Thermal tolerance, metabolic scope, and performance of meagre, *Argyrosomus regius*, reared under high water temperatures

**Authors**

Stavrakidis-Zachou Orestis\(^1\)\(^2\), Konstadia Lika\(^1\), Pavlidis Michail\(^1\), Aleka Tsalafouta\(^1\), Asaad H. Mohamed\(^3\), Papandroulakis Nikos\(^2\)

\(^1\)Department of Biology, University of Crete, Heraklion, 71003, Crete, Greece

\(^2\)Institute of Marine Biology, Biotechnology and Aquaculture, Hellenic Centre for Marine Research, Heraklion, 71500, Crete, Greece

\(^3\)Beacon Development, King Abdullah University of Science and Technology, Thuwal, 23955-6900, Saudi Arabia.

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**Abstract**

This article reports on the thermal tolerance, metabolic capacity and performance of juvenile meagre (*Argyrosomus regius*) reared under three high water temperatures (24, 29 and 34°C) for three months. The analysis includes the thermal effects on the growth performance, metabolism and physiology of meagre, including a range of molecular, haematological, metabolic, enzymatic and hormonal indicators, as well as the effects on the proximate composition and ingestion speed. Meagre performs best between 24 and 29°C while the temperature of 34°C is very close to the upper end of its temperature tolerance range. At 34°C meagre exhibits a poor growth performance and physiological status, increased blood clotting, high mortality rates and a diminished capacity for aerobic metabolism, as indicated by its low aerobic scope (129 mg kg\(^{-1}\) h\(^{-1}\)). Meagre may tolerate short exposures to high temperatures after sufficient acclimation (Critical thermal maximum of 37.5°C after acclimation to 29°C) but its overall performance declines under prolonged exposure, suggesting that this emerging aquaculture species may be vulnerable to global warming. Our work corroborates previous findings on the thermal preferences of the species, identifies critical biological thresholds, and provides insights into the effects of prolonged exposure to high temperature regimes.

**Keywords**

*Argyrosomus regius*, temperature tolerance, growth performance, thermal stress
1. Introduction

Temperature is a major driver of physiological and behavioural change in fish, affecting, among others, their appetite, growth, metabolic activity, development, and reproduction (Crozier et al., 2013). Given that under climate change the global mean temperature is expected to increase by up to 5°C by the end of the century (IPCC, 2014), it is imperative to understand the effects of rising temperatures on fish and identify critical biological thresholds. This is particularly important for aquaculture species due to the potential economic ramifications for the dependent communities and their livelihoods.

A case in point is meagre (Argyrosomus regius), a migratory marine species with widespread distribution in the Mediterranean and eastern Atlantic. Farming of meagre is becoming increasingly popular in the Mediterranean aquaculture and one of the reasons why is that, apart from its high growth potential, it appears rather tolerant to temperature and other environmental fluctuations (Fernández-Alacid et al., 2019). In its natural habitat, it is exposed to temperatures ranging from 14 to 26°C and its biological responses to that range have been well documented (Duncan et al., 2013). Furthermore, experimentation in the 18–30°C range has given the first indications that for juvenile meagre the optimum temperature may lie between 26–30°C (Kir et al., 2017). However, due to the recent introduction of the species in aquaculture production, there is still considerable uncertainty about its biological response to prolonged high temperatures while our understanding of its thermal tolerance remains elusive. Filling this gap in our knowledge is of great importance for aquaculture in the context of climate change as well as the expansion of its farming in southern regions.

In order to evaluate the thermal effects on meagre and pinpoint critical tolerance thresholds, the mechanisms involved in physiological regulation as well as the process of acclimation to new environmental conditions must be taken into account. In general, when fish are exposed to new conditions, they evoke a regulatory/adaptive mechanism known as a stress response. During this initial response, neuro-endocrine paths are activated in the brain which, in turn, mobilize metabolic, haematological, and immunological responses, among others, to cope with the change (Barton, 2002; Schreck and Tort, 2016). Typically, the stress response is manifested in increased concentrations of cortisol, glucose, lactate, and other metabolites for many hours after the exposure. However, in cases of prolonged exposure to the stressor, high concentrations may persist for longer, indicating signs of chronic stress (Yousefi et al., 2016; Deng et al., 2018). In that case, the rapid regulatory mechanisms that are employed at first give way to more permanent modifications that allow the optimization of performance under the new conditions. One approach to understanding such modifications is through the physiological framework of allostasis (Korte et al., 2007; Ramsay and Woods, 2014), which is described by stability through change and involves mechanisms that alter the physiological variables according to the levels of anticipated change. This equips the organisms with the plasticity to cope
with a wide range of environmental changes by adaptive changes to their metabolism and immune and cardiovascular systems. However, because not all allostatic mechanisms are activated at the same rate, a substantial acclimation time is required, and a steady state may not be reached until several weeks after the initial exposure.

During this acclimation period, biochemical, morphological and molecular changes may occur. For instance, haematological parameters such as haematocrit and haemoglobin are known to exhibit a shift under different temperature or oxygen regimes. By upregulating the production of red cells and haemoglobin, fish are able to increase their capacity for aerobic metabolism, a phenomenon well documented as a response to increased temperatures (Samaras et al., 2016). In extreme cases, acclimation mechanisms may involve tissue reorganization and alteration of organ size, as is the case of heart hypertrophy in response to high temperatures (Keen et al., 2016). Moreover, such changes are usually accompanied by higher metabolic rates, as reflected by the increase in respiration (oxygen consumption rate) (Pörtner et al., 2017) and the increase in other plasma metabolites, such as those involved in lipid mobilization and metabolism (cholesterol, lipids, hepatic enzymes), which may also lead to changes in the proximate composition of the fish (Guerreiro et al., 2012; Chatzifotis et al., 2018; Riera-Heredia et al., 2020). Finally, since the upregulation of all these metabolic and biochemical parameters is under genetic control, substantial shifts in gene expression are expected. For instance, the expression of specific proteins called heat shock proteins (HSP), which have a protective role for cell functions, is known to be induced under stress conditions (Roberts et al., 2010).

That being said, it is intuitive that such adaptations cannot take place indefinitely and irrespective of the magnitude of environmental change. There is typically a species- and life stage- specific tolerance range within which organisms are capable of responding in the fashion described above (Pörtner, 2010; Dahlke et al., 2020). If, however, environmental changes exceed certain species-specific thresholds, then the allostatic load becomes excessive and the animals become prone to pathologies and eventually die (Korte et al., 2007). One such threshold commonly used to evaluate the thermal tolerance of fish is the critical thermal maximum (CT_{max}). This threshold represents the maximum temperature that can be tolerated for short periods of time during phases of acute thermal stress and is known to increase when fish are acclimated to high temperatures for a sufficient time (Ern et al., 2016). Specifically for meagre, recent insights into the thermal tolerance range of the species suggest that small juveniles may be able to tolerate temperatures of up to 37°C in acute thermal exposure if acclimated to high temperature conditions (T = 30°C) (Kir et al., 2017).

The present work constitutes an experimental investigation on the thermal tolerance of meagre and an effort to bridge some of the existing knowledge gaps for this emerging aquaculture species.
Specifically, the aim was to evaluate the effects of prolonged exposure to high temperatures and attempt to pinpoint critical tolerance thresholds. This was realized by conducting a three-month thermal trial on juvenile meagre and assessing the effects of three temperature treatments in the 24–34°C range. The assessment was performed by analysing a selected range of the husbandry and physiological parameters discussed above, including hormonal, haematological, biochemical, molecular, and metabolic indicators.

