quantified immediately after collection of blood using hand-held glucose meter (CONTOUR®NEXT ONE blood glucose meter; Ascensia, Germany). Blood lactate levels were measured using Lactate Pro 2 Blood Lactate meter (Arkray, U.K.). The use of these devices for analysing ballan wrasse blood samples has been validated in a previous study (Leclercq et al., 2014c).

Cortisol was quantified from plasma using LC-MS/MS as previously described (Wischhusen et al., 2024). Briefly, to both plasma samples (100 μl) and calibrants (50 μl; 1, 10, 50, 100, 250 ng/ml), 50 μl of d4 cortisol solution was added (50 ng/ml; Merck, Germany). Two rounds of ethyl acetate extraction were then performed by adding 500 μl 1 % (*w/v*) KCl solution, followed by 500 μl ethyl acetate, followed by vortex-mixing, centrifugation, and separation of the upper ethyl acetate phase, which was dried using nitrogen. The extracts were resuspended in 100 μl of 50 % (*v*/v) cold aqueous methanol, centrifuged for 2 mins at 14,000 rpm, with the supernatant transferred to glass LC vials. Samples were analysed using an I-class UPLC attached to a Waters TQ-S triple quadrupole mass spectrometer using the MRM scan mode in positive mode. The column used was aHSS T3 column (2.1 × 5.0 mm, 1.8 μm; Waters, USA), with solvent A consisting of water, 0.1 % (w/v) ammonium formate and 0.1 % (*v*/v) formic acid and solvent B consisting of methanol, 0.1 % (w/v) ammonium formate and 0.1 % (v/v) formic acid.

2.6. Lipid inflammatory mediators

Lipid inflammatory mediators were quantified as previously described (Campos-Sánchez et al., 2022). Briefly, to both plasma samples (200 μl) and calibrants (10 μl), = 10 μl of an internal standard mixture comprising 9 deuterated standards was added, followed by 5 μl of antioxidant solution containing BHT (0.2 ml/ml) and EDTA (0.2 mg/ml) in 50 % (*v*/v) methanol. Ice cold methanol (600 μl) was added into the samples and calibrants, which were left on ice for 15 mins and then centrifuged for 10 mins at 4 °C at 20,000 g. The supernatant was separated, vacuum-dried (40 °C), and resuspended in 800 μl of 10 % (v/v) aqueous methanol. This was followed by acidification with 32 μl of pure acetic acid and loading onto SPE columns (SepPAK tC18 SPE; Waters, USA) that were pre-conditioned with methanol and water. After sample loading, columns were washed twice with water, followed by hexane and then eluted with 1 ml methyl formate. Eluate was dried using nitrogen and resuspended with 40 μl of 50 % (v/v) aqueous methanol. Samples were centrifuged for 2 mins at 13,000 g and transferred to glass LC vials, with 15 μl injected onto a BEH C18 column (2.1 × 100 mm, 1.7 μm; Waters, USA). Samples were analysed using an I-class UPLC attached to a Waters TQ-S triple quadrupole mass spectrometer using the MRM scan mode in negative mode, with solvent A consisting of 80:20 (v/v) water/acetonitrile with 0.02 % (v/v) formic acid, with solvent B consisting of isopropyl alcohol.

2.7. Stress and inflammatory gene expression analysis

RNA was extracted from the collected head kidney and liver tissue samples using TRI Reagent (Sigma-Aldrich, USA). Extracts were quantified using NanoDrop™ 2000 (Thermo Scientific, USA) and the quality was confirmed through gel electrophoresis. cDNA was synthesized using Reverse Transcription Kit (QuantiTect; Qiagen, Germany) following manufacturer's instructions.

The mRNA expression of selected stress and inflammation-related genes in head kidney and liver were quantified through quantitative real-time PCR (qPCR). Reaction components included 5 μl of Luminaris Color HiGreen qPCR Master Mix (ThermoFisher, USA), 0.4 μl of forward primer (10 μM), 0.4 μl of reverse primer (10 μM), 3.2 μl of water and 1 μl of template. Primer details are indicated in Supplementary Table 1 and Supplementary Fig. 1. Thermal cycling conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 sec at 95 °C, 30 sec at 60 °C and 30 sec at 72 °C. qPCR assays were run in qTOWER^3 G (Analytik Jena, Germany). Each qPCR assay included duplicate of each sample and a no-template control which showed no amplification.

Relative gene expression was calculated using by Pfaffl method (Pfaffl, 2001) relative to initial control group. Gene expression levels were normalized using housekeeping genes, *β-actin* and *gapdh2*.

2.8. Liver histology

Collected liver tissues were fixed in 10 % NBF for at least 24 h. Fixed tissue samples were then subjected to standard histological processing including dehydration with ethanol, clearing, impregnation in paraffin, and sectioning at 5 μm thickness. Tissues were then stained with haematoxylin and eosin and then mounted. Stained histological sections were examined under simple compound light microscope. To obtain photomicrographs, Zeiss Axio Scan Z1 slide scanner and Zen Blue microscopy software (Germany) were used.

To analyze for liver intracytoplasmic vacuolation, 5 images from each liver were taken randomly and processed using Fiji ImageJ® (Schindelin et al., 2012). Images were set to 8-bit (grayscale) followed by application of an auto-threshold and watershed. The vacuoles were then automatically analysed using the Analyze Particles function with size set at 20-∞ and circularity at 0.50–1.00. Estimates of vacuole size and number per mm^2 for each fish were based on the average of values obtained from 5 images.

2.9. Statistical analyses

Data for morphometric measurements, plasma stress markers, and

gene expression during the short- and long-term trials were initially subjected to Shapiro-Wilk Test to check for normality of data distribution and Levene's Test for homogeneity of variance. In cases where data were normally distributed and has equal variance, the values among temperature groups and initial control were compared by one-way ANOVA followed by multiple comparisons using the Tukey test. Data with non-normal distribution and unequal variance were compared by Kruskal-Wallis Test and then with multiple pairwise comparisons with Bonferroni adjustment. Differences were considered significant at $P < 0.05$.

For the circulating levels of various lipid inflammatory mediators, the overall effects of temperature were initially examined through Principal Component Analysis conducted using FactoMineR v2.11 and factoextra v1.0.7 packages on R (version 2023.06.1 + 524). The different temperature groups were compared using MANOVA based on Principal Component scores (Abapihi et al., 2021). To further examine the effects of temperature, MANOVA was performed comparing subgroups of lipid mediators (i.e., according to precursor substrate, enzyme and inflammatory activity) among temperature groups. Information on the classification of the lipid inflammatory mediators under different subgroups used for MANOVA is indicated in Supplementary Table 2. Differences were considered significant at $P \leq 0.05$.

3. Results

3.1. Growth and behaviour

The morphological measurements of ballan wrasse at initial sampling and following exposure to various temperatures are shown in Table 1. During the short-term trial, body weight, total length and K did not vary among fish subjected to different temperatures. During the long-term trial, significantly higher body weight and K were observed in fish at 15 °C while those at 7 °C were comparable to initial. A significant increase in total length was seen in both 7 °C and 15 °C fish after the long-term trial. In terms of HSI, short-term trial resulted in similar values among temperature groups however long-term exposure resulted in significantly higher value in 7 °C while 15 °C was similar with that of initial sampling.

In both trials, fish exposed to cold temperatures (6–7 °C) displayed inactive behaviour, primarily staying at the bottom of the tank. Their response to food was also relatively slower. Fish kept at warmer temperatures were swimming and feeding more actively. We observed 100 % survival in all groups during the short-term trial. In the long-trial, survival rate was 98 % at 7 °C and 92 % at 15 °C which are typical values observed in the farm.

