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Seasonal energy investment and metabolic patterns in a farmed fish

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ABSTRACT

The present research focuses on the seasonal changes in the energy content and metabolic patterns of red porgy (*Pagrus pagrus*) sampled in a fish farm in North Evoikos Gulf (Greece). The study was designed in an effort to evaluate the influence of seasonality in several physiological feauteres of high commercial importance that may affect feed intake and growth. We determined glycogen, lipids and proteins levels, and cellular energy allocation (CEA) as a valuable marker of exposure to stress, which integrates available energy (Ea) and energy consumption (Ec). Metabolic patterns and aerobic oxidation potential were based on the determination of *glucose transporter (GLU), carnitine transporter (CTP), L-lactate dehydrogenase (L-LDH), citrate synthase (CS), cytochrome C oxidase subunit IV isoform 1 (COX1)* and *3-hydroxyacyl CoA dehydrogenase (HOAD)* relative gene expression. To integrate metabolic patterns and gene expression, L-LDH, CS, COX and HOAD activities were also determined. For further estimation of biological stores oxidized during seasonal acclimatization, we determined the blood levels of glucose, lipids and lactate. The results indicated seasonal changes in energy content, different patterns in gene expression and reorganization of metabolic patterns during cool acclimatization with increased lipid oxidation. During warm acclimatization, however, energy consumption was mostly based on carbohydrates oxidation. The decrease of E_c and COX1 activity in the warm exposed heart seem to be consistent with the OCLTT hypothesis, suggesting that the heart may be one of the first organs to be limited during seasonal warming. Overall, this study has profiled changes in energetics and metabolic patterns occurring at annual temperatures at which *P. pagrus* is currently farmed, suggesting that this species is living at the upper edge of their thermal window, at least during summer.

1. Introduction

Energy allocation and maintenance of balance between energy input and energy expenditure contributes to the maintenance of basic cellular functions and organism conservation. (Pörtner., 2006; Guderley and Pörtner, 2010; Sokolova, 2013) However, constraints in energy supply can be caused by the seasonal reduction in feeding rate, food availability or changing energy assimilation from the food intake, and are also related to fluctuating energy storages (Fernandes and McMeans, 2019). Moreover, energy provision and consumption may be restricted when fish are exposed beyond their optimum thermal limits. Usually, voluntary food intake increases with moderate elevation of temperature, while it decreases when temperatures are outside the fish's optimal temperature range (Volkoff and Rønnestad, 2020). It has been reported that fish lose appetite and stop ingesting food at temperatures well before the ultimate low and high critical temperature for the species and its lifestage are reached (Pörtner et al., 2004, 2017). On the other hand, low temperature might affect and modulate several physiological processes such as gut transit time, digestive enzyme activity and nutrient digestibility (Miegel et al., 2010). Consequently, seasonal changes in the temperature may determine how much energy fish should obtain (through regulation of feeding behavior and food intake), how much of that energy is acquired (through digestion and absorption), and how much of it can be allocated to key processes such as activity, growth (including development in larvae and juveniles), and reproduction. These temperature-induced physiological changes are followed by metabolic reprogramming and oxidation of energy stores (e.g. lipids, carbohydrates), dependent on the energy demand of tissues. The latter

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aims to maintain energy balance and basal physiological functions enabling fish to withstand thermal stress (Pörtner and Farrell, 2008; Windisch et al., 2011; Jayasundara and Somero, 2013; Sokolova, 2013; Jayasundara et al., 2015; Feidantsis et al., 2018; Sánchez-Nuño et al., 2018a). However, limitation to aerobic performance and metabolic sift to anaerobic component of metabolism when fish are exposed beyond their lower and upper thermal limits causes reduction in ATP turnover and eventually weakens their thermal tolerance and acclimatization (Pörtner and Knust, 2007).

However, the environmental factors causing energetic constraints in nature are complex in space and time and are species specific. Therefore, field studies on the energetic changes in fish can make a significant contribution to understanding whether they face energy constraints seasonally. On the other hand, the Mediterranean Sea has been identified as a ‘‘hotspot’’ for climate change, while further increase in temperature is expected within the next decades altering marine biodiversity and productivity (Galli et al., 2017; IPCC, 2021). Accordingly, taking global warming into account, the results from field studies on the metabolic patterns and energetics can further contribute to the assessment of thermal resistance in relation to their energy requirements and consumption, especially in the high temperature season (Pörtner et al., 2017; Sokolova, 2013). In this context, field studies on farmed fish would be a useful tool to reveal warming-induced effects, as they cannot avoid the seasonal changes in the environmental temperatures. Although several laboratory studies have supported the establishment of physiological mechanisms involved during various exposures and acclimation to simulated field conditions (Pörtner and Knust, 2007; Anestis et al., 2007, 2008), few studies have attempted to link laboratory physiology experiments with ecologically relevant field data on behavior, growth, bioenergetics, and fitness (Donaldson et al., 2008; Reid et al., 1998).

Recent studies on the seasonal expression of several molecular and metabolic indicators in farmed *Sparus aurata* indicated that this species exhibits stress phenomena during prolonged exposure to elevated summer temperatures (Feidantsis et al., 2018, 2021). Apart from *S. aurata*, however, the red porgy *Pagrus pagrus* (Linnaeus, 1758) is also a fish species of high commercial value (Makri et al., 2023) and culture techniques for this economically important species have been developed in the Mediterranean Sea (Hernandez-Cruz et al., 1999; Pavlidis and Mylonas, 2011). Although intensive research has been conducted on *P. pagrus* development, growth, and hatchery, limited knowledge is available regarding its seasonal metabolic patterns and energy allocation in response to seasonal changes of temperature. Temperature levels with a minimum of 11–12 °C and maximum of 26–27 °C are commonly reached from winter to summer, respectively, at the area where this species is farmed (Zgouridou et al., 2022). Similar to our studies on the seasonal stress phenomena in *S. aurata*, the present work aimed to integrate several physiological, biochemical and molecular indices in an effort to examine whether *P. pagrus* exhibits seasonal energetic constraints as well. The latter can further contribute to estimating differences in the thermal tolerance between these two species and to make, in the context of global warming, a better prediction regarding their vulnerability to thermal stress. For the purpose of the present work, we determined: a) the condition indices as it is the gonadosomatic index (GSI), the hepatosomatic index (HSI), the Fulton's condition index (k), the specific growth rate (SGR) and the thermal growth coefficient (TGC). The GSI is an indicator of reproductive activity, while the HSI is a measure of the relative weight of the liver, often used as an estimate of the energy store and energy status of the fish (Wootton et al., 1978). Also, Fulton's condition index (k) can provide information on fish body physiological status reflecting lipid and protein content and may be used for comparing populations living in certain feeding, climate, and other regimes (Pangle and Sutton, 2005; Froese, 2006; Mozsar et al., 2015). Therefore, this condition factor can be used to estimate the feeding activity of species. The SGR is commonly used in fish aquaculture, and it is a coefficient that measures the percentage increase in fish weight per day, while TGC is a measure of daily growth in a given period that takes into account temperature; b) the RNA/DNA ratio since it is recognized as a useful index of nutritional condition and growth of fish and it relates to metabolic activity and protein synthesis at corresponding tissues (Dahlhoff, 2004; Stevenson and Woods, 2006); c) the levels of glycogen, lipids and proteins and the cellular energy allocation (CEA), which integrates the available energy (E_a) and energy consumption (E_c) of an organism, and it has been established as a good marker of exposure to stress (De Coen and Janssen, 1997, 2003); d) the relative gene expression of *glucose transporter* (*GLUT2*), *carnitine transporter* (*CTP*), *L-lactate dehydrogenase* (*L-LDH*), *citrate synthase* (*CS*), *cytochrome C oxidase subunit IV isoform 1* (*COX1*), *3-hydroxyacyl CoA dehydrogenase* (*HOAD*) and *fatty acids binding protein 2* (*fabp2a* and *b* subunits) as indicators of metabolic patterns correlated with the glucose and fatty acid oxidation. Fabp2 is reported to be able to facilitate the uptake and subsequent intracellular transport of diet-derived fatty acids (Alpers et al., 2000; Venold et al., 2013); e) the activities of L-LDH, CS, COX and HOAD in order to integrate the gene expression to metabolic patterns; f) the levels of glucose, lipids and lactate in the blood for further estimation of biological stores oxidized during seasonal acclimatization.

2. Materials and methods

2.1. Animals, experimental procedures, and tissue sampling

The study was conducted in an aquaculture installation at Larymna, Evoikos Gulf, in Greece (Fig. 1A). Based on the annual changes in sea water temperature (Fig. 1Ba), the seasonal profile of sea water temperature was divided into two periods, the first one characterized by decreasing temperatures or cold acclimatization (Sep 15, 2020 until Feb 29, 2021) and the second one by increasing temperature or warm acclimatization (Feb 29, 2021 until Jul 20, 2021) (Fig. 1Bb). Accordingly, individuals were collected within these two periods. In brief, farmed *P. pagrus* grown at the area of aquaculture up to sampling size (mean ± SD weight of 545 ± 6.5 g, N = 10) were collected monthly from the cages and were placed in sea water containing MS-222 to a final concentration of 0.15 g l^{-1} for euthanasia. Fish body weight was measured before dissection. Afterwards fish were dissected, and tissue (heart, liver, white and red muscle) samples were collected and then immediately frozen in liquid nitrogen, transported to the laboratory and maintained at −80 °C until gene expression, biochemical and metabolic analyses. Liver weight was measured after dissection For further analyses, i.e. RNA/DNA, gene expression analysis and protein levels concentration, 10 samples (1 from each animal, N = 10) were employed for each tissue and time point. Using the same sampling scheme, blood sampling was performed to obtain plasma for glucose, lactate, and triglycerides measurements. In order to obtain plasma, whole blood samples were centrifuged at 2000 *g*, for 10 min at 4 °C.

