AQUA EXCEL 2020

AQUAculture infrastructures for EXCELlence in European fish research towards 2020 — AQUAEXCEL2020

D6.2. – Effect of early life oxygen concentration in sea bream

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Executive Summary

Objectives

Identification of potential long-lasting effects on fish performance by exposure of sea bream early rearing stages to a moderate hypoxia challenge.

Rationale:

Environmental conditions encountered by living fish outside their optimum tolerance range, in particular during critical windows of their early life history, can modify their development. Most of these challenges have detrimental effects, but in some cases physiological plasticity allow adaptive responses that become interesting traits for aquaculture production. Thus, trials conducted by AQUAEXCEL[2020] were aimed to identify beneficial adaptive and long-lasting effects of sea bream hypoxia exposure. To achieve this goal, a first experiment was conducted in sea bream juveniles to define the best blood biomarkers for functional phenotyping of severe hypoxia. A biomarker approach was also conducted in a short 3 weeks feeding trial to assess the tissue-specific responsiveness of blood, liver, heart and skeletal muscle during crowding stress and chronic moderate hypoxia (above the limiting oxygen saturation, LOS). Such approach together with data on feed intake and growth performance, confirms the high plasticity and stress resilience of farmed sea bream. Once the main responses to severe and moderate hypoxia exposure were defined on the basis of biometrical, haematological, biochemical and molecular parameters, a second set of experiments took place to assess the potential benefits of hypoxia pre-conditioning during juvenile and larval stages. The challenging protocol included swimming performance tests in metabolic chambers, as a low invasive and informative procedure to assess the metabolic performance of endurance training throughout development. The results opened new perspectives in several productive traits other than growth and welfare (e.g. fillet quality).

Main Results:

Severe hypoxia promotes a shift from aerobic to anaerobic metabolism, reduces basal metabolism and triggers more efficient mitochondrial respiration to increase aerobic energy production.

Moderate hypoxia (above LOS levels) during the juvenile stage reduces feed intake and growth. This effect is potentiated by crowding stress, although feed efficiency is not compromised and to some extent is improved, which reflects the high metabolic and growth plasticity of farmed sea bream.

At the molecular level, different adaptive mechanisms in juvenile fish were outlined, highlighting different tissue responsiveness according to the different tissue capabilities and the nature and severity of the hypoxia stimuli.

Hypoxic pre-conditioning (above LOS levels) during early life does not compromise final juvenile weight and triggers persistent metabolic effects that improve the aerobic scope and swimming performance later in life. This very promising result is one of the first evidence in typically marine fish of early environmental imprinting that might involve different epigenetic mechanisms.

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1. Metabolic adaptation to low oxygen concentrations: Previous AQUAEXCEL project background

The work described in the present Deliverable is a follow up from experiments and results conducted in the AQUAEXCEL project, which focused on the development and application of methodologies for the phenotyping of fish health and welfare status. The key role of mitochondria to cope with adaptive cellular stress in fish and sea bream in particular was evidenced by means of a meta-analysis conducted with the bioinformatics tool "Fish and chips" (online at **fishandchips.toulouse.inra.fr**). "Fish and chips" aggregates fish transcriptome data from more than 350 microarrays available the public domain and a vast array of experimental challenges (Calduch-Giner *et al.*, 2014). This finding led to the development of specific mitochondrial PCR-arrays for gene sequences extracted from the IATS-CSIC transcriptomic and genomic database (**www.nutrigroup-iats.org/seabreamdb**, Calduch-Giner *et al.*, 2013). With this approach it was established that most aquaculture stressors, including nutrient availability and daily routine activities, which involve movements and changes in noise and/or light levels, initiate retrograde signals that affect, among other processes, the transcriptional regulation of cell tissue repair and ATP energy production (Bermejo-Nogales *et al.*, 2014a, 2015). Moreover, the gene expression profile of different enzyme subunits of the mitochondrial respiratory chain is highly regulated in a tissue-specific manner by the type and intensity of environmental stressor. Changes in water temperature also lead to variations in the threshold level for acceptable O_2 concentrations (limiting oxygen saturation, LOS; Remen *et al.*, 2015), and the regulated expression of mitochondrial antioxidant enzymes and molecular chaperones has been found to be essential for the improved tolerance of fish fed with seaweeds to acute hypoxia (Magnoni *et al.*, 2017).

To further understand the adaptive and long-lasting effects of hypoxia exposure in sea bream, a set of different hypoxia challenge tests were conducted in AQUAEXCEL^{2020} to properly define adaptive responses or potential long-term benefits of hypoxia pre-conditioning. As a first step, hematological, biochemical and molecular blood biomarkers were monitored to assess the adaptive responses in fish facing severe hypoxia (20% O_2 saturation). This biomarker approach was extended to other metabolically-relevant tissues (liver, skeletal muscle, heart) for the functional phenotyping of chronic exposure to moderate hypoxia (40% O_2 saturation) in a 3-weeks feeding trial at two different rearing densities, Once the different tissue-contribution to the hypoxia-allostatic load was defined, a third set of experiments of hypoxia pre-conditioning was conducted to assess later fish performance using haematological and hormonal markers, together with a swimming test challenge in metabolic chambers. The final stage of this experimental research included hypoxic training at early life stages, combining two windows of hypoxia exposure, to assess the potential beneficial effects of metabolic priming six month later. The trials conducted in this Deliverable are summarized in **Table 1**.

Table 1. Time schedule of hypoxia tests.

Year/Fish	Challenge test	Targets
2016- Juveniles (230-260 g)	Hypoxia: 1.3 ppm, 8h; 3 ppm, 24h	Blood
2016- Juveniles (40 g)	Hypoxia: 3 ppm at two culture densities (15-30 kg/m^3) for 3 weeks	Blood, liver, heart, skeletal muscle
2017-Juveniles (25 g)	Hypoxia pre-conditioning: 3-4 ppm (6 weeks)	Blood, skeletal muscle, whole animal (swimming tests)
2018- Larva/ juveniles	Hypoxia pre-conditioning: 3-4 ppm larvae/juveniles(2-3 weeks)	Blood, whole animal (swimming tests)

2. Gene expression profiling of whole blood cells during acute hypoxia

Work contained in this section has been published in *Frontiers in Zoology 14:34* (2017). The publication is provided as Annex 2.

Among the abiotic factors, dissolved O_2 is particularly important as the major limiting factor of fish aerobic metabolism. Thus, when regulatory mechanisms are no longer sufficient to maintain the O_2 consumption rate (MO_2), further reductions in MO_2 occur until the termed limiting O_2 saturation (LOS). This threshold is able to maintain a routine metabolic rate in fed fish, and according to the oxystatic theory of feed intake, fish adjust their feed intake to meet dietary O_2 demands (Saravanan *et al.*, 2012). Therefore, fluctuations in tissue O_2 availability is a major factor to guaranty the welfare of farmed fish fed high or low O_2-demanding diets (Remen et al 2015, 2016). This regulation is mediated through O_2 sensors that trigger anaerobic metabolic rates to compensate for the decreasing aerobic ATP production (Luschak and Bagnyukova, 2006; Bermejo-Nogales *et al.*, 2014a). As a result of this, eukaryotic cells switch from mitochondrial oxidative phosphorylation (OXPHOS) to the less efficient anaerobic glycolytic pathway, which induces stress and lactic acidosis (Khacho *et al.*, 2014). Hence, as reported in humans, the hallmarks of muscle adaptation to hypoxia are a decrease in muscle oxidative capacity concomitant with a decrease in aerobic work capacity (Hoppeler & Vogt, 2001; Murray, 2009). This metabolic depression prevents the accumulation of toxic by-products from anaerobic metabolism (Donohoe *et al.*, 1998) and, thereby, hypo-metabolic states should be considered as part of the adaptive response to hypoxia instead of a negative result in hypoxia-tolerant individuals (Gamboa & Andrade, 2012).

Certainly, metabolic suppression is a key adaptive strategy in the hypoxic *Gillichthys mirabilis* to drive the energy resources from growth towards essential metabolic processes for survival (Gracey *et al.*, 2001). However, in *Fundulus grandis*, this contrast with the observation that both cardiac and hepatic tissues display increases in the gene expression of different enzyme subunits of the OXPHOS pathway in response to short-term hypoxia exposure (Everett *et al.*, 2012). Similarly, confounding results have been reported in European sea bass, as early life exposure to moderate hypoxia has long-lasting detrimental effects on growth performance with no improvement of hypoxia tolerance despite of the enhanced expression of glycolytic enzymes (Vanderplancke *et al.*, 2015). Thus, it appears likely that most hypoxia-mediated effects are tissue- or fish species-specific. In this regard, it must be noted that the red blood cells (RBC) of fish and almost all amphibians, reptiles and birds retain a nucleus and functional mitochondria (Stier *et al.*, 2013). These RBCs opens new research opportunities and previous studies have demonstrated that the expression of mitochondrial uncoupling proteins is highly regulated by hypoxia stimuli in sea bream blood

cells (Bermejo-Nogales *et al.*, 2014b). Thus, to go further in the regulation and adaptive responses of hypoxic metabolism in fish, we combined poorly invasive blood transcriptomics based on mitochondrial markers with conventional measures of blood haematology and biochemistry.

2.1 Experimental setup- acute hypoxia

Sea bream juveniles of Atlantic origin (Ferme Marine du Douhet, Bordeaux, France) were reared from early life stages at the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain) under natural photoperiod and temperature conditions at its latitude (40º5'N; 0º10'E). Fish (230-260 g body weight) were distributed in 500-L tanks (16 fish per tank) allocated in a re-circulatory system equipped with physical and biological filters and programmable temperature. The water temperature was maintained at 20-21 °C. Fish were fed daily to visual satiety using a commercial diet (INICIO Forte 824/EFICO Forte 824; BioMar), and all fish were fasted during the hypoxia challenges. The water conditions for the control fish (normoxic fish) remained unchanged, whereas hypoxic fish experienced a gradual decrease in the water O_2 concentration until reaching i) 3.0 ppm (41-42% O_2 saturation; moderate hypoxia, H1) for 24 h or ii) 1.3 ppm (18-19% O_2 saturation; severe hypoxia, H2) for up to 4 h in two different hypoxic tests (**Figure 1**). The two tested water O_2 levels were obtained by the cessation of normal aeration in the tank, achieving an accurate balance between the consumption rates of the animals and the supply of clean and oxygenated water by means of an electrovalve within the established O_2 steady-state condition.

In each test, normoxic or hypoxia-challenged fish were sampled at three different sampling points after decreasing the water O_2 concentration (8 fish per time and condition): i) H1: T0, T1 (24 h), T2 (48 h), and ii) H2: T0, T1 (7 h), T2 (11 h). One blood aliquot (150 µL) was directly collected into a microtube containing 500 µL of stabilising lysis solution (REAL Total RNA Spin Blood Kit, Durviz) and stored at -80 °C until total RNA extraction. Other aliquots were processed for haematocrit (Hc) and haemoglobin (Hb) determinations. The remaining blood was centrifuged at 3,000 x g for 20 min at 4 °C, and the plasma samples were frozen and stored at -20 °C until biochemical and hormonal analyses were performed.

Hc was measured using heparinised capillary tubes centrifuged at 1,500 x g for 30 min in a Sigma 1-14 centrifuge. The Hb concentration was assessed using a Hemocue Hb 201+. Plasma glucose was analysed using the glucose oxidase method. Blood lactate was measured in deproteinised samples (perchloric acid 8%) using an enzymatic method based on the use of lactate dehydrogenase. Total antioxidant capacity in plasma samples was measured using a commercial kit (Cayman Chemical) adapted to 96-well microplates. This assay relies on the ability of the antioxidants in the samples to inhibit the oxidation of ABTS (2,2'-azino-di-[3-ethylbenzothiazoline sulphonate]) to the ABTS radical cation by metamyoglobin, a derivatised form of myoglobin. The capacity of the sample to prevent ABTS oxidation was compared with that of Trolox (water-soluble tocopherol analogue) and quantified as mM Trolox equivalents. Plasma cortisol levels were analysed using an EIA kit (RE52061 m IBL, International GmbH). The limit of detection of the assay was 2.46 ng/mL with intra- and inter-assay coefficients of variation lower that 3% and 5%, respectively. Plasma insulin-like growth factors (Igf) were extracted using acid-ethanol cryoprecipitation (Shimizu *et al.*, 2000), and the concentration was measured using a generic fish Igf-I RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis *et al.*, 2004). The sensitivity and midrange of the assay were 0.05 and 0.7-0.8 ng/mL, respectively.

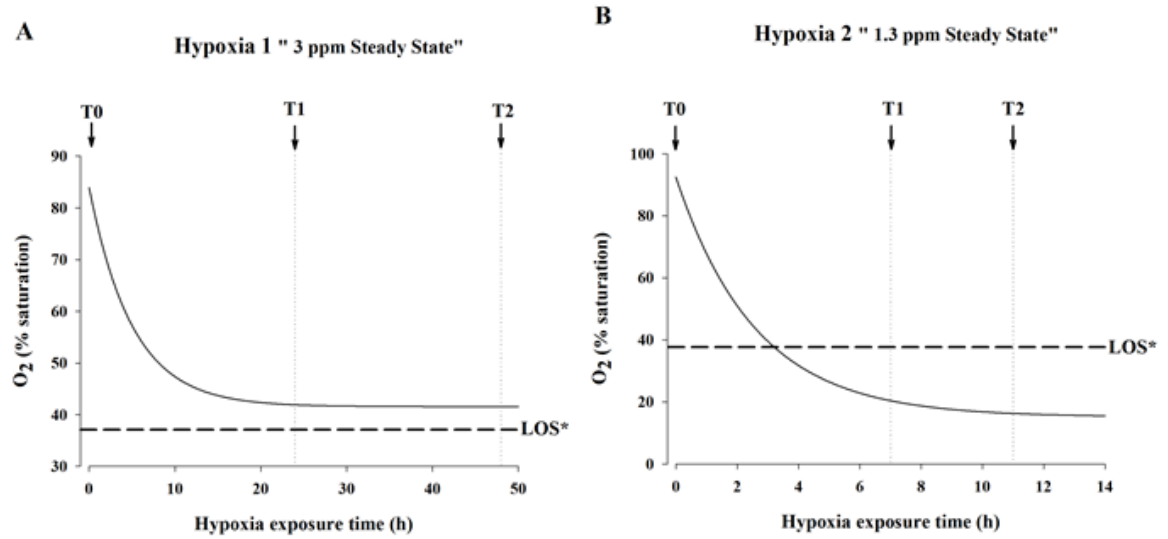


Figure 1. Water O_2 kinetics in fish exposed to hypoxic conditions. The steady state was set at (A) 41-42 % O_2 saturation (3.0 ppm, H1) or (B) 18-19 % O_2 saturation (1.3 ppm, H2). Sampling points (T0, T1 and T2) are indicated by arrow heads. LOS is calculated according to Remen *et al.* (2015).

Total RNA from total blood cells was extracted using the REAL Total RNA Spin Blood Kit including a DNase step. The RNA yield was >2.5 µg, with absorbance measures ($A_{260/280}$) of 1.9-2.1. The cDNA was synthesised using the High-Capacity cDNA Archive Kit (Applied Biosystems) with random decamers and 500 ng of total RNA in a final volume of 100 µL. Reverse transcription (RT) reactions were incubated for 10 min at 25 °C and 2 h at 37 °C. Samples were then used for qPCR assays by means of the IATS-Nutrigroup analytical platform. Diluted RT reactions in 25 µL volume were combined with a SYBR Green Master Mix (Bio-Rad) and specific primers at a final concentration of 0.9 µM. The 96-well PCR-array layout was designed for the simultaneous profiling of a panel of 85 mitochondrial genes under uniform cycling conditions and associated with different biological processes, such as molecular chaperones (7), antioxidant defence (8), transcription factors (5), outer and inner membrane translocation (8), mitochondrial dynamics and apoptosis (10), fatty acid oxidation and the tricarboxylic acid cycle (5), OXPHOS (41) and respiration uncoupling (1). PCR condition of IATS-Nutrigroup analytical platform and primer nucleotide sequences are available in Methods and Supplementary material sections of Annex 2.

2.2 Effects of acute hypoxia in blood haematology and biochemistry

Over the course of the first hypoxia test (H1, 41-42% O_2 saturation), measurements of haematological parameters and plasma glucose and lactate levels remained unaltered in both normoxic (>85% O_2 saturation) and hypoxia-challenged fish (**Figures 2A**, **2C**, **2E**, **2G**, respectively). In contrast, these parameters significantly increased in fish exposed to severe hypoxia (H2, 18-19% O_2 saturation) (**Figures 2B, 2D, 2F, 2H**). The same trend was observed for total plasma antioxidant activity and plasma cortisol levels (**Figures 3A, 3B**), though the cortisol increase was more pronounced at the last sampling point. The opposite regulation was observed for circulating Igf-I, although a statistically significant effect was observed at the last sampling point (**Figure 3C**). No variations in all the parameters studied were observed in fish maintained under normoxic conditions in the H2 test.

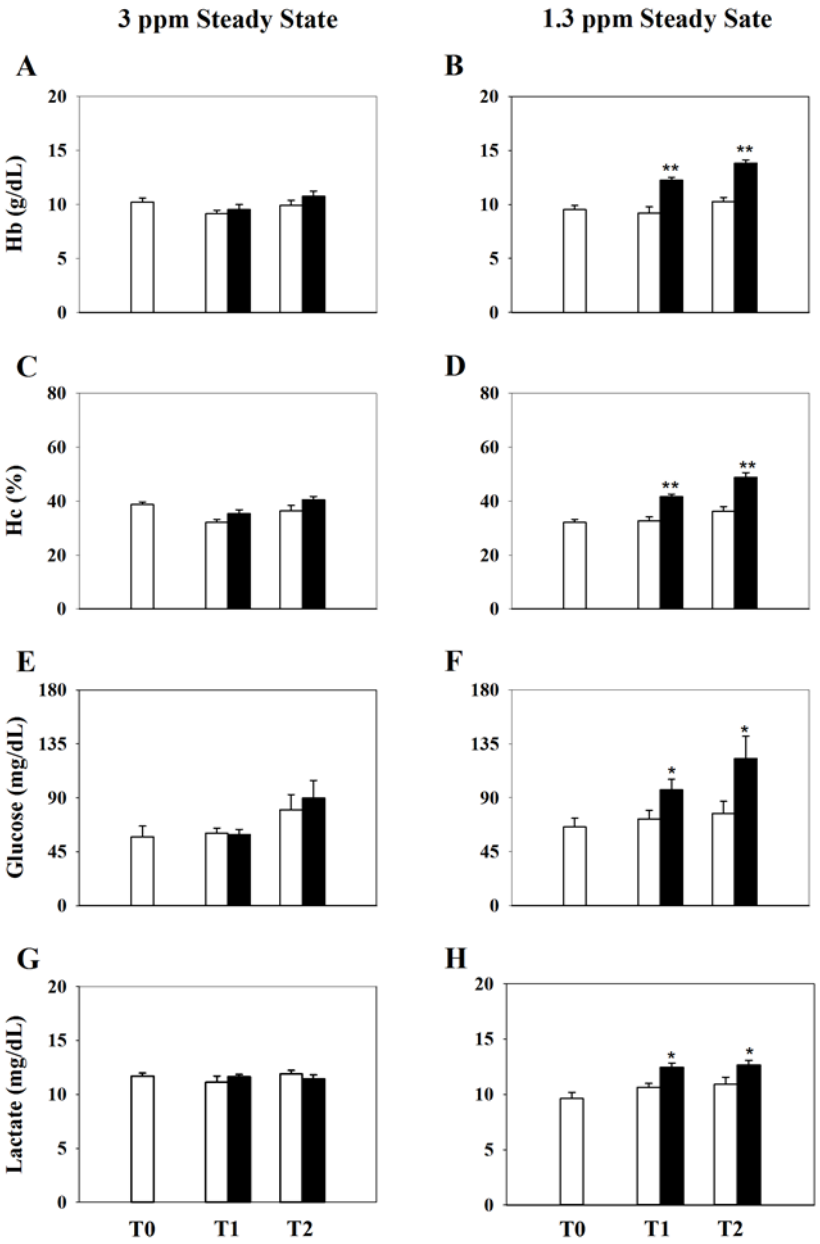


Figure 2. Effects of normoxia (white bars) and hypoxia (black bars) on blood haematology and biochemistry. Hypoxia levels were set above (A, C, E, G; H1) or below the LOS (B, D, F, H; H2). Data are the mean ± SEM (n = 7-8). Statistically significant differences between normoxic and hypoxic fish are indicated (*P <0.05, **P <0.01; two-way analysis of variance (ANOVA) followed by the Holm-Sidak test).

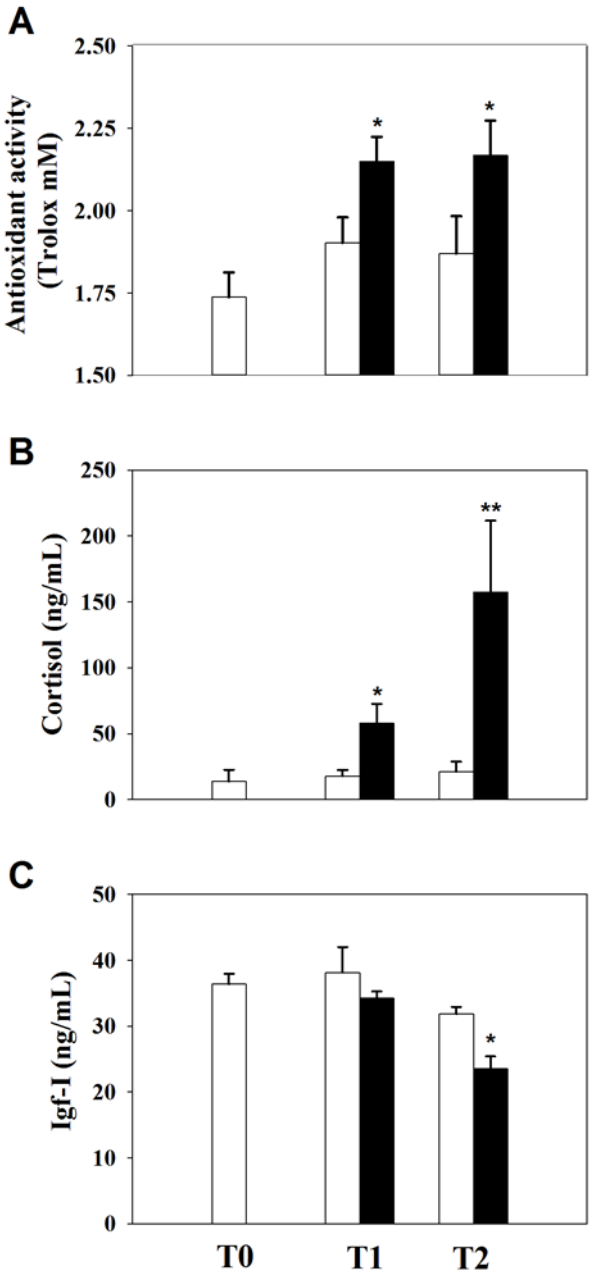


Figure 3. Effects of normoxia (white bars) and hypoxia below the LOS (H2, black bars) on plasma parameters. Antioxidant activity (A), cortisol (B) and Igf-I (C). Data are the mean ± SEM (n = 7-8). Statistically significant differences between normoxic and hypoxic fish are indicated (*P <0.05, **P <0.01; two-way analysis of variance (ANOVA) followed by the Holm-Sidak test).

Thus, all measured haematological and biochemical parameters remained mostly unaltered in fish maintained at 20-21 °C and 41-42% O_2 saturation. In contrast, the rapid and pronounced increase in Hc, Hb and plasma glucose and lactate levels after exposure to severe hypoxia (18-19% O_2 saturation) for 4 h could reflect an increase in blood O_2-carrying capacity associated in the short term with erythrocyte release from a storage organ or with a reduction in plasma volume rather than the formation of new Hb (Wood & Johansen, 1972; Soivio *et al.*, 1980). This finding likely reflects metabolic changes mediated by O_2 sensors that drive the shift of the redox cellular status of NADH to a more reduced form with a rapid recycling of NAD+ to NADH. Certainly, hypoxic situations must improve and adjust the metabolic and O_2-carrying capacities of challenged fish to cope and reach internal

homeostasis (Storey, 2015). The trigger observed in plasma antioxidant capacity after acute and severe hypoxia demonstrates a general decrease in metabolic rates that also reflects the aerobic/anaerobic shift of metabolism (Dalla Via *et al.*, 1998; Virani *et al.*, 2000).
The increase in plasma cortisol levels observed after severe hypoxia indicates a stressful scenario in the experimental model. Other common features of hypoxic and stress conditions include a decrease in plasma Igf-I levels and concomitant growth inhibition (Mommsen *et al.*, 1999; Dyer *et al.*, 2004). In this sense, a characteristic response in challenged sea bream produced by crowding, and presumably also through hypoxia, is the overall down-regulated expression of hepatic igfs and growth hormone receptors (Saera-Vila *et al.*, 2009). However, the precise mechanisms underlying these Gh/Igf-mediated effects remain mostly unexplored in fish.

2.3. Effects of acute hypoxia in blood transcriptomics

Based on the results of hormonal and metabolic parameters, gene expression profiling of whole blood cells was restricted to the last sampling point of the severe hypoxia experiment (H2). The relative gene expression and fold-changes (FC) of differentially expressed genes (41 out of 84) are summarised in **Table 2**. The complete expression table of all analysed genes is provided in Table 1 of Annex 2. The overall response to severe hypoxia involved a repression of gene expression. This response was mediated by antioxidant enzymes (*gpx*1, *gst3*, and sod2), the transcription factor *nrf1*, outer and inner membrane translocases (*tom70*, *tom22*, *tim44, tim10*, and *tim9*), markers of mitochondrial dynamics and apoptosis (*mfn2, miffb, miro1a, miro2*, and *aifm1*), fatty acid β-oxidation (*acaa2* and *hadh*), tricarboxylic acid cycle (*cs*), respiration uncoupling (*ucp2*) and respiratory enzyme subunits of Complex I (*ndufa3, ndufa4, ndufa7, ndufb5*, and *ndufs7*), Complex II (*sdha, sdhaf1*, and *sdhaf2*), Complex III (*uqcrc1, uqcrc2*, and *uqcrh*) and Complex V (*atp5c1, atp5g1, atp5l*, and *atpaf2*), encoded by either mitochondrial or nuclear DNA. The nuclear-encoded assembly factors of Complex IV (*sco1, surf1*, and *cox15*) were also significantly down-regulated, but the opposite trend was observed for catalytic (*coxi*) and regulatory (*cox5a2* and *cox8b*) enzyme subunits of mitochondrial or nuclear origin, respectively. This up-regulation was also observed for the transcription factor *pgc1ß* and the outer membrane translocase *tom34*. The molecular chaperones were the only factors that did not significantly change under hypoxic conditions, although the overall trend was a down-regulation in hypoxic fish.

Table 2. Relative gene expression of differentially expressed mitochondrial-related genes in total blood cells of sea bream exposed to normoxic (O_2 saturation >85 %) and hypoxic (1.3 ppm, O_2 saturation = 18-19 %) conditions. Data are the mean ± SEM (n = 7-8). Statistically significant differences between normoxic and hypoxic fish are indicated (*P <0.05, **P <0.01; Student's *t*-test). Gene names of mitochondrial-encoded catalytic subunits of the OXPHOS pathway are highlighted in bold and italics. Gene names of nuclear-encoded catalytic subunits of the OXPHOS pathway are highlighted in bold. Gene names of nuclear-encoded regulatory subunits are presented in normal font. Gene names of nuclear-encoded assembly factors are in italics. Square symbols are used for up- (red) and down-differentially expressed genes (green).

Gene name	Symbol	Relative expression		FC
		Control	Hypoxia	
Antioxidant enzymes				
Glutathione peroxidase 1	*gpx1*	18.26 ± 1.33	13.90 ± 1.25*	-1.31
Glutathione S-transferase 3	*gst3*	0.17 ± 0.06	0.02 ± 0.01*	-9.68
Superoxide dismutase [Mn]	*sod2*	0.55 ± 0.05	0.28 ± 0.05**	-1.97
Transcription factors				
Nuclear respiratory factor 1	*nrf1*	0.22 ± 0.05	0.10 ± 0.01*	-2.21
Proliferator-activated receptor gamma coactivator 1 beta	*pgc1β*	0.28 ± 0.09	0.80 ± 0.14*	2.85
Outer membrane translocases (TOM complex)				
Mitochondrial import receptor subunit Tom70	*tom70*	0.65 ± 0.04	0.36 ± 0.06**	-1.79
Mitochondrial import receptor subunit Tom34	*tom34*	0.55 ± 0.02	0.76 ± 0.10*	1.39
Mitochondrial import receptor subunit Tom22	*tom22*	0.23 ± 0.03	0.09 ± 0.02**	-2.47
Inner membrane translocases (TIM complex)				
Mitochondrial import inner membrane translocase subunit 44	*tim44*	0.16 ± 0.05	0.05 ± 0.02*	-3.62
Mitochondrial import inner membrane translocase subunit Tim10	*tim10*	0.19 ± 0.06	0.06 ± 0.02*	-3.20
Mitochondrial import inner membrane translocase subunit Tim9	*tim9*	0.13 ± 0.03	0.05 ± 0.01*	-2.55
Mitochondrial dynamics and apoptosis				
Mitofusin 2	*mfn2*	0.21 ± 0.02	0.12 ± 0.01*	-1.72
Mitochondrial fission factor homolog B	*miffb*	0.35 ± 0.04	0.19 ± 0.03*	-1.82
Mitochondrial Rho GTPase 1	*miro1a*	0.12 ± 0.04	0.03 ± 0.01*	-3.70
Mitochondrial Rho GTPase 2	*miro2*	0.31 ± 0.05	0.12 ± 0.02**	-2.69
Apoptosis-related protein 1	*aifm1*	0.23 ± 0.02	0.15 ± 0.03**	-1.53
FA oxidation & TCA				
3-ketoacyl-CoA thiolase	*acaa2*	0.43 ± 0.06	0.18 ± 0.03*	-2.38
Hydroxyacyl-CoA dehydrogenase	*hadh*	0.82 ± 0.03	0.53 ± 0.08**	-1.56
Citrate synthase	*cs*	0.62 ± 0.03	0.47 ± 0.05**	-1.32
OXPHOS (Complex I)				
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3	*ndufa3*	0.81 ± 0.06	0.56 ± 0.07*	-1.46
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	*ndufa4*	1.10 ± 0.07	0.78 ± 0.04**	-1.40
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	*ndufa7*	0.21 ± 0.02	0.12 ± 0.01**	-1.69
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5	*ndufb5*	0.52 ± 0.02	0.32 ± 0.05**	-1.59
NADH dehydrogenase iron-sulfur protein 7	*ndufs7*	0.60 ± 0.04	0.42 ± 0.05**	-1.42
OXPHOS (Complex II)				
Succinate dehydrogenase [ubiquinone] flavoprotein subunit	*sdha*	0.27 ± 0.04	0.13 ± 0.02*	-2.10
Succinate dehydrogenase assembly factor 1	*sdhaf1*	0.20 ± 0.06	0.07 ± 0.02*	-2.99
Succinate dehydrogenase assembly factor 2	*sdhaf2*	0.19 ± 0.04	0.07 ± 0.02*	-2.61
OXPHOS (Complex III)				
Cytochrome b-c1 complex subunit 1	*uqcrc1*	0.31 ± 0.06	0.14 ± 0.02*	-2.14
Cytochrome b-c1 complex subunit 2	*uqcrc2*	0.64 ± 0.06	0.33 ± 0.04**	-1.96
Cytochrome b-c1 complex subunit 6	*uqcrh*	0.27 ± 0.05	0.16 ± 0.02*	-1.72
OXPHOS (Complex IV)				
Cytochrome c oxidase subunit I	*coxi*	2.52 ± 0.37	4.69 ± 1.34*	1.86
Cytochrome c oxidase subunit 5A, mitochondrial-like isoform 2	*cox5a2*	0.04 ± 0.01	0.09 ± 0.02**	2.48
Cytochrome c oxidase subunit 8B	*cox8b*	1.02 ± 0.10	1.39 ± 0.16*	1.37
SCO1 protein homolog, mitochondrial	*sco1*	0.19 ± 0.06	0.05 ± 0.02*	-3.83
Surfeit locus protein 1	*surf1*	0.22 ± 0.04	0.09 ± 0.01*	-2.47
Cytochrome c oxidase assembly protein COX15 homolog	*cox15*	0.15 ± 0.02	0.10 ± 0.01**	-1.48
OXPHOS (Complex V)				
ATP synthase subunit gamma	*atp5c1*	0.36 ± 0.04	0.19 ± 0.04*	-1.83
ATP synthase lipid-binding protein	*atp5g1*	0.61 ± 0.06	0.36 ± 0.06*	-1.68
ATP synthase subunit g	*atp5l*	0.64 ± 0.06	0.42 ± 0.05*	-1.53
Mitochondrial F1 complex assembly factor 2	*atpaf2*	0.04 ± 0.01	0.01 ± 0.00*	-6.02
Respiration uncoupling				
Uncoupling protein 2	*ucp2*	0.12 ± 0.05	0.02 ± 0.02*	-6.26

Most mitochondrial proteins are encoded by nuclear DNA; thus, a healthy metabolic mitochondria phenotype is highly dependent on the protein import system, which involves two assembly complexes: the translocases of the outer membrane (TOM complex) and the translocases of the inner membrane (TIM complex). Thus, as demonstrated in mammalian cells (Ljubicic *et al.*, 2010), the TOM/TIM complex is highly inducible and regulated at both transcriptional and post-transcriptional levels under conditions of chronic stress or energy deficit to ensure the maintenance of adequate mitochondrial protein import rates. Similarly, sea bream juveniles exhibit a clear up-regulation in the gene expression of hepatic protein subunits of the TOM/TIM complex in response to aerobic energy stimuli after exposure to cyclic decreases in water temperature (Bermejo-Nogales *et al.*, 2014a). Conversely, severe hypoxia induced a pronounced down-regulation of *tom70* and *tom22* subunits in whole blood cells concurrent with decreases in mRNAs encoding protein subunits of TIM23 (*tim44*) and TIM22 (*tim10* and *tim9*) complexes. In addition, co-expression analyses (Faou & Hoogenraad, 2012) revealed the up-regulation of *tom34*, which acts as a co-chaperone of the Hsp70/Hsp90 complex, inhibiting mitochondrial protein translocation when expressed in excess. Taken together, these findings suggest an orchestration in hypoxic fish of the TOM/TIM complex that could enable adjustments in mitochondrial protein translocation to reduce plasma oxidative capacity and the risk of oxidative stress. This feature is consistent with the down-regulated expression of markers of ROS production and scavenging, including *ucp2*, mitochondrial superoxide dismutase (*sod2*), enzymes of the glutathione system (*gpx1* and *gst3*) and enzymes of fatty acid β-oxidation and TCA (*acaa2, hadh*, and *cs*). Importantly, the same trend was observed for mitochondrial (*hsp10*, *dnaja3a*, *dnajc20*, *hsp60*, and *grp-75*) and endoplasmic reticulum (*grp-170*) molecular chaperones, suggesting that proper protein folding was primarily assured in the blood cells of sea bream under the depressed metabolism induced by hypoxia exposure.

Mitochondrial dynamics is an essential process that adapts mitochondria morphology to the bioenergetics requirements of the cell and involves the balance of two opposing procedures (fusion and fission), but it is also greatly affected by the organelles used by the mitochondria to move inside the cells. The functionality of these organelles favours the redistribution of mitochondria within the cell to ensure high oxidative capacity under conditions of high energy demand, enabling the removal of dysfunctional or damaged mitochondria. This mechanism is highly conserved from yeast to mammals, and the molecular identity of major components of the fusion (*mfn1* and *mfn2*) and fission (*fis1* and *miffb*) system, as well as those of the MIRO system (*miro1a* and *miro2*) has been characterized in sea bream. Severe hypoxia significantly repressed the expression of most components of this biological process (*mfn2*, *miffb*, *miro1a*, and *miro2*), including the well-known mitochondrial apoptotic factor *aifm1*. The transcriptional regulator pgc1ß is essential for proper metabolic tuning in stress situations, contributing to the maintenance of the basal expression of mitochondrial and metabolic-related genes. However, in our experimental model, the opposite regulation was observed for *pgc1ß* and *mfn2*, suggesting that the up-regulated expression of *pgc1ß* was more a consequence than the cause of the overall repressed expression of mitochondria-related genes. This notion was supported by the observation that the mitochondrial transcription factor *nrf1*, another target gene of *pgc1ß*, was also down-regulated in hypoxia-challenged fish. Notably, despite the overlapping gene expression of pgc1ß and its homologue pgc1α, the compensation of Pgc1α or Pgc1ß functions was not completely observed in Pgc1α or Pgc1ß knockout rodents (Arany *et al.*, 2005; Lelliott *et al.*, 2006). In the case of sea bream blood cells, this effect is more exacerbated because *pgc1α* mRNAs were almost undetectable in both normoxic and hypoxic fish, although the expression of this gene at noticeable levels has previously been reported in other tissues of this fish species (Bermejo-Nogales *et al.*, 2014a). Whether this effect is part of the evolutionary pressure to select the conservation of functional mitochondria in the nucleated RBCs of non-mammalian vertebrates remains to be established.

The ultimate effector for coping with changes in energy needs and aerobic ATP production is the regulation of the OXPHOS pathway, which comprises five enzyme complexes (I-V) with catalytic enzymatic subunits encoded by both nuclear or mitochondrial DNA, whereas the

enzyme subunits with regulatory or assembly properties are strictly of nuclear origin (Bermejo-Nogales *et al.*, 2015). Gene expression profiling of liver, skeletal muscle and cardiac muscle tissues revealed that both the direction and magnitude of change in the enzymatic activities of the OXPHOS pathway is highly dependent on the metabolic capabilities of each tissue (Bermejo-Nogales *et al.*, 2014a). Thus far, the molecular fingerprinting of the OXPHOS pathway remained primarily unexplored in blood cells, evidencing the general depletion of several components of Complexes I, II, III and V in response to severe hypoxia. Assembly factors of Complex IV (*sco1*, *surf1* and *cox15*), that were also down-regulated, play an important role in energy production, and mutations or defects in these molecules produce adverse effects in the appropriate function of the OXPHOS pathway in mammals (Antonicka *et al.*, 2003; Stiburek *et al.*, 2005; Smith *et al.*, 2005). However, this observation contrasted with the overall overexpression of catalytic and regulatory subunits of Complex IV, which was statistically significant for the catalytic *coxi* and the regulatory *cox5a2* and *cox8b* subunits. CoxI protein is encoded by mitochondrial DNA and represents one of the largest subunits of Complex IV, which contains the bimetallic centre where O_2 binds and is reduced to H_2O (Lenka *et al.*, 1998). In addition, the observed increase in the gene expression of Cox5a and Cox8 family subunits highlights their importance during the completion of the holocomplex monomer, which contains the functional structure of the cytochrome c binding site (Ghezzi & Zeviani, 2012). Therefore, the net effect should be a reduced mitochondrial ATP production due to the overall suppression of mRNAs encoding the enzyme subunits of Complexes I, II, III and IV, although the opposite regulation of the catalytic/regulatory components of Complex IV should be accompanied by subsequent mechanisms that allow a better exploitation of available O_2 in the most energetically favourable way. Modifications in mitochondrial properties also occur in other vertebrates, and the hypo-metabolic steady-state observed in overwintering frogs occurred during hypoxic submergence by increases in mitochondrial O_2 affinity and a reduction in resting and active respiration rates in mitochondria isolated from skeletal muscle (St-Pierre *et al.*, 2000). Similarly, early studies in the freshwater European eel suggest that the efficiency of OXPHOS is increased after acclimation to high hydrostatic pressure, decreasing the enzymatic activity of Complex II in red muscle, whereas that of Complex IV is significantly increased (Theron *et al.*, 2000). This situation would enable a reduction in the electron leak and the optimisation of the respiratory chain. Similarly, studies in sea bream have revealed that the gene expression ratio of the enzyme subunits of Complexes I and IV is altered in heart and liver tissue during the recovery state after severe hypoxia exposure (Magnoni *et al.*, 2017). Thus, variations in the mitochondrial efficiency of ATP production exist among individuals, populations and environments, and even within the same individual over time. This spatial and temporal variability in mitochondrial machinery adds an additional layer of complexity to the regulation of energy metabolism, and the maintenance of aerobic metabolism is becoming recognised as a primary hypoxia survival strategy in most organisms, including fish (Rogers *et al.*, 2016).

2.4. Main outcomes- acute hypoxia phenotyping

In a previous AQUAEXCEL study (Remen *et al.*, 2015), the threshold level of LOS determined in 400-g fish varied between 17% O_2 saturation at 12 °C and 36% O_2 saturation at 20 °C. Therefore, these O_2 concentrations can be considered as the lower limit for acceptable decreases in O_2 concentration with respect to the physiological function and welfare of farmed sea bream. Accordingly, data on blood biochemistry and haematology in fish exposed to O_2 concentrations above the theoretically LOS did not significantly vary after 24 h of hypoxia challenge. In contrast, a consistent response, exacerbated over time, was observed for blood parameters measured few hours after exposure to O_2 concentrations below the LOS. In this case, the gene expression profile of whole blood cells was analysed, and the molecular signatures of hypoxic fish revealed important changes consistent with a reduced but more efficient aerobic ATP production.

Changes in plasma antioxidant capacity and circulating levels of hormones and metabolites supported reduced energy needs with the aerobic/anaerobic shift. These results were further confirmed by gene expression profiling of a wide representation of mitochondrial-related markers, including antioxidant enzymes and molecular chaperones, effectors of mitochondrial dynamics and apoptosis, and key components of the respiratory chain, suggesting that the mitochondrial bioenergetic of fish blood cells is finely adjusted at the transcriptional level by drastic changes in water O_2 concentrations. Moreover, the induced gene expression of catalytic and regulatory enzyme subunits of Complex IV should be considered an adaptive process to ensure reduced but more efficient aerobic ATP production, which was consistent with a reduced mitochondrial respiration uncoupling (decreased *ucp2* expression).

3. Sea bream resilience to moderate hypoxia and high stocking density

Work contained in this section has been submitted for publication in ***Frontiers in Physiology***. The original manuscript is provided as Annex 3.

In most aquaculture scenarios, the adaptive features in response to hypoxia are commonly associated to increases in temperature and high stocking rearing densities, which in turn can compromise water quality resulting in impaired fish growth and immunity (**Person-Le Ruyet *et al.*, 2008**; **Vikeså *et al.*, 2017**). Indeed, beneficial effects on growth performance have been reported in Arctic charr or meagre with the preservation of water quality at high stocking densities (**Jørgensen *et al.*, 1993**; **Millán-Cubillo *et al.*, 2016**). The opposite is also true and the impact of crowding stress is minimized when O_2 concentrations are not below LOS (**Araújo-Luna *et al.*, 2018**). Unravelling the combined effects of hypoxia and high rearing density are, thereby, crucial to warrant fish welfare during intensive farming conditions. Thus, we aimed to assess the effect of two different initial stocking densities (9.5 kg/m^3 and 19 kg/m^3, increasing up to 30 kg/m^3 during the challenge) and O_2 saturation levels (85%, 42-43% O_2 saturation) in a 3-weeks trial with fast growing sea bream juveniles. For this purpose, the different tissue contribution of blood, liver, skeletal muscle and heart to the homeostatic load was monitored by data on growth performance, haematology and blood biochemistry in combination with blood and tissue transcriptomic analysis of selected biomarkers of growth, energy and lipid metabolism.

3.1 Experimental setup- sea bream stress resilience

Twelve days prior to the start of the experimental trial, juvenile fish (~34 g average body weight) were randomly distributed in twelve 90-L tanks coupled to a re-circulatory system equipped with physical and biological filters, and programmable temperature and O_2 devices (Figure 4). Water temperature was daily monitored and maintained at 25-27 ºC. Fish were arbitrarily allocated to constitute two different initial stocking densities (six tanks per condition) fed daily to visual satiety with a commercial diet (EFICO Forte 824, BioMar): i) LD (low density, 25 fish/tank, 9.5 kg/m^3) and ii) HD (high density, 50 fish/tank, 19 kg/m^3). After the acclimation period, the water parameters of three tanks of each initial stocking density were kept unchanged, constituting the normoxic (>5.5 ppm O_2; >85% O_2 saturation) groups of each experimental condition (LDN, low density normoxia; HDN, high density normoxia). Fish maintained in the remaining six tanks experienced a gradual decrease in the water O_2 level until reaching 3.0 ppm (42-43% O_2 saturation), constituting the hypoxic groups of each experimental condition (LDH, low density hypoxia; HDH, high density hypoxia). The normal range of variation in O_2 concentrations was marked by a rapid drop 15-30 minutes after feeding (3.8-4 ppm normoxic groups; 2.3 ppm hypoxic groups), with a rapid restoration of

reference values in less than 1 hour by the automatic entrance of clean water from the main reservoir tank. This system allowed maintaining unionized ammonia below toxic levels (<0.50 mg/L) in both HDN and HDH groups.

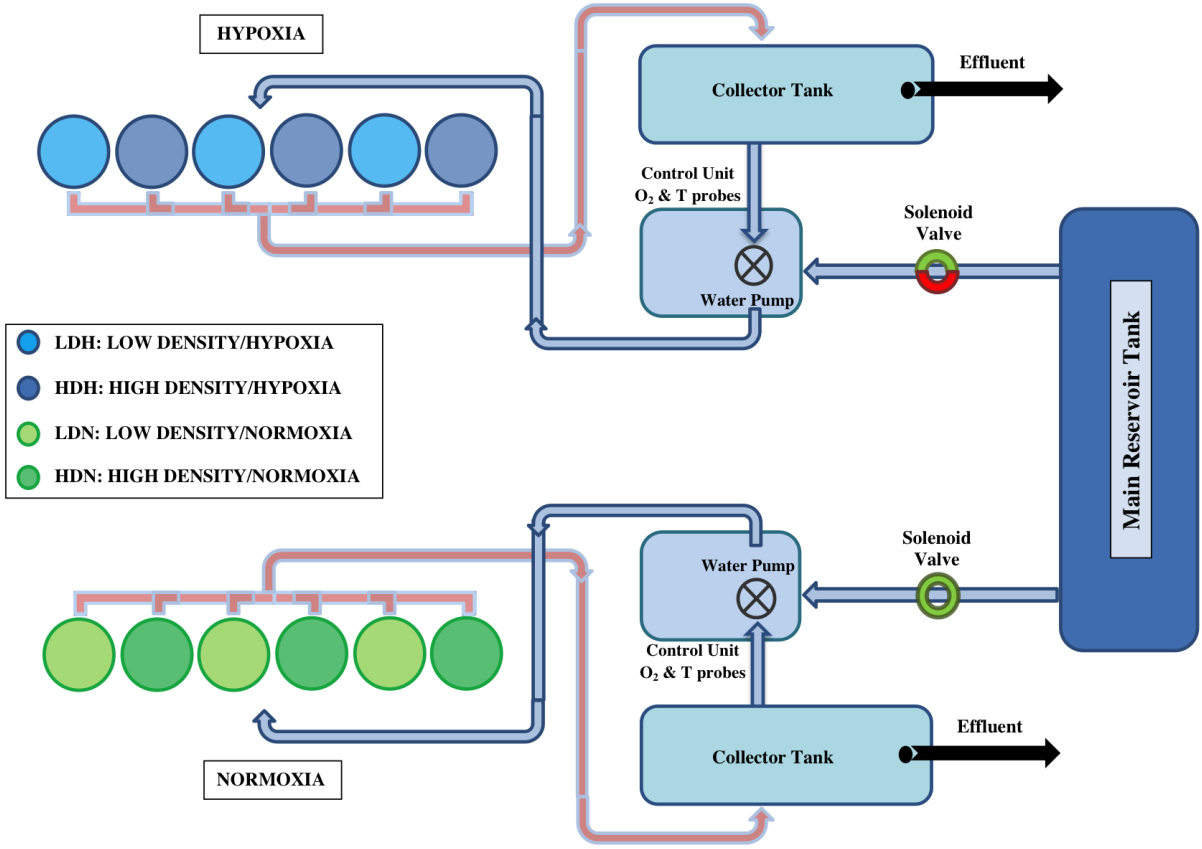


Figure 4. Experimental setup used to control dissolved O_2 levels in the experimental tanks to implement hypoxic conditions under different stocking densities. The O_2 steady-state was set at ~3.0 ppm (42-43% O_2 saturation) in fish kept under hypoxia (LOS), whereas fish maintained in normoxia were at a concentration of >5.5 ppm (>85 % O_2 saturation).