Table 1
Morphometry of ballan wrasse exposed to different temperatures during short- and long-term trial. Values indicate mean ± SD of 12 to 15. In each trial, values within each row that share a common superscript are not significantly different (Kruskal-Wallis Test, $P > 0.05$).

	Short-term				Long-term		
	Initial	6 °C	10 °C	14 °C	Initial	7 °C	15 °C
Body Weight (g)	22.2 ± 3.5	21.7 ± 3.0	20.4 ± 2.5	19.3 ± 2.1	8.5 ± 1.6[b]	9.2 ± 0.6[b]	15.4 ± 3.5[a]
Total Length (cm)	10.5 ± 0.3	10.5 ± 0.4	10.3 ± 0.4	10.2 ± 0.6	8.2 ± 0.5[b]	9.2 ± 1.4[a]	9.6 ± 0.6[a]
K	1.9 ± 0.2	1.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.2	1.5 ± 0.1[b]	1.3 ± 0.4[b]	1.7 ± 0.2[a]
HSI[a] (%)	1.0 ± 0.3	1.2 ± 0.5	1.0 ± 0.3	1.2 ± 0.4	0.8 ± 0.3[b]	1.9 ± 0.4[a]	0.9 ± 0.2[b]

[a] HSI = hepatosomatic index.

3.2. Plasma stress markers

The circulating levels of primary (cortisol) and secondary (glucose and lactate) stress markers are shown in Fig. 1. The levels of cortisol and glucose did not vary among temperature groups and initial sampling during the short-term trial (Fig. 1A,B). Lactate levels were comparable among temperature groups however, when compared to initial sampling, 6 °C had significantly lower value (Fig. 1C). During long-term trial, the levels of cortisol, glucose and lactate were significantly higher in fish exposed to 7 °C compared to 15 °C (Fig. 1D-F).

3.3. Plasma lipid inflammatory mediators

The overall effects of short-term and long-term exposure to different temperatures on the circulating levels of lipid inflammatory mediators are shown through the Principal Component Analysis (PCA) illustrated in Fig. 2. The normalized inflammatory mediators of ballan wrasse during short-term trial (Fig. 2A) showed 76 % of the variance explained along PC1 although no distinct clustering was observed. Long-term exposure to different temperatures (Fig. 2B) showed that, along PC1 (63 % variance explained), fish exposed to 7 °C tended to cluster distinctly from initial sampling while 15 °C overlaps with both 7 °C and initial values. The complete list of lipid inflammatory mediators detected in ballan wrasse plasma during the short-term and long-term trials are shown in Supplementary Tables 3 and 4.

Comparison of overall levels of lipid inflammatory mediators (MANOVA F = 3.2986, $P = 0.0274$) showed significant differences among temperature groups during the long-term trial but not in short-term trial (MANOVA F = 0.6494, $P = 0.6903$) as shown in Table 2. During long-term trial, levels at 7 °C appeared to be significantly different from both 14 °C and initial.

To further examine the observed significant variation in long-term trial, the temperature groups were compared using MANOVA based on different categories of lipid inflammatory mediators according to inflammatory activity (i.e., anti- or pro-inflammatory), which were further classified according to synthesizing enzyme and precursor substrate as shown in Table 3. We noted that 15 °C and initial showed no significant difference in terms of overall anti- and pro-inflammatory lipid mediators. Significant differences among temperature groups were found in anti-inflammatory lipid mediators wherein 7 °C was significantly different compared with 15 °C and initial.

Among the anti-inflammatory mediators, those that were synthesized by cyclooxygenase (COX) showed significant differences in all groups while no variation was found among those synthesized by lipoxygenase (LOX). In terms of lipid mediators derived from DHA, 7 °C was significantly different compared to 15 °C and initial while, in terms of those derived from EPA, initial fish significantly differed from those at 7 °C and 15 °C. The top lipid inflammatory mediators that showed highest statistical difference among groups during long-term trial are shown in Fig. 3. Plasma level of 5-HEPE was significantly lower after long-term exposure to 7 °C although it was comparable with 15 °C (Fig. 3A). For PGE3, significantly lower levels were obtained in both fish subjected at 7 °C and 15 °C after long-term exposure compared with initial ones (Fig. 3B). ResolvinD1 was lower in 7 °C compared to 15 °C and initial (Fig. 3C).

3.4. Expression of stress and inflammation-related genes

The expression of selected genes related to stress response in the head kidney of ballan wrasse is shown in Fig. 4. For *cytochrome P450 11a1* (*cyp11a1*), all temperature groups showed significantly higher values than initial control during short-term trial (Fig. 4A) however expression values were comparable among all treatments during long-term trial (Fig. 4F). No significant variations among groups and in both short-term and long-term trials were observed in the expression levels of *cytochrome P450 11a1* (*cyp11c1*; Fig. 4B,G) and *nuclear receptor*

