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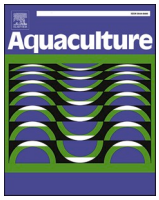

Digestive and metabolic consequences of on-growing greater amberjack (*Seriola dumerili*) juveniles at different temperatures. *In-vivo* and *ex-vivo* assessment

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A R T I C L E I N F O

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A B S T R A C T

Aiming to elucidate the effects of temperature on different aspects of the fish digestive physiology that may affect the growth, we examined, firstly, variations in growth performance, feed intake, digestive enzyme activities, expression of transport related genes, metabolites in plasma and liver, and oxidative stress response in juveniles of greater amberjack (*Seriola dumerili*) growing during two months at 18, 22 and 26 °C of water temperature. Secondly, we analysed the epithelial function of the intestinal mucosa by electrophysiological *ex-vivo* experiments. As expected, body growth increased with increasing temperature in the tested range. Highest relative feed intake was observed at 26 °C, but the food conversion ratio was the same at 22 and 26 °C and less favourable at 18 °C. Digestive proteases activities were similar at 22 and 26 °C, while an evident effect of temperature was observed on lipids digestive capacity, being lipase activity undetectable at 18 °C. Electrophysiological assays revealed a relationship between temperature and intestinal mucosa plasticity. Temperature increase promoted epithelial functionality through higher tissue resistance and short-circuit current in mid-intestine at 26 °C, as well as better electrogenic amino acids transport. On the other hand, mRNA expression of peptide transporters tended to be higher in fish that grew at 18 °C, probably to reinforce the transport capacity. Plasma circulating levels of metabolites demonstrated higher energy and protein mobilization with the increasing temperature, where a hypometabolic state was denoted by lower cortisol levels at 18 °C together with an apparent switch from lipids to carbohydrate usage as energy source and increased oxidative stress in the liver at the lowest temperature. Altogether indicates that the tested temperatures are within the tolerance range for the species, although 22 and 26 °C appear as optimal temperatures for on-growing greater amberjack juveniles. Changes in nutrient digestion and absorption induced by temperature are related with both hydrolytic activity and remodelling of the intestinal mucosa. Impairing growth capacity and initial evidences of welfare compromise were observed at 18 °C.

1. Introduction

Water temperature is a primary factor influencing physiological responses in aquatic ectotherm animals. In fish, environmental temperature modulates different aspects related to feeding, such as the voluntary feed intake (Buentello et al., 2000; Pérez-Casanova et al., 2009; Nguyen et al., 2023), the feed transit throughout the digestive tract (Miegel et al., 2010; Fernández-Montero et al., 2018; Kounna et al.,

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2021), the activity of digestive enzymes (Yúfera et al., 2019; Volkoff and Rønnestad, 2020; Das et al., 2021), the retention efficiency of nutrients (Peres and Oliva-Teles, 1999; Moreira et al., 2008) and the activation of some metabolic pathways linked to appetite (Pham et al., 2022), among others. All these activities, along with the energy expenditure (Clarke and Johnston, 1999; Pirozzi and Booth, 2009; Tirsgaard et al., 2015), ultimately define feed utilization and growth rate (Brett, 1979; Glencross and Bermudes, 2012).

The efficiency of the gastrointestinal tract to convert ingested nutrients into valid molecules for synthesizing new tissues depends in the first instance on the work of digestive enzymes. Many *in-vitro* studies have characterized the digestive enzymes of fish in relation to variations in environmental factors like gastrointestinal luminal pH or water temperature (*i.e.* Tanji et al., 1988; Alarcón et al., 1998; García-Carreño et al., 2002; de la Parra et al., 2007; Gilannejad et al., 2017; Navarro-Guillén et al., 2022). In spite of its convenience when comparing different environmental conditions, relatively few studies use actual physiological temperatures (Chen et al., 2006; Miegel et al., 2010; Bowyer et al., 2012, 2014) or luminal pH (Yúfera et al., 2014, 2019; Dias et al., 2021) for the determination of the enzymatic activities in experiments with living fish.

Protein deposition rate in muscle also relies on the absorption capacity of the intestine, which depends in part on amino acid and peptide transporters. In addition, to accomplish its absorptive function, it is essential to maintain the intestinal epithelial integrity and adequate cell to cell junctions. The electrophysiological assays with the Ussing chamber enable the measurement of two key parameters that define epithelial function: short-circuit current (Isc) and tissue resistance. Tissue resistance, regarded as the electrical manifestation of barrier function, reflects the integrity of the tissue. Meanwhile, Isc provides an accurate measure of net ion transport across the epithelium when the tissue is short-circuited, serving as a precise indicator of the tissue's net absorptive or secretory capacity (Clarke, 2009). Paracellular selective permeability of the intestinal mucosa is also mediated by several elements, among them, the functional proteins claudins and occludins have a crucial role in the complex that maintains tight junctions among epithelial cells (Horowitz et al., 2023). In fish, claudins RNA expression in the intestine has been mainly studied in relation to the use of new ingredients in fish feeds (Estensoro et al., 2016; Barany et al., 2021; Fonseca et al., 2023) and to environmental salinity (Kolosov et al., 2013; Bossus et al., 2015), but the effect of temperature has not been yet examined.

After nutrients absorption, energy requirements to attain physiological homeostasis make up the appropriate balance between metabolites mobilization and reserves storage. In this sense, the environmental temperature could modulate these processes (Pelusio et al., 2021; Khieokhajonkhet et al., 2023). Likewise, thermal stress can lead to oxidative stress by increased levels of Reactive Oxygen Species (ROS) and the organism's incapacity to detoxify the ROS active species or repair of oxidative damage (Madeira et al., 2013).

In a scenario of a rising trend of global temperatures, it is necessary to predict not only the growth potential but also the physiological responses within the expected water temperatures in areas where presumably a species may be cultured. Within the specific range of thermal tolerance, metabolic rates exhibit a progressive increase with the temperature increment up to a maximum, decreasing quickly subsequently to reach sub-lethal and lethal levels. Nevertheless, not all the metabolic activities exhibit the same optimal temperature and hence growth becomes the consequence of a trade-off among different processes that are changing at increasing temperatures. Elucidating the consequences on these processes related to feeding and growth would help to detect vulnerabilities and the adaptive capacity of fish to cope with different temperatures.

The potential effect of temperature is particularly noticeable in fast growing fish, as the greater amberjack (*Seriola dumerili*), a species of great interest for the aquaculture industry at global level (https://www.fao.org/fishery/en/culturedspecies/seriola_dumerili/en; accessed May 2024). This species inhabits temperate and sub-tropical waters around the world preferring temperatures from 17 to 28 °C (Nakada, 2000; Wells and Rooker, 2004; Froese and Pauly, 2024). Different aspects related to digestion and feed utilization in this species have been addressed in several studies (Fernández-Montero et al., 2018; Navarro-Guillén et al., 2019, 2022, 2023a, 2023b; Tomás-Vidal et al., 2019; Pérez et al., 2020; Yokoyama et al., 2020; Gamberoni et al., 2021). On the other hand, effects of water temperature on some aspects of feeding physiology and growth have also been studied in *S. dumerili* (Fernández-Montero et al., 2018; Navarro-Guillén et al., 2023a, 2023b) and other *Seriola* species (Pirozzi and Booth, 2009; Miegel et al., 2010; Bowyer et al., 2014; Larios-Soriano et al., 2018; Fukada et al., 2023).

We hypothesize that variations in fish feed conversion efficiency and growth induced by temperature do not depend exclusively on changes in metabolic rates according to the Arrhenius function of enzymes associated with hydrolysis and absorption of nutrients, but it is also the consequence of a combined strategy among digestion, absorption, and nutrient availability in plasma. Therefore, the objective of the present study is to elucidate the mechanisms underlining changes in digestion and absorption capacity in relation to ambient temperature. For that, we have examined the consequences of rearing temperature on relevant aspects of the digestive physiology and metabolism of greater amberjack juveniles using two different approaches. Firstly, by assessing the interrelations between growth, digestive functionality, metabolic response and wellbeing with an *in-vivo* long-term experiment performed at three different temperatures. Secondly, by assessing the permeability characteristics of the intestinal mucosa with electrophysiological *ex-vivo* experiments performed with fishes from the same experiment. The final aim was to elucidate the mechanisms behind and beyond feed utilization that justify the growth differences as well as to provide a useful informative basis to the productive sector.

2. Materials and methods

2.1. Fish rearing and experimental design

Fish were provided by Futuna Blue España SL and were maintained at the ICMAN experimental facilities (REGA ES110280000311). Juvenile fish ($n = 380$; body weight 23.35 ± 0.76 g, mean and SEM) were randomly distributed into three RAS units, each one with three 900-L tanks. Each RAS unit was maintained at one of the three experimental temperatures tested (18, 22 and 26 °C). This is the prevalent range of surface temperatures in the Mediterranean and North Atlantic areas in which this species is farmed during the last decade. Fish were acclimatised from 22 °C to final experimental temperatures along one week, at a rate of ±0.5 °C per day. In all tanks, the water salinity was 34 $g{\cdot}L^{-1}$, and the pH ranged between 7.8 and 8.0, oxygen saturation was maintained above 87 % and $NH_3 < 0.03$ $mg{\cdot}L^{-1}$. The photoperiod was adjusted to 12 h light and 12 h dark (illumination period from 08:00 to 20:00 h). Fish were fed by hand with a commercial feed (Skretting – Europa 22: Protein 55.45 %, Lipids 17.72 %; including fish meal, wheat gluten, wheat, fish oil, and lecithin as main ingredients) until apparent satiation three times a day during the light period (at 8:00, 12:00 and 16:00 h), usually it took less than 5 min. The amount (g) of supplied feed was recorded for the calculation of feed intake. Fish were maintained under these on-growing conditions for 58 days after acclimatization.

