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RESEARCH ARTICLE

Early exposure to chronic hypoxia induces short- and long-term regulation of hemoglobin gene expression in European sea bass (*Dicentrarchus labrax*)

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ABSTRACT

European sea bass (*Dicentrarchus labrax*) inhabits coastal waters and may be exposed to hypoxia at different life stages, requiring physiological and behavioral adaptation. In the present study, we attempted to determine whether regulation of hemoglobin (Hb) gene expression plays a role in the physiological response to chronic moderate hypoxia in whole larvae and hematopoietic tissues (head kidney and spleen) of juveniles. We also tested the hypothesis that hypoxia exposure at the larval stage could induce a long-term effect on the regulation of Hb gene expression. For this purpose, *D. labrax* were exposed to a non-lethal hypoxic condition (40% air saturation) at the larval stage from 28 to 50 days post-hatching (dph) and/or at the juvenile stage from 196 to 296 dph. Data obtained from larvae indicate that hypoxia induced a subtype-specific regulation of Hb gene expression, with a significant decrease of MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* and increase of MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* transcript levels. Hypoxia did not induce regulation of Hb gene expression in juveniles, except in the head kidney for those that experienced hypoxia at the larval stage. The latter exhibited a significant hypoxia-induced stimulation of MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* gene expression, associated with stimulation of the *PHD-3* gene involved in the hypoxia-inducible factor oxygen-sensing pathway. We conclude that subtype- and stage-specific regulation of Hb gene expression plays a role in the physiological response of *D. labrax* to cope with hypoxia and that early exposure to low oxygen concentration has a long-term effect on this response.

KEY WORDS: Hypoxia, Hemoglobin, Early exposure, European sea bass

INTRODUCTION

In the context of global change, low dissolved oxygen (O_2) concentration (hypoxia) has become a major problem for marine fish species (Breitburg et al., 2009; Roessig et al., 2004; Somero, 2012). Under the combined effects of eutrophication and global warming, hypoxic conditions are especially prevalent in estuarine and coastal regions, towards which fish larvae may drift as a result of tidal currents (Pihl et al., 1992). Oxygen depletion is likely to affect survival and the success of larval development, which is considered to be the most sensitive stage in the fish life cycle (Ishibashi et al., 2007; Levin et al., 2009). Hypoxia is characterized by the degree of O_2 depletion and the exposure period, with different impacts on species and individuals depending on their coping capacities (Ekau et al., 2010). Fish acclimatization to hypoxia consists of behavioral, physiological, biochemical and molecular adjustments (Ekau et al., 2010; Richards, 2011; Zhu et al., 2013). Physiological responses to low-oxygen conditions include modifications of the respiratory cascade to enhance O_2 extraction and transport (Nilsson, 2007), as well as metabolic regulation in order to limit O_2 demands (Hopkins and Powell, 2001). In fish, as in other vertebrates, hemoglobins (Hb) are involved in O_2 transport from respiratory organs to tissues, and contribute to overcoming the effects of reduced oxygen availability (Gollock et al., 2006; Richards, 2011). Hb molecules consist of two alpha- and two beta-globin subunits and each subunit contains a heme group responsible for the binding of O_2. Teleost fish species have the characteristic of expressing several Hbα and Hbβ genes, which are grouped into two unlinked clusters termed 'MN' (gene cluster flanked by the *mpg* and *nprl3* genes) and 'LA' (cluster flanked by the *lcmt1* and *aqp8* genes) located on two separate chromosomes resulting from the teleost-specific genome duplication (Hardison, 2008; Opazo et al., 2013). The gene products give rise to multiple Hb isoforms adapted to different metabolic demands and/or environmental O_2 availability throughout the different ontogenic stages (Weber and Jensen, 1988; Opazo et al., 2013). No common pattern emerged from studies that investigated the effect of hypoxia on Hb proteins and transcripts levels (Lai et al., 2006; Roesner et al., 2006, 2008; Val et al., 2015; Wawrowski et al., 2011; Zi-sheng et al., 2011). The hypoxia response of Hb seems to depend on the fish species, the degree of stress and the ontogenetic stage.

The physiological response of fish to hypoxia can also depend on their previous environmental experience (Ho and Burggren, 2012; Robertson et al., 2014). This historical dimension of hypoxia tolerance can be related to developmental plasticity, i.e. the ability of organisms to respond to changes in the environment by developing phenotypes that allow them to better cope with these conditions (Bateson et al., 2014). Molecular data obtained in zebrafish (*Danio rerio*) suggest that the developmental plasticity induced by hypoxia may result from a long-lasting regulation of the hypoxia-inducible factor-1 (HIF-1) pathway (Robertson et al., 2014). In vertebrates including fish, the transcription factor HIF-1 is a molecular oxygen sensor that regulates the expression of target genes in order to ensure survival in hypoxic conditions (Gracey et al., 2001; Nikinmaa and Rees, 2005). Wawrowski et al. (2011) suggested that HIF-1 regulates the expression of Hb genes in Japanese medaka (*Oryzias latipes*), by binding to hypoxia-responsive elements (HRE). To date, however, involvement of Hb

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List of abbreviations

CJ	control juvenile group
CL	control larval group
dph	days post-hatching
ef1α	elongation factor-1, alpha isoform
Hb	hemoglobin
Hct	hematocrit
HIF	hypoxia-inducible factor
HJ	hypoxia juvenile group
HL	hypoxia larval group
PHD-3	prolyl hydroxylase domain-containing protein 3

genes in the long-term regulation of the HIF pathway by hypoxia has not been studied.