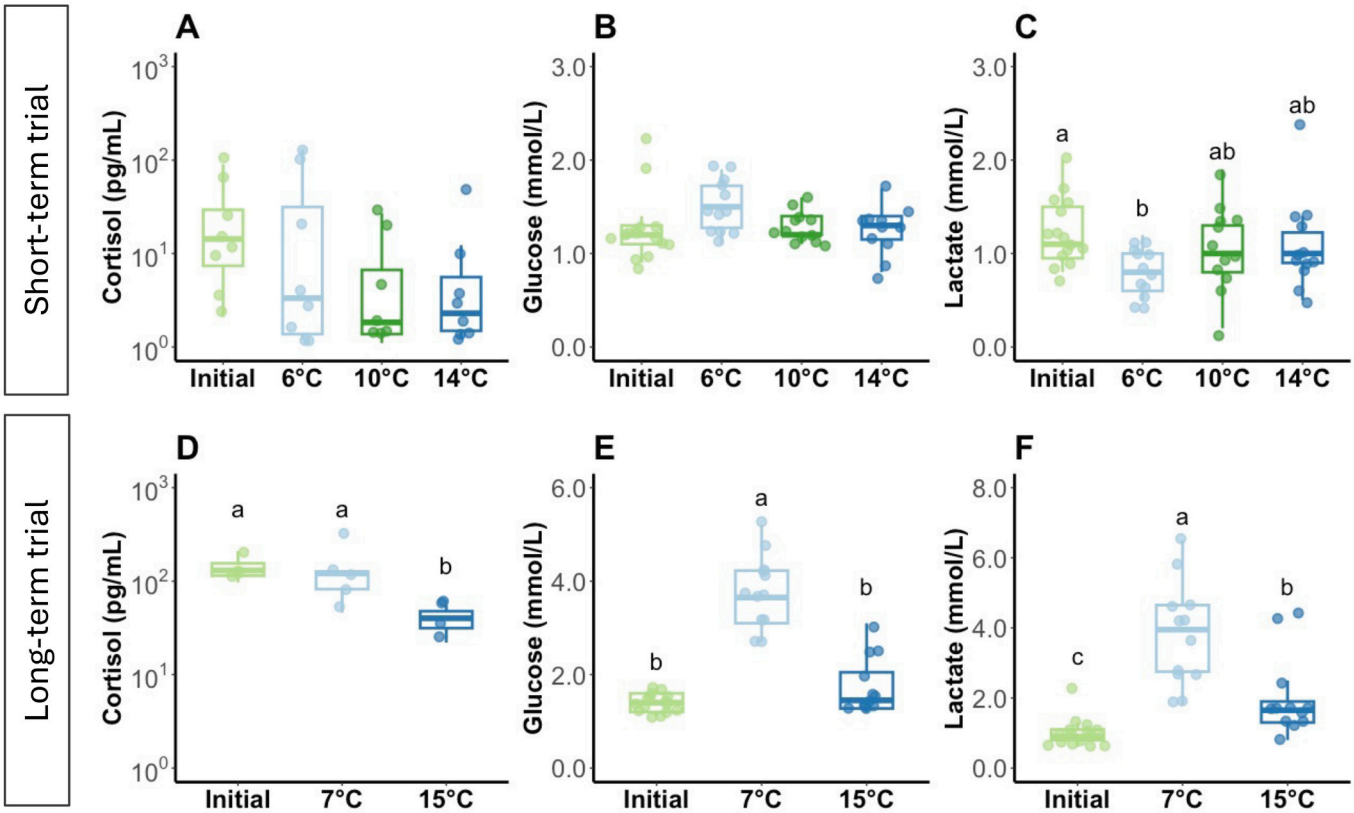

Fig. 1. Circulating levels of cortisol (A,D), glucose (B,E), and lactate (C,F) in ballan wrasse subjected to different temperatures during short-term (A-C) and long-term (D—F) trial. Values that share the same superscript letter are not significantly different ($P > 0.05$).

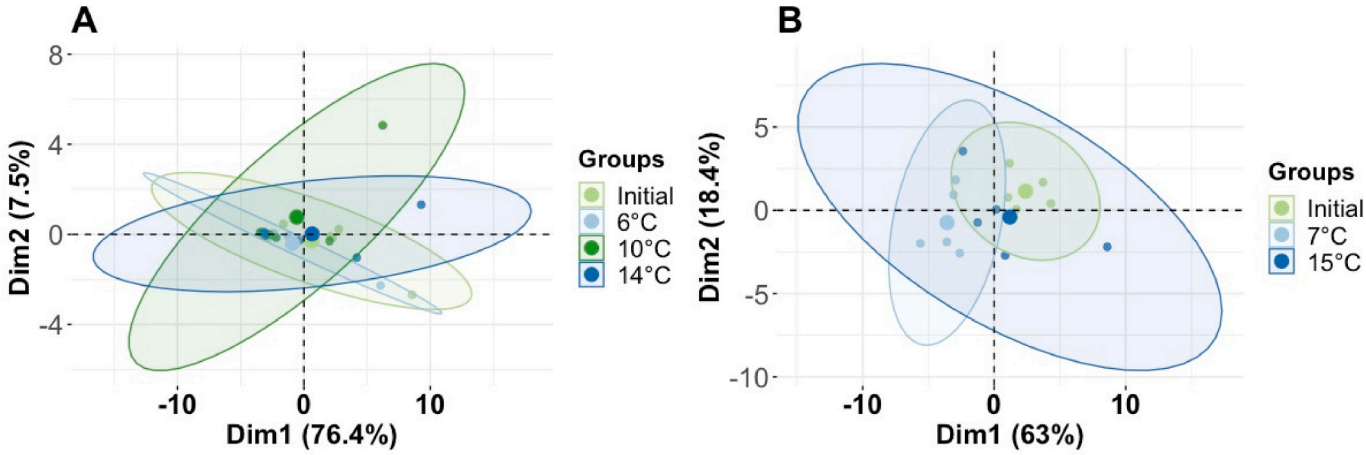

Fig. 2. Principal component analyses of circulating levels of lipid inflammatory mediators in ballan wrasse grown at different temperatures during short-term (A) and long-term (B) trials.

Table 2
MANOVA comparing overall levels of lipid inflammatory mediators (PC scores) among temperature groups during short-term and long-term trials.

Trial	MANOVA					Pairwise Comparisons[a]	
	Pillai	approx F	num Df	den Df	Pr(>F)	Treatments	Pr(>F)
Short-term	0.1627	0.6494	6	44	0.6903		*NA*
						Initial – 7 °C	**0.0035**
Long-term	0.7095	3.2986	4	24	**0.0274**	Initial – 15 °C	0.2691
						7 °C – 15 °C	**0.0386**

[a] NA indicate that pairwise comparisons were not conducted due to non-significance observed in the MANOVA ($P < 0.05$).

Table 3
MANOVA comparing the plasma levels of various sub-groups of lipid inflammatory mediators in ballan wrasse subjected to different temperatures during the long-term trial.

Factor	MANOVA					Pairwise Comparisons[a]	
	Pillai	approx F	num Df	den Df	Pr(>F)	Treatments	Pr(>F)
						Initial – 7 °C	**0.0210**
Anti-inflammatory	1.9372	12.3320	20	8	**0.0006**	Initial – 15 °C	0.0618
						7 °C – 15 °C	**0.0384**
						Initial – 7 °C	**0.0001**
EPA-derived	1.3581	5.2895	8	20	**0.0012**	Initial – 15 °C	**0.0001**
						7 °C – 15 °C	0.2173
						Initial – 7 °C	**0.0034**
COX-synthesized	0.9799	5.7633	4	24	**0.0021**	Initial – 15 °C	**0.0468**
						7 °C – 15 °C	**0.0271**
						Initial – 7 °C	**0.0316**
DHA-derived	1.4073	3.1655	12	16	**0.0169**	Initial – 15 °C	0.2646
						7 °C – 15 °C	**0.0382**
LOX-synthesized	1.5241	2.4019	16	12	0.0651		NA
						Initial – 7 °C	0.1021
Pro-inflammatory	1.8001	3.6027	20	8	**0.0341**	Initial – 15 °C	0.3220
						7 °C – 15 °C	0.1538
						Initial – 7 °C	0.1260
LOX-synthesized	1.6480	3.5112	16	12	**0.0165**	Initial – 15 °C	0.1424
						7 °C – 15 °C	0.1085
ARA-derived	1.2899	2.4223	12	16	0.0502		NA
LA-derived	0.8445	1.8272	8	20	0.1309		NA
COX-synthesized	0.2689	0.9320	4	24	0.4621		NA

[a] NA indicates that pairwise comparisons were not conducted due to non-significance observed in the MANOVA ($P > 0.05$).

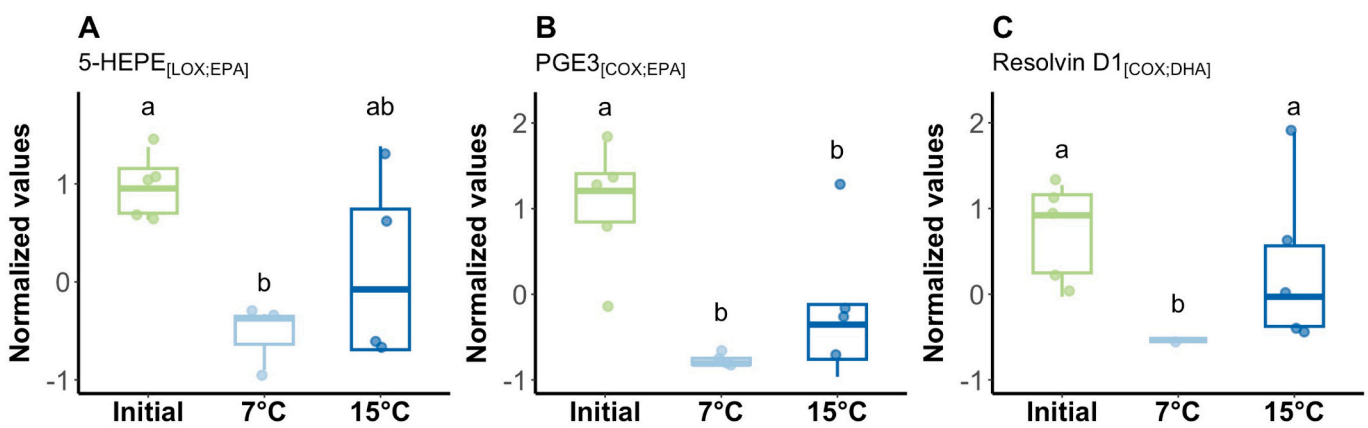

Fig. 3. Top lipid anti-inflammatory mediators that showed significant variation among temperature groups during long-term trial: 5-HEPE (A), PGE3(B) and Resolvin D1(C). The enzymes responsible for their biosynthesis (LOX or COX) and the precursor substrate (EPA or DHA) are indicated in the subscript. Boxes that share a common letter are not significantly different ($P < 0.05$).