2. Materials and Methods

2.1. Fish rearing and sampling

The trial was conducted in a marine RAS at the Institute of Marine Biology, Biotechnology, and Aquaculture (IMBBC) between Oct 2018 and Feb 2019. Juvenile meagre were obtained from the institute’s pilot-scale cage farm (Souda Bay, Crete) and transferred to the land-based facilities. Antibiotic (oxytetracyclin) and antiparasitic (freshwater bath) treatments were performed before the fish were distributed to 2m³ cylindroconical tanks. The temperature in the tanks was adjusted to the ambient temperature at the cage farm (23°C) and fish were acclimated for two weeks before the trial during which behaviour was monitored, mortalities removed, and feed provided by hand according to appetite. On day one of the trial, all fish were anesthetized, individually measured for total length (TL) and weight (W), averaging 22.4 cm and 149 g, respectively, and re-distributed in the tanks (60 per tank). From that day onwards, the temperature in each treatment was raised at a rate of 0.7°C per day until the experimental temperatures of 24, 29, and 34°C were reached, thus forming the T1, T2, and T3 treatments. Each treatment was performed in three replicate tanks, all of which were connected to a RAS, following the established experimental protocol in fish growth studies (Thorarensen et al., 2015).

Fish were fed by hand twice per day until visual satiation, and any uneaten feed was collected and measured at the end of the day. A commercial feed with 45% crude protein and 16% crude fat was provided (provider, IRIDA). Photoperiod was adjusted to a 12L:12D-hour cycle and water renewal in the RAS ranged from 10–20% per day. The temperature and dissolved oxygen in the tanks were monitored continuously by automated loggers (Hach-Lange SC1000) while additional oxygen was provided when oxygen saturation dropped below 80%. Water quality parameters such as pH, ammonia, nitrite and nitrate were monitored on a weekly basis by means of manual measurements with a pH-meter and photometric methods (Hach). These parameters did not exceed the standard safe limits for the duration of the trial.

Samplings occurred on a monthly basis, which led to three samplings at 30, 60, and 90 days after each experimental group reached their respective experimental temperature. In each sampling, all fish
were anaesthetized and measured individually for weight and total length before being returned to their tanks. In addition, 15 fish per treatment (five per replicate) were used for blood collection from the caudal vein via heparinized syringes. Next, the fish were sacrificed by a blow to the head and samples were taken from their heart, liver and spleen. Heart and liver weight were measured to the nearest 0.01g and the tissue samples were then frozen in liquid nitrogen and stored at -80°C. Plasma was stored at -20°C after determination of haematological parameters and centrifugation of blood at 2000g for 10 minutes. Additionally, at the end of the trial, five fish per treatment were sacrificed for determination of the whole-body proximate composition.

Throughout the experiment, mortalities were recorded and weighed before being removed from the tanks. Although temperature in the T1 and T2 treatments was maintained throughout the trial, in the case of T3, animal welfare considerations (high mortalities) led to the decision to reduce the experimental temperature by 0.5°C during the last month of the trial. Upon completion of the trial, the temperature was raised again to 34°C for an additional 10 days and further data regarding mortality rates were collected.

The trial was approved by the Ethics Committee of the IMBBC and the relevant veterinary authorities (Ref Number 255,344) and was conducted in certified laboratories (EL91-BIOexp-04) in accordance with legal regulations (EU Directive 2010/63).

2.2. Growth performance and somatic indices

The following calculations for the absolute growth rate (AGR), feed conversion ratio (FCR), condition factor (CF), hepatosomatic index (HSI), and cardiosomatic index (CSI) were used for the evaluation of the growth performance and changes in relative organ size:

\[
AGR = \frac{BW_t - BW_i}{t}
\]

\[
FCR = \frac{FI}{BW_t - BW_i}
\]

\[
CF = \frac{BW_t}{L_t}
\]

\[
HSI = \frac{LW_t}{BW_t} \times 100
\]

\[
CSI = \frac{HW_t}{BW_t} \times 100
\]
where $BW_t$, $LW_t$, $HW_t$, the whole body, liver, and heart weight (g), respectively, at time $t$, $L_t$ the total length (cm) at time $t$, $BW_i$ the initial whole-body weight (g), $FI$ the feed intake (g) and $t$ the period (days) between samplings.

2.3. Analytical procedures

For evaluating haematological parameters, haematocrit (Hct) was measured with the use of capillary tubes in a microcentrifuge and haemoglobin (Hb) via the use of a commercial kit (Sigma-Aldrich).

With respect to biochemical parameters, the concentration of glucose, triglycerides, proteins, lactate, and cholesterol as well as the activity of diagnostic enzymes (AST, ASP, ALT) in plasma, were also measured with commercial kits (BIOSIS, Sigma-Aldrich) using spectrophotometry. Blood pH was recorded with a penameter. In addition, the enzymatic activity of the antioxidant enzymes glutathione peroxidase (GPx) and superoxide dismutase (SOD) were measured in the liver (kits by Cayman Chemical). Finally, cortisol concentrations were quantified with a competitive assay (ELISA kit, Neogen Life Sciences), previously validated (Samaras et al., 2016). The analyses were performed in the Laboratory of Fish Physiology (Dept. Biology, University of Crete).

With respect to the proximate composition analysis, the Dumas method was used for the determination of total protein, in which the samples were burned at 800°C to convert all nitrogen forms to nitrogen oxides and in turn reduced to nitrogen gas ($N_2$). Regarding total lipids, they were determined according to Folch et al. (1957). Finally, the samples were dried at 90°C to determine moisture and subsequently burned in a muffle furnace at 600°C for seven hours to measure the ash content.

2.4. Determination of metabolic rate

The metabolic performance of meagre was assessed via the Standard and Maximum Metabolic Rates (SMR and MMR, respectively) and determination of the Aerobic Scope (AS) for the three treatments. The metabolic trial was performed in an intermittent-flow respirometer (Loligo Systems) using up to four 2L metabolic chambers simultaneously, and the SMR and MMR were determined according to the methodologies described in Chabot et al. (2016), and Norin and Clark (2016). Briefly, for the MMR, the fish were placed in a 50 L bucket, chased for five minutes, and then quickly transferred to the metabolic chambers. The oxygen consumption rate ($MO_2$) in the next three minutes was assumed to correspond to the MMR. Subsequently, the fish were left in the chambers undisturbed for 24 hours, during which time recordings of $MO_2$ were taken at 10-15-minute intervals. To avoid stressing the fish, the chambers were covered with a black curtain, which prohibited light entering and provided visual
isolation between the fish. For determination of the SMR, the 0.2 quantile method (q0.2) for the measurements obtained over the 24 hours was used (Chabot et al., 2016). In order to account for the prerequisites of measuring this rate, the trial was performed after the second month of the experiment, thus ensuring temperature-acclimated fish, and the fish were fasted for 48 hours prior to the measurement. Finally, the AS was calculated as the difference between MMR and SMR for each treatment.

2.5. Gene expression

The expression of genes coding for GR (glucocorticoid receptor), HSP70, and HSP90 was studied via quantitative real-time polymerase reaction (qPCR) in liver and spleen samples. The focus of this analysis was to identify potential long-term effects of different temperatures on gene expression. For this reason, and while considering a compromise between information gain and practicalities, the two tissues were sampled from five individuals at the end of the trial, after temperature acclimation had been completed. The expression of the three target genes (GR, HSP70, HSP90) was calculated relative to that of a reference gene. For the reference gene, while three potential genes were assessed (β-actin, ribosomal RNA S18, and eEF1-α), β-actin was chosen for the normalization as it exhibited the most stable expression across all samples based on genorm analysis (Vandesompele et al., 2002). The analysis was performed using a qPCR thermocycler (CFX connect real-time, Bio-rad) with KAPA SYBR FAST Universal (KAPA Biosystems) according to the manufacturer’s instructions. The qPCR steps included initial denaturation at 95°C for 3 min followed by 35 cycles consisting of 95°C for 15 sec, 60°C for 30 sec, and 72°C for 2 sec. Subsequently, a melt-curve was performed at 65°C with a 0.5°C degree increment up to 90°C. A standard curve of serial dilutions (1:5, 1:25, 1:125, 1:625) of pooled cDNA samples was constructed for each gene. The primers for each gene were described by our group and are listed in Table 1.