2.2. Sampling and analytical procedure

At the end of the experiment, fish were sampled at 16:05 and 20:05, (fifteen individuals per temperature at each sampling time, 5 fish per tank). Fish were anaesthetized (250 ppm), and then euthanized (600 ppm) by 2-phenoxyethanol overdose. Blood was obtained from the caudal vessels with heparinised syringes and centrifuged at 10,000*g* for 3 min at 4 °C and plasma samples were stored at −80 °C until

biochemical analysis. After determination of body biometrical characteristics, the visceral package was dissected to calculate viscerosomatic and hepatosomatic indexes. Then, a liver sample was immediately snap-frozen in liquid nitrogen and stored at −80 °C for the posterior analyses of metabolites and oxidative stress biomarkers. Likewise, the digestive tract was removed and stored at −20 °C for further analysis of digestive enzymes. The remaining fish in the tanks were kept under the same experimental conditions for another 1 to 3 days but were unfed for the last 24 h before being sampled for the electrophysiological measurements. Specifically, 10 fish per treatment (3–4 fish per tank) were collected for the electrophysiological characterization of the intestine in Ussing chambers.

All experimental procedures complied with the Guidelines of the European Union Council (2010/63/EU) for the use and experimentation of laboratory animals and were reviewed and approved by the Spanish National Research Council (CSIC) bioethical committee and Spanish National Veterinary Authority (REF: 02/07/2019/107).

2.3. Growth performance and biometric measurements

Every two weeks, 5 fish per tank were sampled to check their body weight. At the end of the experiment, 15 fish per tank were sampled to assess growth performance (weight gain, feed intake, feed conversion ratio) and analytical determinations.

The following parameters were assessed:

$$\text{Weight gain (WG; } g) = \text{FBW–IBW}.$$

$$\text{Specific growth rate } \left(\text{SGR; \%day}^{-1}\right) = \left(\text{LnFBW} - \text{LnIBW}\right) x\ 100/\text{days}.$$

$$\text{Voluntary feed intake (VFI; \%GMBW)} = \text{Total weight of feed ingested/GMBW } x \text{ days}.$$

$$\text{Feed conversion ratio (FCR)} = \text{weight of consumed feed } (g)/\text{WG}.$$

$$\text{Hepatosomatic index (HSI; \%)} = (\text{liver weight } (g)/\text{FBW})\ x\ 100.$$

$$\text{Viscerosomatic index (VSI; \%)} = \text{total viscera weight} g\ x\ 100/\text{FBW}.$$

$$\text{Fulton's condition factor } (K) = \left(\text{FBW}/\text{FL}^3\right) x\ 100.$$

Being, IBW (g): initial body weight; FBW (g): final body weigh; GMBW (g); geometric mean of IBW and FBW; FL (cm): fish fork length. VFI has been calculated as the average of the four two-weeks periods of the experiment.

2.4. Digestive tract pH measurements

Luminal pH within the gastrointestinal tract was measured in freshly thawed tract using a pH microelectrode with a tip diameter of 1.7 mm (Thermo Orion, Thermo Fisher Scientific Inc), following the procedure described in Yúfera et al. (2012, 2019). Briefly, the tip of the microelectrode was inserted in small slits made in the stomach and middle intestine. For the pH measurements, the room temperature was adjusted at the same experimental water temperatures (18, 22 and 26 °C). The microelectrode was calibrated before each fish measurement, using standard buffer solutions at pH 4 and 7.

2.5. Digestive enzymes activities

Activity of digestive enzymes (trypsin, chymotrypsin, leucine-aminopeptidase (leu-aminopeptidase), pepsin and lipase) was analysed at the actual physiological temperature and gut luminal pH found at the corresponding sampling points. For the determinations, the complete digestive tract of five individuals from each tank and sampling point were dissected, and immediately frozen at −80 °C. Enzyme extracts were prepared for enzyme activity measurements from these samples. Stomach and intestine samples were dissected and homogenized separately. Samples were mechanically homogenized in distilled water (1:5 v:w) using an Ultra-Turrax® Homogenizer (IKA®-Werke, Germany) and centrifuged for 20 min at 10,000*g* at 4 °C (Eppendorf 5417R, Germany). The supernatants from the stomach samples were analysed for pepsin activity, and the supernatants from the intestine samples were analysed for trypsin, chymotrypsin, leu-aminopeptidase and lipase activities. The enzymatic reactions were performed at the temperature at which the fish had grown (18, 22 or 26 °C). Pepsin activity in stomach extracts was determined by the method of Anson (1938), but the enzymatic reaction was buffered at pH 2.5 as well as at the specific gastric pH determined in the stomach (Yúfera et al., 2012). Trypsin and chymotrypsin were analysed as described in detail in Navarro-Guillén et al. (2022). Lipase and leu-aminopeptidase were measured as described in Perera and Yúfera (2017) and (Navarro-Guillén et al., 2022).

2.6. Plasma and liver metabolites analyses

For the analysis of plasma metabolite levels (glucose, lactate, triacylglycerides, cholesterol, total protein and cortisol), blood was collected from the caudal peduncle with ammonium-heparinized syringes (Sigma, H-6279, 25,000 units/3 mL of saline 0.9 % NaCl). For the analysis of tissue metabolite levels (triacylglycerides, lactate, glucose and glycogen), representative biopsies from the liver were homogenized by mechanical disruption with 7.5 vol. (*w/v*) of ice-cool 0.6 N $HClO_4$ and neutralized with the addition of the same volume of 1 M KHCO3. The homogenates were then centrifuged (30 min, 13,000 rpm, 4 °C) and the supernatants recovered in different aliquots and stored at −80 °C. Before centrifugation, an aliquot of each homogenate was obtained for triacylglycerides determinations.

Glucose, lactate, triacylglycerides concentrations in plasma and liver were measured using commercial kits from Spinreact (Barcelona, Spain) (Glucose-HK Ref. 1,001,200; Lactate Ref. 1,001,330; Triglycerides ref. 1,001,311). Plasma cholesterol was determined by a commercial kit from Spinreact (Cholesterol-LQ Ref. 41,021), whereas plasma total proteins was analysed using the bicinchoninic acid method and a commercial kit (Pierce BCA Protein Assay Kit, ref. 23,225) with bovine serum albumin as standard, as described by Moore (1968). Standards and samples were measured in duplicate. Plasma cortisol levels were measured with a commercial Cortisol Enzyme Immunoassay Kit from ARBORASSAYS (NCAL International Standard Kit, DETECTX, K003) following the manufacturer's instructions. Liver glycogen levels were assessed using the method from Keppler and Decker (1974). All the assays were run on an Automated Microplate Reader (PowerWave 340, BioTek Instrument Inc., Winooski, USA) controlled by KCjunior™ software.

2.7. Oxidative stress biomarkers analyses

Oxidative stress biomarkers (protein carbonylation, PC; catalase activity, CAT; lipid peroxidation, LPO; total antioxidant capacity, TAC; and mitochondrial reactive oxygen species, mtROS) were analysed in the liver of fish collected at 16:05 of the final sampling (n = 15 per treatment). For the analyses of PC, CAT, LPO and TAC samples were homogenized in ultra-pure water using an Ultra-Turrax® Homogenizer (IKA®-Werke, Germany). One aliquot containing 4 % butylated hydroxytoluene (BHT) in methanol was used for the determination of LPO. The remaining homogenate was diluted (1:1) in 0.2 M K-phosphate buffer, pH 7.4, and centrifuged for 10 min at 10,000 g (4 °C). The post-mitochondrial supernatant (PMS) was kept at −80 °C for the analysis of PC, CAT and TAC. CAT was determined by measuring decomposition of the substrate H_2O at 240 nm (Clairborne, 1985). PC was measured by the reaction of 2,4-dinitrophenylhydrazine (DNPH) with carbonyl

groups, according to the DNPH alkaline method (Mesquita et al., 2014) and the results were expressed in nmol carbonyl per mg protein. LPO was determined spectrophotometrically by measuring thiobarbituric acid-reactive substances (TBARS) (Bird and Draper, 1984) and the results were expressed in nmol TBARS/mg protein. TAC was assessed following the protocol described by Erel (2004), using colored 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{+}$). This method is based on the colorless molecule ABTS, which is oxidized to a characteristic blue-green $ABTS^{+}$. This change in color was measured as a change in absorbance at 660 nm and the assay was calibrated with Trolox. Results were expressed in mmol Trolox equivalent per mg protein.

