Introduction

Teleost species inhabit contrasting marine and freshwater environments across the planet and face a wide variety of environmental conditions in terms of water temperature, salinity and dissolved oxygen concentration (Helfman et al. 1997; von der Heyden et al. 2015). Specifically, several fish species have offshore spawning and larval migration toward coastal areas. As a consequence, genetic adaptation or physiological acclimatization has been observed, especially to adjust oxygen demand (metabolism) and supply (oxygen uptake and transport) to environmental oxygen availability at the different stages of their life-cycle (Ishibashi et al. 2007; McKenzie et al. 2008). Several authors have suggested that the ability of fish to colonize a wide range of habitats has evolved together with the molecular and functional modulation of the hemoglobin system (Weber 2000; Verde et al. 2002; Marinakis et al. 2003; Verde et al. 2006; Verde et al. 2008). Hemoglobin (Hb) in vertebrates, including fish, consists of a tetrameric molecule composed of twin α and β globin subunits ($\alpha_2\beta_2$), each of which contain a heme group that can reversibly bind oxygen. While genes coding for α and β globins in mammals are clustered on one chromosome, teleost α and β globin genes (Hbα and Hbβ genes) are grouped within two clusters labeled “MN” and “LA” located on two separate chromosomes resulting from the teleost-specific genome duplication (TGD) (Hardison 2008; Opazo et al. 2013). The cluster names are derived from their flanking genes: N-methylpurine-DNA glycosylase (*mpg*) and Nitrogen permease regulator-like 3 (*nprl3*) for cluster “MN”, and Leucine carboxyl methyltransferase 1 (*lcmt1*) and Aquaporin-8 (*aqp8*) for cluster “LA”. Both physical extent and gene content of the teleost globins have been altered by gene loss and by chromosomal or tandem duplications (Opazo et al. 2013) and the

number of Hb genes varies from a single pseudogene in icefishes (Near et al. 2006) to 36 genes (including 9 pseudogenes) in Atlantic salmon (Quinn et al. 2010).

The multiplicity of Hb genes in many fish species is considered as an advantage that allows them to cope with a variable oxygen demand throughout ontogenic development as well as with temporal and spatial alterations in environmental oxygen availability (Perutz 1983; Rutjes et al. 2007). The strong adaptability has been shown to involve differential expression of Hb genes throughout the fish life cycle (Maruyama et al. 2004; Tiedke et al. 2011) and differential regulation under hypoxia or extreme conditions (Campo et al. 2008; Feng et al. 2014). Furthermore, the diversified Hb forms may exhibit different functional properties in terms of oxygen affinity and responsiveness to regulation by phosphates (ATP, GTP), temperature and pH (Bohr and Root effects) (Weber 1990).

European sea bass is an economically important farmed species with a natural distribution ranging in North Atlantic from the south of Norway (60°N) to the west coast of Morocco (30°N) and throughout the Mediterranean and Black Sea (Tortonese 1986; Perez-Ruzafa and Marcos 2014). The metabolic scope was shown to be susceptible to reduced oxygen availability and elevated temperatures, and hypoxia caused morphological changes and altered expression of genes coding for oxygen-dependent molecules in the gills of European sea bass (Claireaux and Lagardère 1999; Rinaldi et al. 2005). Multiple Hbs proteins have been documented by gel electrophoresis, and a partial globin with antimicrobial properties has been reported in European sea bass (Perez and Maclean 1976) (Terova et al. 2011). The recently published European sea bass genome sequence is a valuable source of information to investigate the globin genes (Tine et al. 2014). Starting from this genome assembly, the objectives of the present study were to clarify the organization of the genomic

"MN" and "LA" clusters in European sea bass, in order to identify Hb genes and the predicted globin proteins expressed from embryonic to adult stages in this species.