Animals received proper care in compliance with the “Guidelines for the Care and Use of Laboratory Animals” published by US National Institutes of Health (NIH publication No 85–23, revised in 1996) and the “Principles of laboratory animal care” published by the Greek Government (160/1991) based on EU regulations (86/609). The protocol as well as surgery and sacrifice conditions were approved by the Committee on the Ethics of Animal Experiments of the Directorate of Veterinary Services of the Prefecture of Thessaloniki, Greece under the license number EL 54 BIO 05.

2.2. Analytical procedures

2.2.1. Condition indices

The gonadosomatic index (GSI), hepatosomatic index (HSI) and Fulton's condition index (k) were estimated as follows.

- GSI = (W_g/W_f) x 100, where W_g and W_f correspond to the gonad and the gonad-free body weight, respectively.

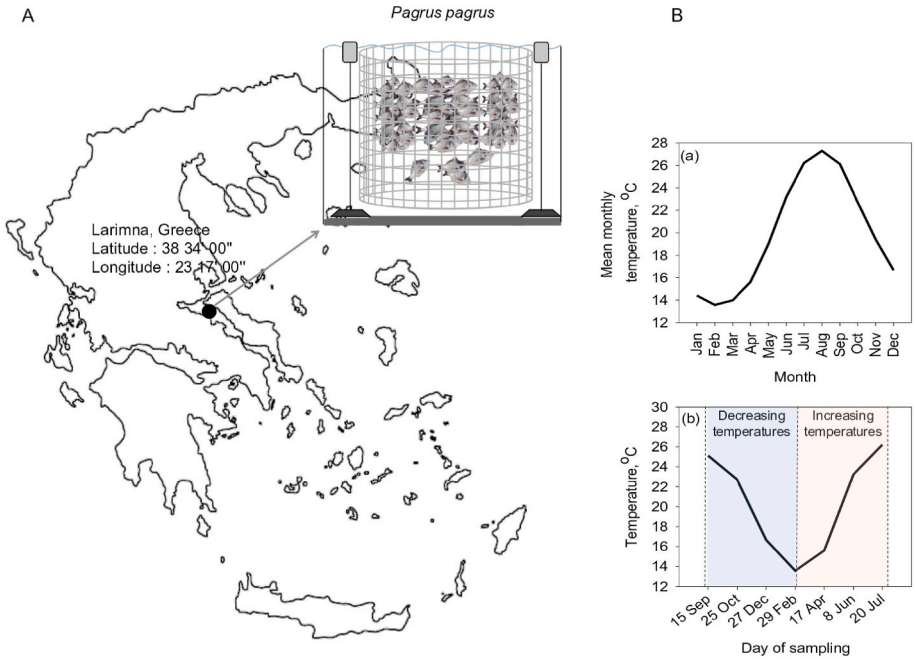

Fig. 1. (A) Study area in Larymna - North Euboean Gulf, Greece; B (a) seasonal variations of sea water temperatures in the study area and B (b) sea water temperature during fish sampling. Light blue rectangle depicts decreasing sea water temperature, while light red rectangle depicts increasing sea water temperature.

- HSI = (W_l/W) x 100, where W_l and W is the liver weight and total weight of the fish (in g), respectively.
- Fulton's condition index (k) was calculated as K = 100 X W/L^3, where W is the fish weight (g) and L the total length (cm).

The thermal growth coefficient (TGC) and specific growth rate (SGR) were measured with following formulas:

$$TGC = \left[\frac{\sqrt[3]{W_t} - \sqrt[3]{W_0}}{T \times t}\right] \times 1000$$

$$SGR = \left[\frac{\ln W_t - \ln W_0}{t}\right] \times 100$$

where T is temperature in °C, W_t is the fish body weight (g), W_0 the initial body weight (g), and t the number of days between each weighing. SGR and TGC were calculated using the average body weight of the 10 fish sampled at each time point.

2.2.2. RNA/DNA ratio

For the determination of RNA/DNA ratio, 0.2 g sample of the examined tissues was placed in buffer RLT Plus (Qiagen, Germany) and AllPrep DNA/RNA Mini Kit (Qiagen, Germany) was used for the simultaneous extraction of total RNA and genomic DNA in each homogenized tissue sample. The homogenate was embedded in lysis buffer and passed through two columns: at first through a silica column that binds DNA and secondly through a second column in order for the RNA to be selectively bind. In this manner, the identical sample was utilized for the extraction of both nucleic acids, eliminating erroneous results in case of different tissue parts examination. Flow through the DNA column was eventually diluted in 50 μL of Qiagen elution buffer, whereas flow through of the RNA column was diluted in 50 μL ultrapure water and their concentrations were measured in a UV–Vis spectrophotometer (Quawell Q5000, Quawell Technology, China). Each measurement carried out used mean value for the estimation of the RNA/DNA ratio.

2.2.3. Cellular energy allocation (CEA)

CEA, which results from the E_a/E_c ratio, represents the organism's net energy budget, is vital for understanding the organism's metabolic balance (Verslycke et al., 2004). For CEA calculation, we determined the available energy (E_a, as the sum of energy reserves such as carbohydrates, lipids, and proteins) and aerobic energy consumption (E_c, measured as ETS activity). ETS capacity as an attempt at correlates of *in vivo* energy use was determined according to the protocol described by Haider et al. (2017), while total energy reserve was determined according to the protocol described by Gnaiger (1983) according to which measured protein, lipid and carbohydrate were transformed the into energy equivalents (respective energy of combustion, i.e., 24 kJ g^{-1} for proteins, 39.5 kJ g^{-1} for lipids and 17.5 kJ g^{-1} for carbohydrates).

2.2.4. Gene expression analyses

Quantitative real-time PCR for an estimation of gene expression profiles was performed on all four examined tissues for eight different genes. Initially, RNA was extracted from homogenized tissues using the Nucleozol purification kit (Macherey-Nagel, Germany) following the manufacturer's recommended protocol. Furthermore, one μL of extracted RNA of approximate concentration 100 ng μL^{-1} were subjected to first strand cDNA synthesis using the Prime Script kit (Takara, Japan) applying the oligodT primers option. The expression levels of *GLUT2, CTP, L-LDH, CS, COX, HOAD* and *Fabp2a/2b* were determined in a real time PCR and primer pairs for each amplified gene are presented in Table 1. Since the newly designed primers were based on closely related species, PCR products were also run in an agarose gel after electrophoresis to confirm the validity of the product.

PCR was performed in a PCR max Eco 48 instrument using the AMPLIFYME SYBR Universal Mix kit (blirt, Poland) in 10-μL reactions containing 5 μL 2x AMPLIFYME ready for use, 0.3 pmol of each forward and reverse primer, 1 μL cDNA of approximate concentration 50 ng μL^{-1} and ultrapure water up to the final volume of 10 μL. Conditions were all the same, i.e., 2 min at 95 °C and 40 cycles of 5 s at 94 °C, 10 s at 60 °C and 20 s at 72 °C. Housekeeping genes used as reference for quantification were the elongation factor (EF-1) and the ribosomal gene L13a (Table 1). Relative quantification was calculated from the comparison of the investigated target genes cycle threshold values (C_T) with the two aforementioned genes $2^{-\Delta\Delta Ct}$ quantification methodology (Livak and Schmittgen, 2001).

Table 1
Primer pairs used for the gene expression analysis.