For mtROS determination, the samples were homogenized in ice-cold mitochondria isolation buffer (225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 4 mM HEPES, pH 7.2). Then, the homogenate was centrifuged for 10 min at 600 *g* and 4 °C. The supernatant was picked off and centrifuged again for 10 min, at 11,000 g and 4 °C. The pellet was resuspended in buffer containing 250 mM sucrose and 5 mM HEPES (pH 7.2). mtROS production was assessed by the dihydrodichlorofluorescein diacetate method, H(2)DCF-DA (da Silva et al., 2015; van der Toorn et al., 2009) and the results were expressed as Relative Fluorescence Units (RFU) per mg mitochondrial protein.

The protein content of PMS and mtROS samples was determined according to the Bradford method (Bradford, 1976) using bovine serum albumin as standard. All biomarkers were determined in 96 well flat bottom microplates using a temperature-controlled microplate reader (Synergy H1, BioTek Instrument, Inc., USA) as a standard.

2.8. Intestinal electrophysiology

Intestinal sections from the anterior and mid intestine were isolated, washed with gassed saline and mounted within five minutes of sacrifice on tissue holders of 0.25 cm^2 or 0.3 cm^2 between two half-Ussing chambers (P2400 or P2300, Physiological Instruments, San Diego, CA, USA) holding 2 mL of physiological saline with the following composition (mmol L^{-1}): NaCl 161, KCl 6, $NaHCO_3$ 7, $MgSO_4$ 1, $CaCl_2$ 2.5, Na_2HPO_4 2.5, HEPES 5, glucose 5, pH 7.8 as described by Molina-Roque et al. (2022) and Fonseca et al. (2023). For the *ex-vivo* assays, the tissues were kept at their matching culture temperature of either 18, 22 or 26 °C, and gassed bilaterally with humidified air. All intestinal sections (anterior and mid-intestine preparations) of a single tank were run simultaneously. The transepithelial potential (TEP, mV) was referenced (grounded) to the apical side (mucosa). Short-circuit current (Isc, μA cm^{-2}) was monitored by clamping the epithelia to 0 mV. Voltage clamping and current injections were performed using VCC MC8 and VCC MC6 voltage-clamp amplifiers (Physiologic Instruments, Reno, USA). Bioelectrical parameters for each tissue were recorded continuously onto Labscribe4 running in a MacIntosh computer using IWorx188 and Lab-Trax-4 data acquisition systems from the time of mounting for 90 min. Epithelial resistance (Rt, Ω cm^{-2}) was manually calculated (using Ohm's law) from the current deflections induced by bilateral +2 mV pulses of 3 s every minute. The apical side of the preparation was considered as the ground. Therefore, negative currents are absorptive, while secretory currents are positive.

For the analyses of electrogenic amino acid transport, once Isc of the mid intestine achieved a steady-state, the apical side of the preparation was stimulated with an essential amino acid mixture (M 5550 MEM [50×], Sigma-Aldrich). The amino acid pool consists of a complex mixture of essential amino acids: L-Arginine•HCl, L-Cystine•2HCl, L-Histidine•HCl•H_2O, L-Isoleucine, L-Leucine, L-Lysine•HCl, L-Methionine, L-Phenylalanine, L-Threonine, L-Tryptophan, L-Tyrosine, L-Valine. The presence of amino acids stimulates the epithelium, and generates a change in the current due to the cotransport of amino acids with ions (Broer, 2008 and several references therein). In the greater amberjack intestine, the response is concentration-dependent, and plateaus within 30 min of amino acid addition. Therefore, sequential effects of different concentrations can be quantified in each epithelial preparation, and sequential concentrations of 4, 8, and 16 mM were used here. For data presentation, Delta Isc (μA cm^{-2}) was calculated as the difference between pre-stimulation values and the steady-state current measured after each stimulation.

2.9. Molecular analyses of peptides transporters and claudins

Six amino acids and peptides transporters have been considered. In spite of the attempts to analyse several claudins and occludins, only partial fragments of four claudin-encoding genes were successfully amplified from greater amberjack intestinal tissues.

Samples of discrete sections of the intestine were obtained for gene expression analysis and preserved in an appropriate volume (1/10, *w/v*) of RNAlater (Ambion) from the same fish used for electrophysiological protocols. Specifically, we used 3 individuals of each of the 3 tanks for each tested temperature, that is 9 individual tissues (from each intestinal region) per temperature. In the qPCR we assayed 9 individual cDNAs per temperature and section of the intestine. Total RNA was extracted with the Total RNA Kit I (E.Z.N.A., Omega Bio-tek, Norcross, GA, USA), including a DNase supplementary treatment (DNA-free Kit - RNase-Free DNase I Set, Omega Bio-tek, Norcross, GA, USA) following the manufacturer instructions. Ratios of 260/280 around 2 and 260/230 of 2–2.2 (NanoPhotometer NP80, IMPLEN GmbH, Munich, Germany) confirmed the purity of the RNA and the lack of protein contamination of the extracts After assessing the RNA quantity and quality, reverse transcription of RNA into cDNA was carried out using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Barrington, IL, USA) with 500 ng of total RNA in a reaction volume of 20 μL. Real-time qPCR amplifications were performed in duplicate with a final volume of 6 μL with 3 μL PerfeCTaSYBR Green SuperMix, ROX (Quanta BioSciences, MA, USA), approximately 1 ng cDNA (based on RNA input in the cDNA synthesis), and 0.3 μM of each forward and reverse primers (Table 1). Amplifications were performed in 384-well plates using the BIO-RAD CFX connect Real-time system (Bio-Rad Laboratories, Hercules, CA, USA) with the following protocol: denaturation and enzyme activation step at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, and primer-pair specific annealing temperature (60 °C) for 10 s. After the amplification phase, a temperature-determining dissociation step was carried out, gradually increasing 5 °C every 15 s, between the 60–95 °C range. All samples were run in parallel using 18S ribosomal RNA, β-actin, and α-tubulin to normalise expression. Based on the performance of the three putative normaliser genes assessed using NormFinder (Andersen et al., 2004), the relative gene expression was calculated as the ratio of the gene of interest to the geometric mean of β-actin and α-tubulin for each individual sample, using the values obtained by the standard curve method (Larionov et al., 2005). During primer testing, a 10-fold serial dilution (from 100 to 0.001 pg) standard curve was generated for each primer pair from a cDNA pool that included all the intestinal samples to estimate qPCR efficiencies, and linearity. Amplifications were performed in 384 well plates that included individual samples and standard curves (100 to 0.001 pg in a 10-fold serial dilution generated with purified target product) to calculate the cycle threshold value as a logarithmic function of the number of copies generated, defined arbitrarily as one copy for the most diluted standard. All calibration curves exhibited correlation coefficients $R^2 > 0.99$, and the corresponding real-time PCR efficiencies ranged between 95 % and 99 %. Individual sample relative expression was calculated normalizing the expression of the gene of interest by the geometric mean of the reference/housekeeping genes (see above). Assayed genes, respective primer sequences and other details are given in Table 1.

2.10. Statistical analysis

Significant differences in biometric and physiological parameters between fish grown at different water temperatures were determined

Table 1
Primer pairs used for expression analysis in intestinal tissue of greater amberjack and qPCR parameters for the genes used for the expression analysis.

Gene	Primer	Sequence 5′ to 3’	NCBI Access No	Ta (°C)	Size (bp)
18S	sq18SF-1	GACTCAACACGGGAAACCTC	AY850370	60	139
	sq18SR-1	AGACAAATCGCTCCACCAAC			
EF1α	sdef1aF1	CCCAAGTTCGTCAAGTCTGG	KP455300	60	76
	sdef1aR1	GGGCTTCTGTGGAATGAGTT			
β-actin	sdactbF1	AGGTTCCGTTGCCCAGAG	KX570957	60	85
	sdactbR1	TGCTGTTGTAGGTGGTCTCG			
α-tubulin	XF_alpha_tubulin_FW	CCATACAACTCCATCCTGACC	XM_022762947.1	60	141
	XF_alpha_tubulin_RV	CAGCTTGTTGAGGTTGGTGT			
SLC15a1	sdSLC15-1F2	TGTGCTTATTGTCGCTGAGG	XM_022746601.1	60	164
	sdSLC15-1R2	GCTCCTTCTGGGCAAACTG			
SLC15a2	sdSLC15-2F2	CTGTTGCATTTGGGAATGTG	XM_022741102.1	60	111
	sdSLC15-2R2	AGATGATGAAGACGGCCAAC			
SLC15a4	sdSLC15a4F1	TGTTTGAAGCGTTTCCACTG	XM_022757647.1	60	94
	sdSLC14a4R1	ATGGCACCACTCCTCAAGTC			
SLC3a1	sdSLC3-a1F2	TCAATGCAGGCTTCAACAAC	XM_022763639.1	60	194
	sdSLC3-a1R2	ACACTGGCATCAGAGTGGA			
SLC3a2	sdSLC3-a2F2	TCCACAGCAGACTTCCCAGT	XM_022758349.1	60	109
	sdSLC3-a2R2	GGCACCATCTTTAGCCCATA			
SLC7a5	sdSLC7-a5F1	TTCCTCATCGTCGTCTCCTT	XM_022746216.1	60	176
	sdSLC7-a5R1	ACCACCTCCATCACCTTCTG			
Cld11	sd-cldn11F2	GCGAGGGTCTGATGTCTTTC	XM_022767762.1	60	114
	sd-cldn11R2	GGGTCCTGATGTCGTTGC			
Cld12	sd-cldn12F2	CTACTCCACCCGCTCACG	XM_022766158.1	60	99
	sdcldn12R2	TCAATGTGCCGAGGTTTACA			
Cld19	sd-cldn19F2	AAGAAACAACAAACACATACATACGA	XM_022745164.1	60	91
	sd-cldn19R2	GGCTACTTGGAAACAACCACA			
Cld20	sd-cldn20F2	ATGCCACCATTCCTCTGTTC	XM_022750463.1	60	113
	sd-cldn20R2	ATCAGCGTTCACTTGTCAGC			

Ta = annealing temperature.

using One-way analysis of variance (ANOVA), followed by Tukey’s multiple comparison test in case of significance ($p \leq 0.05$). Differences of electrophysiological parameters and molecular expression were determined using Two-way ANOVA, considering intestinal region and temperature as independent factors. All the data were tested for normality of distribution and homogeneity of variance before the analyses. Differences were considered statistically significant when $p \leq 0.05$. Data were visualized as mean ± standard deviation (SD) or standard error of the mean (SEM).

