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Channel catfish hemoglobin genes: Identification, phylogenetic and syntenic analysis, and specific induction in response to heat stress

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ABSTRACT

Hemoglobins transport oxygen from gill to inner organs in fish, and this process is affected by temperature, one of the major environmental factors for fish. The hemoglobin gene clusters have been well studied in humans and several model fish species, but remain largely unknown in catfish. Here, eight α- and six β-hemoglobin genes were identified and characterized in channel catfish. Genomic synteny analysis showed that these hemoglobin genes were separated into two unlinked clusters, the MN cluster containing six α- and six β-hemoglobin genes, and the LA cluster consisting of two α-hemoglobin genes. Channel catfish hemoglobin genes were ubiquitously expressed in all the 10 tested tissues from healthy fish, but exhibited higher expression level in spleen, head kidney, and trunk kidney. In response to heat stress, hemoglobin genes, especially MN *Hbα4*, MN *Hbα5*, MN *Hbα6*, MN *Hbβ4*, MN *Hbβ5*, MN *Hbβ6*, LA *Hbα1*, and LA *Hbα2*, presumably the embryonic hemoglobin genes, were drastically up-regulated in the gill and head kidney of heat-tolerant fishes, but not in these tissues of the heat-intolerant fish, suggesting the importance of the embryonic hemoglobin genes in coping with the low oxygen conditions under heat stress.

1. Introduction

Fish are ectotherms, whose body temperature is virtually identical to environmental temperature. Therefore, any rise in the ambient water temperature due to natural variation or human activity would directly increase their body temperature. The elevated temperatures not only reduce the availability of dissolved oxygen in water, but also accelerate the metabolic processes, alter the respiration rate and enhance the oxygen consumption, eventually place a burden on the oxygen transport system of fish (Portner, 2001).

In teleost, hemoglobin, the best-known member of respiratory proteins, plays a critical role in reversibly binding oxygen and transporting oxygen from gill to peripheral tissues. The hemoglobin molecule is made up of two α- and two β-subunits, each having a prosthetic group called heme, which is bound to oxygen. The heme-oxygen bond is exothermic, so increasing temperature could weaken the bond and eventually decrease the hemoglobin's affinity for oxygen (Schmidt-Nielsen, 1997). However, there are exceptions, crucian carp (*Carassius carassius*) hemoglobin increases its oxygen affinity in response to increased temperature, especially at pH levels below 7.0 (Kamshilov and Kamshilova, 2007).

Like other vertebrates, fish has multiple hemoglobin genes including embryonic and adult α-hemoglobin (*Hbα*) and β-hemoglobin (*Hbβ*) chains. To date, the genomic organization of the fish *Hbα–Hbβ* clusters has been investigated in fugu (*Takifugu rubripes*) (Flint et al., 2001; Gillemans et al., 2003), medaka (*Oryzias latipes*) (Maruyama et al., 2004a,b; Hardison, 2008), platyfish (*Xiphophorus maculatus*) (Hardison, 2008; Patel et al., 2008), zebrafish (*Denio rario*) (Brownlie et al., 2003), three-spined stickleback (*Gasterosteus aculeatus*) (Wetten et al., 2010), tilapia (*Oreochromis niloticus*) (Opazo et al., 2013), Atlantic salmon (*Salmo salar*) (Quinn et al., 2010), and Atlantic cod (*Gadus morhua*) (Borza et al., 2009; Wetten et al., 2010). In these studies, the adult *Hbα* and *Hbβ* genes, along with the embryonic hemoglobin genes, were observed to be clustered adjacently in the genome. Furthermore, in most of these fish species, two hemoglobin gene clusters were found to be located on separate chromosomes or linkage groups, which support the hypothesis that the teleost lineage experienced whole genome duplication (WGD) events subsequent to the divergence from tetrapod (Taylor et al., 2001).

Channel catfish (*Ictalurus punctatus*), a commercially important freshwater fish in North America, is a temperate species. It has the

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ability to cope with seasonal temperature changes from near freezing temperature during winter in the north to over 36 °C in summer in the south. Channel catfish exhibits a greater adaptability, geographic range, and a larger amount of genetic variation than other catfish species such as blue catfish (*I. furcatus*), black bullhead (*Ameiurus melas*), and white catfish (*A. catus*) in North America (Taylor et al., 1984). In previous studies, a variable electrophoretic pattern of hemoglobins were observed in various catfish species including channel catfish (Taylor et al., 1984), but the genomic organization of the catfish α- and β-hemoglobin clusters and their respective encoding genes remained unknown.

Changes in gene expression under heat stress have been extensively studied in many teleost fish. Many genes were up-regulated in response to heat stress, including heat shock protein genes (Hori et al., 2010; Quinn et al., 2011b; Dalvi et al., 2012), ribosomal protein genes (Aursnes et al., 2011), and hemoglobin genes (Imsland et al., 1997; Methling et al., 2010; Quinn et al., 2011a). To date, several channel catfish hemoglobin genes have been identified (Skow, 1971; Taylor et al., 1984; Yeh et al., 2006; Chen et al., 2010), but systematic analysis of the whole hemoglobin repertoire was still unavailable. A recent RNA-seq analysis of global gene expression profiling in catfish following heat stress suggested that various short read assemblies representing hemoglobin genes were noted, but gene identities of such short reads could not be fully addressed (Liu et al., 2013). In the present study, we identified and characterized the channel catfish hemoglobin clusters containing a total of eight *Hbα* genes and six *Hbβ* genes. Here we report the identification, phylogenetic and syntenic analysis, and expression profiling of the hemoglobin genes in response to heat stress challenge.

2. Materials and methods

2.1. Identification and sequence analysis of hemoglobin genes

To identify the hemoglobin genes, RNA-seq and whole genome sequence databases of channel catfish were searched using available zebrafish and tilapia hemoglobins as queries. The RNA-seq database was generated from the transcriptome assembly of expressed short reads of a doubled haploid channel catfish (Liu et al., 2012). The quality of the transcriptome assembly obtained from RNA-seq database was confirmed by comparison with the draft catfish whole genome sequences (unpublished), which also originated from sequencing a doubled haploid channel catfish (Waldbieser et al., 2010). The transcripts were translated using FGENESH (Salamov and Solovyev, 2000) and ORF Finder (Sayers et al., 2012). The translated proteins were verified by protein BLAST (BLASTP) (Altschul et al., 1997) against NCBI non-redundant protein database. Functional domains were identified using SMART v 7 (Letunic et al., 2012).

2.2. Assessments of genomic synteny

To identify conserved syntenic regions, the genes in the upstream and downstream of hemoglobin gene clusters were annotated. Initial orthologous predictions were derived from Ensembl Compara Database (Flicek et al., 2013) and were visualized using the program Genomicus v 72.01 (Muffato et al., 2010). The genes lying upstream and downstream of the annotated hemoglobin genes were identified using FGENESH, and their identities determined by BLASTP searches against the non-redundant protein database.

2.3. Sequence alignment and phylogenetic analysis

The deduced amino acid sequences of *Hbα* and *Hbβ* genes were separately aligned using MUSCLE v 3.8 (Edgar, 2004) and the L-INS-i, G-INS-i, and E-INS-i of MAFFT v 6.8 (Katoh et al., 2005). The best-scoring multiple alignment was selected by MUMSA for phylogenetic tree construction (Lassmann and Sonnhammer, 2005, 2006). The phylogenetic tree of the *Hbα* and *Hbβ* genes was constructed by using both maximum likelihood (ML) and Bayesian methods. The best-fitting models were selected using the Akaike Information Criterion with correction for small sample size (AICc) in Treefinder software (v March 2011) (Jobb et al., 2004). Simulations of 1000 bootstraps were performed to provide statistical support of the phylogenetic tree. Bayesian phylogenies were conducted in MrBayes v 3.2.1 (Ronquist et al., 2012), running four simultaneous chains for 10,000,000 generation runs of four chains, sampling every 1000 generations, under a mixed model with gamma distribution of rate and invariant site categories of amino acid substitution and using default priors. Support for the nodes and parameter estimates were derived from a majority rule consensus of the last 2500 trees. MrBayes analyses were run by using the CIPRES Portal (Miller et al., 2009).

2.4. Heat stress challenge and tissue sampling

All experimental protocols concerning the use of catfish were approved by the Institutional Animal Care and Use Committee (IACUC) at Auburn University. The heat stress experiments were conducted at the hatchery of the Auburn University Fish Genetics Research Unit. The fish and heat treatment were previously reported (Liu et al., 2013). Briefly, tanks were set up with a constant flow system with fresh pond water at 24 ± 0.5 °C and ambient oxygen level (8.9–9.2 ppm). A total of 300 fingerling catfish (Average length: 13.20 ± 1.04 cm, average weight: 13.26 ± 2.69 g) were transferred to an experimental tank (Length: 3.2 m, width: 50 cm and depth: 1 m) and left to acclimate for 72 h at ambient temperature. After acclimation and before the heat stress treatment, 45 fish were removed to another tank serving as a control group with the water temperature kept at 24 °C. The water temperature in the treatment tank was increased by 4 °C h^{-1} until it reached 32 °C, and then the water temperature was increased at 1 °C h^{-1} until it reached 36 °C. Dissolved oxygen was allowed to fluctuate naturally and decreased from approximately 8.5 ppm to a minimum of 6.8 ppm during the trial.

When the water temperature reached 36 °C, the temperature was held constant and the fishes were closely monitored for signs of stress. The first 45 (intolerant group) and the last 45 (tolerant group) individuals showing loss of balance were quickly removed from the tank for sampling, similarly as described in Arctic charr (Quinn et al., 2011a). Fish were euthanized by MS-222 exposure at a concentration of 300 ppm. The fish were weighed and their body lengths were measured.

Ten tissues including brain, gill, heart, liver, head kidney, trunk kidney, spleen, intestine, muscle, and skin were dissected from the 45 fish of intolerant and of tolerant group, respectively. For each group, the same tissues of 15 individuals were pooled together (3 pools of 15 fish each) and immediately immersed in RNA*later* Solution (Invitrogen, Carlsbad, CA, USA). Similarly, the 10 tissues from 45 fish (3 pools of 15 fish each) of the control group were collected. All samples were stored at 4 °C overnight to allow RNA*later* Solution to thoroughly penetrate the tissues, removed supernatant, and then moved to −80 °C until RNA extraction.

2.5. Quantitative real-time RT-PCR analysis

Total RNA was extracted using the TRIzol Reagent (Qiagen, Germantown, MD, USA) following manufacturer's instructions, and then quantified using UV-spectrophotometer. First strand cDNA synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA) was used for determination of gene expression by quantitative real-time RT-PCR (qRT-PCR). The qRT-PCR was performed using EvaGreen Supermix (Bio-Rad) on the CFX Real Time PCR Detection System (Bio-Rad) following manufacturer's protocol. The primers used in qRT-PCR were designed using primer 3 plus (Untergasser et al., 2012) and listed in Table 1. The 18S rRNA gene was used as an internal control for

normalization of expression levels (Small et al., 2008; Qin et al., 2012; Zhou et al., 2012). To examine tissue expression of the hemoglobin genes, total RNA of all the ten tissues collected from control fish was used for qRT-PCR with the gill arbitrarily chosen as the calibrator. To assess the expression of hemoglobin genes in response to heat stress, total RNA of the gill and head kidney from intolerant fish and tolerant fish were chosen for the qRT-PCR analysis.