European sea bass (*Dicentrarchus labrax*) is a species of high value that inhabits coastal nurseries and is therefore liable to be exposed to hypoxic events (Dufour et al., 2009; Jennings and Pawson, 1992). While effects of moderate hypoxia on metabolic and physiological parameters have been investigated in this species at larval and adult stages (Dupont-Prinet et al., 2010; Vanderplancke et al., 2015), the regulation of Hb under low oxygen availability remains unknown. The recent characterization of 14 Hb genes in *D. labrax* constitutes a valuable source of information for the investigation of Hb system regulation at different life stages (Cadiz et al., 2017). Hb gene expression in *D. labrax* showed stage-specific patterns, with some genes expressed during the early life stage (MN-*Hbα3–5* and MN-*Hbβ4–6*), while others (LA-*Hbα1*,*2*, LA-*Hbβ1*, MN-*Hbα1*,*2* and MN-*Hbβ1*–3) are principally expressed at juvenile or adult stages. Cadiz et al. (2017) also revealed that most Hb genes were mainly expressed in hematopoietic tissues (head kidney and spleen) at the adult stage in *D. labrax*. In this context, the first objective of the present study was to assess the regulation by moderate hypoxia of Hb gene expression in whole larvae and hematopoietic tissues (head kidney and spleen) of juvenile *D. labrax*. The second objective was to determine whether exposure of larval fish to moderate hypoxic conditions could have a long-term impact on hypoxia-induced regulation of Hb gene expression. Special attention was also paid to the expression of prolyl hydroxylase domain-containing protein 3 (*PHD-3* or *Egln3*), which plays a role in the HIF-1 signaling pathway.

MATERIALS AND METHODS

Animal rearing and treatments

Dicentrarchus labrax (Linnaeus 1758) larvae were reared under normal oxygen conditions in six tanks at 15±0.4°C water temperature and 35±0.2‰ salinity. They were fed daily with *Artemia* according to Zambonino et al. (1996) until the end of larval development. From 28 to 50 days post-hatching (dph), water oxygenation in three of the tanks (hypoxia larval group, HL) was reduced to approximately 40% air saturation (2.95 mg O_2 l^{-1}), while in the other three tanks (control larval group, CL) it was maintained at 100% saturation (7.35 mg O_2 l^{-1}). Hypoxia was generated by bubbling N_2 in a gas equilibration column placed upstream of the experimental tank. Dissolved O_2 was monitored daily using an Odeon oxygen meter (Odeon Classic OPTOD, Caudan, France). Other water quality parameters (salinity, temperature and pH) were also checked daily in each tank during the experiment. It had previously been determined that the level of hypoxia did not induce mortality; thus, genetic selection during larval exposure was avoided. It was not possible to evaluate feed ingestion during this experimental phase. At the end of hypoxia exposure (50 dph), larvae were returned to normal oxygen conditions and replicate tanks were pooled in one 1 m^3 tank per treatment (normoxia; 15±0.4°C). Fish were then fed with a commercial diet (NeoSupra, Coopérative Le Gouessant, Lamballe, Côtes-d'Armor, France). At 166 dph, 60 fish from each of the hypoxia (HL) and control (CL) tanks were selected and tagged subcutaneously (passive integrated transponder, PIT-tag) for individual identification. Fish were fed with NeoGrower commercial diet (Coopérative Le Gouessant) and pooled in the same 2 m^3 tank. From 196 to 296 dph, fish were separated into two tanks, with 30 juveniles from each larval group in each tank. Water oxygenation in one of the tanks was reduced to 40% air saturation (hypoxia juvenile group, HJ), while it was maintained at 100% saturation in the other (control juvenile group, CJ).

Growth monitoring

Larval growth was evaluated for 150 larvae per group (i.e. HL and CL, 50 larvae from each tank) from the beginning (30 dph) to the end of hypoxia exposure (50 dph). Larvae were killed with an excess of anesthetic (200 mg l^{-1} tricaine methanesulfonate, TMS; Pharmaq, Fordingbridge, Hampshire, UK) and transferred to formaldehyde for fixation (4%) until individual mass measurement. Each juvenile from the HJ and CJ groups was weighed at 196 and 296 dph after light anesthesia (10 mg l^{-1} TMS). Growth was estimated by calculating the relative growth rate (RGR):

$$\mathrm{RGR} = \frac{(\text{final mass} - \text{initial mass})}{\text{initial mass}}.$$

Larval and juvenile sampling

Larvae for RNA extraction were sampled at 50 dph, i.e. just before the hypoxic groups were returned to normal oxygenation conditions. Six pools of eight larvae were taken from each treatment (two pools from each tank) and killed with an excess of anesthetic (200 mg l^{-1} TMS). They were transferred into Eppendorf reaction tubes containing RNAlater (Qiagen, Hilden, Germany) and placed at 4°C for 24 h, then kept at −20°C until total RNA extraction.

Sampling of juveniles was performed at 296 dph from fish left undisturbed and unfed for 24 h. Sixteen juveniles from each of the HJ and CJ groups (i.e. eight fish from each of the HL and CL larval groups) were randomly selected and lightly anesthetized (20 mg l^{-1} TMS). A 1 ml sample of blood was drawn from their caudal vein using a heparinized syringe and divided between two tubes for analysis of hematocrit (Hct) and Hb concentration. The fish were then killed with an excess of anesthetic (500 mg l^{-1} TMS) and tissues (spleen and head kidney) were immediately dissected. Blood was removed rapidly to reduce red blood cell contamination from tissues by rinsing with saline solution (0.01% NaCl). Spleen and head kidney were rapidly placed in RNAlater (Qiagen) for RNA extraction.