After twenty-two days under these experimental conditions and following overnight fasting, twelve fish (four per tank) per experimental condition (LDN, LDH, HDN, HDH) were anaesthetized with 3-aminobenzoic acid ethyl ester (100 mg/L), weighed and blood was taken from caudal vessels with EDTA-treated syringes. All lethal samples were collected between 10:00 am and 12:00 am to reduce the biologic variability due to circadian rhythms and postprandial-mediated effects. Prior to tissue collection, fish were killed by cervical section. Liver and viscera were weighed, and representative biopsies of liver, muscle and complete hearts were immediately snap-frozen in liquid nitrogen and stored at -80ºC until extraction of total RNA. One blood aliquot (25 µL) was directly collected into a microtube containing 500 µL of stabilizing lysis solution (REAL total RNA spin blood kit) and stored at -80 ºC until total RNA extraction. Other aliquots were processed for Hc, and Hb using the same procedure as previously detailed. Counts of RBC were made in a Neubauer chamber, using an isotonic solution (1% NaCl). Plasma biochemical and hormonal analysis were conducted as previously detailed. Plasma growth hormone (Gh) was determined by a homologous sea bream RIA as reported elsewhere (**Martínez-Barbera *et al.*, 1995**). The sensitivity and midrange (ED50) of the assay where 0.15 and 1.8 ng/mL, respectively.

Total RNA from liver, white muscle and heart was extracted using a MagMax-96 total RNA isolation kit (Life Technologies), whereas total RNA from total blood cells was extracted using the REAL total RNA spin blood kit including a DNase step. The RNA yield in all tissues was >3.5 µg, with absorbance measures ($A_{260/280}$) of 1.9-2.1. Synthesis of cDNA was performed as previously detailed. The 96-well PCR-array layout was designed for the simultaneous profiling of a panel of 43 (liver), 44 (white muscle and total blood cells) or 34 (heart) genes (Table 1 of Annex 3)., including markers of GH/IGF system (13), lipid metabolism (10), energy sensing and oxidative metabolism (12), antioxidant defense and tissue repair (10), muscle growth and cell differentiation (8), respiration uncoupling (3), xenobiotic metabolism (2), nuclear receptors (3), transmembrane translocation (8), mitochondrial dynamics and apoptosis (5), as well as OXPHOS (22). Diluted RT samples 25 µL volume were used for qPCR assays by means of the IATS-Nutrigroup analytical platform using the same procedure as previously detailed. Primer nucleotide sequences are presented in Supplemental Table 1 of Annex 3. Fluorescence data acquired during the extension phase were normalized by the delta-delta C_T method (**Livak and Schmittgen, 2001**) using *actb* in the liver, white muscle and heart, or *cox4a* in total blood cells, as the housekeeping gene due to its stability among different experimental conditions. For multi-gene analysis, data on gene expression were in reference to the expression level of *cs* in the liver, *igfr2* in the white muscle, *gcr* in the heart, and *tim8a* in total blood cells of LDN fish, for which a value of 1 was arbitrarily assigned.

3.2 Combined effects of moderate hypoxia and stocking density in growth performance and blood parameters

Data on feed intake, growth and somatic indexes (hepatosomatic index, HSI; mesenteric fat index, MSI) are shown in **Table 3**. Two-way ANOVA revealed an O_2 effect with an overall growth reduction under hypoxic conditions. This general impairment of feed intake and growth is further evidenced in fish kept at the highest density, though feed efficiency was improved in moderate hypoxia and more especially in fish kept at HD (HDH group).

Data on blood haematology and biochemistry are shown in **Table 4.** The results show a significant effect of O_2 concentration, with a generalized increase in Hb, Hc, RBC content, mean corpuscular Hb (MCH), cortisol and Gh plasma levels, as well as a widespread decrease in MCH concentration (MCHC), mean corpuscular volume and plasma lactate levels. Overall this feature was more accused in fish maintained under LD conditions. In contrast, the rearing density effect was mostly reduced to plasma cortisol levels, which showed a pronounced rise in HD fish that was exacerbated by hypoxic conditions. Noticeably, significant O_2 concentration and rearing density interactions were found for cortisol, but also for Hc, MCHC, MCH and total antioxidant activity.

Growth impairments due to long-term hypoxia exposure have been noticed in a wide-range of farmed fish, including turbot, European sea bass and Atlantic salmon (**Pichavant *et al.*, 2001**; **Remen *et al.*, 2016**; **Cadiz *et al.*, 2017**; **Vikeså *et al.*, 2017**). As reported herein in sea bream, a primary response was the inhibition of feed intake which would favor a hypo-metabolic state with a reduced ROS production and risk of oxidative stress. This is supported by lowered plasma levels of lactate, which would reflect in hypoxic fish, and in a lower extent in HDN, a low basal metabolism rather than a shift of aerobic to anaerobic metabolism. This metabolic re-adjustment has also been reported in sea bream juveniles facing multiple sensorial stressors in a model of chronic stress that mimic daily aquaculture operations (**Bermejo-Nogales *et al.*, 2014a**). Thus, according with the oxystatic theory, fish finely adjust feed intake and basal metabolism to available O_2, prioritizing feed efficiency at the expenses of maximum growth under restricted mitochondrial respiration. This seems to be especially effective in sea bream, a protandrous hermaphroditic fish with a high metabolic plasticity that makes feasible to be eurytherm, euryhalin and euryoxic thanks, at least in part, to a permissive regulation of feed intake which allows to cope an efficient energy metabolism at

slow growth rates. As a proof of this, the best feed efficiency and hormonal signatures for fast and efficient growth generally occurs before the achievement of maximum growth at the greater ration size (**Brett, 1979**; **Pérez-Sánchez *et al.*, 1995**). This also applies at the cellular level, where the maximum ATP yield per molecule of O_2 (P/O ratio) is highly dependent on ration size, as evidenced the increased P/O ratio of king penguins during periods of food shortage (**Monternier *et al.*, 2014**).

Most of the hypoxia-mediated effects are accompanied by an enhanced O_2-carrying capacity denoted by a swelling, formation and/or release of new erythrocytes together with plasma volume reduction (**Gallaugher and Farrell, 1998**). This was also inferred from our haematological data with changes in the measured Hc and corpuscular concentrations of Hb, which were affected by O_2 concentration and secondly by rearing density. Conversely, changes in HSI, reflecting the amount of lipid and glycogen depots, were more informative of feed intake rather that hypoxic condition, though it is difficult to disclose the main factor. At the hormonal level, this is also inferred from the measurements of circulating levels of cortisol and Gh, which are well-known regulators of metabolic rates by their involvement on mitochondria function (**Bergan-Roller & Sheridan, 2018**). In fact, cortisol is a well marker of crowding stress in sea bream, being enhanced the responsiveness of the hypothalamic-pituitary-adrenal axis by fasting or restricted feeding (**Sangiao-Alvarellos *et al.*, 2005**). This agrees with the observation that the greater circulating concentration of cortisol was achieved herein in the HDH group, which also experienced a higher feed intake inhibition. However, this system cannot be continuously refed and glucocorticoid receptors in rodents and perhaps other animal models translocate cortisol into mitochondria to reduce mitochondrial activity and the risk of oxidative stress (**Du *et al.*, 2009**). Thus, in the absence of a cortisol response, chronic cold-thermal stress up-regulates OXPHOS in sea bream, whereas the cortisol rise in fish facing multiple aquaculture sensorial stressors is accompanied by a pronounced transcriptional repression of all the hepatic complex units of the mitochondrial respiratory chain (**Bermejo-Nogales *et al.*, 2014a**). The aerobic scope and gene expression profiling of mitochondria is also highly regulated at the nutritional level by synthetic and natural dietary oils (**Pérez-Sánchez *et al.*, 2013**; **Martos-Sitcha *et al.*, 2018**), and the suppression of heptanoate effects upon exercise endurance is viewed as a protective measure to counteract disproportionate oxidative metabolic rates in fish fed fast energy-delivery nutrients (short/medium chain fatty acids). In other words, stimulatory and inhibitory effects of energy metabolism coexist as a trade-off mechanism that might reflect the changing energy needs rather than the availability of metabolic fuels. Accordingly, the increased circulating levels of Gh in hypoxic/crowded fish will reflect a reduced feed intake and energy demand rather than a minor capacity to combat oxidative stress, as referenced in fish and other animal models overexpressing GH (**McKenzie *et al.*, 2003**; **Almeida *et al.*, 2013**).

Table 3. Effects of rearing density and dissolved O_2 concentration on sea bream growth performance on a 21-days feeding trial. Values on body weight, feed intake, growth and feed efficiency are the mean ± SEM of triplicate tanks. Values on tissue biometric indexes are the mean ± SEM of 12 fish (4 fish per replicate tank). P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with O_2 concentration for a given rearing density (SNK test, P <0.05).
[1]Weight gain (%) = (100 x body weigh increase)/initial body weight
[2]Specific growth rate = 100 x (ln final body weight - ln initial body weight)/days

	LD		HD		P-value		
	Normoxia	Hypoxia	Normoxia	Hypoxia	[O_2]	Density	Interaction
Initial body weight (g)	34.54 ± 1.11	34.22 ± 0.27	34.32 ± 0.34	33.25 ± 0.45	0.305	0.376	0.571
Final body weight (g)	56.04 ± 1.89	51.65 ± 0.71	54.02 ± 0.50	48.54 ± 1.05**	0.003	0.059	0.651
Feed intake (g DM/fish)	23.78 ± 1.63	18.52 ± 0.7*	24.57 ± 1.06	17.54 ± 0.47**	<0.001	0.932	0.427
Weight gain (%)[1]	62.21 ± 0.31	50.94 ± 1.34**	57.43 ± 1.42	45.97 ± 1.31**	<0.001	0.003	0.941
SGR (%)[2]	2.30 ± 0.01	1.96 ± 0.04**	2.16 ± 0.04	1.80 ± 0.04**	<0.001	0.004	0.832
FE (%)[3]	0.91 ± 0.03	0.94 ± 0.02	0.80 ± 0.02	0.87 ± 0.01*	0.039	0.003	0.445
Liver weight (g)	0.94 ± 0.07	0.67 ± 0.03**	0.90 ± 0.06	0.63 ± 0.03***	<0.001	0.436	0.987
Viscera weight (g)	4.41 ± 0.28	3.84 ± 0.18	4.42 ± 0.19	3.68 ± 0.10**	0.002	0.690	0.681
HSI (%)[4]	1.64 ± 0.07	1.33 ± 0.06**	1.58 ± 0.07	1.25 ± 0.06**	<0.001	0.281	0.866
VSI (%)[5]	7.78 ± 0.29	7.65 ± 0.25	7.87 ± 0.24	7.38 ± 0.22	0.224	0.720	0.487

[3]Feed efficiency = wet weight gain/dry feed intake
[4]Hepatosomatic index = (100 x liver weight)/fish weight
[5]Viscerosomatix index = (100 x viscera weight)/fish weight

Table 4. Effects of rearing density and dissolved O_2 concentration on blood haematology and plasma levels of metabolites, hormones and total antioxidant capacity. Values are the mean ± SEM of 10-12 fish (4 fish per replicate tank). P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with O_2 concentration for a given rearing density (SNK test, P <0.05).

	LD		HD		P-value		
	Normoxia	Hypoxia	Normoxia	Hypoxia	[O_2]	Density	Interaction
Haemoglobin (g/dl)	7.18 ± 0.24	7.73 ± 0.21	7.38 ± 0.14	7.77 ± 0.26	0.041	0.591	0.718
Haematocrit (%)	22.18 ± 1.10	32.91 ± 1.65***	28.27 ± 1.77	29.90 ± 1.39	<0.001	0.313	0.004
RBC x 10^{-6} (cells/µl)[1]	2.45 ± 0.07	2.74 ± 0.07**	2.38 ± 0.06	2.82 ± 0.08***	<0.001	0.924	0.345
MCHC (pg/10µm^3)[2]	34.07 ± 1.12	24.00 ± 1.18***	26.62 ± 1.73	26.46 ± 1.10	<0.001	0.067	<0.001
MCH (pg/cell)[3]	89.79 ± 4.21	116.6 ± 4.46**	116.5 ± 8.28	109.5 ± 7.21	0.118	0.123	0.010
MCV (µm^3)[4]	29.50 ± 1.02	28.33 ± 0.76	31.36 ± 0.93	27.73 ± 0.96*	0.014	0.501	0.194
Glucose (mg/dl)	54.39 ± 1.58	52.17 ± 2.44	58.04 ± 1.78	52.73 ± 2.79	0.091	0.339	0.482
Lactate (mg/dl)	16.30 ± 2.78	4.81 ± 1.41**	10.22 ± 3.06	4.99 ± 0.84	0.001	0.225	0.199
TAA (mM Trolox)[5]	1.34 ± 0.04	1.45 ± 0.04	1.48 ± 0.03	1.43 ± 0.03	0.447	0.104	0.026
Cortisol (ng/ml)	23.40 ± 5.67	21.08 ± 5.32	35.69 ± 11.15	79.25 ± 9.05**	0.036	<0.001	0.027
Growth hormone (ng/ml)	2.34 ± 0.83	6.71 ± 1.17*	5.39 ± 1.29	8.33 ± 4.20	0.069	0.337	0.767
Insulin-like growth factor-I (ng/ml)	46.06 ± 4.76	46.59 ± 4.77	45.78 ± 2.27	41.03 ± 6.29	0.659	0.544	0.582

[1]Red blood cells
[2]Mean corpuscular haemoglobin concentration
[3]Mean corpuscular haemoglobin
[4]Mean corpuscular volume
[5]Total antioxidant activity

3.3 Combined effects of moderate hypoxia and stocking density in tissue gene expression profiling

Results of gene expression profiling in hepatic selected genes are presented in Suppl. Table 2 of Annex 3. Among them, 22 out of 43 genes were affected by at least one of the experimental factors or by its interaction, being 11 differentially expressed (DE) in response to O_2 concentration. Relative expression of markers from GH/IGF system (*ghr-i*), oxidative metabolism (*nd2*), and antioxidant defence and tissue repair (*gpx4*, *prdx5*) was significantly down-regulated by moderate hypoxia in LDH and HDH groups. In addition, several genes of lipid metabolism (*elovl1*, *fads2* and *scd1b*) were up-regulated in the LD group, whereas markers of oxidative metabolism (*nd5*), and antioxidant defence and tissue repair (*gr*, *sod2*, *grp-75*) were down-regulated in fish kept at HD conditions. Stocking density also affected 11 genes related with the GH/IGF system (*ghr-i*, *ghr-ii*, *igf-i*), lipid metabolism (*elovl6*, *fads2*, *scd1a*, *scd1b*, *lpl*), oxidative metabolism (*ucp1*, *pgc1α*) and antioxidant defence and tissue repair (*grp-75*). A statistically significant interaction of O_2 concentration and rearing density was found for *igf-ii*, *fads2*, *scd1a*, *scd1b*, *pgc1ß*, *gr*, *prdx3* and *grp-170* genes.

In white skeletal muscle, 20 out of 44 DE genes were affected at least by one of the experimental condition or even by their interaction (Suppl. Table 3 of Annex 3). Markers of the GH/IGF system were mostly affected by stocking density (*ghr-i*, *igf-ii*, *igfbp3*, *igfbp5b*, *igfbp6b*, *insr*, *igfr1*) rather than by O_2 concentration (*igfr2*). Moderate hypoxia up-regulated *myod2* expression as the sole effect on genes related to muscle growth and cell differentiation. In contrast, a relative high number of genes related to energy sensing, oxidative metabolism, and antioxidant defence and tissue repair were down-regulated by low O_2 concentrations (*sirt1*, *ucp3*, *hif-1α, prdx5*, *sod2*) or up-regulated in HD conditions (*sirt4*, *sirt7*, *coxi*, *hif-1α*, *gpx4*). Additionally, a significant interaction between O_2 concentration and rearing density is reported for *cpt1a* and *grp-170*.

In heart, changes in O_2 saturation and stocking density triggered significant differences in 19 out of 34 genes presented in the array (Suppl. Table 4 of Annex 3). Up to 13 genes, including markers of the GH/IGF system (*ghr-i*), energy sensing and oxidative metabolism (*sirt1*, *sirt5*, *sirt6*, *sirt7*, *cs*, *nd5*, *pgc1α*, *pgc1ß*, *hif-1α*) and antioxidant defence and tissue repair (*cat*, *prdx5*, *sod2*) were down-regulated under moderate hypoxia, especially in HD conditions. The xenobiotic metabolism marker *cyp1a1* was up-regulated by hypoxia in both LD and HD. Stocking density also down-regulated the expression of several genes involved in energy sensing and oxidative metabolism (*sirt3*, *sirt5*, *cs*, *nd2*) as well as antioxidant defence and tissue repair (*gr*, *prdx3*, *prdx5*, *grp-170*, *grp-75*), preferentially under low O_2 concentrations.

In total blood cells, only 5 out of 44 genes were DE mainly by the interaction among different experimental conditions (Suppl. Table 5 of Annex 3), being responsive to the stress challenge enzyme subunits of Complex I (*ndufaf2*) and Complex IV (*coxi*, *coxii*, *cox6a2*, *cox15*) of the mitochondrial respiratory chain.

In order to assess the differential contribution of the DE genes in the physiological response to moderate hypoxia and rearing density, the tissue (liver, white skeletal muscle, heart) gene expression dataset was jointly analysed by Partial Least-Squares Discriminant Analysis (PLS-DA). Results from total blood cells were not included due to its low contribution to the total variance. The contribution of differential genes along liver, white muscle and heart tissues was assessed by means of Variable Importance in Projection (VIP) measurements. A VIP score > 1.1 was considered to be an adequate threshold to determine discriminant variables in the PLS-DA model (**Wold *et al.*, 2001**; **Li *et al.*, 2012**; **Kieffer *et al.*, 2016**). The resulting discriminant model was based on six components, which explained (R^2) 95% and predicted (Q^2) 65% of total variance (**Figure 5A**). Of these, the first three components showed cumulative values for R^2 and Q^2 of 83.6% and 49.3%, respectively. A clear separation between normoxic (LDN, HDN) and hypoxic (LDH, HDH) groups was observed

along the first component that explained 28.89% of total variance (**Figures 5B, 5C**). Component 2 (29.27% of variance) clearly separated LDN and HDN normoxic groups (*Figure 2B*), whereas component 3 (25.42% of variance) discriminated LDH and HDH hypoxic groups (**Figure 5C**).

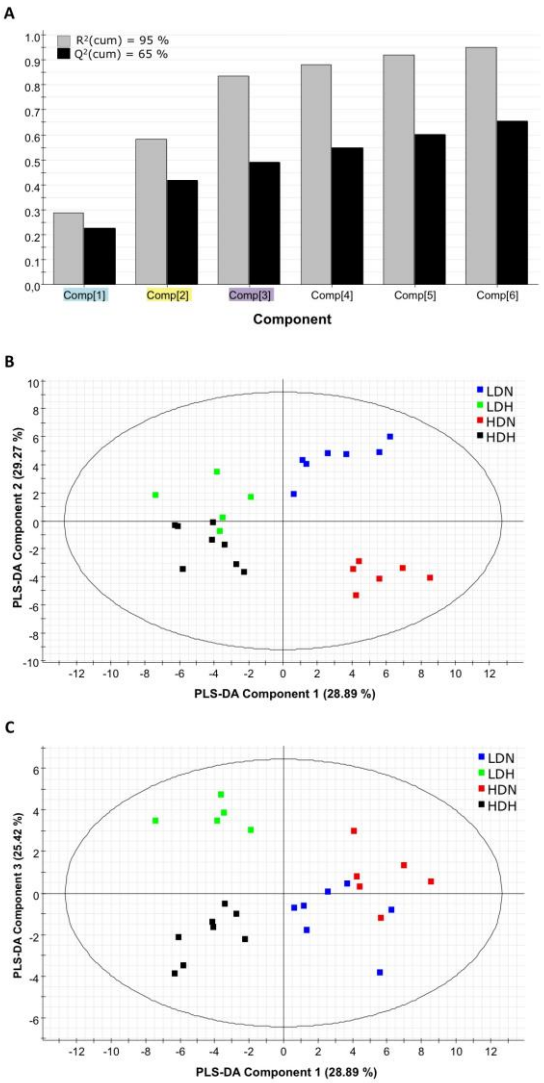
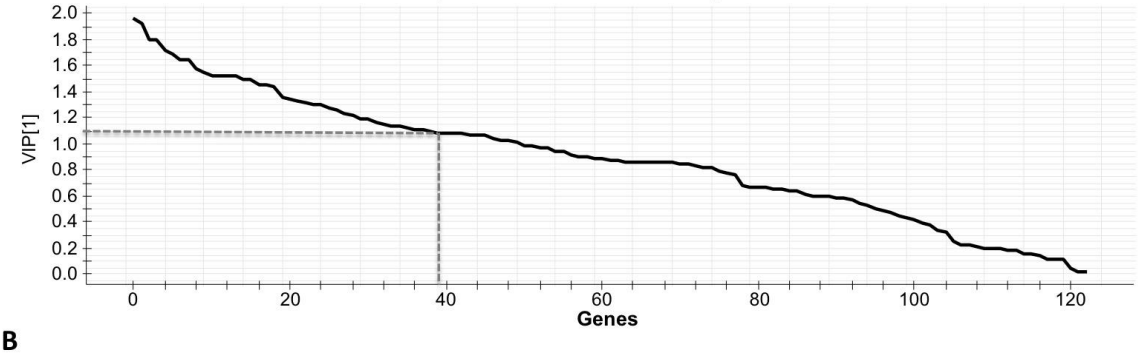


Figure 5. A) Graphical representation of the goodness-of-fit of the PLS-DA model. B) Two-dimensional PLS-DA score plot representing the distribution of the samples between the first two components in the model. C) Two-dimensional PLS-DA score plot representing the distribution of the samples between the first and third components in the model. R^2(cum): explained variance; Q^2(cum): predicted variance; LDN: low density normoxia; LDH: low density hypoxia; HDN: high density normoxia; HDH: high density hypoxia.

Genes with a contribution to VIP > 1.1 in component 1 were a total of 39, with a main contribution of heart (19) and liver (14) genes involved in energy sensing and oxidative metabolism (14), antioxidant defence and tissue repair (12) and OXPHOS (**Figure 6**). When the second component was also considered, a total of 44 genes presented VIP values > 1.1 (**Figure 7**), and 11 out of the 21 new genes (highlighted in yellow) were from white skeletal muscle. Energy sensing and oxidative metabolism (12), antioxidant defence and tissue repair (11), GH/IGF system (11) and OXPHOS (6) were the main categories. Considering the VIP

values from the 3 main components (**Figure 8**), most of the genes due to component 3 contribution (highlighted in purple) were related to lipid metabolism.

A

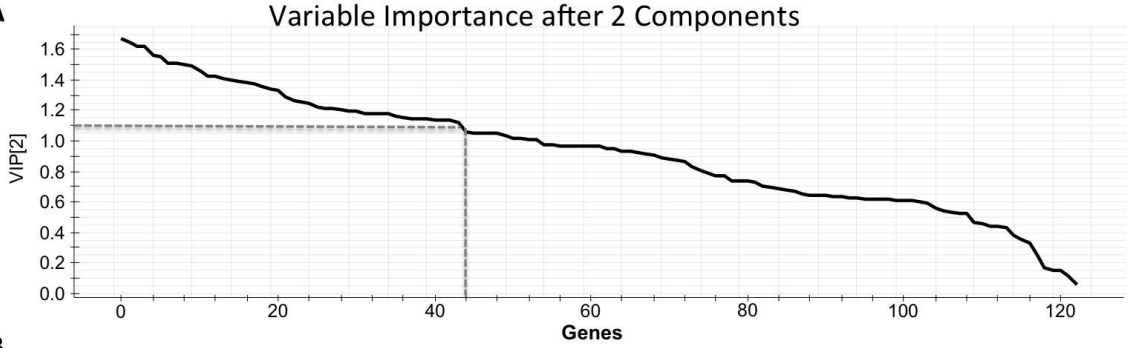


B

Order	Gene (tissue)	VIP (Component 1)	LD		HD		P-value		
			Normoxia	Hypoxia	Normoxia	Hypoxia	[O2]	Density	Interaction
1	*prdx5* (Liv)	1.964	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
2	*nd2* (Liv)	1.926	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
3	*nd5* (Heart)	1.801	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
4	*Mn-sod / sod2* (Heart)	1.799	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
5	*mthsp70/grp-75/mortalin* (Heart)	1.718	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
6	*Mn-sod / sod2* (Liv)	1.691	1.20 ± 0.05	1.02 ± 0.08	1.30 ± 0.09	0.94 ± 0.08*	0.001	0.955	0.280
7	*cs* (Heart)	1.653	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
8	*hif-1α* (Heart)	1.651	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
9	*gpx4* (Liv)	1.584	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
10	*pgc1α* (Heart)	1.553	2.55 ± 0.15	2.28 ± 0.15	2.74 ± 0.26	2.08 ± 0.10*	0.012	0.966	0.274
11	*nd2* (Heart)	1.529	68.14 ± 4.68	70.41 ± 5.69	64.19 ± 5.02	52.61 ± 2.44*	0.322	0.026	0.145
12	*nd5* (Liv)	1.525	16.51 ± 0.83	14.41 ± 0.73	18.11 ± 1.54	14.20 ± 1.11*	0.010	0.533	0.416
13	*igfr2* (WM)	1.521	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
14	*sirt1* (Heart)	1.518	0.22 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.18 ± 0.01*	0.023	0.219	0.572
15	*ucp3* (WM)	1.498	11.65 ± 1.78	6.43 ± 0.93*	16.89 ± 4.46	6.91 ± 0.74*	0.006	0.272	0.359
16	*sirt5* (Heart)	1.496	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
17	*prdx5* (WM)	1.460	28.69 ± 1.98	20.68 ± 1.10**	27.18 ± 2.14	22.48 ± 1.14*	<0.001	0.933	0.329
18	*prdx5* (Heart)	1.449	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
19	*gr* (Liv)	1.441	0.36 ± 0.02	0.34 ± 0.02	0.42 ± 0.04	0.28 ± 0.02**	0.006	0.929	0.050
20	*pgc1β* (Heart)	1.362	0.97 ± 0.04	0.85 ± 0.06	0.96 ± 0.05	0.84 ± 0.05	0.026	0.834	0.983
21	*sirt7* (Heart)	1.350	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.05 ± 0.00*	0.003	0.608	0.395
22	*grp-170* (Heart)	1.337	0.91 ± 0.06	0.92 ± 0.04	0.90 ± 0.05	0.77 ± 0.03*	0.191	0.070	0.103
23	*sirt2* (Liv)	1.316	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
24	*sirt6* (Heart)	1.309	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00*	0.042	0.211	0.099
25	*cyp1a1* (Heart)	1.297	2.58 ± 0.25	3.63 ± 0.32*	2.71 ± 0.23	3.79 ± 0.32*	<0.001	0.601	0.960
26	*coxi* (Heart)	1.268	329.3 ± 18.4	310.7 ± 14.8	332.4 ± 27.5	278.2 ± 19.3	0.086	0.480	0.393
27	*prdx3* (Liv)	1.258	0.82 ± 0.05	0.87 ± 0.05	0.94 ± 0.07	0.72 ± 0.07*	0.178	0.787	0.030
28	*pgc1β* (Liv)	1.239	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
29	*sirt2* (Heart)	1.215	0.34 ± 0.02	0.31 ± 0.02	0.33 ± 0.03	0.28 ± 0.01	0.081	0.454	0.644
30	*elovl1* (Liv)	1.194	10.63 ± 0.46	13.41 ± 0.79*	10.53 ± 0.66	12.69 ± 1.01	0.003	0.593	0.682
31	*sirt4* (Heart)	1.190	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
32	*ghr-i* (Liv)	1.161	3.06 ± 0.27	2.18 ± 0.26*	3.70 ± 0.36	2.79 ± 0.35*	0.007	0.049	0.977
33	*mthsp70/grp-75/mortalin* (Liv)	1.146	0.56 ± 0.04	0.55 ± 0.04	0.80 ± 0.10	0.55 ± 0.05*	0.033	0.044	0.053
34	*sirt2* (WM)	1.141	1.45 ± 0.06	1.49 ± 0.08	1.61 ± 0.14	1.43 ± 0.06	0.475	0.605	0.259
35	*elovl4* (Liv)	1.132	0.44 ± 0.05	0.49 ± 0.03	0.52 ± 0.05	0.41 ± 0.03	0.480	0.948	0.057
36	*sirt1* (Liv)	1.127	0.10 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.067	0.089	0.467
37	*grp-170* (WM)	1.111	1.80 ± 0.08	1.93 ± 0.12	2.09 ± 0.12	1.72 ± 0.11*	0.270	0.705	0.025
38	*igfr1* (WM)	1.107	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
39	*ghr-i* (Heart)	1.102	0.39 ± 0.01	0.35 ± 0.02	0.38 ± 0.02	0.30 ± 0.03	0.021	0.257	0.507

Figure 6. A) Graphical representation of the variable importance (VIP) scores after component 1. B) Ranking list of genes showing VIP score values above 1.1 and their relative gene expression. Values on relative expression are the mean ± SEM of 8 fish (2-3 fish per replicate tank). P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with O_2 concentration for a given rearing density (SNK test, P <0.05).

A

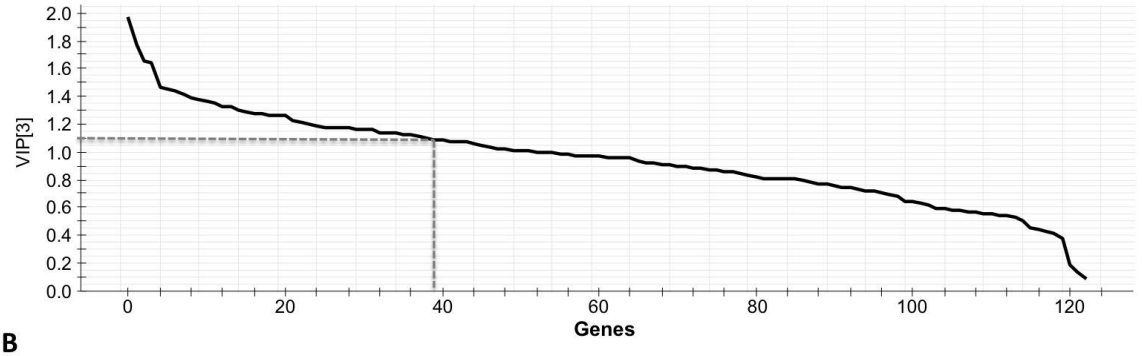


B

Order	Gene (tissue)	VIP (Component 1+2)	LD		HD		P-value		
			Normoxia	Hypoxia	Normoxia	Hypoxia	[O2]	Density	Interaction
1	*cs* (Heart)	1.675	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
2	*ghr-i* (WM)	1.644	6.42 ± 0.57	5.63 ± 0.20	4.14 ± 0.40	4.84 ± 0.51	0.915	0.002	0.101
3	*ucp1* (Liv)	1.618	23.04 ± 2.14	19.01 ± 1.99	17.55 ± 2.17	15.98 ± 1.46	0.218	0.002	0.527
4	*igfbp6b* (WM)	1.617	0.53 ± 0.05	0.59 ± 0.06	1.01 ± 0.10	0.65 ± 0.05*	0.056	0.001	0.009
5	*gpx4* (WM)	1.565	1.78 ± 0.48	1.01 ± 0.39	0.78 ± 0.21	0.43 ± 0.08	0.135	0.038	0.559
6	*Mn-sod / sod2* (Heart)	1.552	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
7	*igfr1* (WM)	1.512	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
8	*igfbp5b* (WM)	1.510	5.16 ± 0.39	6.21 ± 0.35	7.08 ± 0.47	6.18 ± 0.29	0.846	0.019	0.016
9	*pgc1β* (Liv)	1.505	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
10	*ghr-ii* (Liv)	1.491	2.39 ± 0.25	2.62 ± 0.35	4.00 ± 0.59	2.70 ± 0.26	0.182	0.032	0.059
11	*prdx5* (Heart)	1.460	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
12	*insr* (WM)	1.423	3.17 ± 0.15	3.26 ± 0.29	4.30 ± 0.43	3.28 ± 0.17*	0.105	0.050	0.057
13	*lpl* (Liv)	1.421	5.77 ± 0.78	6.57 ± 0.56	8.08 ± 0.99	8.75 ± 0.82	0.361	0.010	0.949
14	*elovl6* (Liv)	1.409	0.89 ± 0.12	0.89 ± 0.14	0.52 ± 0.06	0.60 ± 0.07	0.683	0.004	0.713
15	*gpx4* (Liv)	1.402	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
16	*prdx5* (Liv)	1.389	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
17	*nd5* (Heart)	1.385	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
18	*pgc1α* (Liv)	1.372	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.02	0.12 ± 0.02	0.304	0.010	0.558
19	*nd2* (Liv)	1.358	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
20	*mthsp70/grp-75/mortalin* (Heart)	1.338	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
21	*hif-1α* (Heart)	1.332	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
22	*igf-ii* (Liv)	1.287	2.85 ± 0.44	3.73 ± 0.53	5.37 ± 0.57	3.31 ± 0.76*	0.336	0.094	0.022
23	*pgc1α* (WM)	1.264	0.67 ± 0.21	0.30 ± 0.09	0.35 ± 0.07	0.27 ± 0.07	0.096	0.183	0.273
24	*sirt1* (Liv)	1.252	0.10 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.067	0.089	0.467
25	*sirt2* (Liv)	1.245	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
26	*mthsp70/grp-75/mortalin* (Liv)	1.223	0.56 ± 0.04	0.55 ± 0.04	0.80 ± 0.10	0.55 ± 0.05*	0.033	0.044	0.053
27	*igfr2* (WM)	1.209	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
28	*nd2* (Heart)	1.209	68.14 ± 4.68	70.41 ± 5.69	64.19 ± 5.02	52.61 ± 2.44*	0.322	0.026	0.145
29	*ucp3* (WM)	1.204	11.65 ± 1.78	6.43 ± 0.93*	16.89 ± 4.46	6.91 ± 0.74*	0.006	0.272	0.359
30	*prdx3* (Heart)	1.199	2.14 ± 0.15	2.08 ± 0.17	1.97 ± 0.13	1.61 ± 0.14	0.168	0.041	0.305
31	*Mn-sod / sod2* (Liv)	1.192	1.20 ± 0.05	1.02 ± 0.08	1.30 ± 0.09	0.94 ± 0.08*	0.001	0.955	0.280
32	*sirt5* (Heart)	1.182	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
33	*igf-i* (Liv)	1.180	9.51 ± 0.58	9.26 ± 1.20	13.29 ± 1.86	11.94 ± 0.79	0.514	0.013	0.654
34	*hif-1α* (WM)	1.178	5.45 ± 0.35	4.55 ± 0.30	6.67 ± 0.69	5.51 ± 0.31	0.028	0.020	0.773
35	*sirt1* (WM)	1.177	0.73 ± 0.04	0.70 ± 0.04	0.84 ± 0.04	0.72 ± 0.03*	0.050	0.104	0.263
36	*mthsp70/grp-75/mortalin* (WM)	1.158	5.58 ± 0.27	5.86 ± 0.43	6.96 ± 0.65	5.83 ± 0.47	0.380	0.163	0.147
37	*igfbp3* (WM)	1.156	9.33 ± 0.89	11.68 ± 1.03	13.17 ± 0.99	14.97 ± 2.18	0.143	0.015	0.845
38	*nd5* (Liv)	1.149	16.51 ± 0.83	14.41 ± 0.73	18.11 ± 1.54	14.20 ± 1.11*	0.010	0.533	0.416
39	*sirt4* (Heart)	1.145	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
40	*igf-i* (WM)	1.144	0.76 ± 0.09	0.67 ± 0.06	0.65 ± 0.11	0.63 ± 0.08	0.533	0.387	0.657
41	*gr* (Heart)	1.140	0.42 ± 0.03	0.40 ± 0.03	0.37 ± 0.01	0.33 ± 0.01*	0.136	0.012	0.574
42	*sirt1* (Heart)	1.138	0.22 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.18 ± 0.01*	0.023	0.219	0.572
43	*cpt1a* (Heart)	1.138	1.63 ± 0.15	1.28 ± 0.12	1.23 ± 0.12	1.21 ± 0.10	0.142	0.071	0.189
44	*pgc1α* (Heart)	1.119	2.55 ± 0.15	2.28 ± 0.15	2.74 ± 0.26	2.08 ± 0.10*	0.012	0.966	0.274

Figure 7. A) Graphical representation of the variable importance (VIP) scores after component 2. B) Ranking list of genes showing VIP score values above 1.1 and their relative gene expression. Cells shaded in blue highlight genes detected as VIP after component 1; cells shaded in yellow highlight genes detected as VIP after component 2. For further details, see legend on Figure 6.

A



B

Order	Gene (tissue)	VIP (Component 1+2+3)	LD		HD		P-value		
			Normoxia	Hypoxia	Normoxia	Hypoxia	[O2]	Density	Interaction
1	*fads2* (Liv)	1.966	3.82 ± 0.42	9.19 ± 0.97***	5.10 ± 0.60	4.98 ± 0.66	<0.001	0.048	<0.001
2	*cpt1a* (WM)	1.764	9.05 ± 1.02	6.72 ± 0.43*	7.44 ± 0.46	10.74 ± 1.25*	0.564	0.155	0.002
3	*scd1b* (Liv)	1.657	0.22 ± 0.03	0.52 ± 0.13*	0.22 ± 0.05	0.19 ± 0.02	0.076	0.024	0.026
4	*scd1a* (Liv)	1.639	0.43 ± 0.09	1.15 ± 0.19**	0.53 ± 0.23	0.56 ± 0.14	0.042	0.018	0.050
5	*pgc1β* (Liv)	1.462	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
6	*pgc1α* (Liv)	1.456	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.02	0.12 ± 0.02	0.304	0.010	0.558
7	*cs* (Heart)	1.435	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
8	*coxi* (WM)	1.414	740.2 ± 58.4	732.6 ± 26.9	834.2 ± 61.6	957.9 ± 57.1	0.271	0.005	0.214
9	*ghr-i* (WM)	1.385	6.42 ± 0.57	5.63 ± 0.20	4.14 ± 0.40	4.84 ± 0.51	0.915	0.002	0.101
10	*elovl6* (Liv)	1.376	0.89 ± 0.12	0.89 ± 0.14	0.52 ± 0.06	0.60 ± 0.07	0.683	0.004	0.713
11	*ucp1* (Liv)	1.362	23.04 ± 2.14	19.01 ± 1.99	17.55 ± 2.17	15.98 ± 1.46	0.218	0.002	0.527
12	*igfbp6b* (WM)	1.349	0.53 ± 0.05	0.59 ± 0.06	1.01 ± 0.10	0.65 ± 0.05*	0.056	0.001	0.009
13	*Mn-sod / sod2* (Heart)	1.326	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
14	*ghr-ii* (Liv)	1.324	2.39 ± 0.25	2.62 ± 0.35	4.00 ± 0.59	2.70 ± 0.26	0.182	0.032	0.059
15	*gpx4* (WM)	1.306	1.78 ± 0.48	1.01 ± 0.39	0.78 ± 0.21	0.43 ± 0.08	0.135	0.038	0.559
16	*prdx5* (Heart)	1.286	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
17	*igfbp5b* (WM)	1.273	5.16 ± 0.39	6.21 ± 0.35	7.08 ± 0.47	6.18 ± 0.29	0.846	0.019	0.016
18	*igfr2* (WM)	1.273	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
19	*igfr1* (WM)	1.266	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
20	*igf-ii* (Liv)	1.265	2.85 ± 0.44	3.73 ± 0.53	5.37 ± 0.57	3.31 ± 0.76*	0.336	0.094	0.022
21	*lpl* (Liv)	1.260	5.77 ± 0.78	6.57 ± 0.56	8.08 ± 0.99	8.75 ± 0.82	0.361	0.010	0.949
22	*grp-170* (Liv)	1.224	1.03 ± 0.08	1.41 ± 0.17	1.37 ± 0.19	1.11 ± 0.08	0.635	0.878	0.023
23	*mthsp70/grp-75/mortalin* (Heart)	1.209	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
24	*insr* (WM)	1.194	3.17 ± 0.15	3.26 ± 0.29	4.30 ± 0.43	3.28 ± 0.17*	0.105	0.050	0.057
25	*cpt1a* (Heart)	1.185	1.63 ± 0.15	1.28 ± 0.12	1.23 ± 0.12	1.21 ± 0.10	0.142	0.071	0.189
26	*prdx5* (Liv)	1.179	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
27	*nd5* (Heart)	1.178	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
28	*gpx4* (Liv)	1.176	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
29	*sirt4* (WM)	1.170	0.15 ± 0.01	0.14 ± 0.01	0.17 ± 0.02	0.18 ± 0.01	0.672	0.025	0.343
30	*sirt2* (Liv)	1.166	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
31	*sirt5* (Heart)	1.159	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
32	*pgc1α* (WM)	1.157	0.67 ± 0.21	0.30 ± 0.09	0.35 ± 0.07	0.27 ± 0.07	0.096	0.183	0.273
33	*nd2* (Liv)	1.139	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
34	*sirt4* (Liv)	1.132	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.520	1.000	0.608
35	*sirt4* (Heart)	1.131	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
36	*coxii* (Liv)	1.125	44.07 ± 2.34	37.08 ± 1.90*	39.36 ± 3.29	39.94 ± 2.30	0.205	0.711	0.137
37	*sirt7* (WM)	1.117	0.24 ± 0.01	0.25 ± 0.02	0.27 ± 0.01	0.28 ± 0.02	0.801	0.047	0.898
38	*hif-1α* (Heart)	1.112	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
39	*hif-1α* (WM)	1.101	5.45 ± 0.35	4.55 ± 0.30	6.67 ± 0.69	5.51 ± 0.31	0.028	0.020	0.773

Figure 8. A) Graphical representation of the variable importance (VIP) scores after component 3. B) Ranking list of genes showing VIP score values above 1.1 and their relative gene expression. Cells shaded in blue highlight genes detected as VIP after component 1; cells shaded in yellow highlight genes detected as VIP after component 2; cells shaded in purple highlight genes detected as VIP after component 3. For further details, see legend on Figure 6.

From the gene expression profiling of key metabolic biomarkers, it is noteworthy that liver, white skeletal muscle and heart remained responsive at long-term to changing O_2 and rearing density, whereas the expression pattern of blood cells was mostly unaltered with the imposed stress stimuli of medium intensity. In previous studies in sea bream and other animal models, liver and cardiac muscle are highly responsive to hypoxia (**Everett *et al.*, 2012**; **Hermes-Lima *et al.*, 2015**; **Magnoni *et al.*, 2017**), and genes of these two tissues highly contribute herein to separate normoxic and hypoxic fish along the first component that predicts more than 28% of total variance. One of the most relevant genes participating in this discriminant feature is the *hif-1α*, a well-documented regulator of O_2 homeostasis. This transcriptional factor acts at a multi-regulatory level, managing the hypoxic responsiveness of a vast array of transcribed proteins including antioxidant enzymes. Concretely, herein, we show a clear down-regulation of *hif-1α* that was coincident with the repressed expression of other down-stream markers of antioxidant defence and tissue repair (*prdx5*, *sod2*, *mortalin*, *gpx4*, *gr*, *grp-170*, *prdx3*). This intriguing result can be cautiously interpreted since Hif-1 is mostly regulated at the post-translational level, though this finding should be understood as a steady-state in which O_2 availability is reduced but maintained high enough to preserve aerobic metabolism at a relatively high level. This fact is supported by a reduced expression of *cs* and associated enzyme subunits of Complex I (*nd2*, *nd5*), markers of mitochondria abundance and Krebs cycle activity (**Larsen *et al.*, 2012**; **Magnoni *et al.*, 2017**). In addition to that, several *sirts* (*sirt1, 2, 5, 6, 7*) of liver or cardiac muscle were overall down-regulated in hypoxic fish, especially in the case of HDH fish. These NAD^+-dependent deacetylases are energy sensors that act in sea bream as a link between nutrition and energy metabolism in different growth models with nutrients and genetic variables as source of variation (**Simó-Mirabet *et al.*, 2017a**; **2017b**; **2018**). This was extended herein to hypoxia/crowding stress, which indicates that most of the envisaged adaptive responses should include changes in the acetylation status of both nuclear histones, and cytoplasmic and mitochondrial metabolic enzymes.

The second component of the PLS-DA (R^2=29.27%) differentiated normoxic fish held at different stocking densities. White skeletal muscle clearly promoted this separation mainly by the expression pattern of genes related to GH/IGF system (*ghr-i*, *igfbp6b*, *igfbp5b*, *insr*, *igfbp3*, *igf-i*). Components of liver and muscle GH/IGF system are differentially regulated by nutrients and seasonal environmental cues (reviewed by **Pérez-Sánchez *et al.*, 2018**), but herein this observation becomes especially relevant for muscle *ghr-i* that highly contributed to discriminate the detrimental growth effects of crowding stress from those more related to hypoxia or water quality. Likewise, genes of *igfbp* repertoire highly contribute to this differentiation, though the discriminant role of Igfbp counterparts (*igfbp6b* > *igfpb5b* > *igfpb3)* were mostly reduced to skeletal muscle and *Igfbp3/5/6* clade. The overall depressed expression of the muscle *Igfbp* clade in HD fish is consistent with inhibitory rather than stimulatory growth-promoting effects, which also involves the regulation of insulin and Igfbp receptors with important implications on the final arrangements of carbohydrate, growth and energy metabolism (**Reindl & Sheridan, 2012**; **Vélez *et al.*, 2017**). Indeed, fish are the first group in the vertebrate tree in which there is evidence of distinct insulin and Igf receptors, though certain cross-reactivity between ligand and receptors of insulin and Ifgs occurs and the specific-mediated effects are sometimes confounding. However, it is well-recognized that insulin stimulates Hif-1, whereas intermittent hypoxia induces insulin resistance in mice (**Poulain *et al.*, 2017**). It is also conclusive that the muscle expression of *igfr1 and igfr2* are especially responsive to hypoxia, but importantly *insr* in sea bream seems to be more receptive to crowding stress rather than hypoxic stress stimuli, though it remains to be established the functional relevance of this differential responsiveness to environmental stressors.

The third component of the multivariate approach (R^2=25.42%) discriminated the effect of stocking density in fish exposed to moderate hypoxia, with a marked contribution of hepatic fatty desaturases with Δ6 (*fads2*) or Δ9 (*scd1a*, *scd1b*) activities due to its strong and specific induction in LDH fish. A muscle marker of FA oxidation (*cpt1a*) was also consistently up-

regulated in this group, but this response was opposite to that found in HDH group, which is indicative of the different regulation of muscle lipid catabolism by hypoxia in fish stocked at standard or high densities. Likewise, the major discriminant capacity of other factors related to lipid metabolism (*elovl6*) was achieved between normoxic fish held at LD and HD. Previous studies, in sea bream (**Benedito-Palos *et al.*, 2013; 2014**) and European sea bass (**Rimoldi *et al.*, 2016**) have also evidenced an important effect of ration size on the hepatic and muscle regulation of most of the lipid biomarkers assessed, but again it is difficult to disclose what is the main factor (feed intake or the imposed stress condition). However, as a general rule, stressors enhance the demand of specific nutrients and hypoxia in particular promote the cellular uptake of extracellular unsaturated fatty acids in mice cell lines (**Ackerman *et al.*, 2018**). Moreover, in hypoxic stress, cancer cells enhance lipid synthesis that is important for membrane biosynthesis and energy storage for cell survival and proliferation (**Huang *et al.*, 2014**). All this together supports the pronounced stimulation of *fads2* and *scd* desaturases in our stress model, which will promote the increase of the unsaturation index of structural lipids as previously reported during feed restriction in sea bream (**Benedito-Palos *et al.*, 2013**).

3.4 Main outcomes- hypoxia and high stocking density phenotyping

The combined effects of moderate hypoxia and rearing density in a 3-week feeding trial highlighted a different contribution of target tissues to the homeostatic load in challenged fish. The re-adjustment of several biological functions was evidenced, and most of the hypoxia-mediated effects on growth performance and energy metabolism were exacerbated in fish held at HD. Interestingly, the integrated data on blood haematology, biochemistry and hormonal profiling evidenced a hypo-metabolic state with the enhancement of O_2-carrying capacity, accompanied by a more efficient energy metabolism that could be considered a convenient feature, though it has been achieved at the expense of a reduction in voluntary feed intake leading to lower growth rates. Transcriptional results also support these findings, and the tissue-specific orchestration of stress response reflected the different metabolic capacities of targeted tissues. Indeed, the number of DE genes in response to studied stress stimuli varied across tissues (liver ≥ heart > skeletal muscle > blood). As a practical issue, the validity of blood for a non-invasive monitoring at the molecular level of hypoxia responses becomes limited to severe hypoxia, whereas the liver, heart and skeletal appears more sensitive, contributing to differentiate hypoxia and crowding stress responses.

4. Transient effects of hypoxia pre-conditioning during juvenile stages on metabolic condition and swimming performance

Environmental conditions encountered by living organisms during critical windows of early life stages can affect their development. This concept is termed developmental plasticity, and although the resulting modification of traits derived from an early challenge can often end in detrimental features, some of them can derive in adaptive changes. Indeed, adaptive developmental plasticity allows organisms to tune their phenotypes in response to certain environmental conditions in order to produce animals that are able to better tolerate similar conditions at later life stages. Thus, the application of metabolic priming becomes a tool of interest with a great potential for aquaculture production.