subfamily 3 group C member 1 (*nr3c1*; Fig. 4D,I). *Hydroxysteroid 11-beta dehydrogenase 2* (*11β-hsd2*) did not vary among temperature groups after short-term exposure (Fig. 4C) while long-term exposure resulted in significantly lower expression in 14 °C compared to 6 °C and initial values (Fig. 4H). A significantly higher expression of the *nuclear receptor subfamily 3 group C member 2* (*nr3c2*) gene was observed at 10 °C compared to 6 °C, 14 °C and initial values during the short-term trial (Fig. 4E) while expression was significantly lower at 7 °C and 14 °C compared to initial during the long-term trial (Fig. 4J).

Selected inflammation-related genes in the head kidney of ballan wrasse were quantified in both short-term and long-term trial and are shown in Fig. 5. The expression of *interleukin 1 beta* (*il-1β*) was similar among groups after short exposure (Fig. 5A) while expression was significantly higher at 7 °C compared with 15 °C after long exposure to different temperatures (Fig. 5D). In the case of *interleukin 6* (*il6*) and *suppressor of cytokine signalling 1* (*socs1*), expression values showed an increase after short-term exposure to different temperatures (Fig. 5B,C) but showed no variation among temperature groups after the long-term trial (Fig. 5E,F).

The expression levels of genes that code for cyclooxygenase (*cox*) and lipoxygenase (*lox*), the enzymes responsible for biosynthesizing lipid inflammatory mediators, were analysed in the liver of ballan wrasse subjected to different temperatures (Fig. 6). The mean values of *cox1* did not show significant difference among temperature groups during the short-term trial (Fig. 6A).

3.5. Liver histology

Representative images of ballan wrasse liver histology during short-term and long-term trial are shown in Fig. 7. In the short-term trial, liver in all groups looked relatively normal with hepatocytes containing distinct and round nuclei and clearly separated by borders. Hepatocytes in the initial control fish had nuclei that are centrally located (Fig. 7A) while some nuclei in 6 °C and 10 °C are displayed on the periphery (Fig. 7B,C), denoting intracytoplasmic fat accumulation. In addition, the hepatocytes in 6 °C and 10 °C appear to be larger than in 14 °C (Fig. 7D). During the long-term trial, hepatocytes at the initial stage and at 7 °C are polyhedral in shape and have distinct borders (Fig. 7E,F). However,

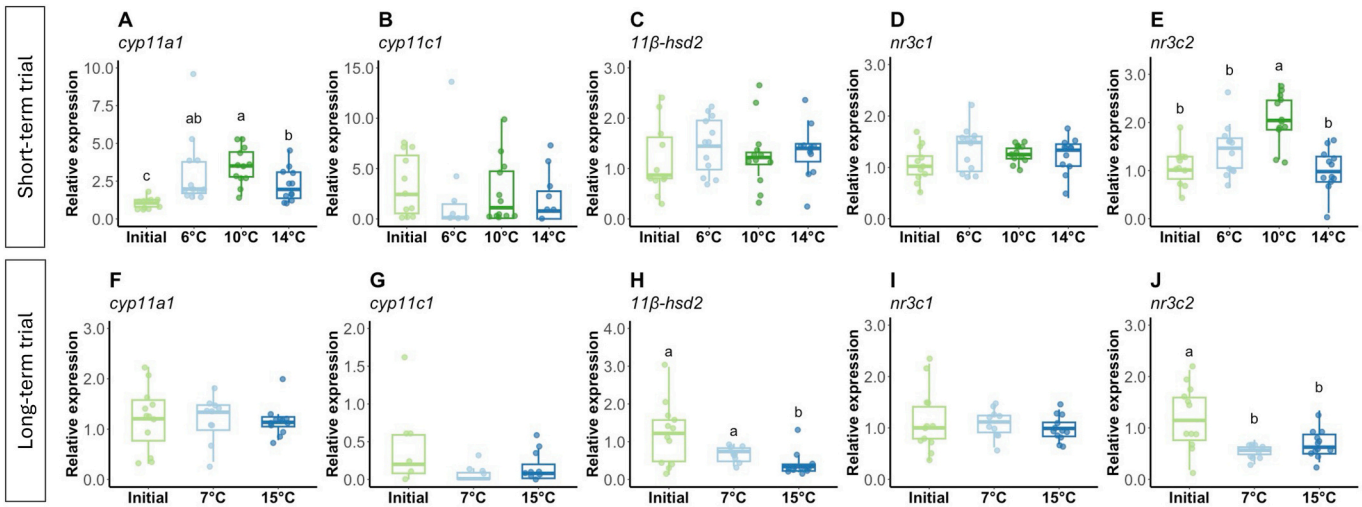

Fig. 4. Expression of selected stress-related genes in the head kidney of ballan wrasse subjected to different temperatures during short-term (A-E) and long-term (F-J) trials. Boxes that share a common superscript are not significantly different ($P > 0.05$).