The present work was performed within IFREMER facilities in accordance with French and European policies and guidelines of the French Animal Care Committee (agreement number: APAFIS#5173).

Hematological parameters

Hct was determined from blood samples after centrifugation at 13,000 ***g*** in capillary tubes. Hb concentration was measured

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using the cyanomethemoglobin method. Blood samples were added to Drabkin's reagent (Sigma-Aldrich Co., St Louis, MO, USA) and compared with Hb standards (Pointe Scientific, Inc., Canton, MI, USA). Optical density was recorded at 540 nm in a spectrophotometer.

RNA extraction and cDNA synthesis

Total RNA for analysis of gene expression was extracted from the samples (whole larvae and tissues from juveniles) using Extract-all reagent (Eurobio, Courtaboeuf, Essonne, France) combined with the Zymo Direct-zol™ RNA MiniPrep Kit, following recommendations from the supplier. Genomic DNA was removed using the DNA-free Kit (MoBio Laboratories Inc., Carlsbad, CA, USA). The quantity, purity and quality of RNA were assessed using a ND-1000 NanoDrop® spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA) and by electrophoresis using an Agilent Bioanalyser 2100 (Agilent Technologies Inc., Santa Clara, CA, USA). All samples showed an RNA integrity number (RIN) higher than 7 and thus could be used for reverse transcription-quantitative PCR (RT-qPCR) analysis. RNA samples were stored at −80°C until use.

Synthesis of cDNA was carried out using 500 ng of DNase-treated total RNA with an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). The total reaction was carried out in a final volume of 20 µl containing 5 µl (500 ng) of sample, 4 µl 5× iScript™ Reaction Mix containing oligo(dT), random primers and RNaseA inhibitor, 1 µl iScript™ Reverse transcriptase and 10 µl RNase/DNase-free water. The cDNA synthesis reaction was incubated for 5 min at 25°C followed by 30 min at 42°C and terminated by incubation for 5 min at 85°C to inactivate the enzyme. Reverse transcription (RT) was performed using a Thermo-cycler TC-152 (Techne Barloworld Scientific, Stone, Staffordshire, UK). cDNA was stored at −20°C until use. Controls without reverse transcriptase were performed on each sample (same reaction mix except the reverse transcriptase).

RT-qPCR analysis

The analysis of gene expression in the whole larvae and in the tissues of European sea bass was carried out by RT-qPCR using the primers listed in Table 1. All *D. labrax* Hb genes and the *PHD-3* gene were investigated at the larval stage. At the juvenile stage, we focused on genes whose expression was influenced by hypoxia at the larval stage (i.e. *PHD-3*, LA-*Hbα1*, LA-*Hbβ1*, MN-*Hbα2*, MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5*) and genes that were highly expressed in the head kidney and spleen at the juvenile stage, i.e. MN-*Hbα1* and MN-*Hbβ1* (Cadiz et al., 2017).

Gene expression was quantified using the iCycler MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). Standard curves were estimated for each primer pair using serial dilutions (from 1/10 to 1/270) of a pool of cDNA. Efficiencies of qPCR for each pair of primers ranged from 95% to 100% with R^2>0.99. Each sample was run in triplicate in a final well volume of 15 µl containing 5 µl cDNA (1/30 dilution) and 10 µl of reaction mix, composed of 0.5 µl of each primer (10 mmol l^{-1}), 1.5 µl RNase/DNase-free water and 7.5 µl iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.) containing antibody-mediated hot-start iTaq DNA polymerase, dNTPs, $MgCl_2$, SYBR® Green I dye, enhancers, stabilizers and fluorescein. Negative controls (non-template control) were systematically included in each plate. Reverse transcriptase-negative controls were also used in order to ensure the absence of residual DNA contamination. The qPCR profiles contained an initial activation step at 95°C for 2 min, followed by 39 cycles of 5 s at 95°C and 20 s at the annealing temperature (60°C for *PHD-3*, LA-*Hbα1*, LA-*Hbα2*, LA-*Hbβ1*, MN-*Hbα1*, MN-*Hbα2*, MN-*Hbβ1*, MN-*Hbβ2*, MN-*Hbβ3*, MN-*Hbβ4* and reference genes; 62°C for MN-*Hbβ5* and MN-*Hbβ6*; and 70°C for MN-*Hbα3*, MN-*Hbα4* and MN-*Hbα5*). After the amplification phase, a melting curve was performed to confirm the amplification of a single product in each reaction.

For each sample, the corresponding Cq (quantification cycle) value was determined automatically using the Gene Expression Module of the CFX Manager software (Bio-Rad Laboratories Inc.). Cq is the number of cycles required to yield a detectable fluorescence signal. The relative quantity of messenger was normalized with the ΔΔCt method using the same CFX Manager software. Reference genes were used to correct for loading differences or other sampling variations present in each sample. The *ef1α* (elongation factor 1-alpha) gene was chosen as the reference gene for whole larvae, while the *28S* gene was used as the reference gene in spleen and head kidney tissues from juvenile fish. These reference genes were used as they did not show any significant variation of expression between samples (relative standard deviation <5% among samples).