Quantification cycle values (Cq) values generated by qRT-PCR were converted into Microsoft Excel, then transferred and analyzed in linear regression software LinRegPCR (Ramakers et al., 2003; Ruijter et al., 2009). Expression difference between control and treatment groups was assessed for statistical significance using a pairwise fixed reallocation randomization test within the Relative Expression Software Tool 384 v.1 (Pfaffl et al., 2002). The fold-change of the channel catfish hemoglobin gene after different treatments was made into a graphical representation; differential regulation was considered significant at $p < 0.05$.

3. Results

3.1. Identification of catfish hemoglobin gene clusters

The hemoglobin genes of catfish were located in two separate clusters on different chromosomes. Such clusters, as referred to as the MN cluster and the LA cluster by Hardison (2008) and Opazo et al. (2013), were clearly delineated by distinct sets of flanking loci. The MN cluster of hemoglobin genes was flanked leftward by the genes *N-methylpurine-DNA glycosylase* (*mpg*) and *Nitrogen permease regulator-like 3* (*nprl3*), while the LA cluster was flanked rightward by the genes *Leucine carboxyl methyltransferase 1* (*lcmt1*) and *Aquaporin-8* (*aqp8*) (Fig. 1). The hemoglobin genes within each cluster were annotated following the nomenclature of Opazo et al. (2013), i.e. the individual hemoglobin genes were numbered from left to right in the MN cluster and from right to left in the LA cluster. The hemoglobin gene in the leftmost position of the MN cluster was named as MN *Hbβ1*, followed by MN *Hbα1*, *Hbβ2*, *Hbα2*, *Hbβ3*, *Hbα3*, *Hbβ4*, *Hbα4*, *Hbβ5*, *Hbα5*, *Hbβ6*, and *Hbα6*.

A total of 12 hemoglobin genes were found in the MN cluster in the catfish genome. Of the 12 genes, six genes are encoded by one strand whereas the other six genes are encoded by the complementary strand DNA (Fig. 1). These genes span a physical size of 65.4 kb, from the start codon of the first hemoglobin gene to the stop codon of the last hemoglobin gene. The six catfish *Hbα* genes and six *Hbβ* genes were organized head-to-head (α1–β1, α2–β2, α3–β3, α4–β5, and α5–β6), tail-to-tail (α1–β2, α2–β3, β4–α5, β5–α5, and β6–α6), and head-to-tail (α5–α6).

The genes flanking the catfish MN cluster hemoglobin genes were completely conserved between the catfish and zebrafish. The six flanking genes on the left of the MN cluster hemoglobin genes were *fam100A* (Family with sequence similarity 100 member A), *mgrn1* (Mahogunin ring finger 1), *aanat* (Aralkylamine N-acetyltransferase), *rhbdf1a* (Rhomboid family member 1a), *mpg*, and *nprl3*. These six flanking genes were identified within a 119-kb region leftwards of the MN cluster of the α-β hemoglobins. Similarly, the flanking genes on the right, *kank2* (KN motif and ankyrin repeat domain-containing protein 2) and *dock6* (Dedicator of cytokinesis 6) were found to be conserved between catfish and zebrafish, suggesting their orthologous relationships. These two genes were identified from the 68-kb region rightward from the MN cluster of the hemoglobin genes.

Two hemoglobin genes, *Hba1* and *Hba2*, were found from the LA cluster in the catfish genome (Fig. 1). They were organized within a 5.7 kb region head to tail. No *Hbβ* genes were found in the LA cluster in catfish. The flanking genes of the LA cluster were well conserved between catfish and zebrafish, with *foxj1* (Forkhead box j1) and *rhbdf1b* genes being identified from a 68-kb region on the left flanking position, and *arhgap17* (Rho GTPase activating protein 17), *lcmt*, *aqp8a* and *aqp8b* genes being identified from a 46-kb region on the right flanking position, suggesting their orthologous relationships.

Table 1
Primers used for the study of catfish hemoglobin genes.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
LA_Hbα1	GTTGGAGAGGCCGTCAATAA	GGCGATCACCACTAGGATGT
LA_Hbα2	TCTCGGGAAGAAGATTGTGG	CCAAATGACAAGCTAAAGTC
MN_Hbα1	AACCCTGGTCAAGGCTTTCT	AATAACGGTTCTGCCATGCT
MN_Hbα2	TCGTCAAAGACCTTTGGGCCA	TTTCACTTGGGCTGATCCAG
MN_Hbα3	AACCTTTGGGCCAAGATCGCT	CAATTACGCTTCCGTGCTTT
MN_Hbα4	GGACTGACTTGAGCTTTGGC	CACGTTGTGGGCTAGGATCT
MN_Hbα5	GTCTACCCGCAGACCAAGAC	GAGCAACCCATTTGTCAGGT
MN_Hbα6	AGGATAAAGCCGCAGTGAAG	CTTCCTCACCTGGACAGAGC
MN_Hbβ1	TCACAATGGTGAGACAATCG	ACGCTCTGGACAACGCAGTG
MN_Hbβ2	CCTTTGGGGAAAGATCAACC	CCGAACTTTGCGGCCAGAGT
MN_Hbβ3	CCTGTGGGGAAAGATCAACC	CCGAAATTTGCGGCCAGAGT
MN_Hbβ4	GGACGTCTTTTCCAAGATCGAC	CAGCAACCTTGGGGTTTCCA
MN_Hbβ5	CATCCAGGACCTCTTTTCCA	ACTACCAAGCCATGGGCAGC
MN_Hbβ6	GCTGTTGGCTGACTGCATTA	CGGAGACTATGACCGCCAGG
18s rRNA	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG

3.2. Phylogenetic analysis of the catfish hemoglobin genes

In order to analyze the evolutionary relationship of hemoglobin genes between channel catfish and other teleost species, phylogenetic trees were conducted using amino acid sequences from various taxa including zebrafish, fugu, green-spotted puffer, medaka, platyfish, stickleback, tilapia, salmon, and cod retrieved from Ensembl database and from Opazo et al (2013). The amino acid sequences of tetrapod and cartilaginous fishes were also included. The L-INS-i alignment of hemoglobin protein sequences was selected for phylogenetic construction because of its highest MUMSA score after comparing it with the other different alignment methods. Based on the AICc, the best-fitting substitution models of LG for *Hbα* and WAG for *Hbβ* were selected in the ML analysis, and the mixed substitution model for *Hbα* and *Hbβ* was applied in MrBayes analysis. The phylogenetic trees suggested that both *Hbα* and *Hbβ* genes of teleost were polyphyletic relative to counterparts of tetrapod (Fig. 2 and 3). The *Hbα* and *Hbβ* genes in the same clade formed monophyletic groups, which reflect their paralogous characteristics.

In the case of *Hbα* genes, a clade of hemoglobin genes consisting of a subset of genes stemmed from the LA cluster were placed sister to chicken (*Gallus gallus*) $Hb\alpha^D$ and platypus (*Ornithorhynchus anatinus*) $Hb\alpha^K$, however all other fish hemoglobin genes were clustered into other monophyletic groups. Channel catfish *Hbα* genes were clustered into the same clade to their homologous genes and always showed the closest relationship to the zebrafish *Hbα* genes.

In the case of *Hbβ* genes, the teleost *Hbβ* could be arranged into four separate clades, three of which consist of MN clusters, and one of which from LA cluster. The six *Hbβ* genes of channel catfish were distributed into the three MN clades, *Hbβ1* in clade 1, *Hbβ2* and *Hbβ3* in clade 2, and *Hbβ4*, *Hbβ5 and Hbβ6* in clade 3. Channel catfish *Hbβ1* was placed sister to its orthologous gene of zebrafish MN *Hbβ1*, and channel catfish *Hbβ4*, *Hbβ5* and *Hbβ6* were clustered together and also placed sister to orthologous genes of zebrafish.

3.3. Sequence analysis of the channel catfish hemoglobin genes

The complete gene sequences of the 14 catfish hemoglobin genes have been deposited to GenBank with consecutive accession numbers of KF471099–KF471112. As shown in Figs. 4 and 5, all the fourteen channel catfish hemoglobin genes displayed an archetypal structure of two introns and three exons, which encoded the predicted eight *Hbα* proteins of 143 amino acids and six *Hbβ* proteins of 147 or 148 amino acids. Interestingly, *Hbα6* gene in the MN cluster encoded 133 amino acids, which were ten amino acids shorter than other *Hbα* genes at

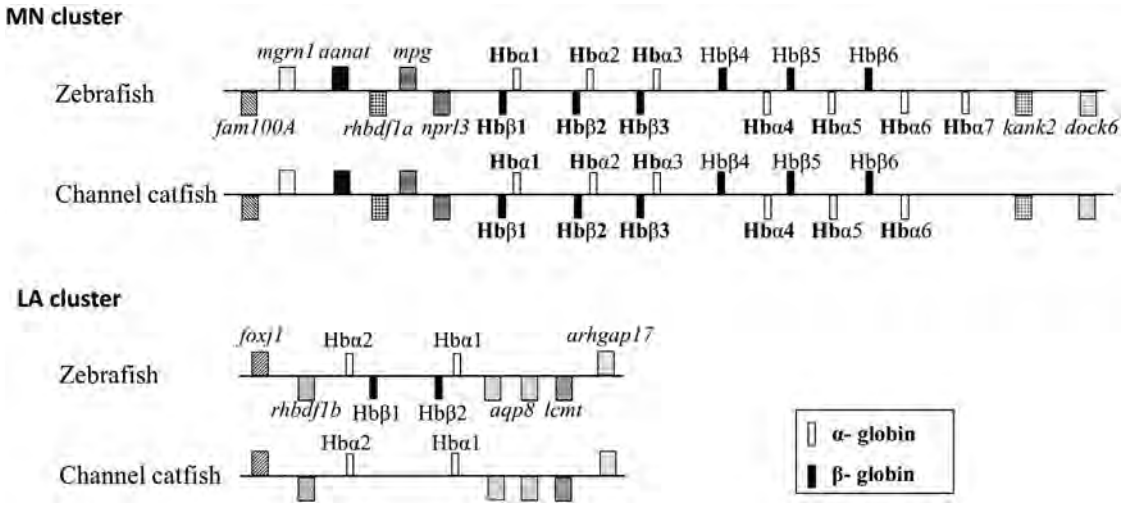


Fig. 1. Conserved synteny in the MN and LA cluster of channel catfish and zebrafish. Genomic synteny of the channel catfish MN and LA hemoglobin clusters is compared with the orthologous loci in zebrafish. The boxed genes above and below line indicate forward and reverse transcriptional orientations, respectively. The flanking genes on MN cluster from left to right are *fam100A* (Family with sequence similarity 100 member A), *mgrn1* (Mahogunin ring finger 1), *aanat* (Aralkylamine N-acetyltransferase), *rhbdf1a* (Rhomboid family member 1a), *mpg* (N-methylpurine-DNA glycosylase), *nprl3* (Nitrogen permease regulator-like 3), *kank2* (KN motif and ankyrin repeat domain-containing protein 2), and *dock6* (Dedicator of cytokinesis 6). The flanking genes on LA cluster from left to right are *foxj1* (Forkhead box J1), *rhbdf1b* (Rhomboid family member 1b), *aqp8a* (Aquaporin-8a), aqp8b (Aquaporin-8b), *lcmt* (Leucine carboxyl methyltransferase 1), and *arhgap17* (Rho GTPase activating protein 17).

the C-terminus. Amino acid sequence of the eight catfish *Hbα* genes shared a relatively low level of similarities (23% identity and 52% similarity), and as a whole they exhibited the identity of 23% and the similarity of 28% to the amino acid sequence of zebrafish Mn *Hbα1*. Amino acid sequence of the six *Hbβ* genes displayed a higher level of identity of 49% and the similarity of 82%, and as a whole they were 42% identical and 79% similar to amino acid sequence of zebrafish MN *Hbβ1*.