In zebrafish, for instance, it has been suggested that early hypoxia exposure may increase the subsequent hypoxia tolerance at adult stage (**Robertson *et al.*, 2014**). Similar long-term beneficial effects remain to be determined in marine water species. In European sea bass, the combined effect of hypoxia and temperature during the larval period resulted in adverse

carry-over effects in juvenile performance, including the prevalence of opercular abnormalities (**Cádiz *et al.*, 2018**). It this is the case of sea bream remains mostly unexplored, and we first assessed during the juvenile window stage the prevalence of potential benefits of hypoxia pre-conditioning upon metabolism and swimming performance, taking advantage of the new knowledge generated in previous sections.

4.1 Experimental setup- hypoxia pre-conditioning at juvenile stages

Juvenile fish (~24 g average body weight) were distributed in 90-L tanks of re-circulatory system previously detailed in **Figure 4**. Water temperature was daily monitored and maintained at 25-26ºC. After 5 days of acclimation period, the water parameters of six tanks were kept unchanged, constituting the normoxic (>5.5 ppm O_2; >85% O_2 saturation) group. Fish maintained in the remaining six tanks experienced a gradual decrease in the water O_2 level until LOS (3.0 ppm, 42-43% O_2 saturation), constituting the hypoxic group (LOS). This hypoxic challenge lasted for six weeks, being fed all LOS fish to visual satiety one time per day with a commercial diet (EFICO Forte 824, BioMar). Three tanks of normoxic fish were also fed close to satiety, constituting the Normoxia group. The feeding level of the three remaining tanks was adjusted to that of the LOS group (Normoxia pair-fed).

At day 46, 6-10 fish (2-3 per tank) per experimental condition and following overnight fasting were anaesthetized with 3-aminobenzoic acid ethyl ester, weighed and sampled for blood and tissues. Liver and viscera were weighed, and representative biopsies of tissues (liver, skeletal muscle, hear, gills, anterior and posterior intestine) were immediately snap-frozen in liquid nitrogen and stored at -80ºC for RNA extraction. Blood aliquots were processed for Hc and Hb using the same procedures as previously detailed. Plasma triglycerides (TG) were determined using lipase/glycerol kinase/glycerol-3-phosphate oxidase reagent (ThermoFisher Scientific). Quantitation of non-esterified fatty acids in plasma was conducted by means of the *in vitro* enzymatic colorimetric method of NEFA C test kit (Wako Chemicals GmbH). Other plasma biochemical and hormonal analysis were conducted as previously detailed.

At this stage, all the remaining fish were weighed and normoxia conditions were restored for all groups. This recovery period lasted for three weeks, and all fish were daily fed to visual satiety. During this period, swimming performance test were run at three time points: at the end of hypoxia challenge (T1, for all three experimental groups), one week after hypoxia pre-conditioning (T2, for Normoxia and LOS groups) and 3 weeks after hypoxia pre-conditioning (T3, Normoxia ad libitum and LOS). A schematic representation of the experimental procedure is detailed in **Figure 9**.

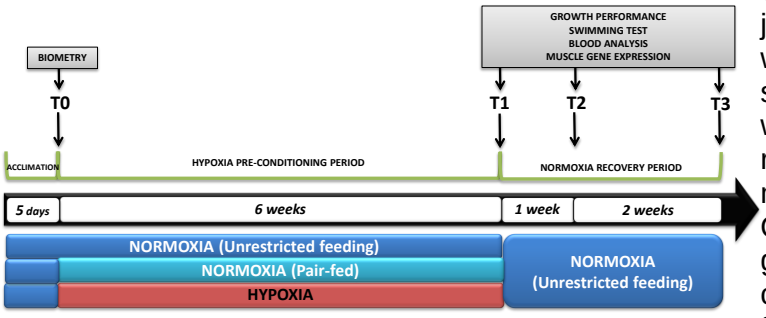


Figure 9. Experimental procedure of the hypoxia pre-conditioning of juvenile fish. The O_2 steady-state was set at ~3.0 ppm (42-43% O_2 saturation) in fish kept at LOS, whereas pair- fed and non-restricted normoxic fed fish were maintained above 5.5 ppm (>85 % O_2 saturation). After 6 weeks, all groups were restored to normoxia condition under non-restricted feeding for a 3-weeks extended period.

Fish metabolic rates and swimming performance were assessed in an intermittent-closed swim tunnel respirometer with a swimming chamber measuring 10 x 10 x 40 cm (10 L water volume, Model PA10500, Loligo® Systems; **Figure 10**). The swim tunnel was submerged into a water bath that served as a water reservoir for flushing the respirometer after each closed respirometry run (flush pump: Eheim 1048, 10 L/min). The water bath was connected by a second flush pump (Eheim 1250, 20 L/min) to a 100 L-reservoir tank coupled to the recirculatory system that provided water O_2 content close to LOS for all swimming performance tests. Unionized ammonia, nitrites and nitrates were at undetectable concentrations along experiments. A thruster within the respirometer was used to generate a swimming current. Water velocities in the swimming section were calibrated at the experimental temperature using a hand-held digital flow meter (Handheld flowtherm NT, Höntzsch GmbH) prior to experimentation, and ordered by the controller Movitrac® LTE 0.37kW/0.5HP (SEW Eurodrive). Respirometry runs and chamber flushing were controlled with the DAQ-M instrument (Loligo® Systems) connected to a PC equipped with AutoResp™ software (Loligo® Systems). Water temperature and O_2 saturation within the respirometer was measured using a Witrox 1 single channel oxygenmeter (Loligo® Systems), equipped with a needle-type fibre optical micro-sensor (NTH, PreSens-Precision Sensing GmbH) and a temperature probe (HTF50 Pt1000 1/3DIN, S+S Regeltechnik GmbH), suspended into the water current within the respirometer. Sensors were calibrated according to the manufacturer's instructions. The oxygen meter's analog output was also fed into the DAQ-M interface and processed using AutoResp™ software.

Figure 10. Loligo® System swimming chamber with respirometer.

Prior each exercise test, overnight fasted fish were slightly anaesthetized with MS-222 (100 mg/L seawater) to guarantee a safe transfer into the swim tunnel respirometer. Fish were then allowed to recover and acclimate to the swimming chamber at a swimming speed of 0.50-1.0 body lengths per second (BL/s) until their O_2 consumption rate (MO_2, $mgO_2/kg/h$) levelled out to a constant low plateau. This typically happened after 30-45 min, reaching values around 240-250 $mgO_2/kg/h$. Swim trials were performed between 9.00 and 16.00 h, but no differences associated circadian rhythms were observed. Trials started with a swimming velocity of 1.0 BL/s. Then, swimming velocity was increased in 0.50 BL/s steps per interval until the fish was exhausted. A fish was considered exhausted when it rested at the back grid of the swimming chamber for at least 10 s. Each swimming interval at a given velocity lasted 5 min, consisting in "flush-wait-measurement" cycles (a 60 s flush interval to exchange the respirometer water = "flush"; a 30 s mixing phase in closed mode = "wait"; and a 210 s MO_2 measuring period in closed mode). During the measurement interval, O_2 saturation of the swim tunnel water was recorded every second. MO_2 was automatically

calculated by the AutoResp^{TM} software from linear decreases in chamber O_2 saturation using the appropriate constants for O_2 solubility in seawater (salinity, temperature and barometric pressure). The critical swimming speed (U_{crit}) was calculated using the equation previously described by **Brett (1964)**:

$$U_{crit} = u_{high} + [(t_{exh}/t_{inter}) \cdot u_{incr}]$$

being u_{high} the highest swimming velocity maintained for the entire time interval (BL/s); t_{exh} the time interval spent at the exhaustion velocity (min); t_{inter} the time interval at each swimming speed (5 min); and u_{incr} the velocity increment in each new interval (0.50 BL/s).

After the exercise test, fish were anesthetized and blood was withdrawn for biochemical and hormonal analysis as previously detailed. Fish were then killed by cervical section and representative biopsies of skeletal muscle were extracted for lactate determination and liquid nitrogen storage for subsequent RNA extraction and RNA-Seq analysis.

4.2 Growth and swimming performance after hypoxia pre-conditioning and normoxia recovery

During the hypoxia pre-conditioning period, normoxic unrestricted fed fish grew faster (78.69 g *vs* 66.06 g; SGR 2.59 % *vs* 2.23 %) than LOS fish (**Table 5**). The same trend was stated in the previous section in a 3 weeks factorial model of hypoxia and stocking densities. However, when the ration size in normoxia was lowered to that of the LOS group (Normoxia Pair-fed), biometric parameters were undistinguishable for both groups, pointing out that the differences in growth performance between normoxic and hypoxic groups can be mostly attributed to a reduced feed intake, which becomes especially evident in hypoxic crowded fish (see section 3).

As also shown in **Table 5**, the return to normoxia values in the LOS group rapidly increased the voluntary feed intake at the same rates as the other two experimental groups under non-restricted feeding. Thus, the feed intake and percentage of weight gain one week after hypoxia pre-conditioning was almost the same in all three groups. Along the two following weeks, feed intake remained almost equal among experimental groups, though some compensatory growth was observed in Normoxia pair-fed and LOS groups, with a statistically significant increase of weight gain and SGR. Nonetheless, this compensatory growth was not enough to achieve in three weeks the same final body weight than that of the normoxic group fed to satiety along the entire experimental period.

Table 5. Effects of feeding regime and O_2 availability on sea bream growth performance during hypoxia pre-conditioning and the subsequent normoxic-unrestricted feeding period. Values on body weight, feed intake, growth and feed efficiency are the mean ± SEM of replicate tanks. P-values are the result of one-way ANOVA. Different superscript letters indicate significant differences between experimental groups (SNK test, P <0.05).

	Normoxia	Normoxia Pair-fed	LOS	P-value
T0-T1 period				
Initial body weight (g)	24.58 ± 0.11	24.1 ± 0.10	24.19 ± 0.03	0.112
Final body weight (g)	78.69 ± 0.79 [b]	66.13 ± 1.41[a]	66.06 ± 0.97[a]	<0.001
Feed intake (g DM/fish)	53.36 ± 0.15[b]	40.77 ± 0.22[a]	40.08 ± 0.84[a]	<0.001
Weight gain (%)[1]	220.30 ± 2.03[b]	174.51 ± 4.50[a]	173.22 ± 3.86[a]	<0.001
SGR (%)[2]	2.59 ± 0.01[b]	2.24 ± 0.04[a]	2.23 ± 0.03[a]	<0.001
FE (%)[3]	1.014 ± 0.009	1.031 ± 0.030	1.044 ± 0.008	0.285
T1-T2 period				
Initial body weight (g)	78.69 ± 0.67[b]	66.13 ± 1.41[a]	66.06 ± 0.97[a]	<0.001
Final body weight (g)	98.76 ± 1.20[b]	83.50 ± 0.50[a]	82.00 ± 1.14[a]	<0.001
Feed intake (g DM/fish)	21.34 ± 1.14	21.31 ± 0.85	19.59 ± 0.53	0.239
Weight gain (%)[1]	25.00 ± 0.58	26.50 ± 1.50	22.60 ± 1.63	0.292
SGR (%)[2]	2.48 ± 0.05	2.61 ± 0.16	2.27 ± 0.15	0.351
FE (%)[3]	0.92 ± 0.04	0.89 ± 0.01	0.83 ± 0.06	0.552
T2-T3 period				
Initial body weight (g)	98.76 ± 1.20b	83.50 ± 0.50a	82.00 ± 1.14a	<0.001
Final body weight (g)	126.5 ± 1.30b	114.7 ± 0.33a	111.3 ± 1.81a	0.001
Feed intake (g DM/fish)	37.62 ± 1.28	36.57 ± 1.50	35.66 ± 0.50	0.329
Weight gain (%)1	28.54 ± 0.46a	37.22 ± 1.33b	35.50 ± 0.85b	0.001
SGR (%)2	1.79 ± 0.03a	2.26 ± 0.07b	2.19 ± 0.04b	<0.001
FE (%)3	0.82 ± 0.02	0.89 ± 0.01	0.87 ± 0.01	0.103

[1]Weight gain (%) = (100 x body weigh increase)/initial body weight
[2]Specific growth rate = 100 x (ln final body weight - ln initial body weight)/days
[3]Feed efficiency = wet weight gain/dry feed intake
[4]Hepatosomatic index = (100 x liver weight)/fish weight
[5]Viscerosomatix index = (100 x viscera weight)/fish weight

Blood parameters did not provide a clear group differentiation at the end of the hypoxia pre-conditioning period, except for plasma lactate levels, that were significantly decreased in Normoxia pair-fed and LOS fish (**Table 6**). Normoxia pair-fed fish also evidenced lower Hb levels. These features would be indicative of a state of hypo-metabolism, which was reinforced by a lowest concentration of circulating free fatty acids (lower tissue lipolytic rates) in LOS group. In contrast, these animals showed the highest concentration of circulating free fatty acids after exhaustive exercise at T1 (**Table 7**), which would support a better swimming performance in a state of low energy demand, as evidenced by lowered circulating cortisol and Gh levels, a trend that was also shown in exercised fish following one week of hypoxia pre-conditioning (T2). Exercise also induced higher accumulation of muscular lactate in LOS fish after the pre-conditioning period (T1), a feature that was retained in those animals in subsequent swimming tests conducted at one (T2) or three weeks (T3) after hypoxia-pre-conditioning. Plasma lactate levels were also increased in LOS fish after exercise at T2 and T3, as an indication of a higher activation of anaerobic metabolism in hypoxia pre-conditioned fish.

Table 6. Effects of feeding regime and O_2 availability on blood haematology and biochemistry at the end of hypoxia pre-conditioning period. Values are the mean ± SEM of 6-10 fish (2-3 fish per replicate tank). P-values are the result of one-way ANOVA. Different superscript letters indicate significant differences between experimental groups (SNK test, P <0.05).

T1 sampling time	Normoxia	Normoxia Pair-fed	LOS	P-value
Haemoglobin (g)	8.36 ± 0.38^{b}	6.43 ± 0.64^{a}	7.88 ± 0.22^{b}	0.011
Haematocrit (%)	34.67 ± 1.24	33.67 ± 0.99	31.00 ± 1.41	0.175
Glucose (mg/dl)	57.14 ± 5.98	55.73 ± 2.29	56.86 ± 2.35	0.493
Lactate (mg/dl)	14.14 ± 0.15^{b}	6.32 ± 0.57^{a}	4.18 ± 0.77^{a}	<0.001
Triglycerides (mg/dl)	2.80 ± 0.28	4.02 ± 0.34	3.02 ± 0.46	0.128
Free fatty acids (nmol/µl)	0.426 ± 0.052^{ab}	0.595 ± 0.045^{b}	0.388 ± 0.045^{a}	0.029
Cortisol (ng/ml)	24.12 ± 5.43	29.27 ± 10.56	14.29 ± 4.71	0.270
Growth hormone (ng/ml)	9.19 ± 3.94	12.42 ± 5.30	13.90 ± 4.87	0.752
Insulin-like growth factor-I (ng/ml)	57.89 ± 4.49	60.56 ± 3.42	51.56 ± 3.54	0.285

Table 7. Effects of hypoxia pre-conditioning and subsequent recovery (1 or 3 weeks) on blood and tissue biochemistry after swimming exercise until exhaustion. Values are the mean ± SEM of 6-7 fish (2-3 fish per replicate tank). Statistically significant differences between experimental groups are indicated (*P <0.05, **P <0.01, ***P <0.001; Student's *t*-test).

T1 sampling time	Normoxia	LOS	P-value
Glucose (mg/dl)	215.8 ± 12.34	179.9 ± 28.10	0.227
Lactate (mg/dl)	103.7 ± 4.41	92.27 ± 5.06	0.120
Muscular lactate (mg/g bm)	44.91 ± 0.75	56.10 ± 1.56***	<0.001
Triglycerides (mg/dl)	2.99 ± 0.49	1.82 ± 0.15	0.187
Free fatty acids (nmol/µl)	0.497 ± 0.06	1.142 ± 0.32*	0.047
Cortisol (ng/ml)	235.0 ± 23.74	160.3 ± 41.65	0.151
Growth hormone (ng/ml)	16.17 ± 7.13	9.02 ± 2.28	0.529
Insulin-like growth factor-I (ng/ml)	77.34 ± 5.65	61.49 ± 4.76	0.058
T2 sampling time			
Glucose (mg/dl)	225.6 ± 10.18	168.6 ± 17.39*	0.018
Lactate (mg/dl)	104.1 ± 6.57	124.1 ± 2.64*	0.017
Muscular lactate (mg/g bm)	50.65 ± 1.86	59.27 ± 3.13*	0.039
Triglycerides (mg/dl)	2.51 ± 0.55	1.88 ± 0.24	0.323
Free fatty acids (nmol/µl)	0.423 ± 0.031	0.789 ± 0.301	0.253
Cortisol (ng/ml)	293.5 ± 41.24	165.3 ± 46.59	0.066
Growth hormone (ng/ml)	21.85 ± 3.51	10.51 ± 2.84*	0.031
Insulin-like growth factor-I (ng/ml)	84.73 ± 7.83	65.72 ± 7.09	0.102
T3 sampling time			
Glucose (mg/dl)	211.9 ± 11.76	210.5 ± 13.54	0.936
Lactate (mg/dl)	104.5 ± 7.19	126.4 ± 4.59*	0.025
Muscular lactate (mg/g bm)	51.46 ± 2.03	59.01 ± 1.72*	0.015
Triglycerides (mg/dl)	2.66 ± 0.32	2.75 ± 0.25	0.836
Free fatty acids (nmol/µl)	0.537 ± 0.119	0.635 ± 0.114	0.549
Cortisol (ng/ml)	235.2 ± 44.61	246.7 ± 19.39	0.818
Growth hormone (ng/ml)	11.13 ± 4.76	11.99 ± 2.43	0.874
Insulin-like growth factor-I (ng/ml)	72.17 ± 6.14	71.98 ± 6.20	0.983

Swimming tests conducted at the end of the pre-conditioning period (T1) displayed similar MO_2 in fish coming from the normoxic or LOS groups (**Figure 11A**), but the latter fish were able to attain a much higher U_{crit} (**Figure 11D**). After one week in normoxia and unrestricted feeding (T2), the MO_2 consumption in the swimming test was sustainably higher for fish from the LOS group at water speeds higher than 4.5 BL/s, and the maximum rate of O_2 consumption (maximum metabolic rate, MMR) was also higher for this group (**Figure 11B**). This feature was accompanied by a much higher U_{crit} for hypoxia pre-conditioned fish (**Figure 11E**). Both results were indicative of an improvement of the aerobic scope and the swimming performance in the group of hypoxia pre-conditioning. This better performance was still reported after three weeks of return to normoxia (T3), though differences with the normoxic control group were diminished with respect to the previous T2 test (**Figures 11C, 11F**).

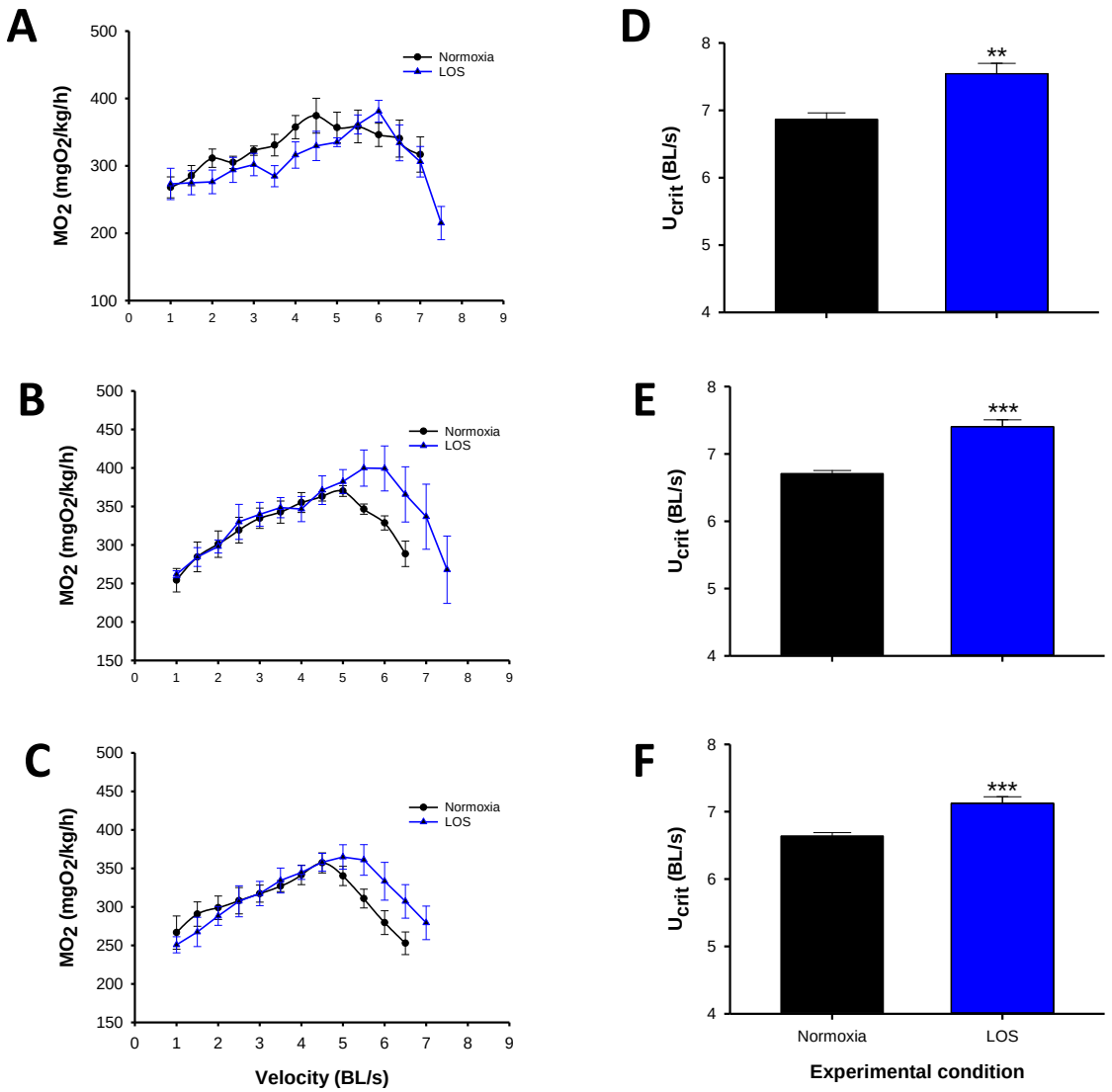


Figure 11. Effects of hypoxia pre-conditioning and subsequent recovery over swimming performance. (**A, B, C**) O_2 consumption rates for Normoxia (black) and LOS (blue) fish at T1, T2 and T3, respectively. (**D, E, F**) critical swimming speed (U_{crit}) at T1, T2 and T3, respectively. Values are the mean ± SEM of 6-7 fish (2-3 fish per replicate tank). Asterisks indicate significant differences between experimental groups (t-test; ** P <0.01, *** P <0.001).

4.3 Main outcomes- hypoxia pre-conditioning at juvenile stages

Hypoxia pre-conditioning during juvenile stages reduced feed intake and growth, but some compensatory catch-up growth was evidenced thereafter with the return to normoxia under unrestricted feeding. At the end of trial, fish coming from LOS conditions still had a lower mean weight but their better growth during the recovery period suggested that similar body weight should be shortly achieved.

The exercise test under low O_2 concentration was aimed to evaluate the swimming performance after hypoxia pre-conditioning, and the results highlighted a higher resistance to exercise exhaustion. Nevertheless, this improved trait tended to disappear progressively over time. Hence, at 3 weeks after hypoxia pre-conditioning, the differences in blood parameters were less evident. Wide-transcriptomic profiling of skeletal muscle (RNA-seq) after exercise exhaustion also confirmed this issue (unpublished/non-delivered results), and the number of DE genes between both groups (Normoxia and LOS) consistently decreased during the normoxia recovery period.

Therefore, a transient improvement in swimming performance has been achieved by means of low O_2 metabolic priming in sea bream juveniles. However, effects on swimming and metabolic performance seem to be diluted over time, and we tested in a final set of experiments if this can be prolonged by earlier life interventions.

5. Potential benefits of metabolic endurance during early life stages to cope with more robust juvenile fish

5.1 Experimental setup of early life hypoxia pre-conditioning

Larvae of sea bream at 45 days post-hatching (dph) and weaned with inert diet were provided by Piscimar (Burriana, Spain) and transferred to IATS-CSIC facilities. The specimens were acclimated in 500-L tanks under natural photoperiod and temperature conditions for 15 days. Feeding was carried out to visual satiety seven times every day with commercial fish diets (Gemma wean 0.2, 0.3, 0.5 Skretting) and water quality was daily checked. After the acclimatization period, 60 dph larvae (mean weight 137 mg) were distributed in six 90-L tanks (n = 150 larvae per tank). The level of dissolved O_2 was maintained in all tanks at 5.8 ppm for two days, and then the O_2 level was gradually decreased in 3 tanks up to 3.8 ppm after 24 h. This low O_2 concentration (3.6-3.8 ppm) was maintained for 21 days (60-81 dph) and normoxia was restored for all tanks up to 112 dph. Then, fish from each tank were distributed in other two tanks (n = 40 fish per tank, mean weight 6.95 g) and O_2 levels were lowered for half of them to 3.6-3.8 ppm during 15 days in order to get four experimental groups according to the normoxic (N) or hypoxic (H) conditions during the 60-81 dph and 112-127 dph time windows (**Figure 12**): NNN, NNH, HNN and HNH. At this stage, overnight fasted fish (n = 12 for each experimental condition) were retrieved, anaesthetized with MS-222 and blood was taken from caudal vessels with EDTA-treated syringes. Blood aliquots were processed as described elsewhere for Hc and Hb. After centrifugation (3,000 x g, 20 min, 4 ºC), plasma samples were frozen and stored at -20 °C until determination of glucose and lactate levels as previously detailed. Other aliquots were processed as described elsewhere for Hc and Hb.

Then, all groups were returned to normoxia for 15 days, and 50 randomly selected fish (mean weight 24.4 g) per experimental condition were individually tagged in the dorsal muscle with passive integrated transponders (PIT-tags, ID-100A 1.25 Nano Transponder). These fish were reared together in 3000-L tanks (“common garden”) for 3 months until final assessment of swimming performance, following the procedure detailed in Section 4.1 under moderate hypoxic conditions (3.6-3.8 ppm) at 23-24ºC.

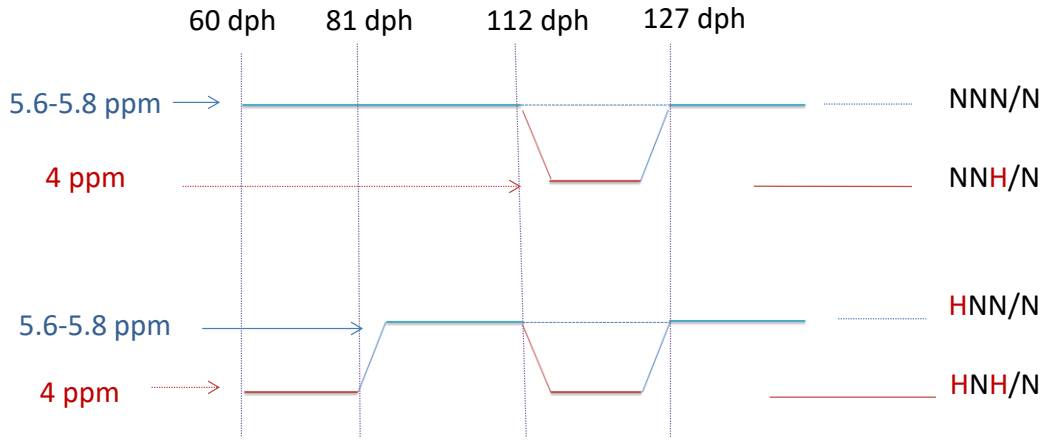
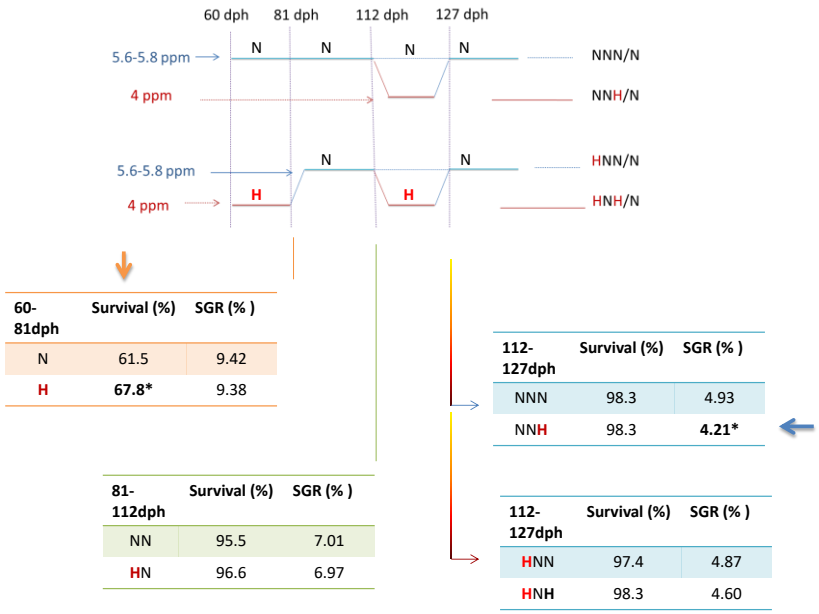


Figure 12. Experimental procedure of the metabolic priming of sea bream early life stages to low O_2 concentration in two time windows (60-81 dph and/or 112-127 dph). The steady-state was set at ~3.6-3.8 ppm O_2 in fish kept under hypoxia (H), whereas fish maintained in normoxia (N) were at a concentration of > 5.5 ppm O_2 . Recovery of normoxic conditions for all groups (/N) lasted for 3 months in common garden until final swimming performance tests.

5.2 Early effects on growth and metabolic performance

Fish grew at very high rates at the beginning of trial (SGR >9) and exposure of fish to reduced O_2 concentrations during the 60-200 dph period did not have a negative impact of growth. Moreover, the reduction of O_2 availability increased survival rates more than 10% in NH group (**Figure 13**). A possible explanation is that hypoxia protocol helps to minimize the oxidative stress of the disproportionate growth of early life stages. This is supported by the observation that other sparid fish, such as common dentex, have increased growth but also very high mortalities, which do not make feasible its farming at industrial scale.

Noticeably, with the reduction of SGR until 5%, mortality rates were mostly negligible and hypoxia priming minimized the reduction of growth in fish exposed again at low O2 concentrations in HNH group. In contrast, a significant reduction of SGR was found in NNH group in comparison to NNN.



60-81dph	Survival (%)	SGR (%)
N	61.5	9.42
H	**67.8***	9.38

81-112dph	Survival (%)	SGR (%)
NN	95.5	7.01
HN	96.6	6.97

112-127dph	Survival (%)	SGR (%)
NNN	98.3	4.93
NNH	98.3	**4.21***

112-127dph	Survival (%)	SGR (%)
HNN	97.4	4.87
HNH	98.3	4.60

Figure 13. Survival rates and SGR for each experimental group at each normoxia (N) /hypoxia (H) time window.

After the second hypoxia challenge (112-127 dph), plasma Hb levels clearly reflected the O_2 concentration, being the measured values in NNN and HNN fish significantly lower than those found in NNH and HNH fish (**Figure 14A**). In contrast, plasma lactate levels highly revealed the early hypoxia exposure, with the lowest levels in NNN and NNH fish (**Figure 14B**)**.** This apparent controversial finding would be indicative of a higher basal metabolism in HNN and HNH. Likewise, acute embryonic anoxia exposure favors the development of a dominant and aggressive phenotype in adult zebrafish (**Ivy *et al.*, 2017**). If this is also indicative of a different behavior in sea bream remains unclear, though as reported below a different swimming performance was evidenced as a persistent phenotypic trait in fish early exposed to moderate hypoxia.

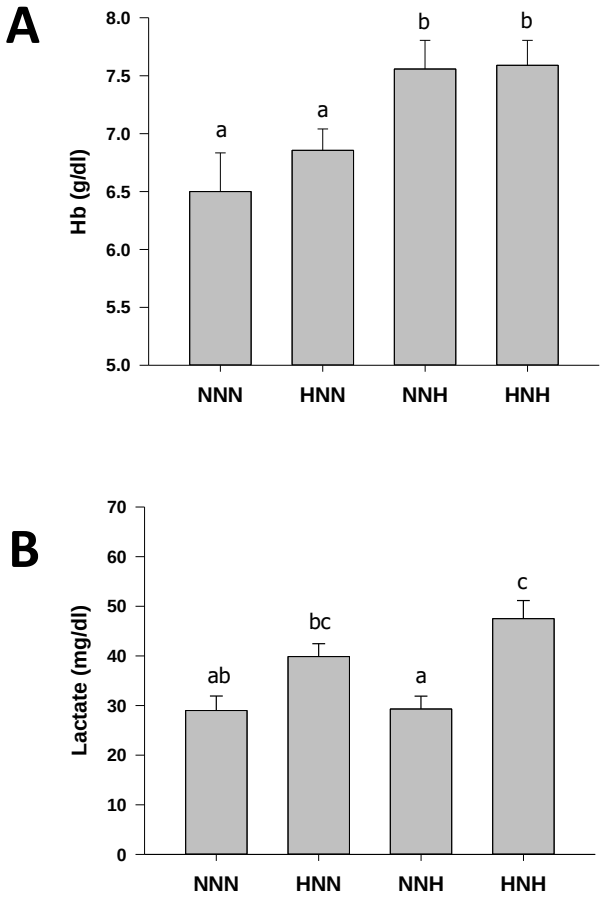


Figure 14. Blood physiological hallmarks in response to early (60-81 dph) and late (112-127 dph) hypoxia priming. (**A**) Blood hemoglobin levels. (**B**) Plasma lactate levels. All values are the mean ± SEM of 12 fish. Different superscript letters indicate significant differences between experimental groups (one-way ANOVA followed by SNK test, P <0.05).

5.3 Persistence of early hypoxia pre-conditioning in sea bream

After the second exposure to low O2 concentrations, fish continued to grow normally with a final body weight close to 60 g at the last recording time. At this stage, no significant differences were found among groups, and all fish were put together for graph simplicity.

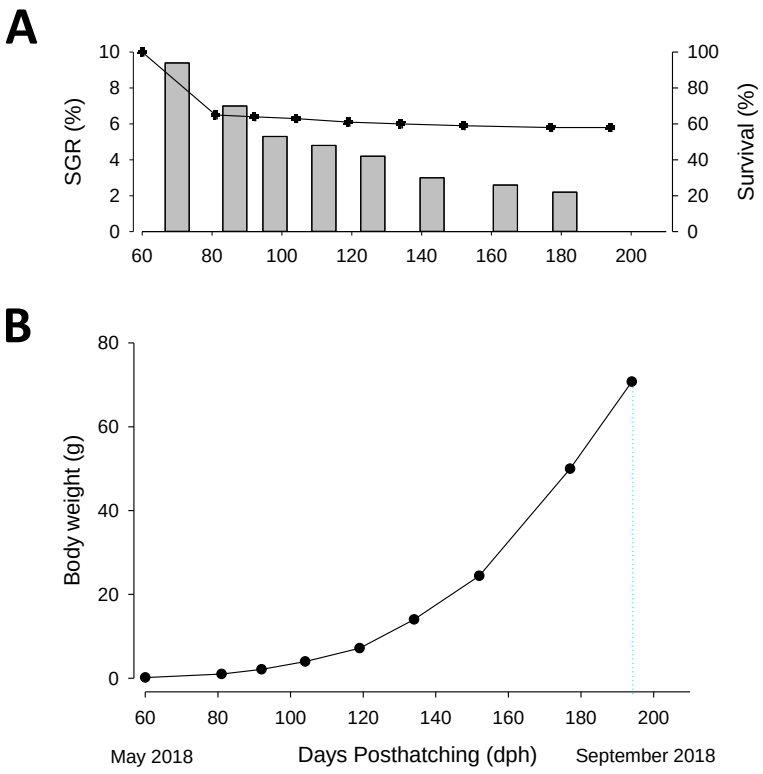


Figure 15. (**A**) Data on growth and survival rates through all the experimental period (60-200 dph). (**B**) Body weight evolution.

Also, the four experimental groups attained similar U_{crit} (around 5.5-5.9 BL/s) and MMR values (290-325 $mgO_2/kg/h$) in the swimming test (**Table 8**). However, when data were analysed more in depth, two main metabolic patterns with different rates of MO2 at high speed levels was observed. This corresponded to two merged groups: NNN-NNH (N ini) and HNN-HNH (H ini). Such approach revealed a different aerobic scope for the two groups, and although the MO_2 were quite variable at each swimming speed (**Figure 16**), it became evident that at water speeds of 4.0 BL/s or higher, fish early primed with low O_2 concentrations showed significantly lowered MO_2. This is indicative of an increased anaerobic contribution to the swimming activity. If this is related to different fish behaviour in normally exercised fish remains unclear, and further studies are underway to determine if this feature is associated to productive traits related to fish exercise and fillet quality.

Table 8. Performance parameters (U_{crit} and MMR) of experimental fish forced to swimming till exhaustion in a swim tunnel respirometer. No significant differences were found betwwen experimental groups Values represent the mean and SEM of 5-6 fish. One-way ANOVA analysis did not evidence significant (P <0.05) differences between experimental groups.

Group	U_{crit}		MMR	
	(BL/s)	SEM	($mgO_2/kg/h$)	SEM
NNN	5.85	0.20	323.45	13.83
HNN	5.79	0.08	289.16	20.30
NNH	5.57	0.13	306.30	11.35
HNH	5.48	0.10	307.03	5.01

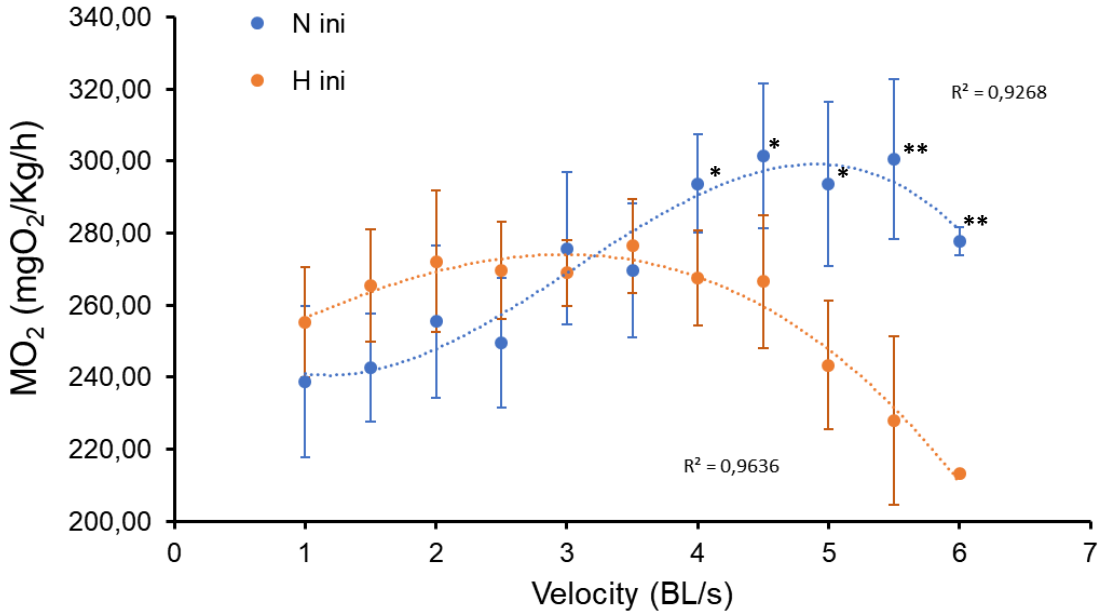


Figure 16. Effect of swimming speed and 60-81 dph priming on O_2 consumption rates (MO_2). Values are the mean ± SEM of 10 fish. Polynomial regression of the MO_2 dynamic of each experimental group is represented for clarification. Asterisks indicate indicate significant differences between experimental groups at a given velocity (*t*-test, * P <0.05, ** P <0.01).

5.4 Main outcomes- hypoxia pre-conditioning at early stages

Exposure to low O_2 levels at early life stages (60-81 dph) did not result in any detrimental trait in the later juvenile performance. Moreover, convenient endurance features have been reported up to five months after the first challenging stimulus. Plasma lactate emerged as a fair indicator of this earlier metabolic priming, with increased levels in “H ini” fish (HNN-HNH). This feature should be envisaged not only as a better adaptation to shift from aerobic to anaerobic metabolism, but also as a putative indicator of different fish behavior in tank/cages, which become of potential interest for fillet quality traits. This was supported by the results of swimming performanc, which evidenced a higher contribution of anaerobic metabolism at high speed. If this implies a different endurance training or recovery time after exhaustive exercise remains to be determined in current studies.

Concluding remarks

Long-lasting effects of hypoxia exposure have been assessed in order to identify metabolic indicators that ensure proper adaptive responses to low O_2 concentrations in sea bream juveniles.

Severe hypoxia promotes a shift from aerobic to anaerobic metabolism, reduces basal metabolism and triggers more efficient mitochondrial respiration to increase aerobic energy production.

Moderate hypoxia (above LOS levels) during the juvenile stage reduces feed intake and growth. This effect is potentiated by crowding stress, although feed efficiency is not compromised and to some extent it is improved, which reflects the high metabolic and growth plasticity of farmed sea bream.

At the molecular level, different adaptive mechanisms in juvenile fish were outlined, highlighting different tissue responsiveness according to the different tissue capabilities and the nature and severity of the hypoxia stimuli.

Hypoxic pre-conditioning (above LOS levels) during early life does not compromise final juvenile weight and triggers persistent metabolic effects that improve the aerobic scope and swimming performance later in life This very promising result is one of the first evidence in typically marine fish of early environmental imprinting that might involve different epigenetic mechanisms.

References

Ackerman, D., Tumanov, S., Qiu, B., Michalopoulou, E., Spata, M., Azzam, A., Xie, H., Simon, M. C., & Kamphorst, J.J. (2018). Triglycerides promote lipid homeostasis during hypoxic stress by balancing fatty acid saturation. *Cell Rep.,* 24, 2596-2605.

Almeida, D. V., Bianchini, A., & Marins, L. F. (2013). Growth hormone overexpression generates an unfavorable phenotype in juvenile transgenic zebrafish under hypoxic conditions. *Gen. Comp. Endocrinol.,* 194, 102-109.

Antonicka, H., Mattman, A., Carlson, C. G., Glerum, D. M., Hoffbuhr, K. C., Leary, S. C., Kennaway, N. G., & Shoubridge, E. A. (2003). Mutations in COX15 produce a defect in the mitochondrial heme biosynthetic pathway, causing early-onset fatal hypertrophic cardiomyopathy. *Am. J. Hum. Genet.,* 72, 101-114.

Arany, Z., He, H., Lin, J., Hoyer, K., Handschin, C., Toka, O., Ahmad, F., Matsui, T., Chin, S., Wu, P.-H., Rybkin, I. I., Shelton, J. M., Manieri, M., Cinti, S., Schoen, F. J., Bassel-Duby. R., Rosenzweig, A., Ingwall. J. S., & Spiegelman, B. M. (2005). Transcriptional coactivator PGC-1α controls the energy state and contractile function of cardiac muscle. *Cell Metab*., 1, 259-271.

Araújo-Luna, R., Ribeiro, L., Bergheim, A., & Pousão-Ferreira, P. (2018). The impact of different rearing condition on gilthead seabream welfare: Dissolved oxygen levels and stocking densities. *Aquacult. Res.,* 49, 3845-3855.

Benedito-Palos, L., Calduch-Giner, J. A., Ballester-Lozano, G. F., & Pérez-Sánchez, J. (2013). Effect of ration size on fillet fatty acid composition, phospholipid allostasis and mRNA expression patterns of lipid regulatory genes in gilthead sea bream (*Sparus aurata*). *British Journal of Nutrition,* 109, 1175-1187.

Benedito-Palos, L., Ballester-Lozano, G., & Pérez-Sánchez, J. (2014). Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). *Gene*, 547, 34-42.

Bergan-Roller, H. E., & Sheridan, M. A. (2018). The growth hormone signaling system: Insights into coordinating the anabolic and catabolic actions of growth hormone. *Gen. Comp. Endocrinol.,* 258, 119-133.

Bermejo-Nogales, A., Calduch-Giner, J. A., & Pérez-Sánchez, J. (2014a). Tissue-specific gene expression and functional regulation of uncoupling protein 2 (UCP2) by hypoxia and nutrient availability in gilthead sea bream (*Sparus aurata*): implications on the physiological significance of UCP1-3 variants. Fish Physiol Biochem. 2014b;40:751-762.

Bermejo-Nogales, A., Nederlof, M., Benedito-Palos, L., Ballester-Lozano, G. F., Folkedal, O., Olsen, R. E., Sitjà-Bobadilla, A., & Pérez-Sánchez, J. (2014b). Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata*) to environmental stress: new insights in fish mitochondrial phenotyping. *Gen Comp Endocrinol.* 205, 305-315.

Bermejo-Nogales, A., Calduch-Giner, J. A., & Pérez-Sánchez, J. (2015). Unraveling the molecular signatures of oxidative phosphorylation to cope with the nutritionally changing metabolic capabilities of liver and muscle tissues in farmed fish. *PLoS ONE*, 10, e0122889.

Brett, J. R., (1979). “Environmental factors and growth”, in *Fish Physiology*, Vol. VIII, eds W.S. Hoar, D.J. Randall, J.R. Brett (London: Academic Press), 599-675.

Cadiz, L., Servili, A., Quazuguel, P., Madec, L., Zambonino-Infante, J. L., & Mazurais, D. (2017). Early exposure to chronic hypoxia induces short-and long-term regulation of hemoglobin gene expression in European sea bass (*Dicentrarchus labrax*). *J. Exp. Biol.,* 220, 3119-3126.

Cadiz, L., Zambonino-Infante, J.-L., Quazuguel, P., Madec, L., Le Delliou, H., & Mazurais, D. (2018). Metabolic response to hypoxia in European sea bass (*Dicentrarchus labrax*) displays developmental plasticity. *Comparative Biochemistry and Physiology,* 215, 1-9.

Calduch-Giner, J. A., Bermejo-Nogales, A., Benedito-Palos, L., Estensoro, I., Ballester-Lozano, G., Sitjà-Bobadilla, A., & Pérez-Sánchez, J. (2013). Deep sequencing for *de novo* construction of a marine fish (*Sparus aurata*) transcriptome database with a large

coverage of protein-coding transcripts. *BMC Genomics*, 14, 178.
Calduch-Giner, J. A., Echasseriau, Y., Crespo, D., Baron, D., Planas, J. V., Prunet, P., & Pérez-Sánchez, J. (2014). Transcriptional assessment by microarray analysis and large-scale meta-analysis of the metabolic capacity of cardiac and skeletal muscle tissues to cope with reduced nutrient availability in gilthead sea bream (*Sparus aurata* L.). *Mar. Biotechnol.*, 16, 423-435.
Dalla Via, J., Van den Thillart, G., Cattani, O., & Cortesi, P. (1998). Behavioural responses and biochemical correlates in *Solea solea* to gradual hypoxic exposure. *Can. J. Zool.*, 76, 2108-2113.
Donohoe, P. H., West, T. G., & Boutilier, R. G. (1998). Respiratory, metabolic and acid–base correlates of aerobic metabolic rate reduction in overwintering frogs. *Am. J. Physiol.*, 43, R704-R710.
Du, J., Wang, Y., Hunter, R., Wei, Y., Blumenthal, R., Falke, C., Khairova, R., Zhou, R., Yuan, P., Machado-Vieirea, R., McEwen, B. S., & Manjia, H. K. (2009). Dynamic regulation of mitochondrial function by glucocorticoids. *Proc. Natl. Acad. Sci. U. S. A.,* 106, 3543-3548.
Dyer, A. R., Upton, Z., Stone, D., Thomas, P. M., Soole, K. L., Higgs, N., Quinn, K., & Carragher, J. F. (2004). Development and validation of a radioimmunoassay for fish insulin-like growth factor I (IGF-I) and the effect of aquaculture related stressors on circulating IGF-I levels. *Gen. Comp. Endocrinol*., 135, 268-275.
Everett, M. V., Antal, C. E., & Crawford, D. L. (2012). The effect of short-term hypoxic exposure on metabolic gene expression. *J. Exp. Zool*. A., 317, 9-23.
Faou, P., & Hoogenraad, N. J. (2012). Tom34: a cytosolic cochaperone of the Hsp90/Hsp70 protein complex involved in mitochondrial protein import. *BBA-Mol. Cell. Res.*, 1823, 348-357.
Gallaugher, P., & Farrell, A. P. (1998). “Hematocrit and blood oxygen-carrying capacity”, in *Fish physiology*, Vol. 17, eds S. F. Perry, and B. L. Tufts (New York, NY: Academic Press), 185-227.
Gamboa, J. L., & Andrade, F. H. (2012). Muscle endurance and mitochondrial function after chronic normobaric hypoxia: contrast of respiratory and limb muscles. *Pflug. Arch. Eur. J. Physiol.*, 463, 327-338.
Ghezzi, D., & Zeviani, M. (2012). Assembly factors of human mitochondrial respiratory chain complexes: Physiology and Pathophysiology. In: Kadenbach B, editor. Mitochondrial Oxidative Phosphorylation. Springer New York p. 65-106.
Gracey, A. Y., Troll, J. V., & Somero, G. N. (2001). Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proc. Natl. Acad. Sci. USA*, 98, 1993-1998.
Hermes-Lima, M., Moreira, D. C., Rivera-Ingraham, G. A., Giraud-Billoud, M., Genaro-Mattos, T. C., & Campos, É. G. (2015). Preparation for oxidative stress under hypoxia and metabolic depression: revisiting the proposal two decades later. *Free Radic. Biol. Med., 89*, 1122-1143.
Hoppeler, H., & Vogt, M. (2001). Muscle tissue adaptations to hypoxia. *J. Exp. Biol.*, 204, 3133-3139.
Huang, D., Li, C., & Zhang, H. (2014). Hypoxia and cancer cell metabolism. *Acta Biochim. Biophys. Sin.,* 46, 214-219.
Ivy, C. M., Robertson, C. E., & Bernier, N. J. (2017). Acute embryonic anoxia exposure favours the development of a dominant and aggressive phenotype in adult zebrafish. *Proc. Royal Society B*, 284, 20161868.
Jørgensen, E. H., Christiansen, J. S., & Jobling, M. (1993). Effects of stocking density on food intake, growth performance and oxygen consumption in Arctic charr (*Salvelinus alpinus*). *Aquaculture, 110*, 191-204.
Khacho, M., Tarabay, M., Patten, D., Khacho, P., MacLaurin, J. G., Guadagno, J., Bergeron, R., Cregan, S. P., Harper, M.-E., Park, D. S., & Slack, R. S. (2014). Acidosis overrides oxygen deprivation to maintain mitochondrial function and cell survival. *Nat. Commun*., 5, 3550.