Table 1 Primer sequences used in this study.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR1_FWD</td>
<td>GCTACGACAGCACCTGCC</td>
</tr>
<tr>
<td>GR1_REV</td>
<td>CATGAGGAAGAGCAGGAGCACTG</td>
</tr>
<tr>
<td>HSP70_FWD</td>
<td>TCGAAGTCTCCATCCTGACCA</td>
</tr>
<tr>
<td>HSP70_REV</td>
<td>CCACAAAGTTGTCCACATGCGG</td>
</tr>
<tr>
<td>HSP90_FWD</td>
<td>CTTTGCCTCCAGGAGAGATC</td>
</tr>
<tr>
<td>HSP90_REV</td>
<td>GGCATTGGAGATCAAACCTCCTGAGG</td>
</tr>
</tbody>
</table>
2.6. Critical Thermal Maximum

The critical thermal maximum (CT\textsubscript{max}) was determined using critical thermal methodology (Bennett and Betinger, 1997; Pörtner et al., 2017). This experiment was performed at the end of the third month for treatments T1 and T2 but not T3 due to the insufficient number of available fish. In total, 15 fish were used for each treatment. In general, the methodology described in Ern et al. (2016) was applied. The fish were placed in groups of five in an insulated 50L container which had been filled with water from the tank at the respective experimental temperature. Moderate aeration was provided to ensure adequate circulation and oxygen levels in the container as well as to avoid temperature stratification. Subsequently, the temperature in the container was increased at a rate of 0.5 ± 0.1°C min\textsuperscript{-1}, and the temperature of each fish reaching the endpoint of loss of equilibrium (LOE) was recorded. The LOE was defined as the inability to maintain dorso-ventral orientation. The fish were then returned to the experimental tanks for recovery, with no mortalities being observed over the next few hours. The CT\textsubscript{max} was defined as the mean temperature at which the fish reached the endpoint.

2.7. Gastric Emptying Time

A gastric emptying time (GET) analysis was conducted at the end of the trial to assess the differences in the digestion speed among treatments. The fish were deprived of feed for 48 hours prior to the experiment to ensure that their stomachs were completely emptied and were then fed to satiation. At specified time intervals after feeding, a number of fish were euthanized by a blow to the head, their stomachs removed, and the wet stomach content (stomach residue) measured to the nearest 0.01g. Stomach residue was also dried in a furnace to calculate the dry weight. The weight of the fish was also recorded, and the stomach residue expressed as a percentage of body weight. At each time point (0, 2, 4, 6, 8, 12, 16, 20, 24, 32 hours post feed), four fish were used. Due to the high number of mortalities in the highest temperature treatment, the number of surviving fish was not sufficient to perform a GET analysis in T3. Therefore, the experiment was performed for the T1 and T2 treatments.

2.8. Statistical analysis

The statistical analysis was performed in SPSS software, version 22. The results are given as mean values followed by the standard deviation. The effects of temperature and experimental duration on the growth and physiological variables were evaluated via analysis of variance (ANOVA) at a $P < 0.05$ level of significance. To evaluate the effects of temperature and time on the growth performance indicators, a two-way ANOVA was performed, while a nested design (two-way nested ANOVA) was implemented for the haematological, metabolic, hormonal, and enzymatic variables to account for potential tank effects. In every case, tank effects were found to be non-significant. Furthermore, due
to the large number of variables, a principal component analysis (PCA) was performed. The aim was to reduce the dimensionality of the analysis to a small number of principal components while maintaining explanatory power for a large portion of the observed variance. The sampling adequacy for the PCA was verified with the Kaiser-Meyer-Olkin measure (KMO>0.5) and Bartlett’s test of sphericity (p<0.05) was used to assess the adequacy of the correlation structure. The number of components was determined using the Kaiser criterion (eigenvalues greater than 1), and a cut-off point of 0.3 was set for primary factor loadings for the variables considered in the analysis. A Varimax factor rotation was performed on the identified principal components. For the molecular metabolic rate, CT_{max}, and proximate composition analyses, one-way ANOVA was performed. Analysis data were checked for meeting the assumption criteria using the Kolmogorov-Smirnov test for normality and Levene’s test for homogeneity of variance. In cases where the assumptions were violated, the data were log-transformed before analysis, or the non-parametric Kruskal-Wallis test was performed. When statistically significant differences were observed, Tukey’s multiple comparisons test was performed to determine the differences between groups.

3. Results

3.1. Growth performance

Temperature had a significant effect on the growth performance of meagre, with T3 differing consistently from T1 and T2 throughout the trial, and T1 and T2 exhibiting differences after the second month (Table 2). In addition, time had a significant effect on some of the considered variables while significant interactions were also found.
Table 2 Growth performance indicators and somatic indices for meagre under three temperature treatments (T1 = 24°C, T2 = 29°C, and T3 = 34°C). The values represent the averages between replicates and the standard deviation. Different letters denote statistically significant differences between temperatures in each sampling (month), and different numbers denote differences between samplings at each temperature at P < 0.05 (2-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>1st month</th>
<th>2nd month</th>
<th>3rd month</th>
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<tbody>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>233.0±5.3</td>
<td>238.7±13.0</td>
<td>154.2±4.4</td>
</tr>
<tr>
<td>Total length (cm)</td>
<td>27.3±2.6</td>
<td>27.7±0.1</td>
<td>25.0±0.2</td>
</tr>
<tr>
<td>AGR (g d⁻¹)</td>
<td>2.34±0.14</td>
<td>2.09±0.06</td>
<td>0.12±0.1</td>
</tr>
<tr>
<td>FCR</td>
<td>1.02±0.14</td>
<td>1.3±0.1</td>
<td>-2.8±2.18</td>
</tr>
<tr>
<td>CF</td>
<td>1.14±0.01</td>
<td>1.12±0.01</td>
<td>0.99±0.03</td>
</tr>
<tr>
<td>Mortality (% d⁻¹)</td>
<td>0.00±0.0</td>
<td>0.00±0.0</td>
<td>0.71±0.1</td>
</tr>
<tr>
<td>HSI (%)</td>
<td>2.17±0.27</td>
<td>1.73±0.09</td>
<td>1.49±0.2</td>
</tr>
<tr>
<td>CSI (%)</td>
<td>0.15±0.01</td>
<td>0.13±0.01</td>
<td>0.17±0.03</td>
</tr>
</tbody>
</table>
In terms of growth rate, it was strongly affected by temperature and time, and a significant interaction was found (temperature: $F_{2,18} = 277.4, P < 0.001$; time: $F_{2,18} = 12.1; P < 0.001$; interaction: $F_{4,18} = 9.6; P < 0.001$). Tukey’s multiple comparisons test indicated that treatment T1 had a significantly higher growth rate in the second and third months compared to the other treatments. From their initial 149 g, fish had doubled in size by the end of the second month and reached a final size of 379 g in the third month. This was followed by T2, where fish showed appreciable growth during the first month, which slowed down slightly in the second month and considerably during the last month. Similarly, length increased from an initial 22.4 cm to 33.3 cm for T1 and to 30.8 cm for T2 by the end of the trial. Differences between the two treatments were negligible in the first month but became significant in the second sampling. On the other hand, T3 was consistently different from the other two groups in all samplings; fish performed poorly, with the growth rate being marginally above zero for the first sampling and exhibiting negative values at the second and third samplings. The CF exhibited the same trend as the growth rate, with statistical significance being detected for the temperature and time effects, and marginal significance for the interaction (temperature: $F_{2,18} = 134.1, P < 0.001$; time: $F_{2,18} = 26.5; P < 0.001$; interaction: $F_{4,18} = 3.5; P = 0.037$). The CF did not differ between T1 and T2 but was significantly lower for T3 in all three samplings. Moreover, it had the tendency to decrease over time from 0.99 in the first month to 0.86 in the third.

![Figure 1](image-url)

*Figure 1* Daily feed intake progression over the course of the trial. Points denote mean values among replicates and bars express the standard deviation.