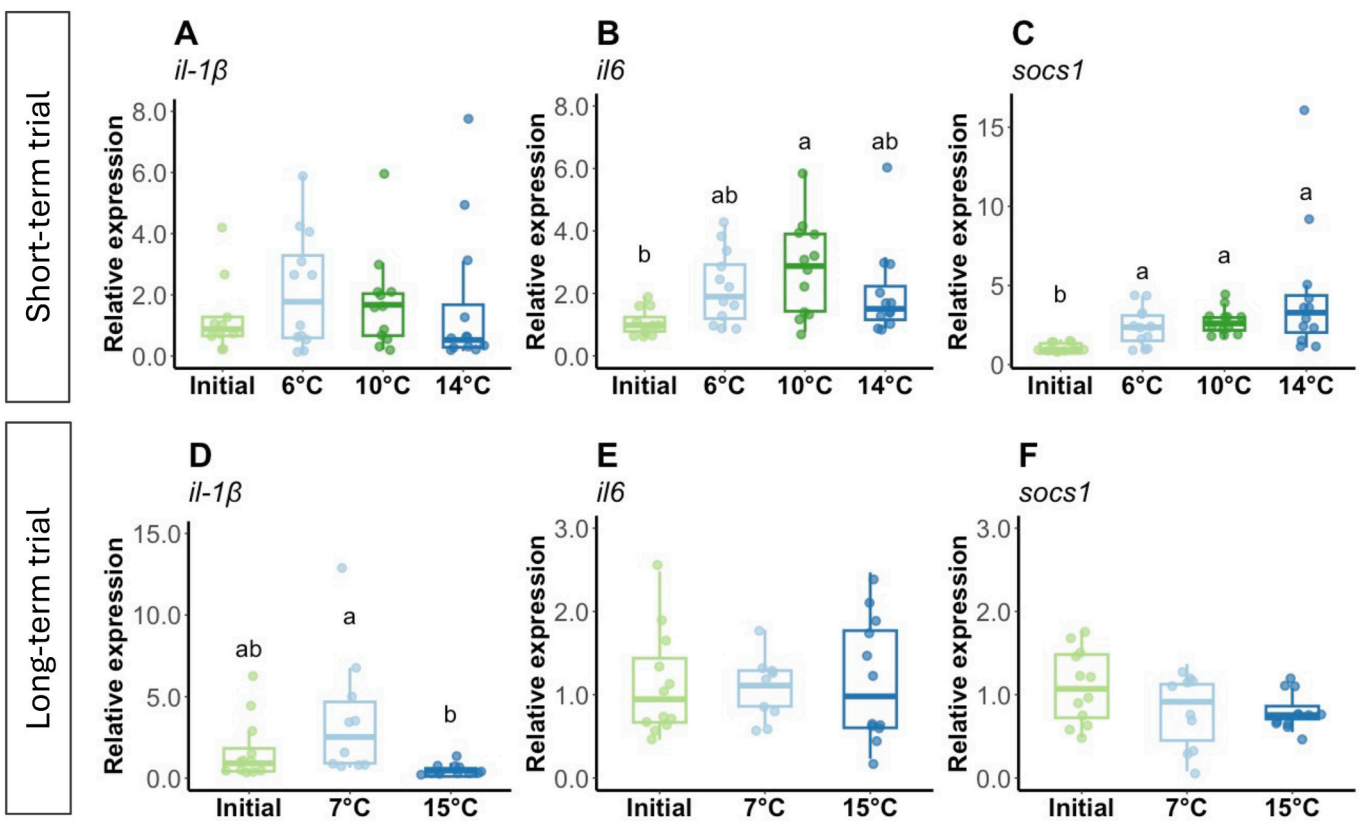

Fig. 5. Expression of selected inflammation-related genes in the head kidney of ballan wrasse subjected to different temperatures during short-term (A-C) and long-term (D—F) trials. Boxes that share a common superscript are not significantly different ($P > 0.05$).

hepatocytes at 7 °C appear enlarged and show a displacement of nuclei (Fig. 7F). Hepatocytes at 15 °C also have rounded and distinct nuclei however they appear smaller and the cytoplasm stains darker compared with the initial and 7 °C reflecting low fat accumulation (Fig. 7G).

Intracytoplasmic vacuolation in liver hepatocytes was analysed and shown in Fig. 8. During the short-term trial, vacuole number (Fig. 8A) and size (Fig. 8B) did not vary between temperature groups. It can be seen on probability density graphs that the estimated vacuole number (Fig. 8E) and size (Fig. 8F) for each temperature groups noticeably overlap. However, during the long-term trial, vacuole counts are significantly higher in 7 °C than in 15 °C (Fig. 8C,G). In terms of vacuole size, 7 °C showed high data variation although it did not significantly differ from 15 °C and initial. Vacuole size at 15 °C is significantly smaller than initial.

4. Discussion

Effective health and welfare management of ballan wrasse, a species widely used as biological control in salmon farms, requires a thorough understanding of their thermal physiology especially during winter

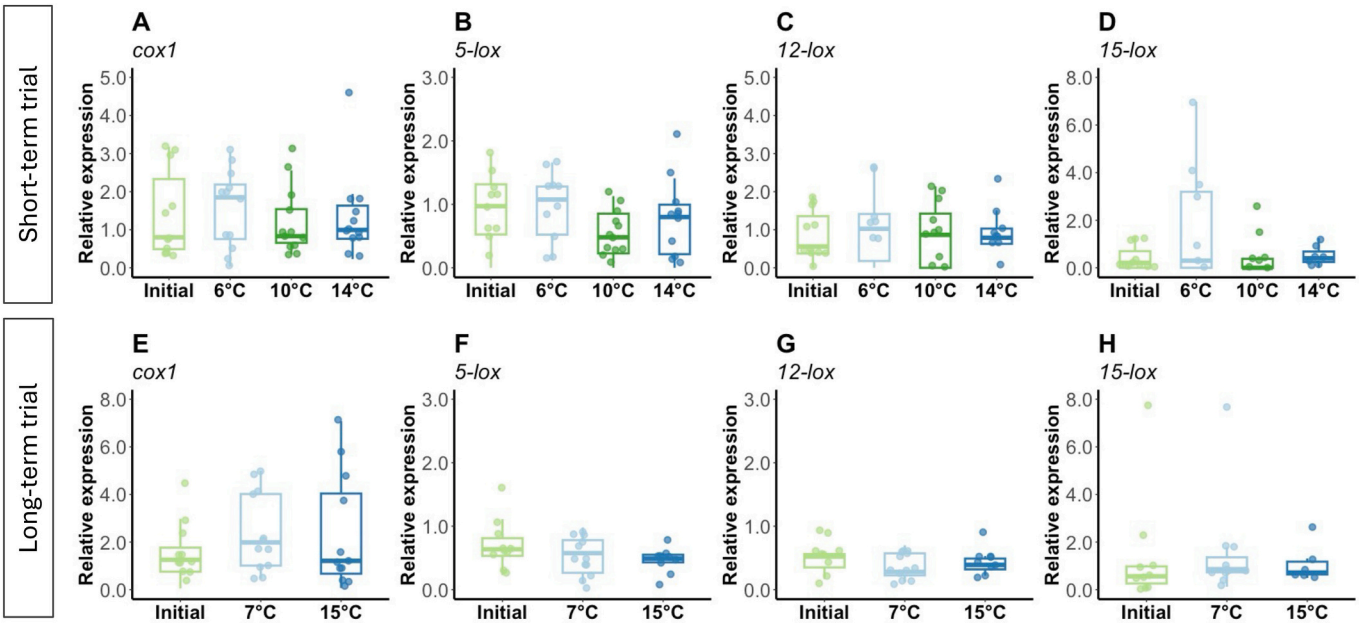

Fig. 6. Expression of *cyclooxygenase* and *lipoxygenase* genes in the liver of ballan wrasse subjected to different temperatures during short-term (A-D) and long-term (*E*-H) trials. Boxes that share a common superscript are not significantly different ($P > 0.05$).