Kieffer, D. A., Piccolo, B. D., Vaziri, N. D., Liu, S., Lau, W.L., Khazaeli, M., Nazertehrani, S., Moore, M. E., Marco, M. L., Martin, R. J., & Adams, S. H. (2016). Resistant starch alters gut microbiome and metabolomic profiles concurrent with amelioration of chronic kidney disease in rats. *Am. J. Physiol. Renal. Physiol.,* 310, F857-F871.

Larsen, S., Nielsen, J., Hansen, C. N., Nielsen, L. B., Wibrand, F., Stride, N., Schroder, H. D., Boushel, R., Helge, J. W., Dela, F., & Hey-Mogensen, M. (2012). Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J. Physiol., 590*, 3349-3360.

Lelliott, C., Medina-Gomez, G., Petrovic, N., Kis, A., Feldmann, H. M., Bjursell, M., Parker, N., Curtis, K., Campbell, M., Hu, P., Zhang, D., Litwin, S. E., Zaha, V. G., Fountain, K. T., Boudina, S., Jimenez-Linan, M., Blount, M., Lopez, M., Meirhaeghe, A., Bohlooly-Y, M., Storlien, L., Strömstedt, M., Snaith, M., Oresic, M., Abel, E. D., Cannon, B., & Vidal-Puig, A. (2006). Ablation of PGC-1b results in defective mitochondrial activity, thermogenesis, hepatic function, and cardiac performance. *PLoS Biol.*, 4, e369.

Lenka, N., Vijayasarathy, C., Mullick, J., & Avadhani, N. G. (1998). Structural organization and transcription regulation of nuclear genes encoding the mammalian cytochrome c oxidase complex. *Prog. Nucl. Res. Molec. Biol.*, 61, 309-344.

Li, H., Ma, M. L., Luo, S., Zhang, R. M., Han, P., & Hu, W. (2012). Metabolic responses to ethanol in *Saccharomyces cerevisiae* using a gas chromatography tandem mass spectrometry-based metabolomics approach. *Int. J. Biochem. Cell. Biol.,* 44, 1087- 1096.

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ method. *Methods,* 25, 402-408.

Ljubicic, V., Joseph, A. M., Saleem, A., Uguccioni, G., Collu-Marchese, M., Lai, R. Y., Nguyen, L., M.-D., & Hood, D. A. (2010). Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: effects of exercise and aging. *BBA-Gen. Subj.*, 1800, 223-234.

Lushchak, V. I., & Bagnyukova, T. V. (2006). Effects of different environmental oxygen levels on free radical processes in fish. *Comp. Biochem. Physiol. B.*, 144, 283–289.

Magnoni, L., Martos-Sitcha, J. A., Queiroz, A., Calduch-Giner, J. A., Magalhães Gonçalves, J. F., Rocha, C. M. R., Abreu, H. T., Schrama, J. W., Ozorio, R. O. A., & Pérez-Sánchez, J. (2017). Dietary supplementation of heat-treated *Gracilaria* and *Ulva* seaweeds enhanced acute hypoxia tolerance in gilthead seabream (*Sparus aurata*). *Biol. Open*., 6, 897-908.

Martínez-Barberá, J. P., Pendón, C., Martí-Palanca, H., Calduch-Giner, J. A., Rodríguez, R. B., Valdivia, M. M., & Pérez-Sánchez, J. (1995). The use of recombinant gilthead sea bream (*Sparus aurata*) growth hormone for radioiodination and standard preparation in radioimmunoassay. *Comp. Biochem. Physiol. A.,* 110, 335-340.

Martos-Sitcha, J. A., Simó-Mirabet, P., Piazzon, M. C., de las Heras, V., Calduch-Giner, J. A., Puyalto, M., Tinsley, J., Makol, A., Sitjà-Bobadilla, A., & Pérez-Sánchez, J. (2018). Dietary sodium heptanoate helps to improve feed efficiency, growth hormone status and swimming performance in gilthead sea bream (*Sparus aurata*). *Aquac. Nutr.,* 24, 1638-1651.

McKenzie, D. J., Martinez, R., Morales, A., Acosta, J., Morales, R., Taylor, E. W., Steffensen, J. F., & Estrada, M. P. (2003). Effects of growth hormone transgenesis on metabolic rate, exercise performance and hypoxia tolerance in tilapia hybrids. *J. Fish Biol*., 63, 398-409.

Millán-Cubillo, A. F., Martos-Sitcha, J. A., Ruiz-Jarabo, I., Cárdenas, S., & Mancera, J. M. (2016). Low stocking density negatively affects growth, metabolism and stress pathways in juvenile specimens of meagre (*Argyrosomus regius*, Asso 1801). *Aquaculture, 451*, 87-92.

Mommsen, T. P., Vijayan, M. M., & Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish.*, 9, 211-268.

Monternier, P. A., Marmillot, V., Rouanet, J. L., & Roussel, D. (2014). Mitochondrial phenotypic flexibility enhances energy savings during winter fast in king penguin chicks. *J. Exp. Biol.,* 217, 2691-2697.

Murray, A. J. (2009). Metabolic adaptation of skeletal muscle to high altitude hypoxia: how

new technologies could resolve the controversies. *Genome Med.*, 1, 117.
Pérez-Sánchez, J., Martí-Palanca, H., & Kaushik, S. (1995). Ration size and protein intake affect growth hormone (GH) levels, hepatic GH-binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, gilthead sea bream (*Sparus aurata*). *J. Nutr.,* 125, 546-552.
Pérez-Sánchez, J., Borrel, M., Bermejo-Nogales, A., Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J. A., & Kaushik, S. (2013). Dietary oils mediate cortisol kinetics and the hepatic expression profile of stress responsive genes in juveniles of gilthead sea bream (*Sparus aurata*) exposed to crowding stress. *Comp. Biochem. Physiol. D,* 8, 123-130.
Pérez-Sánchez, J., Simó-Mirabet, P., Naya, F., Martos-Sitcha, J.A., Perera, E., Bermejo-Nogales, A., Benedito-Palos, L., & Calduch-Giner, J. A. (2018). Somatotropic axis regulation unravels the differential effect of nutritional and environmental factors in growth performance of marine farmed fish. *Front. Endocrinol*., 9, 687.
Person-Le Ruyet, J., Labbé, L., Le Bayon, N., Sévère, A., Le Roux, A., Le Delliou, H., & Quemener, L. (2008). Combined effects of water quality and stocking density on welfare and growth of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Living. Resour., 21*, 185-195.
Pichavant, K., Person-Le-Ruyet, J., Bayon, N. L., Severe, A., Roux, A. L., & Boeuf, G. (2001). Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. *J. Fish Biol.,* 59, 875-883.
Poulain, L., Mathieu, H., Thomas, A., Borel, A. L., Remy, C., Levy, P., Arnaud, C., & Dematteis, M. (2017). Intermittent hypoxia-induced insulin resistance is associated with alterations in white fat distribution. *Sci. Rep.,* 7, 11180.
Reindl, K. M., & Sheridan, M. A. (2012). Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. *Comp. Biochem. Physiol. A, 163*, 231-245.
Remen, M., Nederlof, M. A. J., Folkedal, O., Thorsheim, G., Sitjà-Bobadilla, A., Pérez-Sánchez, J., Oppedal, F., & Olsen, R. E. (2015). Effect of temperature on the metabolism, behavior and oxygen requirements of *Sparus aurata*. *Aquacult. Env. Interac.*,7, 115-123.
Remen, M., Sievers, M., Torgersen, T., & Oppedal F. (2016). The oxygen threshold for maximal feed intake of Atlantic salmon post-smolts is highly temperature-dependent. *Aquaculture*, 464, 582-592.
Rimoldi, S., Benedito-Palos, L., Terova, G., & Pérez-Sánchez, J. (2016). Wide-targeted gene expression infers tissue-specific molecular signatures of lipid metabolism in fed and fasted fish. *Rev. Fish Biol. Fish.,* 26, 93-108.
Robertson, C. E., Wright P. A., Koblitz L., & Bernier N. J. (2014). Hypoxia-inducible factor-1 mediates adaptive developmental plasticity of hypoxia tolerance in zebrafish, *Danio rerio*. *Proc. Biol. Sci.*, 281, 20140637.
Rogers, N. J., Urbina, M. A., Reardon, E. E., McKenzie, D. J., & Wilson, R. W. (2016). A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (Pcrit). *Conserv. Physiol.*, 4, cow012.
Saera-Vila, A., Calduch-Giner, J. A., Prunet, P., & Pérez-Sanchez, J. (2009). Dynamics of liver GH/IGF axis and selected stress markers in juvenile gilthead sea bream (*Sparus aurata*) exposed to acute confinement: differential stress response of growth hormone receptors. *Comp. Biochem. Physiol. A*, 154, 197–203.
Sangiao-Alvarellos, S., Guzmán, J. M., Láiz-Carrión, R., Míguez, J. M., Martín Del Río, M. P., Mancera, J. M., & Soengas, J. L. (2005). Interactive effects of high stocking density and food deprivation on carbohydrate metabolism in several tissues of gilthead sea bream *Sparus auratus*. *J. Exp. Zool. A, 303*, 761-775.
Saravanan, S., Geurden, I., Figueiredo-Silva, A. C., Kaushik, S. J., Haidar, M. N., Verreth, J. A., & Schrama, J. W. (2012). Control of voluntary feed intake in fish: a role for dietary oxygen demand in Nile tilapia (*Oreochromis niloticus*) fed diets with different macronutrient profiles. *Br. J. Nutr*., 108:1519-1529.
Shimizu, M., Swanson, P., Fukada, H,, Hara, A., & Dickhoff, W. W. (2000). Comparison of extraction methods and assay validation for salmon insulin-like growth factor-I using commercially available components. *Gen. Comp. Endocrinol.*, 119, 26–36.

Simó-Mirabet, P., Bermejo-Nogales, A., Calduch-Giner, J. A., & Pérez-Sánchez, J. (2017a). Tissue-specific gene expression and fasting regulation of sirtuin family in gilthead sea bream (*Sparus aurata*). *J. Comp. Physiol. B, 187*, 153-163.

Simó-Mirabet, P., Piazzon, M. C., Calduch-Giner, J. A., Ortiz, Á., Puyalto, M., Sitjà-Bobadilla, A., & Pérez-Sánchez, J. (2017b). Sodium salt medium-chain fatty acids and *Bacillus*-based probiotic strategies to improve growth and intestinal health of gilthead sea bream *(Sparus aurata*). *PeerJ, 5*: e4001.

Simó-Mirabet, P., Perera, E., Calduch-Giner, J. A., Afonso, J. M., & Pérez-Sánchez, J. (2018). Co-expression analysis of sirtuins and related metabolic biomarkers in juveniles of gilthead sea bream (*Sparus aurata*) with differences in growth performance. *Front. Physiol,. 9, 608.*

Smith, D., Gray, J., Mitchell, L., Antholine, W. E., & Hosler, J. P. (2005). Assembly of cytochrome-c oxidase in the absence of assembly protein Surf1p leads to loss of the active site heme. *J. Biol. Chem.* 280, 17652**–**17656.

Soivio, A., Nikinmaa, M., & Westman, K. (1980). The blood oxygen binding properties of hypoxic *Salmo gairdneri*. *J Comp Physiol.*, 136, 83-87.

Stiburek, L., Vesela, K., Hansikova, H., Pecina, P., Tesarova, M., Cerna, L., Houstek, J., & Zeman, J. (2005). Tissue-specific cytochrome c oxidase assembly defects due to mutations in SCO2 and SURF1. *Biochem. J.*, 392, 625**–**632.

Stier, A., Bize, P., Schull, Q., Zoll, J., Singh, F., Geny, B., Gros, F., Royer, C., Massemin, S., & Criscuolo, F. (2013). Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. *Front. Zool.*, 10, 33.

Storey, K. B. (2015). Regulation of hypometabolism: insights into epigenetic controls. *J. Exp. Biol.*, 218, 150-159.

St-Pierre, J., Brand, M. D., & Boutilier, R. G. (2000). The effect of metabolic depression on proton leak rate in mitochondria from hibernating frogs. *J. Exp. Biol.*, 203, 1469-1476.

Theron, M., Guerrero, F., & Sebert, P. (2000). Improvement in the efficiency of oxidative phosphorylation in the freshwater eel acclimated to 10.1 MPa hydrostatic pressure. *J. Exp. Biol.*, 203, 3019-3023.

Vanderplancke, G., Claireaux, G., Quazuguel, P., Madec, L., Ferraresso, S., Sévère, A., Zambonino-Infante, J. L., & Mazurais, D. (2015). Hypoxic episode during the larval period has long-term effects on European sea bass juveniles (*Dicentrarchus labrax*). *Marine Biol.*, 162, 367-376.

Vega-Rubín de Celis, S., Gómez-Requeni, P., & Pérez-Sánchez, J. (2004). Production and characterization of recombinantly derived peptides and antibodies for accurate determinations of somatolactin, growth hormone and insulin-like growth factor-I in European sea bass (*Dicentrarchus labrax*). *Gen. Comp. Endocrinol.*, 139, 266-277.

Vélez, E. J., Lutfi, E., Azizi, S., Perelló, M., Salmerón, C., Riera-Codina, M., Ibarz, J., Fernández-Borràs, J., Blasco, J., Capilla, E., Navarro, I., & Gutiérrez, J. (2017). Understanding fish muscle growth regulation to optimize aquaculture production. *Aquaculture,* 467, 28-40.

Vikeså, V., Nankervis, L., & Hevrøy, E. M. (2017). High dietary energy level stimulates growth hormone receptor and feed utilization in large Atlantic salmon (*Salmo salar* L.) under hypoxic conditions. *Aquac. Nutr.,* 23, 1193-1203.

Virani, N. A., & Rees, B. B. (2000). Oxygen consumption, blood lactate and inter-individual variation in the gulf killifish, *Fundulus grandis*, during hypoxia and recovery. *Comp. Biochem. Physiol. A*, 126, 397-405.

Wold, S., Sjöström, M., & Eriksson, L. (2001). PLS-regression: a basic tool of chemometrics. *Chemometr. Intell. Lab. Syst.*, 58, 109-130.

Wood, S. C., & Johansen, K. (1972). Adaptation to hypoxia by increased HbO_2 affinity and decreased red cell ATP concentration. *Nat. New. Biol.*, 237, 278-279.

Glossary

AQUAEXCEL^{2020}: AQUAculture Infrastructures for EXCELlence in European Fish Research towards 2020

BL/s: Body lengths per second

DE: Differentially expressed

dph: Days post-hatching

FE: Feed efficiency

Gh: Growth hormone

Hb: Haemoglobin

Hc: Haematocrit

HSI: Hepatosomatic index

Igf: Insulin-like growth factor

LOS: Limiting oxygen saturation

MCH: Mean corpuscular haemoglobin

MCHC: Mean corpuscular haemoglobin concentration

MCV: Mean corpuscular volume

MMR: maximum metabolic rate

MO_2: Oxygen consumption rate

MSI: Mesenteric fat index

OXPHOS: Oxidative phosphorylation

PLS-DA: Partial least-squares discriminant analysis

RBC: Red blood cells

SGR: Specific growth rate

TAA: Total antioxidant activity

TG: Triglycerides

U_{crit}: Critical swimming speed

VIP: Variable importance in projection

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Annex 1: Check list

Deliverable Check list (to be checked by the “Deliverable leader”)

	Check list		Comments
BEFORE	I have checked the due date and have planned completion in due time	Yes	*Please inform Management Team of any foreseen delays*
	The title corresponds to the title in the DOW	Yes	*If not please inform the Management Team with justification*
	The dissemination level corresponds to that indicated in the DOW	Yes	
	The contributors (authors) correspond to those indicated in the DOW	Yes	
	The Table of Contents has been validated with the Activity Leader	Yes	*Please validate the Table of Content with your Activity Leader before drafting the deliverable*
	I am using the AQUAEXCEL2020 deliverable template (title page, styles etc)	Yes	*Available in “Useful Documents” on the collaborative workspace*
The draft is ready			
AFTER	I have written a good summary at the beginning of the Deliverable	Yes	*A 1-2 pages maximum summary is mandatory (not formal but really informative on the content of the Deliverable)*
	The deliverable has been reviewed by all contributors (authors)	Yes	*Make sure all contributors have reviewed and approved the final version of the deliverable. You should leave sufficient time for this validation.*
	I have done a spell check and had the English verified	Yes	
	I have sent the final version to the WP Leader, to the 2nd Reviewer and to the Project coordinator (cc to the project manager) for approval	Yes	*Send the final draft to your WPLeader, the 2nd Reviewer and the coordinator with cc to the project manager on the 1st day of the due month and leave 2 weeks for feedback. Inform the reviewers of the changes (if any) you have made to address their comments. Once validated by the 2 reviewers and the coordinator, send the final version to the Project Manager who will then submit it to the EC.*

Annex 2: Open Access scientific publication

Background

Among the abiotic factors, dissolved oxygen (O_2) is particularly important as the major limiting factor of fish aerobic metabolism [1, 2]. When regulatory mechanisms are no longer sufficient to maintain the O_2 consumption rate (MO_2), further reductions in MO_2 occur at a certain level of O_2 saturation [3]. This threshold is termed the limiting oxygen saturation (LOS) in fed fish able to maintain a routine metabolic rate, and according to the oxystatic control theory of feed intake, fish adjust their feed intake to meet dietary O_2 demands [4]. Therefore, changes in LOS, produced by fluctuations in O_2 solubility associated with variations in water temperature, should be considered and regulated to ensure a non-compromised physiological function and guarantee the welfare of farmed fish fed high or low O_2-demanding diets [5, 6]. This regulation is mediated through O_2 sensors that trigger anaerobic metabolic rates to compensate for the decreasing aerobic ATP production [7, 8]. For this purpose, eukaryotic cells switch from mitochondrial oxidative phosphorylation (OXPHOS) to the less efficient anaerobic glycolytic pathway, which induces stress and lactic acidosis (reviewed in [9]). The hallmarks of human muscle adaptation to hypoxia are a decrease in muscle oxidative capacity concomitant with a decrease in aerobic work capacity [10, 11]. In this regard, hypo-metabolic states should be considered as part of the adaptive response to hypoxia instead of a negative result in hypoxia-tolerant individuals [12] since this metabolic depression prevents the accumulation of toxic by-products from anaerobic metabolism [13].

In fish, microarray gene expression profiling of liver and skeletal muscle demonstrated that metabolic suppression is a key adaptive strategy in the hypoxic goby fish, *Gillichthys mirabilis,* to drive energy resources from growth towards metabolic processes that are essential for hypoxia survival [14]. However, in *Fundulus grandis,* both cardiac and hepatic tissues displayed increases in the gene expression of different enzyme subunits of the OXPHOS pathway in response to short-term hypoxic exposure [15]. Similarly, confounding results have been reported in European sea bass (*Dicentrarchus labrax*), as early life exposure to moderate hypoxia has long-lasting detrimental effects on growth performance with no improvement of hypoxia tolerance in juvenile fish despite the enhanced expression of glycolytic enzymes, which are target genes of hypoxia-inducible factors [16]. Whether this response is tissue- or fish species-specific remains unclear. Importantly, the red blood cells (RBC) of fish and almost all amphibians, reptiles and birds retain a nucleus and functional mitochondria [17]. These RBCs present new research opportunities, and previous research attempts have demonstrated that the expression of mitochondrial uncoupling proteins is highly regulated by hypoxia stimuli in the whole blood cells of gilthead sea bream (*Sparus aurata*) [8]. Certainly, fish microarray meta-analysis revealed that mitochondria are particularly sensitive to cellular stress triggered by a wide range of nutritional and environmental stress stimuli [18]. Hence, a PCR-array containing 88 mitochondrial-related markers has been useful to examine changes in hepatic and muscle metabolism in response to short-term fasting [19] or aquaculture stressors that mimic thermal stress and daily operational farming activities in gilthead sea bream [20]. There is little information on blood transcriptomics, and the aim of the present study was to provide new insights into the regulation and adaptive responses of hypoxic metabolism in fish, combining non-invasive transcriptional approaches based on mitochondrial markers with conventional measures of blood haematology and biochemistry. This type of approach is crucial to determine whether samples collected without sacrificing animals provide a reliable measure of mitochondrial functioning and energy metabolism at the level of the whole organism.

Methods

Animal care

Juvenile gilthead sea bream of Atlantic origin (Ferme Marine du Douhet, Bordeaux, France) were reared from early life stages at the indoor experimental facilities of the Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain) under natural photoperiod and temperature conditions at our latitude (40°5′N; 0°10′E). Seawater was pumped ashore (open system) and filtered through a 10-μm filter. The O_2 content of water effluents under standard conditions remained consistently higher than 85% saturation, and unionised ammonia under both control and experimental conditions remained below toxic levels (<0.02 mg/L). For sampling, the fish were anaesthetised using 3-aminobenzoic acid ethyl ester (100 mg/L), and blood was drawn from caudal vessels using EDTA-treated syringes.

Experimental setup and sampling

Juvenile fish of 230–260 g body weight were distributed in 500-L tanks (16 fish per tank) allocated in a recirculatory system equipped with physical and biological filters and programmable temperature. The water temperature was maintained at 20–21 °C. Fish were fed daily to visual satiety using a commercial diet (INICIO Forte 824/EFICO Forte 824; BioMar, Palencia, Spain), and all fish were fasted during the hypoxia challenges. The water conditions for the control fish (normoxic fish) remained unchanged, whereas hypoxic fish experienced a gradual decrease in the water O_2 concentration until reaching i) 3.0 ppm (41–42% O_2 saturation; moderate hypoxia, H1) for 24 h or ii) 1.3 ppm (18–19% O_2 saturation; severe hypoxia, H2) for up to 4 h in two different

hypoxic tests (Fig. 1). Both low dissolved O_2 levels tested were obtained by the cessation of normal aeration in the tank, achieving an accurate balance between the consumption rates of the animals and the supply of clean and oxygenated water by means of an electrovalve within the established O_2 steady-state condition.

In each test, normoxic or hypoxia-challenged fish were sampled at three different sampling points after decreasing the water O_2 concentration (8 fish per time and condition): i) H1: T0, T1 (24 h), T2 (48 h), and ii) H2: T0, T1 (7 h), T2 (11 h). One blood aliquot (150 μL) was directly collected into a microtube containing 500 μL of stabilising lysis solution (REAL Total RNA Spin Blood Kit, Durviz, Valencia, Spain) and stored at −80 °C until total RNA extraction. Other aliquots were processed for haematocrit (Hc) and haemoglobin (Hb) determinations.

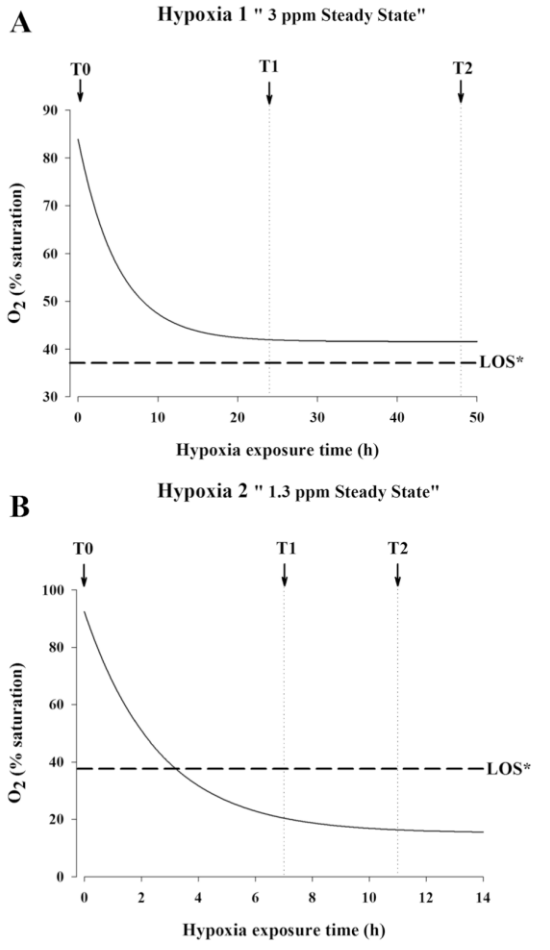

Fig. 1 Water O_2 kinetics in fish exposed to hypoxic conditions. The steady-state was set at (**a**) 41–42% O_2 saturation (3 ppm) or (**b**) 18–19% O_2 saturation (1.3 ppm). Sampling points (T0, T1 and T2) are indicated with arrowheads. LOS was calculated according to Remen et al. [5]

The remaining blood was centrifuged at 3000 × *g* for 20 min at 4 °C, and the plasma samples were frozen and stored at −20 °C until biochemical and hormonal analyses were performed.

Blood biochemistry and hormonal parameters

Hc was measured using heparinised capillary tubes centrifuged at 1500 × *g* for 30 min in a Sigma 1–14 centrifuge (Sigma, Osterode am Harz, Germany). The Hb concentration was assessed using a Hemocue Hb 201+ (Hemocue, Ängelholm, Sweden). Plasma glucose was analysed using the glucose oxidase method (Thermo Electron, Louisville, CO, USA). Blood lactate was measured in deproteinised samples (perchloric acid 8%) using an enzymatic method based on the use of lactate dehydrogenase (Instruchemie, Delfzijl, The Netherlands). Total antioxidant capacity in plasma samples was measured using a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) adapted to 96-well microplates. This assay relies on the ability of the antioxidants in the samples to inhibit the oxidation of ABTS (2,2′-azino-di-[3-ethylbenzothiazoline sulphonate]) to the ABTS radical cation by metamyoglobin, a derivatised form of myoglobin. The capacity of the sample to prevent ABTS oxidation was compared with that of Trolox (water-soluble tocopherol analogue) and quantified as mM Trolox equivalents. Plasma cortisol levels were analysed using an EIA kit (Kit RE52061 m IBL, International GmbH, Germany). The limit of detection of the assay was 2.46 ng/mL with intra- and inter-assay coefficients of variation lower that 3% and 5%, respectively. Plasma insulin-like growth factors (Igf) were extracted using acid-ethanol cryoprecipitation [21], and the concentration was measured using a generic fish Igf-I RIA validated for Mediterranean perciform fish [22]. The sensitivity and midrange of the assay were 0.05 and 0.7–0.8 ng/mL, respectively.

Gene expression analysis

Total RNA from total blood cells was extracted using the REAL Total RNA Spin Blood Kit (Durviz) including a DNase step. The RNA yield was >2.5 μg, with absorbance measures ($A_{260/280}$) of 1.9–2.1. The cDNA was synthesised using the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) with random decamers and 500 ng of total RNA in a final volume of 100 μL. Reverse transcription (RT) reactions were incubated for 10 min at 25 °C and 2 h at 37 °C. Negative control reactions were run without the RT enzyme. qPCR was performed using an Eppendorf Mastercycler Ep Realplex Real-Time Detection System (Eppendorf, Wesseling-Berzdorf, Germany). Diluted RT reactions were conveniently used for qPCR assays in 25 μL volume in combination with a SYBR Green Master

Mix (Bio-Rad, Hercules, CA, USA) and specific primers at a final concentration of 0.9 μM (Additional file 1: Table S1). The 96-well PCR-array layout was designed for the simultaneous profiling of a panel of 85 mitochondrial genes under uniform cycling conditions and associated with different biological processes, such as molecular chaperones (7), antioxidant defence (8), transcription factors (5), outer and inner membrane translocation (8), mitochondrial dynamics and apoptosis (10), fatty acid oxidation and the tricarboxylic acid cycle (5), OXPHOS (41) and respiration uncoupling (1). The programme used for PCR amplification included an initial denaturation step at 95 °C for 3 min, followed by 40 cycles of denaturation for 15 s at 95 °C and annealing/extension for 60 s at 60 °C. All the pipetting operations were conducted using an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (>92%) was assessed, and the specificity of the reactions was verified through an analysis of melting curves (ramping rates of 0.5 °C/10 s over a temperature range of 55–95 °C) and linearity of serial dilutions of the RT reactions (>0.99). Fluorescence data acquired during the extension phase were normalised using the delta-delta C_T method [23]. A range of potential housekeeping genes (*β-actin*, *cox4a*, *elongation factor 1*, *α-tubulin* and *18S rRNA*) was initially tested for gene expression stability using Genorm software. The most stable gene in relation to different experimental conditions (normoxia and hypoxia) was *cox4a* (M score = 0.31); therefore, this gene was used as the housekeeping gene in the normalisation procedure. For multi-gene analysis, the data on gene expression were in reference to the expression level of *sod1* obtained in normoxic fish, for which a value of 1 was arbitrarily assigned (Table 1).

This manuscript follows the ZFIN Zebrafish Nomenclature Guidelines for gene and protein names and symbols (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines).

Statistical analysis

The data on biochemical and hormonal parameters were analysed using two-way analysis of variance (ANOVA), followed by the Holm-Sidak test. The data on gene expression were analysed using Student's *t* test. The significance level was set at $P < 0.05$. All analyses were performed using SigmaPlot Version 13 for Windows.

Results

Hypoxic effects on blood haematology and biochemistry

Over the course of the first hypoxia test (H1, 41–42% O_2 saturation), measurements of haematological parameters and plasma glucose and lactate levels remained unaltered in both normoxic (>85% O_2 saturation) and hypoxia-challenged fish (Fig. 2a, c, e and g, respectively). In contrast, these parameters significantly increased in fish exposed to severe hypoxia (H2, 18–19% O_2 saturation) (Fig. 2b, d, f and h). The same trend was observed for total plasma antioxidant activity and plasma cortisol levels (Fig. 3a, b), although the cortisol increase was more pronounced at the last sampling point. The opposite regulation was observed for circulating Igf-I, although a statistically significant effect was observed at the last sampling point (Fig. 3c). No variations in all the parameters studied were observed in fish maintained under normoxic conditions in sub-experiment H2.

Hypoxic effects in whole blood cell gene expression profiling

Based on the results of hormonal and metabolic parameters, gene expression profiling of whole blood cells was restricted to the last sampling point of the severe hypoxia experiment (H2). The relative gene expression and fold-changes (FC) of mitochondrial-related genes are summarised in Table 1. For easier interpretation and visualisation of the results, the FC of differentially expressed genes is indicated using square symbols in red (up-regulated) or green (down-regulated). With the exception of *pgc1α,* all the genes included in the array were detected in all samples analysed. Among these genes, 41 out of 84 were differentially expressed, and the overall response involved repressed expression in response to severe hypoxia. This response was mediated by antioxidant enzymes (*gpx1*, *gst3*, and *sod2*), the transcription factor *nrf1*, outer and inner membrane translocases (*tom70*, *tom22*, *tim44*, *tim10*, and *tim9*), markers of mitochondrial dynamics and apoptosis (*mfn2*, *miffb*, *miro1a*, *miro2*, and *aifm1*), fatty acid β-oxidation (*acaa2* and *hadh),* tricarboxylic acid cycle (*cs*), respiration uncoupling (*ucp2*) and respiratory enzyme subunits of Complex I (*ndufa3*, *ndufa4*, *ndufa7*, *ndufb5*, and *ndufs7*), Complex II (*sdha*, *sdhaf1*, and *sdhaf2*), Complex III (*uqcrc1*, *uqcrc2*, and *uqcrh*) and Complex V (*atp5c1*, *atp5g1*, *atp5l*, and *atpaf2*), encoded by either mitochondrial or nuclear DNA. The nuclear-encoded assembly factors of Complex IV (*sco1*, *surf1*, and *cox15*) were also significantly down-regulated, but the opposite trend was observed for catalytic (*coxi*) and regulatory *(cox5a2* and *cox8b*) enzyme subunits of mitochondrial or nuclear origin, respectively. This up-regulation was also observed for the transcription factor *pgc1ß* and the outer membrane translocase *tom34*. The molecular chaperones were the only factors that did not significantly change under hypoxic conditions, although the overall trend was a down-regulation in hypoxic fish.

Table 1 Relative gene expression of mitochondrial-related genes in total blood cells

Gene name	Symbol	Relative expression		FC
		Control	Hypoxia	
Molecular chaperones				
10 kDa heat shock protein	*hsp10*	0.40 ± 0.03	0.30 ± 0.07	-1.34
40 kDa heat shock protein DnaJ (Hsp40) homolog, subfamily A, member 3A	*dnaja3a*	0.17 ± 0.02	0.12 ± 0.02	-1.49
Iron-sulfur cluster co-chaperone protein HscB	*dnajc20*	0.06 ± 0.01	0.05 ± 0.01	-1.26
60 kDa heat shock protein	*hsp60*	0.19 ± 0.02	0.15 ± 0.03	-1.25
75 kDa Glucose-regulated protein	*grp-75*	0.30 ± 0.03	0.23 ± 0.03	-1.34
Derlin-1	*der-1*	0.19 ± 0.02	0.23 ± 0.04	1.19
Glucose-regulated protein, 170 kDa	*grp-170*	0.36 ± 0.06	0.27 ± 0.04	-1.35
Antioxidant enzymes				
Catalase	*cat*	4.85 ± 0.52	3.94 ± 0.38	-1.23
Glutathione peroxidase 1	*gpx1*	18.26 ± 1.33	13.90 ± 1.25*	-1.31
Glutathione reductase	*gr*	0.38 ± 0.06	0.26 ± 0.03	-1.47
Glutathione S-transferase 3	*gst3*	0.17 ± 0.06	0.02 ± 0.01*	-9.68
Peroxiredoxin 3	*prdx3*	0.23 ± 0.04	0.21 ± 0.03	-1.14
Peroxiredoxin 5	*prdx5*	0.34 ± 0.04	0.26 ± 0.03	-1.30
Superoxide dismutase [Cu-Zn]	*sod1*	1.08 ± 0.08	1.12 ± 0.11	1.05
Superoxide dismutase [Mn]	*sod2*	0.55 ± 0.05	0.28 ± 0.05**	-1.97
Transcription factors				
GA-binding protein alpha chain	*gabpa*	0.31 ± 0.06	0.18 ± 0.02	1.66
Mitochondrial transcription factor A	*mt-tfa*	0.13 ± 0.02	0.09 ± 0.02	-1.52
Nuclear respiratory factor 1	*nrf1*	0.22 ± 0.05	0.10 ± 0.01*	-2.21
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1a*	*nd*	*nd*	-
Proliferator-activated receptor gamma coactivator 1 beta	*pgc1β*	0.28 ± 0.09	0.80 ± 0.14*	2.85
Outer membrane translocases (TOM complex)				
Mitochondrial import receptor subunit Tom70	*tom70*	0.65 ± 0.04	0.36 ± 0.06**	-1.79
Mitochondrial import receptor subunit Tom34	*tom34*	0.55 ± 0.02	0.76 ± 0.10*	1.39
Mitochondrial import receptor subunit Tom22	*tom22*	0.23 ± 0.03	0.09 ± 0.02**	-2.47
Inner membrane translocases (TIM complex)				
Mitochondrial import inner membrane translocase subunit 44	*tim44*	0.16 ± 0.05	0.05 ± 0.02*	-3.62
Mitochondrial import inner membrane translocase subunit 23	*tim23*	0.28 ± 0.02	0.28 ± 0.04	1.02
Mitochondrial import inner membrane translocase subunit Tim8A	*tim8A*	0.83 ± 0.06	1.14 ± 0.22	1.38
Mitochondrial import inner membrane translocase subunit Tim10	*tim10*	0.19 ± 0.06	0.06 ± 0.02*	-3.20
Mitochondrial import inner membrane translocase subunit Tim9	*tim9*	0.13 ± 0.03	0.05 ± 0.01*	-2.55
Mitochondrial dynamics and apoptosis				
Mitochondrial fission 1 protein	*fis1*	0.63 ± 0.04	0.72 ± 0.09	1.14
Mitofusin 1	*mfn1*	0.06 ± 0.01	0.08 ± 0.02	1.37
Mitofusin 2	*mfn2*	0.21 ± 0.02	0.12 ± 0.01*	-1.72
Mitochondrial fission factor homolog B	*miffb*	0.35 ± 0.04	0.19 ± 0.03*	-1.82
Mitochondrial Rho GTPase 1	*miro1a*	0.12 ± 0.04	0.03 ± 0.01*	-3.70
Mitochondrial Rho GTPase 2	*miro2*	0.31 ± 0.05	0.12 ± 0.02**	-2.69
Apoptosis-related protein 1	*aifm1*	0.23 ± 0.02	0.15 ± 0.03**	-1.53
Apoptosis-related protein 3	*aifm3*	0.28 ± 0.05	0.24 ± 0.05	-1.15
Apoptosis regulator BAX	*bax*	0.33 ± 0.04	0.31 ± 0.02	-1.06
Bcl-2-like protein 1	*bclx*	0.65 ± 0.08	0.52 ± 0.07	-1.25
FA oxidation & TCA				
3-ketoacyl-CoA thiolase	*acaa2*	0.43 ± 0.06	0.18 ± 0.03*	-2.38
Carnitine palmitoyltransferase 1A	*cpt1a*	0.53 ± 0.07	0.33 ± 0.05	-1.59
Enoyl-CoA hydratase	*ech*	0.16 ± 0.01	0.17 ± 0.03	1.07
Hydroxyacyl-CoA dehydrogenase	*hadh*	0.82 ± 0.03	0.53 ± 0.08**	-1.56
Citrate synthase	*cs*	0.62 ± 0.03	0.47 ± 0.05**	-1.32
OXPHOS (Complex I)				
NADH-ubiquinone oxidoreductase chain 2	*nd2*	4.95 ± 0.60	5.07 ± 1.49	1.02
NADH-ubiquinone oxidoreductase chain 5	*nd5*	0.98 ± 0.26	0.80 ± 0.21	-1.22
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1	*ndufa1*	0.83 ± 0.06	0.77 ± 0.10	-1.08
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3	*ndufa3*	0.81 ± 0.06	0.56 ± 0.07*	-1.46
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	*ndufa4*	1.10 ± 0.07	0.78 ± 0.04**	-1.40
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	*ndufa7*	0.21 ± 0.02	0.12 ± 0.01**	-1.69
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5	*ndufb5*	0.52 ± 0.02	0.32 ± 0.05**	-1.59
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 10	*ndufb10*	0.59 ± 0.03	0.75 ± 0.11	1.28
NADH dehydrogenase iron-sulfur protein 2	*ndufs2*	0.54 ± 0.04	0.44 ± 0.04	-1.23
NADH dehydrogenase iron-sulfur protein 7	*ndufs7*	0.60 ± 0.04	0.42 ± 0.05**	-1.42
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2	*ndufaf2*	0.46 ± 0.05	0.32 ± 0.05	-1.43
OXPHOS (Complex II)				
Succinate dehydrogenase [ubiquinone] flavoprotein subunit	*sdha*	0.27 ± 0.04	0.13 ± 0.02*	-2.10
Succinate dehydrogenase cytochrome b560 subunit	*sdhc*	0.16 ± 0.02	0.19 ± 0.03	1.20
Succinate dehydrogenase [ubiquinone] cytochrome b small subunit B	*sdhd*	0.13 ± 0.02	0.17 ± 0.01	1.29
Succinate dehydrogenase assembly factor 1	*sdhaf1*	0.20 ± 0.06	0.07 ± 0.02*	-2.99
Succinate dehydrogenase assembly factor 2	*sdhaf2*	0.19 ± 0.04	0.07 ± 0.02*	-2.61
OXPHOS (Complex III)				
Cytochrome b	*cyb*	3.81 ± 0.42	5.08 ± 1.37	1.33
Cytochrome b-c1 complex subunit Rieske	*uqcrfs1*	0.12 ± 0.03	0.06 ± 0.01	-2.01
Cytochrome b-c1 complex subunit 1	*uqcrc1*	0.31 ± 0.06	0.14 ± 0.02*	-2.14
Cytochrome b-c1 complex subunit 2	*uqcrc2*	0.64 ± 0.06	0.33 ± 0.04**	-1.96
Cytochrome b-c1 complex subunit 6	*uqcrh*	0.27 ± 0.05	0.16 ± 0.02*	-1.72
Cytochrome b-c1 complex subunit 8	*uqcrq*	2.21 ± 0.26	2.09 ± 0.32	-1.06
Cytochrome b-c1 complex subunit 9	*uqcr10*	0.46 ± 0.02	0.40 ± 0.05	-1.15
Ubiquinol-cytochrome c reductase complex chaperone CBP3 homolog	*uqcc*	0.17 ± 0.02	0.11 ± 0.02	-1.50
OXPHOS (Complex IV)				
Cytochrome c oxidase subunit I	*coxi*	2.52 ± 0.37	4.69 ± 1.34*	1.86
Cytochrome c oxidase subunit II	*coxii*	0.96 ± 0.09	1.28 ± 0.31	1.33
Cytochrome c oxidase subunit III	*coxiii*	1.44 ± 0.18	1.76 ± 0.46	1.22
Cytochrome c oxidase subunit 4 isoform 1	*cox4a*	0.59 ± 0.00	0.59 ± 0.00	1.00
Cytochrome c oxidase subunit 5A, mitochondrial-like isoform 2	*cox5a2*	0.04 ± 0.01	0.09 ± 0.02**	2.48
Cytochrome c oxidase subunit 6A isoform 2	*cox6a2*	0.79 ± 0.07	1.09 ± 0.22	1.38
Cytochrome c oxidase subunit 6C-1	*cox6c1*	0.44 ± 0.06	0.35 ± 0.03	-1.25
Cytochrome c oxidase subunit 7B	*cox7b*	0.68 ± 0.09	0.78 ± 0.08	1.14
Cytochrome c oxidase subunit 8B	*cox8b*	1.02 ± 0.10	1.39 ± 0.16*	1.37
SCO1 protein homolog, mitochondrial	*sco1*	0.19 ± 0.06	0.05 ± 0.02*	-3.83
Surfeit locus protein 1	*surf1*	0.22 ± 0.04	0.09 ± 0.01*	-2.47
Cytochrome c oxidase assembly protein COX15 homolog	*cox15*	0.15 ± 0.02	0.10 ± 0.01**	-1.48
OXPHOS (Complex V)				
ATP synthase subunit gamma	*atp5c1*	0.36 ± 0.04	0.19 ± 0.04*	-1.83
ATP synthase subunit beta	*atp5b*	0.40 ± 0.05	0.29 ± 0.04	-1.39
ATP synthase lipid-binding protein	*atp5g1*	0.61 ± 0.06	0.36 ± 0.06*	-1.68
ATP synthase subunit g	*atp5l*	0.64 ± 0.06	0.42 ± 0.05*	-1.53
Mitochondrial F1 complex assembly factor 2	*atpaf2*	0.04 ± 0.01	0.01 ± 0.00*	-6.02
Respiration uncoupling				
Uncoupling protein 2	*ucp2*	0.12 ± 0.05	0.02 ± 0.02*	-6.26

Gilthead sea breams were exposed to normoxic (oxygen saturation > 85%) and hypoxic (1.3 ppm, oxygen saturation = 18–19%) conditions. Data are presented as the mean ± SEM (n = 7–8). Statistically significant differences between normoxic and hypoxic fish are indicated (*P < 0.05, **P < 0.01; Student's t test). *nd*: non-detected. Gene names of mitochondrial-encoded catalytic subunits of the OXPHOS pathway are highlighted in bold and italicised. Gene names of nuclear-encoded catalytic subunits of the OXPHOS pathway are highlighted in bold. Gene names of nuclear-encoded regulatory subunits are presented in normal font. Gene names of nuclear-encoded assembly factors are italicised. Square symbols are used for up- (red) and down-regulated genes (green)

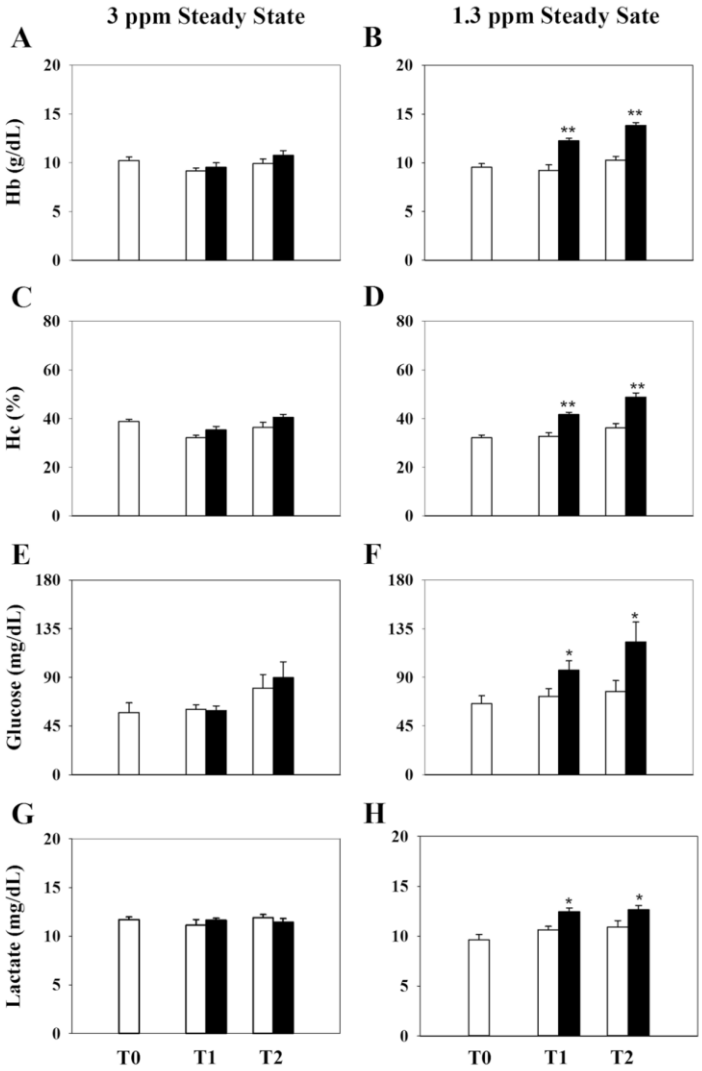

Fig. 2 Effects of normoxia (*white bars*) and hypoxia (*black bars*) on blood haematology and biochemistry. Hypoxia levels were set above (**a**, **c**, **e**, **g**) or below (**b**, **d**, **f**, **h**) the LOS. Data are presented as the mean ± SEM (n = 7–8). Statistically significant differences between normoxic and hypoxic fish are indicated (*P < 0.05, **P < 0.01; two-way analysis of variance (ANOVA) followed by the Holm-Sidak test)

Discussion

Studies in gilthead sea bream have indicated that the response of this species to a progressive decline in O_2 concentration is to reduce its swimming activity, indicative of an increasing metabolic stress and/or a coping strategy to prolong survival time when hypoxia cannot be avoided [5]. In the same study, the threshold level of LOS determined in 400-g fish varied from 17% O_2 saturation at 12 °C and 36% O_2 saturation at 20 °C. These O_2 concentrations can be implemented in aquaculture as a lower limit for acceptable decreases in O_2 concentration with respect to the physiological function and welfare of farmed gilthead sea bream. Therefore, as further explained below, it is not surprising that data on blood biochemistry and haematology in fish exposed to O_2 concentrations above the LOS did not significantly vary after 24 h of hypoxia challenge. In contrast, a consistent response, exacerbated over time, was observed for blood parameters measured a few hours after exposure to O_2 concentrations below the LOS. In this case, the gene expression profile of whole blood cells was analysed, and the molecular signatures of hypoxic fish revealed important changes consistent with reduced but more efficient aerobic ATP production.