With respect to feed consumption, appetite did not differ between T1 and T2, as can be seen from the daily feed intake (*Figure 1*). In both groups, feed intake amounted to approximately 1.5% of fish weight daily for the first few weeks and gradually decreased to 1.1% as the fish increased in size. However,
appetite was dramatically lower for T3. While the first weeks were characterized by a high voluntary feed intake due to the gradual increase in temperature, appetite reduced drastically once the experimental temperature was reached. For the following two months, daily feed intake was lower than 0.5% of fish weight. However, there was a considerable increase in feed intake instantly after the reduction of temperature to 33.5°C, which led to an average daily feed intake of 0.7% of weight for the third month. With respect to FCR, temperature had a strong effect ($F_{2,18} = 14.2, P < 0.001$) but the effect of time was insignificant, as was the interaction (time: $F_{2,18} = 1.9; P < 0.174$; interaction: $F_{4,18} = 0.8; P = 0.515$). Consistent with the trends in feed consumption and growth, the T1 treatment exhibited the lowest FCR, with values ranging from 1 to 1.3 over the three-month period (Table 2). For T2, it was significantly higher in the second and third month (FCR of 1.9 and 2.3) but not in the first month. For T3, the combination of negligible or negative growth and the high mortality rate led to FCR exhibiting negative values for the duration of the trial.

3.2. Mortality

Mortality was particularly pronounced for T3. Although it was negligible for T1 and T2, fish in T3 exhibited a high mortality rate, more than half of the initial population had died by the time of the second sampling. The first fish started to die approximately one week after the experimental temperature was reached, resulting in a daily mortality rate of 0.7 (% d$^{-1}$) for the first month. This rate increased to 2.8% for the second month while the decrease of temperature by 0.5°C during the third month reduced mortality to 0.8% d$^{-1}$. Upon completion of the trial, the fish that had survived represented less than 10% of the initial population. At that point, the temperature was raised to 34°C again, as described in methods. This resulted in a high mortality rate of $6.1 \pm 0.6\% \text{ d}^{-1}$, which led to the last surviving fish perishing within ten days after the completion of the trial. While the exact cause of death could not be determined, many of the fish that perished developed blood clots in the dorsal area of their musculature above the spine (Figure 2-b). These sizeable clots caused deformations that could be spotted as bulges on either the left or the right side of the fish for up to three days before they died (Figure 2-a). The fish that developed them were generally less active than the rest but could still feed and maintain dorso-ventral orientation. Additionally, and irrespective of the developing clots, the spleen and gills of all fish that died exhibited a notable discolouration (Figure 2-c).
Figure 2 Blot clot formations on meagre at T3 as observed externally (a) and upon dissection of the fish (b), and the discolouration of spleen and gills (c).

3.3. Somatic indices

Regarding the somatic indices, the effect of temperature on the hepatosomatic index (HSI) was significant ($F_{2,18} = 0.20.9$, $P = 0.008$) but not time (time: $F_{2,18} = 0.9; P = 0.488$; interaction: $F_{4,18} = 0.7; P = 0.682$). The index appeared highly conservative for T1, at approximately 2.2% during the whole trial, and ranged between 1.7 ± 0.1 - 2 ± 0.2% for T2 (Table 2). In contrast, HSI was significantly lower for T3, taking values between 1.5 ± 0.2% and 1.2 ± 0.2%. The cardiosomatic index was significantly affected by temperature ($F_{2,18} = 0.72.7$, $P = 0.001$), and a significant interaction was also found (time: $F_{2,18} = 1.7; P = 0.291$; interaction: $F_{4,18} = 6.7; P = 0.012$). Specifically, it showed little fluctuation over the three months for T1 and T2, with no significant differences between the treatments at any sampling. For T1 it ranged between 0.13 ± 0.01 and 0.16 ± 0.02%, and for T2 between 0.12 ± 0.02 and 0.14 ± 0.01%. Similar values were calculated for T3 during the first month (0.17 ± 0.03%). However, CSI for T3 increased significantly and differed from the other two treatments for the second and third samplings, with values of 0.3 ± 0.07 and 0.26 ± 0.02%, respectively.
3.4. Proximate composition

Finally, temperature had a significant effect on the whole-body proximate composition, specifically on moisture ($F_{2,12} = 119.2; P < 0.001$), total proteins ($F_{2,12} = 127.7; P < 0.001$), and total lipids ($F_{2,12} = 16.9; P < 0.001$) while the ash content was not significantly affected (Table 3). Moisture positively correlated with temperature, with all treatments being significantly different from each other. For T3, moisture content was higher by almost 10% compared to T1, while T2 exhibited intermediate values. The opposite trend was observed for total proteins, which decreased from 17 ± 0.3% in T1, to 13.6 ± 0.6% in T2, to 10.3 ± 0.7% in T3. Total lipids also decreased with temperature, from 8.0 ± 0.8% in T1 to 5.4 ± 0.7% in T3. Total lipids for T2 showed intermediate values which were not, however, significantly different from the other two treatments. Finally, the ash content did not differ between treatments and ranged between 2.1 – 3.2%.

Table 3 Whole-body proximate composition for meagre under three temperature treatments (T1 = 24°C, T2 = 29°C, and T3 = 34°C). The values represent averages and the standard deviation. Different letters denote statistically significant differences between treatments at $P < 0.05$ (1-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>T1</th>
<th>T2</th>
<th>T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture (%)</td>
<td>72.2±1.1</td>
<td>76.8±0.8</td>
<td>80.9±0.9</td>
</tr>
<tr>
<td>Total proteins (%)</td>
<td>17.3±0.3</td>
<td>13.6±0.8</td>
<td>10.3±0.7</td>
</tr>
<tr>
<td>Total lipids (%)</td>
<td>8.0±0.8</td>
<td>6.9±0.5</td>
<td>5.4±0.7</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>2.7±0.6</td>
<td>2.1±1.1</td>
<td>3.2±0.8</td>
</tr>
</tbody>
</table>

3.5. Physiological variables

Overall, most of the physiological variables showed discrete differences between the highest temperature and the other two treatments, while for some of them there were also significant differences between T1 and T2. In addition, while in most cases time did not yield significant effects, significant interactions were observed for many of the considered variables. The results for the haematological, biochemical, and enzymatic variables are given in Table 4.
Table 4 Haematological, biochemical, hormonal, and enzymatic parameters of meagre under three temperature treatments (T1 = 24°C, T2 = 29°C and T3 = 34°C). The values represent averages between replicates and the standard deviation. Different letters denote statistically significant differences between temperatures in each sampling (month), and different numbers denote differences between samplings at each temperature at P < 0.05 (2-way nested ANOVA); ND: not determined.

<table>
<thead>
<tr>
<th></th>
<th>1st month</th>
<th>2nd month</th>
<th>3rd month</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T1</td>
<td>T2</td>
<td>T3</td>
</tr>
<tr>
<td>Haematocrit (%)</td>
<td>31.7±1.4a</td>
<td>26.1±1.0b</td>
<td>28.2±2.8b</td>
</tr>
<tr>
<td>Haemoglobin (g dL⁻¹)</td>
<td>ND</td>
<td>4.8±0.6a</td>
<td>2.2±0.7a</td>
</tr>
<tr>
<td>Triglycerides (mmol L⁻¹)</td>
<td>2.9±0.4a</td>
<td>5.2±0.9a</td>
<td>4.4±1.7a</td>
</tr>
<tr>
<td>Glucose (mmol L⁻¹)</td>
<td>4.2±0.6a</td>
<td>4.5±1.1a</td>
<td>5.2±0.4a</td>
</tr>
<tr>
<td>Cholesterol (mmol L⁻¹)</td>
<td>3.3±0.6a</td>
<td>3.3±0.9a</td>
<td>3.4±1.2a</td>
</tr>
<tr>
<td>Total proteins (g dL⁻¹)</td>
<td>3.6±0.3a</td>
<td>3.9±0.0a</td>
<td>3.4±0.3a</td>
</tr>
<tr>
<td>Lactate (mmol L⁻¹)</td>
<td>1.6±0.4a</td>
<td>2.8±0.5a</td>
<td>1.9±0.3a</td>
</tr>
<tr>
<td>Cortisol (ng ml⁻¹)</td>
<td>1.3±0.8a</td>
<td>1.3±0.7a</td>
<td>1.9±0.5a</td>
</tr>
<tr>
<td>Blood pH</td>
<td>7.5±0.04a</td>
<td>7.47±0.03a</td>
<td>7.36±0.04b</td>
</tr>
<tr>
<td>G Px (nmol ml⁻¹ min⁻¹)</td>
<td>594±94a</td>
<td>838±97a</td>
<td>321±113a</td>
</tr>
<tr>
<td>SOD (U mg⁻¹)</td>
<td>3.5±2.6</td>
<td>3.1±1.5</td>
<td>3.2±3</td>
</tr>
<tr>
<td>ALT (U L⁻¹)</td>
<td>54.5±31.2</td>
<td>47.3±54.0</td>
<td>34.7±35.4</td>
</tr>
<tr>
<td>AST (U L⁻¹)</td>
<td>43.8±32.0</td>
<td>73.2±30.1</td>
<td>65.2±14.1</td>
</tr>
</tbody>
</table>
3.5.1. Haematological variables