3. Results

3.1. Growth and biometric parameters

Survival at the end of the experiment was 100 % in all cases. Fish maintained at 18 °C clearly grew more slowly from the beginning of the experiment, and SGR progressively increased at 22 and 26 °C (Fig. 1; Table 2). Concordantly, final body weight and weight gain at the end of the experiment increased with the increase of water temperature from 18 to 26 °C (Table 2). Voluntary feed intake was higher in fish fed at 26 °C than at the other two temperatures ($p < 0.05$). Contrarily, the feed conversion ratio was higher at 18 °C ($p < 0.05$) and similar at 22 and 26 °C. The hepatosomatic index was lower at 22 °C and similar at the other two temperatures. No differences in the viscerosomatic index and in the condition factor were observed among temperatures (Table 2).

3.2. Digestive enzymes activities

Samples for digestive analyses were taken at two postprandial times in order to cover the enzymatic activity while the stomach and intestine are filled with the digesta. The values presented here correspond to the averages of all samples from both sampling points (16:05 and 20:05 h) since no significant differences were found between sampling points.

Luminal pH of the digestive tract at both sampling times ranged between 4.57 and 5.18 in the stomach and between 6.76 and 6.99 in the intestine for the three temperatures (mean values per sampling time and

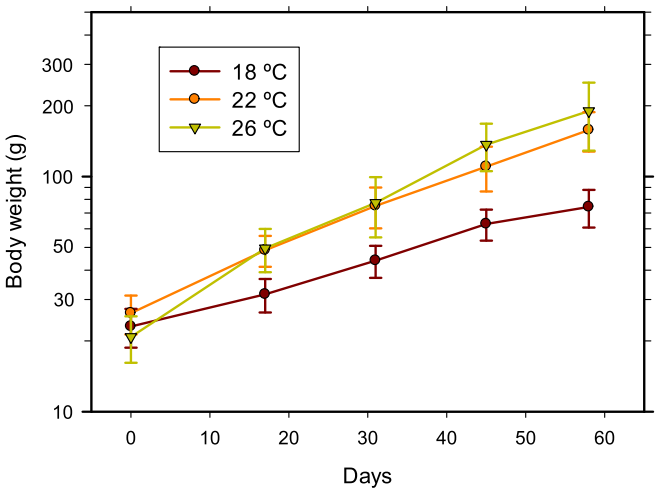

Fig. 1. Growth curves of greater amberjack juveniles on-grown at the three tested temperatures. Values are given as the mean ± SD

temperatures). Therefore, we considered the average pH values of 4.8 for determination of pepsin activity and of 6.9 for the determination of the activity of the rest of enzymes working in the intestine. For comparison reasons, pepsin activity was also analysed by buffering at standard 2.5 pH.

Digestive enzymes activities recorded at the three temperatures are shown in Fig. 2. Trypsin activity was higher in fish maintained at 22 °C than at 18 and 26 °C ($p < 0.05$) while chymotrypsin activity was higher at 26 °C ($p < 0.05$). No statistical differences were detected for the leu-aminopeptidase activities among the three temperatures. Lipase activity was similar in fish growing at 22 °C and 26 °C ($p > 0.05$). At 18 °C the lipase activity was not detected. No pepsin activity was detected at the actual physiological pH at any of the three temperatures. When measured at standard conditions (pH 2.5, and 37 °C of temperature), the

Table 2
Growth performance parameters (mean ± SD) of greater amberjack juveniles grown at the three tested water temperatures.

Temperature	18 °C	22 °C	26 °C
IBW	23.04 ± 4.33	26.23 ± 4.93	20.78 ± 4.64
FBW	74.25 ± 13.54 ^{c}	157.79 ± 30.06 ^{b}	189.88 ± 60.84 ^{a}
WG	51.24 ± 4.42 ^{c}	130.31 ± 4.75 ^{b}	169.10 ± 21.08 ^{a}
SGR	2.02 ± 0.10 ^{b}	3.00 ± 0.22 ^{b}	3.78 ± 0.14 ^{a}
VFI	3.29 ± 0.09 ^{b}	3.78 ± 0.27 ^{b}	5.37 ± 0.41^{a}
FCR	1.47 ± 0.11^{a}	1.02 ± 0.07 ^{b}	1.05 ± 0.09 ^{b}
VSI	10.59 ± 1.12	10.13 ± 1.14	11.07 ± 2.03
HSI	1.49 ± 0.21 ^{a}	1.17 ± 0.22 ^{b}	1.39 ± 0.20 ^{a}
K	1.96 ± 0.18	2.05 ± 0.17	1.99 ± 0.21

Different superscript letters in the same row indicate significant differences among treatments (one-way ANOVA; $p < 0.05$).
IBW = initial body weight, FBW = final body weight, WG = weight gain, SGR = specific growth rate, VFI = voluntary feed intake, FCR = feed conversion ratio, VSI = viscerosomatic index, HSI = hepatosomatic index, K = Fulton's condition factor.

mean values of pepsin activity were quite similar at the three temperatures ranging between 1.07 and 1.09 U·g tissue^{-1} ($p > 0.05$).

3.3. Metabolites in plasma and liver

Levels of metabolites analysed in plasma from fish grown at the three temperatures are shown in Fig. 3. As in the case of enzymatic activities, no statistical differences were detected between values measured at both sampling times and results have been considered together. Glucose levels was similar at 18 and 22 °C and higher at 26 °C ($p < 0.05$), whereas protein levels progressively increased with the increase of temperature ($p < 0.05$). Cortisol level was lower at 18 °C than at the other two temperatures. The rest of measured metabolic markers (triacylglycerides, lactate and cholesterol) were statistically not affected by temperature ($P > 0.05$).

In liver, as in the case of plasma metabolites, there were no statistical differences between values measured at both sampling times for any parameter and the results were considered together. Triacylglycerides values exhibited a decreasing trend with the temperature increase, being statistically higher at 18 °C than at 26 °C ($p < 0.05$). Contrarily, hepatic glucose and glycogen exhibited increasing trend, being lower at 18 °C than two other two temperatures ($p < 0.05$). On the other hand, lactate was not affected by the water temperature (Fig. 4).

3.4. Oxidative stress biomarkers

Oxidative stress biomarkers were analysed in fish from first sampling only. All values were higher at 18 °C than at the other temperatures, except for mROS that were similar among treatments (Fig. 5).

3.5. Intestinal integrity

In vitro experiments on epithelial electrophysiology performed with intestinal tissues showed that tissue integrity was temperature-dependent but mainly depended on the intestinal segment considered. Specifically, trans-epithelial resistance was lower in the mid-intestine than in the anterior intestine at 18 and 22 °C ($P < 0.05$) but not at 26 °C, and no significant differences among temperatures were detected (Fig. 6A). Short-circuit current was significantly lower in the mid-intestine than in the anterior intestine at 26 °C, and this value was also lower than the measurements obtained at 18 and 22 °C (Fig. 6B). The electrogenic amino acid transport was dependent on amino acid concentration, and on the combined effect of amino acid concentration and temperature. The values were higher at 26 °C than at the other temperatures but only statistically significant at the highest amino acid concentration (Fig. 6C).