The putative key residues of structural basis for the Root effect in fishes including Ser2α, Val2β, Trp4β, Ser90β, Glu95β, and Try146β were conserved in the channel catfish hemoglobins except in LA *Hbα2*, where Ser2*α* was absent. However, many amino acid residues involved in the Root effect in other fish species such as Glu140α, Ser94β, Lys144β, Gln145β, and His147β were not observed in the catfish hemoglobin genes. Instead, they were substituted by Asp140α in the LA *Hbα2* and the MN *Hbα1*, Cys94β, Ser144β, and Arg145β in the MN *Hbβ1*, and Gln147β in the MN *Hbβ4* and MN *Hbβ5*, and Phe147 in the MN *Hbβ1* respectively.

The *C*-terminal His residue in *Hbβ*, which is deemed to account for over 50% of the Bohr effect, was only conserved in channel catfish *Hbβ2* and *Hbβ3* that are 148 amino acids, one amino acid longer than all the other *Hbβ* genes of catfish. There were no His residue in the *C*-terminus of *Hbβ1*, *Hbβ4*, *Hbβ5*, and *Hbβ6*, and the His residue was substituted by Phe, Gln, Gln and Tyr in *Hbβ1*, *Hbβ4*, *Hbβ5*, and *Hbβ6*, respectively.

Cysteine residues have been reported to hold and release nitric oxide, giving the blood pigment the ability to regulate local levels of nitric oxide in the circulatory system according to needs. The channel catfish hemoglobin genes possess Cys20 in the MN *Hbα6*, Cys27 and Cys31 in the LA *Hbα1*, Cys108 and Cys115 in the LA *Hbα2*, Cys94 and Cys110 in MN *Hbβ1*, Cys32 and Cys110 in *Hbβ4*, *Hbβ5*, and *Hbβ6* of MN cluster.

3.4. Tissue expression of hemoglobin genes

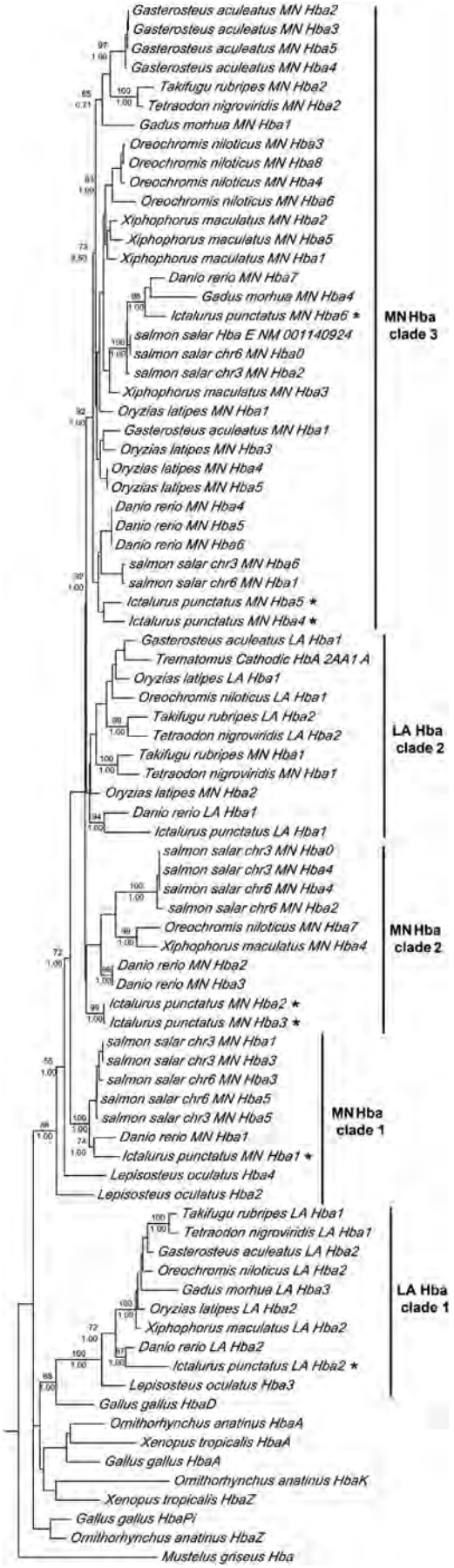
In order to assess the tissue expression profiles of hemoglobin genes, qRT-PCR was conducted to determine patterns of tissue expression. As shown in Figs. 6 and 7, all the eight *Hbα* genes and six *Hbβ* genes were widely expressed in all 10 tested tissues. However, large differences were observed in the expression of the hemoglobin genes among the tested tissues. Among the 10 tested tissues, the highest expression of *Hbα* genes was observed in trunk kidney, spleen, head kidney, skin, intestine, and muscle. Much lower expression levels were observed in the brain, gill, liver, and heart (Fig. 6). A similar expression pattern was observed with *Hbβ* genes except that the highest expression was observed in the spleen (Fig. 7).

Large variations were observed in the expression levels of the eight *Hbα* genes and six *Hbβ* genes in various tissues. In general, MN *Hbα6* was expressed at very high levels in all the tested tissues, followed by LA *Hbα1*, LA *Hbα2*, and MN *Hbα1*. MN *Hbα2* and MN *Hbα3* were expressed at much lower levels almost in all the tested tissues except in the spleen where all the *Hbα* genes were expressed at relatively high levels.

3.5. Expression of hemoglobin genes after heat stress

A study of the response to heat stress was conducted. Expression of the eight *Hbα* genes and six *Hbβ* genes after heat stress were determined by qRT-PCR to compare expression of these genes within pooled samples of heat sensitive and heat tolerant samples, with two tissues: gill and head kidney. The expression of the 14 genes in the gill is shown in Fig. 8. In the gill, all the *Hbα* and *Hbβ* genes except MN *Hbα2* and MN *Hbβ2* were significantly up-regulated in the tolerant fish. However, the extent of up-regulation varied greatly among the genes. MN *Hbα4*, MN *Hbβ4*, MN *Hbβ5*, and MN *Hbβ6* were drastically up-regulated in the tolerant fish, up 400-fold to almost 2000-fold (Fig. 8). In addition, LA *Hbα1*, MN *Hbα1*, MN *Hbα5* and MN *Hbα6* were also dramatically up-regulated in the tolerant fish, up 10–30 times (Fig. 8). In spite of the drastic up-regulation of the hemoglobin genes in the tolerant fish, most of these genes were down-regulated in the intolerant fish, and many of these significantly down-regulated in sensitive fish (Fig. 8).

The comparison of expression of the 14 hemoglobin genes in the head kidney after heat stress between tolerant and intolerant fish is shown in Fig. 9. With tolerant fish, the expression patterns in the head kidney were similar to those in the gill, i.e., drastic up-regulation of MN *Hbα4*, MN *Hbα5*, MN *Hbα6*, MN *Hbβ4*, MN *Hbβ5*, and MN *Hbβ6*. Significant up-regulation was also observed with all the other hemoglobin genes except MN *Hbα2* and MN *Hbα3*, where up-regulation was also observed in the head kidney, but not statistically significant. However,



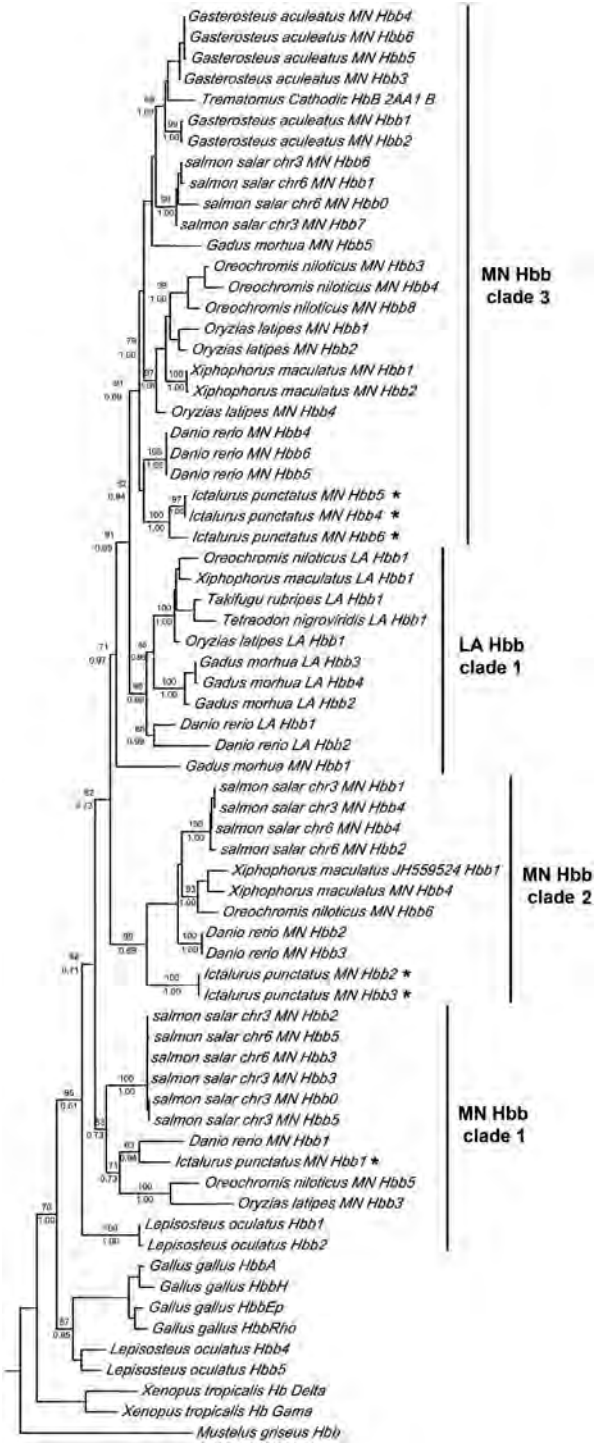


Fig. 3. Phylogenetic tree of beta hemoglobin genes. Details of phylogenetic analysis are provided in section 2. Sequences were retrieved from various databases and amino acid sequences were used for the construction of the phylogenetic tree. Values on the nodes denote bootstrap support values (above) and Bayesian posterior probabilities (below). Channel catfish hemoglobin genes were marked with asterisks.

expression patterns of many of these hemoglobin genes with sensitive fish were quite different from those in the gill. Their expression was also significantly up-regulated, in strong contrast to the situation in

Fig. 2. Phylogenetic tree of alpha hemoglobin genes. Details of phylogenetic analysis are provided in the section of Materials and Methods. Sequences were retrieved from various databases and amino acid sequences were used for the construction of the phylogenetic tree. Values on the nodes denote bootstrap support values (above) and Bayesian posterior probabilities (below). Channel catfish hemoglobin genes were marked with asterisks.