With respect to the haematological variables, haematocrit (Hct) was significantly affected by temperature ($F_{2,108} = 418.8, P < 0.001$) but not time ($F_{2,108} = 1.6, P = 0.303$) while a significant interaction was found ($F_{4,108} = 5.9, P = 0.016$). Specifically, Hct ranged from $32 \pm 1.4$ to $35 \pm 2\%$ during the trial, and Tukey’s test showed that it differed from T2 in the first ($26. \pm 1\%$) but not in the subsequent months. However, marked differences appeared for T3, which was significantly lower than the other two treatments, exhibiting values of $18 \pm 2\%$ in the second and third months. Similar effects were detected for haemoglobin (temperature: $F_{2,108} = 107.8, P < 0.001$; time: $F_{2,108} = 3.4; P = 0.139$; interaction: $F_{4,108} = 5.3; P = 0.04$) which reached values of $7.3 \pm 1.4$ and $7.2 \pm 0.6$ g dl$^{-1}$ for T1 and T2, respectively, by the end of the trial. However, haemoglobin was consistently lower for T3 for all samplings ($2.2 \pm 0.7, 2.5 \pm 0.1$ and $2.6 \pm 0.9$ g dl$^{-1}$ for the three samplings, respectively).

3.5.2. Biochemical variables

Regarding the biochemical parameters, triglyceride concentration was affected by temperature and time, and a significant interaction was found (temperature: $F_{2,108} = 85, P = 0.001$; time: $F_{2,108} = 26.4; P = 0.005$; interaction: $F_{4,108} = 18.9; P < 0.001$). For T2 and T3, triglyceride concentration was higher in the first month compared to T1 ($5.2 \pm 0.9, 5.2 \pm 1.9$, and $2.9 \pm 0.4$ mmoles L$^{-1}$, respectively) but for the rest of the trial, T1 and T2 remained similar, with only T3 exhibiting substantially lower concentrations ($3.3 \pm 0.6$ and $1.8 \pm 0.4$ mmoles L$^{-1}$ for second and third month, respectively). Glucose levels were significantly affected by temperature ($F_{2,108} = 16, P = 0.05$), with T2 exhibiting higher values than the other two treatments. Specifically, in the second month, T2 was almost double ($8.9 \pm 1.6$ mmoles L$^{-1}$) compared to the other treatments and remained substantially higher also in the third ($6.9 \pm 0.9$ mmoles L$^{-1}$). However, differences within samplings for each treatment were not significant (time: $F_{2,108} = 3.2; P = 0.15$; interaction: $F_{4,108} = 4.5; P = 0.3$). Cholesterol was also affected significantly by temperature while an interaction was also found (temperature: $F_{2,108} = 47.6, P = 0.002$; time: $F_{2,108} = 0.5; P = 0.616$; interaction: $F_{4,108} = 5.7; P = 0.018$). There were no significant differences between T1 and T2 in all samplings, with cholesterol concentration ranging from $3.3$ to $4.6$ mmoles L$^{-1}$, but T3 exhibited significantly lower values for the second and third month ($2.3 \pm 0.6$ and $2.2 \pm 0.8$ mmoles L$^{-1}$, respectively).

Likewise, protein concentration was affected by treatment ($F_{2,108} = 7.1, P = 0.048$), and T3 exhibited values significantly lower than T1 and T2 for the first two months. However, time and the interaction were found marginally insignificant (time: $F_{2,108} = 6.6; P = 0.054$; interaction: $F_{4,108} = 3.7; P = 0.054$). The same pattern
was observed for blood pH ($F_{2,108} = 10.8, P = 0.024$), with T3 being significantly lower than the other treatments during the first two months and with no significant effects for time and the interaction (time: $F_{2,108} = 0.2; P = 0.193$; interaction: $F_{4,108} = 2.2; P = 0.218$). Finally, treatment, time, and their interaction had significant effects on lactate concentrations (temperature: $F_{2,108} = 79, P = 0.001$; time: $F_{2,108} = 18; P = 0.01$; interaction: $F_{4,108} = 3.99; P = 0.046$). Specifically, lactate levels remained unchanged for T1 throughout the trial (1.4 ± 0.2 - 1.8 ± 0.5 mmol L$^{-1}$) but were consistently higher for T2 in all samplings, exhibiting the highest concentration in the third month (3.6 ± 0.5 mmol L$^{-1}$). Similarly, for T3, lactate differed from T1 in the second month (2.9 ± 0.6 mmol L$^{-1}$) and from both T1 and T2 in the third month (2.5 ± 0.3 mmol L$^{-1}$).

### 3.5.3. Enzymatic variables

The activity of SOD appeared stable during the trial with values ranging from 3.1 ± 1.2 to 4.5 ± 1.3 U L$^{-1}$ and no significant differences were found between treatments. For GPx, temperature had a significant effect (temperature: $F_{2,108} = 86.5, P = 0.001$; time: $F_{2,108} = 2.2; P = 0.082$). All treatments differed in the first month, and for the other two samplings, T1 and T2 were similar but T3 lower by a factor of four. A significant interaction was also found ($F_{4,108} = 4.6; P = 0.02$). Finally, hepatic enzyme concentrations (ALT, AST, ALP) showed a high variability, which resulted in no significant treatment or time effects.

### 3.5.4. Principal Component Analysis

Regarding the PCA, the KMO measure was calculated at 0.822, above the commonly recommended value (0.5) for sampling adequacy, and Bartlett’s test of sphericity was also found significant ($P < 0.01$). Based on the criterion for primary factor loading above 0.3, 13 variables were found suitable for the PCA, namely, Hct, Hb, HSI, CSI, cortisol, glucose, triglycerides, cholesterol, lactate, total proteins, blood pH, GPx, and SOD, while, according to the criterion of eigenvalue greater than 1, two principal components were determined as statistically significant (eigenvalues 4.397 and 1.801, respectively). Cumulatively, these explained 47.7% of the variance, with the first component accounting for 33.8% and the second for 13.9%. Therefore, the PCA reduced the dimensionality of the physiological analysis considerably by distilling the relationships between the 13 physiological measures in two components (Figure 3).

Specifically, as seen by the rotated component matrix and the corresponding plot (Figure 3-a), the first component correlated predominantly with the haematological parameters (Hct and Hb), the somatic indices (HSI and CSI), cortisol, and GPx, while the second with variables associated with anaerobic metabolism, such as lactate, glucose, and SOD. The CSI and cortisol correlated negatively with the first component while Hct, Hb, GPx, and HSI correlated positively. The remaining variables (triglycerides,
cholesterol, blood pH, and total proteins) showed substantial cross-loading values between the two components, and thus did not contribute to a clear pattern. Finally, plotting the scores of the two components for each individual yielded a marked clustering of the data points based on the temperature treatment (Figure 3-b). Specifically, the first principal component separated the data into two clusters, one corresponding to individuals from T3 and the other to individuals from the other two treatments. Considering the main variables contributing to principal component 1, such a distinction was already seen in 3.3, 3.5.1, and 3.5.2, where T3 exhibited consistently higher CSI and cortisol values compared to T1 and T2 but lower values for the haematological parameters and HSI. On the other hand, the second component tended to separate data between T1 and T2 but not as clearly as principal component 1. That being said, individuals at T2 generally scored higher on the principal component 2 compared to T1, indicating higher levels of anaerobic metabolism for T2. Moreover, the overlap occurred predominantly between samples of the first month (not shown), with a clearer separation between T1 and T2 in subsequent months, confirming the existence of significant interaction effects between temperature and time for several of the considered variables, as already shown in 3.5.2.