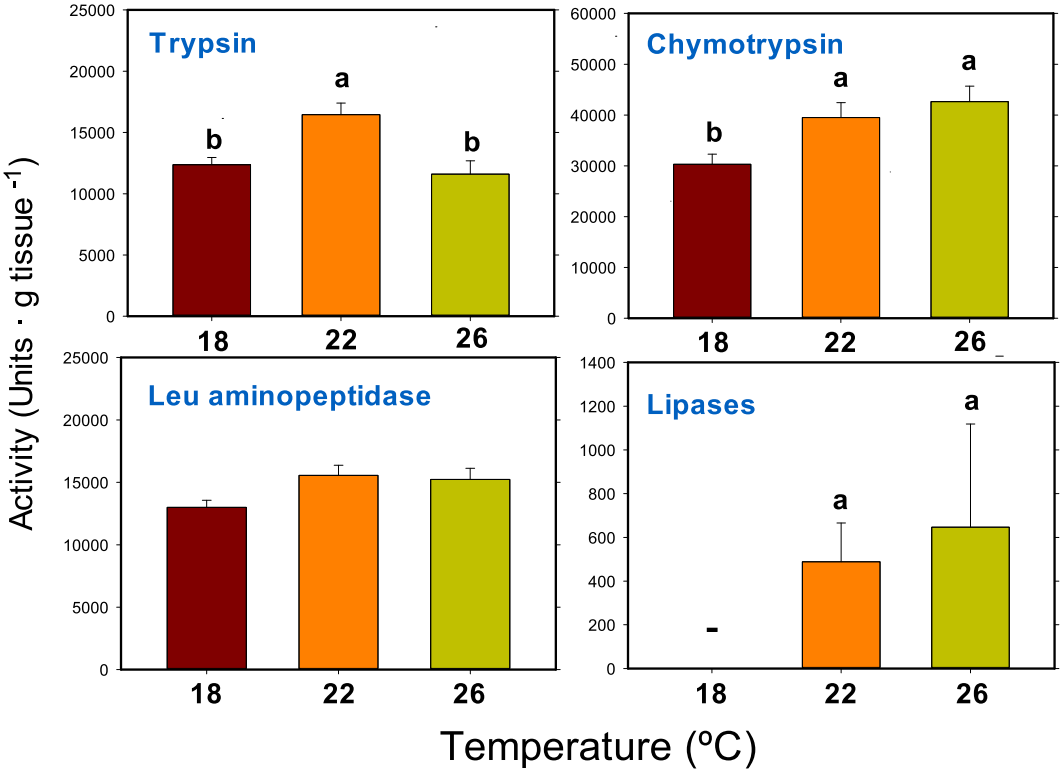

Fig. 2. Enzymatic activities measured in the intestine of greater amberjack juveniles on-grown at the three tested temperatures (mean ± SEM). Different superscript letters indicate significant differences (one-way ANOVA; $p < 0.05$).

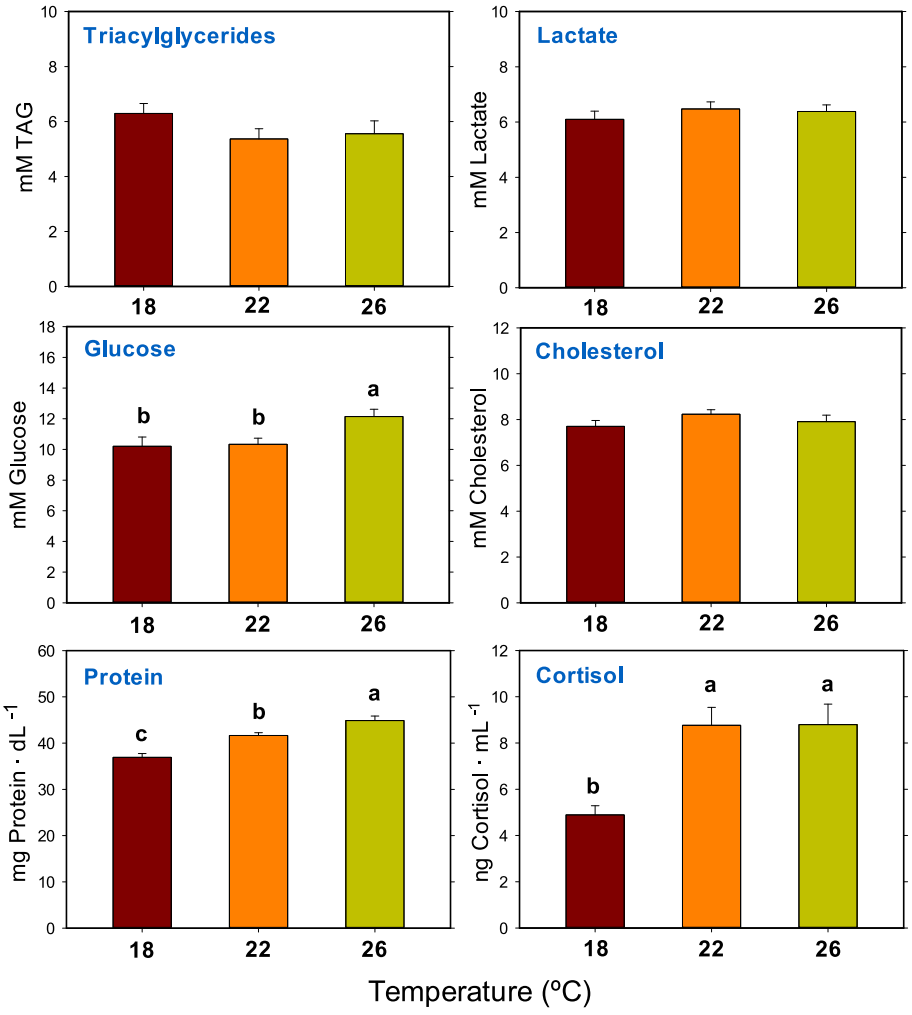

Fig. 3. Plasma metabolites of greater amberjack juveniles on-grown at the three tested temperatures (mean ± SEM). Different superscript letters indicate significant differences (one-way ANOVA; $p < 0.05$).

3.6. Gene expression of amino acids-oligopeptides transporters and claudins

Expression of peptide transport *slc3a1* was not affected by temperature, but was significantly higher in the mid-intestine than in the anterior intestine. Expression of *slc3a2* was affected by temperature, and was similar in both intestinal segments. Expression of *slc7a5* was affected by the temperature in the anterior intestine, being higher at 18 °C, but not in the mid-intestine. Regarding the di- and tri-peptides cotransporters of slc15 family, expression of *slc15a1* was unaffected by temperature but significantly higher in the anterior intestine. Expression of *slc15a2* was also unaffected by temperature but significantly higher in the mid-intestine. The expression of *slc15a4* was unaffected by both temperature and the intestinal region (Fig. 7).

On the other hand, RNA expressions of both *cld11* and *cld12* were higher in the anterior intestine, and significantly decreased with the temperature increase in both intestinal segments. Expression of *cld20* was also affected by both factors but in a different manner, being higher at 26 °C in the anterior intestine and at 18 °C in the mid intestine. Finally, *cld19* was not affected by either temperature or intestinal segment (Fig. 8).

4. Discussion

Commercial on-growing of greater amberjack is currently being done in sea cages and is therefore highly dependent on the environmental water temperature. There are also attempts to on-growing this species in indoor RAS facilities in which water temperature could be settled with a certain economic cost. In both cases, it is necessary to know the response of digestion under the expected or programmed temperatures, and how this response may affect the final growth and welfare. Tested temperatures in this study are within the tolerance range described so far for this species (Nakada, 2000; Takakuwa et al., 2006; Fernández-Montero et al., 2018; Yokoyama et al., 2020) and no critical or drastic reactions were expected. In this study, the most evident result is that growth rate increased with the increase of temperature. As it was expected, 26 °C seems to be still a comfortable temperature for growing juveniles of this species. The growth rates recorded in this experiment are quite similar to those found in other studies within the same body size range and temperatures (Fernández-Montero et al., 2018; Yokoyama et al., 2020; Takakuwa et al., 2022). The question is how the biomass increment is being obtained at the different temperatures, what is contributing to a better weight gain and what are the possible vulnerabilities related to changes of temperature. It is evident that associated changes in metabolic cost, not examined here, affect directly the growth capacity. In the present study we have focused mainly on the feeding and digestion.

Water temperature modulates each physiological process related to acquisition of nutrients and their utilization by the growing body, from feed intake to tissue building and muscle growth (ingestion, digestion, absorption, transport by blood, liver reserves, nutrient deposition in muscle). In our study, specific voluntary feed intake was clearly higher at 26 °C (5.3 % *W*/W) but was similar at the other two tested

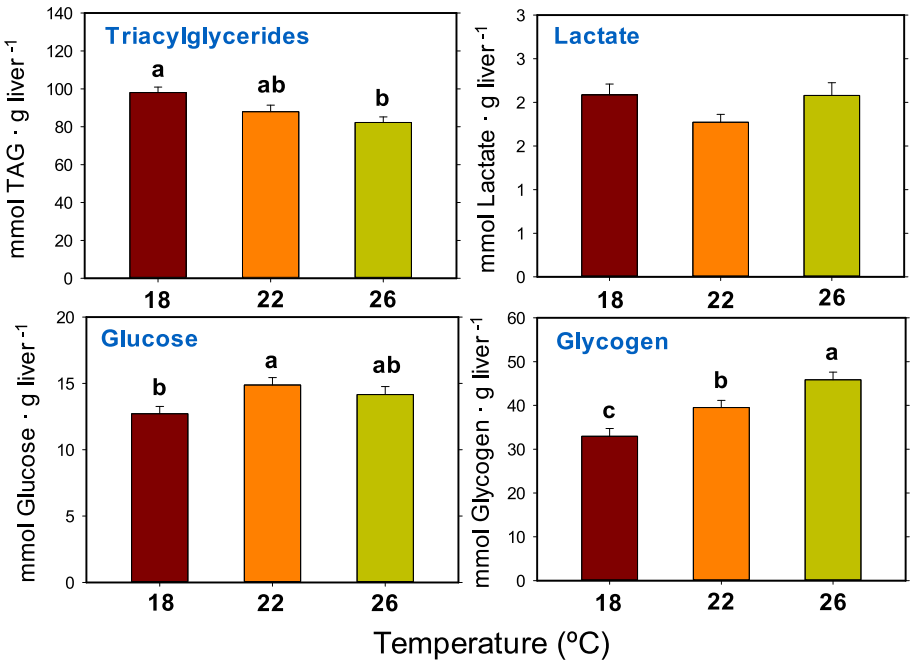

Fig. 4. Hepatic metabolites of greater amberjack juveniles on-grown at the three tested temperatures (mean ± SEM). Different superscript letters indicate significant differences (one-way ANOVA; $p < 0.05$).