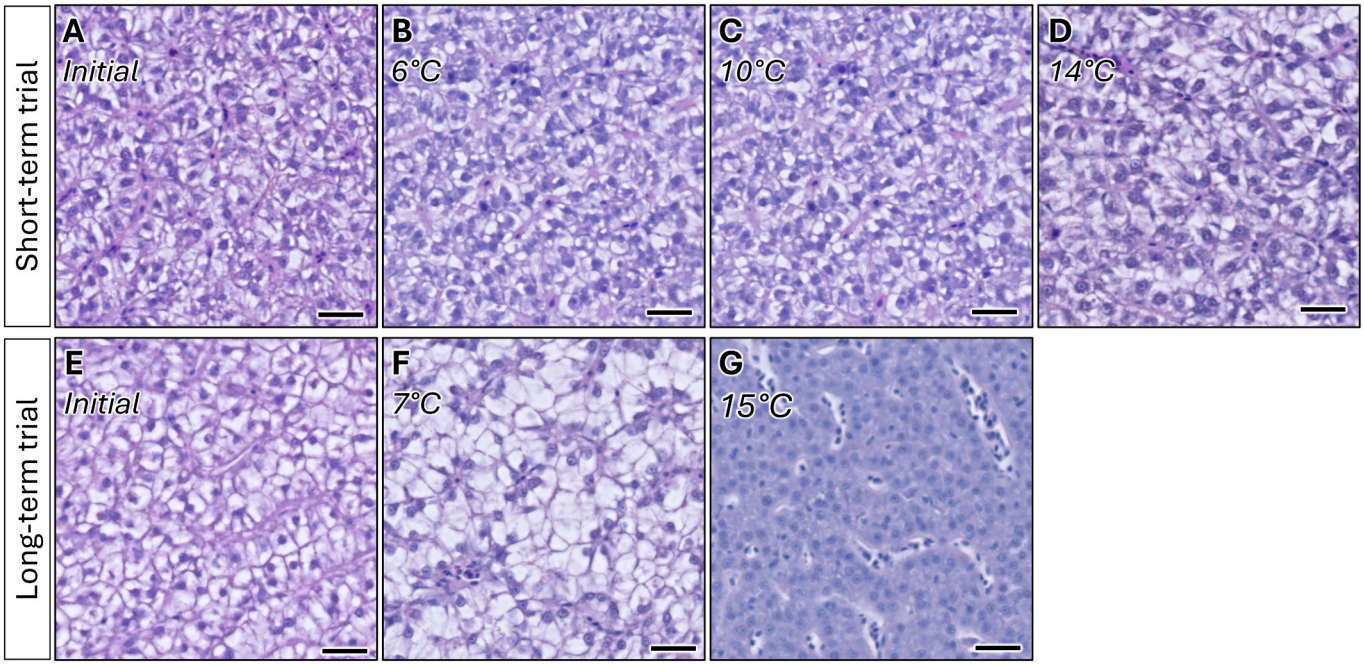

Fig. 7. Representative histological sections of liver in ballan wrasse held at different temperatures during short-term (A-D) and long-term (E-G) trials. Sections are stained with H&E. Scale bar = 20 μm.

where they are observed to exhibit dormancy. In the current study, ballan wrasse were subjected to temperatures encompassing winter and summer conditions for short-term (8 days) and long-term (2.5 months) periods. Tertiary stress response and a shift in basal inflammatory activity were observed after long-term exposure to low temperature. Changes in the liver histology of ballan wrasse were also noted at low temperature.

Short-term exposure of ballan wrasse to 6, 10, or 14 °C did not show significant changes in body weight, length, or K. Plasma stress markers (cortisol, glucose, lactate) and expression of genes related to cortisol synthesis and activity (*cyp11c1, 11β-hsd2, nr3c1*) also showed no significant differences among temperature groups. A previous study in ballan wrasse exposed to an acute stressor (1-min air exposure) showed an increase in cortisol and glucose levels 30 min post-exposure, followed by a return to baseline levels within a few hours (i.e., 4 h for cortisol, 1–2 h for glucose, and 8 h for lactate) (Leclercq et al., 2014c). In Atlantic salmon acclimated to 3 °C, exposure to cold stress (4 h at 0 °C) resulted in increased plasma glucose but not cortisol within 6 h, followed by a return to baseline after 1 day of recovery (Vadboncoeur et al., 2023). However, longer exposure to cold stress (24 h at 0 °C) led to elevated plasma glucose levels 5 days post-exposure and increased cortisol levels 6 h post-exposure, which then returned to baseline after 1 day of

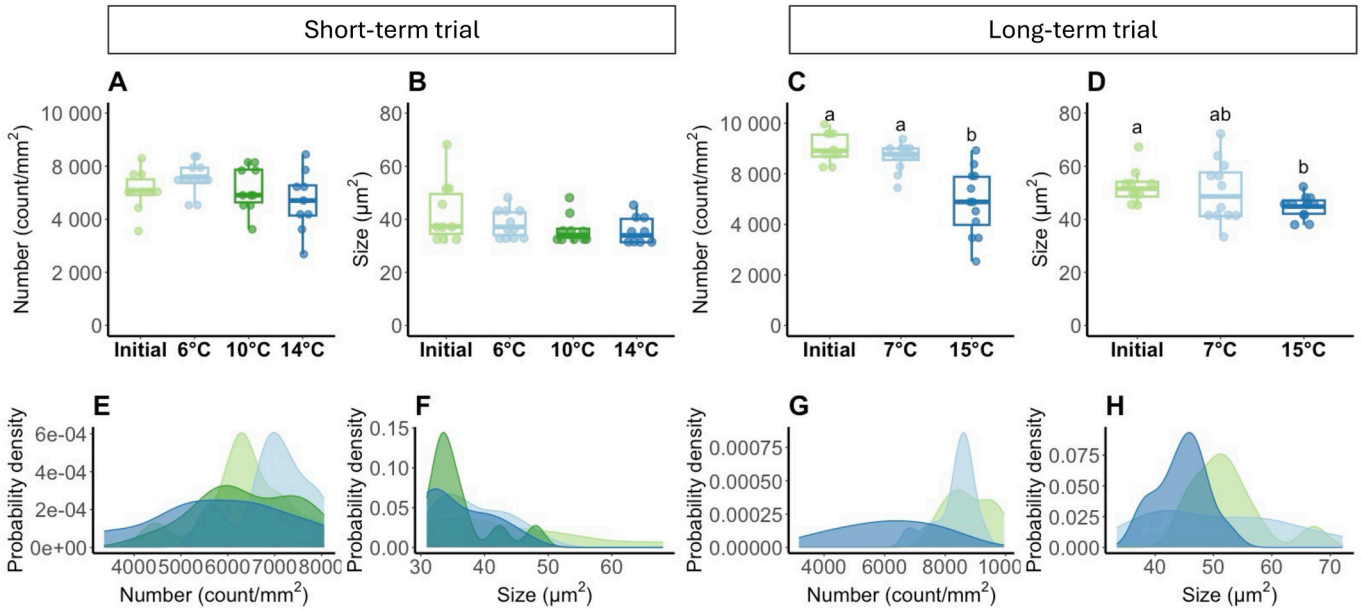

Fig. 8. Vacuolation in the liver of ballan wrasse during short-term trial (A,B,E,F) and long-term trial (C,D,G,H). Vacuoles number (A,C) and size (B,D) were estimated. The probability densities of vacuole number (E,G) and size (F,H) were also calculated. Boxes that share a common superscript are not significantly different ($P < 0.05$).

recovery (Vadboncoeur et al., 2023). These observations are consistent with thermal stress response model in fish wherein plasma stress markers spike in response to acute stress but rapidly return to baseline levels upon recovery or acclimation (Reid et al., 2022). Moreover, these highlight that the kinetics of circulating cortisol and glucose vary depending on the severity or type of stress. In the present study, it is possible that an initial stress response (i.e., increase in cortisol and glucose) occurred at an earlier point but fish were immediately able to re-establish homeostasis given the 8 days of acclimation.