Gene targeted	Primer sequence (5′-3′)	Amplified product length	Reference
glut2	F: GCTTGGTTGGATGCCTATGT R: AGGACTCTGTTGCCGCTTT	88 bp	This study (NCBI Reference Sequence: XM030416074)
ctp	F: AACCTCATCAACTTCCACATC R: TCCAAATTCGTCTCAATCATCC	168 bp	This study (NCBI Reference Sequence: XM_030426203.1
l-ldh	F: ATCCCGAACATCATCGTCAAGTA R: TTGATAACCTCGTAGGCTCC	368 bp	This study (NCBI Reference Sequence: XM_030425767.1)
cs	F: TCCAGGAGGTGACGAGCC R: GTGACCAGCAGCCAGAAGAG	51 bp	Bermejo-Nogales et al. (2014)
cox	F: ACCCTGAGTCCAGAGCAGAAGTCC R: AGCCAGTGAAGCCGATGAGAAAGAAC	187 bp	Bermejo-Nogales et al. (2014)
hoad	F: TCACTTCTTCAACCCAGTCC R: GTTGACAATGAATCCCGGTG	154 bp	This study (NCBI Reference Sequence: XM_030431227.1)
Fabp2a	*F: GCTGGCTGCTCACGACAAC* *R: CGTGATCAGTTTGGTCTAAGC*	*325 bp*	Kaitetzidou et al. (2015)
Fabp2b	*F: CCGCAACGACAACTATGATAAG* *R: TGGACTCTTTGATGTGAAACTTG*	*131 bp*	Kaitetzidou et al. (2015)
ef-1	F: CCCGCCTCTGTTGCCTTCG R: CAGCAGTGTGGTTCCGTTAGC	135 bp	Bermejo-Nogales et al. (2014)
l13a	F: TCTGGAGGACTGTCAGGGGCATGC R: AGACGCACAATCTTAAGAGCAG	148 bp	Kaitetzidou et al. (2015)

2.2.5. Determination of enzyme activities in the tissue homogenates

The enzymatic activities (V_{max}) of L-LDH (EC 1.1.1.27), CS (EC 4.1.3.7), and HOAD (EC1.1.1.35) and cytochrome *c* oxidase (CIV, EC 1.9.3.1) were spectrophotometrically determined at 18 °C according to well-established protocols for fish tissues (Moon and Mommsen, 1987; Sidell et al., 1987; Singer and Ballantyne, 1989; Driedzic and Almeida-Val, 1996; Hunter-Manseau et al., 2019).

2.2.6. Determination of glucose, lactate and triglycerides in the plasma

Lactate, glucose and triglycerides were determined in plasma sampled from fish using commercial kits from Spinreact, Spain.

2.3. Statistics

The statistical analysis of results was performed using SPSS 22.0. Comparisons among samples were made by one-way analysis of variance (ANOVA), attributing significance to 5% confidence level ($p < 0.05$). The Bonferroni test, followed by Dunn's post-test, was employed to perform post-hoc comparisons.

3. Results

3.1. Seasonal profile of sea water temperature

Fig. 1Ba depicts the mean monthly sea water temperature recorded during the last decade in the fish farm where the samplings were performed. As shown, temperature decreases up to the middle of March and starts increasing after the middle of April until July and August. During seasonal samplings, the lowest temperature of sea water was recorded in February (12.9 °C), while the highest temperature was recorded in July (26,2 °C) (Fig. 1Bb).

3.2. Seasonal changes in condition and biochemical indices

The annual change in the (SGR) seems to follow that of sea water temperature, exhibiting a significant decrease up to mid-March. However, a continuous and sharp increase was observed afterwards, with the highest annual values observed in mid-July and August (Fig. 2).

Similar to SGR, the TGC followed the changes in the sea water temperature during cooling. In contrast to SGR, however, the changes in TGC seem to follow two distinct periods of increase. A sharp increase up to early May was followed by a slower increase up until August.

HSI exhibited no changes (Fig. 3a), while the Fulton's Factor exhibited a gradual decrease from September to February. Thereafter, it progressively increased from February until June (Fig. 3b). Regarding GSI, it exhibited a significant increase from December until the middle of April, followed by a significant decrease from June to July (Fig. 3c).

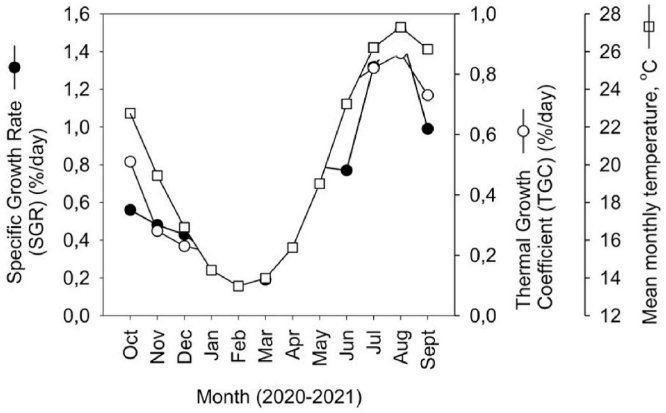

Fig. 2. Seasonal changes in specific growth rate (SGR) and thermal growth coefficient (TGC) of *Pagrus pagrus* in comparison to mean monthly sea water temperature.

The RNA/DNA ratio in the heart exhibited two different significant peaks. An increase was observed between September and December, followed by a decrease in February. Thereafter, it progressively increased again until June, followed by a significant decrease in July (Fig. 4a). In the red muscle, the RNA/DNA ratio peaked from October to December, while it showed a downward trend until the middle of April, and it peaked again in July (Fig. 4b). The RNA/DNA ratio patterns in the white muscle were similar to those observed in red muscle (Fig. 4c), while in the liver a gradual increase was observed between February and July when the highest RNA/DNA ratio value was recorded (Fig. 4d).

3.3. Seasonal changes in energetic stores and available energy (E_a), energy consumption (E_c) and CEA

3.3.1. Energetic stores

Total lipid content showed two peaks in the heart, the first one late in December and the second early in June (Fig. 5Aa). In red and white muscle, the content of total lipids increased significantly by December, gradually decreased until April and thereafter remained stable until July (Fig. 5Ab and 5Ac, respectively). A continuous decrease in the total lipids was observed from September to the middle of April in the liver,

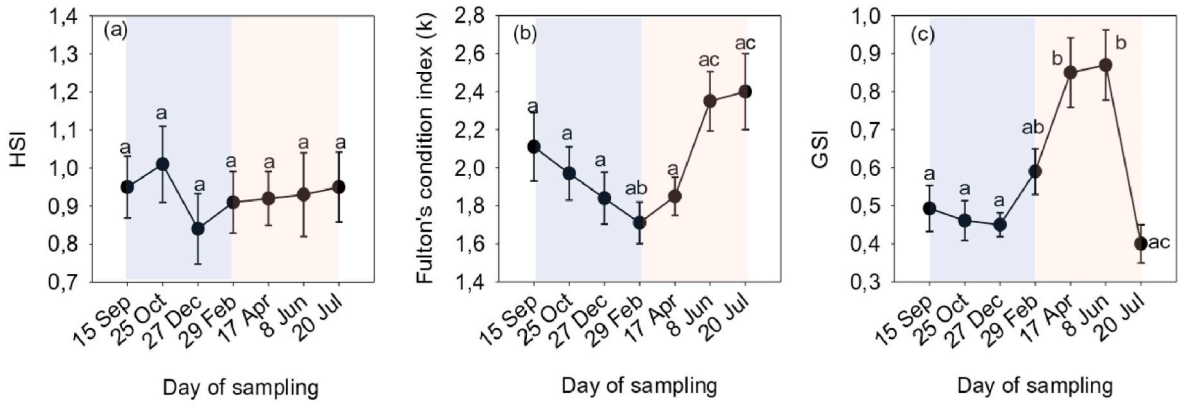

Fig. 3. Seasonal variations of (a) HIS, (b) Fulton's condition index and (c) GSI of *Pagrus pagrus*. Light blue rectangle depicts decreasing sea water temperature, while light red rectangle depicts increasing sea water temperature. n = 10 preparations from different animals. Lower-case letters indicate significant differences ($p < 0.05$) between different sampling dates.

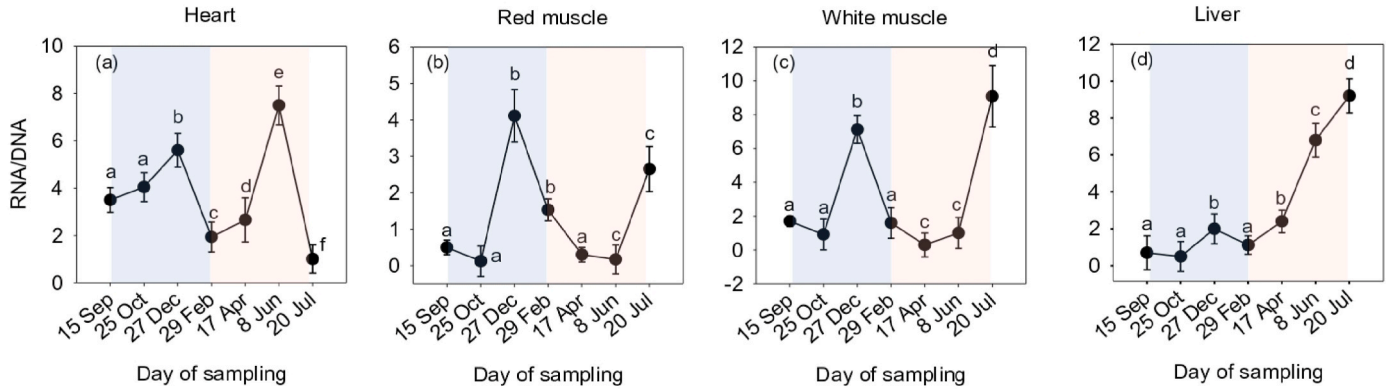

Fig. 4. Seasonal variations of RNA/DNA ratio in the heart (a), red muscle (b), white muscle (c) and liver (d) of *Pagrus pagrus*. Light blue rectangle depicts decreasing sea water temperature, while light red rectangle depicts increasing sea water temperature. n = 10 preparations from different animals. Lower-case letters indicate significant differences ($p < 0.05$) between different sampling dates.

followed by a progressive increase (Fig. 5Ad).