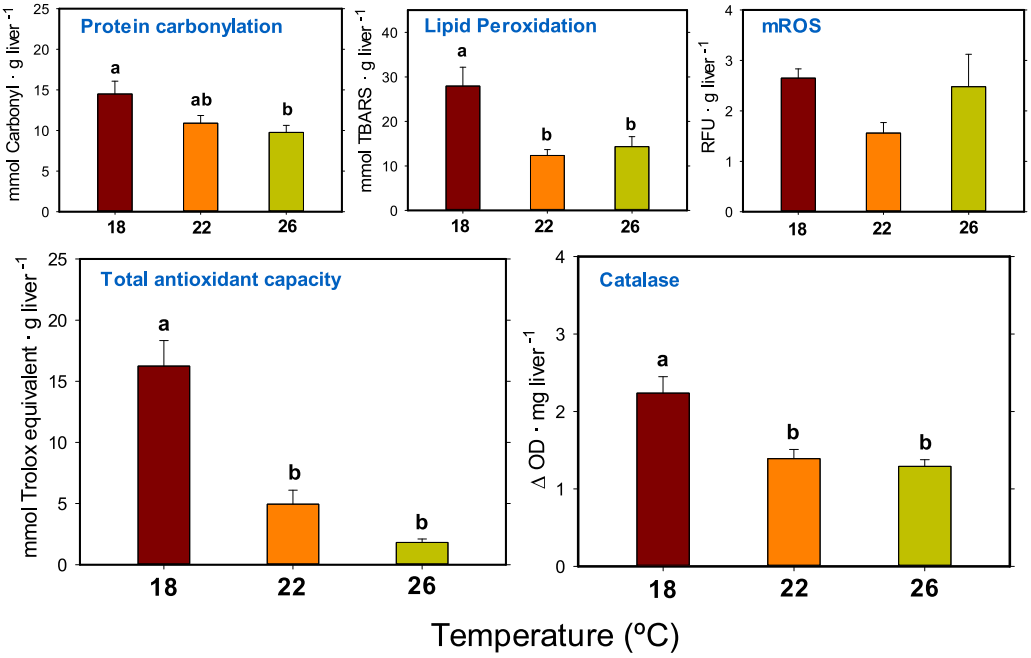

Fig. 5. Oxidative stress biomarkers in liver of greater amberjack on-grown at the three tested temperatures (mean ± SEM). mROS: mitochondrial reactive oxygen species. Different superscript letters indicate significant differences (one-way ANOVA; $p < 0.05$).

temperatures. That is, the growth difference between 18 and 22 °C cannot be justified by changes of total daily feed ingestion. This response is concordant with results of a previous short-term experiment (Navarro-Guillén et al., 2023a). However, FCR were similar at 22 and 26 °C (1.02 and 1.05 respectively) and less favourable at 18 °C (1.43). This would explain in part the growth difference between 18 and 22 °C but also indicates that there is no apparent additional energy cost for the higher growth obtained at 26 °C as compared with 22 °C. Interestingly, Yokoyama et al. (2020) found in this species fed on different diets at 27–29 °C, that the daily voluntary feed intake ranged between 3.58 and 4.11 % and the FCR between 1.06 and 1.19, indicating that the optimal temperature for these two parameters began to be exceeded.

Water temperature did affect the digestive enzyme activity, but no excessive differences among temperatures were observed in the level of digestive proteases activities in the intestine (trypsin, chymotrypsin and leu-aminopeptidase). In the stomach, pepsinogen (as analysed at pH 2.5) was available at the three temperatures but pepsin could not be activated at the actual physiological pH measured in the stomach. In fact, *in vitro* characterization of pepsin showed no measurable activity above pH 3.5 (Navarro-Guillén et al., 2022). This relatively poor gastric

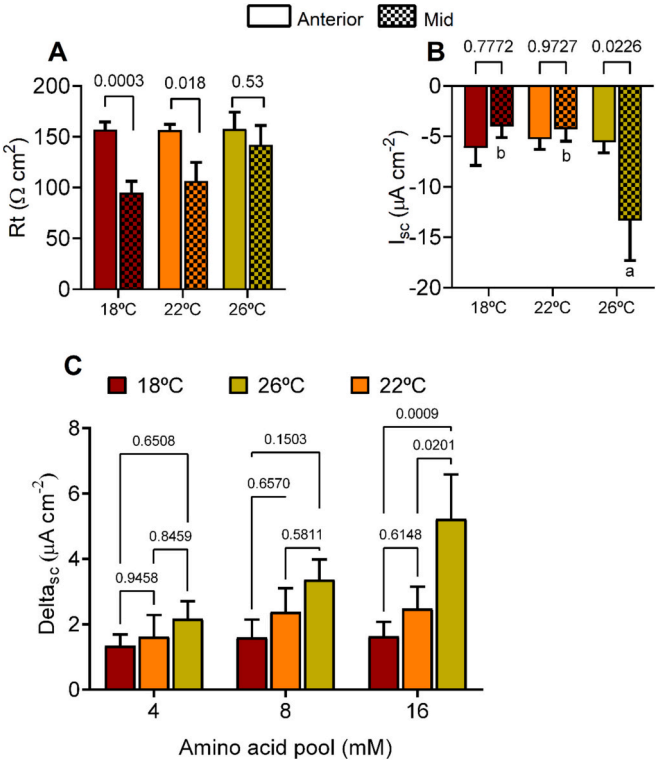

Fig. 6. Trans-epithelial resistance (A), short-circuit current (B) and electrogenic amino acids transport (C) in anterior and medium intestine tissues of greater amberjack on-growing at the three tested temperatures (mean ± SEM). Numbers over bars indicate *P* values among treatments.

acidification was already observed in a previous experiment (Navarro-Guillén et al., 2023a) and could be either due to buffering capacity of this feed or to the quick transfer of chyme to the intestine, but in any case, it did not seriously affect the growth capacity. The positive effect of temperature on the hydrolysis of nutrients was clearly observed in the increase of the lipase activity. The most remarkable fact is that we did not detect any lipase activity at 18 °C. This result is concordant with our previous *in-vitro* analyses that already showed no lipase activity below 20 °C. Likewise, we were not able to detect α-amylase activity at any temperature. We have no clear explanation for this frustrating result, because low carbohydrases activity is still expected in fish species with carnivorous preferences. In fact, we did not detect amylase activity in juveniles of this species in our previous studies (Navarro-Guillén et al., 2022), although we did detect amylase in first-feeding larvae using fluorescence techniques (Gamberoni et al., 2021). Conversely, Bowyer et al. (2014) working with yellowtail kingfish (*Seriola lalandi*) in the temperature range 21 to 27 °C was able to detect amylase at 21 °C, and found the highest trypsin and lipase activities at 24 °C. Nevertheless, it is difficult to obtain reliable comparisons for the enzymatic activities with other studies. Besides the interspecific response variation, the differences in sampling and analytical methodology are skewing the results. In addition, the response pattern against the temperature may vary with the diet composition as observed in yellowtail (*Seriola quinqueradiata*) by Kofuji et al. (2005). In our study, 22 to 26 °C seems to be around of optimal temperatures for the *in-vivo* work of digestive enzymes. This higher enzymatic activity measured at 22 and 26 °C as compared to 18 °C somehow compensates the faster gut transit and shorter residence time of digesta observed at increasing temperatures (Navarro-Guillén et al., 2023a).

In an attempt to elucidate the influence of temperature in nutrient absorption from the lumen we analysed, firstly, the mRNA expression of some transepithelial solute carriers and, secondly, the transport capacity of the intestinal mucosa. Here we analysed the molecular expression of three amino acid (slc3a1, slc3a2, slc7a5) and three oligopeptide (slc15a1, slc15a2, slc15a4) transporters of the SLC gene family. These genes have been described in many fish species (Romano et al., 2014) and aside of their evident relation with the nutritional status (Terova et al., 2009), some of them have been studied in relation to environmental conditions, particularly to changes in water salinity (Bucking and Schulte, 2012; Con et al., 2017; Nitzan et al., 2017), but scarcely in relation to environmental temperature. We found that the expression of these transporters was affected in a different manner by temperature. Overall, but not always, the mRNA expression tended to be higher at 18 °C and lower at 26 °C indicating either a higher transcript turnover at higher temperature or a higher production at 18 °C to reinforce the transport. Concordantly, Jeffries et al. (2018) working on Delta smelt (*Hypomesus transpacificus*) found a decrease of the expression of the ion exchange transporter *slc8b1* by increasing the temperature from 17 to 25 °C, and the expression increased again at higher temperatures up to 27 °C, a response attributed to reaching sub-lethal thermal threshold. In our experimental design, one of the conclusions that comes across very clearly is that the main factor regulating the gene expression of the transporters tested is the intestinal region, with specific effects of temperature in some cases. It remains to be tested if these changes along the intestine (and the specific effects of temperature) are related to changes in luminal pH, or regulated by the specific substrates available in each intestinal region.