Materials and Methods

Genomic organization and structure analysis of Hb gene clusters

The first genome assembly of European sea bass was produced based on high throughput sequencing and physical maps (Tine et al. 2014). This assembly was first annotated by homology at the protein level with five closely related published genomes and then by mapping of an annotated gene model constructed from RNA-Seq experiments. It contains 19 annotations for Hb genes localized in different linkage groups (LG), including two clusters, one composed of 3 Hb genes in LG1B and the other composed of 8 Hb genes in LG8. These clusters harbored the typical flanking genes of Hb clusters (i.e. *lcmt1* and *aqp8* for LG1B and *mpg* and *nprl3* for LG8), and probably corresponded to the LA and MN clusters, respectively (Hardison 2008). However, eight globin homolog genes were also detected in several unordered genomic contigs (UN), suggesting a potential incomplete or imperfect reconstitution of the genomic clusters in the published assembly. This is not surprising given the perfect homology that some exons of different Hb genes can display and the chromosomal clustering of these genes. In an attempt to improve the original assembly of the Hb cluster regions, especially with the goal of integrating the unplaced contigs containing Hb genes, we used an iterative process of assembly, based on sequential homology searches among long sequences of the plasmids used for building the initial physical map of the sea bass genome

(http://seabass.mpipz.de/index.html?org=European+seabass&db=dicLab1&hgsid=3779). We first built a blast database containing the original Sanger sequences of the plasmids (500–1000 bp). We then blasted this database with the sequences of the flanking genes *kank2* and *aqp8* to retrieve homologous but overlapping plasmids to walk inward the Hb clusters. Once the first plasmids were merged into the gene sequences, we used this growing assembly as the query for another round of homology search, until we reached the other end of the cluster, i.e. the flanking gene on the other side of the Hb clusters in other species. The rationale for this approach was to benefit from the larger length of these sequences compared with those of NGS reads for resolving assembly ambiguities. Indeed, some parts of the Hb genes can be strictly identical sometimes over more than 200 bp but never on longer stretch of sequence. Moreover, the plasmids contained genomic inserts up to several Kb long and were sequenced from both ends. We first built a (frame) scaffold of overlapping plasmid sequences by taking into account the pairing information between the two sequence ends of each cloned genomic fragment insert (sometime spanning a 5 Kb region of genome). We then plot the whole genome shotgun (WGS) short reads on that frame with BWA-MEM. The alignment of short reads was then manually curated with Geneious 9.1.7 over the two Hb clusters to remove inconsistently mapped pairs of reads, i.e. those without the two mates matching in this genomic region. Finally we reconstituted a consensus sequence based on the short reads and compare it to that obtained from the plasmids sequences only. This approach made it possible to reorganize the two globin clusters (LA and MN cluster sequences available in Supplementary file 1). Predicted exon-intron structures of genes were investigated using Genscan software (http://genes.mit.edu/GENSCAN.html) (Burge and Karlin 1997). The sequences of the Hb genes have been published in GenBank under accession numbers KX196178–KX196191. In order to validate the Hb gene succession

in the MN Cluster, PCR primers were designed at different locations in this cluster and allowed to confirm the predicted amplicon sizes on genomic DNA (Supplementary file 2). Our LA cluster assembly was similar to that resulting from the automatic LG1B assembly published in the databases (http://seabass.mpipz.de/cgi-bin/hgGateway). Then based on the transcript sequences predicted from this new assembly we amplified cDNA that we cloned and sequenced (GenBank accession numbers: KY425658-KY425671), thereby confirming the biological existence of the new gene sequences we built.

Sequence alignment and phylogenetic analysis

Hbα and Hbβ amino acid sequences inferred by the translation of the European sea bass Hb cDNAs cloned in the present study were aligned using ClustalW along with sequences listed in a previous work (Opazo et al. 2013). Human and elasmobranch species were included as outgroup for alignment. To reconstruct the Hb phylogenies, the best model of evolution was assessed from this protein sequence alignment by means of the ProtTest 2.4 program (Abascal et al. 2005). Based on the Akaike information criterion (AIC), LG model was chosen as transition probability matrices (Le and Gascuel 2008), calculated as distance of amino acid substitution per site. We reconstructed Hb phylogeny including Hbα and Hbβ genes using maximum likelihood and Bayesian approaches. Maximum Likelihood phylogeny inference as implemented in MEGA v. 6 (Tamura et al. 2013) was used with 1000 replicates for the bootstrap test. The proportion of invariant sites was evaluated during the analysis, and the range of variation rate across site was determined by a gamma distribution with 4 categories. A Bayesian Markov Chain Monte Carlo (MCMC) analysis was performed using MrBayes software for testing evolutionary hypotheses in which the tree was weighted proportionally to their posterior probability. Two independent assays were performed for Hb