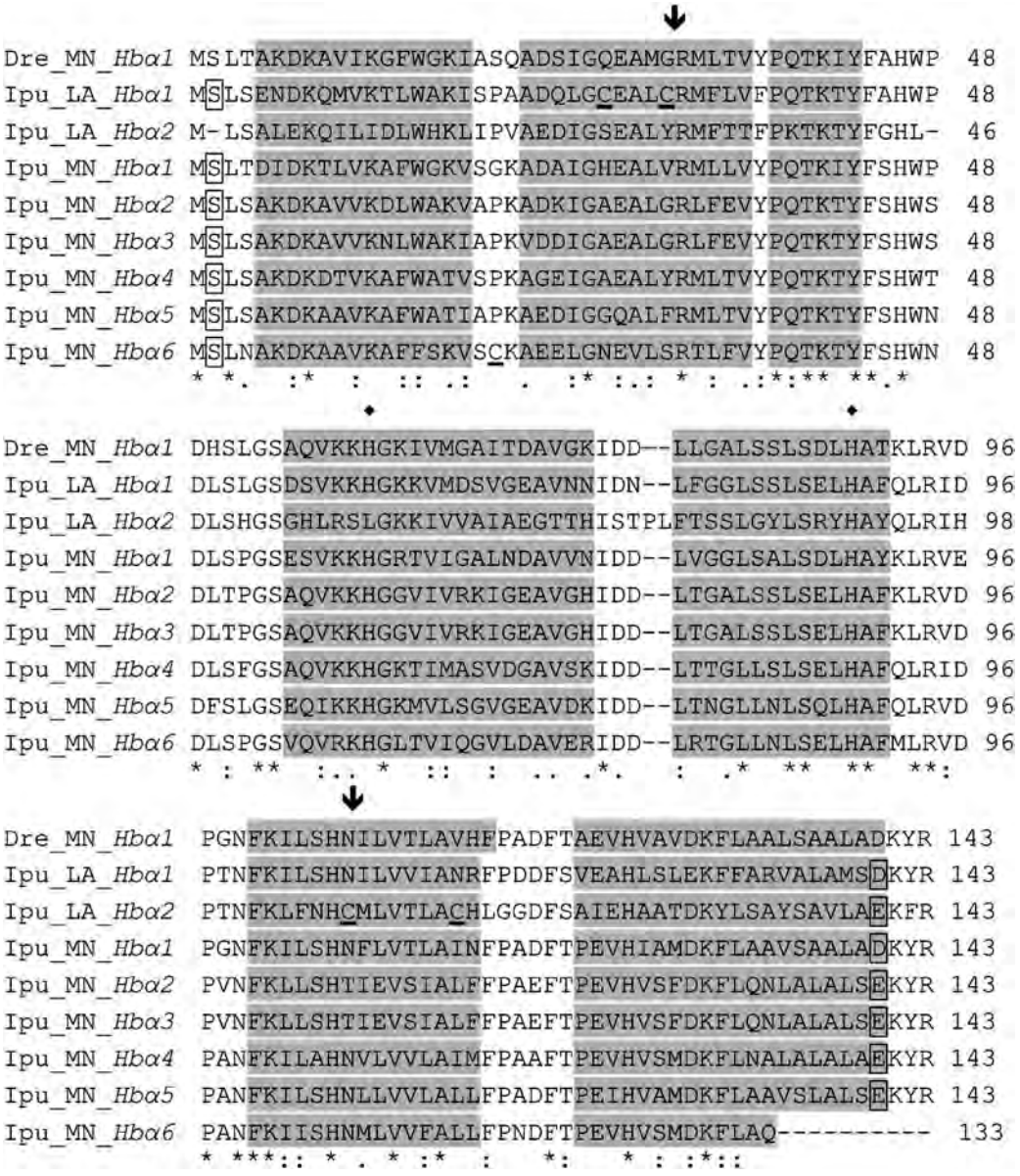


Fig. 4. Amino acid sequence alignment of the *Hbα* of zebrafish (Dre) and channel catfish (Ipu). The arrow shows the division between different exons. The amino acid similarity between all proteins is given as follows: asterisks indicate identical amino acids, colons or dots indicate similar amino acids and empty space represent absence or low degree of similarity. The gray background indicates predicated α-helical (from A to H, without D) contents of the subunits. The solid diamonds designated the heme-oxygen binding sites. The boxed amino acids are those involved in the Root effect. The underline cysteine residues (C) are involved in nitric oxide transport.

the gill, where almost all the genes were down-regulated with sensitive fish. In the head kidney, only three genes were down-regulated, i.e., MN *Hbα2*, MN *Hbα3*, and MN *Hbβ1* (Fig. 9).

4. Discussion

In the present study, we identified and characterized the hemoglobin gene repertoire in channel catfish with eight *Hbα* genes and six *Hbβ* genes, which adds to the much smaller and incomplete list of the hemoglobin genes characterized previously (Skow, 1971; Taylor et al., 1984; Yeh et al., 2006; Chen et al., 2010). The hemoglobin genes were located in two unlinked clusters: the LA cluster and the MN cluster. The two clusters are inferred to be on different chromosomes in the catfish genome (Ninwichian et al., 2012), consistent with those in the other teleost fishes with sequenced genomes (Flint et al., 2001; Gillemans et al., 2003; Maruyama et al., 2004a,b; Hardison, 2008; Borza et al., 2009; Quinn et al., 2010; Wetten et al., 2010; Opazo et al., 2013). In channel catfish, the LA cluster had two *Hbα* genes in linkage group 5, and the MN cluster contained six *Hbα* genes and six *Hbβ* genes in linkage group 23 (Ninwichian et al., 2012).

Channel catfish possessed more hemoglobin genes (14) than fugu (5), green spotted puffer (5), stickleback (13), platyfish (13), cod (9), medaka (12), and Atlantic croaker (10), but fewer hemoglobin genes than zebrafish (17), tilapia (18), common carp (16) and salmon (27) (Shelly and C.P., 1997; Flint et al., 2001; Gillemans et al., 2003; Maruyama et al., 2004a,b; Hardison, 2008; Borza et al., 2009; Quinn et al., 2010; Wetten et al., 2010; Opazo et al., 2013). However, salmon and common carp are generally regarded as tetraploid species, and therefore, their diploid equivalents would contain fewer hemoglobin genes than channel catfish. It has been proposed that the capacity of fish to colonize a wide range of habitats is directly related to their hemoglobin system and especially to their hemoglobin gene copies (Verde et al., 2006b). Zebrafish, medaka, stickleback, salmon, carp, and tilapia show a wide distribution and a diversified living environment, which could possibly reflect their adaptability offered by the large hemoglobin gene repertoire. The variation in hemoglobin gene copy number may be

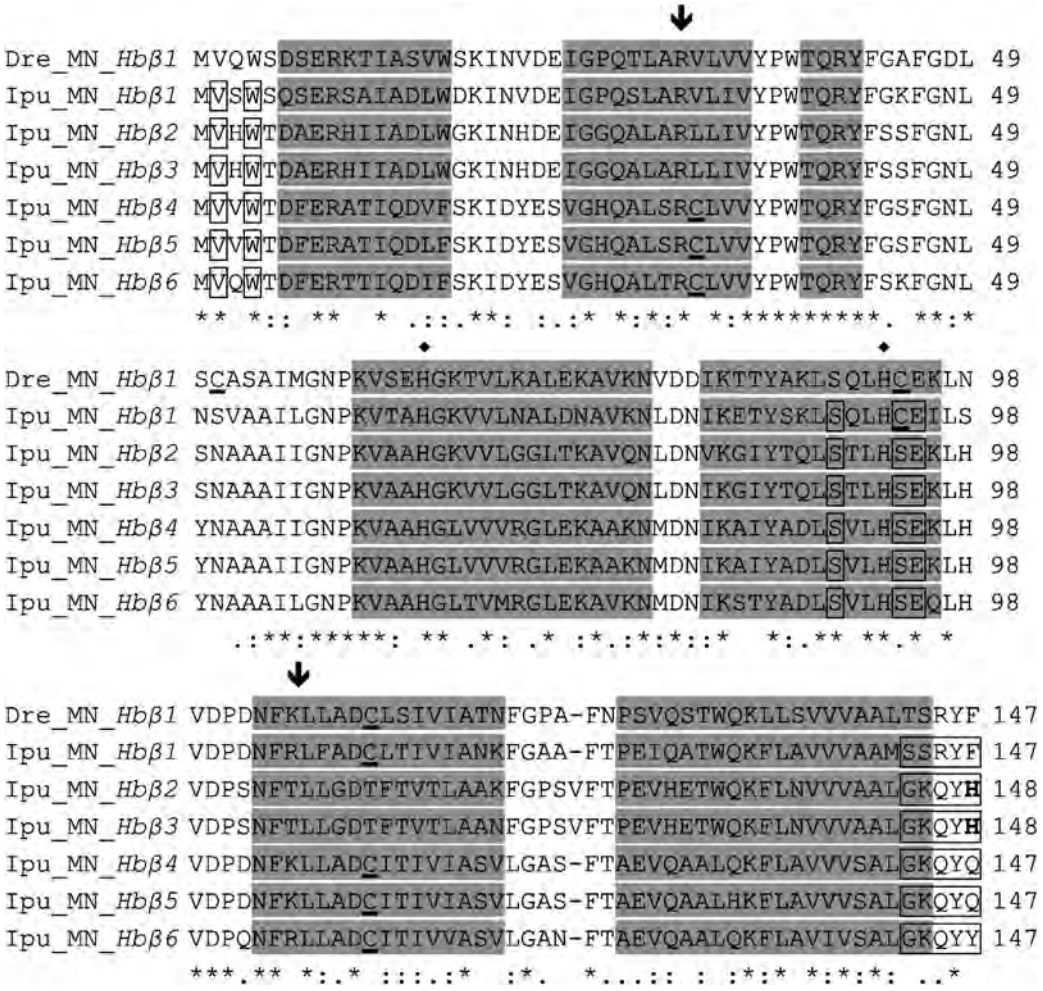


Fig. 5. Amino acid sequence alignment of the *Hbβ3* of zebrafish (Dre) and channel catfish (Ipu). The arrow shows the division between different exons. The amino acid similarity between all proteins is given as follows: asterisks indicate identical amino acids, colons or dots indicate similar amino acids and empty space represent absence or low degree of similarity. The gray background indicates predicated α-helical (from A–H, without D) contents of the subunits. The solid diamonds designated the heme-oxygen binding sites. The boxed amino acids are those involved in the Root effect. The bold amino acid letters (H) are amino acids involved in the Bohr effect. The underline cysteine residues (C) are involved in nitric oxide transport.