Statistical analyses

Statistical analyses were performed using STATISTICA software version 10 (http://statsoft.fr/). All data were log-transformed to fit a normal distribution. For all analyses, variables were checked for normality (Shapiro test) and equality of variances (Levene test). Significant differences between O_2 treatments at the larval stage in growth and gene expression were analyzed by one-way ANOVA. Two-way ANOVA were used to determine the effects of juvenile exposure and O_2 larval treatment on growth, hematological parameters and gene expression. Tukey's test (P<0.05) was used for *post hoc* comparisons. All figures were drawn using GraphPad Prism® (v.5.0b) software.

RESULTS

Growth at larval and juvenile stages

Larval growth was monitored from the beginning (23 dph) to the end (50 dph) of hypoxia exposure. Relative growth rates were significantly lower in larvae exposed to the hypoxic treatment (one-way ANOVA: P=0.002; Table 2). Similarly, juveniles exposed to hypoxia between 196 and 259 dph displayed a significantly lower relative growth rate compared with the normoxic treatment (two-way ANOVA: $P=10^{-6}$). No significant interaction with the conditions encountered during the larval rearing phase was observed (Table 2).

Larval gene expression patterns under moderate hypoxia

The relative levels of 14 Hb transcripts (seven *Hbα* genes and seven *Hbβ* genes) were compared between *D. labrax* larvae exposed or not to moderate hypoxia treatment (40% air saturation) (Fig. 1; Table S1). The transcript levels of LA-*Hbα1*, LA-*Hbβ1* and MN-*Hbα2* genes were significantly higher (7-fold, 6-fold and 14-fold, respectively) in larvae exposed to hypoxia ($P=2\times10^{-5}$, 1×10^{-5} and 1×10^{-6}, respectively). Conversely, MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* transcript levels were reduced (3-fold, 5-fold and 4-fold, respectively) in larvae under hypoxia ($P=4\times10^{-4}$, 1×10^{-4} and 1×10^{-4}, respectively). Other Hb genes (LA-*Hbα2*, MN-*Hbα4*, MN-*Hbα5* and MN-*Hbβ6*) did not exhibit any differential expression or were not sufficiently expressed (Cq>33 for MN-*Hbα1*, MN-*Hbβ1*, MN-*Hbβ2* and MN-*Hbβ3*) to be analyzed. The expression level of the *PHD-3* gene, involved in the HIF pathway, was significantly

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Table 1. Specific primers used for real-time amplification of *Hb*, *PHD-3* and reference (*28S* and *ef1α*) genes

Gene	Accession no.	Forward primer sequences (5′–3′)	Reverse primer sequences (5′–3′)
LA-*Hbα1*	KX196178*	CAGTGGGACAGGATCTTGAAGT	GGTGATGGGTGGAATCAATC
LA-*Hbα2*	KX196180*	TTTCCCATGAGAGAGCAGGT	TCAGATGCGCTTCTTAGGATGT
MN-*Hbα1*	KX196190*	GGCCAGGATGCTGACTGTA	CCAGCAAGGTCATCCATCTT
MN-*Hbα2*	KX196188*	CCTGCCAACTTCAAGATTCTG	TTTCTCAGACAAGGCACGAG
MN-*Hbα3*	KX196184*	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTC
MN-*Hbα4*	KX196183*	ACAGACAAGATGACCAGTCTCACT	GCCAATGTCCTCTGCCTTT
MN-*Hbα5*	KX196181*	ACAGACAAGATGACCAGTCTCACA	GCCAATGTCCTCTGCCTTC
LA-*Hbβ1*	KX196179*	CCCGACAACTTCAAACTGCT	CCTGCGTCTCTGGTGTGAAG
MN-*Hbβ1*	KX196191*	TGATTTGAGCAAAGATCCTGAA	CATGGACGACATCAAGAACG
MN-*Hbβ2*	KX196189*	GTCAGCCAGCAGCCTGAAAT	GCAGCTCTTTCCAGGTGTCT
MN-*Hbβ3*	KX196187*	CAGAAGCTTTGGCAAGAGTG	GCTGCTACTTTGGCGTTACC
MN-*Hbβ4*	KX196186*	GTCGTTTACCCCTGGACTCA	GTTTTGCGACCATCGGATTT
MN-*Hbβ5*	KX196185*	ACCATCCAGGACATCTTCTCT	GTTTTGCGACCAACGGATTC
MN-*Hbβ6*	KX196182*	ACCATCCAGGACATTTTCTCC	GTTTTGCGACCAACGGATTC
PHD-3	DLPD06823‡	TCCTACTCCACCAGGTACGC	GCAGTCATGTTTGCTCTCCA
28S	AH011863.2*	CAAGAACATCCAGCTGCTGAC	GGTGATATGTCGGCCATAAA
ef1α	AJ866727.1*	CTGGAGGGCAGTGAAAAGAT	CATCAAGAGCCTCCAGCAGT

*NCBI accession number; ‡University of Padova accession number.

higher (3-fold induction) under the hypoxic than under the control condition (P=2×10$^{−5}$).

Hematological parameters in juvenile fish under moderate hypoxia

At the juvenile stage, Hct was increased under mild hypoxia, while Hb protein levels in the blood remained unchanged (Table 3). Oxygen conditions experienced at the larval stage did not influence these blood parameters (two-way ANOVA; P=0.32).