alignment: LG was used as the substitution matrix, the site heterogeneity model was also a gamma distribution with 4 categories, and the number of MCMC replicates was 10^6. The final results were summarized in the best tree after discarding the first 25% of them. All phylogenetic trees were viewed by FigTree v.1.4.3 software (http://tree.bio.ed.ac.uk/software/figtree/).

Animals

Eggs, juveniles (3 months post hatching) and adults (18 months post hatching) of European sea bass were obtained from a commercial hatchery (Aquastream, Ploemeur, France). Eggs were transferred to the wet laboratories in one 60-l incubator with open flow and aerated sea water kept at 15 ± 0.4°C. After hatching, larvae were fed daily with Artemia until the end of larval development (Zambonino-Infante et al. 1996). In order to investigate the gene expression pattern during embryonic and larval development, pools (in triplicate) of eggs and larvae between 20 and 50 mg (containing five to several hundred individuals depending on developmental stage) were sampled at two embryonic (12 and 60 h post fertilization) and six larval stages (5, 13, 19, 26, 33, 45 days post hatching). Juvenile (n = 3 per triplicate) and adult fishes (n = 3) were killed by lethal doses of anesthesia (Eugenol 0.05%, Sigma–Aldrich Saint-Louis, Missouri, USA). Adult tissues (brain, gills, spleen, head kidney, heart, intestine and liver) were immediately dissected. Blood was quickly removed to reduce red blood cell contamination from tissues by rinsing with saline solution (NaCl 0.01%). The pools of eggs and larvae as well as whole juvenile and adult tissues were transferred into microtubes containing *RNAlater* (Qiagen, Hilden, Germany) and placed at 4°C until total RNA extraction. Present work was performed in accordance with French and European policies and guidelines of the French Animal Care Committee (ACCF). No ethical approval for this study

was necessary since fish were reared in optimal conditions until they were sacrificed. Fish were killed with overdose of Eugenol (500mg/L) by prolonged immersion. Fish were left in the solution for 10 min at least following cessation of opercular movements. Fish were next decapitated to ensure brain death.

RNA extraction and cDNA synthesis

Total RNA for analysis of gene expression was extracted from the samples 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). Only samples with a RNA integrity number (RIN) greater than 7 were measured using real-time quantitative PCR (qPCR). RNA samples were stored at –80°C until use.

Synthesis of cDNA was carried out using 0.5 µg of DNase-treated total RNA with an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc.; Hercules, CA, USA). Total reaction was carried out in a final volume of 20 µL containing 5 µL of sample, 4 µL 5x 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. RT negative controls were performed on each sample.

Quantitative real-time RT-PCR analysis

The analysis of globin gene expression at different developmental stages and in different tissues of European sea bass was carried out by quantitative RT-PCR using the primers given in table 1. 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. Primers were designed using Primer 3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi).

Expression of the Hb genes was quantified using the iCycler MyiQ™ Single Color Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). 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 mM), 1.5 µL RNase/DNase free water, 7.5 µL iQ™ SYBR® Green Supermix (Bio-Rad Laboratories Inc.) containing antibody-mediated hot-started 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. RT negative controls were also used as template 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: 5 s at 95°C and 20 s at annealing temperature (60°C for 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*). Annealing temperatures for each couple of primers were fixed by testing PCR condition on plasmids containing the different Hb cDNAs. Following this procedure, we determined qPCR condition allowing specific amplification of each Hb cDNA subtype. 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 "Gene Expression Module" of CFX Manager software (Bio-Rad Laboratories Inc.). Cq is the cycle number 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 18S gene was chosen as reference gene for eggs, larvae and juvenile tissues, while elongation factor 1-alpha (*ef1α*) and 18S were used as reference genes in adult tissues. These reference genes were used since they did not show any significant variation of expression between samples (relative standard deviation <5% among samples). Significant differences of expression between developmental stages were analyzed by one-way ANOVA using Tukey'HSD as post-hoc test ($p<0.05$). Expression data were Log transformed to fit a normal distribution. Heat map was used to illustrate relative expression of each Hb gene during the fish development and among tissues. To this end, normalized data were subjected to hierarchical clustering (Distance metric selection: Pearson uncentered) and a heat map was generated using MeV software (Multi Experiment Viewer; http://www.tm4.org).

