Chronic impact of exposure to low dissolved oxygen on the physiology of *Dicentrarchus labrax* and *Sparus aurata* and its effects on the acute stress response.

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Abstract

Dissolved oxygen (DO) saturation in the water is a crucial factor in fish performance and welfare. Exposure to low DO can affect a wide variety of functions such as metabolic rate and physiological adaptations including hematological, hormonal, biochemical and osmoregulatory alterations in blood and plasma. In the present study European sea bass, *Dicentrarchus labrax* and gilthead seabream, *Sparus aurata* were reared for approximately 3 months at different levels of DO saturation, namely 40-60%, 60-80% and 80-100% at a temperature of 26.5°C. Both species showed reduced performance at the lowest DO regime compared to the highest, as well as a reduced aerobic capacity as indicated by the aerobic scope and the post-stress lactate concentrations. Blood samples were collected before and after exposure to an acute chasing and confinement stress. Hematocrit, hemoglobin and mean corpuscular hemoglobin concentration were affected by DO saturation in *D. labrax* but not in *S.*

aurata. Cortisol levels in fish plasma and scales were similar between different DO regimes in both species, while in plasma it was increased after exposure to acute stress. Moreover, in both species post-stress levels of osmolality and lactate were higher at the lowest DO examined, indicative of osmoregulatory imbalance. Based on multivariate analysis glucose and lactate were highly affected by acute stress in low oxygen saturation in *D. labrax*, while osmolality was mostly affected in *S. aurata*. Overall, this study provided a detailed insight in the effects of DO in the physiology of *D. labrax* and *S. aurata*.

Keywords: dissolved oxygen; European sea bass; gilthead seabream; metabolic rate; stress

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Introduction

Oxygen is vital for aerobic respiration and energy production in vertebrates. In the aquatic environment, oxygen availability is lower than in the air, while temporal and spatial variability in its concentrations is common (Makridis et al., 2018; Mavropoulou et al., 2020). This variability is considered to have driven the development of evolutionary adaptation mechanisms in many aquatic vertebrates (reviewed in Farrell and Richards, 2009; Pollock et al., 2007). Moreover, in a changing environment, due to climate change, the elevated sea water temperature may set new challenges for respiration (Pörtner et al., 2017; Pörtner and Knust, 2007). These challenges can be even greater for animals reared under extensive aquaculture conditions, since during the warm months of the year, a combination of reduced oxygen capacity of the warm water with the reduced circulation of water through the net of the cage due to fouling and the increased oxygen demand by the fish can make oxygen scarce (Johansson et al., 2006; Makridis et al., 2018; Solstorm et al., 2018). Hypoxia is a shortage of oxygen, usually defined as dissolved oxygen concentrations below 2–3 mg O_2 L^{-1} (Farrell and Richards, 2009). In Mediterranean aquaculture cases of dissolved oxygen (DO) saturation levels below 40% are not uncommon (Makridis et al., 2018).

Available oxygen can affect the rate at which oxygen is consumed, and therefore the capacity for aerobic metabolism. Especially in ectotherm vertebrates, the most commonly used estimation of metabolism at a whole animal level is measuring the rate of oxygen consumption. This is often called indirect calorimetry, because it is based on the notion that the consumption of oxygen is attributed to catabolic functions, and therefore it is related to metabolic rate (Nelson, 2016). Three biologically different parameters are of major importance when considering metabolic rate. The first is called standard metabolic rate (SMR) and it reflects the minimum metabolic rate required for

the subsistence of the organism. By definition the measurement of SMR requires that the animal is at rest, it is not developing, it is showing minimum muscular activity and additionally it is in a post-absorptive, yet not starving, state (Chabot et al., 2016). On the contrary, maximum (or active) metabolic rate (MMR) refers to the oxygen uptake at the maximum aerobic muscular activity, while the difference between the MMR and SMR is referred to as "aerobic scope" and represents the energy requirements that can be fulfilled through aerobic metabolism (Brauner and Richards, 2020; Chabot et al., 2016; Norin and Clark, 2016).

Dissolved oxygen has long been identified as a factor that can regulate metabolic rate in fish (Fry, 1971, 1947). Novel methodologies in aquatic respirometry have confirmed the initial hypothesis that as the availability of oxygen decreases, the SMR of an organism remains constant while the MMR and aerobic scope are being reduced until they reach a point where MMR equals SMR (Bergstedt et al., 2021; Brauner and Richards, 2020; Claireaux and Lagardère, 1999; Garduño Paz et al., 2020; Hvas and Oppedal, 2019; Neill et al., 1994; Pang et al., 2021). This oxygen level is usually referred to as critical O_2 concentration (or tension depending on the methodology of measurement), below which oxygen is not adequate to fuel basic functional needs and therefore SMR is restricted by it and start to decrease (Claireaux et al., 2000; Claireaux and Chabot, 2016; Claireaux and Lagardère, 1999; Neill et al., 1994). However, the metabolic rate of fish exposed to chronic rather than acute hypoxia has been less well studied, and it becomes crucial to gain a better insight in this subject, especially in the context of climate change and fish welfare in aquaculture.

Although many fish species pose various adaptations to counteract environmental hypoxia, such as changes in hematological parameters, alterations in the gill lamellae, changes in cardiac output and hyperventilation (Anttila et al., 2015; Farrell, 2007;

Farrell and Richards, 2009; Galhardo et al., 2011; Mignucci et al., 2021; Saroglia et al., 2002;), usually those adaptations come on cost. It is well known that reduced dissolved oxygen (DO) can negatively affect growth, health and welfare in aquaculture fish both due to reduced feeding and altered metabolism (Gamperl et al., 2020; Hansen et al., 2015; Pichavant et al., 2001; Thetmeyer et al., 1999). Changing the morphology of gills to assist ventilation, can have negative effects on the regulation of ionic equilibrium, a phenomenon called osmorespiratory compromise (Giacomin et al., 2019; Onukwufor and Wood, 2018; Saroglia et al., 2009; Wood et al., 2019).

Blood, specifically erythrocytes, is responsible for the transportation of oxygen, and therefore a plethora of adaptations to hypoxia have been described in this tissue. Specifically, although responses to low oxygen availability are species-specific, as well as affected by the acuteness, severity and duration of the hypoxia, commonly reported adaptations include the increase in the number of erythrocytes and hematocrit (Baldisserotto et al., 2008; Farrell and Richards, 2009; Hvas and Oppedal, 2019; Silkin and Silkina, 2005), increase in hemoglobin concentration and/or change in its affinity to oxygen (Campo et al., 2008; Pan et al., 2017; Wells, 2009) and the increase in cardiac output (Farrell and Richards, 2009). However, absence of hematological response, at least as indicated by the common indicators discussed above, has also been reported in fish (Araújo-Luna et al., 2018; Cadiz et al., 2018). In terms of stress physiology, as assessed by common biomarkers, elevations in cortisol, glucose, lactate and osmolality concentration, has been observed during acute hypoxia exposure, while chronic hypoxia did not to affect these indicators (Aboagye and Allen, 2018; Araújo-Luna et al., 2018; Hvas and Oppedal, 2019; Williams et al., 2019). On the other hand, exposure to stressors, such as physical exercise, under hypoxic conditions can lead to altered stress responses (Oldham et al., 2019). Circulating cortisol levels are considered to be

reliable and precise indicators of acute stress in fish (Fanouraki et al., 2011; Sadoul and Geffroy, 2019), but in all species studied thus far, including *Dicentrarchus labrax* and *Sparus aurata*, they cannot indicate chronic stress (Aerts et al., 2015; Carbajal et al., 2019a), unless an additional acute stressor is applied (Madaro et al., 2015; Samaras et al., 2021, 2018). Instead, accumulation of cortisol in fish scales has been shown to reliably indicate exposure to chronic stress in many teleost fish species (Aerts et al., 2015; Carbajal et al., 2019a; Hanke et al., 2020; Laberge et al., 2019; Samaras et al., 2021).

D. labrax and, *S. aurata* production represents the vast majority of fish aquaculture in the Mediterranean (FEAP 2020). Both species are considered tolerant to low oxygen, though the scientific guidelines for optimum performance and welfare have set a threshold of minimum 40% DO saturation (EFSA 2008). However, although these species tolerate low oxygen concentrations, reduction in performance such as growth and feeding (Pichavant et al., 2001; Naya-Catala et al., 2021) as well as alterations in physiology (Perez-Jimenez et al., 2012) and haematology (Berillis et al., 2016; Araujo-Luna et al. 2018) are observed.