![Figure 3 PCA for meagre reared under three temperature treatments (T1 = 24°C, T2 = 29°C, and T3 = 34°C). a) The loading plot of principal component 1 vs principal component 2. The component loadings per considered variable are included in the rotated component matrix and sorted by size. b) The scores of principal components 1 and 2 plotted for each fish and coloured according to temperature treatment.]

3.6. Metabolic rate

Regarding the metabolic rate of meagre, an almost linear increase of SMR was observed as temperature increased from 91.4 ± 21 mg kg⁻¹ h⁻¹ at 24°C to 189.8 ± 43.6 mg kg⁻¹ h⁻¹ at 34°C (Figure 4). However, MMR showed small differences between the first two treatments (351.7 ± 55.5 mg kg⁻¹ h⁻¹ and 366.6 ± 42.6 mg...
kg$^{-1}$h$^{-1}$ for T1 and T2, respectively), with only that of the T3 being lower (318 ± 48.8 mg kg$^{-1}$ h$^{-1}$). This resulted in the AS differing significantly ($F_{2,33} = 53.6; P < 0.001$) between all groups, being the highest for T1 at 260.3 mg kg$^{-1}$ h$^{-1}$, lowest for T3 at 129 mg kg$^{-1}$ h$^{-1}$, and intermediate for T2 (233.7 mg kg$^{-1}$ h$^{-1}$).

![Figure 4](image)

*Figure 4 Standard and maximum metabolic rate (SMR and MMR) of meagre as a function of temperature. Bars indicate the standard deviation.*

3.7. Gene expression

Considering the relative gene expression, the most significant differences were recorded for the GR gene in both liver ($F_{2,12} = 38.7; P < 0.001$; Figure 5, a-c) and spleen ($F_{2,12} = 71.6; P < 0.001$; Figure 5, d-f). In both tissues, it was significantly downregulated for T3. Specifically, GR expression decreased twofold in liver for T3 compared to the other two treatments, while for spleen, all treatments differed between them, with T2 exhibiting the highest expression and T3 the lowest. Regarding the HSP70 gene, there was no difference in the expression pattern in the liver while in the spleen it was significantly downregulated for T3 compared to T1 and T2 ($F_{2,12} = 19.5; P < 0.001$). Furthermore, for HSP90, there seemed to be a trend for upregulation in both tissues for the T3 treatment, yet differences were not significant.
3.8. CT<sub>max</sub> and GET

Finally, the critical thermal maximum and the gastric emptying time experiments on T1 and T2 showed a positive relationship of acclimation temperature with CT<sub>max</sub> and digestion speed (Figure 6-a). Specifically, the CT<sub>max</sub> was substantially higher for T2 as opposed to T1, with the LOE reaching 33.9 ± 0.5°C for T1 and 37.1 ± 0.6°C for T2. Likewise, the GET exhibited differences between the T1 and T2 treatments, with the latter being faster (Figure 6-b). The voluntary feed intake at the beginning of the experiment was similar for both treatments but notable differences started appearing after the first four hours; specifically, halving of the stomach content required approximately 6 hours for T1 and 4 hours for T2. Moreover, the T2 group reached 0% stomach residue by the 24<sup>th</sup> hour, while for T1, small quantities were still present until the completion of gastric emptying at 32 hours.
Figure 6 Critical thermal maximum (a) and gastric emptying time (b) for meagre at T1 (24 °C) and T2 (29 °C). The box plot represents the interquartile range (1st and 3rd quartile), the whiskers the minimum and maximum values, and the horizontal line the median. The vertical bars indicate the standard deviation.

4. Discussion

The investigation into the thermal preferences and tolerance of meagre conducted in this study corroborates findings from other studies while also providing novel information regarding the species’ biological responses towards the upper end of its thermal tolerance range.

With respect to growth performance, literature on meagre growing under constant temperatures is scarce due to the species having only recently received attention. However, recent experimental work suggests that the optimum temperature for juvenile growth lies between 23–27°C, with Kounna et al. (2021) reporting similar SGR values (1.4–1.6% d⁻¹) at 23 and 26°C. Moreover, there are several studies of production systems under seasonal temperature regimes typically found in Mediterranean countries. For instance, growth trials in earthen ponds under natural photoperiod and temperature suggest that, like our findings, the highest growth for meagre occurs in the high summer temperatures (20–25°C) where values of SGR of 1.76% d⁻¹ have been reported (Vargas-Chacoff et al., 2014). High growth rates of 2.55% d⁻¹ (expressed as daily increment in weight) have also been reported for meagre grown in ponds in Egypt under higher year-round temperatures (15–32°C) (El-Shebly et al., 2007). In another case, Fountoulaki et al. (2017) report growth rates of 0.5% d⁻¹ and FCR values greater than two for juvenile meagre grown in marine cages. However, these values refer to growth under natural seasonal photoperiods and temperatures that ranged from 16 to 26°C. Therefore, the lower biological performance compared with our findings at 24°C could be attributed to the low winter temperatures, which are suboptimal for growth.
In fact, the same authors report values of voluntary feed intake of between 1.1 and 1.3% of fish weight daily for the high temperature season (25°C), which are consistent with the findings of our trial.

According to Kir et al. (2017), the optimal temperature for juvenile meagre is between 26–30°C, yet their analysis was not accompanied by growth performance data. Our findings suggest that the optimum temperature for growth is indeed in the 24–29°C area although it is most likely closer to the lower end of that range. Indicative of this is the long-term effects that temperature had on the 29°C treatment. Although during the first month, growth was similar at 24°C and 29°C, eventually the performance of the latter group deteriorated over the course of the trial. It therefore appears that although fish can survive and grow well in high temperatures, prolonged exposure to them has an additive effect that reduces their overall performance, which is an important observation in the context of climate change. The thermal stress effects were particularly pronounced at 34°C, where appetite was significantly reduced and growth ceased, reflecting the high energetic costs at that temperature. These costs were also evident by changes in the proximate composition of the fish in relation to temperature. At 34°C, the moisture content had significantly increased while total lipids and proteins declined, trends that are consistent with starvation experiments (Chatzifotis et al., 2018; Shirvan et al., 2020) where catabolism of adipose and muscle tissues occurs to fuel basic metabolism. Due to appetite typically declining at high temperatures while the energetic demands increase, the same pattern in body composition has been documented as a response to increasing temperatures for a number of species such the E. seabass (Person-Le et al., 2004), the cobia (Rachycentron canadum) (Sun and Chen, 2014), and the spotted seabass (Lateolabrax maculatus) (Cai et al., 2020). On the other hand, changes in body composition tend to be insignificant in the absence of thermal stress, as was shown for meagre grown at temperatures within the 17–26°C range (Kounna et al., 2021). In fact, the body composition at 24°C in the present study was similar to that reported by Kounna et al. (2021) and also by Lozano et al. (2017) at 23.5°C. Therefore, the changes in body composition observed at the 29°C treatment indicate a depletion of energy reserves at that temperature, which was further exacerbated at 34°C.

Furthermore, mortality was particularly high at 34°C. For the first weeks after the fish reached the experimental temperature there were only isolated incidences of mortality, arguably due to the activation of regulatory mechanisms that allow fish to cope with stress for a limited amount of time. After this period, however, the failure of these mechanisms led to a high and rather constant mortality rate. This resulted in about 50% of the fish perishing within the first two months. Moreover, many of the fish that perished had developed blood clots. Although fish mortality attributed to clots has not been explicitly
linked with high temperature, stress is known to induce clots that lead to mortalities, while for some species, a positive correlation has been established between temperature and thrombocyte density (Casillas and Smith, 1977; Tavares-Dias and Oliveira, 2009). Interestingly, mortality was notably reduced once the temperature decreased by 0.5°C during the third month while it drastically increased after the completion of the trial, when temperature was raised again to 34°C. This indicates that the survival thresholds at the upper end of the temperature tolerance range for the species are extremely narrow and even the smallest temperature changes can have large-scale effects on the organism. Although the species is considered eurythermal and substantial mortalities have not been reported for temperatures up to 32°C (El-Shebly et al., 2007), the low capacity of meagre to cope with high temperature variations has also been highlighted elsewhere (Kir et al., 2017).