Results

Genomic organization of globin gene clusters

The MN and LA clusters in European sea bass were shown to contain a total of seven Hbα and seven Hbβ genes (Fig1). The MN cluster included five Hbα genes annotated MN-*Hbα1* to MN-*Hbα5* and six Hbβ genes annotated MN-*Hbβ1* to MN-*Hbβ6* following the suggested nomenclature (Opazo et al. 2013). The cluster consisted of about 42 kb from the

first codon of MN-*Hbα5* to the stop codon of MN-*Hbβ1*. Whereas stickleback (*Gasterosteus aculeatus*) MN-Hbα and β genes were found on reverse and forward strands respectively, these genes were indifferently located on either strands in European sea bass (Fig1). A gene exhibiting high homology with the CCDC (Coiled-coil domain-containing protein) gene family was predicted on the reverse strand between MN-*Hbβ4* and MN-*Hbβ5*.

The LA cluster included two Hbα and one Hbβ genes, annotated LA-*Hbα1*, LA-*Hbα2* and LA-*Hbβ1* (Fig1). These three genes spanned a sequence length of about 14 kb from the first codon of LA-*Hbα2* to the stop codon of LA-*Hbα1*. The Hbα genes of the LA cluster were encoded by one strand whereas *Hbβ1* was encoded by the reverse strand. LA-*Hbα1* and LA-*Hbβ1* were organized head to head whereas LA-*Hbα2*and LA-*Hbβ1* were arranged tail to tail. The organization of the LA-cluster was conserved between the European sea bass and tilapia (*Oreochromis niloticus*).

Structure of European sea bass hemoglobin genes

The complete gene sequences and deduced coding sequences were confirmed by cDNA cloning. The European sea bass Hbα and Hbβ genes typically consist of three exons separated by two introns (Table 2). While all the Hbβ genes coded for proteins of 147 aa, the size of the proteins encoded by Hbα genes ranged between 141 and 144 aa.

Analysis of putative key residues in European sea bass Hb

The histidine (His) residues deemed to be implicated in oxygen binding were conserved in the predicted Hbα and Hbβ globins (Figs2 and 3). The His residue at position 122 of Hbα globins seems to be involved in the Bohr effect (Bellelli and Brunori 2011) and was found in all Hbα sequences of European sea bass. Similarly, the C-terminal His residue of Hbβ responsible for the Bohr effect was also present in all Hbβ sequences, except for MN-*Hbβ3* where it is substituted by a Phe residue. European sea bass Hbα and Hbβ genes possessed all the putative residues responsible for the Root effect, such as Val2β, Ser2α, Trp4β, Ser90β, Ser94β, Glu95β, Asp95α, Asp101β, Glu140α, Arg144β, Gln145β, Tyr146β and His147β (Bonaventura et al. 2004), except MN-Hbβ3 which was devoid of Ser94β and Arg144β.

Phylogenetic analysis of European sea bass Hb

Phylogenetic analysis was performed based on the deduced Hbα and Hbβ amino acid sequences of the European sea bass and other teleost and non-teleost species (Supplementary file 3). The data set was used to calculate the best Hb tree using maximum likelihood (ML) approach and the Bayesian MCMC methods. Since the two methods led to similar phylogenies, we only present the tree obtained with the Bayesian approach (Figs4 and 5) where the nodes appeared to be more robust, i.e. posterior probabilities above 0.5. The phylogenetic analysis shed light on the ortho- versus paralogy relationships of the *D. labrax* genes with those of other species. The tree allowed to group Hbα in many clades, two

of them composed with genes from the LA cluster. Hbβ are grouped in four major clades including three clades related to the MN cluster and one related to the LA cluster.