Gene expression patterns in the head kidney and spleen of juveniles

We found that moderate hypoxia at the juvenile stage only induced significant up-regulation of LA-*Hbα1*, LA-*Hbβ1* and MN-*Hbα2* genes in the head kidney of juveniles that have been previously exposed to larval hypoxia (Fig. 2; Table S2). Such effects were not observed in the spleen tissue (Fig. 3; Table S3). The mRNA levels of MN-*Hbα1* and MN-*Hbβ1* remained unchanged under moderate hypoxia in the head kidney and spleen tissues. The transcript levels of MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* genes were too low to identify any potential differential expression in the head kidney or spleen tissues. Hypoxia also induced a stimulation of *PHD-3* expression, limited to the head kidney of juveniles that had experienced a low oxygen concentration at the larval stage (Fig. 2; Table S2).

DISCUSSION

As an inhabitant of shallow marine areas, *D. labrax* is expected to be exposed to environmental fluctuations including hypoxia events. In order to cope with oscillations in water P_{O_2}, marine fish species have developed adaptive mechanisms that often depend, to a varying extent, on changes in gene expression (Gracey et al., 2001; van der Meer et al., 2005). Our results show that the regulation of Hb genes in response to moderate chronic hypoxia is specific to the developmental stage considered, with the involvement of particular genes, and also depends on an individual's life history (previous exposure to a hypoxic environment). We recognize that transcriptional changes in Hb gene expression observed in the present study may not reflect the concentration of the related subunit in Hb tetramers. It is in fact acknowledged that Hb tetramer assembly is tightly coordinated, as any alpha subunits formed in excess are broken down if not incorporated to alpha–beta dimers (Kong et al., 2004). Therefore, while the present gene expression analysis gives precious insights related to mechanisms involved in the hypoxia response, it is difficult to make definitive statements about their functional significance.

At the larval stage, the impact of hypoxia on growth reflected a sensitivity of the organism to the oxygen conditions. The highest regulation of Hb gene expression in response to hypoxia was found at this stage. Transcriptional regulation of Hb expression therefore appears to be a crucial element of the response to mild hypoxia during larval development in *D. labrax*. During hypoxia, the levels of regulation reached 14-fold, 7-fold and 6-fold induction for whole-body MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* transcripts, respectively, while MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* were down-regulated 3.5-fold, 4.5-fold and 4-fold, respectively. However, the expression abundance of these genes also needs to be taken into account. As MN-*Hbα3*, MN-*Hbβ4* and MN-*Hbβ5* transcripts are more abundant than MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* at the larval stage (Cadiz et al., 2017), the net balance of the Hb regulation observed in the present study tends towards a decrease in global Hb gene expression in sea

Table 2. Relative growth rate of larvae and juvenile *Dicentrarchus labrax* under normoxia and moderate hypoxia

Larval phase			Juvenile phase						
			CJ		HJ		*P*-value		
CL	HL	*P*-value	CL	HL	CL	HL	Larval	Juvenile	Larval×juvenile
9.4	5.9	**0.002**	1.28	1.16	0.96	0.98	0.26	**1×10$^{−6}$**	0.07

Larval growth was monitored in hypoxia (HL) and control (CL) groups. The *P*-value was calculated from a one-way ANOVA (n=150 larvae for each group). Growth of juveniles exposed (HJ, n=60) or not (CJ, n=60) to hypoxia was evaluated for fish previously exposed (HL, n=30 in HJ and CJ groups) or not (CL, n=30 in HJ and CJ groups) to larval hypoxia. The *P*-value for larval/juvenile oxygenation was calculated from a two-way ANOVA.

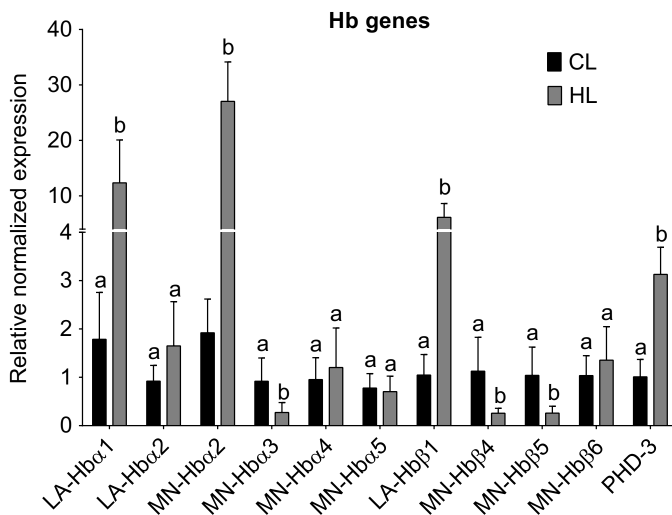

Fig. 1. Expression pattern of hemoglobin (Hb) genes (LA-*Hbα1,2*, LA-*Hbβ1*, MN-*Hbα2–5* and MN-*Hbβ4–6*) and *PHD-3* under normoxia (CL) and moderate hypoxia (HL, 40% air saturation) in the whole larvae (50 dph). The *y*-axis shows expression levels normalized to those of *ef1α*, and presented relative to the normoxia group (×1). Significant differences for each gene between groups are identified with different letters (one-way ANOVA followed by Tukey's test).