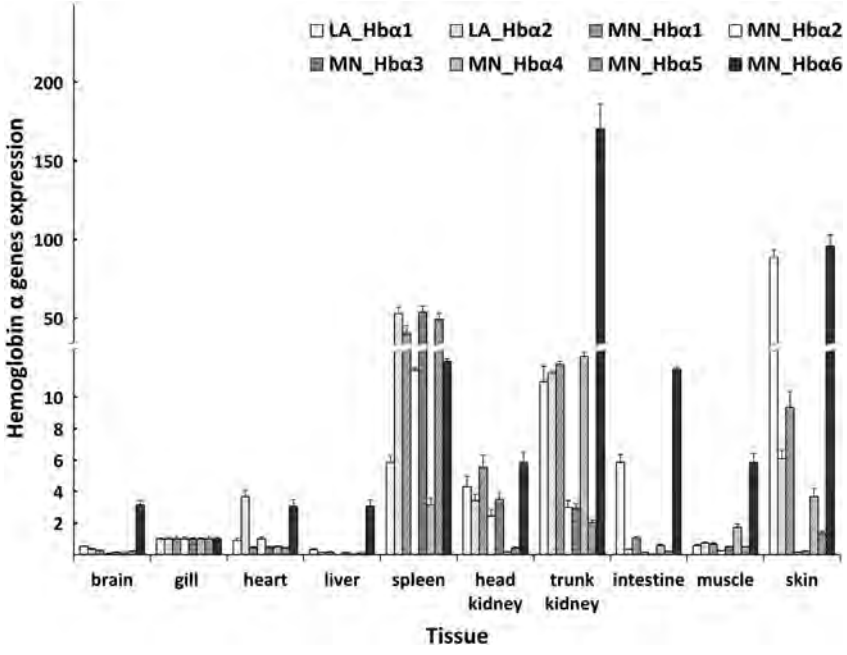


Fig. 6. Analysis of expression of *Hbα* genes in various tissues of catfish, as determined by using qRT-PCR. The Y-axis represents relative expression as normalized with expression levels of the 18S rRNA. Expression levels are expressed relative to that of the gill tissue (1×).

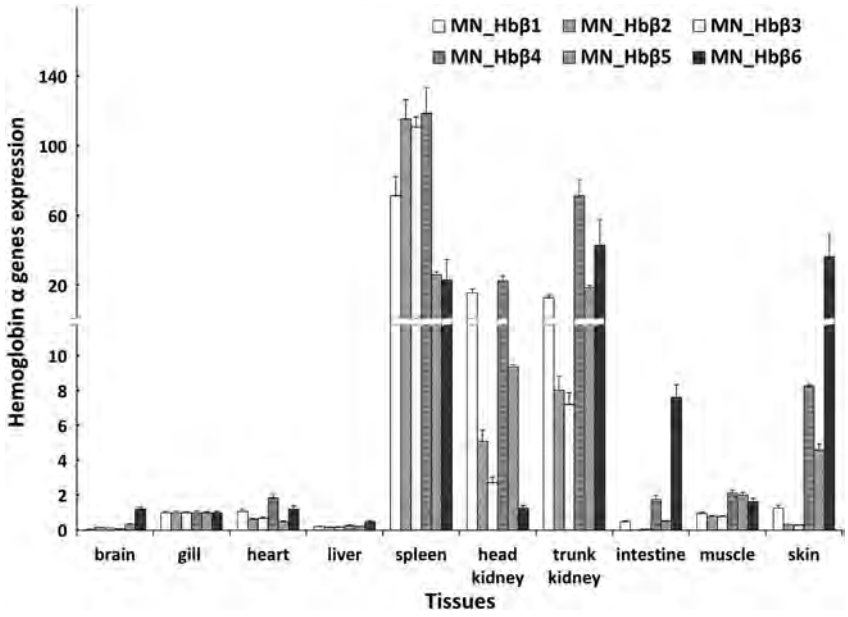


Fig. 7. Analysis of expression of *Hbβ* genes in various tissues of catfish, as determined by using qRT-PCR. The Y-axis represents relative expression as normalized with expression levels of the 18S rRNA. Expression levels are expressed relative to that of the gill tissue (1×).

a source of regulatory variation affecting physiological difference in blood oxygen transport and aerobic energy metabolism (Hoffmann and Storz, 2007). Apparently channel catfish is widely distributed in North America and is among the hardiest fish, especially in dealing with extreme temperatures, which might be reflective of its large hemoglobin gene repertoire.

The genomic organization of the channel catfish hemoglobin genes is highly similar to that of zebrafish. Accordingly, we annotated these genes the way Opazo et al. (2013) did. The highly conserved gene order and orientation of the hemoglobin genes and their flanking genes allowed establish of orthologies, and thereby allowing for proper annotation. However, phylogenetic analysis indicated that the genes carrying the same names may not be orthologous. For instance, MN *Hbα1* of catfish is placed in the same clade with the zebrafish MN *Hbα1*, but the same clade contained five paralogous genes in Atlantic salmon. Similarly, MN *Hbα4* and MN *Hbα5* of catfish are in the same clade with the zebrafish MN *Hbα4* and MN *Hbα5* genes, but apparently these genes were paralogous, perhaps derived from lineage-specific duplications. It is obvious from the phylogenetic analysis that MN *Hbα2* and MN *Hbα3* are paralogous duplicates, MN *Hbα4* and MN *Hbα5* are paralogous duplicates. Similarly, among the six catfish beta hemoglobin genes, MN *Hbβ4*, MN *Hbβ5*, and MN *Hbβ6* are paralogous, and MN *Hbβ2* and MN *Hbβ3* are paralogous, as they share the highest similarities within species.

One striking feature of the catfish hemoglobin gene organization is the loss of the *Hbβ* genes in the LA cluster. The overall flanking gene

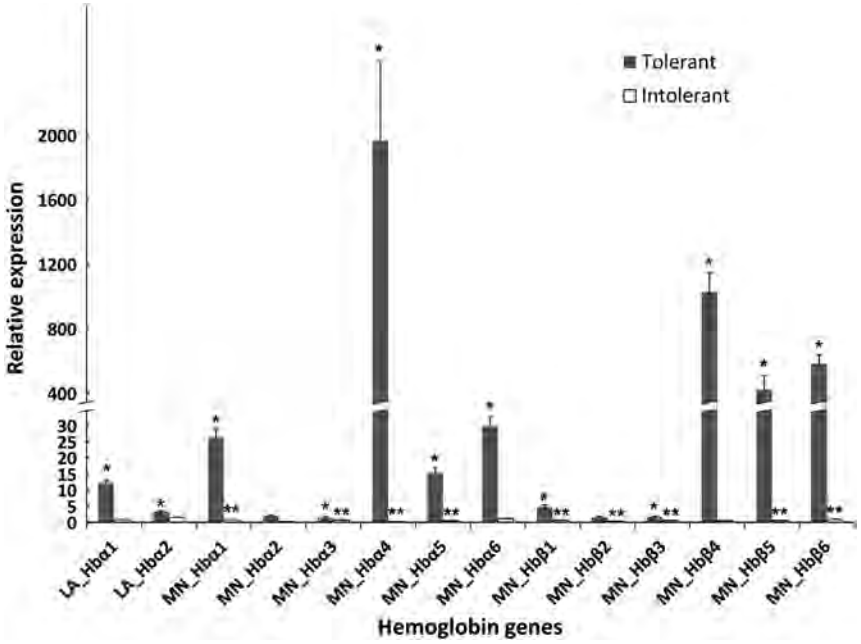


Fig. 8. Expression of hemoglobin genes in the gill in heat tolerant and intolerant fish. Relative hemoglobin gene expression was expressed as fold change over control samples taken at the same time intervals as normalized to change in expression in the 18S rRNA control. The bars indicated mean expression of 3 tested pools (15 fish each) ± SE. Single asterisks indicate statistical significance ($P < 0.05$) for up-regulated genes, and double asterisks indicate statistical significance ($P < 0.05$) for down-regulated genes.

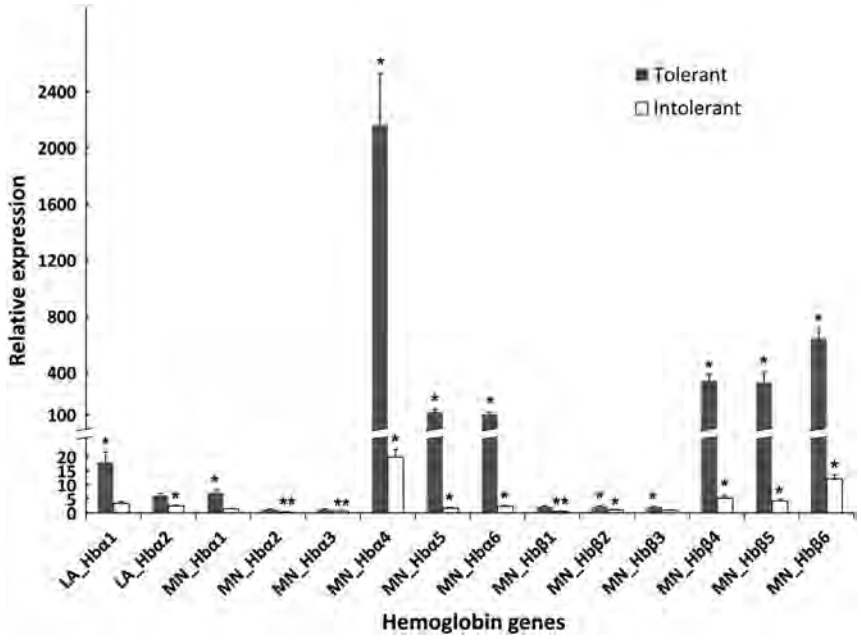


Fig. 9. Expression of hemoglobin genes in the head kidney in heat tolerant and intolerant fish. Relative hemoglobin gene expression was expressed as fold change over control samples taken at the same time intervals as normalized to change in expression in the 18S rRNA control. The bars indicated mean expression of 3 tested pools (15 fish each) ± SE. Single asterisks indicate statistical significance ($P < 0.05$) for up-regulated genes, and double asterisks indicate statistical significance ($P < 0.05$) for down-regulated genes.

order of the LA cluster is well conserved, confirming the orthologies of the LA cluster. However, with the catfish LA cluster, only LA *Hbα1* and LA *Hbα2* genes were found without *Hbβ* gene in the cluster (Fig. 1). Previous studies have shown that teleost fish may have gone through a third round of whole genome duplication 300–400 million years ago, and therefore there are two hemoglobin gene clusters in the genome. Specific gene loss after the whole genome duplication has been previously reported in the LA cluster. For instance, in Atlantic salmon, all the hemoglobin genes from one of the duplicated LA clusters were lost after the fourth round of whole genome duplication (Quinn et al., 2010). It has been documented that the pre-WGD hemoglobin gene cluster of teleost fish contained at least two *Hbα* genes and two *Hbβ* genes, and after WGD but before divergence among different teleost species one or both of two ancestral *Hbβ* paralogs in the LA cluster could be lost (Opazo et al., 2013). Similar to the situation in catfish, the *Hbβ* genes in the LA cluster were also lost in stickleback in teleost species (Hardison, 2008; Opazo et al., 2013), suggesting that both of the two LA *Hbβ* paralogs were lost in specific lineages after WGD (Hoffmann and Storz, 2007; Quinn et al., 2010; Opazo et al., 2013).