While each Hbβ clade included at least one Hbβ globin from European sea bass, only four of the five Hbα clades contained one or more European sea bass Hbα globins. Analysis of the MN clades revealed that European sea bass MN-Hbα3-5 on one hand and MN-Hbβ2, MN-Hbβ4-6 on the other clustered together in the MN-Hbα and MN-Hbβ clades, respectively, that is consistent with the high coding sequence similarities (Supplementary file 4).

Expression pattern of Hb genes during developmental stages

The temporal expression patterns of the Hb genes varied considerably, but some common features can be noted (Fig6, table 3). During embryogenesis (12 h and 60 h post-fertilization; hpf) relative expression levels of both Hbα and Hbβ genes were very low or not detected (Cq>30) (Supplementary file 5). Several genes (notably LA-*Hbα2*, MN-*Hbα2*, MN-*Hbβ2*, MN-*Hbα3*, MN-*Hbβ5*, MN-*Hbβ4* and MN-*Hbα5)* exhibited a substantial increase in expression between 60 hpf and 5 days post hatching (dph). The expression levels of several genes (LA-*Hbα2*, LA-*Hbβ1*, MN-*Hbβ1*, MN-*Hbα2,* MN-*Hbβ4,* MN-*Hbα5,* MN-*Hbα4 and* MN-*Hbβ6*) increased exponentially between 5 and 45 dph, with a key step between 26 dph and 45 dph. After 45 dph, expression increased substantially for some genes (LA-*Hbβ1*, MN-*Hbα2,* LA-*Hbα1*, MN-*Hbα1*, MN-*Hbβ2* and MN-*Hbα2)* during the juvenile stage, while it dropped significantly for others (MN-*Hbβ4,* MN-*Hbα5,* MN-*Hbα4* and MN-*Hbβ6* genes). MN-*Hbβ3* was very weakly expressed (Cq>35) at all the developmental stages investigated.

Tissue expression pattern of Hb genes

Based on the order of Cq value magnitude, MN-*Hbα1* and MN-*Hbβ1* exhibited the highest expression levels in the adult fish, irrespective of the tissue considered (Supplementary file 6). In contrast, genes MN-*Hbα3*, MN-*Hbα4* and MN-*Hbα5*, as well as MN-*Hbβ4*, MN-*Hbβ5* and MN-*Hbβ6*, were expressed at very low levels in the different adult tissues investigated. MN-*Hbα1*, MN-*Hbβ1* and MN-*Hbβ3* showed the highest expression levels in the head kidney and lesser levels in the spleen (Fig7, Table 4). MN-*Hbα2* and MN-*Hbβ2* genes showed the highest transcript levels in spleen. Several Hb genes were expressed at low levels in other non-hematopoietic tissues such as brain, heart, intestine and gills.

Discussion

In order to gain knowledge about Hb genes in the European sea bass, we assembled and confirm by transcript sequencing fourteen Hb genes that we assigned onto two genomic LA and MN clusters localized respectively on the linking group (LG) 1B (Sequence ID: emb|CBXY010008361.1|) and LG 8 (Sequence IDs: emb|CBXY010016043.1|-emb|CBXY010016046.1|) of the first assembly of the European sea bass genome (Tine et al. 2014).