Living organisms are characterised by continuous switching between resting and active states, which includes long resting periods with low ATP production

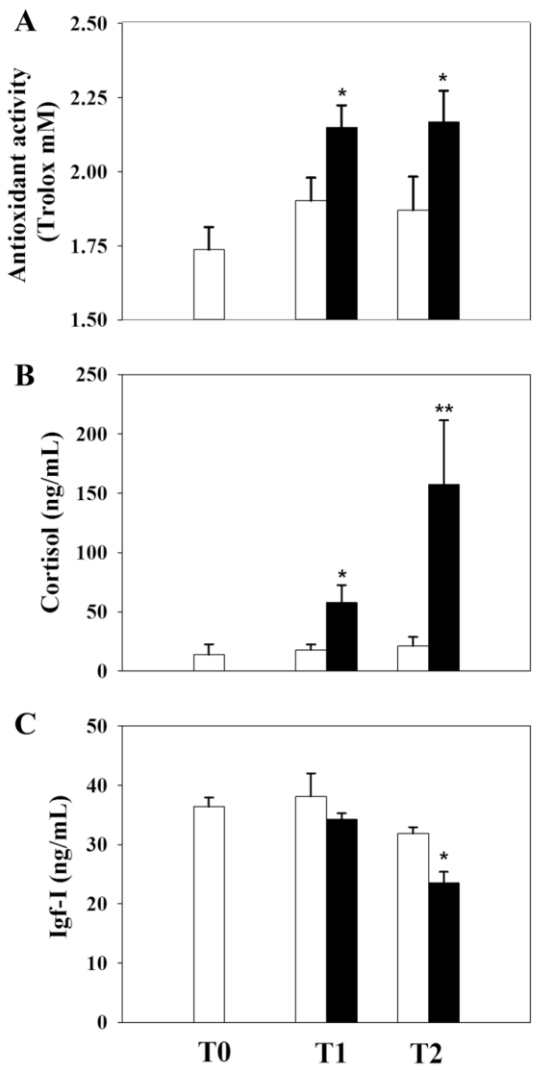

Fig. 3 Effects of normoxia (*white bars*) and hypoxia (*black bars*) below the LOS on plasma parameters. Antioxidant activity (**a**), cortisol (**b**) and Igf-I (**c**). Data are presented as the mean ± SEM (n = 7–8). Statistically significant differences between normoxic and hypoxic fish are indicated (*P < 0.05, **P < 0.01; two-way analysis of variance (ANOVA) followed by the Holm-Sidak test)

[24]. Similarly, the response to hypoxia has two main aspects characterised by defence and rescue, where the early defence stage is achieved by reducing energy needs (hypo-metabolic state) and the dependence on aerobic metabolism [25]. In the case of gilthead sea bream, the antioxidant defences in fish fed diets supplemented with methionine and white tea were insufficient to avoid oxidative stress under moderate hypoxia induced by 40% O_2 saturation at 22–23 °C [26]. However, LOS increases with decreasing temperature [6], and the results of the present study showed that all measured haematological and biochemical parameters remained mostly unaltered in fish maintained at 20–21 °C and 41–42% O_2 saturation. In contrast, a pronounced increase in Hc, Hb and plasma glucose and lactate levels was reported after exposure to severe hypoxia (18–19% O_2 saturation) for 4 h under steady-state conditions. Indeed, this rapid response could reflect an increase in blood O_2-carrying capacity [27] associated in the short term with erythrocyte release from a storage organ or with a reduction in plasma volume rather than the formation of new Hb [28]. Consistent with [8], this finding likely reflects metabolic changes mediated by O_2 sensors that drive the shift of the redox cellular status of NADH to a more reduced form with a rapid recycling of NAD^+ to NADH. Certainly, hypoxic situations must improve and adjust the metabolic and O_2-carrying capacities of challenged fish to cope and reach internal homeostasis [29]. The trigger observed in plasma antioxidant capacity after acute and severe hypoxia demonstrates a general decrease in metabolic rates that also reflects the aerobic/anaerobic shift of metabolism [25, 30, 31].

The increase in plasma cortisol levels observed after severe hypoxia indicates a stressful scenario in the experimental model used in the present study. Other common features of hypoxic and stress conditions include a decrease in plasma Igf-I levels and concomitant growth inhibition [32, 33]. In this sense, a characteristic response in challenged gilthead sea bream produced by crowding, and presumably also through hypoxia, is the overall down-regulated expression of hepatic *igf*s and growth hormone receptors [34]. Studies in rodents support the involvement of the Gh/Igf system in the regulation of key antioxidant enzymes, ROS production and scavenging as well mitochondrial biogenesis and activity [35, 36]. However, thus far, the precise mechanisms underlying these Gh/Igf-mediated effects remain unexplored in fish. Moreover, confounding results have been reported for the aerobic/anaerobic shift during hypoxia exposure, although studies in the euryoxic mudsucker *Gillichthys mirabilis* showed a tissue-specific gene regulation resulting in suppressed protein synthesis in skeletal muscle and enhanced anaerobic ATP production in the liver tissue [14]. Similarly, zebra fish (*Danio rerio*) embryos survive during severe hypoxia (0–5% O_2 saturation) through changes in the gene and protein expression of master regulators of O_2 homeostasis, such as the hypoxia-inducible factor 1 alpha (*hif*-1α/Hif-*1α*) [37–40]. Additionally, long-term adaptive responses in the gene expression of several pathways related to cell architecture, cell division and energy metabolism have been underlined in the gills of adult hypoxic fish [41]. In our experimental model, this hypothesis was perfectly consistent with hypoxic-mediated effects on mitochondrial-related markers of blood cells (see below).

Most mitochondrial proteins are encoded by nuclear DNA; thus, a healthy metabolic mitochondria phenotype is highly dependent on the protein import system, which involves two assembly complexes: the translocases of the outer membrane (TOM complex) and the translocases of the inner membrane (TIM complex) (see [42, 43] for review). Thus, as demonstrated in mammalian cells [44], the TOM/TIM complex is highly inducible and regulated at both transcriptional and post-transcriptional levels under conditions of chronic stress or energy deficit to ensure the maintenance of adequate mitochondrial protein import rates. Similarly, juveniles of gilthead sea bream exhibit a clear up-regulation in the gene expression of hepatic protein subunits of the TOM/TIM complex in response to aerobic energy stimuli after exposure to cyclic decreases in water temperature [20]. Conversely, the present study demonstrated that severe hypoxia induced a pronounced down-regulation of *tom70* and *tom22* subunits in whole blood cells concurrent with decreases in mRNAs encoding protein subunits of TIM23 (*tim44*) and TIM22 (*tim10* and *tim9*) complexes. In addition, co-expression analyses revealed the up-regulation of *tom34*, which acts as a co-chaperone of the Hsp70/Hsp90 complex, inhibiting mitochondrial protein translocation when expressed in excess [45]. Taken together, these findings suggest in hypoxic fish an orchestration of the TOM/TIM complex that could enable adjustments in mitochondrial protein translocation to reduce plasma oxidative capacity and the risk of oxidative stress, a feature that is consistent with the down-regulated expression of markers of ROS production and scavenging, including *ucp2*, mitochondrial superoxide dismutase (*sod2*), enzymes of the glutathione system (*gpx1* and *gst3*) and enzymes of fatty acid β-oxidation and TCA (*acaa2*, *hadh*, and *cs*). Importantly, the same trend was observed for mitochondrial (*hsp10*, *dnaja3a*, *dnajc20*, *hsp60*, and *grp-75*) and endoplasmic reticulum (*grp-170*) molecular chaperones, suggesting that proper protein folding was primarily assured in the blood cells of gilthead sea bream under the depressed metabolism induced by hypoxia exposure. Similarly, severe hypoxia did not induce the gene expression of heat shock proteins in rainbow trout (*Oncorhynchus mykiss*) RBCs cultured in vitro when the hypoxia challenge was not accompanied by a heat shock treatment [46].

Mitochondrial dynamics is an essential process that adapts mitochondria morphology to the bioenergetics requirements of the cell (see [47] for review). The mechanism of this biological process involves the balance of two opposing procedures (fusion and fission), but it is also greatly affected by the "railways" used by the mitochondria to move inside the cells. The functionality of these organelles favours the redistribution of mitochondria within the cell to ensure high oxidative capacity under conditions of high energy demand, enabling the removal of dysfunctional or damaged mitochondria. This mechanism is highly conserved from yeast to mammals [48], and the molecular identity of major components of the fusion (*mfn1* and *mfn2*) and fission (*fis1* and *miffb*) system, as well as those of the MIRO system (*miro1a* and *miro2*) has been characterised in gilthead sea bream and uploaded to public database repositories [20]. Nevertheless, experimental evidences demonstrated that the gene expression of some of these effectors is highly induced in response to aerobic stimuli after cold-water exposure. In contrast, in the present study, severe hypoxia significantly repressed the expression of most components of this biological process (*mfn2, miffb, miro1a,* and *miro2*), including the well-known mitochondrial apoptotic factor *aifm1*. Consistently, the knockout of the transcriptional regulator *pgc1ß* is associated with a selective reduction in the expression in mice [49]. The lack of Pgc1*ß* also impaired the thermogenic response of adipose tissue and hepatic lipid metabolism in response to high fat dietary loads [50]. Therefore, Pgc1*ß* is essential for proper metabolic tuning in stress situations, contributing to the maintenance of the basal expression of mitochondrial and metabolic-related genes. However, in the present experimental model, the opposite regulation was observed for *pgc1ß* and *mfn2*, suggesting that the up-regulated expression of *pgc1ß* was more a consequence than the cause of the overall repressed expression of mitochondria-related genes. This notion was supported by the observation that the mitochondrial transcription factor *nrf1*, another target gene of *pgc1ß* [50], was also down-regulated in hypoxia-challenged fish. Notably, despite the overlapping gene expression of *pgc1ß* and its homologue *pgc1α*, the compensation of Pgc1α or Pgc1ß functions was not completely observed in Pgc1α or Pgc1ß knockout rodents [51–53]. In the case of gilthead sea bream blood cells, this effect is more exacerbated because *pgc1α* mRNAs were almost undetectable in both normoxic and hypoxic fish, although the expression of this gene at noticeable levels has previously been reported in other tissues of this fish species [20]. Whether this effect is part of the evolutionary pressure to select the conservation of functional mitochondria in the nucleated RBCs of non-mammalian vertebrates remains to be established [17].

The ultimate effector for coping with changes in energy needs and aerobic ATP production is the regulation of the OXPHOS pathway, which comprises five enzyme complexes (I-V) with catalytic enzymatic subunits encoded by both nuclear or mitochondrial DNA, whereas the enzyme subunits with regulatory or assembly properties are strictly of nuclear origin [19]. Changes in the enzymatic activities of the OXPHOS pathway have been studied for many years both in mammals and fish

(e.g., [54–56]). Little is known at the molecular level, although gene expression profiling of liver, skeletal muscle and cardiac muscle tissues revealed that both the direction and magnitude of change is highly dependent on the metabolic capabilities of each tissue [8]. Thus far, the molecular fingerprinting of the OXPHOS pathway remains primarily unexplored in blood cells, and this is the first to address the specific regulation of this pathway in response to environmental stressors, evidenced by the general depletion of several components of Complexes I, II, III and V in response to severe hypoxic stimuli. Assembly factors of Complex IV (*sco1*, *surf1* and *cox15)* were also down-regulated in the present experimental model. These enzyme subunits play an important role in energy production, and mutations or defects in these molecules produce adverse effects in the appropriate function of the OXPHOS pathway in mammals [57–61]. However, this observation contrasted with the overall overexpression of catalytic and regulatory subunits of Complex IV, which was statistically significant for the catalytic *coxi* and the regulatory *cox5a2* and *cox8b* subunits. CoxI protein is encoded by mitochondrial DNA and represents one of the largest subunits of Complex IV, which contains the bimetallic centre where O_2 binds and is reduced to H_2O [62, 63]. In addition, the observed increase in the gene expression of Cox5a and Cox8 family subunits highlights their importance during the completion of the holocomplex monomer, which contains the functional structure of the cytochrome *c* binding site (see [64] for review). Therefore, we hypothesised that the net effect should be a reduced mitochondrial ATP production due to the overall suppression of mRNAs encoding the enzyme subunits of Complexes I, II, III and IV, although the opposite regulation of the catalytic/regulatory components of Complex IV should be accompanied by subsequent mechanisms that allow a better exploitation of available oxygen in the most energetically favorable way. Modifications in mitochondrial properties also occur in other vertebrates, and the hypo-metabolic steady-state observed in overwintering frogs (*Rana temporaria*) occurred during hypoxic submergence by increases in mitochondrial O_2 affinity and a reduction in resting (state 4) and active (state 3) respiration rates in mitochondria isolated from skeletal muscle [65]. Similarly, early studies in the freshwater European eel (*Anguilla anguilla*) suggest that the efficiency of OXPHOS is increased after acclimation to high hydrostatic pressure, decreasing the enzymatic activity of Complex II in red muscle, whereas that of Complex IV is significantly increased [66]. This situation would enable a reduction in the electron leak and the optimisation of the respiratory chain. Similarly, more recent studies in gilthead sea bream have revealed that the gene expression ratio of the enzyme subunits of Complexes I and IV is altered in heart and liver tissue during the recovery state after severe hypoxia exposure [67]. Thus, as reviewed by [68], it is now evident that variations in the mitochondrial efficiency of ATP production exist among individuals, populations and environments, and even within the same individual over time. This spatial and temporal variability in mitochondrial machinery adds an

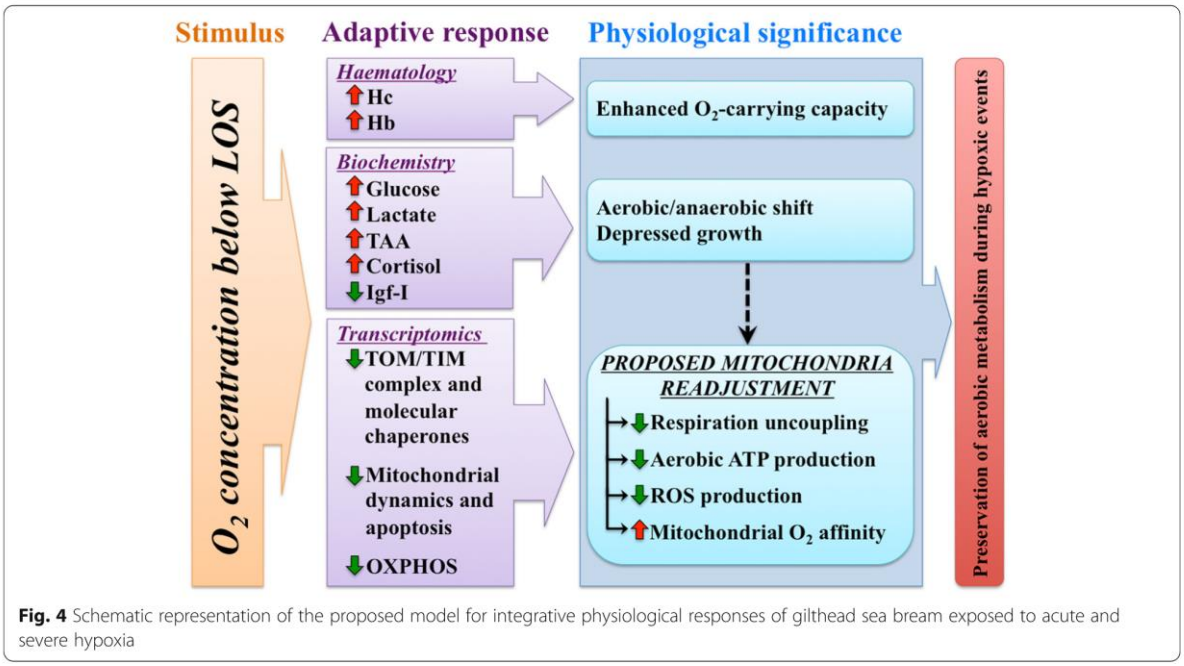

Fig. 4 Schematic representation of the proposed model for integrative physiological responses of gilthead sea bream exposed to acute and severe hypoxia

additional layer of complexity to the regulation of energy metabolism, and the maintenance of aerobic metabolism is becoming recognised as a primary hypoxia survival strategy in most organisms, including fish [69]. Even so, the usage of transcriptomic analysis with other experimental approaches related to mitochondrial activity and respiration would be necessary for the better understanding about the proposed re-adjustment of mitochondrial function in hypoxia-challenged fish.

Conclusions and future perspectives

As summarized in Fig. 4, the integrated data on blood haematology, biochemistry and transcriptomics in response to water O_2 concentrations below the LOS highlighted an enhanced O_2-carrying capacity as a result of higher Hc and Hb concentrations in response to strong hypoxic stimuli. Changes in plasma antioxidant capacity, as well as hormone and metabolite levels supported reduced energy needs and also reflected an aerobic/anaerobic shift. These results were further confirmed by gene expression profiling of a wide representation of mitochondrial-related markers, including antioxidant enzymes and molecular chaperones, effectors of mitochondrial dynamics and apoptosis, and key components of the respiratory chain, suggesting that the mitochondrial bioenergetics of fish blood cells are finely adjusted at the transcriptional level through changes in water O_2 concentrations. The induced gene expression profiles of catalytic and regulatory enzyme subunits of Complex IV should be considered an adaptive process to ensure reduced but more efficient aerobic ATP production consistent with reduced respiration uncoupling, as suggested by the decreased expression of *ucp2*. These results indicate that the gilthead sea bream is a highly euryoxic fish. Further studies are underway to determine the resilience of gilthead sea bream to high rearing densities and low O_2 concentrations, exploring the potential benefits of hypoxic preconditioning for improving the aerobic scope and swimming metabolic activity of farmed fish.

Additional file

Additional file 1: Table S1. Forward (F) and reverse (R) primers used for real-time PCR. (DOCX 50 kb)

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Availability of data and materials
The datasets supporting the conclusions of this study are included within the article and its additional Supporting Information file.

Authors' contributions
JACG and JPS conceived and designed the study. ABN, JACG and JAMS performed the experimental procedures. JAMS, JACG and JPS analysed and interpreted the data. JAMS and JPS drafted the original manuscript. All authors reviewed, edited and approved the final manuscript.

Ethics approval
All procedures were approved by the Ethics and Animal Welfare Committee of Institute of Aquaculture Torre de la Sal and carried out according to the National (Royal Decree RD53/2013) and the current EU legislation (2010/63/EU) on the handling of experimental fish.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

Publisher's Note

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References

1. Fry FEJ. Effects of the environment on animal activity. Univ Tor Stud Biol Ser. 1947;68:5–62.
2. Fry FEJ. The effect of environmental factors on the physiology of fish. In: Hroar WS, Randall DJ, editors. Fish physiology. Environmental relations and behavior 6. New York: Academic Press, Elsevier; 1971. p. 1–87.
3. Pörtner HO, Grieshaber MK. Critical PO2 (s) in oxyconforming and oxyregulating animals gas exchange, metabolic rate and the mode of energy production. In: Bicudo JEPW, editor. The vertebrate gas transport cascade adaptations to environment and mode of life. Boca Raton: CRC Press; 1993. p. 330–57.
4. Saravanan S, Geurden I, Figueiredo-Silva AC, Kaushik SJ, Haidar MN, Verreth JA, Schrama JW. Control of voluntary feed intake in fish: a role for dietary oxygen demand in Nile tilapia (*Oreochromis niloticus*) fed diets with different macronutrient profiles. Br J Nutr. 2012;108:1519–29.
5. Remen M, Nederlof MAJ, Folkedal O, Thorsheim G, Sitjà-Bobadilla A, Pérez-Sánchez J, Oppedal F, Olsen RE. Effect of temperature on the metabolism, behavior and oxygen requirements of *Sparus aurata*. Aquacult Env Interac. 2015;7:115–23.
6. Remen M, Sievers M, Torgersen T, Oppedal F. The oxygen threshold for maximal feed intake of Atlantic salmon post-smolts is highly temperature-dependent. Aquaculture. 2016;464:582–92.
7. Lushchak VI, Bagnyukova TV. Effects of different environmental oxygen levels on free radical processes in fish. Comp Biochem Physiol B-Biochem Mol Biol. 2006;144:283–9.
8. Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. Tissue-specific gene expression and functional regulation of uncoupling protein 2 (UCP2) by hypoxia and nutrient availability in gilthead sea bream (*Sparus aurata*): implications on the physiological significance of UCP1-3 variants. Fish Physiol Biochem. 2014;40:751–62.
9. Khacho M, Tarabay M, Patten D, Khacho P, MacLaurin JG, Guadagno J, Bergeron R, Cregan SP, Harper M-E, Park DS, Slack RS. Acidosis overrides oxygen deprivation to maintain mitochondrial function and cell survival. Nat Commun. 2014;5:3550.

10. Hoppeler H, Vogt M. Muscle tissue adaptations to hypoxia. J Exp Biol. 2001; 204:3133–9.
11. Murray AJ. Metabolic adaptation of skeletal muscle to high altitude hypoxia: how new technologies could resolve the controversies. Genome Med. 2009; 1:117.
12. Gamboa JL, Andrade FH. Muscle endurance and mitochondrial function after chronic normobaric hypoxia: contrast of respiratory and limb muscles. Pflug Arch Eur J Physiol. 2012;463:327–38.
13. Donohoe PH, West TG, Boutilier RG. Respiratory, metabolic and acid–base correlates of aerobic metabolic rate reduction in overwintering frogs. Am J Physiol-Reg I. 1998;43:R704–10.
14. Gracey AY, Troll JV, Somero GN. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. Proc Natl Acad Sci U S A. 2001;98: 1993–8.
15. Everett MV, Antal CE, Crawford DL. The effect of short-term hypoxic exposure on metabolic gene expression. J Exp Zool Part A. 2012;317:9–23.
16. Vanderplancke G, Claireaux G, Quazuguel P, Madec L, Ferraresso S, Sévère A, Zambonino-Infante JL, Mazurais D. Hypoxic episode during the larval period has long-term effects on European sea bass juveniles (*Dicentrarchus labrax*). Mar Biol. 2015;162:367–76.
17. Stier A, Bize P, Schull Q, Zoll J, Singh F, Geny B, Gros F, Royer C, Massemin S, Criscuolo F. Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. Front Zool. 2013;10:33.
18. Calduch-Giner JA, Echasseriau Y, Crespo D, Baron D, Planas JV, Prunet P, Pérez-Sánchez J. Transcriptional assessment by microarray analysis and large-scale meta-analysis of the metabolic capacity of cardiac and skeletal muscle tissues to cope with reduced nutrient availability in gilthead sea bream (*Sparus aurata* L.). Mar Biotechnol. 2014;16:423–35.
19. Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. Unraveling the molecular signatures of oxidative phosphorylation to cope with the nutritially changing metabolic capabilities of liver and muscle tissues in farmed fish. PLoS One. 2015;10(4):e0122889.
20. Bermejo-Nogales A, Nederlof M, Benedito-Palos L, Ballester-Lozano GF, Folkedal O, Olsen RE, Sitjà-Bobadilla A, Pérez-Sánchez J. Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata*) to environmental stress: new insights in fish mitochondrial phenotyping. Gen Comp Endocrinol. 2014a;205:305–15.
21. Shimizu M, Swanson P, Fukada H, Hara A, Dickhoff WW. Comparison of extraction methods and assay validation for salmon insulin-like growth factor-I using commercially available components. Gen Comp Endocrinol. 2000;119:26–36.
22. Vega-Rubín de Celis S, Gómez-Requeni P, Pérez-Sánchez J. Production and characterization of recombinantly derived peptides and antibodies for accurate determinations of somatolactin, growth hormone and insulin-like growth factor-I in European sea bass (*Dicentrarchus labrax*). Gen Comp Endocrinol. 2004;139:266–77.
23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods. 2001;25:402–8.
24. Kadenbach B, Ramzan R, Wen L, Vogt S. New extension of the Mitchell theory for oxidative phosphorylation in mitochondria of living organism. BBA-Gen Subj. 1800;2010:205–12.
25. Hochachka PW, Buck LT, Doll CJ, Land SC. Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proc Natl Acad Sci U S A. 1996;93:9493–8.
26. Pérez-Jiménez A, Peres H, Rubio VC, Oliva-Teles A. The effect of hypoxia on intermediary metabolism and oxidative status in gilthead sea bream (*Sparus aurata*) fed on diets supplemented with methionine and white tea. Comp Biochem Physiol C-Toxicol Pharmacol. 2012;155:506–16.
27. Wood SC, Johansen K. Adaptation to hypoxia by increased HbO_2 affinity and decreased red cell ATP concentration. Nat New Biol. 1972;237:278–9.
28. Soivio A, Nikinmaa M, Westman K. The blood oxygen binding properties of hypoxic *Salmo gairdneri*. J Comp Physiol. 1980;136(1):83–7.
29. Storey KB. Regulation of hypometabolism: insights into epigenetic controls. J Exp Biol. 2015;218(1):150–9.
30. Dalla Via J, Van den Thillart G, Cattani O, Cortesi P. Behavioural responses and biochemical correlates in *Solea solea* to gradual hypoxic exposure. Can J Zool. 1998;76:2108–13.
31. Virani NA, Rees BB. Oxygen consumption, blood lactate and inter-individual variation in the gulf killifish, *Fundulus grandis*, during hypoxia and recovery. Comp Biochem Physiol A-Mol Integr Physiol. 2000;126:397–405.
32. Mommsen TP, Vijayan MM, Moon TW. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev Fish Biol Fish. 1999;9: 211–68.
33. Dyer AR, Upton Z, Stone D, Thomas PM, Soole KL, Higgs N, Quinn K, Carragher JF. Development and validation of a radioimmunoassay for fish insulin-like growth factor I (IGF-I) and the effect of aquaculture related stressors on circulating IGF-I levels. Gen Comp Endocrinol. 2004;135:268–75.
34. Saera-Vila A, Calduch-Giner JA, Prunet P, Pérez-Sanchez J. Dynamics of liver GH/IGF axis and selected stress markers in juvenile gilthead sea bream (*Sparus aurata*) exposed to acute confinement: differential stress response of growth hormone receptors. Comp Biochem Physiol A-Mol Integr Physiol. 2009;154:197–203.
35. Brown-Borg HM, Rakoczy SG, Romanick MA, Kennedy MA. Effects of growth hormone and insulin like growth factor-1 on hepatocyte antioxidative enzymes. Exp Biol Med. 2002;227:94–104.
36. Brown-Borg HM, Rakoczy SG. Growth hormone administration to long-living dwarf mice alters multiple components of the antioxidative defense system. Mech Aging Dev. 2003;124:1013–24.
37. Ton C, Stamatiou D, Liew CC. Gene expression profile of zebrafish exposed to hypoxia during development. Physiol Genomics. 2003;13:97–106.
38. Woods IG, Imam FB. Transcriptome analysis of severe hypoxic stress during development in zebrafish. Genomics data. 2015;6:83–8.
39. Robertson CE, Wright PA, Köblitz L, Bernier NJ. Hypoxia-inducible factor-1 mediates adaptive developmental plasticity of hypoxia tolerance in zebrafish, *Danio rerio*. P Royal Soc Lond B Bio. 2014;281(1786):20140637.
40. Köblitz L, Fiechtner B, Baus K, Lussnig R, Pelster B. Developmental expression and hypoxic induction of hypoxia inducible transcription factors in the zebrafish. PLoS One. 2015;10(6):e0128938.
41. van der Meer DL, van den Thillart GE, Witte F, de Bakker MA, Besser J, Richardson MK, Spaink HP, Leito JTD, Bagowski CP. Gene expression profiling of the long-term adaptive response to hypoxia in the gills of adult zebrafish. Am J Physiol-Reg I. 2005;289:1512–9.
42. Pfanner N, Meijer M. Mitochondrial biogenesis: the tom and Tim machine. Curr Biol. 2010;7:100–3.
43. Smits P, Smeitink J, van den Heuvel L. Mitochondrial translation and beyond: processes implicated in combined oxidative phosphorylation deficiencies. J Biomed Biotechnol. 2010;2010:737385.
44. Ljubicic V, Joseph AM, Saleem A, Uguccioni G, Collu-Marchese M, Lai RY, Nguyen LM-D, Hood D. A. Transcriptional and post-transcriptional regulation of mitochondrial biogenesis in skeletal muscle: effects of exercise and aging. BBA-Gen Subj. 2010;1800:223–34.
45. Faou P, Hoogenraad NJ. Tom34: a cytosolic cochaperone of the Hsp90/ Hsp70 protein complex involved in mitochondrial protein import. BBA-Mol Cell Res. 2012;1823:348–57.
46. Currie S, Tufts BL, Moyes CD. Influence of bioenergetic stress on heat shock protein gene expression in nucleated red blood cells of fish. Am J of Physiol-Reg I. 1999;276:990–6.
47. Ferree A, Shirihai O. Mitochondrial dynamics: the intersection of form and function. In: Kadenbach B, editor. Mitochondrial oxidative Phosphorylation. New York: Springer; 2012. p. 13–40.
48. Anesti V, Scorrano L. The relationship between mitochondrial shape and function and the cytoskeleton. BBA–Bioenerg. 2006;1757:692–9.
49. Liesa M, Borda-d'Água B, Medina-Gómez G, Lelliott CJ, Paz JC, Rojo M, Palacín M, Vidal-Puig A, Zorzano A. Mitochondrial fusion is increased by the nuclear coactivator PGC-1β. PLoS One. 2008;3(10):e3613.
50. Patti ME, Butte AJ, Crunkhorn S, Cusi K, Berria R, Kashyap S, Miyazaki Y, Kohane I, Costello M, Saccone R, Landaker EJ, Goldfine AB, Mun E, DeFronzo R, Finlayson J, Kahn CR, Mandarino LJ. Coordinated reduction of genes of oxidative metabolism in humans with insulin resistance and diabetes: potential role of PGC1 and NRF1. P Nat Acad Sci USA. 2003;100:8466–71.
51. Arany Z, He H, Lin J, Hoyer K, Handschin C, Toka O, Ahmad F, Matsui T, Chin S, Wu P-H, Rybkin II, Shelton JM, Manieri M, Cinti S, Schoen FJ, Bassel-Duby R, Rosenzweig A, Ingwall JS, Spiegelman BM. Transcriptional coactivator PGC-1α controls the energy state and contractile function of cardiac muscle. Cell Metab 2005;1:259-271.
52. Leone TC, Lehman JJ, Finck BN, Schaeffer PJ, Wende AR, Boudina S, Courtois M, Wozniak DF, Sambandam N, Bernal-Mizrachi C, Chen Z, Holloszy JO, Medeiros DM, Schmidt RE, Saffitz JE, Abel ED, Semenkovich CF, Kelly DP. PGC-1α deficiency causes multi-system energy metabolic derangements: muscle dysfunction, abnormal weight control and hepatic steatosis. PLoS Biol. 2005;3(4):e101.

53. Lelliott C, Medina-Gomez G, Petrovic N, Kis A, Feldmann HM, Bjursell M, Parker N, Curtis K, Campbell M, Hu P, Zhang D, Litwin SE, Zaha VG, Fountain KT, Boudina S, Jimenez-Linan M, Blount M, Lopez M, Meirhaeghe A, Bohlooly-Y M, Storlien L, Strömstedt M, Snaith M, Oresic M, Abel ED, Cannon B, Vidal-Puig A. Ablation of PGC-1b results in defective mitochondrial activity, thermogenesis, hepatic function, and cardiac performance. PLoS Biol. 2006; 4(11):e369.
54. Holloszy JO. Biochemical adaptations in muscle effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. J Biol Chem. 1967;242:2278–82.
55. Zerbetto E, Vergani L, Dabbeni-Sala F. Quantification of muscle mitochondrial oxidative phosphorylation enzymes via histochemical staining of blue native polyacrylamide gels. Electrophoresis. 1997;18:2059–64.
56. Guderley H. Metabolic responses to low temperature in fish muscle. Biol Rev. 2004;79:409–27.
57. Zhu Z, Yao J, Johns T, Fu K, De Bie I, Macmillan C, Cuthbert AP, Newbold RF, Wang J, Chevrette M, Brown GK, Brown RM, Shoubridge EA. SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. Nat Genet. 1998;20:337–43.
58. Antonicka H, Mattman A, Carlson CG, Glerum DM, Hoffbuhr KC, Leary SC, Kennaway NG, Shoubridge EA. Mutations in COX15 produce a defect in the mitochondrial heme biosynthetic pathway, causing early-onset fatal hypertrophic cardiomyopathy. Am J Hum Genet. 2003;72:101–14.
59. Williams SL, Valnot I, Rustin P, Taanman JW. Cytochrome c oxidase subassemblies in fibroblast cultures from patients carrying mutations in COX10, SCO1, or SURF1. J Biol Chem. 2004;279:7462–9.
60. Stiburek L, Vesela K, Hansikova H, Pecina P, Tesarova M, Cerna L, Houstek J, Zeman J. Tissue-specific cytochrome c oxidase assembly defects due to mutations in SCO2 and SURF1. Biochem J. 2005;392:625–32.
61. Smith D, Gray J, Mitchell L, Antholine WE, Hosler JP. Assembly of cytochrome-c oxidase in the absence of assembly protein Surf1p leads to loss of the active site heme. J Biol Chem. 2005;280:17652–6.
62. García-Horsman JA, Barquera B, Rumbley J, Ma J, Gennis RB. The superfamily of heme-copper respiratory oxidases. J Bacteriol. 1994;176(18):5587.
63. Lenka N, Vijayasarathy C, Mullick J, Avadhani NG. Structural organization and transcription regulation of nuclear genes encoding the mammalian cytochrome c oxidase complex. Prog Nucl Res Molec Biol. 1998;61:309–44.
64. Ghezzi D, Zeviani M. Assembly factors of human mitochondrial respiratory chain complexes: physiology and Pathophysiology. In: Kadenbach B, editor. Mitochondrial oxidative Phosphorylation. New York: Springer; 2012. p. 65–106.
65. St-Pierre J, Brand MD, Boutilier RG. The effect of metabolic depression on proton leak rate in mitochondria from hibernating frogs. J Exp Biol. 2000; 203:1469–76.
66. Theron M, Guerrero F, Sebert P. Improvement in the efficiency of oxidative phosphorylation in the freshwater eel acclimated to 10.1 MPa hydrostatic pressure. J Exp Biol. 2000;203:3019–23.
67. Magnoni L, Martos-Sitcha JA, Queiroz A, Calduch-Giner JA, Magalhães Gonçalves JF, Rocha CMR, Abreu HT, Schrama JW, Ozorio ROA, Pérez-Sánchez J. Dietary supplementation of heat-treated *Gracilaria* and *Ulva* seaweeds enhanced acute hypoxia tolerance in gilthead Seabream (*Sparus aurata*). Biol Open. 2017;6(6):897–908. doi:10.1242/bio.024299.
68. Salin K, Auer SK, Rey B, Selman C, Metcalfe NB. Variation in the link between oxygen consumption and ATP production, and its relevance for animal performance. Proc R Soc B. 2015;282:20151028.
69. Rogers NJ, Urbina MA, Reardon EE, McKenzie DJ, Wilson RW. A new analysis of hypoxia tolerance in fishes using a database of critical oxygen level (Pcrit). Conserv Physiol. 2016;4(1):cow012.

Annex 3: Manuscript submitted to Frontiers in Physiology (Open Access)

Tissue-specific Orchestration of Gilthead Sea Bream Resilience to Hypoxia and High Stocking Density

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The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

JM-S, JC-G and JP-S: conceived and designed the study; JM-S, PS-M and VH: carried out experimental procedures; JM-S and JP-S: wrote the original draft; All authors analysed and interpreted the data, reviewed, edited and approved the final manuscript.

Keywords

haematology, Hypometabolism, hypoxia, Limiting oxygen saturation, Sparus aurata, stocking density, Tissue-specific transcriptomics

Abstract

Word count: 350

Two different levels of O2 concentration (normoxia: 75-85% O2 saturation; moderate hypoxia: 42-43% O2 saturation) and stocking densities (LD: 9.5, and HD: 19 kg/m3) were assessed on gilthead sea bream in a 3-weeks feeding trial. Reduced O2 availability had a negative impact on feed intake and growth rates, which was exacerbated by HD despite of the improvement in feed efficiency. Blood physiological hallmarks disclosed the enhancement in O2-carrying capacity in fish maintained under moderate hypoxia. This feature was related to a hypo-metabolic state to cope with a chronic and widespread environmental O2 reduction, which was accompanied by a differential regulation of circulating cortisol and growth hormone levels. Customized PCR-arrays were used for the simultaneous gene expression profiling of 34-44 selected markers of liver, white skeletal muscle, heart and blood cells, informing about the stress- and metabolic-tissue condition. The number of differentially expressed genes ranged between 22-19 in liver, heart and white skeletal muscle to 5 in total blood cells. Partial Least-Squares Discriminant Analysis (PLS-DA) explains (R2) and predicts (Q2) up to 95% and 65% of total variance, respectively. The first component (R2=28.89%) gathers fish on the basis of O2 availability, and liver and cardiac genes on the category of energy sensing and oxidative metabolism (cs, hif-1α, pgc1α, pgc1β, sirts 1-2-4-5-6-7), antioxidant defence and tissue repair (prdx5, sod2, mortalin, gpx4, gr, grp-170, prdx3) and oxidative phosphorylation (nd2, nd5, coxi) highly contribute to this separation. The second component (R2=29.27%) differentiates normoxic fish at different stocking densities, and the white muscle clearly promotes this separation by a high over-representation of genes related to GH/IGF system (ghr-i, igfbp6b, igfbp5b, insr, igfbp3, igf i). The third component (R2=25.42%) discriminates the effect of stocking density in fish exposed to moderate hypoxia by means of hepatic fatty acid desaturases (fads2, scd1a, scd1b) and muscle markers of fatty acid oxidation (cpt1a). All these findings disclose the different contribution of analysed tissues (liver≥ heart > muscle > blood) and specific genes to the hypoxic- and crowding stress-mediated responses. This new knowledge will contribute to better explain and understand the different stress resilience of farmed fish across individuals and species.

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Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: Yes

Please provide the complete ethics statement for your manuscript. Note that the statement will be directly added to the manuscript file for peer-review, and should include the following information:

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All procedures decribed here were approved by the Ethics and Animal Welfare Committee of Institute of Aquaculture Torre de la Sal and carried out according to national (Royal Decree RD53/2013) and EU legislation (2010/63/EU) on the handling of animals for experiments.

Data availability statement

Generated Statement: The datasets for this manuscript are not publicly available because The datasets generated for this study are available on request to the corresponding authors. Requests to access the datasets should be directed to The datasets generated for this study are available on request to the corresponding authors.

Tissue-specific Orchestration of Gilthead Sea Bream Resilience to Hypoxia and High Stocking Density

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Running head title: stress resilience in *Sparus aurata*

Keywords: Haematology, Hypometabolism, Hypoxia, Limiting oxygen saturation, *Sparus aurata*, Stocking density, Tissue-specific transcriptomics

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Abstract

Two different levels of O_2 concentration (normoxia: 75-85% O_2 saturation; moderate hypoxia: 42-43% O_2 saturation) and stocking densities (LD: 9.5, and HD: 19 kg/m^3) were assessed on gilthead sea bream in a 3-weeks feeding trial. Reduced O_2 availability had a negative impact on feed intake and growth rates, which was exacerbated by HD despite of the improvement in feed efficiency. Blood physiological hallmarks disclosed the enhancement in O_2-carrying capacity in fish maintained under moderate hypoxia. This feature was related to a hypo-metabolic state to cope with a chronic and widespread environmental O_2 reduction, which was accompanied by a differential regulation of circulating cortisol and growth hormone levels. Customized PCR-arrays were used for the simultaneous gene expression profiling of 34-44 selected markers of liver, white skeletal muscle, heart and blood cells, informing about the stress- and metabolic-tissue condition. The number of differentially expressed genes ranged between 22-19 in liver, heart and white skeletal muscle to 5 in total blood cells. Partial Least-Squares Discriminant Analysis (PLS-DA) explains (R^2) and predicts (Q^2) up to 95% and 65% of total variance, respectively. The first component (R^2=28.89%) gathers fish on the basis of O_2 availability, and liver and cardiac genes on the category of energy sensing and oxidative metabolism (*cs*, *hif-1α*, *pgc1α*, *pgc1β*, *sirts 1-2-4-5-6-7*), antioxidant defence and tissue repair (*prdx5*, *sod2*, *mortalin*, *gpx4*, *gr*, *grp-170*, *prdx3*) and oxidative phosphorylation (*nd2*, *nd5*, *coxi*) highly contribute to this separation. The second component (R^2=29.27%) differentiates normoxic fish at different stocking densities, and the white muscle clearly promotes this separation by a high over-representation of genes related to GH/IGF system (*ghr-i*, *igfbp6b*, *igfbp5b*, *insr*, *igfbp3*, *igf-i*). The third component (R^2=25.42%) discriminates the effect of stocking density in fish exposed to moderate hypoxia by means of hepatic fatty acid desaturases (*fads2*, *scd1a*, *scd1b)* and muscle markers of fatty acid oxidation (*cpt1a)*. All these findings disclose the different contribution of analysed tissues (liver ≥ heart > muscle > blood) and specific genes to the hypoxic- and crowding stress-mediated responses. This new knowledge will contribute to better explain and understand the different stress resilience of farmed fish across individuals and species.

1. Introduction

Several attempts have been made over the course of last years to monitor the ecological and physiological impacts of a reduced O_2 availability in aquatic environments (Ekau et al., 2010; Richards, 2011; Zhu et al., 2013; Deutsch et al., 2015). The magnitude and orchestration of adaptive responses will reflect the duration and intensity of hypoxic stimuli (Martos-Sitcha et al., 2017; Cadiz et al., 2018), being defined the limiting O_2 saturation (LOS) as the threshold level where regulatory mechanisms are no longer sufficient to maintain O_2 consumption without compromising any physiological function (Remen et al., 2015; 2016). To minimize the hypoxia impact, fish reduce feed intake and reorganize its metabolism to limit the tissue O_2 demand (Hopkins and Powell, 2001; Bermejo-Nogales et al., 2014a), which allows to preserve aerobic metabolism by means of a restricted mitochondrial respiration and a shift in substrate preferences, as it has been reported in humans and rodents during the metabolic adaption of skeletal muscle to high altitude hypoxia (Murray, 2009). Other adaptive responses include changes in the production and scavenging of reactive oxygen species (ROS) (Lushchak and Bagnyukova, 2006; Bermejo-Nogales et al., 2014b), gill surface functionality (Nilsson, 2007) and haemoglobin (Hb)-O_2 binding characteristics (Jensen and Weber, 1982; Nikinmaa, 2001). In most aquaculture scenarios, these adaptive features are commonly associated to increases in temperature and high stocking rearing densities (Person-Le Ruyet et al., 2008; Vikeså et al., 2017), which in turn can compromise water quality resulting in impaired fish growth and immunity (Pickering, 1993; Van Weerd and Komen, 1998; Montero et al., 1999; Ashley, 2007). Indeed, beneficial effects on growth performance have been reported in Arctic charr (*Salvelinus alpinus*, Jørgensen et al., 1993) or meagre (*Argyrosomus regius*, Millán-Cubillo et al., 2016) with the preservation of water quality at high stocking densities. The opposite is also true and early studies pointed out that the impact of crowding stress is minimized when O_2 concentrations are not below LOS (Ruer et al., 1987; Araújo-Luna et al., 2018). Unravelling the combined effects of hypoxia and high rearing density are, thereby, crucial to warrant fish welfare during intensive farming in a scenario of global change with a greater stratification and deoxygenation of oceans (Keeling et al., 2009; Helm et al., 2011; Schmidtko et al., 2017).

Progress towards a more sustainable and environmentally friendly aquaculture requires important investments in both conventional and new methodologies for a less invasive and more refined phenotyping of individual farmed fish. Main achievements so far include the use of acoustic telemetry or stand-alone biosensors for the non-disturbing monitoring of feeding behavior or metabolic capabilities (Føre et al., 2017; Martos-Sitcha et al., submitted). In addition to that, major progress has been done with the advent of wide-holistic omics based on functional genomics, proteomics, metabolomics and metagenomics as powerful toolsets for the development of a highly technified aquaculture in both salmonid and non-salmonid fish (Yáñez et al., 2015; Martin and Król, 2017; Martínez-Porchas and Vargas-Albores, 2017; Alfaro and Young, 2018; Rodrigues et al., 2018). Such approaches are increasingly used in gilthead sea bream (*Sparus aurata*), a highly and economically important cultured fish species in all the Mediterranean area. Thus, a first draft genome based on genetic-linkage maps (Pauletto et al., 2018) and other current genome initiatives will contribute to have major progress in selective breeding and epigenetic research in gilthead sea bream. Also, in this species, important research efforts have been conducted to define a reference pattern for skin/intestine mucus proteome (Estensoro

et al., 2016; Pérez-Sánchez et al., 2017), gut microbiota (Piazzon et al., 2017) or serum metabolome (Gil-Solsona et al., 2019). Moreover, the use of high-density microarrays (Calduch-Giner et al., 2010; 2012; 2014), pathway-focused PCR-arrays (Benedito-Palos et al., 2014; 2016; Bermejo-Nogales et al., 2014a; 2015; Pérez-Sánchez et al., 2015; Magnoni et al., 2017; Martos-Sitcha et al., 2017) and more recently NGS (Piazzon et al., 2019, submitted) have contributed to define tissue-specific gene expression patterns in response to nutritional, environmental and parasite challenges. Current progress in this way has been reviewed to unravel the differential regulation of somatotropic axis at the systemic and local tissue-level by nutritional and environmental factors in marine fish and gilthead sea bream in particular (Pérez-Sánchez et al., 2018). Likewise, the synchronization of the molecular clock of sea bream larvae, involving more than 2,500 genes with a clear circadian rhythmicity, has been proposed as certification of juvenile quality later in life (Yúfera et al., 2017). In the present study, we aim to go further on the definition of criteria of fish welfare and quality, regarding in depth the effect of two different stocking densities (9.5 kg/m^3, 19 kg/m^3) and O_2 saturation levels (85%, 42-43% O_2 saturation) in a 3-weeks trial with fast growing juveniles of gilthead sea bream. The analysed parameters included the gene expression pattern of a set of growth and metabolic markers of liver, skeletal muscle, heart and blood cells in combination with data on growth performance, as well as blood haematology and biochemistry. The working hypothesis is that each tissue contributes differentially to the homeostatic load achievement, helping the generated knowledge to better exploit the plasticity and stress resilience of gilthead sea bream.

2. Material and Methods

2.1. Animal care

Gilthead sea bream juveniles of Atlantic origin (Ferme Marine du Douhet, Bordeaux, France) were reared from early life stages in the indoor experimental facilities of Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain) under natural photoperiod and temperature conditions at our latitude (40º5'N; 0º10'E). Sea water was pumped ashore (open system) and filtered through a 10-µm filter. The O_2 content of water effluents in standard conditions was always higher than 85% saturation, and unionized ammonia remained below 0.02 mg/L.

All procedures described here and elsewhere were carried out according to national (Royal Decree RD53/2013) and EU legislation (2010/63/EU) on the handling of animals for experimentation.

2.2. Experimental set-up and sampling

Twelve days prior to the start of the experimental trial, juvenile fish (~34 g average body weight) were randomly distributed in twelve 90 L tanks coupled to a re-circulatory system equipped with physical and biological filters, and programmable temperature and O_2 devices (*Figure 1*). Water temperature was daily monitored and maintained at 25-27ºC. Fish were arbitrarily allocated to constitute two different initial stocking densities (six tanks per condition) fed daily to visual satiety with a commercial diet (EFICO Forte 824, BioMar, Palencia, Spain): i) LD (low density, 25 fish/tank, 9.5 kg/m^3) and ii) HD (high density, 50 fish/tank, 19 kg/m^3). After the acclimation period, the water parameters of three tanks of each initial stocking density were kept unchanged, constituting the normoxic (>5.5 ppm O_2; >85% O_2 saturation)

groups of each experimental condition (LDN, low density normoxia; HDN, high density normoxia). Fish maintained in the remaining six tanks experienced a gradual decrease in the water O_2 level until reaching 3.0 ppm (42-43% O_2 saturation), constituting the hypoxic groups of each experimental condition (LDH, low density hypoxia; HDH, high density hypoxia). The normal range of variation in O_2 concentrations was marked by a rapid drop (3.8-4 ppm normoxic groups; 2.3 ppm hypoxic groups) 15-30 minutes after feeding, with a rapid restoration of reference values in less than 1 hour by the automatic entrance of clean water from the main reservoir tank. This system allowed maintaining unionized ammonia below toxic levels (<0.50 mg/L) in both HDN and HDH groups.