To date several fish species have been clearly demonstrated their hemoglobin gene tetramers, such as three tetramers Hb1 ($\alpha^1{}_2\beta^1{}_2$), Hb2 ($\alpha^2{}_2\beta^2{}_2$), and Hb3 ($\alpha^1{}_2\beta^2{}_2$) in adult cod (Verde et al., 2006a), and three tetramers CI ($\alpha^1\alpha^2\beta^1{}_2$), CII ($\alpha^1\alpha^2\beta^1\beta^2$), and CIII ($\alpha^1\alpha^2\beta^2{}_2$) in common carp (Ohkudo et al., 1994). However the exact compositions of catfish hemoglobin tetramers, as well as oxygen affinities of distinct hemoglobin are unknown. There was also unclear evidence for a switch of distinct hemoglobin genes on the genetic levels. So it was still unknown about how the channel catfish LA *Hbα1* and LA *Hbα2* constitute the hemoglobin tetramer to perform the function of oxygen transportation. Due to the character of distinct hemoglobin gene switch from embryo to larval, larval to adult, the channel catfish LA *Hbα1* and LA *Hbα2* genes, which were inferred to be expressed in the embryonic and larval stage, could combine the other *Hbβ* genes in the MN cluster to constitute hemoglobin tetramer, or could not constitute tetramer with other *Hbβ* gene and just acted as a single genes to absorb oxygen from blood to organ tissues.

The orientation of hemoglobin genes in the LA cluster varies among various fish. In channel catfish, the LA cluster genes showed the same forward orientation arrangement as in zebrafish, tilapia, medaka, platyfish, and stickleback, but were different from that in fugu and green spotted pufferfish with the reverse orientation. In Atlantic cod, the mixed orientation with both forward and reverse orientation was observed (Hardison, 2008; Opazo et al., 2013). The orientation of hemoglobin genes in the MN cluster of channel catfish was same as that of zebrafish, but different from that of several other fish species with sequenced genomes such as medaka, fugu, Tetraodon, carp, Atlantic salmon, and stickleback (Hardison, 2008; Opazo et al., 2013). In the teleost such as Atlantic salmon, carp and zabrafish, a finding has been reported that adult *Hbα* gene is adjacently linked to adult *Hbβ* genes with a head to head orientation in transcriptional polarity (McMorrow et al., 1996; Chan et al., 1997; Miyata and Aoki, 1997; Chu et al., 2006).

Although teleost fishes harbor various numbers of hemoglobin genes, their gene structures are highly conserved with three exons and two introns, as reported in carp (Takeshita et al., 1984; Miyata and Aoki, 1997), medaka (Maruyama et al., 2004a; Wawrowski et al., 2011), zebrafish (Chan et al., 1997; Tiedke et al., 2011), Atlantic cod (Borza et al., 2009; Halldorsdottir and Arnason, 2009; Wetten et al., 2010), and flounder (Lu et al., 2011).

Not only the gene structure and organization are highly conserved, but important amino acid residues are also well conserved among various hemoglobin genes. For instance, Root effect is functional properties of fish hemoglobin genes (Vergara et al., 2010). It has been shown that channel catfish exhibited weak Root effect, and the channel catfish hemoglobin genes retained almost half the number of key residues of the structural basis for the Root effect.

The Bohr effect, which means hemoglobin–oxygen affinity decreases with carbon dioxide content increasing, is another phenomena for teleost fish (Gillen and Riggs, 1977). It is widely accepted that overall histidine content in the hemoglobin molecule correlates with the Bohr effect, with the *C*-terminal histidine residue accounting for up to 50% of the effect (Shih et al., 1993). In channel catfish, only MN *Hbβ2* and MN *Hbβ3* had a histidine in the 146th amino acid, and the histidine in others *Hbβ* genes were substituted by glutamine and phenylalanine, suggesting the reduced Bohr effect with catfish.

In teleost, embryonic hemoglobin genes have been identified in rainbow trout, zebrafish, tilapia and medaka, and they were quite different from adult hemoglobin genes (Chan et al., 1997; Brownlie et al., 2003; Maruyama et al., 2004a,b; Tiedke et al., 2011; Opazo et al., 2013).

However, the embryonic and adult hemoglobin genes were identified using various methodologies among various species. For example, the rainbow trout and zebrafish embryonic and adult hemoglobin genes were identified based on the difference in molecular characteristics and the specificity in developmental expression profile (Maruyama et al., 1999; Ganis et al., 2012). However, the medaka hemoglobin genes were identified purely based on molecular phylogenetic analysis, as compared with the embryonic and adult hemoglobin genes of rainbow trout and zebrafish (Maruyama et al., 2004b). In zebrafish, hemoglobin gene expression has been shown to exhibit a clear developmental stage-specific expression pattern. Different genes can be active at the embryonic, fetal or adult stages to generate slightly different forms of hemoglobin genes (Maruyama et al., 2004b; Ganis et al., 2012). MN *Hbα1*, MN *Hbα2*, MN *Hbα3*, MN *Hbβ1*, MN *Hbβ2* and MN *Hbβ3* genes were nearly exclusively expressed in mature and adult stage (1 year of age), LA *Hbβ2* gene was exclusively expressed in embryonic stage, and others including LA *Hbα1*, LA *Hbα2*, LA *Hbβ1*, MN *Hbα4*, MN *Hbα5*, MN *Hbα6*, MN *Hbα7*, MN *Hbβ4*, MN *Hbβ5* and MN *Hbβ6* were expressed through embryonic and larval stage (Ganis et al., 2012). As the objectives of this study were to determine expression of hemoglobin genes under heat stress, we did not have samples representing different developmental stages to determine their expression patterns. Therefore, we could only make inferences based on structural similarities through phylogenetic analysis (Fig. 2 and 3). Channel catfish LA *Hbα1*, LA *Hbα2*, MN *Hbα4*, MN *Hbα5*, MN *Hbα6*, MN *Hbβ4*, MN *Hbβ5* and MN *Hbβ6* were grouped together with zebrafish embryonic hemoglobin genes; and channel catfish MN *Hbα1*, MN *Hbα2*, MN *Hbα3*, MN *Hbβ1*, MN *Hbβ2* and MN *Hbβ3* were grouped together with the zebrafish adult hemoglobin genes. Furthermore, they formed a monophyletic group among all the teleost hemoglobin genes previously investigated. The adult hemoglobin genes were clearly discriminated in phylogeny from the embryonic hemoglobin genes group. Therefore, for the sake of discussion, here infer, based on the phylogeny analysis, that channel catfish LA *Hbα1*, LA *Hbα2*, MN *Hbα4*, MN *Hbα5*, MN *Hbα6*, MN *Hbβ4*, MN *Hbβ5* and MN *Hbβ6* may be normally expressed in embryonic/larval stage, and channel catfish MN *Hbα1*, MN *Hbα2*, MN *Hbα3*, MN *Hbβ1*, MN *Hbβ2* and MN *Hbβ3* may be normally expressed in adult stage. Such speculations need to be confirmed in future studies.

The results of qRT-PCR of hemoglobin expression in various tested tissues showed that spleen, head kidney, and trunk kidney are the major tissues expressing the eight *Hbα* genes and six *Hbβ* genes. Previous histological studies in fish revealed that the kidney and the spleen are the main organs forming blood (Boomker, 1979; Lu et al., 2011), and the cells forming the erythroid and granuloid lineage are mostly found in kidney, while thrombocytes and monocytes are formed in head kidney and spleen in these species (Boomker, 1981a,b). It has been previously demonstrated that the initial embryonic hemoglobin expression is involved in primitive hematopoiesis which is located in intermediated cell mass, while adult hemoglobin in definitive hematopoiesis which includes spleen and kidney after hatch (Maruyama et al., 1999, 2002). In this study, all hemoglobin genes, regardless of the embryonic or adult hemoglobin genes, were highly expressed in spleen and kidney. In addition to spleen and kidney, LA *Hbα1*, LA *Hbα2*, MN *Hbα1*, MN *Hbα6*, MN *Hbβ4*, MN *Hbβ5* and MN *Hbβ6*, which presumably the embryonic hemoglobin genes, were also highly expressed in the skin. It is possible that the tested catfish were fingerlings which tend to have thinner skin and breathe through their skin may have favored more efficient oxygen uptake (McDonald and McMahon, 1977; Oikawa and Itazawa, 1985; Rombough, 2007).

It was noted that MN *Hbα6* and *MN Hbβ6* displayed very high levels of expression in all tested tissues. It is possible that MN *Hbα6* and MN *Hbβ6*, inferred to be embryonic hemoglobin genes in catfish, were highly expressed in larval stage, so it would transport much more oxygen to fulfill the demands of growth and metabolism in the larval fish (Becker, 1983; Smith, 1985). Therefore, it appeared that MN *Hbα6* and *MN Hbβ6* are crucially important for oxygen transport under heat stress conditions. Similarly, LA *Hbα1*, also inferred to be embryonic, was expressed in a moderate level in intestine. The posterior intestine of channel catfish still keeps the respiratory function in the fingerling stage, with histological structures of short microvilli and goblet cells, similar to the situation of Callichthyidae species that are intestinal respiratory species (Krementz and Chapman, 1975; Sis et al., 1979; McMahon and Burggren, 1987; Podkowa and Goniakowska-Witalinska, 2002). Channel catfish and Callichthyidae species are from the same order Siluriformes (Sullivan et al., 2006). It is not surprising that channel catfish fingerlings have conserved the intestinal respiration. The relatively high expression of LA *Hbα1* could be reflective of the intestinal respiration.

The LA *Hbα2* was expressed at a low level in the catfish heart. It is known that cardiac muscle is a highly oxidative tissue, and suboptimal delivery of oxygen to working cardiac muscle would be expected to impact heart contractility and pressure generation (Pelster and Burggren, 1996). The channel catfish sinus venosus and atrium oxygen demands rely on the luminal venous blood supply, and oxygen demands in ventricle rely on the superficial coronary vessels (Farrel et al., 2012). However, myoglobin, which could absorb oxygen from luminal venous blood and store oxygen, has been shown to be necessary for cardiac function during development (Vlecken et al., 2009). LA *Hba2* could be speculated to be responsible for the oxygen transport from the blood in coronary vessels to the ventricle tissues.