The total carbohydrate levels displayed a sharp increase from April to July in the heart (Fig. 5Ba), while such an increase was also observed from September to December in the red muscle. After a decrease in February, these levels remained stable until July (Fig. 5Bb). In the white muscle, changes in total carbohydrate levels were similar to those observed in the heart (Figure 5Bc). In the liver, carbohydrate levels exhibited changes similar to those observed for lipids (Fig. 5Bd).

In the heart, the content of proteins decreased significantly from December to February, followed by a sharp increase early in June (Fig. 5Ca). In contrast, the content of proteins in the red muscle increased significantly from December to April, followed by a progressive decrease until July (Fig. 5Cb). In the white muscle, significant increases were observed mainly from February to July (Fig. 5Cc), while no significant seasonal changes were observed in the liver (Fig. 5Cd).

3.3.2. *Energy available (E_a), energy consumption (E_c) and cellular energy allocation (CEA)*

As shown in Fig. 6Aa, E_a displayed two peaks in the heart, one in December and a second early in June. In the red muscle, a progressive increase in Ea was observed by September, followed by a gradual decrease (Fig. 6Ab). In the white muscle a significant decrease until February was followed by a gradual increase in E_a by July (Fig. 6Ac). In the liver, a sharp decrease was observed from September to April, followed by an increase (Fig. 6Ad).

Regarding energy consumption, a significant increase from October to December and from February to April was observed in the heart (Fig. 6Ba). In the red muscle, E_c increased progressively and peaked late in February, while it returned to precooling levels in July (Fig. 6Bb). In the white muscle, the main changes in E_c were observed from February to June (Figure 6Bc). A sharp decrease in E_c was observed between September to December and returned to precooling levels in July (Fig. 6Bd).

After a significant decrease from October to December, CEA remained at low levels by the middle of April, followed by a marked increase in July (Fig. 6Ca). In the red muscle, CEA values fluctuated exhibiting a significant decrease between October and February. Nevertheless, the lowest CEA values were found in the summer months (Fig. 6Cb). In the white muscle, CEA values progressively decreased and exhibited low values early in June. Thereafter, they increased sharply giving the higher values in the middle of summer (Fig. 6Cd). In the liver, CEA values were higher between December and early April and recovered to precooling levels in July (Fig. 6Cd).

3.4. *Seasonal changes in gene expression and enzymatic activities*

3.4.1. *Seasonal changes in Glu, CPT and fabp relative mRNA expression*

Glu Transporter mRNA expression levels exhibited an increase in the heart from September to February, followed by decreased levels in June (Fig. 7Aa). In the red muscle, a significant increase was observed in December. Thereafter, levels decreased in February and increased again in April (Fig. 7Ab). In the white muscle, mRNA levels increased in December and gradually decreased in April, followed by a sharp increase until July (Fig. 7Ac). In the liver, the highest levels were observed late in

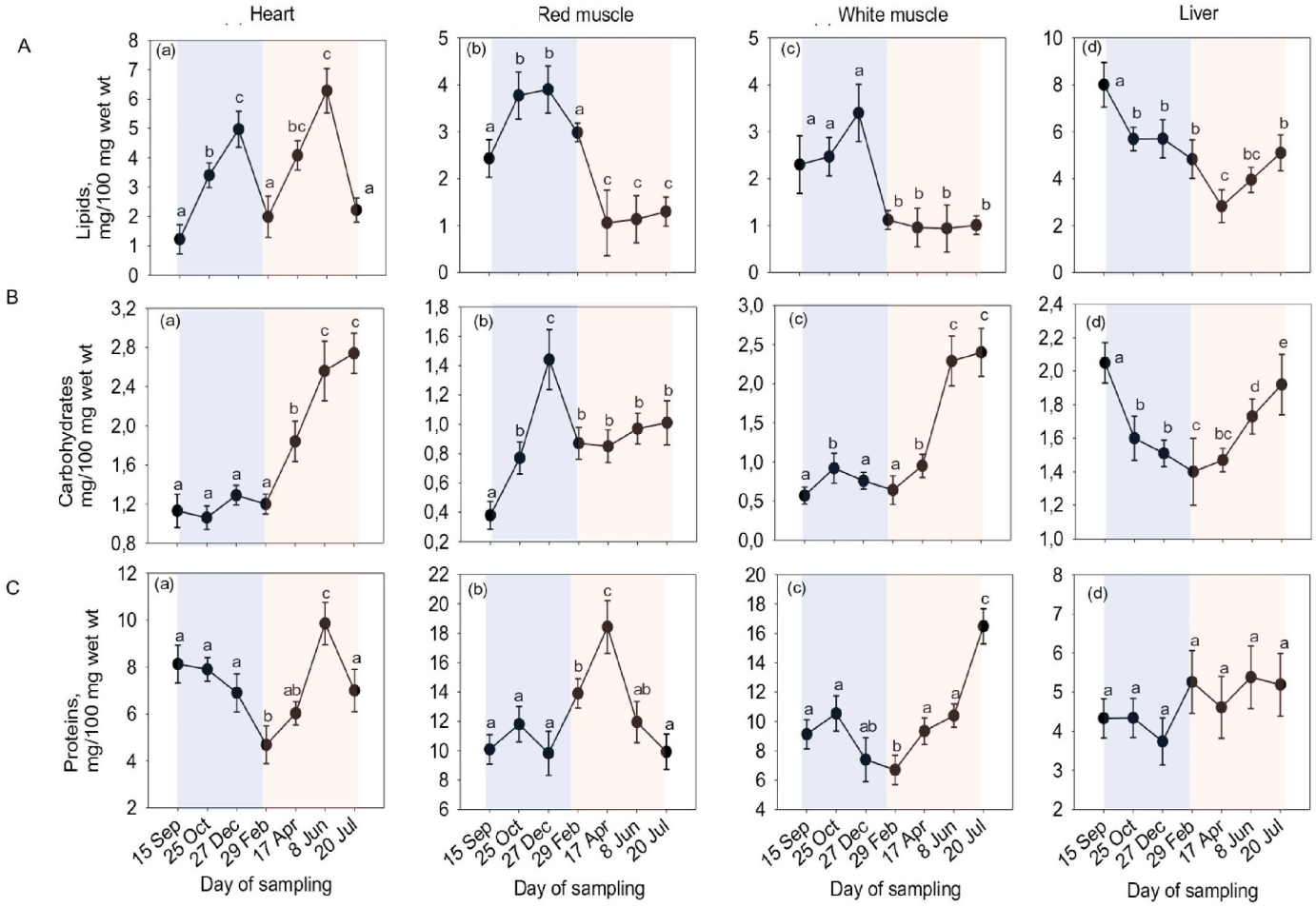

Fig. 5. Seasonal variations of (A) lipid, (B) carbohydrate and (C) protein concentrations in the heart (a), red muscle (b), white muscle (c) and liver (d) of *Pagrus pagrus*. Light blue rectangle depicts decreasing sea water temperature, while light red rectangle depicts increasing sea water temperature. n = 10 preparations from different animals. Lower-case letters indicate significant differences ($p < 0.05$) between different sampling dates.

December, followed by a gradual decrease reaching the lowest levels early in June (Fig. 7Ad).

In the heart *CPT* mRNA expression levels exhibited a significant increase in December and thereafter gradually decreased to precooling levels until July (Fig. 7Ba). In the red muscle, the same pattern in mRNA levels of expression was observed (Fig. 7Bb). However, in the white muscle a slight increase from September to December was followed by levels peaking in April and thereafter decreasing (Figure 7Bc). In the liver, *CPT* mRNA expression levels exhibited the highest values late in December, followed by a gradual increase until April, while thereafter levels returned to those observed during precooling (Fig. 7Bd).

In general, both *Fabp* isoforms mRNA expression levels in the heart exhibited a significant increase with decreasing sea water temperature, exhibiting their lowest in April and thereafter gradually increased (Fig. 7Ca). In the red muscle, *Fabp2a* and *Fabp2b* mRNA expression depicted their highest levels during decreasing sea water temperature, and from December and February, respectively, they gradually decreased (Fig. 7Cb). In the white muscle, both *Fabp* mRNA isoforms expression exhibited their highest levels in October, their lowest in December and a gradual increase from February (Fig. 7Cc). On the other hand, in the liver, *Fabp* mRNA expression levels decreased in October and December, thereafter increased and in general remained at these levels (Fig. 7Cd).

3.4.2. *Seasonal changes in the mRNA expression levels and activity levels of intermediate metabolism enzymes*

In the heart, *LDH* mRNA expression levels gradually increased until the highest levels were observed late in December. Thereafter, levels gradually decreased. L-LDH enzymatic activity levels followed the same pattern, however peaking in February (Fig. 8Aa). In the red muscle, *LDH* mRNA expression levels increased gradually from September to February, when these levels peaked, and thereafter gradually decreased. However, L-LDH enzymatic activity levels in this tissue showed a continuous increase until July (Fig. 8Ab). In the white muscle, a significant increase in *LDH* mRNA expression levels was observed from September to December, followed by a gradual decrease. L-LDH enzymatic activity levels in the white muscle exhibited a pattern similar to that of the red muscle (Fig. 8Ac). In liver, *LDH* mRNA expression levels increased from September to April and decreased during the summer period from June to July. L-LDH enzymatic activity exhibited a decline from September to December followed by increased levels in April (Fig. 8Ad).