All the above underline the necessity to better understand metabolic needs and physiological alterations occurring to these species when exposed to chronic hypoxia, especially in order to get a better insight in the robustness and welfare of aquaculture fish. In this context, the current study aimed to examine the effects of chronic exposure to different levels of DO in the water in body weight growth, metabolic rate, hematological and physiological status and stress response in *D. labrax*, and *S. aurata* and highlight the differences in their response to low DO. To do so, groups of fish from both species were reared under three different levels of DO, *i.e.* 40-60%, 60-80% and 80-100% oxygen saturation, at a temperature of 26.5°C and measurements of metabolic

rate, as well as blood and plasma hematological, hormonal and biochemical parameters under basal and post-stress conditions were performed.

Materials & Methods

Fish husbandry and dissolved oxygen

In total 315 fish from each species were used, randomly distributed in 9 tanks (35 fish/tank). The mean (± SD) initial weight for *D. labrax* was 88.00 (± 0.82) g and for *S. aurata* 79.23 (± 1.01) g. The rearing system consisted of three recirculating aquaculture systems (RAS) with fully controlled rearing conditions in three tanks of 500 L in each RAS. Three different DO saturation levels were applied to each RAS in triplicates, with the DO in each RAS being between 40-60%, 60-80% and 80-100%, hereinafter referred to as 40%, 60% and 80% groups, respectively. The rearing temperature was set at 26.5°C to stimulate summer conditions in the Mediterranean (Samaras et al., 2016; Androulidakis & Krestenitis 2022; Garcia-Monteiro et al., 2022) and salinity was 38. The targeted oxygen saturation levels were reached by the natural oxygen consumption of fish and controlled within the above-mentioned ranges through an automatic oxygen monitoring and provision control system (SENECT® AQUACULTURE / CONTROL). This system automatically supplied oxygen when DO was reaching the lowest limit and stopped providing oxygen once the desired levels were reached. This was achieved by real-time monitoring of the oxygen levels in each tank. The pH of the water and the concentrations of NH_4, NO_2 and NO_3 were monitored daily (Table 1). Fish were hand-fed to apparent satiation three times a day for 7 days a week during the trial period, while the experiments lasted for 81 days for *S. aurata* and 95 days for *D. labrax*. At the end of the rearing period fish were sampled for body

weight measurements, as well as blood and scale sampling. The experiment was conducted at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (HCMR) Heraklion, Greece.

Measuring metabolic rate

In the present study the guidelines for reporting methods to estimate metabolic rates (Killen et al., 2021) have been followed and are presented in detail in Supplementary Table 1. Metabolic rate was determined by measuring the oxygen consumption of fish using an intermittent-flow respiratory system, provided by the Fish Physiology Laboratory, Department of Biology, University of Crete. This system includes glass metabolic chambers, oxygen monitoring systems, circulating pumps and specialized software for the recording of oxygen concentration and calculation of metabolic rate (Loligo® Systems, DK-8800, Viborg, Denmark). Specifically, 2.1 L chambers were used, with a total water volume, including the tubing, of 3.586 L. Water for the metabolic rate system was obtained from the experimental tanks through a recirculation system in order to maintain the same oxygen saturation conditions as the experimental groups. Therefore, fish in the metabolic chambers shared the same "flush" water. Apart from this, the metabolic rate system included two circulating routes using water pumps; the first containing the oxygen sensor and being constantly circulating water, while the second was the flushing system working periodically in order to provide "new and clean" water to the chambers. The duration of the measurement cycles was selected in order to allow for reliable estimation of oxygen consumption but at the same time avoid reducing oxygen saturation substantially below the oxygen regimes that fish had been reared at (Svendsen et al., 2016). Therefore, each measuring cycle consisted of 450-500 s of flushing (corresponding to 10 - 12 times recycling of water), 30 s of waiting and

45 - 50 s of measuring. During the measuring phase, each chamber was a closed system, isolated from the flush tank, since the respective pumping circulation was shut-off, while only water from the chamber was circulating through the sensor.

The oxygen probes of the respirometry system were calibrated every 3 days using sodium sulphite solution (20 g L^{-1}) to obtain 0% saturation and air bubbling through an aerator to obtain 100% saturation levels using water in beakers with no water flow. Moreover, every day the performance of the probe was tested against a portable oxygen meter (Hach 40d). The water temperature was set at 26°C during the respirometry measurements. The microbial background respiration was calculated before and after each experimental day by performing three measurement circles of 1,000 sec each and since the activity was constant the average value was subtracted from the final value as described by Svendsen et al. (2016). The background respiration was below 5% of SMR.

Fish were fasted for 1.5 days before being tested for metabolic rate. Respiratory experiments started in the morning and lasted for approximately 22 hours. In the beginning three fish were immediately individually captured from the holding tank and were individually chased for 5-minutes with a net in order to induce exhaustion and estimate MMR. Subsequently their weight was measured without exposing them to air in pre-weighted buckets with water, and the fish were placed in the metabolic chamber to initiate the measurements (exposure to air for less than 5 seconds). Chambers were covered with an opaque plastic material throughout the duration of the measurements to avoid visual stress to fish, while additionally it did not allow visual contact between the fish being tested. In total three fish from one replicate tank were used in each measuring trial. Between trials, the water in the chambers was changed and flushed for 1 hour in order to provide a fresh environment for the next batch of fish. Between

oxygen treatments the measurements were stopped, the system was cleaned with fresh water and dried for 2 days before refilling with water from tanks of the respective oxygen regime.

Calculation of SMR, MMR and AS

In the analysis of metabolic rate (MR) only measurements having a slope higher than 0.9 were used since the rejection rate using this criterion (r^2 > 0.90) was very low (Chabot et al., 2021). SMR, the minimum metabolic rate needed to sustain life at resting, post-absorptive state, is challenging to calculate. In this study the methodology of Chabot et al. (2016) was used. Specifically, the mean of the lowest normal distribution (MLND) and the lowest 0.2 quantile ($q_{0.2}$) methods were calculated for each individual, but since the variation of the MLND curves ($C.V._{MLND}$%) for some individuals was higher than the suggested threshold of 5.4%, the $q_{0.2}$ value was used for all fish, as suggested by Chabot et al. (2016). SMR was calculated in R using the calcSMR script (Chabot et al., 2016). MMR is the highest metabolic rate that the animal can produce under the given environmental conditions. It was calculated as the oxygen consumption rate immediately after the fish was placed in the chamber, and no longer than 3 min after the end of the chasing protocol (Norin and Clark, 2016). AS defines the animal's range for aerobic activities and was calculated by subtracting SMR from MMR. SMR, MMR and AS are expressed as consumed mg of oxygen per fish biomass (kg) per time (h) (mg O_2 kg^{-1} h^{-1}).

Stress challenge

In order to study the acute stress response of fish reared under different DO saturation regimes a standardized acute stress protocol that has been previously evaluated in

various Mediterranean fish species (Fanouraki et al., 2011; Samaras et al., 2016) was applied. Initially, control, *i.e.* unstressed, fish were sampled before the application of any stressor and were euthanized (500 ppm phenoxyethanol, Merck, 80729; followed by decapitation after blood collection) and 1 ml of blood was withdrawn from the caudal vessel via heparinized syringes, to obtain resting levels of the examined bioindicators. Subsequently, fish to be acutely stressed were chased for 5 minutes and confined to 1/3 of the tank volume for 1 hour after which they were euthanized and sampled as described for the control fish. The time point of sampling fish was chosen since both species show the highest cortisol stress response at 1-hour post-stress (Fanouraki et al., 2011; Samaras et al., 2016). The stress protocol was applied to triplicate tanks per treatment (n = 4 fish per tank, N = 3 tanks per treatment).

After blood collection, the whole blood was used for hematocrit and hemoglobin analysis, the remaining blood was centrifuged at 2,000 g for 10 min at 4°C and the supernatant plasma was collected and stored at -20°C until the analysis, which was performed within one month. Scales from the caudal area of fish were also collected from control fish only, since cortisol is scales is not affected by acute stress (Laberge et al., 2019) and stored at -20°C in order to quantify cortisol concentration.

Analytical procedures

Hematocrit was measured in capillary tubes after centrifugation in a hematocrit microcentrifuge, and hemoglobin was determined using a commercial kit (Spinreact, Girona, Spain). Mean corpuscular hemoglobin concentration (MCHC) was calculated according to the formula MCHC = 100*hemoglobin/hematocrit. Plasma glucose, cholesterol, triglycerides, total proteins and lactate concentrations were measured by commercial enzymatic colorimetric kits (Biosis, Greece, for all analyses except lactate;

Spinreact, Spain) following manufacturer's instructions, whereas plasma osmolality was determined using an osmometer (Osmomat 030, Gonotec GmbH, Germany). These plasma parameters are commonly used indicators of stress and welfare (Fanouraki et al., 2011; Samaras et al., 2016), as well as of the physiological and nutritional status of the fish (Peres et al., 2014).