Three genes (2 Hbα and 1 Hbβ) were located adjacent to the conserved *aqp8* and *lcmt1* genes of the LA cluster in teleosts (Opazo et al. 2013). As observed in platyfish, medaka and tilapia, the two α-globin genes were separated by the β-globin gene located on the opposite strand (Opazo et al. 2013). The similar organization of this cluster suggests that the LA α-

and β-globin genes of European sea bass are 1:1 orthologs with those in platyfish, medaka and tilapia, in agreement with the phylogenetic analysis which grouped the three LA genes within the respective LA-clades of these species. The LA clades also included orthologous globin genes from green spotted puffer and fugu, wherein these genes have been reversed in the LA cluster. We noted that the ortholog of sea bass LA-*Hbβ1* seems to have been lost in stickleback, which yet exhibits high overall orthologous sequence homologies with European sea bass (Tine et al. 2014). Our data also revealed that eleven globin genes (five Hbα and six Hbβ) were flanked by *nprl3* and *kank2* genes similar to the MN cluster in teleosts. Intriguingly, a *CCDC106-like* gene was found inserted between MN-*Hbβ4* and MN-*Hbβ5,* and the specific functional role of this potential p53-interacting partner (Zhou et al. 2010) warrants further studies. It is accepted that distinct organization between teleost species in the LA and, especially, MN clusters results from lineage-specific changes in gene content via repeated duplication and deletion events (Opazo et al. 2013). Our phylogenetic analysis suggests that two gene groups (MN-*Hbα3*, MN-*Hbα4*, MN-*Hbα5* and MN-*Hbβ2*, MN-*Hbβ4*, MN-*Hbβ5,* MN-*Hbβ6*) each consist of close paralogous copies and are probably the results of recent duplication events that occurred in the Moronidae lineage. In comparison, MN-*Hbα4* and MN-*Hbβ2* branch deeper in the phylogenetic trees, suggesting that they might be ancestral with regard to their respective paralog copies located at the tips of their branch. In any case, from an evolutionary point of view, the MN cluster seems to be largely more dynamic than the LA one, which was suggested to have been lost in Atlantic salmon (Quinn et al. 2010).

The LA-Hb genes of European sea bass did not show expression patterns specific to the early life stage, contrary to the embryonic Hb genes in medaka, zebrafish and tilapia (Maruyama

et al. 2004; Tiedke et al. 2011; Opazo et al. 2013). However, the three MN-*Hbα3–5* genes and three MN-*Hbβ4–6* genes exhibited high larval expression compared to juvenile and adult levels. Intriguingly, the expression patterns throughout development were similar in the closely linked MN-*Hbα4*, MN-*Hbα5* and MN-*Hbβ6* on one hand and in the neighbour genes MN-Hbα3 and MN-Hbβ5 on the other hand, suggesting that these Hbα and Hbβ genes likely display common elements or/and mechanisms of regulation. With the new assembly we built, it will be now possible to investigate further the existence of such regulation elements. The common feature of the Hb genes significantly expressed just after hatching, was the increased expression from 60 hpf to 5 dph. This data suggests that around the 60 hpf stage oxygen supply by simple diffusion throughout the embryonic tissues may be sufficient to fulfill physiological requirements in European sea bass. The absence of significant Hb gene expression in fertilized eggs of sea bass was consistent with data obtained in another seawater species, the Atlantic cod, but contrasted with the expression profiles of embryonic genes observed in medaka and zebrafish (Maruyama et al. 2004; Wetten et al. 2010; Tiedke et al. 2011). It has been shown that the expression of embryonic Hb genes in zebrafish starts with primitive erythropoiesis, which takes place in the intermediate cell mass around 15 hpf (Kulkeaw and Sugiyama 2012). The stage at which intermediate cell mass develops is not known in European sea bass; however, our data suggested that primitive erythropoiesis resulting in matured erythrocytes that are replete with Hb occurs after 60 hpf. Moreover, European sea bass Hb genes that did not show high mRNA levels during the first stage of larval development (LA-*Hbα1*, LA-*Hbα2*, MN-*Hbα1*, MN-*Hbα2*, LA-*Hbβ1*, MN-*Hbβ1*, MN-*Hbβ2* and MN-*Hbβ3*) showed a progressive increase in gene expression from 26 dph to juvenile/adult stage. Most of these “late expressed” Hb genes exhibited a significant rise in expression between 26 and 33 dph. Interestingly, this