CS mRNA expression in the heart reached its highest levels in late October, decreased slightly from December to April, and fell to the lowest values in June and July. CS enzymatic activity gradually decreased and exhibited its lowest levels in April, and thereafter increased in June and July (Fig. 8Ba). In the red muscle, both *CS* mRNA expression and enzymatic activity levels followed a similar pattern: levels gradually increased, peaked in April and thereafter decreased and exhibited the lowest levels in June and July (Fig. 8Bb). In the white muscle and in the liver, *CS* mRNA expression levels exhibited a pattern similar to that exhibited in the red muscle (Fig. 8Bc and 8Bd, respectively). In the white muscle, CS enzymatic activity exhibited the lowest levels from September to February, and thereafter increased to its

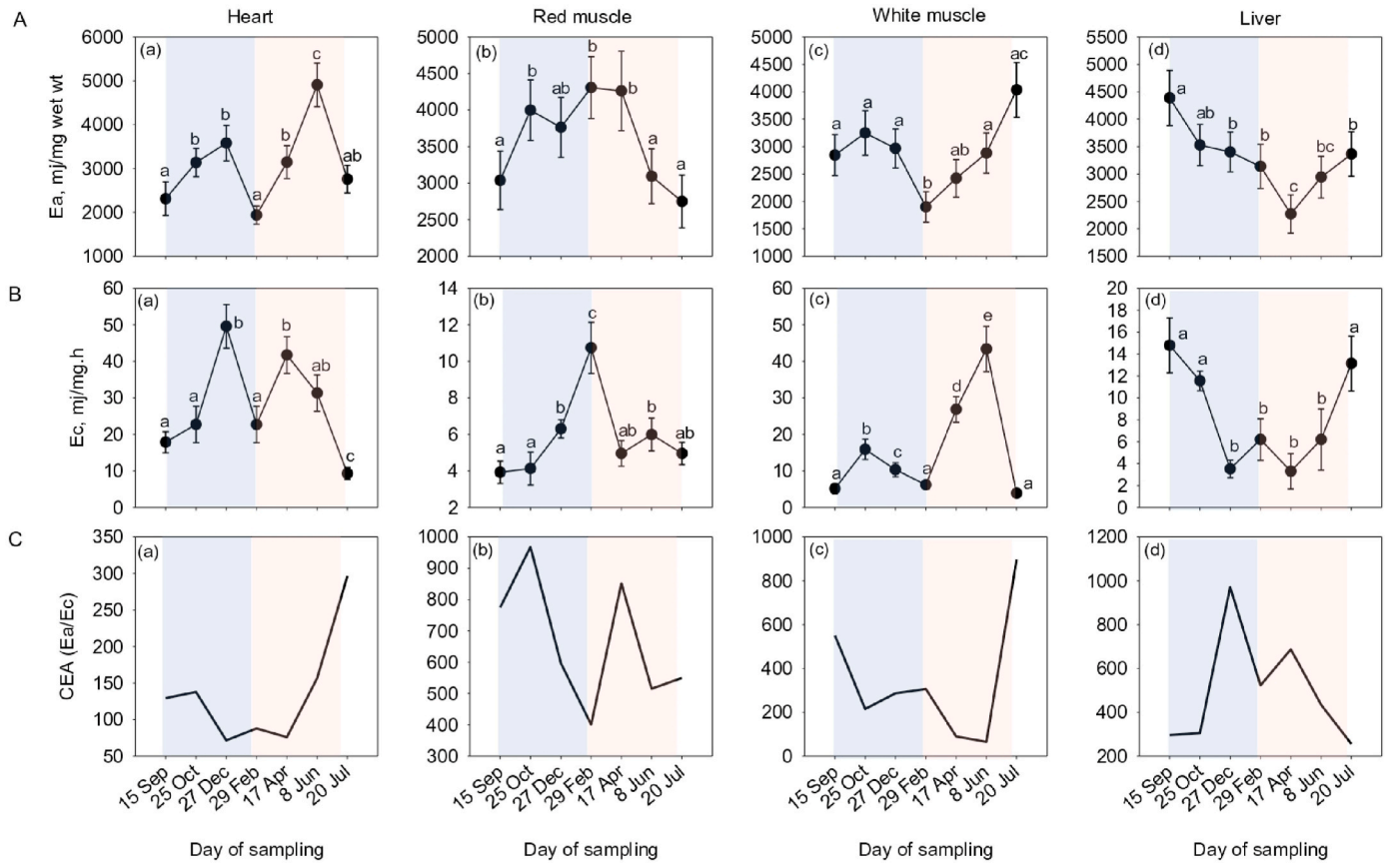

Fig. 6. Seasonal variations of (A) E_a (B) E_c and (C) CEA in the heart (a), red muscle (b), white muscle (c) and liver (d) of *Pagrus pagrus.* Light blue rectangle depicts decreasing sea water temperature, while light red rectangle depicts increasing sea water temperature. n = 10 preparations from different animals. Lower-case letters indicate significant differences ($p < 0.05$) between different sampling dates.

highest levels between April and July (Figure 8Bc). In the liver, CS enzymatic activity levels gradually decreased until late in February and April, and thereafter increased exhibiting the highest levels in June and July (during increased sea water temperature) (Fig. 8Bd).

In the heart, *HOAD* mRNA expression levels and enzymatic levels gradually increased until late in February and April, respectively, and thereafter decreased, exhibiting the lowest values at the highest sea water temperatures (Fig. 8Ca). In the red muscle, *HOAD* mRNA expression exhibited its highest levels late in February, and thereafter gradually decreased during increasing sea water temperature (Fig. 8Cb). A similar pattern was observed in the white muscle, with the difference that *HOAD* mRNA expression levels peaked late in December (Fig. 8Cc). HOAD enzymatic activity in both skeletal muscles, exhibited a gradual decrease until February when the lowest levels were exhibited, and thereafter gradually increased (Fig. 8Cb and 8Cc). In the liver, *HOAD* mRNA expression levels and HOAD enzymatic activity levels exhibited a similar pattern, gradually increasing until late February, and gradually decreasing thereafter (Fig. 8Cd).

In the heart, *COX* mRNA expression levels exhibited an increase late in October with these levels remaining high until April, and thereafter decreased to levels similar to those observed in September. COX enzymatic activity changed opposite to mRNA expression (Fig. 8Da). In the red muscle, *COX* mRNA expression levels gradually increased from September to July. In contrast, COX enzymatic activity levels increased from October to April, and thereafter decreased from April to July, parallel to increasing sea water temperature (Fig. 8Db). In the white muscle, *COX* mRNA expression levels increased late in October, significantly decreased by April, and increased again from April to July. COX enzymatic activity levels showed an increase from September to December, and thereafter gradually decreased to July when the lowest levels were exhibited (Fig. 8Dc). In the liver, *COX* mRNA expression levels increased from October to February (when the highest levels were observed), decreased in April, and again significantly increased in June and July when the highest sea water temperatures were recorded. COX enzymatic activity levels exhibited a pattern similar to that of mRNA expression only until April, when the highest levels were observed. Thereafter, COX enzymatic activity decreased to similar levels of the precooling period (Fig. 8Dd).

3.5. Seasonal changes in blood plasma biomarkers

Glucose levels in blood plasma exhibited a gradual increase between September and April, when the highest value was observed, followed by a significant decrease in June and July, when the highest sea water temperatures were recorded (Fig. 9a). Lipids in blood plasma exhibited a significant decrease from October to late in December, increased thereafter until April and decreased again to its lowest values in June and July (Fig. 9b). Lactate levels in the blood plasma gradually decreased from September to April and increased from June to July (Fig. 9c).