Taken all the observations together, it appeared that heat stress induced high expression of hemoglobin genes could be highly relevant to the observed heat tolerance. Temperature increases tend to aggravate hypoxia and its consequences in ectotherms. On the one hand metabolic activity typically increases, and on the other hand blood oxygen affinity decreases. Moreover, elevations of temperature decrease ambient oxygen tension in water. In this study, eight *Hbα* genes and six *Hbβ* genes were induced under heat stress, and especially in tolerant fish these genes were expressed in much higher levels than in intolerant fish. The hemoglobin genes that were most significantly induced under heat stress conditions in tolerant fish were inferred to be embryonic hemoglobin genes. Highly induced expression of these hemoglobin genes allowed expression of more hemoglobin in tolerant fish to transport oxygen from gill and skin to inner organs, while in intolerant fish, expression of these genes were down-regulated. Such a strong correlation of high expression of hemoglobin genes and tolerance to heat stress suggested the importance of hemoglobin genes in adaptation of catfish to its environment including high temperature and low oxygen situations.

5. Conclusions

Channel catfish possessed six *Hbα* genes and six *Hbβ* genes in the MN cluster, and two *Hbα* genes in the LA cluster. The channel catfish hemoglobin genes were normally expressed in most tested tissues and with higher expression in hematopoietic organ spleen and kidney. A striking observation was that a subset of the 14 hemoglobin genes, particularly the embryonic hemoglobin genes as inferred from phylogenetic analysis, were highly up-regulated in heat tolerant fish, but were down-regulated in heat intolerant fish, suggesting their involvement in offering the heat tolerance to the tested catfish.

Acknowledgments

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References

Altschul, S.F., Madden, T.L, Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Aursnes, I.A., Rishovd, A.L., Karlsen, H.E., Gjoen, T., 2011. Validation of reference genes for quantitative RT-qPCR studies of gene expression in Atlantic cod (*Gadus morhua* l.) during temperature stress. BMC Res. Notes 4, 104.

Becker, G.C., 1983. Fishes of Wisconsin. University of Wisconsin Press, Madison.

Boomker, J., 1979. The haemocytology and histology of the haemopoietic organs of South African fresh water fish. I. The haemopoietic organs of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort J. Vet. Res. 46, 217–222.

Boomker, J., 1981a. The haematology and histology of the haemopoietic organs of South African freshwater fish. III. The leucocytes, plasma cells and macrophages of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort J. Vet. Res. 48, 185–193.

Boomker, J., 1981b. The haemocytology and histology of the haemopoietic organs of South African freshwater fish IV. Ultrastructure of some cells of *Clarias gariepinus* and *Sarotherodon mossambicus*. Onderstepoort J. Vet. Res. 48, 195–205.

Borza, T., Stone, C., Gamperl, A.K., Bowman, S., 2009. Atlantic cod (*Gadus morhua*) hemoglobin genes: multiplicity and polymorphism. BMC Genet. 10, 51.

Brownlie, A., Hersey, C., Oates, A.C., Paw, B.H., Falick, A.M., Witkowska, H.E., Flint, J., Higgs, D., Jessen, J., Bahary, N., Zhu, H., Lin, S., Zon, L., 2003. Characterization of embryonic globin genes of the zebrafish. Dev. Biol. 255, 48–61.

Chan, F.Y., Robinson, J., Brownlie, A., Shivdasani, R.A., Donovan, A., Brugnara, C., Kim, J., Lau, B.C., Witkowska, H.E., Zon, L.I., 1997. Characterization of adult alpha- and beta-globin genes in the zebrafish. Blood 89, 688–700.

Chen, F., Lee, Y., Jiang, Y., Wang, S., Peatman, E., Abernathy, J., Liu, H., Liu, S., Kucuktas, H., Ke, C., Liu, Z., 2010. Identification and characterization of full-length cDNAs in channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*). PloS One 5, e11546.

Chu, W., Wei, Y., Qian, R., Yu, X., Yu, L., 2006. Characterization of the 5′-to-5′linked adult alpha- and beta-globin genes from three sciaenid fish species (*Pseudosciaena crocea*, *Sciaenops ocellatus*, *Nibea miichthioides*). Comp. Biochem. Physiol. Part D Genomics Proteomics 1, 319–327.

Dalvi, R.S., Pal, A.K., Tiwari, L.R., Baruah, K., 2012. Influence of acclimation temperature on the induction of heat-shock protein 70 in the catfish *Horabagrus brachysoma* (Gunther). Fish Physiol. Biochem. 38, 919–927.

Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Farrel, A.P., Farrel, N.D., Jourdan, H., Cox, G.K., 2012. A perspective on the evolution of the coronary circulation in fishes and the transition to terrestrial life. Ontogeny and Phylogeny of the Vertebrate Heart.Springer.

Flicek, P., Ahmed, I., Amode, M.R., Barrell, D., Beal, K., Brent, S., Carvalho-Silva, D., Clapham, P., Coates, G., Fairley, S., Fitzgerald, S., Gil, L., Garcia-Giron, C., Gordon, L., Hourlier, T., Hunt, S., Juettemann, T., Kahari, A.K., Keenan, S., Komorowska, M., Kulesha, E., Longden, I., Maurel, T., McLaren, W.M., Muffato, M., Nag, R., Overduin, B., Pignatelli, M., Pritchard, B., Pritchard, E., Riat, H.S., Ritchie, G.R., Ruffier, M., Schuster, M., Sheppard, D., Sobral, D., Taylor, K., Thormann, A., Trevanion, S., White, S., Wilder, S.P., Aken, B.L., Birney, E., Cunningham, F., Dunham, I., Harrow, J., Herrero, J., Hubbard, T.J., Johnson, N., Kinsella, R., Parker, A., Spudich, G., Yates, A., Zadissa, A., Searle, S.M., 2013. Ensembl 2013. Nucleic Acids Res. 41, D48–D55.

Flint, J., Tufarelli, C., Peden, J., Clark, K., Daniels, R.J., Hardison, R., Miller, W., Philipsen, S., Tan-Un, K.C., McMorrow, T., Frampton, J., Alter, B.P., Frischauf, A.M., Higgs, D.R., 2001. Comparative genome analysis delimits a chromosomal domain and identifies key regulatory elements in the alpha globin cluster. Hum. Mol. Genet. 10, 371–382.

Ganis, J.J., Hsia, N., Trompouki, E., de Jong, J.L., DiBiase, A., Lambert, J.S., Jia, Z., Sabo, P.J., Weaver, M., Sandstrom, R., Stamatoyannopoulos, J.A., Zhou, Y., Zon, L.I., 2012. Zebrafish globin switching occurs in two developmental stages and is controlled by the LCR. Dev. Biol. 366, 185–194.

Gillemans, N., McMorrow, T., Tewari, R., Wai, A.W., Burgtorf, C., Drabek, D., Ventress, N., Langeveld, A., Higgs, D., Tan-Un, K., Grosveld, F., Philipsen, S., 2003. Functional and comparative analysis of globin loci in pufferfish and humans. Blood 101, 2842–2849.

Gillen, R.G., Riggs, A., 1977. The enhancement of the alkaline Bohr effect of some fish hemoglobins with adenosine triphosphate. Arch. Biochem. Biophys. 183, 678–685.

Halldorsdottir, K., Arnason, E., 2009. Organization of a beta and alpha globin gene set in the teleost Atlantic cod, *Gadus morhua*. Biochem. Genet. 47, 817–830.

Hardison, R.C., 2008. Globin genes on the move. J. Biol. 7, 35.

Hoffmann, F.G., Storz, J.F., 2007. The alphaD-globin gene originated via duplication of an embryonic alpha-like globin gene in the ancestor of tetrapod vertebrates. Mol. Biol. Evol. 24, 1982–1990.

Hori, T.S., Gamperl, A.K., Afonso, L.O., Johnson, S.C., Hubert, S., Kimball, J., Bowman, S., Rise, M.L., 2010. Heat-shock responsive genes identified and validated in Atlantic cod (*Gadus morhua*) liver, head kidney and skeletal muscle using genomic techniques. BMC Genomics 11, 72.

Imsland, A.K., Brix, O., Navdal, G., Samuelsen, E.N., 1997. Hemoglobin Genotypes in Turbot (*Scophthalmus maximus* Rafinesque), their oxygen affinity properties and relation with growth. Comp. Biochem. Physiol. A Physiol. 116, 157–165.

Jobb, G., von Haeseler, A., Strimmer, K., 2004. TREEFINDER: a powerful graphical analysis environment for molecular phylogenetics. BMC Evol. Biol. 4, 18.

Kamshilov, I.M., Kamshilova, T.B., 2007. Effect of temperature on functional properties of hemoglobin of crucian carp (*Carassius carassius*). J. Ichthyol. 47, 469–472.

Katoh, K., Kuma, K., Toh, H., Miyata, T., 2005. MAFFT version 5: improvement in accuracy of multiple sequence alignment. Nucleic Acids Res. 33, 511–518.

Krementz, A.B., Chapman, G.B., 1975. Ultrastructure of the posterior half of the intestine of the channel catfish Ictalurus punctatus. J. Morphol. 145, 441–482.

Lassmann, T., Sonnhammer, E.L., 2005. Automatic assessment of alignment quality. Nucleic Acids Res. 33, 7120–7128.

Lassmann, T., Sonnhammer, E.L., 2006. Kalign, Kalignvu and Mumsa: web servers for multiple sequence alignment. Nucleic Acids Res. 34, W596–W599.

Letunic, I., Doerks, T., Bork, P., 2012. SMART 7: recent updates to the protein domain annotation resource. Nucleic Acids Res. 40, D302–D305.

Liu, S., Zhang, Y., Zhou, Z., Waldbieser, G., Sun, F., Lu, J., Zhang, J., Jiang, Y., Zhang, H., Wang, X., Rajendran, K.V., Khoo, L., Kucuktas, H., Peatman, E., Liu, Z., 2012. Efficient assembly and annotation of the transcriptome of catfish by RNA-Seq analysis of a doubled haploid homozygote. BMC Genomics 13, 595.

Liu, S., Wang, X., Sun, F., Zhang, J., Feng, J., Liu, H., Rajendran, K.V., Sun, L., Zhang, Y., Jiang, Y., Peatman, E., Kaltenboeck, L., Kucuktas, H., Liu, Z., 2013. RNA-Seq reveals expression signatures of genes involved in oxygen transport, protein synthesis, folding, and degradation in response to heat stress in catfish. Physiol. Genomics 45, 462–476.

Lu, W., Mayolle, A., Cui, G., Luo, L., Balment, R.J., 2011. Molecular characterization and expression of alpha-globin and beta-globin genes in the euryhaline flounder (*Platichthys flesus*). Evidence-Based Complementary and Alternative Medicine: eCAM. 965153.

Maruyama, K., Yasumasu, S., Iuchi, I., 1999. Characterization and expression of embryonic globin in the rainbow trout, *Oncorhynchus mykiss*: intra-embryonic initiation of erythropoiesis. Develop. Growth Differ. 41, 589–599.

Maruyama, K., Yasumasu, S., Iuchi, I., 2002. Characterization and expression of embryonic and adult globins of the teleost *Oryzias latipes* (medaka). J. Biochem. 132, 581–589.