bass larvae exposed to hypoxia. Further analysis will be necessary in order to determine whether such transcriptional regulation is associated with a decrease in Hb concentration. If so, our data would be consistent with the decrease in Hb concentration reported in *O. mykiss* larvae exposed to moderate hypoxia by Bianchini and Wright (2013). The consequences of the stimulation of MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* gene expression on the composition of the Hb tetramers and on the O_2 affinity of Hb are unknown in *D. labrax* larvae. Thus, we can only suggest that *D. labrax* larvae may react physiologically to the functional characteristics of MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* gene products, which might be a determining factor for survival when faced with the overall decrease in oxygen concentration. As previously suggested in other teleost species, the regulation of Hb gene expression in the hypoxia condition may result from stimulation by the transcription factor HIF-1 (Wawrowski et al., 2011). The absence of functional anti-HIF-1 antibody in *D. labrax* prevented us from measuring HIF content in larvae and thus we cannot really conclude about the role of HIF-1 in the Hb gene regulation studied here. However, mRNA levels of the oxygen sensor gene *PHD-3*, whose expression is known to be stimulated in hypoxia via a HIF-mediated pathway (Aprelikova et al., 2004), were higher in *D. labrax* larvae exposed to hypoxia. These data suggest an activation of the HIF-mediated pathway associated with the regulation of Hb genes in *D. labrax* larvae exposed to chronic hypoxia.

Juvenile sea bass under chronic moderate hypoxia did not exhibit any regulation of Hb gene expression in spleen and head kidney tissues when these juvenile fish had not experienced any hypoxia during their larval stage. Provided that there was no variation of Hb transcripts in blood or non-erythropoietic tissues, this result suggests that the regulation of Hb gene expression was not needed to cope with the moderate hypoxia condition during the juvenile period. In line with these data, Hb protein levels remained unchanged in juveniles under hypoxia despite a significant increase in erythrocyte volume (Hct) in the blood. The erythrocyte volume could increase in hypoxic conditions by adrenergic activation of sodium–proton exchange without an associated increase in Hb protein levels (Nikinmaa, 2001). Although further investigations will be required to evaluate the impact of hypoxia on blood O_2 transport (e.g. blood oxygen affinity), our results are in agreement with previous data obtained in *D. labrax* juveniles, showing that the O_2 carrying capacity remained unchanged under chronic hypoxia similar to the levels used here (Pichavant et al., 2003). Our data indicated that the Hb gene response of juveniles differed from that of larvae when exposed to similar environmental O_2 conditions. The lack of a requirement to regulate Hb gene expression in juveniles may be interpreted as a lower sensitivity to moderate hypoxia compared with larvae. In fish, as in other vertebrates, early life stages are generally considered to be the most sensitive to environmental constraints, including hypoxia (Ishibashi et al., 2007; Levin et al., 2009). Indeed, the correct progress of developmental processes associated with morphogenesis, organogenesis and maturation of physiological functions is crucial for larval survival (and cannot really be postponed), requires intense aerobic metabolism and would probably explain the imperative need for Hb regulation during the larval period. The physiological trade-off adopted by juvenile fish is different, as they can more easily restrain their energy expenditure by limiting food ingestion and growth (as observed in the present study and also reported by Pichavant et al., 2001), and consequently do not really need any significant down-regulation of Hb synthesis when exposed to moderate hypoxia. Furthermore, the apparent lower Hb sensitivity at the juvenile stage may also be explained by a more efficient oxygen extraction/transport system. A comparison of parameters related to food intake, metabolic rate (standard metabolic rate) and oxygen consumption (critical oxygen tension, P_{crit}) at different larval and juvenile stages would represent useful information for a better understanding of the sensitivity to hypoxia during the *D. labrax* life cycle.

The major result of the present study is the fact that Hb regulation in the kidney of *D. labrax* juveniles in response to hypoxia was only observed in fish that were exposed to a low oxygen concentration during the larval stage. These juveniles exhibited a significant stimulation of MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* genes under hypoxia that was not associated with a higher Hb concentration. This can be explained by the fact that the major subtypes of Hb tetramers at the juvenile stage are MN-*Hbα1* and MN-*Hbβ1* (Cadiz

Table 3. Blood hematocrit (Hct) and hemoglobin (Hb) protein levels under normoxia (CJ) and moderate hypoxia (HJ) in *D. labrax* juveniles previously exposed (HL) or not (CL) to hypoxia

	CJ		HJ		*P*-value		
	CL	HL	CL	HL	Larval	Juvenile	Larval×juvenile
Hct (%)	27.28	26.28	35.14	37.14	0.73	**2×10^{-6}**	0.32
Hb (g dl^{-1})	8.5	8.6	8.4	8.3	0.97	0.59	0.88

The *P*-value for larval oxygenation/juvenile oxygenation was calculated from a two-way ANOVA (*n*=8 juveniles for each group).