Total cortisol concentration in plasma and fish scales was analyzed using commercially available ELISA assays (DRG®, International Inc, Germany for *D. labrax*; Neogen Corporation, UK for *S. aurata*) which have been previously validated for use in fish plasma and scales (Samaras et al., 2021, 2017). These two assays quantify cortisol levels at different concentration ranges, specifically between 2.5 and 800 ng ml^{-1} (DRG) and between 0.04 and 10 ng ml^{-1} (Neogen), and therefore are suitable for use with *D. labrax* and *S. aurata* plasma samples, respectively, according to the normal range of cortisol concentration of these species.

For the extraction of cortisol from fish scales the protocol developed and validated by Carbajal et al., (2018) was used. In short, 250 – 300 mg of scales were washed three times with 3 ml of isopropanol in order to get rid of mucus, and possible traces of blood. During the washing procedure scales were vortexed for 2.5 minutes, after which isopropanol was discarded, and the next washing followed. Three washing steps were performed in total, after which scales were air-dried for 24 hours. After that, the scales were minced in a bead mill disruptor (Tissue Lyser II; Qiagen) for 2 min at 30 Hz using 5 mm beads. Then, 40 - 60 mg of the powdered scales were mixed with 1.5 ml of methanol and incubated for 16 hours with continuous mixing. Afterwards, samples were centrifuged at 9,500 g and 1 ml of the supernatant extract was evaporated. Finally, samples were reconstituted using 0.2 ml of Neogen's reconstitution buffer and measured using this assay due to the lower than plasma cortisol levels in the scales of

both species, which were within the measuring range of the current assay. The capacity of the current assay to reliably quantify scales cortisol has been previously evaluated (Carbajal et al., 2018; Samaras et al., 2021).

Statistical analysis

All statistical analysis were performed on SPSS v22.0 (IBM Statistics for Windows; IBM Corp., Armonk, NY, USA), while the figures were created using GraphPad Prism 6.0 (Graph-Pad Software Inc., La Jolla, CA, USA). Results are presented as means ± standard deviation (SD). Data were checked for normality and equality of variances using the Kolmogorov-Smirnoff and Brown-Forsythe tests, respectively. Differences in the SMR, MMR and AS between oxygen treatments were tested using nested ANOVA, with the factor *tank* being nested within the factor *oxygen*. Differences in physiological data were tested using two-way ANOVA with the factors *oxygen* and *stress*. Scale cortisol was examined only in control fish, and therefore tested with one-way nested ANOVA. Correlations in cortisol concentrations between plasma and scales were performed using Pearson's correlation coefficient.

Principal Component Analysis (PCA) in the physiological data was performed using Primer 6 software (Clarke and Gorley, 2006). Data of hematocrit, hemoglobin, cortisol, glucose, lactate, osmolality, cholesterol, triglycerides, and total protein levels were used and were normalized prior to analysis. PCA graphs were created using the "factoextra" package in R studio. Permutational Multivariate Analysis of Variance (PERMANOVA) with *oxygen* and *stress* as fixed factors after calculation of a resemblance matrix of the data using Euclidean distance was performed in order to check for statistically significant differences between groups. Permutation of residuals under a reduced model

and 9,999 permutations was applied, while Monte Carlo p-values were also considered for the significance of differences among factors.

Ethical note

The laboratories of the Hellenic Centre for Marine Research are licensed for breeding and husbandry of animals for scientific purposes (EL 91-BIO-03, EL 91-BIO-04). All procedures involving the handling and treatment of fish were approved by the HCMR Institutional Animal care and use committee in accordance to Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use of experimental animals following the principles of refinement, replacement and reduction in animal experimentation under where approved by the Institutional committee (EL91-BIOexp-04). All experimental procedures were performed by FELASA accredited researchers.

Results

Growth and metabolic rate

In both species the initial weight between treatment groups was similar, however the final weight differed. Specifically, in *D. labrax* the final weight and the SGR (%) was higher at 80% than 60% and 40%, and also higher at 60% than 40% ($F_{2,8} = 27.118$; $p < 0.001$ for weight; $F_{2,8} = 37.891$; $p < 0.001$ for SGR) (Table 1). On the other hand, in *S. aurata* the final weight at the SGR (%) was higher at 60% and 80% than 40% ($F_{2,8} = 243.61$; $p < 0.001$ for weight; $F_{2,8} = 218.54$; $p < 0.001$ for SGR) (Table 1).

Statistical analysis of the SMR data showed that there were no significant differences, among experimental groups, in both *D. labrax* ($F_{2,27} = 0.415$; p = 0.667) and *S. aurata* ($F_{2,27} = 1.923$; p = 0.175) (Figure 1). SMR ranged between 160 (±33) mg O2 kg^{-1} h^{-1} in 60% and 175 (±37) mg O2 kg^{-1} h^{-1} in 40% in *D. labrax*, and between 166 (±38) mg O2

338 kg^{-1} h^{-1} in 60% and 204 (±32) mg O2 kg^{-1} h^{-1} in 40% in *S. aurata*. Species-specific
339 responses were observed in MMR, since in *D. labrax* MMR was significantly affected
340 by water oxygen saturation ($F_{2,27}$ = 14.240; p < 0.001), being higher at 60% and 80%
341 compared to 40% group, while this was not the case in *S. aurata*, where no statistically
342 significant differences were observed ($F_{2,27}$ = 0.940; p = 0.409) (Figure 1). Specifically,
343 MMR ranged between 350 (±75) mg O2 kg^{-1} h^{-1} in 40% and 500 (±73) mg O2 kg^{-1} h^{-1}
344 in 80% in *D. labrax*, and between 394 (±45) mg O2 kg^{-1} h^{-1} in 40% and 445 (±111) mg
345 O2 kg^{-1} h^{-1} in 80% in *S. aurata*.
346 Finally, the aerobic scope (AS) showed to be affected by rearing oxygen saturation in
347 both species (Figure 1). Specifically, in *D. labrax* higher AS was recorded in fish of the
348 60% (317 (±58) mg O2 kg^{-1} h^{-1}) and 80% (337 (±40) mg O2 kg^{-1} h^{-1}) compared to 40%
349 (174 (±50) mg O2 kg^{-1} h^{-1}) group ($F_{2,27}$ = 34.064; p < 0.001). In *S. aurata* AS was also
350 affected by the treatment ($F_{2,27}$ = 5.414; p = 0.014), while post-hoc analysis revealed
351 statistically significant differences only between 40% (190 (± 30) mg O2 kg^{-1} h^{-1}) and
352 80% (266 (±70) mg O2 kg^{-1} h^{-1}) groups (p = 0.012).
353
354 *Hematological parameters*
355 In *D. labrax* mean hematocrit was significantly affected by both factors, *i.e. oxygen*
356 ($F_{2,66}$ = 8.207; p < 0.001) and *stress* ($F_{1,66}$ = 11.89; p = 0.001) (Fig. 2a). Post-hoc
357 analysis revealed that hematocrit was higher in fish reared at 40% (HCT% = 37 (±5) in
358 control and 34 (±5) in stressed fish) compared to 60% (HCT% = 32 (±3) in control and
359 31 (±3) in stressed fish) (p < 0.001) and 80% (HCT% = 35 (±4) and 30 (±3) in stressed
360 fish) (p = 0.011) at both resting and stress levels. Moreover, stressed fish showed lower
361 hematocrit compared to unstressed fish in all oxygen regimes. In hemoglobin
362 concentration a significant interaction between factors was observed ($F_{2,66}$ = 8.482; p <

0.001) (Fig. 2b). Post-hoc analysis showed that the resting hemoglobin mean concentrations of fish at 80% (Hgb = 8.3 (±1.0) g dl^{-1}) were significantly higher than 40% (Hgb = 6.9 (±0.8) g dl^{-1}) ($p = 0.004$) and 60% (Hgb = 7.0 (±0.7) g dl^{-1}) ($p = 0.008$), while a significant decrease in hemoglobin was observed after stress in the 80% group alone (Hgb = 6.7 (±1.0) g dl^{-1}) ($p < 0.001$). MCHC was significantly affected by *oxygen* ($F_{2,66} = 16.36$; $p < 0.001$), being lower at 40% (MCHC = 19 (±3) g dl^{-1} in control and 20 (±2) g dl^{-1} in stressed fish) compared to the two other groups (MCHC = 22 (±2) g dl^{-1} in and 24 (±3) g dl^{-1} in 60% control and stressed fish, respectively and 24 (±3) g dl^{-1} and 23 (±2) g dl^{-1} in 80% control and stressed fish, respectively) (Fig. 2c).