4. Discussion

4.1. Cold acclimatization

Food intake and growth were significantly decreased in *P. pagrus* during progressive acclimatization to cold. Similarly, in *S. aurata*, low temperature (below 13 °C) constrains feeding rate, resulting in growth arrest and metabolic depression (Ibarz et al., 2003, 2010; Sánchez-Nuño et al., 2018b). Decreased feeding indicates a parallel reduction in energy

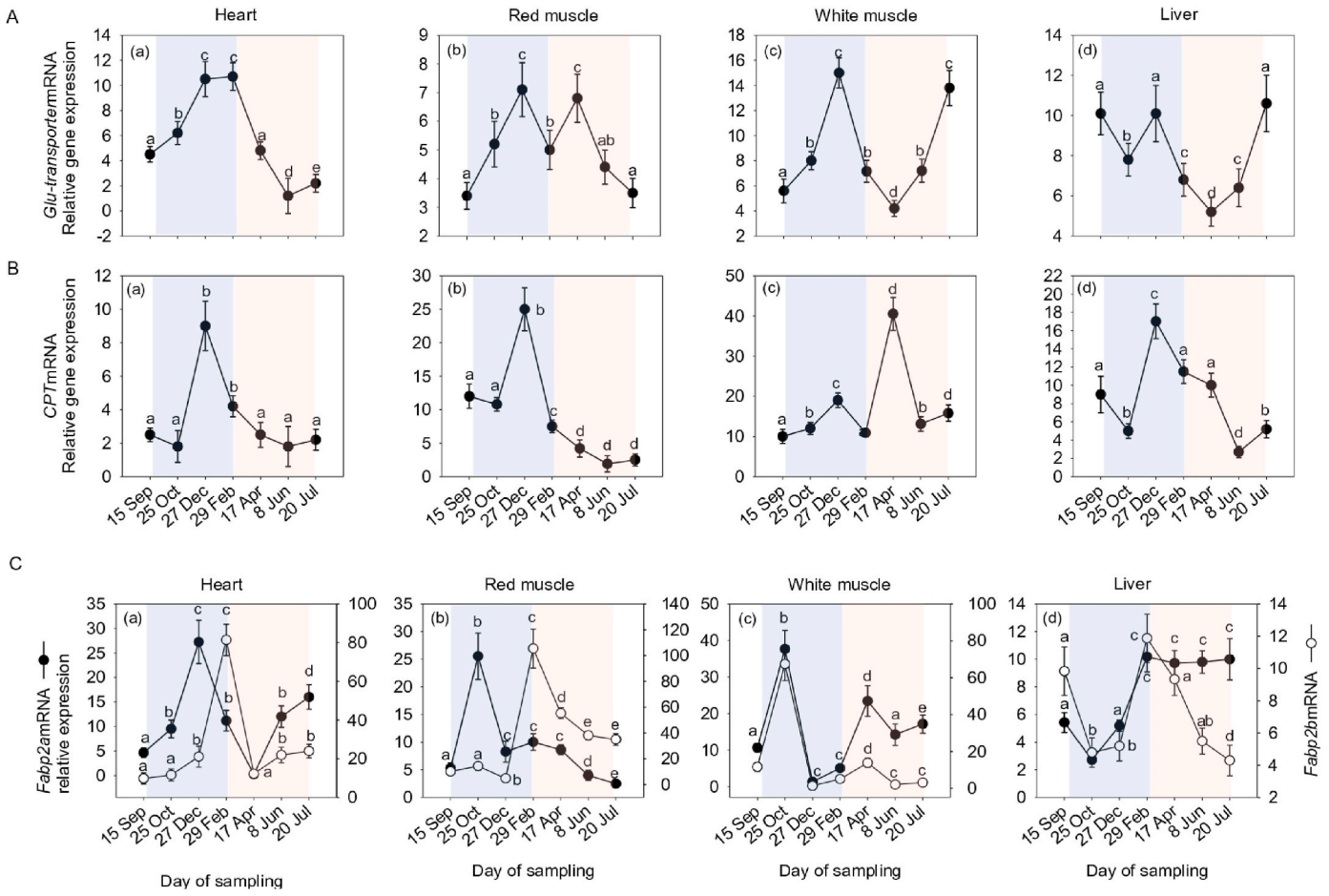

Fig. 7. Seasonal variations of (A) *Glu-transporter*, (B) *CPT* and (C) *Fabp2* relative mRNA expression in the heart (a), red muscle (b), white muscle (c) and liver (d) of *Pagrus pagrus*. Light blue rectangle depicts decreasing sea water temperature, while light red rectangle depicts increasing sea water temperature. n = 10 preparations from different animals. Lower-case letters indicate significant differences ($p < 0.05$) between different sampling dates.

intake, which probably causes nutritional stress and disruption in tissues' energy balance. Under these conditions fish may mobilize energy stores to provide the necessary demands, mainly for maintenance. The present data indicates that seasonal mobilization of energy stores are likely shaped by tissue-specific energy investment (E_a). The increase in lipid levels in the heart, red and white muscle indicated a transiently increasing energy investment during progressive acclimatization until December, supporting an important role of lipid oxidation in providing energy. The increased levels of *CPT* mRNA indicate up regulation of *CTP* gene expression, which in conjunction with the increased expression of fabp2a/b support mobilization of fatty acids to the tissues. On the other hand, the gradual drop in lipids in the liver, in conjunction with increased triglycerides levels in the blood plasma, indicated that liver was the primary source of invested energy. The fabp2a and fabp2b subunits have been identified in several teleost fish species (Kaitetzidou et al., 2015) while *fabp2* gene expression is up regulated by cold and nutritional stimuli (Turkmen et al., 2017; Taşbozan et al., 2022, 2023). However, the exact physiological role of subunits a and b is not well known.

Increased *Glu* mRNA levels in all examined tissues indicate glucose transport along with enhanced glycolytic potential and probably a complementary involvement of carbohydrate oxidation in overall metabolism and energy consumption. In line with this hypothesis, glycogen significantly decreased in the liver and blood glucose levels increased gradually during acclimatization to cold. Activation of glycogenolysis in the liver would provide the energetically demanding tissues with glucose. On the contrary, a significant decrease in the levels of protein were exhibited in the heart mainly after December, indicating a complementary protein mobilization in overall metabolism and energy provision. Distinct strategies are exhibited between different fish species in order to endure periods of food deprivation and starvation. These strategies employ a versatile use of energy reserves (carbohydrates, lipids, and proteins) from different body compartments (Bandeen and Leatherland, 1997). Liver glycogen, formed by stored carbohydrates, is the primary energy source (Navarro and Gutierez, 2005). During prolonged periods of decreased food intake, however, some fish species such as *Anguilla anguilla* (Linnaeus, 1758), *Carassius auratus* (Linnaeus, 1758), and *Pleuronectes platessa* (Linnaeus, 1758) use as a primary fuel source their muscle protein (Czesny et al., 2003). Todgham et al. (2007) have shown that the levels of ubiquitin-conjugated proteins were significantly elevated in several tissues of cold-adapted Antarctic fishes, suggesting temperature impacts on protein homeostasis and compensation for *in-vivo* protein cold-denaturation. The increase in ubiquitin-conjugated proteins and an activation of the autophagy pathway may be involved in recycling destroyed proteins thus refueling intermediary metabolism in *P. pagrus* (Makri et al., 2023). During low food intake, fish may trigger alternative metabolic pathways to complement lipid and glycogen oxidation for energy homeostasis and heart function in the slow lane of prolonged cold acclimatization.

After December, E_c dropped to levels determined in early September in the heart, while a gradual increase in E_c was observed in the red muscle. Overall, the E_c decrease in the heart after December may indicate energy consumption at a low level, while a shift to anaerobic metabolism supplies the energy for maintaining heart function. The