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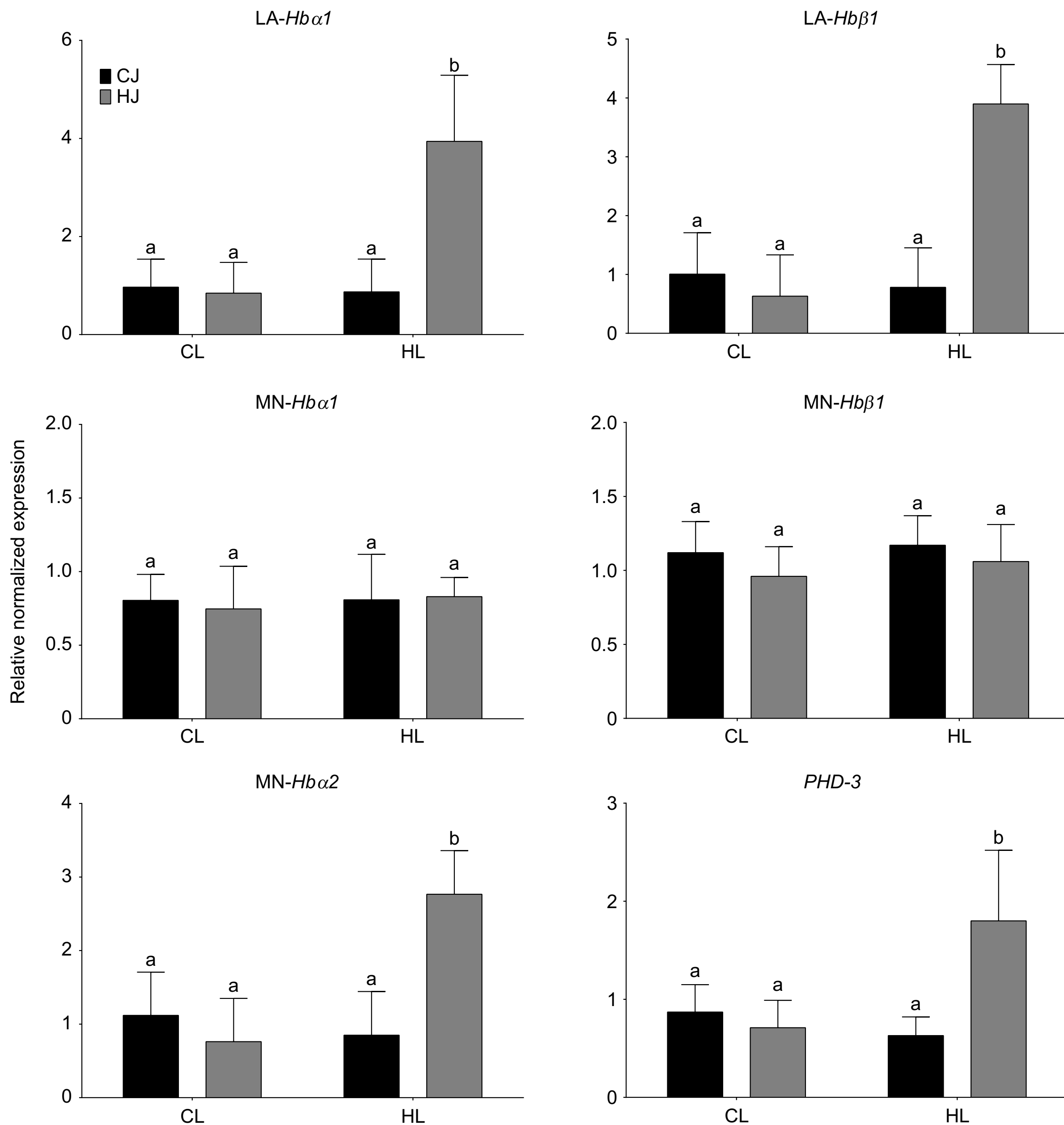

Fig. 2. Expression pattern of Hb genes (LA-*Hbα1*, LA-*Hbβ1*, MN-*Hbα1*, MN-*Hbα2* and MN-*Hbβ1*) and *PHD-3* under normoxia (CJ) and moderate hypoxia (HJ, 40% air saturation) in the head kidney of juveniles previously exposed (HL) or not (CL) to larval hypoxia. The *y*-axis shows expression levels normalized to those of *28S*, and presented relative to the juvenile control group in normoxia (×1). Significant differences for each gene between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

et al., 2017), which were not regulated under moderate hypoxia in head kidney. An alternative explanation could be that such stimulation was only found in the kidney and not in the other major hematopoietic tissue investigated (i.e. the spleen). As mentioned for the larval stage, we cannot really conclude from the present data about the involvement of HIF-1 in the regulation of Hb genes at the juvenile stage. However, it is interesting to note that the stimulation of Hb genes in the hypoxic condition was again associated with the up-regulation of *PHD-3* expression, which could suggest an activation of the HIF-1-mediated pathway.

The significant hypoxia-induced stimulation of *PHD-3* expression may reflect higher hypoxia sensitivity in juveniles that experienced hypoxia at the larval stage. This hypothesis, in contrast to that suggesting beneficial effects of early-life exposure to hypoxia (Robertson et al., 2014), would be in line with previous data obtained in humans demonstrating an increased susceptibility to hypoxia in specific adult muscle related to previous intermittent hypoxia exposure during postnatal development (McDonald et al., 2016). Growth data measured under chronic hypoxia at the juvenile stage did not allow us to conclude about any potential beneficial or negative effects of early-life exposure to hypoxia. As mentioned above for the larval stage, additional information related to the functional characteristics of the different Hb genes will be necessary in order to decipher the physiological and functional consequences of their long-term regulation in the kidney. Considering that MN-*Hbα2*, LA-*Hbα1* and LA-*Hbβ1* could have specific functional