In *S. aurata* it was shown that hematocrit was significantly affected by the interaction of factors *oxygen* and *stress* ($F_{2,65} = 3.165$; $p = 0.049$) (Fig. 2d). Specifically, differences were observed between stressed fish, being significantly higher in fish reared at 60% (HCT% = 28 (±2)) compared to the other two groups (HCT% = 34 (±4) and 33 (±5) in 40% and 80% groups, respectively). Hemoglobin concentration was also significantly affected by the interaction of factors *oxygen* and *stress* ($F_{2,65} = 7.841$; $p < 0.001$) (Fig. 2e). Post-hoc analysis showed that levels of hemoglobin in stressed fish were significantly higher in fish reared at 80% (Hgb = 9.5 (±2.1) g dl^{-1}) compared to 60% (Hgb = 6.9 (±1.3) g dl^{-1}) oxygen saturation ($p = 0.004$), and that stress affected fish at 60% leading to lower levels of hemoglobin compared to resting levels (Hgb = 9.1 (±1.4) g dl^{-1}) ($p = 0.023$). Finally, MCHC was also affected by the interaction of factors ($F_{2,65} = 6.144$; $p < 0.001$), resulting in a significant difference between stressed fish at 40% (MCHC = 23 (±2) g dl^{-1}) and 80% (MCHC = 29 (±6) g dl^{-1}) groups (Fig. 2f).

388 *Cortisol analysis*

389 Analysis of plasma cortisol in both species showed a significant effect of the factor
390 *stress* (*D. labrax*: $F_{2,66} = 143.2$; $p < 0.001$; Fig 3a; *S. aurata*: $F_{1,65} = 4.607$; $p = 0.034$;
391 Fig. 3c), while no differences between oxygen groups were observed. Specifically, it
392 was shown that cortisol levels in stressed fish were higher than the basal, unstressed
393 ones. Cortisol ranged between 184.1 (±169.4) and 248.5 (±152.4) ng ml^{-1} in control and
394 545.5 (±96.0) and 605.4 (±89.3) ng ml^{-1} in stressed *D. labrax* groups and between 8.8
395 (±6.5) and 12.9 (±6.3) ng ml^{-1} in control and 15.4 (±16.5) and 19.1 (±16.6) ng ml^{-1} in
396 stressed *S.aurata* groups. Cortisol concentration in fish scales showed no differences
397 between oxygen saturation treatments (*D. labrax*: $F_{2,22} = 1.036$; $p = 0.372$; Fig 3b; *S.*
398 *aurata*: $F_{2,24} = 2.398$; $p = 0.112$; Fig 3d). Cortisol in scales ranged between 2.7 (±2.4)
399 and 4.3 (±2.8) pg mg^{1} in *D. labrax* groups and between 0.9 (±0.6) and 1.5 (±0.7) pg
400 mg^{-1} in *S.aurata* groups. Correlation analysis between plasma and scale cortisol
401 resulted in positive correlations in both species, reaching levels of statistical
402 significance at 40% and 60% groups in *D. labrax* ($r = 0.74$; $p = 0.037$ and $r = 0.81$; $p =$
403 0.008, respectively) and 60% and 80% groups in *S. aurata* ($r = 0.78$; $p = 0.017$ and $r =$
404 0.70; $p = 0.043$, respectively).

405

406 *Biochemical analysis*

407 In *D. labrax* significant interactions between the factors *treatment* and *stress* were
408 observed in the commonly used acute stress indicators, glucose ($F_{2,66} = 8.419$; $p <$
409 0.001), lactate ($F_{2,66} = 3.663$; $p = 0.031$) and osmolality ($F_{2,66} = 3.682$; $p = 0.031$) (Table
410 2). In all three parameters no differences between treatment were observed in unstressed
411 fish, but stressed fish of 40% group had significantly higher levels of glucose ($p <$
412 0.001) and lactate ($p = 0.039$) than 80% and of osmolality than both 60% ($p < 0.001$)

and 80% (p = 0.017) treatments. On the other hand, the metabolic indicators cholesterol and triglycerides were only affected by the oxygen saturation regime ($F_{2,66}$ = 10.15; p < 0.001 for cholesterol; $F_{2,66}$ = 8.763; p < 0.001 for triglycerides), being in both cases higher at 80% (Table 2). Finally, total proteins concentration was affected by the interaction of factors ($F_{2,66}$ = 4.798; p = 0.011), being higher at 80% compared to other groups in unstressed fish, while a significant decrease after stress was observed in the same treatment group (p < 0.001) (Table 2).

In *S. aurata* circulating concentration of glucose was only significantly affected by the factor *stress* ($F_{1,65}$ = 62.13; p < 0.001), being higher after stress in all treatments (Table 3). Lactate and osmolality concentrations, on the other hand, were significantly affected by the interaction of factors ($F_{2,65}$ = 7.938; p < 0.001 for lactate; $F_{2,65}$ = 9.504; p < 0.001 for osmolality). Post-hoc analysis revealed that although no differences between oxygen treatments existed in the resting levels of lactate and osmolality, after stress fish reared at 40% showed significantly higher concentration than those at 60% and 80% DO saturation (Table 3). In lactate a significant increase was observed after stress only at 40%, while in osmolality in all oxygen treatments. Cholesterol, triglycerides and total proteins levels were significantly affected only by stress, being in all parameters lower after exposure to stress ($F_{1,65}$ = 17.15; p < 0.001 for cholesterol; $F_{1,65}$ = 14.86; p < 0.001 for triglycerides: $F_{1,65}$ = 6.533; p = 0.033 for total proteins) (Table 3).

Principal components analysis on blood and plasma hematological, hormonal and biochemical data

In *D. labrax* PCA analysis revealed that the first two principal components (PC) explained 78.7% of the total variation of the data (58.1% and 24.4% the 1st and 2nd component, respectively). PC1 was mainly influenced by lactate, with an eigenvector

438 of 0.560, while PC2 was mostly affected by triglycerides with an eigenvector of -0.573.
439 PERMANOVA showed that there was a statistically significant interaction between
440 factors *treatment* and *stress* (Pseudo-$F_{2,66}$ = 3.877; p = 0.001) (Fig. 4a). Post-hoc pair-
441 wise tests revealed that in the absence of acute stress all oxygen treatment groups
442 differed between them. In the presence of acute stress, however, only fish from 40%
443 saturation regime were different from those of 60% (p = 0.044) and 80% (p = 0.009).
444 On the other hand, within each oxygen saturation group control fish were significantly
445 different from stressed fish ($p < 0.001$ in all comparisons) (Fig. 4a).
446 In *S. aurata* PCA analysis revealed that the first two principal components (PC)
447 explained 70.1% of the total variation of the data (55.6% and 14.5% the 1st and 2nd
448 component, respectively). PC1 was mainly influenced by osmolality, with an
449 eigenvector of 0.653, while PC2 was mostly affected by glucose with an eigenvector of
450 -0.739. PERMANOVA showed that there was a significant interaction between factors
451 *treatment* and *stress* (Pseudo-$F_{2,65}$ = 4.556; $p < 0.001$) (Fig. 4b). Post-hoc pair-wise tests
452 revealed that in the absence of acute stress no differences were presented between
453 oxygen treatment groups. In the presence of acute stress, however, the different groups
454 were significantly different between them. On the other hand, within each oxygen
455 saturation group control fish were significantly different from stressed fish ($p < 0.001$
456 in all comparisons) (Fig. 4b).
457

458 **Discussion**

459 The current study provides evidence regarding the effect of dissolved oxygen in growth,
460 metabolic rate and aerobic scope, as well as the stress response of *D. labrax* and *S.*
461 *aurata*. DO saturation levels had a significant effect on specific growth rate, being in
462 both species lower at the lowest oxygen saturation. The effect of low DO was, however,

larger in *D. labrax* compared to *S. aurata* as evidenced by the lower growth at 60% vs 80% in the former but not the later species. In *S. aurata*, specifically, growth was only affected at the lowest examined DO range. In general, these results are in accordance with previous studies on both species (Martos-Sitcha et al., 2019; Pichavant et al., 2001; Thetmeyer et al., 1999). In a study in *S. aurata* , however, no differences in final weight were observed between fish reared for 6 weeks at 40-60%, 60-80% or 80-100% DO, but still there was a tendency for higher SGR (%) at the two higher DO regimes compared to the low DO treatment (Araújo-Luna et al., 2018). The differences in growth performance between the study by Araújo-Luna et al., (2018) and the present study could possibly be explained by the shorter period in the former study compared to the present study (6 weeks and 11.5 weeks, respectively), as well as the fact that in that study larger fish, that in general show lower SGR (%) compared to smaller fish, were used compared to the present study (initial weight of 316.3g and 79.3 g, respectively).