The physiological evaluation also corroborated the above observations since at 24°C and 29°C many parameters exhibited similar values. However, some indications of stress were evident at 29°C and were significantly magnified at 34°C where fish exhibited poor overall physiological status. In fact, much like the growth performance findings, the PCA confirmed the differentiation of the three temperature treatments with the most distinct being for the 34°C group. The two principal components identified in the analysis explained over half of the variation in the measurements and pinpointed to key variables that could differentiate each group. In particular, the 34°C treatment was distinct from the other two mainly in terms of the somatic indices, cortisol, and haematological variables while differences in glucose and lactate levels tended to further separate the 24°C and 29°C groups.

Hypotheses on why performance declines at high temperatures point to oxygen limitations in combination with high energetic costs for basic metabolism (Pörtner et al., 2017). Although in the present study oxygen saturation was kept the same (above 80%) for all treatments, the actual oxygen content differed (approximately 6, 5.5, and 5 mg L⁻¹ for 24°C, 29°C and 34°C, respectively) as oxygen saturation is temperature-dependent. Furthermore, such limitations are largely related to physiological restrictions in the capacity to deliver adequate oxygen to the tissues (Pörtner et al., 2017). Prolonged exposure to 34°C resulted in a progressive reduction of HSI with a concomitant increase in CSI. Decrease of HSI is often reported in starvation or thermal stress experiments and is indicative of the depletion of energy reserves (Rossi et al., 2017; Chatzifotis et al., 2018). On the other hand, the increase of the CSI suggests the presence of heart hypertrophy (megalocardia), which is the phenomenon of heart enlargement and is well documented in fish, particularly salmonids, as an adaptive response to hypoxia and elevated temperatures (Gamperl and Farell, 2004; Gamperl et al., 2020). Since temperature effects are linked to
oxygen limitations, the megalocardia at 34°C is indicative of the hypoxic stress these fish were subjected to.

The diminished aerobic capacity at 34°C was further supported by the results of the metabolic rate trial. It was shown that the SMR increased almost linearly from 24 to 34°C, reflecting the increase in energetic costs relating to basic metabolism, which is typically found in fish (Christensen et al., 2020). The MMR reached a maximum at 29°C and significantly decreased at 34°C. The reduction of MMR is a sign of the disruption of the natural mechanisms used to upregulate oxygen uptake under stressful conditions. This indicates that the optimum conditions for the species have long been surpassed and its physiological mechanisms are on the verge of collapsing. Although the decrease in MMR is generally predicted by the oxygen- and capacity- limited thermal tolerance hypothesis (OCLTT) (Pörtner et al., 2017), it has rarely been recorded experimentally for fish. Most species have a very narrow temperature threshold for survivability above that point and therefore it is challenging to obtain such values. The combination of SMR and MMR resulted in the AS being the highest at 24°C and severely diminished (less than half) at 34°C, as predicted by the OCLTT for temperatures close to the upper end of the tolerance range (Jutfelt et al., 2020). The results regarding the metabolic rate align with the findings of Kir et al. (2017), who suggest that the optimal temperature for juvenile meagre is between 26–30°C. However, in that study, much higher oxygen consumption rates were recorded. In particular, rates of 410, 618, and 642 mg·kg⁻¹·h⁻¹ were reported for 22, 26, and 30°C, respectively, which are twofold higher than the ones found here. This could be attributed to several factors, including the size of the fish (3.4 g), since SMR is size-and life stage-dependent, or the measurement protocol. Kir et al. (2017) used a static respirometer and recorded measurements of oxygen consumption only for one hour, and both of these factors are known to overestimate the metabolic rate (Chabot et al., 2016). In contrast, rates similar to those of the present study at 24°C have been reported at 21.5°C (99 and 245 mg kg⁻¹ h⁻¹ for SMR and MMR, respectively) for bigger fish (82.1 g) and similar experimental protocols (Peixoto et al., 2017).

With respect to the haematological, biochemical, enzymatic, and hormonal variables, similar conclusions can be drawn. Neither haematocrit or haemoglobin levels differed among the first two treatments but were substantially lower (half) for the fish at 34°C, when the fish developed anaemia, indicating their inability at that temperature to meet the aerobic demands by upregulating red cell and haemoglobin production. For haemoglobin, there was a trend of increase over time at 24°C and 29°C, yet concentrations did not exceed the typical range reported for spring and summer months, as was also the case for haematocrit (Samaras et al., 2016). The induction of anaemia due to extreme thermal stress as observed
at 34°C has been documented in other species, with even temperature tolerant fish such as the rohu (*Labeo rohita*) (Roychowdhury *et al.*, 2020) and the striped catfish (*Pangasianodon hypophthalmus*) (Shahjahan *et al.*, 2018) exhibiting a substantial decrease of haematological variables (30-50%) at temperatures above 35°C compared to baseline values. Lactate, which is a proxy of anaerobic metabolism, was elevated at both 29°C and 34°C compared to the baseline levels. This is a strong indication that the aerobic capacity was not only diminished at 34°C but also challenged at 29°C. As suggested by the high lactate levels in the plasma and tissues of gilthead sea bream (*Sparus aurata*) exposed to temperatures above 24°C, an increase in lactate is indicative of the internal mismatch between oxygen supply and energy demands which triggers anaerobic metabolic pathways and is typically accompanied with increased activity of glycolytic enzymes (PK, L-LDH) and expression of the hypoxia-induced factor Hisf-1a (Feidantsis *et al.*, 2013, 2020). Similarly, cortisol concentration showed a marked increase at 34°C that was above any baseline value reported for meagre (Fanouraki *et al.*, 2011; Samaras *et al.*, 2016), showing that the fish were under chronic stress for the duration of the trial. Such an increase in plasma cortisol has been well documented for fish under thermal stress (Wang *et al.*, 2019; de Freitas Souza *et al.*, 2020; Yuan *et al.*, 2020) and it has been recently hypothesized that it is linked to the release of ATP and nucleotides into the extracellular space, thus contributing to the cell damage and inflammation typically encountered under stressful conditions (Baldissera *et al.*, 2020; de Freitas Souza *et al.*, 2020). In the contrast, cortisol levels at 24°C fell within the baseline values (0.1-3.2 ng ml⁻¹) reported in Samaras *et al.* (2016) confirming the absence of a stress response at that temperature. In fact, the lowest concentrations appeared after the first month, indicating the considerable amount of time required for complete acclimation to the applied temperature regimes. Notably, at 29°C, fish exhibited slightly higher concentration levels from 24°C, thus pointing to an intermediate level of stress.

The protective mechanisms that fish employ under stressful conditions increase energetic costs (Jager *et al.*, 2014). Consequently, energy reserves need to be mobilised and released into the bloodstream to supply energy for the organism’s vital functions. While glucose is the molecule predominantly used for this purpose and is supplied by the liver via gluconeogenesis, other metabolites such as lipids and proteins may also be used. For this reason, high plasma concentrations of such metabolites, and particularly of glucose, are typically linked to catabolism and are interpreted as signs of stress (Aedo *et al.*, 2019). In the present study, triglycerides, glucose and cholesterol concentrations increased at 29°C compared to 24°C, clearly indicating signs of stress for the former treatment. However, under extreme stress the mechanisms responsible for supplying and utilizing these energy sources become impaired and fail to maintain high concentrations of metabolites in the plasma, leading to their rapid decline. The plummeting of
metabolites such as proteins, triglycerides, and cholesterol at temperatures close to the upper lethal limits has been documented for many species including the rohu (Roychowdhury et al., 2020), the E. seabass (Islam et al., 2020) and the armoured catfish (Hoplosternum littorale) (Rossi et al., 2017). In line with these studies, the concentration of triglycerides, proteins and cholesterol showed a pattern of substantial decrease at 34°C in this study, while glucose concentrations remained at relatively high levels which was also reported by Roychowdhury et al. (2020) and Rossi et al., (2017). Considering that the HSI was very low at 34°C, it is likely that glycogen reserves were depleted. Therefore, as hypothesized by Rossi, et al. (2017) glucose levels could be maintained through hepatic glucose production from free amino acids instead of gluconeogenesis. This is also supported by the diminished protein concentration in the plasma, which may indicate the use of proteins as fuels via proteolysis and the subsequent oxidation of amino acids. Conversely, the metabolite levels at 24°C did not indicate signs of stress, exhibiting comparable values with those reported by Chatzifotis et al. (2018) in control conditions. Specifically, the authors report plasma concentrations of 6.2, 2.9 and 8 mmol L⁻¹ at 20°C for glucose, cholesterol and triglycerides respectively, which are similar to the ones found in this study.