Our *ex-vivo* measurements with Ussing chambers were performed at the culture temperature to ensure an accurate assessment of the intestinal mucosa functionality. We found that the trans-epithelial resistance (Rt, Ω cm^{-2}) was constant and maximum in the anterior intestine at all temperatures. However, in the mid-intestine Rt increased in parallel with temperature to reach levels similar to those to the anterior intestine at 26 °C. The constant level of Rt in the anterior intestine is probably linked to the need to maintain a consistent transcellular barrier against the chyme in this segment, which is still close to the stomach and faces the relatively low pH gastric content, bile, and rapid changes in luminal content (Syakuri et al., 2013). Additionally, the stepwise increase in Rt in the mid-intestine at higher temperatures is likely related to the functional need for better protection in this segment. This need arises only at the highest tested temperature when gut transit is much faster and food can reach the mid-intestine within a few minutes after ingestion (Navarro-Guillén et al., 2023a). Maintaining the integrity of the barrier function in the intestinal epithelium is crucial, as it separates the body's internal and external compartments (Clarke, 2009), to prevent the translocation of luminal contents through the epithelium. In fish intestines, as far as we know, the potential effect of temperature had not been demonstrated, but this approach has been used to evaluate epithelial damage caused by the introduction of plant proteins or alternative protein sources in sea bream (Estensoro et al., 2016; Aragão et al., 2020; Molina-Roque et al., 2022), sea bass (Fonseca et al., 2023), and meagre (Sáenz de Rodrigáñez et al., 2013).

It seems that temperature modulates the physiological plasticity of intestinal epithelial function in the greater amberjack. Thus, in addition to changes in barrier function, we also observed changes in short-circuit current in the mid-intestine, which was notably stronger at 26 °C, pointing to a higher transcellular net transport capacity. Intestinal absorption of protein components (amino acids and small peptides) requires coordinated nutrient and ion transport actions. This interaction connects nutrient sensing with nutrient absorption by regulating ion transport (McCauley et al., 2020). Here, we performed a comparative analysis of the effect of temperature on the mid-intestine of fish and observed a higher electrogenic transport of essential amino acids in fish raised at 26 °C. Interestingly, this coincides with the tissue exhibiting the strongest basal absorptive Isc. When plotting temperature *versus* the slope of response to increasing concentrations of essential amino acids (Fig. 6C), the group raised at 26 °C shows a much steeper slope than projected from the data obtained at 18 and 22 °C, that conforms to an exponential regression where slope $= 8^{-0.5}e^{0.3059\times Temp}$, ($R^2 = 0.9975$).

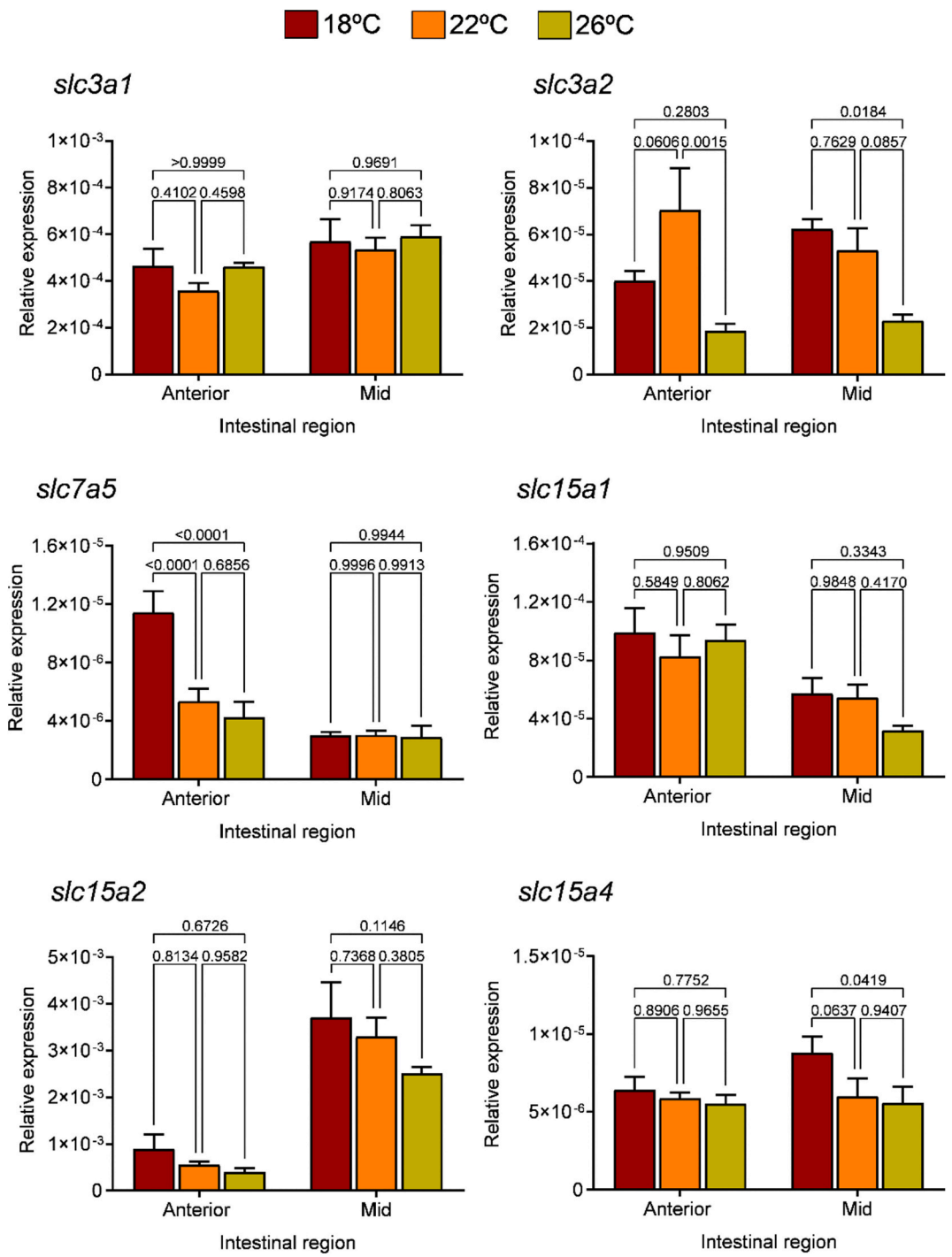

Fig. 7. Gene expression of peptides transporters of greater amberjack on-growing at the three tested temperatures (mean ± SEM). Numbers over bars indicate P values among treatments.

This indicates that the expected functional linearity (based on the Arrhenius plot) is affected by other biological factors, making the intestinal epithelium more effective at amino acid transport at 26 °C, likely due to molecular regulation when the fish were exposed *in vivo*.

Absorptive functionality of intestinal mucosa is also mediated by claudins that are involved in the control of tight junctions among epithelial cells. In the present study, four claudin genes, *cldn-11*, *−12*, *−19*, and *− 20*, were identified in the intestine of greater amberjack. All of them, were previously found in the intestine of other fish species. *Cldn-11* was reported in the intestine of common carp (*Cyprinus carpio*) (Syakuri et al., 2013; Wu et al., 2017), zebrafish (*Danio rerio*) (Clelland and Kelly, 2010; Kumai et al., 2011) and channel catfish (*Ictalurus punctatus*) (Sun et al., 2015), being more expressed in the mid-intestine. *Cldn-12* was reported in Japanese pufferfish (*Fugu rubripes*) (Loh et al., 2004), zebrafish (Clelland and Kelly, 2010; Kumai et al., 2011), goldfish (*Carassius auratus*) (Chasiotis and Kelly, 2012), gilthead seabream (*Sparus aurata*) (Pérez-Sánchez et al., 2015), rice field eel (*Monopterus Albus*) (Shi et al., 2020) and channel catfish (*Ictalurus punctatus*) (Sun et al., 2015; Shi et al., 2021). *Cldn-19* has been found in the intestine of channel catfish (Sun et al., 2015) and sea lamprey (*Petromyzon marinus*) (Kolosov et al., 2020). *Cldn-20* was also reported in intestine of channel catfish (*Ictalurus punctatus*) (Sun et al., 2015).