In line with most published literature, DO saturation levels had no effect on standard metabolic rate (SMR) of fish (Bergstedt et al., 2021; Brauner and Richards, 2020; Garduño Paz et al., 2020; Hvas and Oppedal, 2019; Pang et al., 2021). It should, however, be noted that most of the above-mentioned studies on SMR have exposed fish to acute hypoxia, and not chronic as was applied in the current study. In general, SMR is unaffected by DO due to the fact that various fish species display adaptations that allow fish to maintain oxygen update in a range DO concentrations (Farrell and Richards, 2009). However, there is a critical or limiting oxygen concentration below which basal metabolic needs cannot be covered and SMR starts to reduce (Claireaux and Chabot, 2016; Claireaux and Lagardère, 1999; Neill et al., 1994). In the current study the examined DO regimes were above the limiting or critical oxygen

concentration, which in *D. labrax* has been identified as 20-25% at 25°C (Zhang et al., 2021).

On the other hand, maximum metabolic rate (MMR) and aerobic scope are in general dependent on DO, since reduced DO lowers the capacity for aerobic metabolism (Milinkovitch et al., 2020; Claireaux and Chabot, 2016; Claireaux and Lagardère, 1999; Neill et al., 1994). In the present study aerobic scope was reduced at 40% compared to 80% in both species, while MMR was affected only in *D. labrax*. This larger effect of low DO in MMR in *D. labrax* compared to *S. aurata* could possibly indicate a higher susceptibility to low oxygen when intense activity and therefore maximum aerobic metabolism is concerned. Moreover, the reduced aerobic capacity at the lowest DO regime examined can affect the overall performance of fish, as well as their ability to respond to energetically demanding situations, such as the application of an acute stressor. Indeed, it was shown that based on their overall post-stress levels of physiological and metabolic biomarkers, as seen in the PCA analysis, fish reared at 40% were separated from the rest of the treatments, depicting that their response was different from the other two treatments. Interestingly, in both species lactate concentration after exposure to stress was higher at 40% than the other two oxygen regimes. This result, together with the reduced aerobic scope at this DO regime underlines the increased function of anaerobic metabolism for the production of additional energy due to the lack of oxygen. Similarly, in Atlantic salmon, *Salmo salar*, increased lactate concentrations after exercise were observed under hypoxic compared to normoxic conditions (Oldham et al., 2019).

Examining each physiological parameter separately, the hematological adaptations due to reduced DO in *D. labrax* included an increase in hematocrit, accompanied by a reduction in hemoglobin and MCHC. On the other hand, no clear effect of DO

saturation in hematological parameters in *S. aurata* was observed. This result is in line with the results of another recently published study in *S. aurata* exposed to similar DO saturation levels in terms of hemoglobin and MCHC, but not in terms of hematocrit, which was higher at 80% compared to the other groups (Araújo-Luna et al., 2018). However, it should be noted that crucial differences in the experimental design between the two studies, such as the use of significantly larger animals and the significantly shorter duration in the study of Araújo-Luna et al., (2018) compared to the present study, might have influenced the results.

The absence of effects of DO levels on hematocrit, hemoglobin and MCHC in *S. aurata* compared to *D. labrax* underlines the species-specificity of these responses, which has been reported in other species as well (Baldisserotto et al., 2008; Hvas and Oppedal, 2019), and could be an indication of larger hematological effects on *D. labrax* than *S. aurata*. However, it is common to observe increased concentrations (Baldisserotto et al., 2008; Farrell and Richards, 2009; Hvas and Oppedal, 2019; Silkin and Silkina, 2005), as an adaptation to increase the blood's capacity for oxygen transportation, as was observed in *D. labrax*. On the contrary, the reduced MCHC underlines the fact that an increase in hematocrit does not necessarily mean increased transportation capacity by the blood. MCHC has also been shown to have a species-specific response to low DO (Baldisserotto et al., 2008; Hvas and Oppedal, 2019). It should, however, be mentioned that in the above-mentioned studies the exposure to low oxygen was rapid and short-termed and not chronic as was in the current study.

Moreover, although acute stress has commonly been associated with increases in hematocrit due to the action of catecholamines and contraction of spleen (Wendelaar Bonga, 1997), it seems that this is also a species-specific feature that can also be affected by environmental parameters (Samaras et al., 2016). In *D. labrax*, for instance,

in accordance with the present study, it has been previously shown that when reared at high temperatures (25-26°C), hematocrit is reduced after exposure to acute stress (Samaras et al., 2016). This phenomenon was observed in all groups, regardless of the DO regime.

Regarding cortisol, although it is well known that rapid exposure to hypoxia results in elevated cortisol levels in fish (De Mercado et al., 2018; Morales-Lange et al., 2022), including *D. labrax* (Ferrari et al., 2015), it is not equally well study what the effects of prolonged exposure to low DO are. Data from the current experiment provide evidence that in both species under study DO saturation of 40% did not result in a long-term activation of the cortisol producing stress axis in both species. Specifically, cortisol concentration in plasma was unaffected by DO levels in either basal or post-stress conditions in both species, while post-stress levels of plasma cortisol were significantly elevated compared to basal, however, the increase was not DO saturation-depended. Similar results for basal levels have been obtained in a previous study in *S. aurata* (Araujo-Luna et al. 2018), whereas in *Oncorhynchus kisutch* oxygen saturation of 50% and 60% had no effect on circulating cortisol levels, while in water with oxygen saturation of 25% and 35% cortisol was significantly increased compared to 100% saturation (Martínez et al., 2020). Regarding cortisol in fish scales, no differences were observed between fish reared in different DO regimes. The effects of low DO in cortisol in fish scales are not well-studied, since, to the best of our knowledge, this is the first study to examine it. As far as the accumulation of cortisol in scales due to prolonged activation of the cortisol response axis is concerned, it cannot be ruled out that the lowest DO examined was not severe enough to trigger a prolonged cortisol response, as reported for instance in *O. kisutch* where circulating cortisol was elevated only in fish reared in lower DO, *i.e.* 25% and 35% (Martínez et al., 2020) or that the cortisol

producing axis could have been adapted to the environmental conditions. This, however, should not exclude the possibility DO of 40% acted as an environmental stressor to fish as evidenced by other indicators of stress such as the reduced body weight and lower anaerobic metabolic capacity observed under these conditions.

Moreover, in both species a positive correlation between plasma and scales cortisol was observed, reaching levels of significance at fish of the 40% and 60% group in *D. labrax* and 60% and 80% group in *S. aurata*. Such positive correlations are common in literature (Carbajal et al., 2019a; b; Samaras et al., 2021; 2022), underlying the fact that a major route for cortisol accumulation in the scales is through circulation.

In both species, no differences in mean concentrations of glucose, lactate and osmolality were observed in unstressed fish. Similar results have been reported in *D. labrax* (Pichavant et al., 2001) for all three parameters and *S. aurata* for glucose (Araujo-Luna et al., 2018, Naya-Catala et al., 2021) but not lactate (Martos-Sitcha et al., 2019; Naya-Catala et al., 2021) which was lower at fish exposed to hypoxia. In contrast, post-stress lactate and osmolality concentrations were affected by DO, being higher in the group reared at 40% compared to 80% in both species, while the same was true for glucose in *D. labrax*. The increased response of lactate, an anaerobic metabolism indicator, with the associated reduced MMR in *D. labrax* and AS, in both species at 40%, indicate the reduced aerobic capacity of fish reared at low available oxygen and shift to anaerobic metabolism in order to meet the increased energetic demands set by exposure to chasing acute stress (Oldham et al., 2019; Milinkovitch et al., 2020).

The same pattern was observed in osmolality, where fish reared at 40% DO showed a higher response than those reared at 80%. One of the changes in fish physiology due to exposure to low oxygen, is to increase the permeability of the gills to facilitate oxygen

uptake (Anttila et al., 2015; Araújo-Luna et al., 2018; Farrell and Richards, 2009; Sollid et al., 2003). However, this increased permeability is accompanied by a reduced efficiency of the gills to control for ion transportation, a phenomenon called "osmorespiratory compromise" (Giacomin et al., 2019; Onukwufor and Wood, 2018; Saroglia et al., 2009; Wood et al., 2019). Therefore, a possible explanation for the observed differences in plasma osmolality is that changes in gills' structure and function might lead to a dysfunction of the ionic regulatory system, especially during periods of enhanced energetic demands, such as chasing acute stress. However, currently no direct evidence of fish gill condition and function are available to support or reject this hypothesis.