After twenty-two days under these experimental conditions and following overnight fasting, twelve fish (four per tank) per experimental condition (LDN, LDH, HDN, HDH) were anaesthetized with 3-aminobenzoic acid ethyl ester (100 mg/L), weighed and blood was taken from caudal vessels with EDTA-treated syringes (less than 5 min for all the fish sampled for each tank). All lethal samples were collected between 10.00 am and 12.00 am to reduce the biologic variability due to circadian rhythms and postprandial-mediated effects. One blood aliquot (25 µL) was directly collected into a microtube containing 500 µL of stabilizing lysis solution (REAL total RNA spin blood kit, Durviz, Valencia, Spain) and stored at -80ºC until total RNA extraction. Other aliquots were processed for haematocrit, haemoglobin and red blood cells (RBC) counting. The remaining blood was centrifuged at 3,000 × *g* for 20 min at 4ºC, and plasma samples were frozen and stored at -20ºC until biochemical and hormonal analyses were performed. Prior to tissue collection, fish were killed by cervical section. Liver and viscera were weighed, and representative biopsies of liver, muscle and complete hearts were immediately snap-frozen in liquid nitrogen and stored at -80ºC until extraction of total RNA.

2.3. Blood biochemistry and hormonal parameters

Haematocrit was measured using heparinized capillary tubes centrifuged at 1,500 × *g* for 30 min in a Sigma 1-14 centrifuge (Sigma, Germany). Haemoglobin was assessed using a Hemocue Hb 201+ (Hemocue, Sweden). Counts of RBC were made in a Neubauer chamber, using an isotonic solution (1% NaCl). Plasma glucose was analysed using the glucose oxidase method (Thermo Electron, Louisville, CO, USA). Lactate was measured in deproteinized samples (perchloric acid 8%) by an enzymatic method based on the use of lactate oxidase and peroxidase (SPINREACT S.A., Girona, Spain). Total antioxidant capacity in plasma samples was measured with a commercial kit (Cayman Chemical, Ann Arbor, MI, USA) adapted to 96-well microplates. This assay relies on the ability of antioxidants in the samples to inhibit the oxidation of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS radical cation by metmyoglobin, a derivatized form of myoglobin. The capacity of the sample to prevent ABTS oxidation is compared with that of Trolox (water-soluble tocopherol analogue) and is quantified as mM Trolox equivalents. Plasma cortisol levels were analysed using an EIA kit (Kit RE52061m IBL, International GmbH, Germany). The limit of detection of the assay was 3.01 ng/mL with intra- and inter-assay coefficients of variation lower than 3% and 5%, respectively. Plasma growth hormone (Gh) was determined by a homologous gilthead sea bream RIA as reported elsewhere (Martínez-Barbera et al., 1995). The sensitivity and midrange (ED50) of the assay where 0.15 and 1.8 ng/mL, respectively. Plasma insulin-like growth factors (Igf) were extracted by acid–ethanol cryoprecipitation (Shimizu et al., 2000), and the

concentration of Igf-I was measured by means of a generic fish Igf-I RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis et al., 2004). The sensitivity and midrange of the assay were 0.05 and 0.7–0.8 ng/mL, respectively.

2.4. Gene expression analysis

Total RNA from liver, white muscle and heart was extracted using a MagMax-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, USA), whereas total RNA from total blood cells was extracted using the REAL total RNA spin blood kit including a DNase step. The RNA yield in all tissues was >3.5 µg, with absorbance measures ($A_{260/280}$) of 1.9-2.1. Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) using random decamers and 500 ng of total RNA in a final volume of 100 µL. Reverse transcription (RT) reactions were incubated 10 min at 25ºC and 2 h at 37ºC. Negative control reactions were run without RT.

The 96-well PCR-array layout was designed for the simultaneous profiling of a panel of 43 (liver), 44 (white muscle and total blood cells) or 34 (heart) genes, including markers of GH/IGF system (13), lipid metabolism (10), energy sensing and oxidative metabolism (12), antioxidant defence and tissue repair (10), muscle growth and cell differentiation (8), respiration uncoupling (3), xenobiotic metabolism (2), nuclear receptors (3), transmembrane translocation (8), mitochondrial dynamics and apoptosis (5), as well as OXPHOS (22) (*Table 1*). qPCR reactions were performed using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, USA). Diluted RT reactions were conveniently used for qPCR assays in 25 µL volume in combination with a SYBR Green Master Mix (Bio-Rad, Hercules, CA, USA) and specific primers at a final concentration of 0.9 µM (*Supplemental Table S1*). The program used for PCR amplification included an initial denaturation step at 95ºC for 3 min, followed by 40 cycles of denaturation for 15 s at 95ºC and annealing/extension for 60 s at 60ºC. All the pipetting operations were made by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (>92%) was checked, and the specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5ºC/10s over a temperature range of 55-95ºC) and linearity of serial dilutions of RT reactions (>0.99). Fluorescence data acquired during the extension phase were normalized by the delta-delta C_T method (Livak and Schmittgen, 2001) using *actb* in the liver, white muscle and heart, or *cox4a* in total blood cells, as the housekeeping gene due to its stability among different experimental conditions. For multi-gene analysis, data on gene expression were in reference to the expression level of *cs* in the liver, *igfr2* in the white muscle, *gcr* in the heart, and *tim8a* in total blood cells of LDN fish, for which a value of 1 was arbitrarily assigned (*Supplemental Tables 2-5*, respectively).

This manuscript follows the ZFIN Zebrafish Nomenclature Guidelines for gene and protein names and symbols (https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Guidelines).

253 **2.5. Statistical analysis**

254 Data on growth performance, blood biochemistry and gene expression were analysed
255 by two-way analysis of variance (ANOVA) followed by the Holm-Sidak test. The
256 significance level was set at $P<0.05$. All analyses were performed using the
257 SigmaPlot Version 13 for Windows. To achieve the maximum separation among
258 experimental groups, Partial Least-Squares Discriminant Analysis (PLS-DA) was
259 applied jointly in liver, heart and white muscle, avoiding the inclusion of results from
260 total blood cells due to its low contribution to the total variance. The quality of the
261 PLS-DA model was evaluated by R^2Y and Q^2 parameters, which indicated the fitness
262 and prediction ability, respectively. The contribution of differential genes along liver,
263 white muscle and heart tissues was assessed by means of Variable Importance in
264 Projection (VIP) measurements. A VIP score > 1.1 was considered to be an adequate
265 threshold to determine discriminant variables in the PLS-DA model (Wold et al.,
266 2001; Li et al., 2012; Kieffer et al., 2016).
267
268
269 **3. Results**

270 **3.1. Growth performance**

271 Data on feed intake, growth and somatic indexes (hepatosomatic index, HSI;
272 mesenteric fat index, MSI) are shown in *Table 2*. Two-way ANOVA reveals an O_2
273 effect with an overall reduction under hypoxic conditions. This general impairment of
274 feed intake and growth is further evidenced in fish kept at the highest density, though
275 FE was improved in moderate hypoxia and more especially in fish kept at HD (HDH
276 group).
277
278 **3.2. Blood analysis**

279 Data on blood haematology and biochemistry are shown in *Table 3*. The results show
280 a significant effect of O_2 concentration, with a generalized increase in Hb, Hc, RBC
281 content, MCH, cortisol and Gh plasma levels, as well as a widespread decrease in
282 MCHC, MCV and plasma lactate levels. Overall this feature was more accused in fish
283 maintained under LD conditions. In contrast, the rearing density effect was mostly
284 reduced to plasma cortisol levels, which showed a pronounced rise in HD fish that
285 was exacerbated by hypoxic conditions. Noticeably, significant O_2 concentration and
286 rearing density interactions were found for cortisol, but also for Hc, MCHC, MCH
287 and TAA.
288
289 **3.3. Gene expression profiling**

290 All genes selected for PCR-arrays were found at detectable levels in the four tissues
291 analysed. Results of gene expression profiling in hepatic selected genes are presented
292 in *Suppl. Table 2*. Among them, 22 out of 43 genes where affected by at least one of
293 the experimental factors or by its interaction, being 11 differentially expressed (DE)
294 in response to O_2 concentration. Relative expression of markers from GH/IGF system
295 (*ghr-i*), oxidative metabolism (*nd2*), and antioxidant defence and tissue repair (*gpx4*,
296 *prdx5*) was significantly down-regulated by moderate hypoxia in LDH and HDH
297 groups. In addition, several genes of lipid metabolism (*elovl1*, *fads2* and *scd1b*) were
298 up-regulated in the LD group, whereas markers of oxidative metabolism (*nd5*), and
299 antioxidant defence and tissue repair (*gr*, *sod2*, *grp-75*) were down-regulated in fish
300 kept at HD conditions. Stocking density also affected 11 genes related with the

GH/IGF system (*ghr-i*, *ghr-ii*, *igf-i*), lipid metabolism (*elovl6*, *fads2*, *scd1a*, *scd1b*, *lpl*), oxidative metabolism (*ucp1*, *pgc1α*) and antioxidant defence and tissue repair (*grp-75*). A statistically significant interaction of O_2 concentration and rearing density was found for *igf-ii*, *fads2*, *scd1a*, *scd1b*, *pgc1ß*, *gr*, *prdx3* and *grp-170* genes.

In white skeletal muscle, 20 out of 44 DE genes were affected at least by one of the experimental condition or even by their interaction (*Suppl. Table 3*). Markers of the GH/IGF system were mostly affected by stocking density (*ghr-i*, *igf-ii*, *igfbp3*, *igfbp5b*, *igfbp6b*, *insr*, *igfr1*) rather than by O_2 concentration (*igfr2*). Moderate hypoxia up-regulated *myod2* expression as the sole effect on genes related to muscle growth and cell differentiation. In contrast, a relative high number of genes related to energy sensing, oxidative metabolism, and antioxidant defence and tissue repair were down-regulated by low O_2 concentrations (*sirt1*, *ucp3*, *hif-1α*, *prdx5*, *sod2*) or up-regulated in HD conditions (*sirt4*, *sirt7*, *coxi*, *hif-1α*, *gpx4*). Additionally, a significant interaction between O_2 concentration and rearing density is reported for *cpt1a* and *grp-170*.

In heart, changes in O_2 saturation and stocking density triggered significant differences in 19 out of 34 genes presented in the array (*Suppl. Table 4*). Up to 13 genes, including markers of the GH/IGF system (*ghr-i*), energy sensing and oxidative metabolism (*sirt1*, *sirt5*, *sirt6*, *sirt7*, *cs*, *nd5*, *pgc1α*, *pgc1ß*, *hif-1α*) and antioxidant defence and tissue repair (*cat*, *prdx5*, *sod2*) were down-regulated under moderate hypoxia, especially in HD conditions. The xenobiotic metabolism marker *cyp1a1* was up-regulated by hypoxia in both LD and HD. Stocking density also down-regulated the expression of several genes involved in energy sensing and oxidative metabolism (*sirt3*, *sirt5*, *cs*, *nd2*) as well as antioxidant defence and tissue repair (*gr*, *prdx3*, *prdx5*, *grp-170*, *grp-75*), preferentially under low O_2 concentrations.

In total blood cells, only 5 out of 44 genes were DE mainly by the interaction among different experimental conditions (*Suppl. Table 5*), being responsive to the stress challenge enzyme subunits of Complex I (*ndufaf2*) and Complex IV (*coxi*, *coxii*, *cox6a2*, *cox15*) of the mitochondrial respiratory chain.

In order to assess the differential contribution of the DE genes in the physiological response to moderate hypoxia and rearing density, the tissue (liver, white skeletal muscle, heart) gene expression dataset was analysed by PLS-DA. The discriminant model was based on six components, which explained (R^2) 95% and predicted (Q^2) 65% of total variance (*Figure 2A*). Of these, the first three components showed cumulative values for R^2 and Q^2 of 83.6% and 49.3%, respectively. A clear separation between normoxic (LDN, HDN) and hypoxic (LDH, HDH) groups was observed along the first component that explained 28.89% of total variance (*Figure 2B and 2C*). Component 2 (29.27% of variance) clearly separated LDN and HDN normoxic groups (*Figure 2B*), whereas component 3 (25.42% of variance) discriminated LDH and HDH hypoxic groups (*Figure 2C*).

Genes with a contribution to VIP > 1.1 in component 1 were a total of 39, with a main contribution of heart (19) and liver (14) genes involved in energy sensing and oxidative metabolism (14), antioxidant defence and tissue repair (12) and OXPHOS (*Figure 3*). When the second component was also considered, a total of 44 genes presented VIP values >1.1 (*Figure 4*), and 11 out of the 21 new genes (highlighted in yellow) were from white skeletal muscle. Energy sensing and oxidative metabolism (12), antioxidant defence and tissue repair (11), GH/IGF system (11) and OXPHOS (6) were the main categories. Considering the VIP values from the 3 main

components (*Figure 5*), most of the genes due to component 3 contribution (highlighted in purple) were related to lipid metabolism.

4. Discussion

Hypoxia in aquatic habitats is a common disturbance that is predicted to occur in the future more extensively, more frequently and for longer periods of time (IPCC, 2014), becoming a major aquaculture stressor around the world. This is especially true in the case of intensive fish farming, and unraveling the adaptive hypoxic responses helps to better understand the nature of metabolic disturbances after short- and long-term exposures to challenging O_2 concentrations. Certainly, blood physiological landmarks remain mostly unaltered in juveniles of gilthead sea bream exposed over 24 h to moderate hypoxia (40% O_2 saturation), whereas changes in Hc and circulating levels of Hb, glucose and lactate are reported few hours after acute hypoxia (20% O_2 saturation) (Martos-Sitcha et al., 2017). In the same study, gene expression profiling of total blood cells evidenced a consistent transcriptional response after strong hypoxic insults, which serve to ensure a reduced but more efficient aerobic ATP production during severe hypoxia. Herein, we assessed the combined effects of moderate hypoxia and rearing density in a 3-weeks feeding trial, which highlights a reduced growth and a different contribution of target tissues to the homeostatic load in challenged fish. As discussed below, the ultimate mechanisms remain far to be established, though probably they have a major impact in mitochondrial respiration uncoupling, which varies across life, tissues, individuals and species (Rolfe et al., 1996, Hulbert et al., 2002; 2006). Indeed, as reviewed by Salin et al. (2015), rates of O_2 consumption are not by itself a good marker of energy metabolism as they do not distinguish between the energy used to produce ATP and the energy dissipated through H^+ leakage. These two processes are apparently irreconcilable and natural selection can promote respiration uncoupling, faster growth and/or greater reproductive output in conditions of energy excess, whereas improved energy efficiency and reduced mitochondrial respiration uncoupling becomes a priority with low food availability (Auer et al., 2015). Of course, mitochondrial functioning also varies from one tissue to another, and the efficiency of aerobic energy production differs substantially between pectoralis and gastrocnemius muscles in fasted cold-acclimated birds (Monternier et al., 2017). Likewise, in gilthead sea bream, the expression of mitochondrial uncoupling proteins (UCP2/UCP3) is differentially regulated by feed restriction in glycolytic (white skeletal muscle) and highly oxidative (heart and skeletal red muscle) tissues (Bermejo-Nogales et al., 2014b).

Growth impairments due to long-term hypoxia exposure have been noticed in a wide-range of farmed fish, including turbot, European sea bass and Atlantic salmon (Pichavant et al., 2001; Remen et al., 2016; Cadiz et al., 2017; Vikeså et al., 2017). As reported herein in gilthead sea bream, a primary response is the inhibition of feed intake which would favor a hypo-metabolic state with a reduced ROS production and risk of oxidative stress. This is supported by lowered plasma levels of lactate, which would reflect in hypoxic fish, and in a lower extent in HDN, a low basal metabolism rather than a shift of aerobic to anaerobic metabolism. This metabolic re-adjustment has also been reported in gilthead sea bream juveniles facing multiple sensorial stressors in a model of chronic stress that mimic daily aquaculture operations (Bermejo-Nogales et al., 2014a). Thus, according with the oxystatic theory (Dam and Pauly, 1995; Saravanan et al., 2012), fish finely adjust feed intake and basal metabolism to available O_2, prioritizing feed efficiency at the expenses of maximum

398 growth under restricted mitochondrial respiration. This seems to be especially
399 effective in gilthead sea bream, a protandrous hermaphroditic fish with a high
400 metabolic plasticity that makes feasible to be eurytherm, euryhalin and euryoxic
401 thanks, at least in part, to a permissive regulation of feed intake which allows to cope
402 an efficient energy metabolism at slow growth rates. As a prove of this, the best FE
403 and hormonal signatures for fast and efficient growth generally occurs before the
404 achievement of maximum growth at the greater ration size (Brett, 1979; Pérez-
405 Sánchez et al., 1995). This also applies at the cellular level, where the maximum ATP
406 yield per molecule of O_2 (P/O ratio) is highly dependent on ration size, as evidenced
407 the increased P/O ratio of king penguins during periods of food shortage (Monternier
408 et al., 2014).

409 Most of the hypoxia-mediated effects are accompanied by an enhanced O_2-carrying
410 capacity denoted by a swelling, formation and/or release of new erythrocytes together
411 with plasma volume reduction (Gallaugher and Farrell, 1998). This is also inferred
412 from our haematological data with changes in the measured Hc and corpuscular
413 concentrations of Hb, which were affected by O_2 concentration and secondly by
414 rearing density. Conversely, changes in HSI, reflecting the amount of lipid and
415 glycogen depots, are more informative of feed intake rather that hypoxic condition,
416 though it is difficult to disclose the main factor. At the hormonal level, this is also
417 inferred from the measurements of circulating levels of cortisol and Gh, which are
418 well-known regulators of metabolic rates by their involvement on mitochondria
419 function (see Mommsen et al., 1999; Bergan-Roller and Sheridan, 2018 for review).
420 In fact, cortisol is a well marker of crowding stress in gilthead sea bream (Arends et
421 al., 1999; Skrzynska et al., 2018), being enhanced the responsiveness of the
422 hypothalamic-pituitary-adrenal axis by fasting or restricted feeding (Sangiao-
423 Alvarellos et al., 2005). This agrees with the observation that the greater circulating
424 concentration of cortisol was achieved herein in the HDH group, which also
425 experienced a higher feed intake inhibition. However, this system cannot be
426 continuously refed and glucocorticoid receptors in rodents and perhaps other animal
427 models translocate cortisol into mitochondria to reduce mitochondrial activity and the
428 risk of oxidative stress (Du et al., 2009). Thus, in the absence of a cortisol response,
429 chronic cold-thermal stress up-regulates OXPHOS in gilthead sea bream, whereas the
430 cortisol rise in fish facing multiple aquaculture sensorial stressors is accompanied by a
431 pronounced transcriptional repression of all the hepatic complex units of the
432 mitochondrial respiratory chain (Bermejo-Nogales et al., 2014a). A similar response
433 has been reported after strong hypoxic insults, though in this case the catalytic and
434 regulatory enzyme subunits of Complex IV (the last electron acceptor of respiratory
435 chain involved in the O_2 reduction) were up-regulated, maximizing the use of
436 available O_2 for aerobic ATP generation (Martos-Sitcha et al., 2017). The aerobic
437 scope and gene expression profiling of mitochondria is also highly regulated at the
438 nutritional level by synthetic and natural dietary oils (Pérez-Sánchez et al., 2013;
439 Martos-Sitcha et al., 2018), and the suppression of heptanoate effects upon exercise
440 endurance is viewed as a protective measure to counteract disproportionate oxidative
441 metabolic rates in fish fed fast energy-delivery nutrients (short/medium chain fatty
442 acids). In other words, stimulatory and inhibitory effects of energy metabolism
443 coexist as a trade-off mechanism that might reflect the changing energy needs rather
444 than the availability of metabolic fuels. Accordingly, in the present study, the increased
445 circulating levels of Gh in hypoxic/crowded fish will reflect a reduced feed intake and
446 energy demand rather than a minor capacity to combat oxidative stress, as it is generally

referenced in fish and other animal models overexpressing GH (Brown-Borg et al., 1999, 2000; McKenzie et al., 2003; Almeida et al., 2013).

The gene expression profiling of key metabolic biomarkers also contributes to better understand the search of allostatic load in a challenging environment. Thus, the two-way ANOVA reveals the different involvement of tissues and gene categories into the stress-mediated responses. This observation is reinforced by the use of multivariate analysis, which offers the possibility to identify, at a high level of confidence, the most responsive tissues and biomarkers for a given stress stimuli in a factorial stress design. Using such approach, we are able to explain and to predict a high percentage of total variance, being noteworthy that liver, white skeletal muscle and heart remain responsive at long-term to changing O_2 and rearing density, whereas the expression pattern of blood cells becomes mostly unaltered with the imposed stress stimuli of medium intensity. In previous studies in gilthead sea bream and other animal models, liver and cardiac muscle are highly responsive to hypoxia (Everett et al., 2012; Hermes-Lima et al., 2015; Magnoni et al., 2017), and genes of these two tissues highly contribute herein to separate normoxic and hypoxic fish along the first component that predicts more than 28% of total variance. One of the most relevant genes participating in this discriminant feature is the *hif-1α*, a well-documented regulator of O_2 homeostasis. This transcriptional factor acts at a multi-regulatory level, managing the hypoxic responsiveness of a vast array of transcribed proteins including antioxidant enzymes (Nikinmaa and Rees, 2005; Lushchak and Bagnyukova, 2006). Concretely, herein, we show a clear down-regulation of *hif-1α* that was coincident with the repressed expression of other down-stream markers of antioxidant defence and tissue repair (*prdx5*, *sod2*, *mortalin*, *gpx4*, *gr*, *grp-170*, *prdx3*). This intriguing result can be cautiously interpreted since Hif-1 is mostly regulated at the post-translational level (Ke and Costa, 2006), though this finding should be understood as a steady-state in which O_2 availability is reduced but maintained high enough to preserve aerobic metabolism at a relatively high level. This fact is supported by a reduced expression of *cs* and associated enzyme subunits of Complex I (*nd2*, *nd5*), used successfully in several studies as markers of mitochondria abundance and Krebs cycle activity (Larsen et al., 2012; Magnoni et al., 2017). In addition to that, several *sirts* (*sirt1, 2, 5, 6, 7*) of liver or cardiac muscle were overall down-regulated in hypoxic fish, especially in the case of HDH fish. These NAD^+-dependent deacetylases are energy sensors that act in gilthead sea bream as a link between nutrition and energy metabolism in different growth models with nutrients and genetic variables as source of variation (Simó-Mirabet et al., 2017a; 2017b; 2018). This was extended herein to hypoxia/crowding stress, which indicates that most of the envisaged adaptive responses should include changes in the acetylation status of both nuclear histones, and cytoplasmic and mitochondrial metabolic enzymes.

The second component of our PLS-DA (R^2=29.27%) differentiates normoxic fish held at different stocking densities. In this case, the white skeletal muscle clearly promotes this separation mainly by the expression pattern of genes related to GH/IGF system (*ghr-i*, *igfbp6b*, *igfbp5b*, *insr*, *igfbp3*, *igf-i*). Components of liver and muscle GH/IGF system are differentially regulated by nutrients and seasonal environmental cues (reviewed by Pérez-Sánchez et al., 2018), but herein this observation becomes specially relevant for muscle *ghr-i* that highly contributes to discriminate the detrimental growth effects of crowding stress from those more related to hypoxia or water quality. Likewise, genes of *igfbp* repertoire highly contribute to this

differentiation, though the discriminant role of Igfbp counterparts (*igfbp6b* > *igfpb5b* > *igfpb3)* was mostly reduced to skeletal muscle and *Igfbp3/5/6* clade. In this regard, it is noteworthy that the ancestral *Igfbp* gene was duplicated in tandem during an early stage of vertebrate evolution to produce pairs of *Igfbps* that gave rise in subsequent genome duplication events the two *Igfbp* clades of modern vertebrates (*Igfbp1/2/4*; *Igfbp3/5/6*) (Kajimura et al., 2005; Ning et al., 2006; Shimizu et al., 2017; Garcia de la Serrana and Macqueen, 2018) Additionally, the third and fourth round of whole-genome duplication create the corresponding paralog pairs, being the Igpbp subtypes of *igfbp3/5/6* clade overexpressed in the skeletal muscle whereas those of *igfbp1/2/4* clade are more characteristic of liver in adult animals (Pérez-Sánchez et al., 2018). Functional divergence regarding the growth-inhibitory or growth-promoting action of *igfbp*s have been reported across species and physiological context (Garcia de la Serrana and Macqueen., 2018), but herein the overall depressed expression of the muscle *Igfbp* clade in HD fish is consistent with inhibitory rather than stimulatory growth-promoting effects, which also involves the regulation of insulin and Igfbp receptors with important implications on the final arrangements of carbohydrate, growth and energy metabolism (reviewed by Reindl and Sheridan, 2012; Vélez et al., 2017). Indeed, fish are the first group in the vertebrate tree in which there is evidence of distinct insulin and Igf receptors, though certain cross-reactivity between ligand and receptors of insulin and Ifgs occurs and the specific-mediated effects are sometimes confounding. However, it is well-recognized that insulin stimulates Hif-1, whereas intermittent hypoxia induces insulin resistance in mice (Treins et al., 2002; Poulain et al., 2017). Likewise, *Igfbp1* know-down alleviates the hypoxia-induced growth retardation in zebrafish (Kajimura et al., 2005), whereas the IGFBP4 expression is induced by hypoxia in U87 glioma cells (Minchenko et al., 2016). From our results it is also conclusive that the muscle expression of *igfr1 and igfr2* are specially responsive to hypoxia, but importantly *insr* in gilthead sea bream seems to be more receptive to crowding stress rather than hypoxic stress stimuli, though it remains to be established the functional relevance of this differential responsiveness to environmental stressors.

Finally, the third component of our multivariate approach (R^2=25.42%) discriminates the effect of stocking density in fish exposed to moderate hypoxia, with a marked contribution of hepatic fatty desaturases with Δ6 (*fads2*) or Δ9 (*scd1a*, *scd1b*) activities due to its strong and specific induction in LDH fish. A muscle marker of FA oxidation (*cpt1a*) was also consistently up-regulated in this group, but this response was opposite to that found in HDH group, which is indicative of the different regulation of muscle lipid catabolism by hypoxia in fish stocked at standard or high densities. Likewise, the major discriminant capacity of other factors related to lipid metabolism (*elovl6*) was achieved between normoxic fish held at LD and HD. Meanwhile, other elongases (*elovl5*) with a well-recognized role in the control of hepatic triglyceride storage did not take part of the group separation in the present study, though *elovl5* highly contributes to differentiate two gilthead sea bream strains with differences in growth performance and metabolic capacities (Simó-Mirabet et al., 2018). Previous studies, in gilthead sea bream (Benedito-Palos et al., 2013; 2014) and European sea bass (Rimoldi et al., 2016) have also evidenced an important effect of ration size on the hepatic and muscle regulation of most of the lipid biomarkers assessed in the present study, but again it is difficult to disclose what is the main factor (feed intake or the imposed stress condition) due to the logistic limitations of our experiment design that did not include pair-fed groups. However, as a general rule, stressors enhance the demand of specific nutrients and hypoxia in particular

promote the cellular uptake of extracellular unsaturated fatty acids in mice cell lines (Ackerman et al., 2018). Moreover, in hypoxic stress, cancer cells enhance lipid synthesis that is important for membrane biosynthesis and energy storage for cell survival and proliferation (Huang et al., 2014), being induced this hypoxia lipogenic phenotype via dependent- and HIF1α-independent pathways (Valli et al., 2015). All this together supports the pronounced stimulation of *fads2* and *scd* desaturases in our stress model, which will promote the increase of the unsaturation index of structural lipids as previously reported during feed restriction in gilthead sea bream (Benedito-Palos et al., 2013). In agreement with this, hypoxia stress on HeLa cells leads to significant changes in their membrane lipid profiles, and polyunsaturated phospholipid species are becoming stronger biomarkers for discriminating the effect of hypoxia treatment on membrane fluidity and further membrane-dependent functions (Yu et al., 2014).

5. Concluding Remarks

The findings described herein evidence the re-adjustment of several biological functions in a factorial model of chronic stress, where most of the hypoxia-mediated effects on growth performance and energy metabolism were exacerbated in fish held at HD. The integrated data on blood haematology, biochemistry and hormonal profiling highlights a hypo-metabolic state with the enhancement of O_2-carrying capacity, being this metabolic feature accompanied by a reduction in voluntary feed intake and a more efficient energy metabolism at the expenses of slow growth rates. This notion was supported at the transcriptional level by global changes of tissue-gene expression profiles, which also evidenced tissue-specific orchestration of stress response reflecting the nature and intensity of stress stimuli, but also the different metabolic capacities of targeted tissues. Thus, the number of DE in response to a given stress stimuli varies across the targeted tissues (liver ≥ heart > muscle > blood), but importantly PLS-DA analysis also informs of the different tissue contribution to the allostatic load. Thus, liver and heart mostly contribute to cope with a global hypoxic response involving changes in energy sensing and production as well as antioxidant defence and tissue repair. In contrast, metabolic markers of skeletal muscle with a high over-representation of GH/IGF system mostly contribute to disclose the effects of rearing density not necessarily mediated by low O_2 concentrations. Likewise, lipid metabolism and hepatic fatty acid desaturases are becoming strong biomarkers of crowding stress in hypoxic fish, which reveals the complexity and metabolic plasticity of gilthead sea bream to cope with stress resilience under intensive fish farming.

584 **FIGURE CAPTIONS**
585
586 **Figure 1.** Experimental set-up used to control dissolved O_2 levels in the experimental
587 tanks to implement hypoxic conditions under different stocking densities. The steady-
588 state was set at ~3.0 ppm O_2 (42-43% oxygen saturation) in fish kept under hypoxia
589 (LOS), whereas fish maintained in normoxia a concentration of >5.5 ppm O_2 (>85 %
590 O_2 saturation) was always assured.
591
592 **Figure 2.** A) Graphical representation of the goodness-of-fit of the PLS-DA model.
593 B) Two-dimensional PLS-DA score plot representing the distribution of the samples
594 between the first two components in the model. C) Two-dimensional PLS-DA score
595 plot representing the distribution of the samples between the first and third
596 components in the model. R^2(cum): explained variance; Q^2(cum): predicted variance;
597 LDN: low density normoxia; LDH: low density hypoxia; HDN: high density
598 normoxia; HDH: high density hypoxia.
599
600 **Figure 3.** A) Graphical representation of the variable importance (VIP) scores after
601 component 1. B) Ranking list of genes showing VIP score values above 1.1 and their
602 relative gene expression. Values on relative expression are the mean ± SEM of 8 fish
603 (2-3 fish per replicate tank). P-values are the result of two-way analysis of variance.
604 Asterisks in each row indicate significant differences with oxygen concentration for a
605 given rearing density (SNK test, $P < 0.05$).
606
607 **Figure 4.** A) Graphical representation of the variable importance (VIP) scores after
608 component 2. B) Ranking list of genes showing VIP score values above 1.1 and their
609 relative gene expression. Cells shaded in blue highlight genes detected as VIP after
610 component 1; cells shaded in yellow highlight genes detected as VIP after component
611 2. For further details, see legend on Figure 3.
612
613 **Figure 5.** A) Graphical representation of the variable importance (VIP) scores after
614 component 3. B) Ranking list of genes showing VIP score values above 1.1 and their
615 relative gene expression. Cells shaded in blue highlight genes detected as VIP after
616 component 1; cells shaded in yellow highlight genes detected as VIP after component
617 2; cells shaded in purple highlight genes detected as VIP after component 3. For
618 further details, see legend on Figure 3.

AUTHOR CONTRIBUTIONS

JM-S, JC-G and JP-S: conceived and designed the study; JM-S, PS-M and VH: carried out experimental procedures; JM-S and JP-S: wrote the original draft; All authors analysed and interpreted the data, reviewed, edited and approved the final manuscript.

COMPETING INTERESTS

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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REFERENCES

Ackerman, D., Tumanov, S., Qiu, B., Michalopoulou, E., Spata, M., Azzam, A., et al. (2018). Triglycerides promote lipid homeostasis during hypoxic stress by balancing fatty acid saturation. *Cell Rep.* 24, 2596-2605. doi: 10.1016/j.celrep.2018.08.015

Alfaro, A. C., and Young, T. (2018). Showcasing metabolomics research in aquaculture: a review. *Rev. Aquac.* 10, 135-152. doi: 10.1111/raq.12152

Almeida, D. V., Bianchini, A., and Marins, L. F. (2013). Growth hormone overexpression generates an unfavorable phenotype in juvenile transgenic zebrafish under hypoxic conditions. *Gen. Comp. Endocrinol.* 194, 102-109. doi: 10.1016/j.ygcen.2013.08.017

Araújo-Luna, R., Ribeiro, L., Bergheim, A., and Pousão-Ferreira, P. (2018). The impact of different rearing condition on gilthead seabream welfare: Dissolved oxygen levels and stocking densities. *Aquacult. Res.* 49, 3845-3855. doi: 10.1111/are.13851

Arends, R. J., Mancera, J. M., Munoz, J. L., Bonga, S. W., and Flik, G. (1999). The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *J Endocrinol. 163*, 149-157. doi: 10.1677/joe.0.1630149

Ashley, P. J. (2007). Fish welfare: Current issues in aquaculture. *Appl. Anim. Behav. Sci.* 104, 199-235. doi: 10.1016/j.applanim.2006.09.001

Auer, S. K., Salin, K., Rudolf, A. M., Anderson, G. J., and Metcalfe, N. B. (2015). The optimal combination of standard metabolic rate and aerobic scope for somatic growth depends on food availability. *Funct. Ecol.* 29, 479-486. doi: 10.1111/1365-2435.12396

Benedito-Palos, L., Calduch-Giner, J. A., Ballester-Lozano, G. F., and Pérez-Sánchez, J. (2013). Effect of ration size on fillet fatty acid composition, phospholipid allostasis and mRNA expression patterns of lipid regulatory genes in gilthead sea bream (*Sparus aurata*). *British Journal of Nutrition* 109, 1175-1187. doi: 10.1017/S000711451200311X

Benedito-Palos, L., Ballester-Lozano, G., and Pérez-Sánchez, J. (2014). Wide-gene expression analysis of lipid-relevant genes in nutritionally challenged gilthead sea bream (*Sparus aurata*). *Gene 547*(1), 34-42. doi: 10.1016/j.gene.2014.05.073

Benedito-Palos, L., Ballester-Lozano, G. F., Simó, P., Karalazos, V., Ortiz, A., Calduch-Giner, J. A., et al. (2016). Lasting effects of butyrate and low FM/FO diets on growth performance, blood haematology/biochemistry and molecular growth-related markers in gilthead sea bream (*Sparus aurata*). *Aquaculture* 454, 8-18. doi: 10.1016/j.aquaculture.2015.12.008

Bergan-Roller, H. E., and Sheridan, M. A. (2018). The growth hormone signaling system: Insights into coordinating the anabolic and catabolic actions of growth hormone. *Gen. Comp. Endocrinol.* 258, 119-133. doi: 10.1016/j.ygcen.2017.07.028

Bermejo-Nogales, A., Nederlof, M., Benedito-Palos, L., Ballester-Lozano, G. F., Folkedal, O., Olsen, R. E., Sitjà-Bobadilla, A. and Pérez-Sánchez, J. (2014a). Metabolic and transcriptional responses of gilthead sea bream (*Sparus aurata* L.) to environmental stress: New insights in fish mitochondrial phenotyping. *Gen. Comp. Endocrinol. 205*, 305-315. doi: 10.1016/j.ygcen.2014.04.016

Bermejo-Nogales, A., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2014b). Tissue-specific gene expression and functional regulation of uncoupling protein 2 (UCP2) by hypoxia and nutrient availability in gilthead sea bream (*Sparus aurata*):

693 implications on the physiological significance of UCP1-3 variants. *Fish Physiol.*
694 *Biochem.* 40(3), 751-762. doi: 10.1007/s10695-013-9882-7
695 Bermejo-Nogales, A., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2015). Unraveling
696 the molecular signatures of oxidative phosphorylation to cope with the
697 nutritionally changing metabolic capabilities of liver and muscle tissues in farmed
698 fish. *PLoS ONE* 10: e0122889. doi: 10.1371/journal.pone.0122889
699 Brett, J. R., (1979). "Environmental factors and growth", in *Fish Physiology*, Vol.
700 VIII, eds W.S. Hoar, D.J. Randall, J.R. Brett (London: Academic Press), 599-675.
701 Brown-Borg, H. M., Bode, A. M., and Bartke, A. (1999). Antioxidative mechanisms
702 and plasma growth hormone levels. *Endocrine* 11, 41-48. doi:
703 10.1385/ENDO:11:1:41
704 Brown-Borg, H.M., and Rakoczy, S.G. (2000). Catalase expression in delayed and
705 premature aging mouse models. *Exp. Gerontol.* 35, 199-212. doi: 10.1016/S0531-
706 5565(00)00079-6
707 Cadiz, L., Servili, A., Quazuguel, P., Madec, L., Zambonino-Infante, J. L., and
708 Mazurais, D. (2017). Early exposure to chronic hypoxia induces short-and long-
709 term regulation of hemoglobin gene expression in European sea bass
710 (*Dicentrarchus labrax*). *J. Exp. Biol.* 220, 3119-3126. doi: 10.1242/jeb.160713
711 Cadiz, L., Zambonino-Infante, J.-L., Quazuguel, P., Madec, L., Le Delliou, H., and
712 Mazurais, D. (2018). Metabolic response to hypoxia in European sea bass
713 (*Dicentrarchus labrax*) displays developmental plasticity. *Comparative*
714 *Biochemistry and Physiology* 215, 1-9. doi: 10.1016/j.cbpb.2017.09.005
715 Calduch-Giner, J. A., Davey, G., Saera-Vila, A., Houeix, B., Talbot, A., Prunet, P., et
716 al. (2010). Use of microarray technology to assess the time course of liver stress
717 response after confinement exposure in gilthead sea bream *(Sparus aurata* L.).
718 *BMC Genomics* 11:193. doi: 10.1186/1471-2164-11-193.
719 Calduch-Giner, J. A., Sitjà-Bobadilla, A., Davey, G. C., Cairns, M. T., Kaushik, S.,
720 and Pérez-Sánchez, J. (2012). Dietary vegetable oils do not alter the intestine
721 transcriptome of gilthead sea bream (*Sparus aurata*), but modulate the
722 transcriptomic response to infection with *Enteromyxum leei*. *BMC Genomics*
723 13:470. doi: 10.1186/1471-2164-13-470
724 Calduch-Giner, J. A., Echasseriau, Y., Crespo, D., Baron, D., Planas, J. V., Prunet, P.,
725 et al. (2014). Transcriptional assessment by microarray analysis and large-scale
726 meta-analysis of the metabolic capacity of cardiac and skeletal muscle tissues to
727 cope with reduced nutrient availability in gilthead sea bream (*Sparus aurata* L.).
728 *Mar. Biotechnol.* 16, 423-435. doi: 10.1007/s10126-014-9562-3
729 Dam, A. V., and Pauly, D. (1995). Simulation of the effects of oxygen on food
730 consumption and growth of Nile tilapia, *Oreochromis niloticus* (L.). *Aquacult. Res.*
731 26, 427-440. doi: 10.1111/j.1365-2109.1995.tb00932.x
732 Deutsch, C., Ferrel, A., Seibel, B., Pörtner, H.-O., and Huey, R. (2015) Climate
733 change tightens a metabolic constraint on marine habitats. *Science* 384, 1132-1135.
734 doi: 10.1126/science.aaa1605
735 Du, J., Wang, Y., Hunter, R., Wei, Y., Blumenthal, R., Falke, C., et al. (2009).
736 Dynamic regulation of mitochondrial function by glucocorticoids. *Proc. Natl.*
737 *Acad. Sci. U. S. A. 106*, 3543-3548. doi: 10.1073/pnas.0812671106
738 Ekau, W., Auel, H., Pörtner, H. O., and Gilbert, D. (2010). Impacts of hypoxia on the
739 structure and processes in pelagic communities (zooplankton, macro-invertebrates
740 and fish). *Biogeosciences* 7, 1669-1699. doi: 10.5194/bg-7-1669-2010
741 Estensoro, I., Ballester-Lozano, G. F., Benedito-Palos, L., Grammes, F., Martos-
742 Sitcha, J. A., Mydland, L.-T., et al. (2016). Dietary butyrate helps to restore the

743 intestinal status of a marine teleost (*Sparus aurata*) fed extreme diets low in fish
744 meal and fish oil. *PLoS ONE* 11: e0166564. doi: 10.1371/journal.pone.0166564
745 Everett, M. V., Antal, C. E., and Crawford, D. L. (2012). The effect of short-term
746 hypoxic exposure on metabolic gene expression. *J Exp Zool A Ecol Genet*
747 *Physiol. 317*, 9-23. doi: 10.1002/jez.717
748 Føre, M., Frank, K., Norton, T., Svendsen, E., Alfredsen, J. A., Dempster, T., et al.
749 (2017). Precision fish farming: A new framework to improve production in
750 aquaculture. *Biosyst. Eng.* 173, 176-193. doi:
751 10.1016/j.biosystemseng.2017.10.014
752 Gallaugher, P., and Farrell, A. P. (1998). "Hematocrit and blood oxygen-carrying
753 capacity", in *Fish physiology*, Vol. 17, eds S. F. Perry, and B. L. Tufts (New York,
754 NY: Academic Press), 185-227.
755 Garcia de la Serrana, D., and Macqueen, D. J. (2018). Insulin-like growth factor-
756 binding proteins of teleost fishes. *Front. Endocrinol. 9*: 80. doi:
757 10.3389/fendo.2018.00080
758 Gil-Solsona, R., Calduch-Giner, J. A., Nácher-Mestre, J., Lacalle-Bergeron, L.,
759 Sancho, J. V., Hernández, F., et al. (2019). Contributions of MS metabolomics to
760 gilthead sea bream (*Sparus aurata*) nutrition. Serum fingerprinting of fish fed low
761 fish meal and fish oil diets. *Aquaculture* 498, 503-512. doi:
762 10.1016/j.aquaculture.2018.08.080
763 Helm, K. P., Bindoff, N. L., and Church, J. A. (2011). Observed decreases in oxygen
764 content of the global ocean. *Geophys. Res. Lett.* 38: L23602. doi:
765 10.1029/2011GLO49513
766 Hermes-Lima, M., Moreira, D. C., Rivera-Ingraham, G. A., Giraud-Billoud, M.,
767 Genaro-Mattos, T. C., and Campos, É. G. (2015). Preparation for oxidative stress
768 under hypoxia and metabolic depression: revisiting the proposal two decades
769 later. *Free Radic. Biol. Med. 89*, 1122-1143. doi:
770 10.1016/j.freeradbiomed.2015.07.156
771 Hopkins, S. R., and Powell, F. L. (2001). "Common themes of adaptation to hypoxia",
772 in *Hypoxia*, Vol. 502, eds R.C. Roach, P.D. Wagner, and P.H. Hackett (Boston,
773 MA: Springer), 153-167.
774 Huang, D., Li, C., and Zhang, H. (2014). Hypoxia and cancer cell metabolism. *Acta*
775 *Biochim. Biophys. Sin.* 46, 214-219. doi: 10.1093/abbs/gmt148
776 Hulbert, A. J, Else, P. L., Manolis, S. C., and Brand, M. D. (2002). Proton leak in
777 hepatocytes and liver mitochondria from archosaurs (crocodiles) and allometric
778 relationships for ectotherms. *Journal of Comparative Physiology B* 172, 387-397.
779 doi: 10.1007/s00360-002-0264-1
780 Hulbert, A. J., Turner, N., Hinde, J., Else, P., and Guderley, H. (2006). How might
781 you compare mitochondria from different tissues and different species? *J. Comp.*
782 *Biochem. B* 176, 93-105. doi: 10.1007/s00360-005-0025-z
783 Intergovernmental Panel on Climate Change. (2014). Climate change 2014-Impacts,
784 adaptation and vulnerability: Part A: Global and sectoral aspects: Working group II
785 contribution to the IPCC fifth assessment report. (Cambridge: Cambridge
786 University Press). doi:10.1017/CBO9781107415379
787 Jensen, F. B., and Weber, R. E. (1982). Respiratory properties of tench blood and
788 hemoglobin. Adaptation to hypoxic-hypercapnic water. *Mol. Physiol.* 2, 235-250.
789 Jørgensen, E. H., Christiansen, J. S., and Jobling, M. (1993). Effects of stocking
790 density on food intake, growth performance and oxygen consumption in Arctic
791 charr (*Salvelinus alpinus*). *Aquaculture 110*, 191-204. doi: 10.1016/0044-
792 8486(93)90272-Z

Kajimura, S., Aida, K., and Duan, C. (2005). Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. *Proc. Natl. Acad. Sci. U.S.A.* 102, 1240-1245. doi: 10.1073/pnas.0407443102

Ke, Q., and Costa, M. (2006). Hypoxia-inducible factor-1 (HIF-1). *Mol. Pharmacol. 70*, 1469-1480. doi: 10.1124/mol.106.027029

Keeling, R. F., Körtzinger, A., and Gruber, N. (2009). Ocean deoxygenation in a warming world. *Ann. Rev. Mar. Sci.* 2, 199-229. doi: 10.1146/annurev.marine.010908.163855

Kieffer, D. A., Piccolo, B. D., Vaziri, N. D., Liu, S., Lau, W.L., Khazaeli, M., et al. 2016. Resistant starch alters gut microbiome and metabolomic profiles concurrent with amelioration of chronic kidney disease in rats. *Am. J. Physiol. Renal. Physiol.* 310: F857-F871. doi: 10.1152/ajprenal.00513.2015

Larsen, S., Nielsen, J., Hansen, C. N., Nielsen, L. B., Wibrand, F., Stride, et al. (2012). Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J. Physiol. 590*, 3349-3360. doi: 10.1113/jphysiol.2012.230185

Li, H., Ma, M. L., Luo, S., Zhang, R. M., Han, P., and Hu, W. (2012). Metabolic responses to ethanol in *Saccharomyces cerevisiae* using a gas chromatography tandem mass spectrometry-based metabolomics approach. *Int. J. Biochem. Cell. Biol.* 44, 1087- 1096. doi: 10.1016/j.biocel.2012.03.017

Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_T$ method. *Methods* 25, 402-408. doi: 10.1006/meth.2001.1262

Lushchak, V. I., and Bagnyukova, T. V. (2006). Effects of different environmental oxygen levels on free radical processes in fish. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 144, 283-289. doi: 10.1016/j.cbpb.2006.02.014

Magnoni, L. J., Martos-Sitcha, J. A., Queiroz, A., Calduch-Giner, J. A., Magalhães Gonçalves, J. F., Rocha, C. M. R., et al. (2017). Dietary Supplementation of heat-treated *Gracilaria* and *Ulva* seaweeds enhanced acute hypoxia tolerance in gilthead seabream (*Sparus aurata*). *Biology open* 6, 897-908. doi: 10.1242/bio.024299

Martin, S. A. M., and Król, E. (2017). Nutrigenomics and immune function in fish: new insights from omics technologies. *Dev. Comp. Immunol.* 75, 86-98. doi: 10.1016/j.dci.2017.02.024

Martínez-Barberá, J. P., Pendón, C., Martí-Palanca, H., Calduch-Giner, J. A., Rodríguez, R. B., Valdivia, M. M., et al. (1995). The use of recombinant gilthead sea bream (*Sparus aurata*) growth hormone for radioiodination and standard preparation in radioimmunoassay. *Comp. Biochem. Physiol. A Physiol.* 110, 335-340. doi: 10.1016/0300-9629(94)00178-V

Martínez-Porchas, M., and Vargas-Albores, F. (2017). Microbial metagenomics in aquaculture: a potential tool for a deeper insight into the activity. *Rev. Aquac.* 9, 42-56. doi: 10.1111/raq.12102

Martos-Sitcha, J. A., Bermejo-Nogales, A., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2017). Gene expression profiling of whole blood cells supports a more efficient mitochondrial respiration in hypoxia-challenged gilthead sea bream (*Sparus aurata*). *Front. Zool.* 14: 34. doi: 10.1186/s12983-017-0220-2

Martos-Sitcha, J. A., Simó-Mirabet, P., Piazzon, M. C., de las Heras, V., Calduch-Giner, J. A., Puyalto, M., et al. (2018). Dietary sodium heptanoate helps to improve feed efficiency, growth hormone status and swimming performance in gilthead sea bream (*Sparus aurata*). *Aquac. Nutr.* 24, 1638-1651. doi:

10.1111/anu.12799
McKenzie, D. J., Martinez, R., Morales, A., Acosta, J., Morales, R., Taylor, E. W., et al. (2003). Effects of growth hormone transgenesis on metabolic rate, exercise performance and hypoxia tolerance in tilapia hybrids. *J. Fish Biol.* 63, 398-409. doi: 10.1046/j.1095-8649.2003.00162.x
Millán-Cubillo, A. F., Martos-Sitcha, J. A., Ruiz-Jarabo, I., Cárdenas, S., and Mancera, J. M. (2016). Low stocking density negatively affects growth, metabolism and stress pathways in juvenile specimens of meagre (*Argyrosomus regius*, Asso 1801). *Aquaculture 451*, 87-92. doi: 10.1016/j.aquaculture.2015.08.034
Minchenko, D. O., Kharkova, A. P., Halkin, O. V., Karbovskyi, L. L., and Minchenko, O. H. (2016). Effect of hypoxia on the expression of genes encoding insulin-like growth factors and some related proteins in U87 glioma cells without IRE1 function. *Endocr. Regul.* 50, 43-54. doi: 10.1515/enr-2016-0008
Mommsen, T. P., Vijayan, M. M., and Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Rev. Fish Biol. Fish. 9*, 211-268. doi: 10.1023/A:1008924418720
Monternier, P. A., Marmillot, V., Rouanet, J. L., and Roussel, D. (2014). Mitochondrial phenotypic flexibility enhances energy savings during winter fast in king penguin chicks. *J. Exp. Biol.* 217, 2691-2697. doi: 10.1242/jeb.122671
Monternier, P., Teulier, L., Drai, J., Brouguignon, A., Collin-Chavagnac, D., Hervant, F., et al. (2017). Mitochondrial oxidative phosphorylation efficiency is upregulated during fasting in two major oxidative tissues of ducklings. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 212, 1-8. doi: 10.1016/j.cbpa.2017.06.0178
Montero, D., Izquierdo, M. S., Tort, L., Robaina, L., and Vergara, J. M. (1999). High stocking density produces crowding stress altering some physiological and biochemical parameters in gilthead seabream, *Sparus aurata*, juveniles. *Fish Physiol. Biochem.* 20, 53-60. doi: 10.1023/A:1007719928905
Murray, A. J. (2009). Metabolic adaptation of skeletal muscle to high altitude hypoxia: how new technologies could resolve the controversies. *Genome Med.* 1, 117. doi: 10.1186/gm117
Nikinmaa, M. (2001). Haemoglobin function in vertebrates: evolutionary changes in cellular regulation in hypoxia. *Respir. Physiol.* 128, 317-329. doi: 10.1016/S0034-5687(01)00309-7
Nikinmaa, M., and Rees, B. B. (2005). Oxygen-dependent gene expression in fishes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 288: R1079-R1090. doi: 10.1152/ajpregu.00626.2004
Nilsson, G. E. (2007). Gill remodeling in fish–a new fashion or an ancient secret?. *J. Exp. Biol.* 210, 2403-2409. doi: 10.1242/jeb.000281
Ning, Y., Schuller, A. G., Bradshaw, S., Rotwein, P., Ludwig, T., Frystyk, J., et al. (2006). Diminished growth and enhanced glucose metabolism in triple knockout mice containing mutations of insulin-like growth factor binding protein-3, -4, and -5. *Mol. Endocrinol.* 20, 2173-2186. doi: 10.1210/me.2005-0196
Pauletto, M., Manousaki, T., Ferraresso, S., Babbucci, M., Tsakogiannis, A., Louro, B., et al. (2018). Genomic analysis of *Sparus aurata* reveals the evolutionary dynamics of sex-biased genes in a sequential hermaphrodite fish. *Commun. Biol.* 1, 119. doi: 10.1038/s42003-018-0122-7
Pérez-Sánchez, J., Martí-Palanca, H., and Kaushik, S. (1995). Ration size and protein intake affect growth hormone (GH) levels, hepatic GH-binding and plasma insulin-like growth factor-I immunoreactivity in a marine teleost, gilthead sea bream

(*Sparus aurata*). *J. of Nutr.* 125, 546-552. doi: 10.1093/jn/125.3.546

Pérez-Sánchez, J., Borrel, M., Bermejo-Nogales, A., Benedito-Palos, L., Saera-Vila, A., Calduch-Giner, J. A., et al. (2013). Dietary oils mediate cortisol kinetics and the hepatic expression profile of stress responsive genes in juveniles of gilthead sea bream (*Sparus aurata*) exposed to crowding stress. *Comp. Biochem. Physiol. Part D* 8, 123-130. doi: 10.1016/j.cbd.2013.02.001

Pérez-Sánchez, J., Benedito-Palos, L., Estensoro, I., Petropoulos, Y., Calduch-Giner, J. A., Browdy, C. L., et al. (2015). Effects of dietary NEXT ENHANCE®150 on growth performance and expression of immune and intestinal integrity related genes in gilthead sea bream (*Sparus aurata* L.). *Fish Shellfish Immunol.* 44, 117-128. doi: 10.1016/j.fsi.2015.01.039

Pérez-Sánchez, J., Terova, G., Simó-Mirabet, P., Rimoldi, S., Folkedal, O., Calduch-Giner, J. A., et al. (2017). Skin mucus of gilthead sea bream (*Sparus aurata* L.). Protein mapping and regulation in chronically stressed fish. *Front. Physiol.* 8: 34. doi: 10.3389/fphys.2017.00034

Pérez-Sánchez, J., Simó-Mirabet, P., Naya, F., Martos-Sitcha, J.A., Perera, E., Bermejo-Nogales, A., et al. (2018). Somatotropic axis regulation unravels the differential effect of nutritional and environmental factors in growth performance of marine farmed fish. *Front. Endocrinol.* 9: 687. doi: 10.3389/fendo.2018.00687.