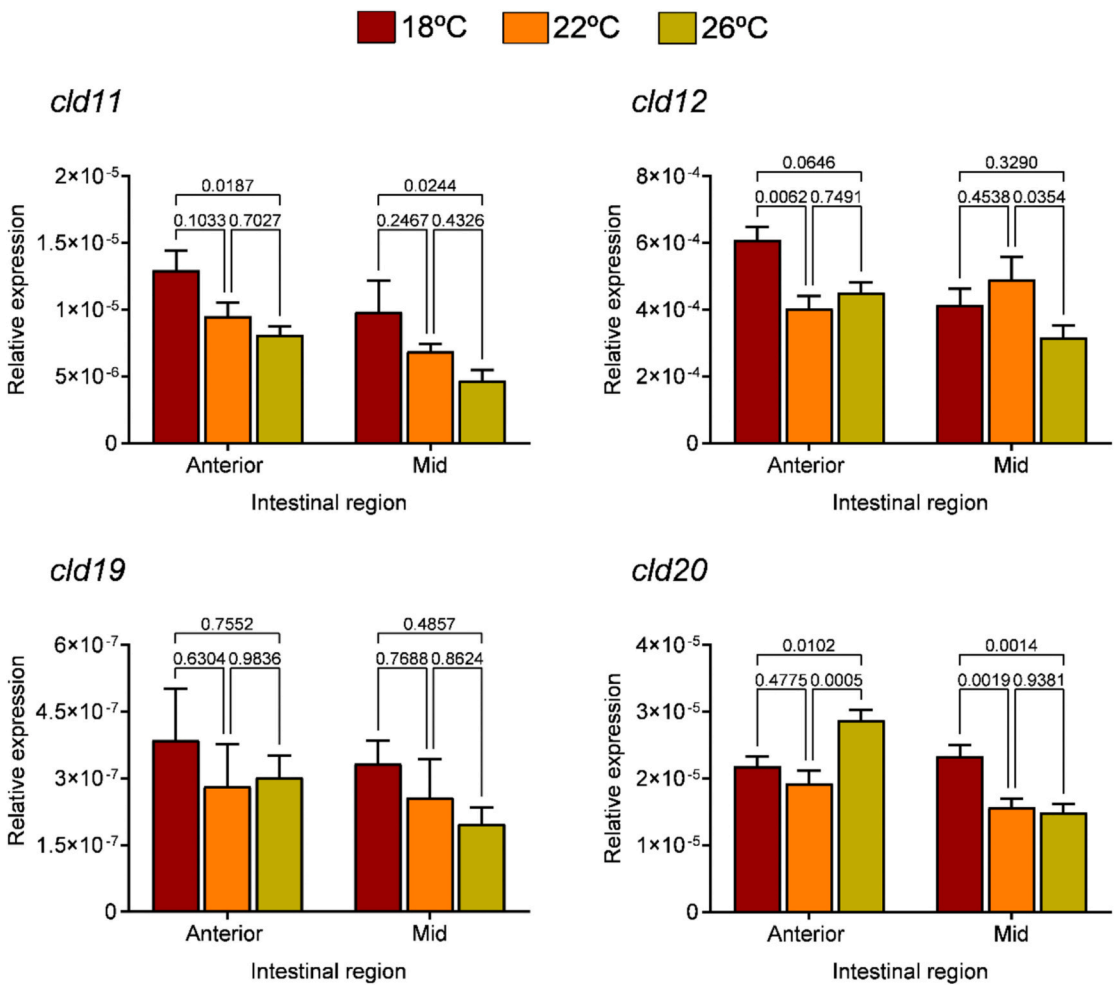

Fig. 8. Gene expression of claudins of greater amberjack on-growing at the three tested temperatures (mean ± SEM). Numbers over bars indicate P values among treatments.

Claudin 11 and 19 are barrier building and decreased ions permeability (Amasheh et al., 2011; Kolosov et al., 2020) while claudin-12 has been proposed to increase or decrease paracellular permeability (Amasheh et al., 2011). Information on the intestinal role of Claudin 20 is scarce, but its gene overexpression in human breast tissue has been associated to decreased trans-epithelial resistance (Martin et al., 2013). In our study, only two claudin genes (*cldn-11* and *cldn-20*) clearly responded to changes in temperature. *Cldn-11* exhibited higher expression in both the anterior and middle intestine at 18 °C, which may indicate a higher selectivity and integrity of the tight junctions. *Cldn-12* and *cldn-19* also showed decreasing trends with the increase of temperature but the difference was only statistically significant in *cldn-12* in the anterior intestine between 18 and 22 °C. In contrast, *cldn-20* had opposite expression patterns in the anterior intestine and the mid-intestine in relation to temperature. It is interesting to remark that in the mid-intestine higher *cldn-20* expression corresponded to the lower recorded trans-epithelial resistance.

Once the nutrients have been absorbed, plasma analyses revealed that only the protein level seems to be affected by the temperature increase as correspond to a higher protein deposition and body growth, in addition to being putatively used as energy sources when in excess (García-Márquez et al., 2023, and references therein). Thus, our results on plasma proteins could also be explained and correlated with growth performance. Moreover, cortisol levels were also higher at 22 and 26 °C but always maintaining very low levels (<10 ng/mL), thus indicating not only a notable well-being in the range of temperatures tested herein, but also suggesting a clear combination between i) a moderate increase in this hormone known to positively modulate voluntary feed intake and feed-anticipatory activity (Bernier et al., 2004) with increasing temperatures, ii) and the orchestration of general physiology to produce a hypometabolic state under sub-optimal environmental conditions (Martos-Sitcha et al., 2019), as shown in fish maintained at the lowest temperature tested. Regarding hepatic metabolites, our results demonstrate a clear effect of temperature in energy reserves in the liver. Thus, higher triacylglycerides content was recorded at the lowest temperature and higher carbohydrates at the highest temperature. That means that at 18 °C the juveniles are mobilizing preferentially carbohydrate reserves as the main source of energy, while at 26 °C seems to be the use of lipids.

Finally, a clear effect of water temperature was also observed in the liver oxidative status. Although no significant differences were recorded in the mitochondrial production of ROS between fish from the three temperatures, the biomarkers for oxidative damage (PC and LPO) and antioxidant defenses (CAT and TAC) were significantly higher in fish that grew at 18 °C. At low temperatures ROS production levels seem not to be the only factor favoring oxidative damage, the enhanced oxygen solubility and a decreased mitochondrial membrane fluidity can also be key factors promoting oxidative stress (Joy et al., 2017). In addition, an increased polyunsaturation in the mitochondrial membranes may boost peroxidation of lipids (Ale et al., 2021). Similarly to what found in the present study, the increase in both, oxidative defenses and damage by low water temperature has been reported for the gilthead seabream (Ibarz et al., 2010), the Brazilian flounder (*Paralichthys orbignyanus*) (Garcia et al., 2015), and the pacu (*Piaractus mesopotamicus*) (Ale et al., 2021). Thus, it appears that antioxidant defenses may be elevated if

temperature decline is not too severe, but with a prolonged temperature exposure, antioxidant defenses are not enough to prevent oxidative damage (Reid et al., 2022). On the other hand, Hao et al. (2024), working with greater amberjack juveniles at elevated temperatures in the range 25 to 31 °C, found that long-term exposure to the higher temperature increased oxidative damage biomarkers and decreased antioxidant defenses in plasma.

5. Conclusions

The present study addresses a comprehensive analysis of the functional modulation of the digestive system by water temperature in greater amberjack juveniles, covering from nutrient hydrolysis, to energy mobilization and mucosa remodelling. Results confirm that the tested temperatures are within the tolerance range for the species, being the most evident and expected result that growth rate increased with the increase of temperature. However, there were observed effects on key aspects of digestive physiology. We found a clear effect of temperature on intestinal hydrolytic capacity, but primarily on the lipases and in a lesser extent in the proteases. In addition to the hydrolytic work of the digestive enzymes, water temperature also modulated the remodelling of the intestinal mucosa. This is the first work assessing the effect of temperature on intestine electrophysiological properties in fish, as well as on the expression levels of peptide transporters and some claudins in greater amberjack intestine. Higher temperature, within the tested range, promoted the epithelial function in the intestine through higher tissue resistance and electrogenic transport of amino acids while, on the other hand, mRNA expression of peptide transporters tended to be higher in fish that grew at 18 °C, probably to reinforce the transport capacity. Regarding fish metabolic plasticity, differences in cortisol levels may be a consequence of variations in metabolic rate with temperature. The use of liver energy reserves was also differently modulated by temperature. At 26 °C fish seems to be using primarily triacylglycerides for covering the energetic demand, while at 18 °C switched to carbohydrates, with the concomitant increase in TAG leading to a higher risk of liver steatosis and oxidative stress.

From a practical view, 22 and 26 °C appear as optimal temperatures for on-growing the species, although surely better growth would be obtained at 26 °C in longer periods. At 18 °C, the low ingestion and the damage derived from the oxidative stress impair the growth capacity and might compromise fish welfare.

CRediT authorship contribution statement

C. Navarro-Guillén: Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **E. Perera:** Writing – review & editing, Validation. **D. Pérez-Hilario:** Validation, Methodology. **J.A. Martos-Sitcha:** Writing – review & editing, Investigation, Formal analysis. **L. Molina-Roque:** Validation, Formal analysis. **S.F. Gregorio:** Validation, Formal analysis. **F. Fonseca:** Validation, Formal analysis. **J. Fuentes:** Writing – review & editing, Validation, Investigation, Formal analysis. **M. Yúfera:** Writing – original draft, Validation, 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

Data will be made available on request.

Acknowledgments

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