Finally, total proteins, cholesterol and triglycerides concentrations were higher at 80% compared to 40% in *D. labrax*, while no differences were observed in *S. aurata*. The increased concentrations of metabolic plasma indicators in *D. labrax* at higher DO could possibly be explained by the better growth and nutritional status of fish in this DO regime, since these parameters are biomarkers of the nutritional status of *D. labrax* (Chatzifotis et al., 2011; Peres et al., 2014). Specifically, the increased feeding rate and higher capacity for aerobic metabolism in energetically demanding periods could have resulted in increased energetic reserves, especially of lipids (Eroldogan et al., 2004; Peres et al., 2014). Moreover, lipid metabolism has been shown to increase in order to facilitate the aerobic energy metabolism during prolonged low oxygen availability (Naya-Catala et al., 2021).

Comparison between the two species can suggest that under non-acute stress conditions there were only few differences in their responses to low oxygen. In general, *D. labrax* was affected by low DO in more of the tested parameters than *S. aurata*, which could possibly suggest a slightly higher susceptibility in this species compared to *S. aurata.*

For instance, growth in 60% compared to 80%, as well as MMR and AS in 40% compared to both the two other DO regimes were negatively affected in *D. labrax* but not *S. aurata*. Moreover, hematological, including hematocrit, hemoglobin and MCHC and biochemical, specifically, cholesterol, triglycerides, and total protein, data were negatively affected by low oxygen in *D. labrax* only. On the other hand, both species showed a similar post-acute stress response, with enhanced lactate and osmolality response at the lowest DO compared to the other two DO regimes. Interestingly, *D. labrax* but not *S. aurata* showed higher amounts of circulating post-stress glucose at 40% compared to the other groups, while, *S. aurata* when exposed to stress utilized non-carbohydrate resources such as cholesterol, triglycerides and total proteins, yet in the same way between different DO groups.

Conclusions

Taken together the current results revealed the effects of water DO saturation on body weight growth, metabolic rate as well as blood and plasma hematological, hormonal and biochemical characteristics of *D. labrax* and *S. aurata*. Few differences were observed under basal conditions between fish reared under different oxygen regimes, showing therefore the ability of both species to adapt to low DO concentrations under such conditions. However, when faced with the energy demanding chasing acute stress reduced aerobic scope and increased lactate levels in fish plasma were observed, which underlie the enhanced function of anaerobic metabolism to cover excess energy demands under low DO regimes.

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642

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948 **Table 1.** Mean (± SD) levels of pH, NH_4, NO_2 and NO_3 in the tank of *Dicentrarchus*
949 *labrax* and *Sparus aurata* reared at 40%, 60% and 80% dissolved oxygen saturation for
950 95 and 81 days, respectively.

	Dicentrarchus labrax			*Sparus aurata*		
	80%	60%	40%	80%	60%	40%
pH	7.62 ± 0.10	7.65 ± 0.12	7.60 ± 0.10	7.64 ± 0.13	7.70 ± 0.17	7.60 ± 0.09
NH_4 (mg l^{-1})	0.20 ± 0.10	0.20 ± 0.11	0.22 ± 0.13	0.19 ± 0.13	0.21 ± 0.1	0.23 ± 0.12
NO_2 (mg l^{-1})	0.36 ± 0.13	0.37 ± 0.11	0.39 ± 0.12	0.37 ± 0.09	0.36 ± 0.11	0.38 ± 0.12
NO_3 (mg l^{-1})	30 ± 13	32 ± 9	34 ± 8	37 ± 10	35 ± 8	36 ± 10

951

952 **Table 2.** Growth performance of *Dicentrarchus labrax* and *Sparus aurata* reared at
953 40%, 60% and 80% dissolved oxygen saturation for 95 and 81 days, respectively.
954 Results are presented as mean ± SD. Different letters indicate significantly different
955 means between oxygen groups in the same species. The level of significance was set at
956 $p < 0.05$.

	Dicentrarchus labrax			*Sparus aurata*		
	80%	60%	40%	80%	60%	40%
Initial weight (g)	88.0±0.4	88.3±1.1	88.2±0.8	80.0±0.6	79.7±0.5	78.1±0.6
Final weight (g)	252.4±4.5^{a}	230.7±7.3^{b}	197.2±0.8^{c}	249.3±2.5^{a}	244.4±4.5^{a}	195.4±2.5^{b}
SGR (%)	1.11±0.02^{a}	1.01±0.05^{b}	0.85±0.03^{c}	1.44±0.01^{a}	1.42±0.01^{a}	1.16±0.01^{b}

957

Table 3. Concentrations of the biochemical indicators glucose, lactate, osmolality, cholesterol, triglycerides and total proteins in control (unstressed) and stressed *Dicentrarchus labrax* plasma reared for 95 days at 40%, 60% and 80% dissolved oxygen saturation groups (n = 12 individuals per treatment, N = 3 tanks per treatment). Results are presented as mean ± SD. Significant differences between oxygen groups in the same stress condition (control or stressed) are indicated by different letters. Significant differences between control and stressed groups in the same oxygen treatment (80%, 60% or 40%) are indicated by asterisks. The level of significance was set at $p < 0.05$.

Parameter	Stress	Oxygen		
		80%	60%	40%
Glucose	Control	3.3 ± 0.6	3.1 ± 0.4	3.5 ± 0.5
(mmol l^{-1})	Stressed	$7.3 \pm 1.4^{a,*}$	$8.7 \pm 1.1^{b,*}$	$9.5 \pm 1.1^{b,*}$
Lactate	Control	4.0 ± 0.5	3.6 ± 0.8	3.2 ± 0.5
(mmol l^{-1})	Stressed	$16.1 \pm 3.6^{a,*}$	$17.4 \pm 3.6^{ab,*}$	$19.2 \pm 3.3^{b,*}$
Osmolality	Control	344 ± 6	341 ± 9	343 ± 10
(mOsmol kg^{-1})	Stressed	$393 \pm 18^{a,*}$	$393 \pm 12^{a,*}$	$409 \pm 15^{b,*}$
Cholesterol	Control	3.8 ± 0.9^{a}	2.8 ± 0.5^{b}	2.8 ± 0.7^{b}
(mmol l^{-1})	Stressed	$3.2 \pm 0.8^{a,*}$	2.9 ± 0.6^{b}	2.7 ± 0.5^{b}
Triglycerides	Control	6.3 ± 1.7^{a}	3.2 ± 1.5^{b}	4.7 ± 1.8^{ab}
(mmol l^{-1})	Stressed	5.2 ± 1.7^{a}	4.1 ± 2.0^{b}	4.9 ± 1.8^{ab}
Total proteins	Control	5.4 ± 0.7^{a}	4.5 ± 0.6^{b}	4.6 ± 0.5^{b}
(g dl^{-1})	Stressed	$4.4 \pm 0.6^{*}$	4.2 ± 0.4	4.5 ± 0.4

968 **Table 4.** Concentrations of the biochemical indicators glucose, lactate, osmolality,
969 cholesterol, triglycerides and total proteins in control (unstressed) and stressed *Sparus*
970 *aurata* plasma reared for 81 days at 40%, 60% and 80% dissolved oxygen concentration
971 groups (n = 12 individuals per treatment, N = 3 tanks per treatment). Results are
972 presented as mean ± SD. Significant differences between oxygen groups in the same
973 stress condition (control or stressed) are indicated by different letters. Significant
974 differences between control and stressed groups in the same oxygen treatment (80%,
975 60% or 40%) are indicated by asterisks. The level of significance was set at $p < 0.05$.

Parameter	**Stress**	**Oxygen**		
		80%	**60%**	**40%**
Glucose	Control	3.9 ± 0.7	3.8 ± 0.7	3.4 ± 0.5
(mmol l^{-1})	Stressed	6.6 ± 1.9*	5.7 ± 2.1*	6.8 ± 1.7*
Lactate	Control	1.2 ± 0.4	1.4 ± 0.6	2.0 ± 1.2
(mmol l^{-1})	Stressed	1.9 ± 1.2^{a}	1.6 ± 1.1^{a}	6.7 ± 4.9^{b,*}
Osmolality	Control	352 ± 7	346 ± 8	352 ± 7
(mOsmol kg^{-1})	Stressed	367 ± 14^{a,*}	360 ± 5^{a,*}	391 ± 20^{b,*}
Cholesterol	Control	7.8 ± 1.3	7.8 ± 2.1	7.9 ± 1.0
(mmol l^{-1})	Stressed	6.7 ± 1.1*	6.1 ± 1.1*	6.5 ± 1.4*
Triglycerides	Control	2.3 ± 1.1	2. 5 ± 0.8	2.1 ± 0.9
(mmol l^{-1})	Stressed	1.8 ± 0.7*	1.3 ± 0.7*	1.6 ± 0.6*
Total proteins	Control	4.5 ± 0.4	4.7 ± 0.6	4.7 ± 0.8
(g dl^{-1})	Stressed	4.4 ± 0.3*	4.2 ± 0.5*	4.3 ± 0.6*

976

977 **Legends to figures**

978

979 **Figure 1**. Standard and maximum metabolic rate and aerobic scope in *Dicentrarchus*
980 *labrax* (a, b, c) and *Sparus aurata* (d, e, f) reared at 80%, 60% and 40% dissolved
981 oxygen saturation groups (n = 9 individuals per treatment, N = 3 tanks per treatment).
982 The y-axis in figures (b) and (e) starts at the value of 200. Results are presented as mean
983 ± SD. In each type of metabolic rate different letters indicate significantly different
984 means between oxygen groups as tested by a nested ANOVA at a significance level of
985 $p < 0.05$.