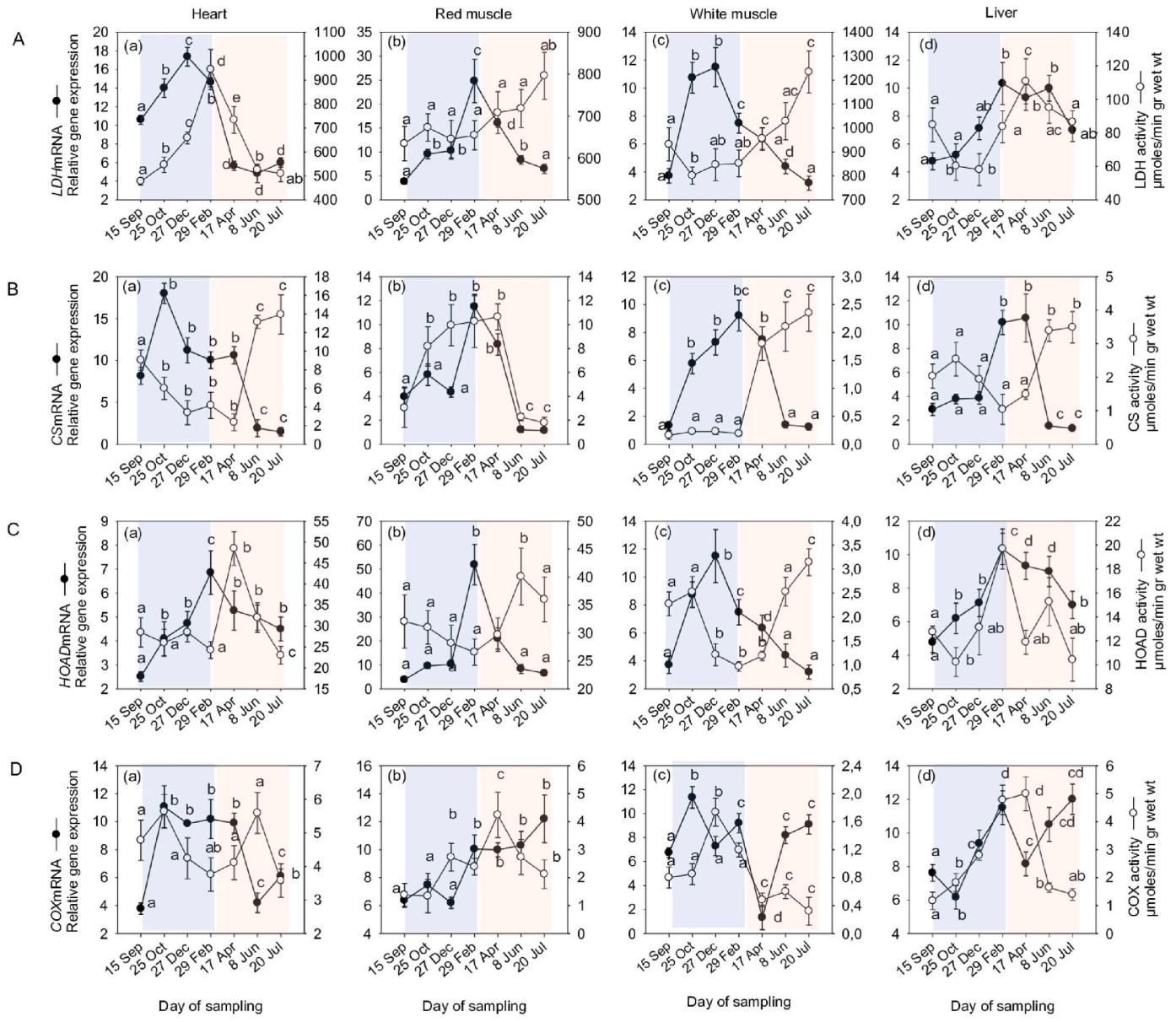

Fig. 8. Seasonal variations of (A) *LDH*, (B) *HOAD*, (C) *CS* and (D) *COX* relative mRNA expression and respectively, (A) L-LDH, (B) HOAD, (C) CS and (D) COX enzymatic activity levels in the heart (a), red muscle (b), white muscle (c) and liver (d) of *Pagrus pagrus*. Light blue rectangle depicts decreasing sea water temperature, while light red rectangle depicts increasing sea water temperature. n = 10 preparations from different animals. Lower-case letters indicate significant differences ($p < 0.05$) between different sampling dates.

pattern of mobilized fuels was in line with the E_c levels revealed (measured as total ETS) which displayed a transient increase in the heart until December. Furthermore, it was closely related to the transient increase in the availability of energy (E_a) indicating increased energy expenditure in the heart. Several investigations revealed that fish seasonal acclimatization is closely related to tissue metabolic reorganization and energy trade-offs (Nathanailides, 1996; Guderley, 1990; Pörtner et al., 2017). Nevertheless, the drop in the ratio E_a/E_c (CEA) until December indicates nutritional stress due to a mismatch between energy availability and energy demand, probably caused by the decrease in feeding rate. The latter might have been responsible for triggering the downward shift of energy availability mainly to the red muscle. Progressive acclimatization to cold is characterized by a hormetic response, including changes in gene expression and metabolic enzymatic activities. This observation was more evident in the heart, where a significant increase in ETS (determined as E_c) and E_a were observed by December. The physiological role of such a phenotypic change is not clear and may represent a preparatory shift for the heart to withstand longer periods of cold during winter. It has been reported that, under chronic temperature changes, teleosts' heart modulates its transcriptome and proteome in order for cellular homeostasis to be restored and their cardiac phenotype to be adjusted to a new thermal optimum (Jayasundara et al., 2015). The tissue damage during cold shock is mitigated by the synthesis of proteins which also assist fish in meeting the physiological demands. The absence of this adaptive response would probably decrease fish endurance when low temperature exposure increased in duration and/or severity (Reid et al., 1998). Therefore, we suggest that the gradual increase in the RNA/DNA ratio detected in the heart, red and white muscle until December may be associated with such adaptive mechanisms.

Energy reserves' mobilization due to nutritional stress is under endocrine control. Specifically, adrenaline and noradrenaline are released into blood circulation by chromaffin cells when internal or external conditions are under suboptimum or stressful (Reid et al., 1998). In fish, adrenaline and noradrenaline, together with cortisol

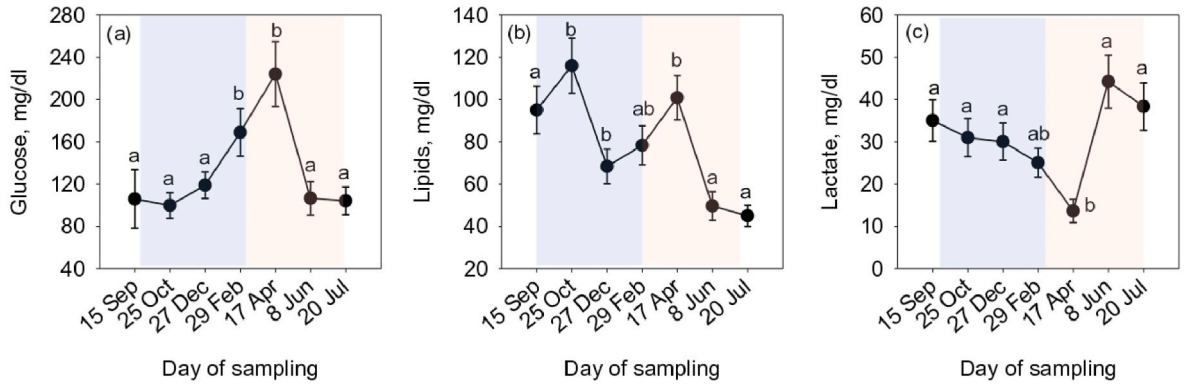

Fig. 9. Seasonal changes in the levels of (a) glucose, (b) lipids and (c) lactate in the blood plasma of *Pagrus pagrus*. Light blue rectangle depicts decreasing sea water temperature, while light red rectangle depicts increasing sea water temperature. n = 10 preparations from different animals. Lower-case letters indicate significant differences ($p < 0.05$) between different sampling dates.

mobilization and elevated glucose production, which is elicited by pathways of glycogenolysis (Iwama et al., 1998; Momoda et al., 2007; Wiseman et al., 2007), synergistically act in order for the energy demand for the "fight of flight" reaction to be met. Cortisol mobilizes gene expression and fuel stores for metabolic homeostasis to be maintained, by directly effecting on metabolism (van der Boon et al., 1991; Vijayan et al., 1991, 1996; Mommsen et al., 1999; Aluru and Vijayan, 2009). An increase in circulating cortisol in the low temperature group of this study may have triggered the metabolic changes resulting oxygen consumption rates increase. Recently, Samaras et al. (2022) have shown that plasma cortisol in *P. major* was inversely related to temperature, being significantly higher at 15 °C compared to 25 °C. It should be highlighted that cortisol is a reliable stress indicator for several fish species under farming conditions (Samaras et al., 2016, 2018, 2021). The latter is in line with the present data regarding the gradual increase in plasma glucose levels during cooling, indicating a close relation between cortisol elevation and carbohydrate mobilization. However, elevated cortisol levels in teleosts' blood plasma caused by both acute and chronic stress is not only associated with elevated plasma glucose but also with the stimulation of anaerobic metabolism (De Boeck et al., 2001; Liew et al., 2013).

Nevertheless, the present data showed that changing transcript patterns of metabolic enzymes and translational phenomena might cause metabolic shifts in the examined tissues during cold acclimatization. As shown, CS and COX activities increased in the red muscle indicating that the increasing transcripts led to translational phenomena during cooling. In contrast, the elevation of *CS* mRNA did not reflect an increase in CS activity in the white muscle and liver. In addition, increased levels of *CS* mRNA were not associated with an increase in the heart enzymatic activity, whereas COX activity remained rather constant, despite a significant increase in *COX* mRNA. Despite increased *LDH* gene transcription, enzymatic activity remained either constant or slightly decreased in the red and white muscle respectively. Overall, these data reflect a higher demand for enhanced aerobic capacity during prolonged acclimatization to cold in the red muscle compared to other tissues. Studies in winter-acclimatized rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) also found increased oxidative capacity in the red muscle, due increased mitochondria cristae and increased oxidative capacity (Guderley and St-Pierre, 2002; Fernandes and McMeans, 2019). These ultrastructural and biochemical properties of mitochondria may expedite activity in the cold (Guderley and St-Pierre, 2002). Similar to our findings, Fangue et al. (2008) reported that cold exposure may significantly increase *COX* mRNA expression in the liver in both southern and northern fish species but not in the case of muscle. Moreover, previous analyses provided evidence that cold exposure of poikilotherms that naturally experience environmental cooling involves the regulation of a very large number of genes (Gracey et al., 2015). Lucassen et al. (2003) reported that in the muscle and the liver, CS and COX (mRNA expression and enzyme activities) changes were more significant in cod populations acclimated to colder temperatures. Probably energy availability is shifted mostly to red muscle to support fish mobility under cold conditions (Blier and Guderley, 1988; Guderley, 1990; Guderley and St-Pierre, 2002; Lucassen et al., 2003). Due to its slow contraction speed and aerobically usage of ubstrates, red muscle is employed for routine activity, such as slow swimming (Johnston et al., 1977; Johnston, 1981).