In addition to changes in metabolic biomarkers, temperature induces oxidative stress due to the increased production of reactive oxygen species (ROS). Organisms typically respond by upregulating antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and glutathione peroxidase (GPX) which aim to detoxify the ROS (Suzuki and Mittler, 2006); a process that has been well documented for fish under thermal stress (Jia et al., 2020; Rossi et al., 2017; Yang et al., 2021) and is also known to be tissue specific (Feidantsis et al., 2018). However, such an increase was not observed in response to temperature for the antioxidant enzymes considered in this study. They remained unaltered at 24, and 29°C while a substantial decrease was observed for GPx at 34°C compared to the other treatments. Antonopoulou et al. (2020) report similar observations for meagre, with no significant differences of SOD, catalase, and GPx levels in several examined tissues for fish reared at 23 and 26°C compared to 20°C. However, the authors report a tissue-specific decrease in SOD and GPx levels at 17°C, suggesting that meagre may have a limited capacity for antioxidant activity at higher temperatures. This is further supported by the marked decrease of GPx at 34°C in the present study indicating the proximity of that temperature to the species upper thermal limit. Such a diminishing performance of antioxidant activity at high temperature extremes has been recently reported for the Nile tilapia (Oreochromis niloticus) (Waheed et al., 2020) and rainbow trout (Oncorhynchus mykiss) (Topal et al., 2021) and is typically attributed to the denaturation of proteins and thus the inactivation of enzymes at high temperatures (Kregel, 2002; Lie et al., 2020).
Like the antioxidant enzyme activity, the upregulation of genes relating to stress response (GR) and protection from thermal stress (HSP70 and HSP90) is a well-documented mechanism that fish employ when exposed to high temperatures (Feidantsis et al., 2015; Jeffries et al., 2018; Zhang et al., 2020; Topal et al., 2021). Moreover, the response seems to be highly tissue-specific (Shin et al., 2018; Bildik et al., 2019; Antonopoulou et al., 2020) which was also the case for the present study. Specifically, while the expression of HSP90 did not differ among treatments and tissues, HSP70 was downregulated at 34°C compared to the other treatments in the spleen but not in the liver. Moreover, GR was downregulated at 34°C in both liver and spleen while it was upregulated at 29°C but only in the spleen. For GR in particular, the upregulation at 29°C compared to 24°C fulfils the expectation of the established stress response pattern (Faught and Vijayan, 2016) and aligns with that of the biochemical parameters discussed previously. In parallel with our findings, Antonopoulou et al. (2020) did not detect differences in the levels of HSP90 in meagre tissues (heart, liver, muscle, intestine) in the 17–26°C range. Moreover, they report increasing levels of HSP70 with temperature in the intestine but not in the liver, which showed similar values between 20–26°C and lower at 17°C. Along with our findings, this indicates that meagre has a limited capacity for upregulation in the tested temperature range. This is reinforced by the decreased expression of HSP70 in the spleen and GR at 34°C, which indicates an impairment of the cellular functions similar to the one discussed for the antioxidant enzymes. The observation aligns with findings on flounder (Paralichthys olivaceus) where fish reared at temperatures beyond the upper tolerance limit showed an initial increase of HSP70 followed by a diminished expression in subsequent weeks due to cellular defence mechanisms eventually being overcome by thermal stress (Kim et al., 2019).

Regarding the gastric evacuation time, a positive relationship between temperature and digestion speed has been documented in many species, including the common sole (Solea solea) (Vinagre et al., 2007), the brown trout (Salmo trutta) (Khan and Seyhan, 2021), and the Nile tilapia (Azaza and Dhraied, 2020). For meagre in particular, Kounna et al. (2021) report a higher GET at 26°C compared to 23°C, with the halving of stomach emptying occurring at 7.4 and 6 hours, respectively. In line with this finding, the digestion speed in the present study was faster at 29°C compared to 24°C, with the stomach emptying halving time being 4 and 6h. This could be an indication that despite signs of stress at 29°C the fish were able to upregulate their gastrointestinal functions, as a mechanism for coping with the increase energetic demands at the higher temperature (De et al., 2016). Finally, with respect to the critical thermal maximum (CT_max) experiment, a positive relationship between thermal tolerance and acclimation temperature was detected. The increase of CT_max with acclimation temperature is a pattern that has been demonstrated numerous times in fish studies (Yilmaz et al., 2019; Zhou et al., 2019; Penny and Pavey, 2021; Sakurai et
That being said, the capacity to increase thermal tolerance seems to plateau at acclimation temperatures close to upper lethal limits, which is the case for stenothermal fish such as many tropical and cold-water species (Illing et al., 2020; Morrison et al., 2020; Nati et al., 2020). However, in our study, CT\text{max} increased significantly between 24–29°C, indicating that meagre has a substantial capacity for thermal acclimation in that temperature range. In combination with the physiological findings, this suggest that under a warming ocean, meagre may be able to cope appreciably with acute climatic events such as heatwaves despite its overall performance being compromised due to prolonged thermal stress. The only previously reported case of CT\text{max} in meagre acclimated to high temperatures comes from Kir et al. (2017). For juveniles of 3.4 g, the authors report a CT\text{max} of 35.91°C for fish acclimated to 26°C, and a CT\text{max} of 36.98°C for acclimation to 30°C. The findings of the present trial suggest a CT\text{max} of 33.9°C for acclimation to 24°C, which fits perfectly below that of Kir et al.’s (2017) 26°C, taking into account the positive relationship between acclimation temperature and critical thermal tolerance (Ern et al., 2016). In addition, like Kir et al.’s (2017) results at 30°C, we report a CT\text{max} of 37.5°C for acclimation at 29°C, which is, to our knowledge, the highest ever recorded CT\text{max} for meagre.

5. Conclusions

This investigation on the thermal preferences and tolerance of meagre revealed that the species performs best between 24 and 29°C with the optimum being closer to the lower end of that range. Moreover, the temperature of 34°C is sharply close to the upper end of its temperature tolerance range. This was reflected by a poor growth performance, blood clot formation, mortalities, and low levels of haematological, biochemical, and enzymatic parameters at 34°C, as well as high levels of stress parameters (cortisol and lactate) in combination with high energetic demands and a reduced capacity for aerobic metabolism (high SMR and low AS). Elevated concentrations in some plasma metabolites at 29°C indicate a high metabolic activity at that temperature and signs of mild stress while the study of the metabolic rate reveals an intermediate capacity for aerobic metabolism and therefore overall biological performance. While additional research is needed to unravel the underlying mechanisms of thermal tolerance, the present work contributes by bridging significant knowledge gaps and identifying critical biological thresholds for this emerging aquaculture species.

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References


metabolism of meagre (*Argyrosomus regius*) under different water temperatures. Fish Physiology and Biochemistry 44, 527–542. https://doi.org/10.1007/s10695-017-0451-3


Khan, U., Seyhan, K., in press. Parameterizing an expanded square root model to account for the effects of temperature, meal size, and body size on gastric evacuation rate in farmed brown trout. Aquaculture Research. https://doi.org/10.1111/are.15318


apoptotic genes, heat shock genes, ER-stress genes and oxidative stress genes. Fish & Shellfish Immunology. https://doi.org/10.1016/j.fsi.2021.05.002


Wang, Y., Han, G., Pham, C.V., Koyanagi, K., Song, Y., Sudo, R., Lauwereyns, J., Cockrem, J.F., Furuse, M., Chowdhury, V.S., 2019. An acute increase in water temperature can increase free amino acid concentrations in the blood, brain, liver, and muscle in goldfish (Carassius auratus). Fish Physiology and Biochemistry 45, 1343–1354. https://doi.org/10.1007/s10695-019-00642-5