986

987 **Figure 2.** Hematocrit (HCT %), hemoglobin (Hgb) and mean corpuscular hemoglobin
988 concentration (MCHC) levels of control (unstressed, black bars) and stressed (grey
989 bars) *Dicentrarchus labrax* (a, b, c) and *Sparus aurata* (d, e, f). reared at 80%, 60% and
990 40% dissolved oxygen saturation groups (n = 12 individuals per treatment, N = 3 tanks
991 per treatment). Results are presented as mean + SD. Different letters indicate
992 significantly different means between oxygen groups in the same stress conditions,
993 while asterisks between control and stressed fish within the same oxygen treatment.
994 Statistical differences were tested by two-way ANOVA with the level of significance
995 being set at $p < 0.05$.

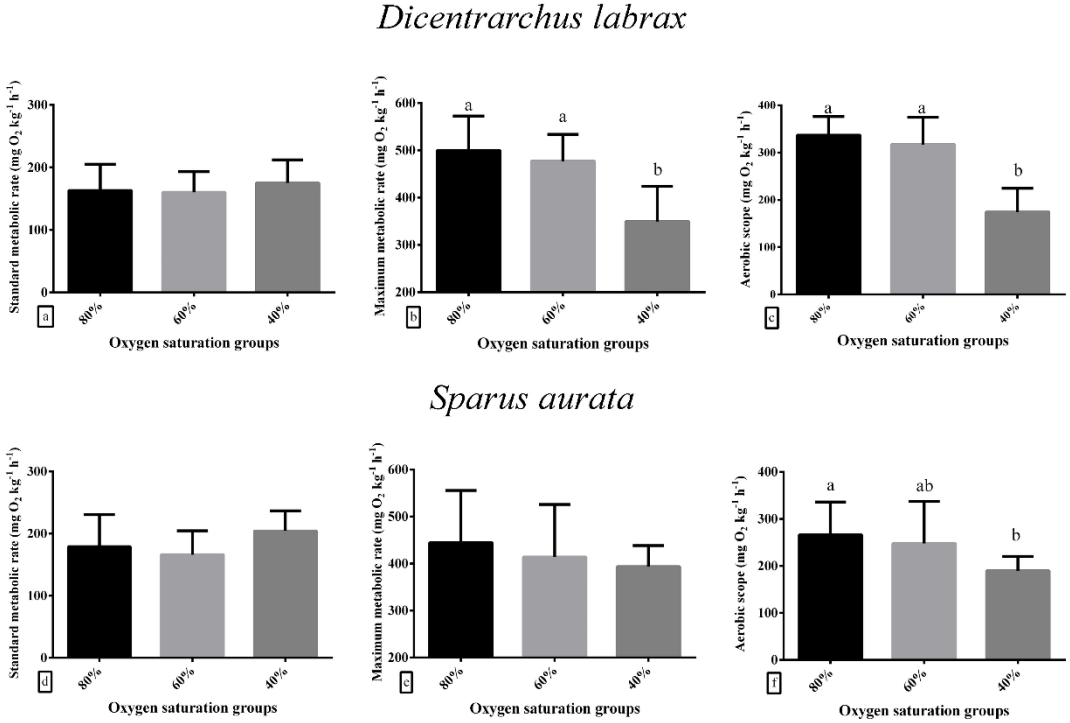
996

997 **Figure 3.** Cortisol concentration in plasma of control (unstressed, black bars) and
998 stressed (grey bars) *Dicentrarchus labrax* (a) and *Sparus aurata* (c) and fish scales only
999 in control *Dicentrarchus labrax* (b) and *Sparus aurata* (d) reared at 80%, 60% and 40%
1000 dissolved oxygen saturation groups (n = 12 for plasma, n = 9 for scales, N = 3 tanks per
1001 treatment). Results are presented as mean + SD. Different letters indicate significantly

different means between oxygen groups in the same stress conditions, while asterisks between control and stressed fish within the same oxygen treatment. Statistical differences were tested by two-way ANOVA for plasma and one-way ANOVA for scales with the level of significance being set at $p < 0.05$.

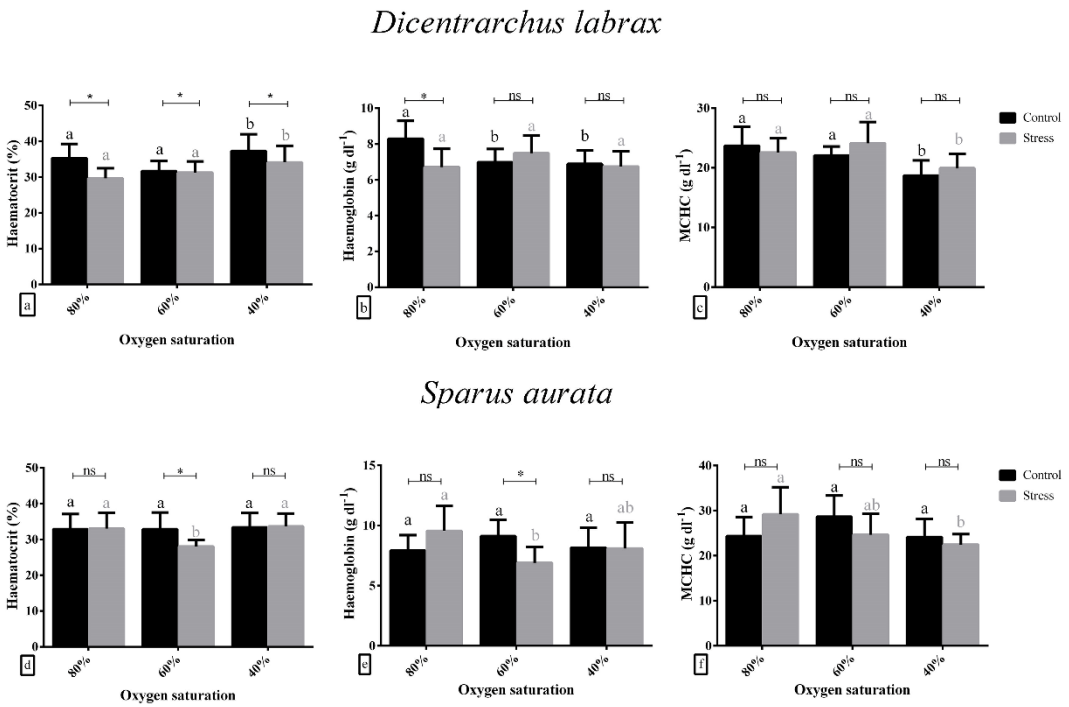
Figure 4. Principal Component Analysis performed on blood and plasma hematological, hormonal and biochemical data of control (unstressed) and stressed (a) *Dicentrarchus labrax* and (b) *Sparus aurata* reared at 80%, 60% and 40% dissolved oxygen saturation. Ellipse represents confidence ellipse. Plots created using the functions fviz_pca_biplot of the facto-extra package in R studio.