In contrast to the red muscle, the gradual increase in *LDH* gene expression and enzymatic activity in the heart indicates a progressive shift to anaerobic metabolism. Such a metabolic response suggests that ATP production may increasingly rely on the anaerobic component of metabolism and may become more important for securing heart activity during cold acclimatization. This anaerobic strengthening in the heart is in line with CS activity changes, which despite the increased transcription of the corresponding gene during cooling, showed a significant decrease. Aligned with these findings, a temperature decrease below 14 °C [lower critical temperature (T_c) of this species] probably triggers *S. aurata* anaerobic metabolism, a fact which is depicted by increased L-LDH activity and L-LDH/CS ratio in the heart and red muscle (Kyprianou et al., 2010; Feidantsis et al., 2018). Oxidative muscles of eurythermal fishes may exhibit limitations in aerobic ATP generation (Johnston et al., 1977; Johnston, 1981). Similar to *S. aurata*, during cold acclimatization there was no lactate accumulation in *P. pagrus* blood plasma, indicating a low Cori cycle activity (Weber et al., 2016). Most likely the *in-situ* usage of lactate in several aerobic tissues such as kidney, brain, heart, red muscle) can result in cellular lactate clearance (Bilinski and Jonas, 1972; Jayasundara and Somero, 2013; Tseng et al., 2014). Probably, during cold acclimation/acclimatization fish heart increases L-LDH activity in order to support lactate oxidation to pyruvate (Jayasundara and Somero, 2013).

While the transcript pattern of the *HOAD* gene seems to be similar in all examined tissues, the patterns of HOAD enzymatic activities suggest different translational phenomena between them, thus supporting different involvements of fatty acid oxidation in the energy consumption. The gradual increase in the *HOAD* mRNA expression until February (when the lowest temperature values were recorded) coincided with the maintenance of enzymatic activity in the heart and red muscle, and an increase in the enzymatic activity in the liver. These data indicate an important role for fatty acid oxidation in aerobic energy consumption during seasonal cooling. The importance of lipid oxidation in energy consumption during cold acclimation or acclimatization has been extensively studied n fish and has been identified as the main energy source oxidized in the fish heart (Ibarz et al., 2003, 2010; Sánchez-Nuño et al., 2018b). Lu et al. (2019) reported that the cold resistance of fish is enhanced through stimulation of lipid catabolism and autophagy.

4.2. Warm acclimatization

Recovery from cold acclimatization is a highly energy-demanding process on account of the numerous reactivated physiological functions. However, TGC indicates a strong dependence of growth on temperature because of Q_{10} phenomena until early May, followed by a significantly lower dependence. The latter was closely related to Fulton's condition index (k), which indicates a sharp reactivation of feeding and energy intake until June. During initial rewarming, a significant fraction of the energy taken in is allocated to gametogenesis, as indicated by the marked increase in GSI and it is in line with the seasonal cycle of gonadal development in *P. pagrus* (Kokokiris et al., 2001). The above data indicates a temperature optimum for the above mentioned physiological processes at about 20°C–22 °C. Ostrowski et al. (2011) have shown that larval red porgy maximum growth, survival, and osmoregulatory abilities occur at approximately 23°C–24 °C.

The pattern of energy allocation to different energy-demanding functions is tissue-specific during progressive acclimatization towards the warm summer months. Rewarming caused a gradual increase in E_a in the heart between February and June. However, the rate of E_c suggests a sharp increase between February and April. The sharp energy consumption increase during the first months of rewarming seems to cover the highly demanding heart activity to deliver oxygen and nutrients. In contrast to cold exposure, E_c returned to levels determined in September in the red muscle, while the increased E_c between February and June in the white muscle supports the highly demanding mechanical activity and mobility of fish. Such a metabolic burst was also well-coordinated with the increased E_c in the heart. During acclimatization to the warmer summer months, CEA exhibited an increase in the heart. However, a significant decrease in E_c in relation to E_a was detected by July. The drop and increase in CEA in the red and white muscle, respectively, provides evidence for entirely different energy investments in tissues and concomitant energy expenditures. It is therefore reasonable to relate the high energy demand for fish mobility and white muscle function during the first months of rewarming to the sharp increase in E_c in the white muscle, causing the detected reduction in CEA in the liver.

Overall, the changes in cardiac gene expression and the related enzymatic activities reveal metabolic remodeling during progressive acclimatization towards warmer summer months. The parallel decrease and increase in the activities of L-LDH and COX, respectively, between February and early June, suggest a shift from the heart anaerobic to the aerobic component of metabolism. The latter is in line with the significant increase in CS activity. However, the lower activity of COX as determined in mid-July coincides well with the detected decrease in E_c in the heart during summer. Regarding the metabolic patterns of ATP production, it seems that there is a coordination between fatty acid and carbohydrate oxidation, which is also tissue-specific. Both lipid and carbohydrate oxidation seem to be involved in ATP production in the heart between February and June, covering the energetic needs for a sharp and quick delivery of oxygen and nutrients during the first stages of functional reactivation. Thereafter, as indicated by the higher levels of CS/HOAD ratio, ATP is mainly based on carbohydrate oxidation.

In the white muscle, the sharp increase in CS activity between February and April indicates increased aerobic capacity. Thereafter, metabolism shifted mainly to glycolysis as indicated by the increasing L-LDH activity and the decreasing CS/LDH ratio. These are in accordance with increased *Glu-transporter* mRNA expression and blood lactate levels during the same period, indicating enhanced glycolytic potential. Overall, the obtained data showed that the energy invested in the white muscle after recovery from winter is associated mainly with carbohydrate stores. Accordingly, the gene expression of *CPT transporter* decreased, and blood lipid levels decreased towards acclimatization to warmer months. Similar to the white muscle, aerobic capacity was increased in the liver during rewarming as indicated by the increase in CS and the decrease in LDH, respectively. The above-mentioned findings also match with the decrease in HOAD activity and the gene expression of *CPT transporter*, indicating that carbohydrates are the main fuels oxidized in the liver. The switch from lipid to carbohydrate stores probably increases warm hardiness, in order to sustain anaerobic metabolism and prepare for the internal hypoxia which usually develops upon warming (Windisch et al., 2011).

Similar to cold acclimatization, the changes in enzymatic activities during warm induced recovery from winter seem to be coordinated by translational phenomena. The increase in CS and COX activities was not attributed to an increased gene transcription but mainly to translation of corresponding mRNA. The latter becomes more evident in the white muscle where the decrease in *CS* mRNA, *LDH* mRNA and *HOAD* mRNA levels were paralleled by increased enzymatic activities during seasonal warm acclimatization. During adaptation of aerobic capacity, translation or protein stability is usually preferred compared to control at the transcriptional level (Lucassen et al., 2003; Duggan et al., 2011). Nonetheless, an accumulation of mRNAs might be beneficial for highly demanding physiological processes during winter recovery, such as the mechanical activity during swimming. The sharp increase in the RNA/DNA ratio estimated herein indicates that an increase in the rate of protein synthesis is involved in this process.

5. Conclusion

Overall, the present study has profiled the changes in *P. pagrus* energetics during seasonal acclimatization and at environmental conditions at which it is currently farmed. Moreover, these changes underline the seasonal metabolic reorganization and how the oxidation of different energy stores may be shifting during cold or warm acclimatization by transcriptional and translational phenomena. On the other hand, as already mentioned above, high summer temperatures are recorded (beyond 26 °C) at the area where *P. pagrus* is farmed (Zgouridou et al., 2022). The results regarding E_c and COX activity decrease in the heart of individuals during summer acclimatization seem to be in line with the general view of the OCLTT hypothesis, suggesting that one of the first organs to be limited during exposure at elevated temperatures may be the heart (Pörtner and Farrell, 2008; Clark and Lioyd, 2009; Eliason et al., 2011; Chung and Schulte, 2020). Several investigations in fish species suggest that mitochondrial failure impacts physiological processes such as cardiac failure and whole-organism thermal limits (Pörtner, 2012; Iftikar and Hickey, 2013; Iftikar et al., 2014). However, apart from COX, other components of mitochondrial metabolism might also be depressed in *P. pagrus* under thermal stress and further research is needed for a better estimation of the upper thermal limits of the organism's physiological performance. Apart from temperature, however, other environmental factors as salinity and levels of dissolved oxygen, may synergistically act to shape the thermal limits of *P. pagrus*. Comparatively, the latter may contribute to the understanding of possible differences in the thermal sensitivity between fish species farmed at the same marine area. As it has been reported elsewhere (Sánchez-Nuño et al., 2018a, 2018b), the data regarding metabolic reorganization and seasonal energy demands of tissues may help fish producers and researchers in shaping and including nutritional dietary plans during seasonal management of fish stocks.

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CRediT authorship contribution statement

Vasiliki Makri: Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition. **Ioannis A. Giantsis:** Writing – review & editing, Writing – original draft, Software, Project administration, Formal analysis, Data curation. **Cosmas Nathanailides:** Writing – review & editing, Writing – original draft, Investigation, Data curation. **Konstantinos Feidantsis:** Writing – review & editing, Writing – original draft, Visualization, Project administration, Methodology, Data curation, Conceptualization. **Efthimia Antonopoulou:** Writing – review & editing, Writing – original draft, Project administration, Investigation, Data curation. **John A. Theodorou:** Writing – review & editing, Writing – original draft, Funding acquisition, Data curation. **Basile Michaelidis:** Writing – review & editing, Writing – original draft, Supervision, Resources, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Vasiliki Makri reports financial support was provided by Hellenic Foundation for Research and Innovation. John A. Theodorou reports financial support was provided by EU-Greece Operational Program of Fisheries. 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.

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