Person-Le Ruyet, J., Labbé, L., Le Bayon, N., Sévère, A., Le Roux, A., Le Delliou, H., et al. (2008). Combined effects of water quality and stocking density on welfare and growth of rainbow trout (*Oncorhynchus mykiss*). *Aquat. Living. Resour. 21*, 185-195. doi: 10.1051/alr:2008024

Piazzon, M. C., Calduch-Giner, J. A., Fouz, B., Estensoro, I., Simó-Mirabet, P., Puyalto, M., et al. (2017). Under control: how a dietary additive can restore the gut microbiome and proteomic profile, and improve disease resilience in a marine teleostean fish fed vegetable diets. *Microbiome* 5: 164. doi: 10.1186/s40168-017-0390-3

Pichavant, K., Person-Le-Ruyet, J., Bayon, N. L., Severe, A., Roux, A. L., and Boeuf, G. (2001). Comparative effects of long-term hypoxia on growth, feeding and oxygen consumption in juvenile turbot and European sea bass. *J. Fish Biol.* 59, 875-883. doi: 10.1006/jfbi.2001.1702

Pickering, A. D. (1993). "Growth and stress in fish production", in *Genetics in Aquaculture,* Vol. 111, eds A.E. Graham Gall and Hongxi Chen (Amsterdam: Elsevier Science Publishers B.V.), 51-63.

Planas, J. V., Méndez, E., Baños, N., Capilla, E., Castillo, J., Navarro, I., et al. (2000). Fish insulin, IGF-I and IGF-II receptors: a phylogenetic approach. *Amer. Zool. 40*, 223-233. doi:10.1093/icb/40.2.223

Poulain, L., Mathieu, H., Thomas, A., Borel, A. L., Remy, C., Levy, P., et al. (2017). Intermittent hypoxia-induced insulin resistance is associated with alterations in white fat distribution. *Sci. Rep.* 7: 11180. doi: 10.1038/s41598-017-11782-0

Reindl, K. M., and Sheridan, M. A. (2012). Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. *Comp. Biochem. Physiol. A Mol. Integr. Physiol. 163*, 231-245. doi: 10.1016/j.cbpa.2012.08.003

Remen, M., Nederlof, M. A. J., Folkedal, O., Thorsheim, G., Sitjà-Bobadilla, A., Pérez-Sánchez, J., Oppedal, F., Olsen, R. E. (2015). Effect of temperature on the metabolism, behavior and oxygen requirements of *Sparus aurata*. *Aquacult. Env. Interac.* 7, 115-123. doi: 10.3354/aei00141

Remen, M., Sievers, M., Torgersen, T., and Oppedal, F. (2016). The oxygen threshold

for maximal feed intake of Atlantic salmon post-smolts is highly temperature-dependent. *Aquaculture 464*, 582-592. doi: 10.1016/j.aquaculture.2016.07.037

Richards, J. G. (2011). Physiological, behavioral and biochemical adaptations of intertidal fishes to hypoxia. J. Exp. Biol. 214, 191-199. doi: 10.1242/jeb.047951

Rimoldi, S., Benedito-Palos, L., Terova, G., and Pérez-Sánchez, J. (2016). Wide-targeted gene expression infers tissue-specific molecular signatures of lipid metabolism in fed and fasted fish. *Rev. Fish Biol. Fish.* 26, 93-108. doi: 10.1007/s11160-015-9408-8

Rodrigues, P. M., Martin, S. A. M., Silva, T. S., Boonanuntanasarn, S., Schrama, D., Moreira, M., et al. (2018). "Proteomics in fish and aquaculture research", in *Proteomics in domestic animals: from farm to systems biology*, eds A. de Almeida, D. Eckersall, and I. Miller (Cham, Switzerland: Springer), 311-338.

Rolfe, D. F., and Brand, M. D. (1996). Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 271, C1380-C1389. doi: 10.1152/ajpcell.1996.271.4.C1380

Ruer, P. M., Cech Jr, J. J., and Doroshov, S. I. (1987). Routine metabolism of the white sturgeon, *Acipenser transmontanus*: effect of population density and hypoxia. *Aquaculture* 62, 45-52. doi: 10.1016/0044-8486(87)90183-9

Salin, K., Auer, S. K., Rey, B., Selman, C., and Metcalfe, N. B. (2015). Variation in the link between oxygen consumption and ATP production, and its relevance for animal performance. *Proc. R. Soc. B* 282: 20151028.

Sangiao-Alvarellos, S., Guzmán, J. M., Láiz-Carrión, R., Míguez, J. M., Martín Del Río, M. P., Mancera, J. M., et al. (2005). Interactive effects of high stocking density and food deprivation on carbohydrate metabolism in several tissues of gilthead sea bream *Sparus auratus*. *J. Exp. Zool. A Comp. Exp. Biol. 303*, 761-775. doi: 10.1002/jez.a.203

Saravanan, S., Geurden, I., Figueiredo-Silva, A. C., Kaushik, S. J., Haidar, M. N., Verreth, J. A., et al. (2012). Control of voluntary feed intake in fish: a role for dietary oxygen demand in Nile tilapia (*Oreochromis niloticus*) fed diets with different macronutrient profiles. *Br. J. Nutr.* 108, 1519-1529. doi: 10.1017/S0007114511006842

Schmidtko, S., Stramma, M., and Visbeck, M. (2017). Decline in global oxygen content during the past five decades. *Nature* 542, 335-339. doi: 10.1038/nature21399

Shimizu, M., Swanson, P., Fukada, H., Hara, A., and Dickhoff, W. W. (2000). Comparison of extraction methods and assay validation for salmon insulin-like growth factor-I using commercially available components. *Gen. Comp. Endocrinol.* 119, 26-36. doi: 10.1006/gcen.2000.7498

Shimizu, M., and Dickhoff, W. W. (2017). Circulating insulin-like growth factor binding proteins in fish: their identities and physiological regulation. *Gen. Comp. Endocrinol.* 252, 150-161. doi: 10.1016/j.ygcen.2017.08.002

Simó-Mirabet, P., Bermejo-Nogales, A., Calduch-Giner, J. A., and Pérez-Sánchez, J. (2017a). Tissue-specific gene expression and fasting regulation of sirtuin family in gilthead sea bream (*Sparus aurata*). *J. Comp. Physiol. B 187*, 153-163. doi: 10.1007/s00360-016-1014-0

Simó-Mirabet, P., Piazzon, M. C., Calduch-Giner, J. A., Ortiz, Á., Puyalto, M., Sitjà-Bobadilla, A., et al. (2017b). Sodium salt medium-chain fatty acids and *Bacillus*-based probiotic strategies to improve growth and intestinal health of gilthead sea bream *(Sparus aurata)*. *PeerJ 5*: e4001. doi: 10.7717/peerj.4001.

Simó-Mirabet, P., Perera, E., Calduch-Giner, J. A., Afonso, J. M., and Pérez-Sánchez,

J. (2018). Co-expression analysis of sirtuins and related metabolic biomarkers in juveniles of gilthead sea bream (*Sparus aurata*) with differences in growth performance. *Front. Physiol. 9:608.* doi: 10.3389/fphys.2018.00608

Skrzynska, A. K., Martos-Sitcha, J. A., Martínez-Rodríguez, G., and Mancera, J. M. (2018). Unraveling vasotocinergic, isotocinergic and stress pathways after food deprivation and high stocking density in the gilthead sea bream. *Comp. Biochem. Physiol. A Mol. Integr. Physiol. 215*, 35-44. doi: 10.1016/j.cbpa.2017.10.012

Treins, C., Giorgetti, S., Murdaca, J., Semenza, G. L., and Van Obberghen, E. (2002). Insulin stimulates hypoxia-inducible factor 1 through a phosphatidylinositol 3-kinase/target of rapamycin-dependent signaling pathway. *J. Biol. Chem.* 277: 27975-27981. doi: 10.1074/jbc.M204152200

Valli, A., Rodriguez, M., Moutsianas, L., Fischer, R., Fedele, V., Huang, H.-L., et al. (2015). Hypoxia induces a lipogenic cancer cell phenotype via HIF1α-dependent and -independent pathways. *Oncotarget* 6, 1920-1941. doi: 10.18632/oncotarget.3058

Van Weerd, J. H., and Komen, J. (1998). The effects of chronic stress on growth in fish: a critical appraisal. *Comp. Biochem. Physiol., Part A Mol. Integr. Physiol.* 120, 107-112. doi: 10.1016/S1095-6433(98)10017-X

Vega-Rubín de Celis, S., Gómez-Requeni, P. and Pérez-Sánchez, J. (2004). Production and characterization of recombinantly derived peptides and antibodies for accurate determinations of somatolactin, growth hormone and insulin-like growth factor-I in European sea bass (*Dicentrarchus labrax*). *Gen. Comp. Endocrinol.* 139, 266-277. doi: 10.1016/j.ygcen.2004.09.017

Vélez, E. J., Lutfi, E., Azizi, S., Perelló, M., Salmerón, C., Riera-Codina, M., et al. (2017). Understanding fish muscle growth regulation to optimize aquaculture production. *Aquaculture* 467, 28-40. doi: 10.1016/j.aquaculture.2016.07.004

Vikeså, V., Nankervis, L., and Hevrøy, E. M. (2017). High dietary energy level stimulates growth hormone receptor and feed utilization in large Atlantic salmon (*Salmo salar* L.) under hypoxic conditions. *Aquac. Nutr.* 23, 1193-1203. doi: 10.1111/anu.12488

Wold, S., Sjöström, M., and Eriksson, L. (2001). PLS-regression: a basic tool of chemometrics. Chemometr. Intell. Lab. Syst. 58, 109-130. doi: 10.1016/S0169-7439(01)00155-1

Yáñez, J. M., Newman, S., and Houston, R. D. (2015). Genomics in aquaculture to better understand species biology and accelerate genetic progress. *Front. Genet.* 6, 128. doi: 10.3389/fgene.2015.00128

Yu, Y., Vidalino, L., Anesi, A., Macchi, P., and Guella, G. (2014). A lipidomics investigation of the induced hypoxia stress on HeLa cells by using MS and NMR techniques. *Mol. Biosyst.* 10, 878-890. doi: 10.1039/C3MB70540D

Yúfera, M., Perera, E., Mata-Sotres, J. A., Calduch-Giner, J., Martínez-Rodríguez, G., and Pérez-Sánchez, J. (2017). The circadian transcriptome of marine fish (*Sparus aurata*) larvae reveals highly synchronized biological processes at the whole organism level. *Sci. Rep.* 7: 12943.

Zhu, C. D., Wang, Z. H., and Yan, B. (2013). Strategies for hypoxia adaptation in fish species: a review. *J. Comp. Physiol. B, Biochem. Syst. Environ. Physiol.* 183, 1005-1013. doi: 10.1007/s00360-013-0762-3.

1040 **Table 1.** Genes included in the liver (†), white muscle (#), heart (‡) and total blood
1041 cells (*) pathway-focused PCR arrays.

Gene name/category	Symbol
GH/IGF system	
Growth hormone receptor I	*ghr-i* †#‡
Growth hormone receptor II	*ghr-ii* †#‡
Insulin-like growth factor-I	*igf-i* †#‡
Insulin-like growth factor-II	*igf-ii* †#‡
Insulin-like growth factor binding protein 1a	*igfbp1a* †
Insulin-like growth factor binding protein 2b	*igfbp2b* †
Insulin-like growth factor binding protein 3	*igfbp3* #
Insulin-like growth factor binding protein 4	*igfbp4* †
Insulin-like growth factor binding protein 5b	*igfbp5b* #
Insulin-like growth factor binding protein 6b	*igfbp6b* #
Insulin receptor	*insr* #
Insulin-like growth factor receptor I	*igfr1* #
Insulin-like growth factor receptor II	*igfr2* #
Energy sensing and oxidative metabolism	
Sirtuin 1	*sirt1* †#‡
Sirtuin 2	*sirt2* †#‡
Sirtuin 3	*sirt3* †#‡
Sirtuin 4	*sirt4* †#‡
Sirtuin 5	*sirt5* †#‡
Sirtuin 6	*sirt6* †#‡
Sirtuin 7	*sirt7* †#‡
Carnitine palmitoyltransferase 1A	*cpt1a* †#‡*
Citrate synthase	*cs* †#‡*
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α* †#‡
Proliferator-activated receptor gamma coactivator 1 beta	*pgc1β* †#‡*
Hypoxia inducible factor-1 alpha	*hif-1α* †#‡
Respiration uncoupling	
Uncoupling protein 1	*ucp1* †
Uncoupling protein 2	*ucp2* *‡
Uncoupling protein 3	*ucp3* #
Xenobiotic metabolism	
Aryl hydrocarbon receptor 1	*ahr1* ‡
Cytochrome P450 1A1	*cyp1a1* ‡

Gene name/category	Symbol
Lipid metabolism	
Elongation of very long chain fatty acids 1	*elovl1* †
Elongation of very long chain fatty acids 4	*elovl4* †
Elongation of very long chain fatty acids 5	*elovl5* †
Elongation of very long chain fatty acids 6	*elovl6* †
Fatty acid desaturase 2	*fads2* †
Stearoyl-CoA desaturase 1a	*scd1a* †
Stearoyl-CoA desaturase 1b	*scd1b* †
Lipoprotein lipase	*lpl* †
Peroxisome proliferator-activated receptor α	*pparα* †
Peroxisomeproliferator-activated receptor γ	*pparγ* †
Antioxidant defence and tissue repair	
Catalase	*cat* †#‡
Glutathione peroxidase 4	*gpx4* †#‡
Glutathione reductase	*gr* †#‡
Peroxiredoxin 3	*prdx3* †#‡*
Peroxiredoxin 5	*prdx5* †#‡*
Superoxide dismutase [Mn]	*Mn-sod/sod2* †#‡*
Glucose-regulated protein, 170 kDa	*grp-170* †#‡
Glucose-regulated protein, 94 kDa	*grp-94* †#‡
70 kDa heat shock protein, mitochondrial	*mthsp70/grp-75/mortalin* †#‡
Glutathione S-transferase 3	*gst3* *
Muscle growth and cell differentiation	
Myoblast determination protein 1	*myod1* #
Myogenic factor MYOD2	*myod2* #
Myogenic factor 5	*myf5* #
Myogenic factor 6	*myf6/mrf4/herculin* #
Myostatin/Growth differentiation factor 8	*mstn/gdf-8* #
Myocyte-specific enhancer factor 2A	*mef2a* #
Myocyte-specific enhancer factor 2C	*mef2c* #
Follistatin	*fst* #
Nuclear receptors	
Glucocorticoid receptor	*gcr* ‡
Estrogen receptor alpha	*er-α* ‡
Nuclear respiratory factor 1	*nrf1* *

Table 1. (*continued*)

Outer and Inner transmembrane translocation (TOM and TIM complex)	
Mitochondrial import receptor subunit Tom70	*tom70* *
Mitochondrial import receptor subunit Tom34	*tom34* *
Mitochondrial import receptor subunit Tom22	*tom22* *
Mitochondrial import inner membrane translocase subunit 44	*tim44* *
Mitochondrial import inner membrane translocase subunit 23	*tim23* *
Mitochondrial import inner membrane translocase subunit Tim8A	*tim8a* *
Mitochondrial import inner membrane translocase subunit Tim10	*tim10* *
Mitochondrial import inner membrane translocase subunit Tim9	*tim9* *

OXPHOS (Complex I)	
NADH-ubiquinone oxidoreductase chain 2	*nd2* †#‡*
NADH-ubiquinone oxidoreductase chain 5	*nd5* †#‡*
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1	*ndufa1* *
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3	*ndufa3* *
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	*ndufa4* *
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	*ndufa7* *
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5	*ndufb5* *
NADH dehydrogenase iron-sulfur protein 2	*ndufs2* *
NADH dehydrogenase iron-sulfur protein 7	*ndufs7* *
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2	*ndufaf2* *

Mitochondrial dynamics and apoptosis	
Mitofusin 2	*mfn2* *
Mitochondrial fission factor homolog B	*miffb* *
Mitochondrial Rho GTPase 1	*miro1a* *
Mitochondrial Rho GTPase 2	*miro2* *
Apoptosis-related protein 1	*aifm1* *

OXPHOS (Complex IV)	
Cytochrome c oxidase subunit I	*coxi* †#‡*
Cytochrome c oxidase subunit II	*coxii* †#‡*
Cytochrome c oxidase subunit III	*coxiii* *
Cytochrome c oxidase subunit 4 isoform 1	*cox4a* *
Cytochrome c oxidase subunit 5A, mitochondrial-like isoform 2	*cox5a2* *
Cytochrome c oxidase subunit 6A isoform 2	*cox6a2* *
Cytochrome c oxidase subunit 6C1	*cox6c1* *
Cytochrome c oxidase subunit 7B	*cox7b* *
Cytochrome c oxidase subunit 8B	*cox8b* *
SCO1 protein homolog, mitochondrial	*sco1* *
Surfeit locus protein 1	*surf1* *
Cytochrome c oxidase assembly protein COX15 homolog	*cox15* *

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Table 2. Effects of rearing density and dissolved oxygen concentration on gilthead sea bream growth performance on a 21-days feeding trial. Values on body weight, feed intake, growth and feed efficiency are the mean ± SEM of triplicate tanks. Values on tissue biometric indexes are the mean ± SEM of 12 fish (4 fish per replicate tank). P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with oxygen concentration for a given rearing density (SNK test, P <0.05).

	LD		HD		P-value		
	Normoxia	Hypoxia	Normoxia	Hypoxia	[O_2]	Density	Interaction
Initial body weight (g)	34.54 ± 1.11	34.22 ± 0.27	34.32 ± 0.34	33.25 ± 0.45	0.305	0.376	0.571
Final body weight (g)	56.04 ± 1.89	51.65 ± 0.71	54.02 ± 0.50	48.54 ± 1.05**	0.003	0.059	0.651
Feed intake (g DM/fish)	23.78 ± 1.63	18.52 ± 0.7*	24.57 ± 1.06	17.54 ± 0.47**	<0.001	0.932	0.427
Weight gain (%)[1]	62.21 ± 0.31	50.94 ± 1.34**	57.43 ± 1.42	45.97 ± 1.31**	<0.001	0.003	0.941
SGR (%)[2]	2.30 ± 0.01	1.96 ± 0.04**	2.16 ± 0.04	1.80 ± 0.04**	<0.001	0.004	0.832
FE (%)[3]	0.91 ± 0.03	0.94 ± 0.02	0.80 ± 0.02	0.87 ± 0.01*	0.039	0.003	0.445
Liver weight (g)	0.94 ± 0.07	0.67 ± 0.03**	0.90 ± 0.06	0.63 ± 0.03***	<0.001	0.436	0.987
Viscera weight (g)	4.41 ± 0.28	3.84 ± 0.18	4.42 ± 0.19	3.68 ± 0.10**	0.002	0.690	0.681
HSI (%)[4]	1.64 ± 0.07	1.33 ± 0.06**	1.58 ± 0.07	1.25 ± 0.06**	<0.001	0.281	0.866
VSI (%)[5]	7.78 ± 0.29	7.65 ± 0.25	7.87 ± 0.24	7.38 ± 0.22	0.224	0.720	0.487

[1]Weight gain (%) = (100 x body weigh increase)/initial body weight
[2]Specific growth rate = 100 x (ln final body weight - ln initial body weight)/days
[3]Feed efficiency = wet weight gain/dry feed intake
[4]Hepatosomatic index = (100 x liver weight)/fish weight
[5]Viscerosomatix index = (100 x viscera weight)/fish weight

Table 3. Effects of rearing density and dissolved oxygen concentration on blood haematology and plasma levels of metabolites, hormones and total antioxidant capacity. Values are the mean ± SEM of 10-12 fish (4 fish per replicate tank). P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with oxygen concentration for a given rearing density (SNK test, P <0.05).

	LD		HD		P-value		
	Normoxia	Hypoxia	Normoxia	Hypoxia	[O_2]	Density	Interaction
Haemoglobin (g)	7.18 ± 0.24	7.73 ± 0.21	7.38 ± 0.14	7.77 ± 0.26	0.041	0.591	0.718
Haematocrit (%)	22.18 ± 1.10	32.91 ± 1.65***	28.27 ± 1.77	29.90 ± 1.39	<0.001	0.313	0.004
RBC x 10^6 (cells/µl)[1]	2.45 ± 0.07	2.74 ± 0.07**	2.38 ± 0.06	2.82 ± 0.08***	<0.001	0.924	0.345
MCHC (pg/10µm^3)[2]	34.07 ± 1.12	24.00 ± 1.18***	26.62 ± 1.73	26.46 ± 1.10	<0.001	0.067	<0.001
MCH (pg/cell)[3]	89.79 ± 4.21	116.6 ± 4.46**	116.5 ± 8.28	109.5 ± 7.21	0.118	0.123	0.010
MCV (µm^3)[4]	29.50 ± 1.02	28.33 ± 0.76	31.36 ± 0.93	27.73 ± 0.96*	0.014	0.501	0.194
Glucose (mg/dl)	54.39 ± 1.58	52.17 ± 2.44	58.04 ± 1.78	52.73 ± 2.79	0.091	0.339	0.482
Lactate (mg/dl)	16.30 ± 2.78	4.81 ± 1.41**	10.22 ± 3.06	4.99 ± 0.84	0.001	0.225	0.199
TAA (mM Trolox)[5]	1.34 ± 0.04	1.45 ± 0.04	1.48 ± 0.03	1.43 ± 0.03	0.447	0.104	0.026
Cortisol (ng/ml)	23.40 ± 5.67	21.08 ± 5.32	35.69 ± 11.15	79.25 ± 9.05**	0.036	<0.001	0.027
Growth hormone (ng/ml)	2.34 ± 0.83	6.71 ± 1.17*	5.39 ± 1.29	8.33 ± 4.20	0.069	0.337	0.767
Insulin-like growth factor-I (ng/ml)	46.06 ± 4.76	46.59 ± 4.77	45.78 ± 2.27	41.03 ± 6.29	0.659	0.544	0.582

[1]Red blood cells
[2]Mean corpuscular haemoglobin concentration
[3]Mean corpuscular haemoglobin
[4]Mean corpuscular volume
[5]Total antioxidant activity

Figure 1.TIFF

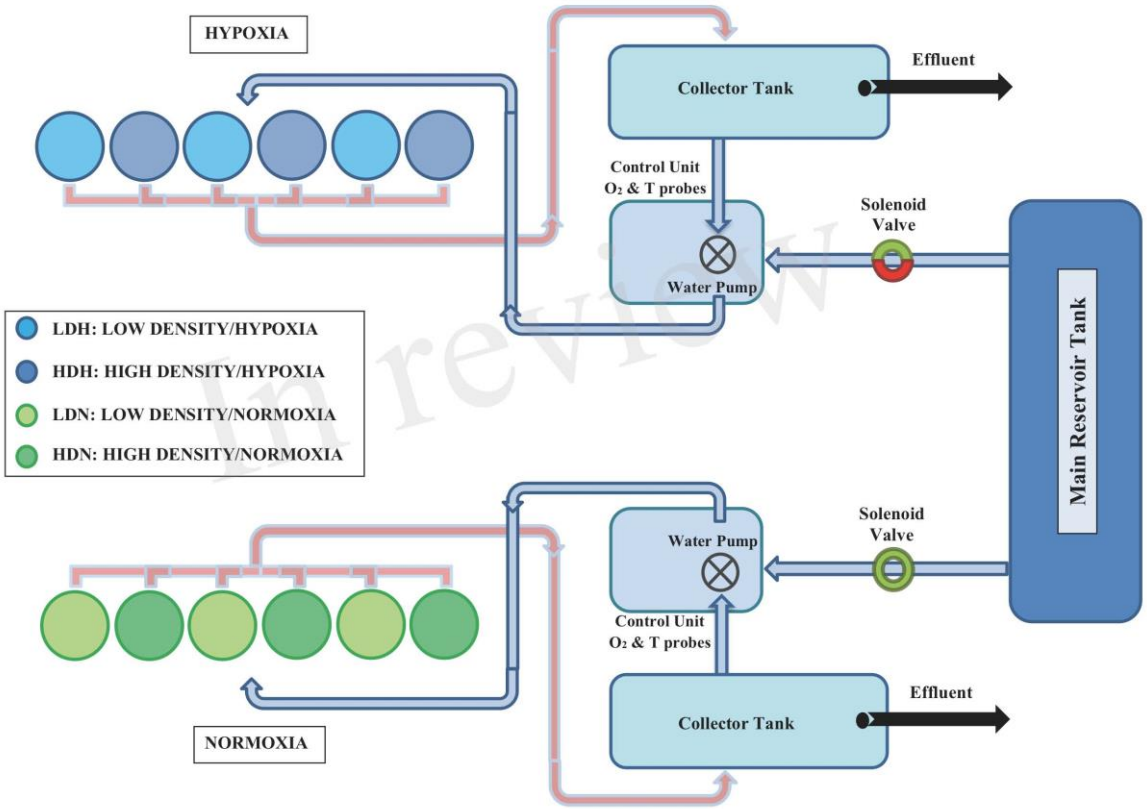
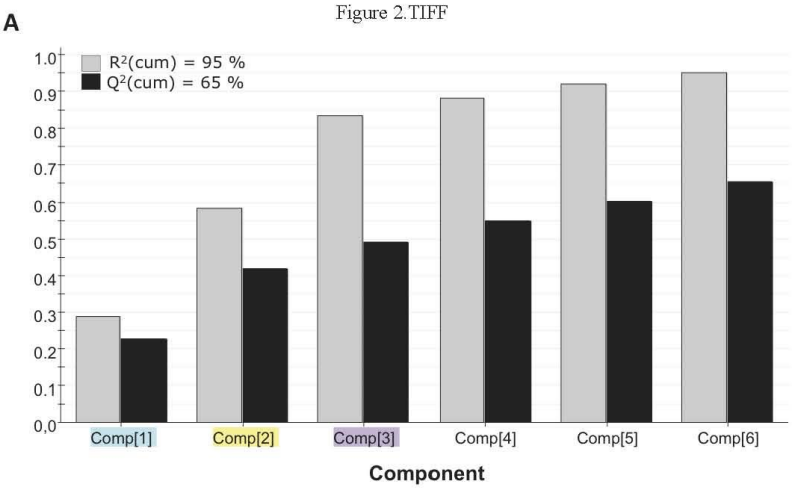
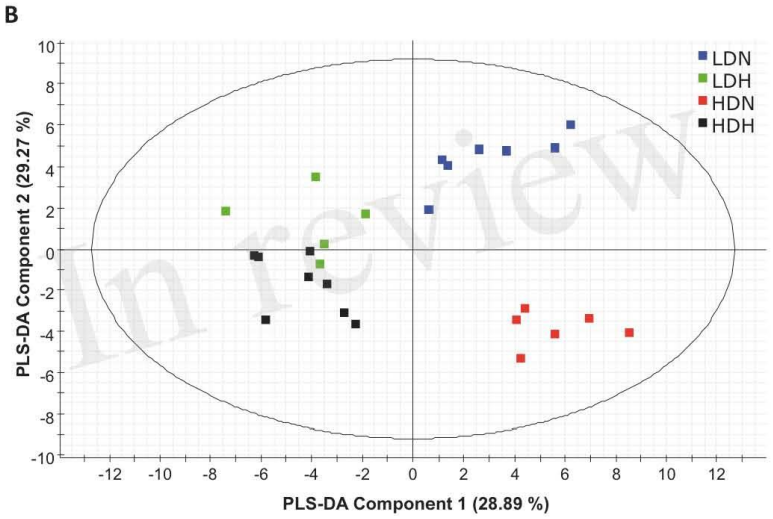


Figure 2.TIFF





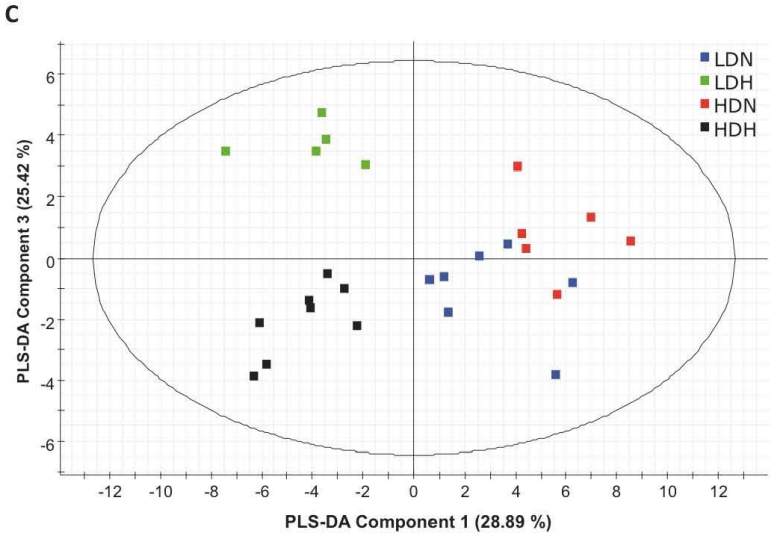
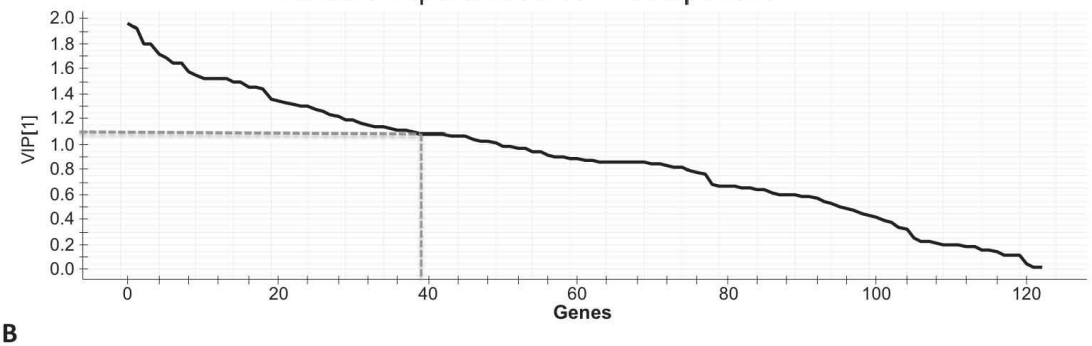


Figure 3.TIFF

A

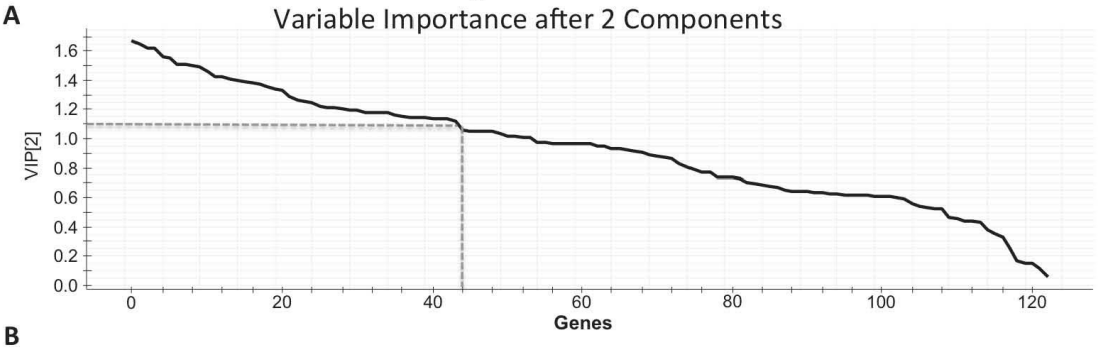


B

Order	Gene (tissue)	VIP (Component 1)	LD		HD		P-value		
			Normoxia	Hypoxia	Normoxia	Hypoxia	[O2]	Density	Interaction
1	*prdx5* (Liv)	1.964	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
2	*nd2* (Liv)	1.926	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
3	*nd5* (Heart)	1.801	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
4	*Mn-sod / sod2* (Heart)	1.799	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
5	*mthsp70/grp-75/mortalin* (Heart)	1.718	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
6	*Mn-sod / sod2* (Liv)	1.691	1.20 ± 0.05	1.02 ± 0.08	1.30 ± 0.09	0.94 ± 0.08*	0.001	0.955	0.280
7	*cs* (Heart)	1.653	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
8	*hif-1α* (Heart)	1.651	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
9	*gpx4* (Liv)	1.584	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
10	*pgc1α* (Heart)	1.553	2.55 ± 0.15	2.28 ± 0.15	2.74 ± 0.26	2.08 ± 0.10*	0.012	0.966	0.274
11	*nd2* (Heart)	1.529	68.14 ± 4.68	70.41 ± 5.69	64.19 ± 5.02	52.61 ± 2.44*	0.322	0.026	0.145
12	*nd5* (Liv)	1.525	16.51 ± 0.83	14.41 ± 0.73	18.11 ± 1.54	14.20 ± 1.11*	0.010	0.533	0.416
13	*igfr2* (WM)	1.521	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
14	*sirt1* (Heart)	1.518	0.22 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.18 ± 0.01*	0.023	0.219	0.572
15	*ucp3* (WM)	1.498	11.65 ± 1.78	6.43 ± 0.93*	16.89 ± 4.46	6.91 ± 0.74*	0.006	0.272	0.359
16	*sirt5* (Heart)	1.496	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
17	*prdx5* (WM)	1.460	28.69 ± 1.98	20.68 ± 1.10**	27.18 ± 2.14	22.48 ± 1.14*	<0.001	0.933	0.329
18	*prdx5* (Heart)	1.449	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
19	*gr* (Liv)	1.441	0.36 ± 0.02	0.34 ± 0.02	0.42 ± 0.04	0.28 ± 0.02**	0.006	0.929	0.050
20	*pgc1β* (Heart)	1.362	0.97 ± 0.04	0.85 ± 0.06	0.96 ± 0.05	0.84 ± 0.05	0.026	0.834	0.983
21	*sirt7* (Heart)	1.350	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.05 ± 0.00*	0.003	0.608	0.395
22	*grp-170* (Heart)	1.337	0.91 ± 0.06	0.92 ± 0.04	0.90 ± 0.05	0.77 ± 0.03*	0.191	0.070	0.103
23	*sirt2* (Liv)	1.316	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
24	*sirt6* (Heart)	1.309	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00*	0.042	0.211	0.099
25	*cyp1a1* (Heart)	1.297	2.58 ± 0.25	3.63 ± 0.32*	2.71 ± 0.23	3.79 ± 0.32*	<0.001	0.601	0.960
26	*coxi* (Heart)	1.268	329.3 ± 18.4	310.7 ± 14.8	332.4 ± 27.5	278.2 ± 19.3	0.086	0.480	0.393
27	*prdx3* (Liv)	1.258	0.82 ± 0.05	0.87 ± 0.05	0.94 ± 0.07	0.72 ± 0.07*	0.178	0.787	0.030
28	*pgc1β* (Liv)	1.239	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
29	*sirt2* (Heart)	1.215	0.34 ± 0.02	0.31 ± 0.02	0.33 ± 0.03	0.28 ± 0.01	0.081	0.454	0.644
30	*elovl1* (Liv)	1.194	10.63 ± 0.46	13.41 ± 0.79*	10.53 ± 0.66	12.69 ± 1.01	0.003	0.593	0.682
31	*sirt4* (Heart)	1.190	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
32	*ghr-i* (Liv)	1.161	3.06 ± 0.27	2.18 ± 0.26*	3.70 ± 0.36	2.79 ± 0.35*	0.007	0.049	0.977
33	*mthsp70/grp-75/mortalin* (Liv)	1.146	0.56 ± 0.04	0.55 ± 0.04	0.80 ± 0.10	0.55 ± 0.05*	0.033	0.044	0.053
34	*sirt2* (WM)	1.141	1.45 ± 0.06	1.49 ± 0.08	1.61 ± 0.14	1.43 ± 0.06	0.475	0.605	0.259
35	*elovl4* (Liv)	1.132	0.44 ± 0.05	0.49 ± 0.03	0.52 ± 0.05	0.41 ± 0.03	0.480	0.948	0.057
36	*sirt1* (Liv)	1.127	0.10 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.067	0.089	0.467
37	*grp-170* (WM)	1.111	1.80 ± 0.08	1.93 ± 0.12	2.09 ± 0.12	1.72 ± 0.11*	0.270	0.705	0.025
38	*igfr1* (WM)	1.107	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
39	*ghr-i* (Heart)	1.102	0.39 ± 0.01	0.35 ± 0.02	0.38 ± 0.02	0.30 ± 0.03	0.021	0.257	0.507

Figure 4.TIFF

A

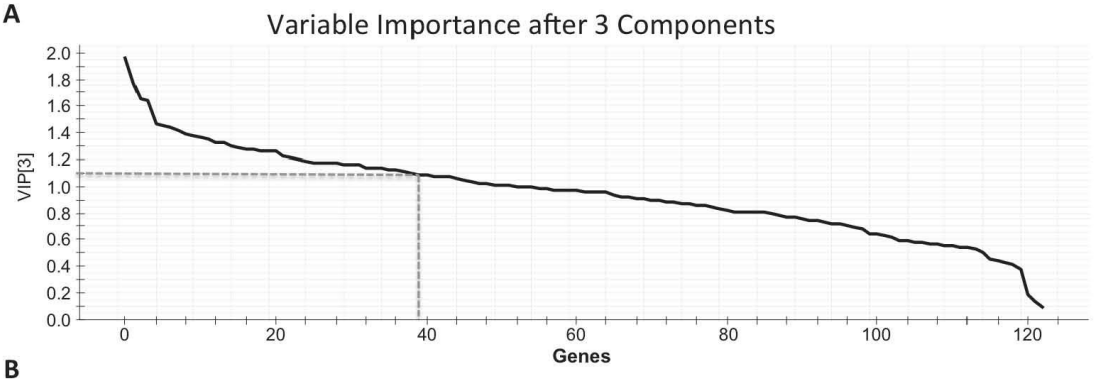


B

Order	Gene (tissue)	VIP (Component 1+2)	LD		HD		P-value		
			Normoxia	Hypoxia	Normoxia	Hypoxia	[O2]	Density	Interaction
1	*cs* (Heart)	1.675	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
2	*ghr-i* (WM)	1.644	6.42 ± 0.57	5.63 ± 0.20	4.14 ± 0.40	4.84 ± 0.51	0.915	0.002	0.101
3	*ucp1* (Liv)	1.618	23.04 ± 2.14	19.01 ± 1.99	17.55 ± 2.17	15.98 ± 1.46	0.218	0.002	0.527
4	*igfbp6b* (WM)	1.617	0.53 ± 0.05	0.59 ± 0.06	1.01 ± 0.10	0.65 ± 0.05*	0.056	0.001	0.009
5	*gpx4* (WM)	1.565	1.78 ± 0.48	1.01 ± 0.39	0.78 ± 0.21	0.43 ± 0.08	0.135	0.038	0.559
6	*Mn-sod / sod2* (Heart)	1.552	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
7	*igfr1* (WM)	1.512	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
8	*igfbp5b* (WM)	1.510	5.16 ± 0.39	6.21 ± 0.35	7.08 ± 0.47	6.18 ± 0.29	0.846	0.019	0.016
9	*pgc1β* (Liv)	1.505	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
10	*ghr-ii* (Liv)	1.491	2.39 ± 0.25	2.62 ± 0.35	4.00 ± 0.59	2.70 ± 0.26	0.182	0.032	0.059
11	*prdx5* (Heart)	1.460	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
12	*insr* (WM)	1.423	3.17 ± 0.15	3.26 ± 0.29	4.30 ± 0.43	3.28 ± 0.17*	0.105	0.050	0.057
13	*lpl* (Liv)	1.421	5.77 ± 0.78	6.57 ± 0.56	8.08 ± 0.99	8.75 ± 0.82	0.361	0.010	0.949
14	*elovl6* (Liv)	1.409	0.89 ± 0.12	0.89 ± 0.14	0.52 ± 0.06	0.60 ± 0.07	0.683	0.004	0.713
15	*gpx4* (Liv)	1.402	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
16	*prdx5* (Liv)	1.389	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
17	*nd5* (Heart)	1.385	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
18	*pgc1α* (Liv)	1.372	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.02	0.12 ± 0.02	0.304	0.010	0.558
19	*nd2* (Liv)	1.358	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
20	*mthsp70/grp-75/mortalin* (Heart)	1.338	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
21	*hif-1α* (Heart)	1.332	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
22	*igf-ii* (Liv)	1.287	2.85 ± 0.44	3.73 ± 0.53	5.37 ± 0.57	3.31 ± 0.76*	0.336	0.094	0.022
23	*pgc1α* (WM)	1.264	0.67 ± 0.21	0.30 ± 0.09	0.35 ± 0.07	0.27 ± 0.07	0.096	0.183	0.273
24	*sirt1* (Liv)	1.252	0.10 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.067	0.089	0.467
25	*sirt2* (Liv)	1.245	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
26	*mthsp70/grp-75/mortalin* (Liv)	1.223	0.56 ± 0.04	0.55 ± 0.04	0.80 ± 0.10	0.55 ± 0.05*	0.033	0.044	0.053
27	*igfr2* (WM)	1.209	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
28	*nd2* (Heart)	1.209	68.14 ± 4.68	70.41 ± 5.69	64.19 ± 5.02	52.61 ± 2.44*	0.322	0.026	0.145
29	*ucp3* (WM)	1.204	11.65 ± 1.78	6.43 ± 0.93*	16.89 ± 4.46	6.91 ± 0.74*	0.006	0.272	0.359
30	*prdx3* (Heart)	1.199	2.14 ± 0.15	2.08 ± 0.17	1.97 ± 0.13	1.61 ± 0.14	0.168	0.041	0.305
31	*Mn-sod / sod2* (Liv)	1.192	1.20 ± 0.05	1.02 ± 0.08	1.30 ± 0.09	0.94 ± 0.08*	0.001	0.955	0.280
32	*sirt5* (Heart)	1.182	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
33	*igf-i* (Liv)	1.180	9.51 ± 0.58	9.26 ± 1.20	13.29 ± 1.86	11.94 ± 0.79	0.514	0.013	0.654
34	*hif-1α* (WM)	1.178	5.45 ± 0.35	4.55 ± 0.30	6.67 ± 0.69	5.51 ± 0.31	0.028	0.020	0.773
35	*sirt1* (WM)	1.177	0.73 ± 0.04	0.70 ± 0.04	0.84 ± 0.04	0.72 ± 0.03*	0.050	0.104	0.263
36	*mthsp70/grp-75/mortalin* (WM)	1.158	5.58 ± 0.27	5.86 ± 0.43	6.96 ± 0.65	5.83 ± 0.47	0.380	0.163	0.147
37	*igfbp3* (WM)	1.156	9.33 ± 0.89	11.68 ± 1.03	13.17 ± 0.99	14.97 ± 2.18	0.143	0.015	0.845
38	*nd5* (Liv)	1.149	16.51 ± 0.83	14.41 ± 0.73	18.11 ± 1.54	14.20 ± 1.11*	0.010	0.533	0.416
39	*sirt4* (Heart)	1.145	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
40	*igf-i* (WM)	1.144	0.76 ± 0.09	0.67 ± 0.06	0.65 ± 0.11	0.63 ± 0.08	0.533	0.387	0.657
41	*gr* (Heart)	1.140	0.42 ± 0.03	0.40 ± 0.03	0.37 ± 0.01	0.33 ± 0.01*	0.136	0.012	0.574
42	*sirt1* (Heart)	1.138	0.22 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.18 ± 0.01*	0.023	0.219	0.572
43	*cpt1a* (Heart)	1.138	1.63 ± 0.15	1.28 ± 0.12	1.23 ± 0.12	1.21 ± 0.10	0.142	0.071	0.189
44	*pgc1α* (Heart)	1.119	2.55 ± 0.15	2.28 ± 0.15	2.74 ± 0.26	2.08 ± 0.10*	0.012	0.966	0.274

Figure 5.TIFF

A



B

Order	Gene (tissue)	VIP (Component 1+2+3)	LD		HD		P-value		
			Normoxia	Hypoxia	Normoxia	Hypoxia	[O2]	Density	Interaction
1	*fads2* (Liv)	1.966	3.82 ± 0.42	9.19 ± 0.97***	5.10 ± 0.60	4.98 ± 0.66	<0.001	0.048	<0.001
2	*cpt1a* (WM)	1.764	9.05 ± 1.02	6.72 ± 0.43*	7.44 ± 0.46	10.74 ± 1.25*	0.564	0.155	0.002
3	*scd1b* (Liv)	1.657	0.22 ± 0.03	0.52 ± 0.13*	0.22 ± 0.05	0.19 ± 0.02	0.076	0.024	0.026
4	*scd1a* (Liv)	1.639	0.43 ± 0.09	1.15 ± 0.19**	0.53 ± 0.23	0.56 ± 0.14	0.042	0.018	0.050
5	*pgc1β* (Liv)	1.462	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
6	*pgc1α* (Liv)	1.456	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.02	0.12 ± 0.02	0.304	0.010	0.558
7	*cs* (Heart)	1.435	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
8	*coxi* (WM)	1.414	740.2 ± 58.4	732.6 ± 26.9	834.2 ± 61.6	957.9 ± 57.1	0.271	0.005	0.214
9	*ghr-i* (WM)	1.385	6.42 ± 0.57	5.63 ± 0.20	4.14 ± 0.40	4.84 ± 0.51	0.915	0.002	0.101
10	*elovl6* (Liv)	1.376	0.89 ± 0.12	0.89 ± 0.14	0.52 ± 0.06	0.60 ± 0.07	0.683	0.004	0.713
11	*ucp1* (Liv)	1.362	23.04 ± 2.14	19.01 ± 1.99	17.55 ± 2.17	15.98 ± 1.46	0.218	0.002	0.527
12	*igfbp6b* (WM)	1.349	0.53 ± 0.05	0.59 ± 0.06	1.01 ± 0.10	0.65 ± 0.05*	0.056	0.001	0.009
13	*Mn-sod / sod2* (Heart)	1.326	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
14	*ghr-ii* (Liv)	1.324	2.39 ± 0.25	2.62 ± 0.35	4.00 ± 0.59	2.70 ± 0.26	0.182	0.032	0.059
15	*gpx4* (WM)	1.306	1.78 ± 0.48	1.01 ± 0.39	0.78 ± 0.21	0.43 ± 0.08	0.135	0.038	0.559
16	*prdx5* (Heart)	1.286	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
17	*igfbp5b* (WM)	1.273	5.16 ± 0.39	6.21 ± 0.35	7.08 ± 0.47	6.18 ± 0.29	0.846	0.019	0.016
18	*igfr2* (WM)	1.273	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
19	*igfr1* (WM)	1.266	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
20	*igf-ii* (Liv)	1.265	2.85 ± 0.44	3.73 ± 0.53	5.37 ± 0.57	3.31 ± 0.76*	0.336	0.094	0.022
21	*lpl* (Liv)	1.260	5.77 ± 0.78	6.57 ± 0.56	8.08 ± 0.99	8.75 ± 0.82	0.361	0.010	0.949
22	*grp-170* (Liv)	1.224	1.03 ± 0.08	1.41 ± 0.17	1.37 ± 0.19	1.11 ± 0.08	0.635	0.878	0.023
23	*mthsp70/grp-75/mortalin* (Heart)	1.209	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
24	*insr* (WM)	1.194	3.17 ± 0.15	3.26 ± 0.29	4.30 ± 0.43	3.28 ± 0.17*	0.105	0.050	0.057
25	*cpt1a* (Heart)	1.185	1.63 ± 0.15	1.28 ± 0.12	1.23 ± 0.12	1.21 ± 0.10	0.142	0.071	0.189
26	*prdx5* (Liv)	1.179	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
27	*nd5* (Heart)	1.178	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
28	*gpx4* (Liv)	1.176	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
29	*sirt4* (WM)	1.170	0.15 ± 0.01	0.14 ± 0.01	0.17 ± 0.02	0.18 ± 0.01	0.672	0.025	0.343
30	*sirt2* (Liv)	1.166	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
31	*sirt5* (Heart)	1.159	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
32	*pgc1α* (WM)	1.157	0.67 ± 0.21	0.30 ± 0.09	0.35 ± 0.07	0.27 ± 0.07	0.096	0.183	0.273
33	*nd2* (Liv)	1.139	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
34	*sirt4* (Liv)	1.132	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.520	1.000	0.608
35	*sirt4* (Heart)	1.131	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
36	*coxii* (Liv)	1.125	44.07 ± 2.34	37.08 ± 1.90*	39.36 ± 3.29	39.94 ± 2.30	0.205	0.711	0.137
37	*sirt7* (WM)	1.117	0.24 ± 0.01	0.25 ± 0.02	0.27 ± 0.01	0.28 ± 0.02	0.801	0.047	0.898
38	*hif-1α* (Heart)	1.112	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
39	*hif-1α* (WM)	1.101	5.45 ± 0.35	4.55 ± 0.30	6.67 ± 0.69	5.51 ± 0.31	0.028	0.020	0.773

Suppl. Table 1. Forward (F) and reverse (R) primers used for real-time PCR.