developmental window was previously associated with a switch of global transcriptomic profile related to a key physiological step in the development of European sea bass larvae (Darias et al. 2008). This increase in Hb gene expression was likely related to the transition from primitive to definitive erythropoiesis, which is associated with the emergence of the hematopoietic function in the kidney and spleen during larval development of teleosts (Brownlie and Zon 1999). Indeed, erythropoietic activity has been detected in the spleen of European sea bass before 40 dph (Quesada et al. 1994). In zebrafish, the switch from primitive to definitive erythropoiesis has been shown to start around 10 dph (Tiedke et al. 2011). The earlier expression of Hb embryonic genes, as well as the earlier period of transition between embryonic and adult Hb expression observed in zebrafish, can be explained by faster developmental processes in this species.

The present investigation revealed that head kidney and, to a lesser extent, spleen are the major tissues in which most of European sea bass Hb genes were expressed at adult stage. Consistently, spleen, kidney (mesonephros) and head kidney (pronephros) are the sites of erythropoiesis in European sea bass (Esteban et al. 1989; Quesada et al. 1994) and other teleosts species, such as catfish (*Ictalurus punctatus*) (Feng et al. 2014) and euryhaline flounder (Platichthys flesus) (Lu et al. 2011). MN-*Hbα1,* MN-*Hbβ1* and to a lesser extent LA-*Hbα2*, MN-*Hbβ3*, MN-*Hbα2* and MN-*Hbβ2* were the most predominantly expressed genes at the adult stage. The expression of three different Hbα and three different Hbβ genes in adult European sea bass is consistent with the identification by gel electrophoresis of five major Hb tetramers which likely comprise different combinations of these subunits (Perez and Maclean 1976). Most of the European sea bass Hb genes exhibited moderate expression level in non-hematopoietic tissues such as brain, heart, liver, intestine and gill. Consistently,

expression of Hb in several vertebrates has been found in many non-erythroid cells (Saha et al. 2014), in which it may facilitate tissue oxygen transport or increase cellular oxygenation (Nishi et al. 2008; Biagioli et al. 2009; Tezel et al. 2010). In channel catfish, the expression of Hb genes in the intestine is associated with the respiratory function of this tissue (Feng et al. 2014). Moreover, antimicrobial activity has also been attributed to Hbβ in gills and skin of channel catfish exposed to parasite (Ullal et al. 2008). Such antimicrobial activity is suggested to be also associated with the increase of Hbβ-like (highly similar to the present MN-*Hbβ1* cDNA sequence) expression in the epithelium of European sea bass submitted to an acute stress (Terova et al. 2011).

Compared to mammals, the functional property of fish Hbs known as Root effect is a decrease in both Hb-oxygen affinity and cooperativity at low blood pH. Even if the Root effect cannot be based solely on the presence or absence of residues, Asp95α and Asp101β, that are considered as the minimal structural requirement for the Root effect (Mazzarella et al. 2006), were conserved in the different European sea bass Hb genes. Moreover, Ser94β, Arg144β and His147β also known to be important amino acid residues involved in Hb-oxygen binding and particularly in the Root effect (Mylvaganam et al. 1996; Bonaventura et al. 2004) were conserved in the different European sea bass Hbβ genes except for MN-*Hbβ3*. The substitution of key functional residues are partially found in *Hbβ1*, *Hbβ4* and *Hbβ5* of catfish (Feng et al. 2014) and in trout Hb I (Gabbianelli et al. 2004). Functional characterization of the European sea bass Hb proteins related to water temperature, oxygen affinity and pH sensitivity will be necessary to determine potential specific functional properties.

Conclusion

Even though the composition the Hb tetramers in European sea bass Hb and their functional properties are not known, present data support the evidence that European sea bass is capable of producing different types of Hb with potential different functional properties over their life cycle. The positive relation between the diversity of Hb genes expressed in fish species and the diversity of their living environments suggests an important role played by these oxygen binding proteins in the ability to cope with environmental constraints. This appears to be particularly relevant in the European sea bass, which inhabits coastal nurseries with large fluctuations in environmental conditions.

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Compliance with Ethical Standards:

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval : All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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