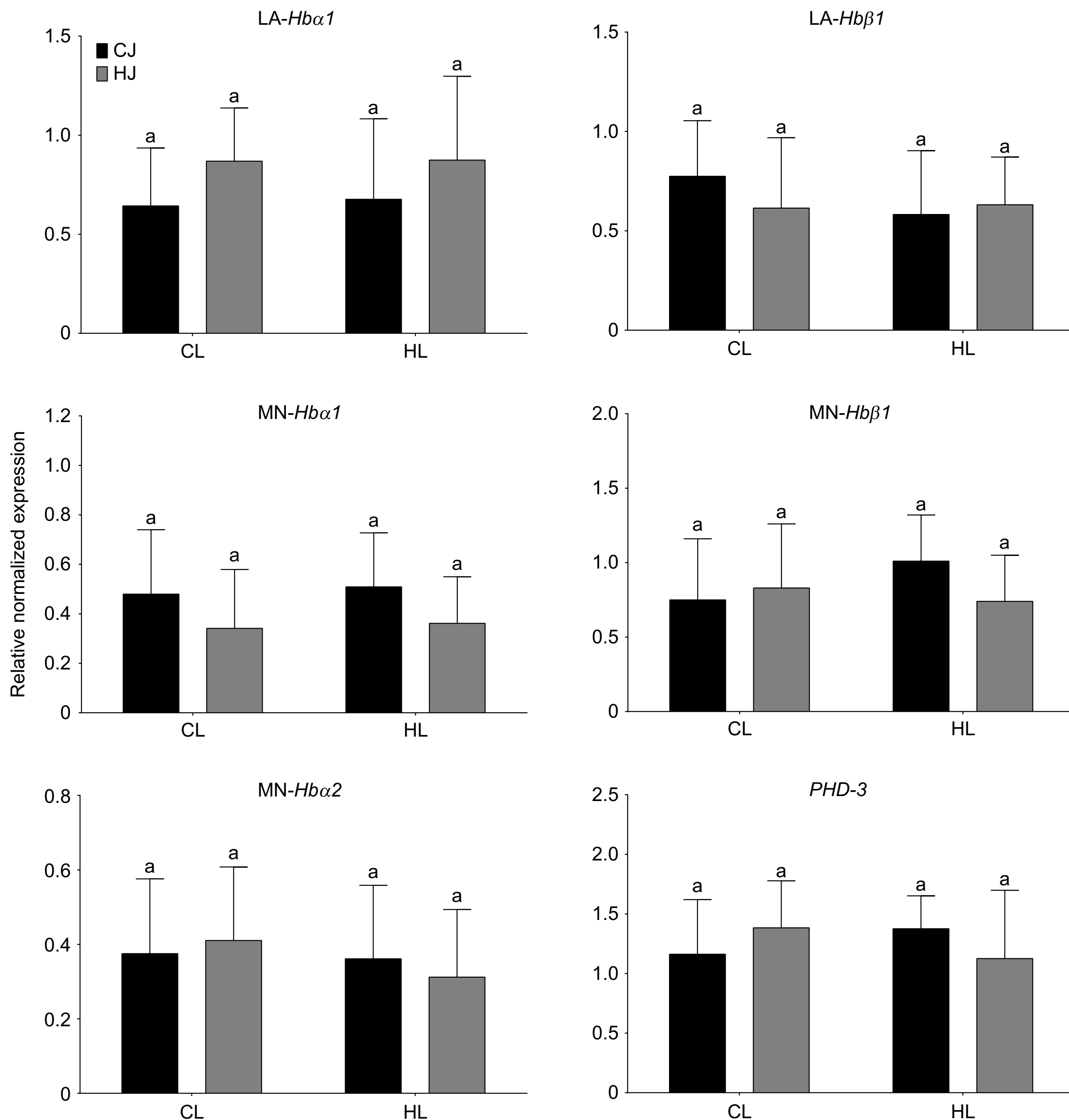

Fig. 3. Expression patterns of Hb genes (LA-*Hbα1*, LA-*Hbβ1*, MN-*Hbα1*, MN-*Hbα2* and MN-*Hbβ1*) and *PHD-3* under normoxia (CJ) and moderate hypoxia (HJ, 40% air saturation) in the spleen of juveniles previously exposed (HL) or not (CL) to larval hypoxia. The *y*-axis shows expression levels normalized to those of *28S*, and presented relative to the juvenile control group in normoxia (×1). Significant differences for each gene between groups are identified with different letters (two-way ANOVA followed by Tukey's test).

characteristics (e.g. binding capacities, Root and Bohr effects), we cannot rule out the possibility that their regulation may be associated with enhanced O_2 transport capacity, allowing fish to cope better with mild hypoxia, as previously suggested by Wawrowski et al. (2011). Moreover, regulation of LA-*Hbα1* and LA-*Hbβ1* expression may impact cellular/physiological functions other than those strictly related to O_2 transport, as these genes were found to be most abundantly expressed in non-hematopoietic tissues (Cadiz et al., 2017).

Conclusions

The present work confirms that the regulation of Hb gene expression is involved in the molecular response to hypoxia in *D. labrax*. Regulation of Hb expression in response to hypoxia appears to be dependent on developmental stage, and involves different specific Hb genes. The characteristics of this response to hypoxia reinforce the idea of distinct functional properties in Hb subtypes, as already suggested for this species (Cadiz et al., 2017). Further investigations are needed, however, to better understand the physiological consequences of this regulation at different life stages. In particular, it remains to be clarified whether the long-term regulation of Hb gene expression induced by early-life exposure to hypoxia actually results in beneficial or negative effects in response to moderate O_2 deprivation during the juvenile period. Finally, additional investigations will be necessary to decipher the molecular processes (e.g. epigenetic) underlying the developmental plasticity of hemoglobin gene regulation.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: D.M.; Formal analysis: L.C.; Investigation: L.C., P.Q., L.M.; Writing - original draft: L.C., A.S., J.-L.Z.-I., D.M.; Writing - review & editing: L.C., D.M.; Supervision: J.-L.Z.-I., D.M.; Funding acquisition: J.-L.Z.-I.

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Supplementary information
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