Short-term exposure of ballan wrasse to 6 °C resulted in a notable behavioural shift, with fish moving from mid- to upper-tank swimming to resting on the bottom. Inactivity in fish is often an energy-saving strategy for winter survival, when food and energy resources are scarce, and is linked to metabolic rate depression (Reeve et al., 2022; Speers-Roesch et al., 2018). Indeed, ballan wrasse exhibit a significant decrease in standard metabolic rate when acclimated to 5–10 °C compared to 15 °C (Yuen et al., 2019). The observed inactivity and metabolic slowdown at low temperatures likely reduces reliance on anaerobic glycolysis and could explain the significant reduction in lactate levels at 6 °C. This aligns with studies showing lower basal lactate levels and lactic dehydrogenase activity in winter months for gilthead sea bream and rainbow trout (*Oncorhynchus mykiss*) (Cordiner and Egginton, 1997; Thibault et al., 1997; Vargas-Chacoff et al., 2009). Furthermore, we observed higher mRNA levels of *cyp11a1* at 6–10 °C, which likely suggests increased steroidogenesis. This gene is responsible for the biosynthesis of pregnenolone, the precursor of all steroid hormones (Tokarz et al., 2015). Steroids play a crucial role in various physiological processes in fish, including metabolism (Tokarz et al., 2015), supporting the notion that ballan wrasse in this study were undergoing physiological (metabolic) acclimation to different temperatures.

We previously speculated that although ballan wrasse can survive winter temperatures below its intrinsic thermal tolerance range (7.9 to 16.8 °C) through metabolic and physiological adaptation, this likely comes at a cost to their health and welfare (Palma et al., 2025). Previous studies in ballan wrasse have shown that circulating cortisol levels do not always reflect the physiological impact of environmental stressors (Gaffney et al., 2024). In such cases, stress tests or challenges can be useful to reveal differences in fish stress response capacity, where stressed fish exhibit a constrained response (Gaffney et al., 2024). Although a stress test was not conducted in the current study, previous work has demonstrated that ballan wrasse have a limited capacity to respond to environmental challenges at 5–6 °C compared to 14–15 °C, based on aerobic scope and critical thermal tolerance range or thermal breadth (Palma et al., 2025; Yuen et al., 2019). Alternatively, the impact of temperature on ballan wrasse can also be elucidated through long-term exposure trials, which was explored in the present study.

During the long-term trial, ballan wrasse at 7 °C showed stunted growth while those at 15 °C exhibited enhanced growth, as evidenced by increases in body weight, total length, and K. The temperature dependence of ballan wrasse growth has been previously documented, with the fastest growth observed at the highest temperature tested (10, 13, and 16 °C) (Cavrois-Rogacki et al., 2019), although these temperatures fall within the species' intrinsic thermal tolerance range (~8 to 17 °C; Palma et al., 2025). Another study comparing growth of ballan wrasse at 6 and 15 °C reported a negative specific growth rate after 4.5 months at 6 °C (Kottmann et al., 2023). The lack or reversal of energetic contribution towards growth at low temperatures (6–7 °C) in current and previous studies marks tertiary stress response in ballan wrasse. This is supported by high basal levels of cortisol, glucose, and lactate observed in ballan wrasse after long-term exposure to 7 °C in the present study. Nile tilapia (*O. niloticus*) subjected to chronic hypoxia for 28 days also displayed elevated glucose and lactic acid levels (Li et al., 2018). In South American catfish (*Rhamdia quelen*), chronic stress results in serum cortisol levels that, while lower than acute stress responses, are significantly higher than control or basal levels (Barcellos et al., 2004). Cortisol has been shown to impair growth by interfering with the GH-IGF signalling as reported in several fish species such as tilapia (*O. mossambicus*) (Pierce et al., 2011), silver seabream (*S. sarba*) (Leung et al., 2008), and rainbow trout (Madison et al., 2015). Taken together, these observations suggest that ballan wrasse exposed to 7 °C over an extended period experience chronic stress which likely contributes to the observed lack of growth during farming and deployment in salmon cages in winter.

It can be noted that despite the high levels of cortisol at 7 °C during the long-term trial, the mRNA levels of genes related to cortisol biosynthesis and bioactivity (*cyp11c1*, *nr3c1*, and *nr3c2*) did not differ between 7 °C and 15 °C. The expression of *nr3c2* which encodes for

mineralocorticoid receptor (MR) was low in both temperatures following the long-term trial. The specific role of MR in fish remains elusive, partly because it deviates from the well-characterized functions and signalling systems observed in mammalian models (Baker and Katsu, 2019; Bury et al., 2003). Evidence from zebrafish suggests that it interacts with the glucocorticoid receptor (encoded by *nr3c1*) in regulating the stress response (Faught and Vijayan, 2018) however the exact mechanism remains unknown and merits further investigation. The expression level of *11β-hsd2* which is responsible for converting cortisol to its inactive form, cortisone, was higher at 7 °C than at 15 °C. This seems to suggest that metabolic clearance of cortisol is enhanced, likely to mitigate the negative impact of high circulating level of cortisol on other physiological processes and functions.

Inflammatory response in fish is generally prompted by pathogenic infection but can also occur in response to trauma or injury (Chen and Nuñez, 2010). In addition, thermal stress has been shown to trigger inflammatory response as shown in gilthead seabream and pikeperch (*S. lucioperca*) exposed to high temperatures (Feidantsis et al., 2020; Liu et al., 2022). During the short-term trial in the current study, the hepatic expression levels of *cox* and *lox* genes are comparable between temperature groups, with circulating levels of lipid inflammatory mediators also not showing any differences, as shown in the PCA plot and MANOVA of PC scores. These observations suggest that short-term exposure to different temperatures, including 7 °C which is outside its intrinsic thermal tolerance range, had no significant impact on its basal inflammatory activity. Although there was increased expression of *il-6*, a pro-inflammatory cytokine, in the head kidney of ballan wrasse, we note that it was observed in all temperature groups. Similarly, higher expression of *socs1*, a cytokine suppressor, was found in all temperature groups. We suspect that these mRNA changes in *il-6* and *socs1* were only prompted as an offshoot of physiological changes (as supported by increased steroidogenesis) brought by temperature change.

Ballan wrasse grown at 6 °C for 4.5 months were previously reported to exhibit emaciation, scale loss, thinning of epidermis and reduction of epidermal mucous cells (Kottmann et al., 2023). We predicted that such thermal conditions (i.e., winter temperature) could prompt an inflammatory response in ballan wrasse. In the present study, we found that the levels of lipid inflammatory mediators at 7 °C showed significant variation compared with 15 °C and initial. The observed statistical variations were more specifically driven by the anti-inflammatory mediators synthesized by COX and derived from DHA and EPA. Individual analysis of these anti-inflammatory mediators (i.e., 5-HEPE, PGE3 and Resolvin D1) shows that the levels are significantly lower at 7 °C compared with 15 °C and initial. In addition, at 7 °C, ballan wrasse showed significantly higher expression of *il-1β*, a pro-inflammatory cytokine, than at 15 °C. These observations seem to suggest an increased capacity for inflammation in ballan wrasse at low temperature in case of infection, tissue damage or other immune stimuli. It is well established that low temperatures suppress the immune function of fish, particularly the adaptive immunity (Abram et al., 2017). It has been suggested that, under cold thermal conditions, fish rely more heavily on innate immunity (Le Morvan et al., 1998; Wentworth et al., 2018). Results from present study seem to support this notion. This is also consistent with previous report in sockeye salmon (*Oncorhynchus nerka*) wherein exposure to colder temperature drives the immune system away from adaptive immune response and towards relying more on innate immune response (Alcorn et al., 2002). It must be noted that cortisol administration in gilthead seabream resulted in the inhibition of gene expression of inflammatory cytokine in the head kidney and liver (Castillo et al., 2009; Teles et al., 2013). Thus, the observed changes in the inflammatory activity in the current study can be attributed to the direct impact of low temperature rather than an indirect effect through stress response.