Gene name	Symbol	Acc. No.		Primer sequences (5' → 3')
70 kDa heat shock protein, mitochondrial	*mthsp70/grp-75/mortalin*	DQ524993	F	TCC GGT GTG GAT CTG ACC AAA GAC
			R	TGT TTA GGC CCA GAA GCA TCC ATG
Apoptosis-related protein 1	*aifm1*	JX975255	F	ACA GAG GAG TCA GGA ACC
			R	GGA GCA GGC AAT GAA GAG
Aryl hydrocarbon receptor 1	*ahr1*	EU254480	F	CCT GGG ACT GAA CGC CGA AG
			R	GCT AAG TGT TGG GAT GTG GTT GG
ß-Actin	*actb*	X89920	F	TCC TGC GGA ATC CAT GAG A
			R	GAC GTC GCA CTT CAT GAT GCT
Carnitine palmitoyltransferase 1A	*cpt1a*	JQ308822	F	GTG CCT TCG TTC GTT CCA TGA TC
			R	TGA TGC TTA TCT GCT GCC TGT TTG
Catalase	*cat*	JQ308823	F	TGG TCG AGA ACT TGA AGG CTG TC
			R	AGG ACG CAG AAA TGG CAG AGG
Citrate synthase	*cs*	JX975229	F	TCC AGG AGG TGA CGA GCC
			R	GTG ACC AGC AGC CAG AAG AG
Cytochrome c oxidase assembly protein COX15 homolog	*cox15*	KC217651	F	CAT ACT AGG TCG CTG GTT AG
			R	GAT TCC GTG AGC CTT GTG
Cytochrome c oxidase subunit 4 isoform 1	*cox4a*	JQ308835	F	ACC CTG AGT CCA GAG CAG AAG TCC
			R	AGC CAG TGA AGC CGA TGA GAA AGA AC
Cytochrome c oxidase subunit 5A, mitochondrial-like isoform 2	*cox5a2*	KC217635	F	CGC CAT CCG CAT CCT TGA
			R	GGC TTC AAC TCT TGG ATC AGG TAG G
Cytochrome c oxidase subunit 6A isoform 2	*cox6a2*	KC217639	F	TGT TGG CTG CTG CGT CAC ATT C
			R	CAG AAT CTT CCA GGT CCT CGC TCC
Cytochrome c oxidase subunit 6C1	*cox6c1*	KC217642	F	TCT CTC TGT CAC TCC TGG CTG CGA TAG
			R	CCT GGG CTC TGT CAC TGC GTA CTT G
Cytochrome c oxidase subunit 7B	*cox7b*	KC217645	F	TCT TCT GTG TGG CTG TGT GGT CAT ACG
			R	TTC CCA ACA GGT GAC AAA TTC CAG GTG AT
Cytochrome c oxidase subunit 8B	*cox8b*	KC217648	F	TCC GCT GGT CCC TGT GGC TAA
			R	CCT CCA CTG ATA TTG TGT TTG GCA GGT TTG
Cytochrome c oxidase subunit I	*coxi*	KC217652	F	GTC CTA CTT CTT CTG TCC CTT CCT GTT CT
			R	AGG TTT CGG TCT GTA AGG AGC ATT GTA ATC
Cytochrome c oxidase subunit II	*coxii*	KC217653	F	ACT GCC TAC ACA GGA CCT TGC C
			R	GTC TGC TTC CAG GAG ACG GAA TTG T
Cytochrome c oxidase subunit III	*coxiii*	KC217654	F	CCA AGC ACA CGC ATA CCA CAT A
			R	GCG GCA ACT GCA CCT GTA

Suppl. Table 1. (Continued)

Gene name	Symbol	Acc. No.		Primer sequences (5' → 3')
Cytochrome P450 1A1	*cyp1a1*	AF011223	F	GCA TCA ACG ACC GCT TCA ACG C
			R	CCT ACA ACC TTC TCA TCC GAC ATC TGG
Elongation of very long chain fatty acids 1	*elovl1*	JX975700	F	CTT CCT ACA CAT CTT CCA CCA CTC
			R	CCA TTC CAC CAG GAG CAA AGG
Elongation of very long chain fatty acids 4	*elovl4*	JX975701	F	CGG TGG CAA TCA TCT TCC
			R	TCA ACT GGC TGT CTG TGT
Elongation of very long chain fatty acids 5	*elovl5*	AY660879	F	CCT CCT GGT GCT CT ACA AT
			R	GTG AGT GTC CTG GCA GTA
Elongation of very long chain fatty acids 6	*elovl6*	JX975702	F	GTG CTG CTC TAC TCC TGG TA
			R	ACG GCA TGG ACC AAG TAG T
Estrogen receptor alpha	*er-α*	AF136979	F	TCT AAG GGT CTG GAG CAC
			R	TCG GTA TAG GGT CGG TTC
Fatty acid desaturase 2	*fads2*	AY055749	F	GCA GGC GGA GAG CGA CGG TCT GTT CC
			R	AGC AGG ATG TGA CCC AGG TGG AGG CAG AAG
Follistatin	*fst*	AY544167	F	GGA CCA GAC AAA CAA CGC ATA TTG
			R	CAT AGA TGA TCC CGT CGT TTC CAC
Glucocorticoid receptor	*gcr*	DQ486890	F	CCA GGA CAG GTG CCG AAC G
			R	TGG AGG AAC TGC TGC TGA ACC
Glucose-regulated protein, 170 kDa	*grp-170*	JQ308821	F	CAG AGG AGG CAG ACA GCA AGA C
			R	TTC TCA GAC TCA GCA TTT CCA GAT TTC
Glucose-regulated protein, 94 kDa	*grp-94*	JQ308820	F	AAG GCA CAG GCT TAC CAG ACA G
			R	CTT CAG CAT CAT CGC CGA CTT TC
Glutathione peroxidase 4	*gpx4*	AM977818	F	TGC GTC TGA TAG GGT CCA CTG TC
			R	GTC TGC CAG TCC TCT GTC GG
Glutathione reductase	*gr*	AJ937873	F	TGT TCA GCC ACC CAC CCA TCG G
			R	GCG TGA TAC ATC GGA GTG AAT GAA GTC TTG
Glutathione S-transferase 3	*gst3*	JQ308828	F	CCA GAT GAT CAG TAC GTG AAG ACC GTC
			R	CTG CTG ATG TGA GGA ATG TAC CGT AAC
Growth hormone receptor I	*ghr-i*	AF438176	F	ACCTGTCAGCCACCACATGA
			R	TCGTGCAGATCTGGGTCGTA
Growth hormone receptor II	*ghr-ii*	AY573601	F	GAGTGAACCCGGCCTGACAG
			R	GCGGTGGTATCTGATTCATGGT
Hypoxia inducible factor-1 alpha	*hif-1α*	JQ308830	F	CAG ATG AGC CTC TAA CTT GTG GAC
			R	TTA GCA AGA ATG GTG GCA AGA TGA G

Suppl. Table 1. (Continued 2)

Gene name	Symbol	Acc. No.		Primer sequences (5' → 3')
Insulin receptor	*insr*	KM522774	F	ACG GAC AGC AAG AAG GCA GAG AAT C
			R	GGC TTC AAC GGT CGG ATC AGG T
Insulin-like growth factor binding protein 1a	*igfbp1a*	KM522771	F	ACA AAC CAA AAC AGT GCG AGT CCT C
			R	CCG TTC CAA GAG TTC ACA CAC CAG
Insulin-like growth factor binding protein 2b	*igfbp2b*	AF377998	F	AGC GAT GTG TCC TGA GAT AGT GAG
			R	GCA CCG TGG CGT GTA GAC C
Insulin-like growth factor binding protein 3	*igfbp3**	MH577191	F	ACA GTG CCG TCC ATC CAA
		MH577192	R	GCT GCC CGT ATT TGT CCA
Insulin-like growth factor binding protein 4	*igfbp4*	KM658998	F	GGC ATC AAA CAC CCG CAC AC
			R	ATC CAC GCA CCA GCA CTT CC
Insulin-like growth factor binding protein 5b	*igfbp5b*	MH577194	F	CGA CAG GGC AGT CAA AGA AGC TAA CC
			R	GTC TCG AAG GCA TGT GAG CAG AAG G
Insulin-like growth factor binding protein 6b	*igfbp6b*	MH577196	F	GAT TGC TCA CTG CGG ATC
			R	GGA GGG ACA GAC CTT GAA
Insulin-like growth factor receptor I	*igfr1*	KM522775	F	TCA ACG ACA AGT ACG ACT ACC GCT GCT
			R	CAC ACT TTC TGG CAC TGG TTG GAG GTC
Insulin-like growth factor receptor II	*igfr2*	KM522776	F	ACA TTC GGG CAG CAC TCC TAA GAT
			R	CCA GTT CAC CTC GTA GCG ACA GTT
Insulin-like growth factor-I	*igf-i*	AY996779	F	TGTCTAGCGCTCTTTCCTTTCA
			R	AGAGGGTGTGGCTACAGGAGATAC
Insulin-like growth factor-II	*igf-ii*	AY996778	F	TGGGATCGTAGAGGAGTGTTGT
			R	CTGTAGAGAGGTGGCCGACA
Lipoprotein lipase	*lpl*	AY495672	F	CGT TGC CAA GTT TGT GAC CTG
			R	AGG GTG TTC TGG TTG TCT GC
Mitochondrial fission factor homolog B	*miffb*	JX975252	F	CGC AGC AGC ATT CCC TTC
			R	CTC GTA CTG GAT TCG GTT CAT CT
Mitochondrial import inner membrane translocase subunit 23	*tim23*	JX975240	F	CAA GTC AGG AAG TGG CGT AA
			R	AGA GCG TAG GCA CCA GAT A
Mitochondrial import inner membrane translocase subunit 44	*tim44*	JX975239	F	GAT GAC CTG GGA CAC ACT GG
			R	TCA CTC CTC TTC CTG AGT CTG G
Mitochondrial import inner membrane translocase subunit Tim10	*tim10*	JX975247	F	TAC CGC CAC ATT ACA AGG AGC
			R	ATC CAG GCA CAC CGA CTC
Mitochondrial import inner membrane translocase subunit Tim8A	*tim8a*	JX975245	F	CGA CAC CAC CCT GAC CAT CAC
			R	CGC CCT TCT GCA CCA TCT GT

Suppl. Table 1. (Continued 3)

Gene name	Symbol	Acc. No.		Primer sequences (5' → 3')
Mitochondrial import inner membrane translocase subunit Tim9	*tim9*	JX975248	F	CGT CAA AGA TTT CAC CAC CAG AGA G
			R	GGA GAC ACG ACT CGG AGC A
Mitochondrial import receptor subunit Tom22	*tom22*	JX975236	F	CGC TCT GGG TGG GTA CTA CCT CCT T
			R	CGA ACA CAA CAG GCA GCA CCA GGA T
Mitochondrial import receptor subunit Tom34	*tom34*	JX975235	F	GCT ACC GCC ACT TCT CCA CAA
			R	TCT GTT TGG TGC CGT TCT GCT
Mitochondrial import receptor subunit Tom70	*tom70*	JX975234	F	GAG TCA GGT GGT CGA TAC A
			R	CCA ATG AGC AGG TAG AAT GTG
Mitochondrial Rho GTPase 1	*aifm1*	JX975255	F	ACA GAG GAG TCA GGA ACC
			R	GGA GCA GGC AAT GAA GAG
Mitochondrial Rho GTPase 2	*aifm1*	JX975255	F	ACA GAG GAG TCA GGA ACC
			R	GGA GCA GGC AAT GAA GAG
Mitofusin 2	*mfn2*	JX975251	F	GGG ATG CCT CAG CCT CAG AAC CT
			R	CTG CCT GCG GAC CTC TTC CAT GTA TT
Myoblast determination protein 1	*myod1*	AF478568	F	ATG GAG CTG TCG GAT ATC TCT TTC
			R	GAA GCA GGG GTC ATC GTA GAA ATC
Myocyte-specific enhancer factor 2A	*mef2a*	KM522777	F	ATG GAC GAG AGG AAC AGG CAG GTT A
			R	GGC TAT CTC ACA GTC ACA TAG TAC GCT CAG
Myocyte-specific enhancer factor 2C	*mef2c*	KM522778	F	TAG CAA CTC CCA CTC TAC CAG GAC AAG
			R	GGA ATA CTC GGC ACC ATA AGA AGT CG
Myogenic factor 5	*myf5*	JN034420	F	GCA TGG TTG ACA GCA ACA GTC CAG TGT
			R	TGT CTT ATC GCC CAA AGT GTC GTT CTT CAT
Myogenic factor 6	*myf6/mrf4/ herculin*	JN034421	F	GCA GCA ATG ACA AAC CAG AGA GAC GGA ACA
			R	GAG GCT GGA GGA CGC CGA AGA TTC A
Myogenic factor MYOD2	*myod2*	AF478569	F	CCA ACT GCT CTG ATG GCA TGA TGG ATT TC
			R	GAC CGT TTG CTT CTC CTG GAC TCG TAT G
Myostatin/Growth differentiation factor 8	*mstn/gdf-8*	AF258448	F	AAG AGC AGA TCA TCT ACG GCA AGA TCC
			R	TCA AGA GCA TCC ACA ACG GTC TAC CA
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex, assembly factor 2	*ndufaf2*	KC217598	F	AGG CAG CAT ACC GAT AGA G
			R	ACT CAT TCT TCA GCA ACT CCT
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1	*ndufa1*	KC217562	F	CGG GTT CCG TGG CAG TGG TA
			R	TCC TGT TCC TGA TAC TCG CTT GTC TCT

Suppl. Table 1. (Continued 4)

Gene name	Symbol	Acc. No.		Primer sequences (5' → 3')
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3	*ndufa3*	KC217564	F	TCG GAG CGT TCC TGA AGA ATG C
			R	GAA GAG CCA TAC CTA TCA GTC CAA TAC CA
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	*ndufa4*	KC217565	F	GCT CGT CTG GGC TTG AGA AAC C
			R	GCT CTG GGT TGT TCT TGC GAT CC
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	*ndufa7*	KC217569	F	CCG AGC CAC AAG TAT GCC AGC AAC TA
			R	AGC CTC CCT GCG TCC ATC TCT G
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5	*ndufb5*	KC217580	F	TGC GTC GGC AGA TGA GGA T
			R	CTT GTT GAG GGT GTT CAC CTG GAA
NADH dehydrogenase iron-sulfur protein 2	*ndufs2*	KC217589	F	GTA TCA GAC GGC TCC AGC AGA C
			R	AGA CCA GCC AAG TGA GCG AAT
NADH dehydrogenase iron-sulfur protein 7	*ndufs7*	KC217594	F	AAC GGA GGA GGC TAC TAC CAC TAC T
			R	CGG TAC GAT TCG GTC ACA ACC TCT AAC
NADH-ubiquinone oxidoreductase chain 2	*nd2*	KC217558	F	TAG GTT GAA TGA CCA TCG TA
			R	GGC TAA GGA GTT GAG GTT
NADH-ubiquinone oxidoreductase chain 5	*nd5*	KC217559	F	CCT AAA CGC CTG AGC CCT GG
			R	GCT GTA AAC GAG GTG GCT AGA AGG
Nuclear respiratory factor 1	*nrf1*	JX975263	F	CAG ATA GTC CTG GCA GAG A
			R	GAC CTG TGG CAT CTT GAA
Peroxiredoxin 3	*prdx3*	GQ252681	F	ATC AAC ACC CCA CGC AAG ACT G
			R	ACC GTT TGG ATC AAT GAG GAA CAG ACC
Peroxiredoxin 5	*prdx5*	GQ252683	F	ATC AAC ACC CCA CGC AAG ACT G
			R	TCC ACA TTG ATC TTC TTC ACG ACT CC
Peroxisome proliferator-activated receptor α	*pparα*	AY590299	F	TCT CTT CAG CCC ACC ATC CC
			R	ATC CCA GCG TGT CGT CTC C
Peroxisomeproliferator-activated receptor γ	*pparγ*	AY590304	F	CGC CGT GGA CCT GTC AGA GC
			R	GGA ATG GAT GGA GGA GGA GGA GAT GG
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α*	JX975264	F	CGT GGG ACA GGT GTA ACC AGG ACT C
			R	ACC AAC CAA GGC AGC ACA CTC TAA TTC T
Proliferator-activated receptor gamma coactivator 1 beta	*pgc1β*	JX975265	F	TCA GAG GAA GAG GCG GAT
			R	GAC ACA GGT GGA GGA TGG
SCO1 protein homolog, mitochondrial	*sco1*	KC217649	F	ACA ACA ACA AGC CCA CCA AGA
			R	GAC AGT GAG TGA ACC CGA AGT AGA T

Suppl. Table 1. (Continued 5)

Gene name	Symbol	Acc. No.		Primer sequences (5' → 3')
Sirtuin1	*sirt1*	KF018666	F	GGT TCC TAC AGT TTC ATC CAG CAG CAC ATC
			R	CCT CAG AAT GGT CCT CGG ATC GGT CTC
Sirtuin2	*sirt2*	KF018667	F	GAA CAA TCC GAC GAC AGC AGT GAA G
			R	AGG TTA CGC AGG AAG TCC ATC TCT
Sirtuin3	*sirt3*	KF018668	F	CTG CCA AGT CCT CAT CCC
			R	CTT CAC CAG ACG AGC CAC
Sirtuin4	*sirt4*	KF018669	F	GGC TGG CGG AGT CGG ATG
			R	TCC TGA ATA CAC CTG TGA CGA AGA C
Sirtuin5	*sirt5*	KF018670	F	CAG ACA TCC TAA CCC GAG CAG AG
			R	CCA CGA GGC AGA GGT CAC A
Sirtuin6	*sirt6*	KF018671	F	ACT CCA CCA CCA CCG ATG TCA A
			R	CTC CTC CTC CTT CAC CTT TCG CTT TG
Sirtuin7	*sirt7*	KF018672	F	CTG GAG CAA CCT CTA AAC TGG AA
			R	CAC CTT CAG ACT GGA GCC TAA
Stearoyl-CoA desaturase 1a	*scd1a*	JQ277703	F	CGG AGG CGG AGG CGT TGG AGA AGA AG
			R	AGG GAG ACG GCG TAC AGG GCA CCT ATA TG
Stearoyl-CoA desaturase 1b	*scd1b*	JQ277704	F	GCT CAA TCT CAC CAC CGC CTT CAT AG
			R	GCT GCC GTC GCC CGT TCT CTG
Superoxide dismutase [Mn]	*Mn-sod/sod2*	JQ308833	F	CCT GAC CTG ACC TAC GAC TAT GG
			R	AGT GCC TCC TGA TAT TTC TCC TCT G
Surfeit locus protein 1	*surf1*	KC217650	F	AGA TGG AAG GTG AAG TGG AGG TGG TC
			R	GCG TTG CTC TGT CTG CCG AAC T
Uncoupling protein 1	*ucp1*	FJ710211	F	GCA CAC TAC CCA ACA TCA CAA G
			R	CGC CGA ACG CAG AAA CAA AG
Uncoupling protein 2	*ucp2*	JQ859959	F	CGG CGG CGT CCT CAG TTG
			R	AAG CAA GTG GTC CCT CTT TGG TCA T
Uncoupling protein 3	*ucp3*	EU555336	F	AGG TGC GAC TGG CTG ACG
			R	TTC GGC ATA CAA CCT CTC CAA AG

(*) Acc. No. MH577191: *igfbp3a*; Acc. No. MH577192: *igfbp3b*. Primers used for *igfbp3* gene expression jointly amplify both *igfbp3a* and *igfbp3b* isoforms.

Suppl. Table 2. Effects of rearing density and dissolved oxygen concentration on gilthead sea bream relative expression of hepatic selected genes on a 21-days feeding trial. Values on relative expression are the mean ± SEM of 8 fish (2-3 fish per replicate tank). P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with oxygen concentration for a given rearing density (SNK test, P <0.05).

Category	Symbol	LD		HD		P-value		
		Normoxia	Hypoxia	Normoxia	Hypoxia	$[O_2]$	Density	Interaction
GH/IGF system	*ghr-i*	3.06 ± 0.27	2.18 ± 0.26*	3.70 ± 0.36	2.79 ± 0.35*	0.007	0.049	0.977
	ghr-ii	2.39 ± 0.25	2.62 ± 0.35	4.00 ± 0.59	2.70 ± 0.26	0.182	0.032	0.059
	igf-i	9.51 ± 0.58	9.26 ± 1.20	13.29 ± 1.86	11.94 ± 0.79	0.514	0.013	0.654
	igf-ii	2.85 ± 0.44	3.73 ± 0.53	5.37 ± 0.57	3.31 ± 0.76*	0.336	0.094	0.022
	igfbp1a	0.03 ± 0.00	0.03 ± 0.00	0.03 ± 0.00	0.04 ± 0.00	0.736	0.174	0.736
	igfbp2b	3.17 ± 0.22	3.29 ± 0.22	3.64 ± 0.40	3.11 ± 0.17	0.459	0.595	0.240
	igfbp4	0.81 ± 0.09	0.79 ± 0.10	0.82 ± 0.13	0.85 ± 0.06	0.975	0.712	0.856
Lipid metabolism	*elovl1*	10.63 ± 0.46	13.41 ± 0.79*	10.53 ± 0.66	12.69 ± 1.01	0.003	0.593	0.682
	elovl4	0.44 ± 0.05	0.49 ± 0.03	0.52 ± 0.05	0.41 ± 0.03	0.480	0.948	0.057
	elovl5	2.67 ± 0.55	3.32 ± 0.65	4.14 ± 1.11	2.17 ± 0.25	0.388	0.827	0.096
	elovl6	0.89 ± 0.12	0.89 ± 0.14	0.52 ± 0.06	0.60 ± 0.07	0.683	0.004	0.713
	fads2	3.82 ± 0.42	9.19 ± 0.97***	5.10 ± 0.60	4.98 ± 0.66	<0.001	0.048	<0.001
	scd1a	0.22 ± 0.03	0.52 ± 0.13*	0.22 ± 0.05	0.19 ± 0.02	0.076	0.024	0.026
	scd1b	0.43 ± 0.09	1.15 ± 0.19**	0.53 ± 0.23	0.56 ± 0.14	0.042	0.018	0.050
	lpl	5.77 ± 0.78	6.57 ± 0.56	8.08 ± 0.99	8.75 ± 0.82	0.361	0.010	0.949
	pparα	2.26 ± 0.26	2.69 ± 0.20	2.29 ± 0.28	2.84 ± 0.24	0.055	0.732	0.807
	pparγ	0.78 ± 0.06	0.83 ± 0.09	0.87 ± 0.09	0.71 ± 0.04	0.456	0.803	0.165
Energy sensing and oxidative metabolism	*sirt1*	0.10 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.067	0.089	0.467
	sirt2	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
	sirt3	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00	0.406	0.780	0.102
	sirt4	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.520	1.000	0.608
	sirt5	0.32 ± 0.02	0.33 ± 0.02	0.33 ± 0.03	0.30 ± 0.02	0.555	0.598	0.231
	sirt6	0.03 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.821	0.821	0.264

	sirt7	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.319	0.188	0.754
	cpt1a	0.71 ± 0.11	0.50 ± 0.04	0.62 ± 0.10	0.59 ± 0.06	0.129	0.965	0.251
	cs	1.02 ± 0.08	0.93 ± 0.05	0.98 ± 0.08	0.93 ± 0.07	0.289	0.756	0.782
	nd2	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
	nd5	16.51 ± 0.83	14.41 ± 0.73	18.11 ± 1.54	14.20 ± 1.11*	0.010	0.533	0.416
	coxi	65.74 ± 4.70	71.34 ± 7.10	76.95 ± 7.56	69.25 ± 3.24	0.860	0.448	0.270
	coxii	44.07 ± 2.34	37.08 ± 1.90*	39.36 ± 3.29	39.94 ± 2.30	0.205	0.711	0.137
	ucp1	23.04 ± 2.14	19.01 ± 1.99	17.55 ± 2.17	15.98 ± 1.46	0.218	0.002	0.527
	pgc1α	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.02	0.12 ± 0.02	0.304	0.010	0.558
	pgc1β	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
	hif-1α	1.12 ± 0.07	1.14 ± 0.09	1.24 ± 0.13	1.05 ± 0.04	0.332	0.862	0.281
Antioxidant defence and tissue repair	*cat*	23.55 ± 1.75	22.09 ± 2.76	21.97 ± 1.98	21.21 ± 1.10	0.582	0.542	0.862
	gpx4	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
	gr	0.36 ± 0.02	0.34 ± 0.02	0.42 ± 0.04	0.28 ± 0.02**	0.006	0.929	0.050
	prdx3	0.82 ± 0.05	0.87 ± 0.05	0.94 ± 0.07	0.72 ± 0.07*	0.178	0.787	0.030
	prdx5	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
	Mn-sod / sod2	1.20 ± 0.05	1.02 ± 0.08	1.30 ± 0.09	0.94 ± 0.08*	0.001	0.955	0.280
	grp-170	1.03 ± 0.08	1.41 ± 0.17	1.37 ± 0.19	1.11 ± 0.08	0.635	0.878	0.023
	grp-94	3.44 ± 0.40	3.90 ± 0.48	3.37 ± 0.46	3.60 ± 0.42	0.456	0.685	0.794
	mthsp70/grp-75/mortalin	0.56 ± 0.04	0.55 ± 0.04	0.80 ± 0.10	0.55 ± 0.05*	0.033	0.044	0.053

Suppl. Table 3. Effects of rearing density and dissolved oxygen concentration on gilthead sea bream relative expression of white muscle selected genes on a 21-days feeding trial. Values on relative expression are the mean ± SEM of 8 fish (2-3 fish per replicate tank). P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with oxygen concentration for a given rearing density (SNK test, P <0.05).

Category	Symbol	LD		HD		P-value		
		Normoxia	Hypoxia	Normoxia	Hypoxia	[O_2]	Density	Interaction
GH/IGF system	*ghr-i*	6.42 ± 0.57	5.63 ± 0.20	4.14 ± 0.40	4.84 ± 0.51	0.915	0.002	0.101
	ghr-ii	2.71 ± 0.41	3.32 ± 0.50	2.83 ± 0.42	3.44 ± 0.71	0.267	0.835	0.996
	igf-i	0.76 ± 0.09	0.67 ± 0.06	0.65 ± 0.11	0.63 ± 0.08	0.533	0.387	0.657
	igf-ii	3.16 ± 0.28	3.50 ± 0.28	2.49 ± 0.27	3.03 ± 0.25	0.114	0.043	0.725
	igfbp3	9.33 ± 0.89	11.68 ± 1.03	13.17 ± 0.99	14.97 ± 2.18	0.143	0.015	0.845
	igfbp5b	5.16 ± 0.39	6.21 ± 0.35	7.08 ± 0.47	6.18 ± 0.29	0.846	0.019	0.016
	igfbp6b	0.53 ± 0.05	0.59 ± 0.06	1.01 ± 0.10	0.65 ± 0.05*	0.056	0.001	0.009
	insr	3.17 ± 0.15	3.26 ± 0.29	4.30 ± 0.43	3.28 ± 0.17*	0.105	0.050	0.057
	igfr1	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
	igfr2	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
Muscle growth and cell differentiation	*myod1*	27.87 ± 2.12	25.73 ± 1.72	31.73 ± 2.85	29.29 ± 2.00	0.309	0.105	0.946
	myod2	10.96 ± 0.82	12.83 ± 0.61	10.78 ± 0.63	13.29 ± 1.03	0.010	0.859	0.688
	myf5	1.41 ± 0.09	1.41 ± 0.11	1.32 ± 0.11	1.63 ± 0.14	0.199	0.572	0.195
	myf6/mrf4	0.85 ± 0.05	0.85 ± 0.04	0.95 ± 0.06	0.92 ± 0.04	0.783	0.093	0.764
	mstn/gdf-8	11.95 ± 1.31	13.93 ± 1.95	9.89 ± 0.36	12.45 ± 1.54	0.125	0.227	0.842
	mef2a	53.53 ± 4.03	57.79 ± 5.17	59.61 ± 3.72	57.52 ± 2.69	0.788	0.473	0.434
	mef2c	14.88 ± 0.89	13.95 ± 0.66	15.38 ± 0.91	14.87 ± 0.74	0.382	0.385	0.796
	fst	1.63 ± 0.06	1.66 ± 0.19	1.39 ± 0.18	1.69 ± 0.16	0.303	0.490	0.396
Energy sensing and oxidative metabolism	*sirt1*	0.73 ± 0.04	0.70 ± 0.04	0.84 ± 0.04	0.72 ± 0.03*	0.050	0.104	0.263
	sirt2	1.45 ± 0.06	1.49 ± 0.08	1.61 ± 0.14	1.43 ± 0.06	0.475	0.605	0.259
	sirt3	0.19 ± 0.01	0.19 ± 0.02	0.20 ± 0.02	0.20 ± 0.01	0.905	0.605	0.780
	sirt4	0.15 ± 0.01	0.14 ± 0.01	0.17 ± 0.02	0.18 ± 0.01	0.672	0.025	0.343
	sirt5	2.15 ± 0.22	2.23 ± 0.17	2.28 ± 0.18	2.34 ± 0.16	0.690	0.522	0.941

	sirt6	0.16 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.17 ± 0.02	0.742	0.541	0.128
	sirt7	0.24 ± 0.01	0.25 ± 0.02	0.27 ± 0.01	0.28 ± 0.02	0.801	0.047	0.898
	cpt1a	9.05 ± 1.02	6.72 ± 0.43*	7.44 ± 0.46	10.74 ± 1.25*	0.564	0.155	0.002
	cs	53.95 ± 3.69	50.43 ± 3.17	53.22 ± 4.00	55.01 ± 2.65	0.802	0.577	0.443
	nd2	251.51 ± 20.79	217.53 ± 15.18	250.45 ± 25.13	231.71 ± 20.24	0.212	0.753	0.715
	nd5	151.75 ± 13.47	125.16 ± 6.09	135.62 ± 8.33	123.75 ± 10.63	0.069	0.395	0.475
	coxi	740.24 ± 58.36	732.57 ± 26.90	834.22 ± 61.65	957.89 ± 57.10	0.271	0.005	0.214
	coxii	471.08 ± 49.13	453.37 ± 26.52	496.06 ± 57.85	474.56 ± 27.28	0.655	0.599	0.965
	ucp3	11.65 ± 1.78	6.43 ± 0.93*	16.89 ± 4.46	6.91 ± 0.74*	0.006	0.272	0.359
	pgc1α	0.67 ± 0.21	0.30 ± 0.09	0.35 ± 0.07	0.27 ± 0.07	0.096	0.183	0.273
	pgc1β	1.30 ± 0.18	1.01 ± 0.06	1.12 ± 0.14	1.15 ± 0.09	0.333	0.849	0.232
	hif-1α	5.45 ± 0.35	4.55 ± 0.30	6.67 ± 0.69	5.51 ± 0.31	0.028	0.020	0.773
Antioxidant defence and tissue repair	*cat*	9.19 ± 0.61	7.64 ± 0.46	9.19 ± 0.80	9.17 ± 0.49	0.203	0.215	0.218
	gpx4	1.78 ± 0.48	1.01 ± 0.39	0.78 ± 0.21	0.43 ± 0.08	0.135	0.038	0.559
	gr	0.99 ± 0.05	0.88 ± 0.07	1.08 ± 0.08	0.98 ± 0.06	0.127	0.137	0.925
	prdx3	10.44 ± 0.97	9.60 ± 0.45	9.96 ± 0.77	9.42 ± 0.94	0.402	0.683	0.857
	prdx5	28.69 ± 1.98	20.68 ± 1.10**	27.18 ± 2.14	22.48 ± 1.14*	<0.001	0.933	0.329
	Mn-sod / sod2	9.81 ± 0.68	8.31 ± 0.33	11.16 ± 1.25	8.92 ± 0.46	0.021	0.211	0.627
	grp-170	1.80 ± 0.08	1.93 ± 0.12	2.09 ± 0.12	1.72 ± 0.11*	0.270	0.705	0.025
	grp-94	4.28 ± 0.28	4.70 ± 0.35	4.76 ± 0.40	4.29 ± 0.31	0.955	0.914	0.205
	mthsp70/grp-75/mortalin	5.58 ± 0.27	5.86 ± 0.43	6.96 ± 0.65	5.83 ± 0.47	0.380	0.163	0.147

Suppl. Table 4. Effects of rearing density and dissolved oxygen concentration on gilthead sea bream relative expression of cardiac selected genes on a 21-days feeding trial. Values on relative expression are the mean ± SEM of 8 fish (2-3 fish per replicate tank). P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with oxygen concentration for a given rearing density (SNK test, P <0.05).

Category	Symbol	LD		HD		P-value		
		Normoxia	Hypoxia	Normoxia	Hypoxia	$[O_2]$	Density	Interaction
GH/IGF system	*ghr-i*	0.39 ± 0.01	0.35 ± 0.02	0.38 ± 0.02	0.30 ± 0.03	0.021	0.257	0.507
	ghr-ii	1.85 ± 0.13	1.77 ± 0.14	2.11 ± 0.08	1.73 ± 0.21	0.128	0.437	0.310
	igf-i	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.635	0.971	0.328
	igf-ii	2.22 ± 0.21	2.26 ± 0.14	2.51 ± 0.17	1.88 ± 0.24*	0.134	0.828	0.088
Energy sensing and oxidative metabolism	*sirt1*	0.22 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.18 ± 0.01*	0.023	0.219	0.572
	sirt2	0.34 ± 0.02	0.31 ± 0.02	0.33 ± 0.03	0.28 ± 0.01	0.081	0.454	0.644
	sirt3	0.10 ± 0.00	0.11 ± 0.01	0.09 ± 0.00	0.09 ± 0.00	0.279	0.036	0.196
	sirt4	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
	sirt5	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
	sirt6	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00*	0.042	0.211	0.099
	sirt7	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.05 ± 0.00*	0.003	0.608	0.395
	cpt1a	1.63 ± 0.15	1.28 ± 0.12	1.23 ± 0.12	1.21 ± 0.10	0.142	0.071	0.189
	cs	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
	nd2	68.14 ± 4.68	70.41 ± 5.69	64.19 ± 5.02	52.61 ± 2.44*	0.322	0.026	0.145
	nd5	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
	coxi	329.33 ± 18.41	310.66 ± 14.83	332.42 ± 27.46	278.19 ± 19.29	0.086	0.480	0.393
	coxii	84.97 ± 5.14	99.58 ± 8.00	83.61 ± 5.07	80.59 ± 5.62	0.355	0.110	0.163
	ucp2	1.88 ± 0.23	1.78 ± 0.18	1.97 ± 0.28	1.62 ± 0.15	0.308	0.876	0.572
	pgc1α	2.55 ± 0.15	2.28 ± 0.15	2.74 ± 0.26	2.08 ± 0.10*	0.012	0.966	0.274
	pgc1β	0.97 ± 0.04	0.85 ± 0.06	0.96 ± 0.05	0.84 ± 0.05	0.026	0.834	0.983
	hif-1α	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
Antioxidant defence and	*cat*	2.06 ± 0.16	1.59 ± 0.10*	1.73 ± 0.10	1.56 ± 0.10	0.001	0.226	0.321
	gpx4	0.09 ± 0.01	0.10 ± 0.03	0.10 ± 0.01	0.09 ± 0.01	0.913	0.916	0.477

tissue repair	*gr*	0.42 ± 0.03	0.40 ± 0.03	0.37 ± 0.01	0.33 ± 0.01*	0.136	0.012	0.574
	prdx3	2.14 ± 0.15	2.08 ± 0.17	1.97 ± 0.13	1.61 ± 0.14	0.168	0.041	0.305
	prdx5	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
	Mn-sod / sod2	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
	grp-170	0.91 ± 0.06	0.92 ± 0.04	0.90 ± 0.05	0.77 ± 0.03*	0.191	0.070	0.103
	grp-94	0.68 ± 0.03	0.73 ± 0.06	0.65 ± 0.05	0.68 ± 0.04	0.402	0.372	0.772
	mthsp70/grp-75/mortalin	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
Xenobiotic metabolism	*ahr1*	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.12 ± 0.01	0.636	0.425	0.408
	cyp1a1	2.58 ± 0.25	3.63 ± 0.32*	2.71 ± 0.23	3.79 ± 0.32*	<0.001	0.601	0.960
Nuclear receptors	*gcr*	1.01 ± 0.05	0.99 ± 0.04	1.05 ± 0.05	0.98 ± 0.09	0.493	0.786	0.708
	erα	0.17 ± 0.02	0.15 ± 0.01	0.19 ± 0.02	0.16 ± 0.01	0.095	0.433	0.809

Suppl. Table 5. Effects of rearing density and dissolved oxygen concentration on gilthead sea bream relative expression of total blood cells genes on a 21-days feeding trial. Values on relative expression are the mean ± SEM of 8 fish (2-3 fish per replicate tank). P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with oxygen concentration for a given rearing density (SNK test, P <0.05).

Category	Symbol	LD		HD		P-value		
		Normoxia	Hypoxia	Normoxia	Hypoxia	$[O_2]$	Density	Interaction
Antioxidant enzymes	*gst3*	0.97 ± 0.07	1.09 ± 0.08	1.03 ± 0.07	0.90 ± 0.11	0.957	0.422	0.141
	prdx3	0.07 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	1.000	0.344	0.231
	prdx5	0.25 ± 0.02	0.23 ± 0.01	0.21 ± 0.03	0.21 ± 0.02	0.643	0.217	0.643
	Mn-sod / sod2	1.33 ± 0.09	1.36 ± 0.07	1.32 ± 0.06	1.22 ± 0.12	0.663	0.398	0.481
Transcription factors	*nrf1*	0.95 ± 0.08	1.00 ± 0.09	1.01 ± 0.06	0.95 ± 0.08	0.942	0.917	0.548
	pgc1ß	1.71 ± 0.14	1.93 ± 0.13	1.95 ± 0.10	1.80 ± 0.11	0.786	0.672	0.139
Outer membrane translocation (*TOM complex*)	*tom70*	1.10 ± 0.08	1.28 ± 0.08	1.20 ± 0.07	1.14 ± 0.09	0.475	0.826	0.174
	tom34	1.06 ± 0.08	1.16 ± 0.08	1.15 ± 0.07	1.05 ± 0.08	0.994	0.845	0.237
	tom22	0.57 ± 0.05	0.65 ± 0.05	0.62 ± 0.05	0.59 ± 0.05	0.655	0.837	0.309
Inner membrane translocation (*TIM complex*)	*tim44*	0.95 ± 0.08	1.12 ± 0.08	1.01 ± 0.06	0.96 ± 0.09	0.489	0.540	0.165
	tim23	0.10 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.911	0.787	0.132
	tim8a	1.02 ± 0.06	1.11 ± 0.06	1.08 ± 0.06	1.01 ± 0.07	0.823	0.719	0.195
	tim10	0.98 ± 0.08	1.13 ± 0.09	1.08 ± 0.07	0.99 ± 0.10	0.753	0.841	0.181
	tim9	1.36 ± 0.12	1.18 ± 0.08	1.14 ± 0.06	1.17 ± 0.09	0.392	0.239	0.276
Mitochondrial dynamics and apoptosis	*mfn2*	0.45 ± 0.03	0.50 ± 0.04	0.50 ± 0.03	0.44 ± 0.04	0.793	0.911	0.142
	miffb	1.10 ± 0.09	1.23 ± 0.08	1.22 ± 0.07	1.14 ± 0.09	0.747	0.860	0.235
	miro1a	0.87 ± 0.08	0.86 ± 0.05	0.92 ± 0.06	0.94 ± 0.07	0.920	0.317	0.802
	miro2	1.17 ± 0.09	1.31 ± 0.08	1.38 ± 0.09	1.18 ± 0.07	0.714	0.670	0.054
	aifm1	0.07 ± 0.00	0.07 ± 0.00	0.08 ± 0.01	0.06 ± 0.00	0.307	0.614	0.120
FA oxidation & TCA	*cpt1a*	1.64 ± 0.14	1.93 ± 0.16	1.75 ± 0.08	1.67 ± 0.13	0.436	0.568	0.170
	cs	1.10 ± 0.06	1.17 ± 0.08	1.13 ± 0.05	1.05 ± 0.06	0.968	0.442	0.226
OXPHOS (*Complex I*)	*nd2*	10.19 ± 0.90	11.80 ± 0.82	11.12 ± 0.85	10.72 ± 0.77	0.476	0.928	0.239
	nd5	2.25 ± 0.18	2.68 ± 0.23	2.80 ± 0.27	2.55 ± 0.28	0.697	0.400	0.175

	ndufa1	1.03 ± 0.06	1.17 ± 0.08	1.08 ± 0.06	1.00 ± 0.08	0.676	0.401	0.120
	ndufa3	0.94 ± 0.05	0.93 ± 0.04	0.94 ± 0.06	0.85 ± 0.05	0.304	0.435	0.421
	ndufa4	1.84 ± 0.10	1.94 ± 0.06	1.97 ± 0.07	1.84 ± 0.07	0.896	0.832	0.145
	ndufa7	0.65 ± 0.05	0.69 ± 0.05	0.69 ± 0.04	0.66 ± 0.06	0.886	0.943	0.520
	ndufb5	0.88 ± 0.06	1.00 ± 0.07	0.91 ± 0.05	0.82 ± 0.07	0.825	0.257	0.114
	ndufs2	1.12 ± 0.07	1.24 ± 0.06	1.23 ± 0.07	1.13 ± 0.08	0.889	0.986	0.151
	ndufs7	0.75 ± 0.04	0.84 ± 0.05	0.83 ± 0.04	0.76 ± 0.05	0.793	0.923	0.088
	ndufaf2	0.11 ± 0.01	0.11 ± 0.01	0.11 ± 0.01	0.07 ± 0.01**	0.034	0.145	0.024
OXPHOS (*Complex IV*)	*coxi*	2.46 ± 0.22	2.95 ± 0.27	2.94 ± 0.27	2.36 ± 0.26	0.869	0.815	0.045
	coxii	1.46 ± 0.12	1.91 ± 0.11*	1.69 ± 0.14	1.46 ± 0.13	0.366	0.395	0.012
	coxiii	3.21 ± 0.23	3.46 ± 0.32	3.89 ± 0.34	3.32 ± 0.44	0.644	0.426	0.239
	cox4a	1.21 ± 0.00	1.21 ± 0.00	1.21 ± 0.00	1.21 ± 0.00	-	-	-
	cox5a2	0.46 ± 0.05	0.45 ± 0.02	0.43 ± 0.02	0.46 ± 0.02	0.763	0.698	0.547
	cox6a2	0.25 ± 0.02	0.29 ± 0.02	0.28 ± 0.02	0.23 ± 0.01*	0.644	0.472	0.047
	cox6c1	0.20 ± 0.02	0.23 ± 0.03	0.22 ± 0.02	0.21 ± 0.03	0.933	0.970	0.461
	cox7b	0.73 ± 0.05	0.71 ± 0.04	0.83 ± 0.06	0.73 ± 0.06	0.272	0.261	0.480
	cox8b	1.14 ± 0.05	1.26 ± 0.07	1.19 ± 0.07	1.21 ± 0.10	0.370	0.980	0.491
	sco1	1.20 ± 0.10	1.36 ± 0.12	1.34 ± 0.12	1.22 ± 0.10	0.837	1.000	0.220
	surf1	0.76 ± 0.06	0.91 ± 0.08	0.90 ± 0.08	0.85 ± 0.08	0.513	0.578	0.207
	cox15	0.04 ± 0.00	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.00	0.672	0.266	0.024
Respiration uncoupling	*ucp2*	0.92 ± 0.10	0.99 ± 0.06	0.98 ± 0.06	0.88 ± 0.09	0.841	0.731	0.285