1012 **Fig. 1**



1013

1014 **Fig. 2**



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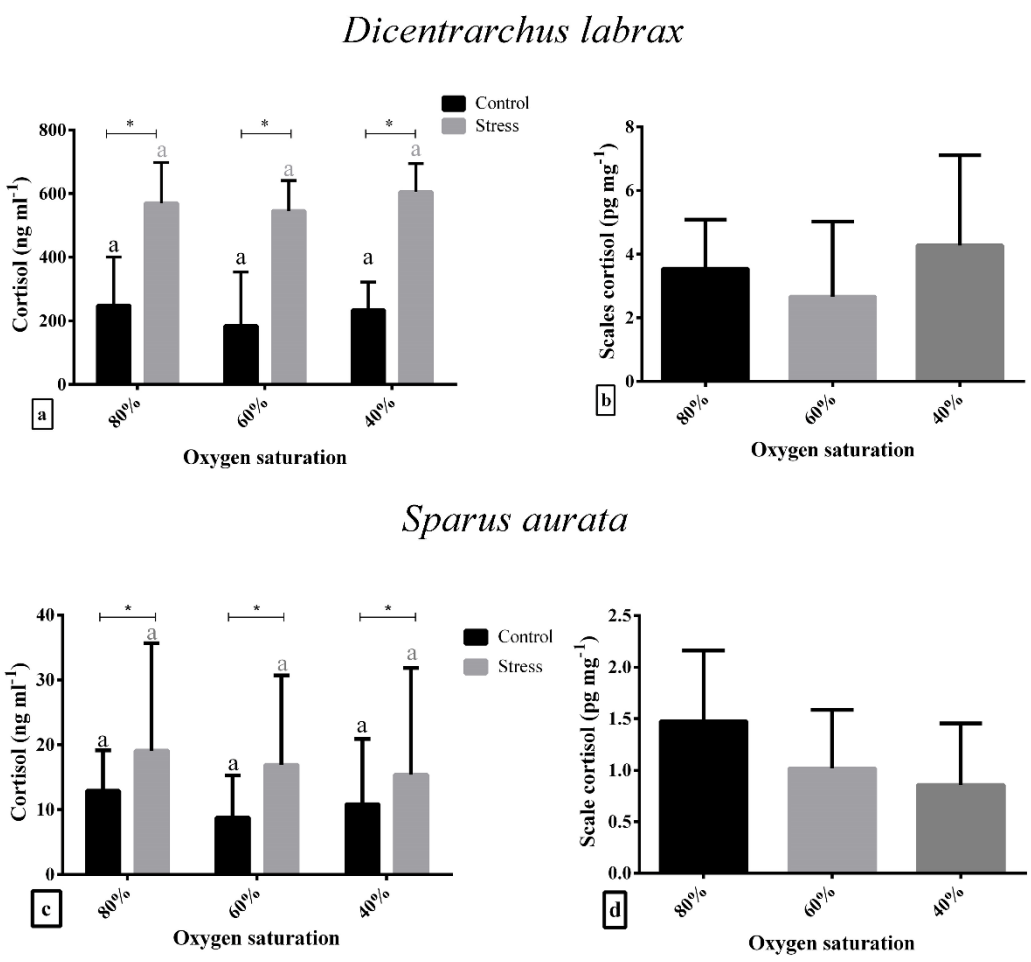
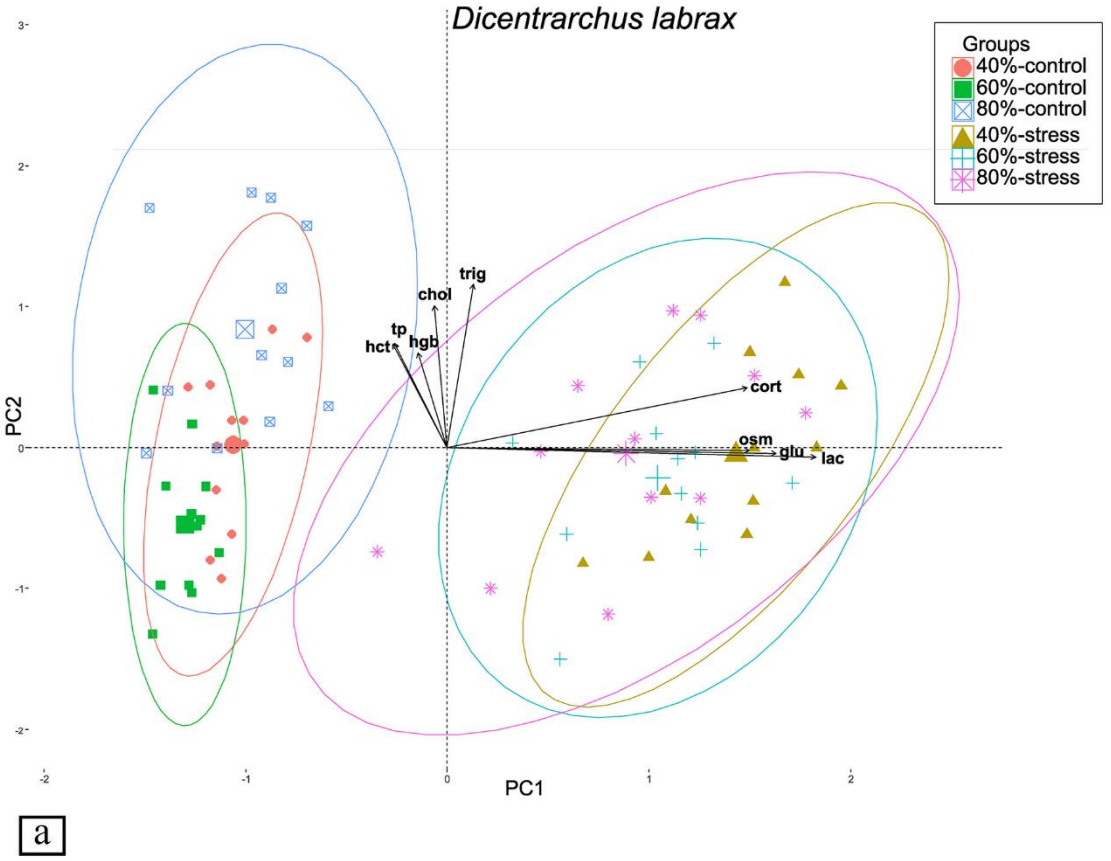
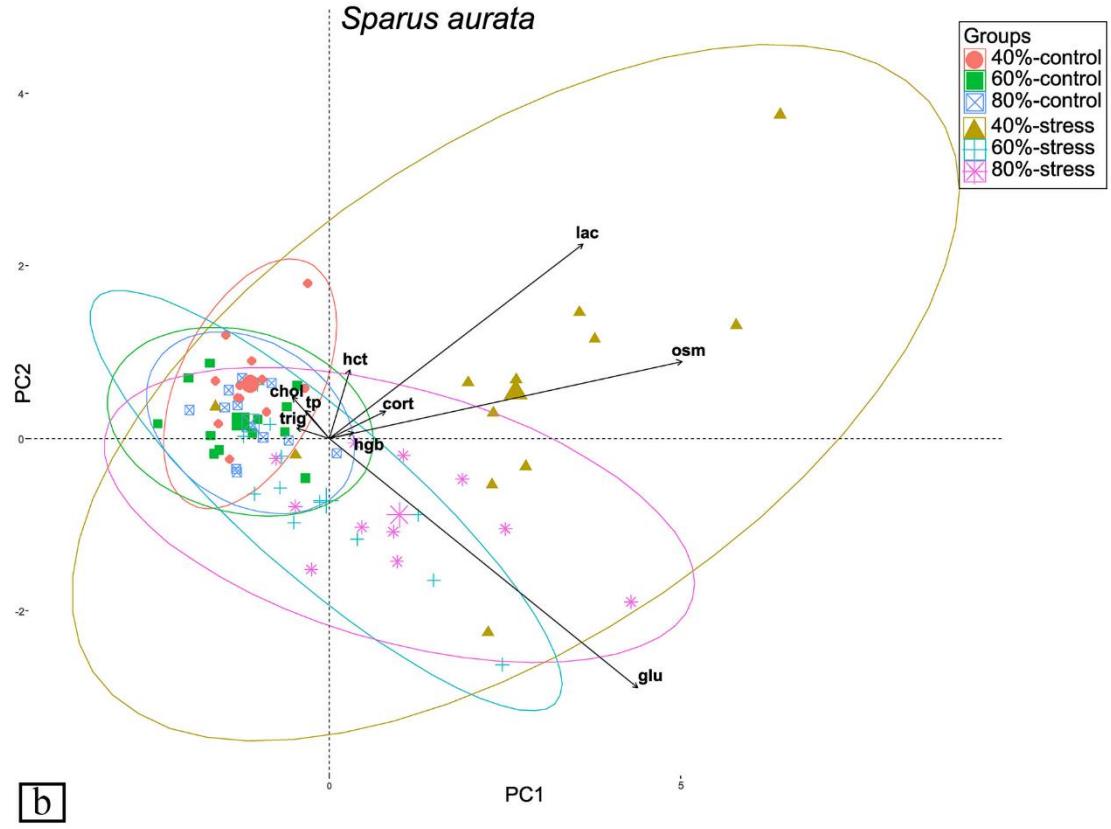
1016 **Fig. 3**



1017

1018 **Fig. 4**





1019