The impact of thermal stress on energy metabolism especially the utilization or storage of energy substrate in the liver has been reported in previous studies. For example, in pikeperch, 2-h heat exposure (30–34 °C from 23 °C ambient) resulted in severe vacuolization and apoptosis in the liver (Liu et al., 2022). Low temperature-induced apoptosis in liver was also previously described in orange spotted grouper (*Epinephelus coioides*) wherein 48-h exposure to 13 °C from ambient 28 °C resulted in upregulation of apoptosis-related genes (*casp3, casp8, casp9* and *BCL-2*) (Sun et al., 2019). In the present-study, since the lower end of the temperature range tested (6–7 °C) falls outside the intrinsic thermal tolerance range of ballan wrasse (Palma et al., 2025), we expected that this could similarly trigger liver damage. On the contrary, fish subjected to 7 °C during the long-term trial did not exhibit hepatocyte shrinkage or apoptosis but intracytoplasmic vacuolation, characterized by swelling and clearing of the cytoplasm. Moreover, long-term exposure to 7 °C was associated with significant increase in HSI. Our observation is consistent with experimental evidence and field observation in goldfish (*Carassius auratus*) and crucian carp (*C. carassius*), which similarly exhibit winter dormancy, wherein winter thermal conditions promote significant increase in HSI (Varis et al., 2016; Vornanen et al., 2011; Yang et al., 2015). Juvenile Tibetan naked carp (*Gymnocypris przewalskii*) also had significantly higher HSI after 15 days of exposure to 4 °C compared to ambient 17 °C (Liu et al., 2023). A previous study on ballan wrasse is also consistent with these observations where rearing at cold condition (6 °C) for 4.5 months is associated with significantly higher HSI than at warm condition (15 °C) (Kottmann et al., 2023). In another study in ballan wrasse where growth was compared at 10 °C, 13 °C or 16 °C, HSI was largest at 10 °C and smallest at 16 °C (Cavrois-Rogacki et al., 2019). However, we note that these temperatures (10 to 16 °C) are within the intrinsic thermal tolerance range of ballan wrasse, it is likely that the increased HSI is a direct impact of low temperature on the energy metabolism of this species rather than a stress response through cortisol signalling. This notion is supported by evidence from rainbow trout wherein chronic cortisol administration lead to significantly reduction in HSI after 1.5 months (Madison et al., 2015).

Exposure to low temperature is associated with increased energy demand in fish which is addressed by altering their energy metabolism (Chen and Nuñez, 2010). In species that exhibit winter dormancy in the wild, their swimming activity is reduced as a form energy saving strategy and some energy is reallocated towards storage to survive winter (Reeve et al., 2022; Speers-Roesch et al., 2018). Freshwater species generally tend to accumulate glycogen in the liver during cold conditions as shown in goldfish and crucian carp (Varis et al., 2016; Vornanen et al., 2011; Yang et al., 2015). Interestingly, in atipa (*Hoplosternum littorale*), a species of freshwater catfish, reared at low temperature (10 °C vs 25 °C ambient) for 3 weeks showed low glycogen but high lipid content in the liver (Rossi et al., 2017). In comparison, marine fish species tend to store lipid rather than glycogen during cold conditions. For example, post-smolt Atlantic salmon held at 5 °C vs 12 °C for 950 degree days exhibited significant accumulation of fat in the liver and intestine (Ruyter et al., 2006). Previous report have shown Atlantic salmon exposed to even lower temperature (3 °C) can develop fatty liver disease (Rojas et al., 2024). In coho salmon (*Oncorhynchus kisutch*), liver glycogen content did not change after two months of exposure to 2.5 °C from ambient 10 °C (Larsen et al., 2001). Similarly, ballan wrasse in the current study likely accumulated lipid in the liver. It should be noted that reallocation of energy towards storage in the liver constrains growth in fish (Fernandes and McMeans, 2019) thus contributes to the lack of growth observed in ballan wrasse at 7 °C during the long-term trial.

The current study provides valuable insights into the physiological response of ballan wrasse to winter conditions however, certain gaps remain that should be addressed in future studies. In the present study, we have shown differences in the response of ballan wrasse after short-term and long-term exposure to winter conditions. A more frequent sampling over a long period (e.g., every month over three months) would further enhance our understanding of how thermal stress response develops in ballan wrasse and other fish. Additionally, other biochemical markers related to thermal stress (e.g., Heat Shock Proteins)

should be investigated. Future studies could also subject ballan wrasse to an immune challenge to demonstrate the full impact of long-term exposure to low temperatures on their immunity. Finally, due to logistical constraints (i.e., limited availability of experimental tanks, chillers and fish from the same population), the experimental design was limited to using only one tank per treatment group, which is a notable limitation of the present study.

The thermal conditions in the field cannot be controlled and therefore interventions should be directed towards improving quality of ballan wrasse stocks deployed in salmon cages so that delousing activity and overall welfare is sustained across seasons. A previous study has pointed out that the response of ballan wrasse to low temperature varies considerably depending on the initial body condition of ballan wrasse (Kottmann et al., 2023). Using bigger size groups of ballan wrasse (e.g., ≥50 g) can provide advantage given their larger energy deposits and, therefore, better ability to cope with stress during winter. Early conditioning (e.g., exposure to varying temperatures) as well as nutritional supplementation prior to deployment can also potentially improve the reliance of ballan wrasse towards thermal stress and hence merits further study (Ciji and Akhtar, 2021; Esmaeili et al., 2022; Robinson et al., 2019). Finally, genetic selection based on low temperature-tolerance offers a potential long-term solution to improve the thermal tolerance of ballan wrasse which is also relevant in addressing issues associated with changing seawater temperatures. However, this first requires the development of reliable genomic markers.

5. Conclusion

The present study showed that low temperature drives various physiological responses in ballan wrasse including stress, inflammation, and liver health that are evident after long-term exposure. Low temperature stimulates stress response including increased primary (cortisol) and secondary (glucose, lactate) plasma stress markers and stunted growth. Long-term exposure to cold thermal conditions also drives the immune system to suppress anti-inflammatory lipid mediators that potentially enhances inflammatory capacity of ballan wrasse during winter. Moreover, low temperature stimulates mobilization of energy substrate to accumulate in the liver that led to increased HSI and hepatic vacuolation. Our results support the field observations that welfare, growth and delousing performance of ballan wrasse is compromised during winter. Findings from the current study highlight the physiological challenges and adaptive responses of ballan wrasse to winter temperatures, providing valuable insights for optimising farming practices and enhancing species' health, welfare and performance during deployment.

CRediT authorship contribution statement

Peter Almaiz Palma: Writing – original draft, Investigation, Formal analysis, Conceptualization. **Richard Broughton:** Writing – review & editing, Investigation, Formal analysis. **Elfred John C. Abacan:** Writing – review & editing, Formal analysis. **Eduardo Jiménez-Fernández:** Writing – review & editing, Resources, Investigation. **Alejandro P. Gutierrez:** Project administration, Funding acquisition, Conceptualization. **Herve Migaud:** Funding acquisition, Conceptualization. **Mónica B. Betancor:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Funding

This study was funded by Innovate UK KTP Ballan Wrasse Broodstock Project KTP12430. P.A.P. was supported by EastBio DTP.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Herve Migaud, Alejandro P. Gutierrez reports financial support was provided by UKRI Innovate UK KTP. Peter Almaiz Palma reports financial support was provided by UKRI-BBSRC EastBio DTP. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

The authors thank Alan Anderson, Arthair Gordon and Irene Le for their assistance in fish husbandry during the trials. The authors also thank Susan Harvey for her assistance during histological processing.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpa.2025.111923.

Data availability

Data will be made available on request.

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