Maruyama, K., Yasumasu, S., Iuchi, I., 2004a. Evolution of globin genes of the medaka *Oryzias latipes* (Euteleostei; Beloniformes; Oryziinae). Mech. Dev. 121, 753–769.

Maruyama, K., Yasumasu, S., Naruse, K., Mitani, H., Shima, A., Iuchi, I., 2004b. Genomic organization and developmental expression of globin genes in the teleost *Oryzias latipes*. Gene 335, 89–100.

McDonald, D.G., McMahon, B.R., 1977. Respiratory development in Arctic char *Salvelinus alpinus* under condtions of normoxia and chronic hypoxia. Can. J. Zool. 55, 1461–1467.

McMahon, B.R., Burggren, W.W., 1987. Respiratory physiology of intestinal air breathing in the teleost fish *Misgurnus anguillicaudatus*. J. Exp. 133.

McMorrow, T., Wagner, A., Deryckere, F., Gannon, F., 1996. Structural organization and sequence analysis of the globin locus in Atlantic salmon. DNA Cell Biol. 15, 407–414.

Methling, C., Aluru, N., Vijayan, M.M., Steffensen, J.F., 2010. Effect of moderate hypoxia at three acclimation temperatures on stress responses in Atlantic cod with different haemoglobin types. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 156, 485–490.

Miller, M.A., Holder, M.T., Vos, R., Midford, P.E., Liebowitz, T., Chan, L., Hoover, P., Warnow, T., 2009. The CIPRES portals. CIPRES (2009-08-04. URP: <http://www.phylo.org/sub_sections/portal>. (Archived by WebCite(r) at <http://www.webcitaction.org/5imQ1JeQa>) (accessed 04.08.09)).

Miyata, M., Aoki, T., 1997. Head-to-head linkage of carp alpha- and beta-globin genes. Biochim. Biophys. Acta 1354, 127–133.

Muffato, M., Louis, A., Poisnel, C.E., Roest, C.M., 2010. Genomicus: a database and a browser to study gene synteny in modern and ancestral genomes. Bioinformatics 26, 1119–11121.

Ninwichian, P., Peatman, E., Liu, H., Kucuktas, H., Somridhivej, B., Liu, S., Li, P., Jiang, Y., Sha, Z., Kaltenboeck, L., Abernathy, J.W., Wang, W., Chen, F., Lee, Y., Wong, L., Wang, S., Lu, J., Liu, Z., 2012. Second-generation genetic linkage map of catfish and its integration with the BAC-based physical map. G3 (Bethesda) 2, 1233–1241.

Ohkudo, N., Watabe, S., Oshiro, T., Takashima, F., Nakajima, H., 1994. Subunit structures of multiple hemoglobins in carp. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol 164, 88.

Oikawa, S., Itazawa, Y., 1985. Gill and body-surface areas of the carp in relation to bodymass, with special reference to the metabolism–size relationship. J. Exp. Biol. 117, 1–14.

Opazo, J.C., Butts, G.T., Nery, M.F., Storz, J.F., Hoffmann, F.G., 2013. Whole-genome duplication and the functional diversification of teleost fish hemoglobins. Mol. Biol. Evol. 30, 140–153.

Patel, V.S., Cooper, S.J., Deakin, J.E., Fulton, B., Graves, T., Warren, W.C., Wilson, R.K., Graves, J.A., 2008. Platypus globin genes and flanking loci suggest a new insertional model for beta-globin evolution in birds and mammals. BMC Biol. 6, 34.

Pelster, B., Burggren, W.W., 1996. Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebrafish (*Danio rerio*). Circ. Res. 79, 358–362.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Podkowa, D., Goniakowska-Witalinska, L., 2002. Adaptations to the air breathing in the posterior intestine of the catfish (*Corydoras aeneus*). The histological and ultralstructural study. 50, 69–82.

Portner, H.O., 2001. Climate change and temperature-dependent biogeography: oxygen limitation of thermal tolerance in animals. Naturwissenschaften 88, 137–146.

Qin, H., Guo, Y., Zhang, C., Zhang, L., Li, M., Guan, P., 2012. The expression of neuroglobin in astrocytoma. Brain Tumor Pathol. 29, 10–16.

Quinn, N.L., Boroevich, K.A., Lubieniecki, K.P., Chow, W., Davidson, E.A., Phillips, R.B., Koop, B.F., Davidson, W.S., 2010. Genomic organization and evolution of the Atlantic salmon hemoglobin repertoire. BMC Genomics 11, 539.

Quinn, N.L., McGowan, C.R., Cooper, G.A., Koop, B.F., Davidson, W.S., 2011a. Identification of genes associated with heat tolerance in Arctic charr exposed to acute thermal stress. Physiol. Genomics 43, 685–696.

Quinn, N.L., McGowan, C.R., Cooper, G.A., Koop, B.F., Davidson, W.S., 2011b. Ribosomal genes and heat shock proteins as putative markers for chronic, sublethal heat stress in Arctic charr: applications for aquaculture and wild fish. Physiol. Genomics 43, 1056–1064.

Ramakers, C., Ruijter, J.M., Deprez, R.H., Moorman, A.F., 2003. Assumption-free analysis of quantitative real-time polymerase chain reaction (PCR) data. Neurosci. Lett. 339, 62–66.
Rombough, P., 2007. The functional ontogeny of the teleost gill: which comes first, gas or ion exchange. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 148, 732–742.
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Hohna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.
Ruijter, J.M., Ramakers, C., Hoogaars, W.M., Karlen, Y., Bakker, O., van den Hoff, M.J., Moorman, A.F., 2009. Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 37, e45.
Salamov, A.A., Solovyev, V.V., 2000. Ab initio gene finding in drosophila genomic DNA. Genome Res. 10.
Sayers, E.W., Barrett, T., Benson, D.A., Bolton, E., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M., Dicuccio, M., Federhen, S., Feolo, M., Fingerman, I.M., Geer, L.Y., Helmberg, W., Kapustin, Y., Krasnov, S., Landsman, D., Lipman, D.J., Lu, Z., Madden, T.L., Madej, T., Maglott, D.R., Marchler-Bauer, A., Miller, V., Karsch-Mizrachi, I., Ostell, J., Panchenko, A., Phan, L., Pruitt, K.D., Schuler, G.D., Sequeira, E., Sherry, S.T., Shumway, M., Sirotkin, K., Slotta, D., Souvorov, A., Starchenko, G., Tatusova, T.A., Wagner, L., Wang, Y., Wilbur, W.J., Yaschenko, E., Ye, J., 2012. Database resources of the National Center for Biotechnology Information. Nucleic Acids Res. 40, D13–D25.
Schmidt-Nielsen, K., 1997. Animal physiology, adaptation and environment, fifth edition. Cambridge University Press.
Shelly, D.A., C.P., M., 1997. Hemoglobin polymorphism in the Atlantic croaker, *Micropogon undulatus*. Comp. Biochem. Physiol. A Physiol. 118, 1419–1428.
Shih, D.T., Luisi, B.F., Miyazaki, G., Perutz, M.F., Nagai, K., 1993. A mutagenic study of the allosteric linkage of His(HC3)146 beta in haemoglobin. J. Mol. Biol. 230, 1291–1296.
Sis, R.F., Ives, P.J., Jones, D.M., Lewis, D.H., Haensly, W.E., 1979. The microscopic anatomy of the oesophagus, stomach and intestine of the channel catfish *Ictalurus punctatus*. J. Fish Biol. 14.
Skow, L.C., 1971. Electrophotetic characterization of the hemoglobins of five species of catfish and on interspecific hybrids. (M.S.Thesis) Abilene Christian College, Abilene, Tex.
Small, B.C., Murdock, C.A., Bilodeau-Bourgeois, A.L., Peterson, B.C., Waldbieser, G.C., 2008. Stability of reference genes for real-time PCR analyses in channel catfish (*Ictalurus punctatus*) tissues under varying physiological conditions. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 151, 296–304.
Smith, S.V., 1985. Physical, chemical and biological characteristics of CO_2 gas flux across the air–water interface. Plant Cell Environ. 8, 387–398.
Sullivan, J.P., Lundberg, J.G., Hardman, M., 2006. A phylogenetic analysis of the major groups of catifshes (Teleostei Siluriformes) using rag1 and rag2 nuclear gene sequences. Mol. Phylogenet. Evol. 41, 636–662.
Takeshita, S., Aoki, T., Fukumaki, Y., Takagi, Y., 1984. Cloning and sequence analysis of a cDNA for the alpha-globin mRNA of carp, *Cyprinus carpio*. Biochim. Biophys. Acta 783, 265–271.
Taylor, P.W., Rogers, W.A., Dunham, R.A., 1984. Comparisons of hemoglobin and hematocrits of blue, brown bullhead, channel, white, crossbred, and hybrid catfishes. Proc. Annu. Conf. Southeast. Assoc. Fish Wildl. Agen. 38, 374–382.
Taylor, J.S., Van de Peer, Y., Braasch, I., Meyer, A., 2001. Comparative genomics provides evidence for an ancient genome duplication event in fish. Philos. Trans. R. Soc. B Biol. Sci. 356, 1661–1679.
Tiedke, J., Gerlach, F., Mitz, S.A., Hankeln, T., Burmester, T., 2011. Ontogeny of globin expression in zebrafish (*Danio rerio*). J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 181, 1011–1021.
Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S.G., 2012. Primer3—new capabilities and interfaces. Nucleic Acids Res. 40, e115.
Verde, C., Balestrieri, M., de Pascale, D., Pagnozzi, D., Lecointre, G., di Prisco, G., 2006a. The oxygen transport system in three species of the boreal fish family Gadidae. Molecular phylogeny of hemoglobin. J. Biol. Chem. 281, 22073–22084.
Verde, C., Parisi, E., di Prisco, G., 2006b. The evolution of thermal adaptation in polar fish. Gene 385, 137–145.
Vergara, A., Vitagliano, L., Merlino, A., Sica, F., Marino, K., Verde, C., di Prisco, G., Mazzarella, L., 2010. An order–disorder transition plays a role in switching off the root effect in fish hemoglobins. J. Biol. Chem. 285, 32568–32575.
Vlecken, D.H., Testerink, J., Ott, E.B., Sakalis, P.A., Jaspers, R.T., Bagowski, C.P., 2009. A critical role for myoglobin in zebrafish development. Int. J. Dev. Biol. 53, 517–524.
Waldbieser, G.C., Bosworth, B.G., Quiniou, S.M., 2010. Production of viable homozygous, doubled haploid channel catfish (*Ictalurus punctatus*). Mar. Biotechnol. (NY) 12, 380–385.
Wawrowski, A., Gerlach, F., Hankeln, T., Burmester, T., 2011. Changes of globin expression in the Japanese medaka (*Oryzias latipes*) in response to acute and chronic hypoxia. J. Comp. Physiol. B Biochem. Syst. Environ. Physiol. 181, 199–208.
Wetten, O.F., Nederbragt, A.J., Wilson, R.C., Jakobsen, K.S., Edvardsen, R.B., Andersen, O., 2010. Genomic organization and gene expression of the multiple globins in Atlantic cod: conservation of globin-flanking genes in chordates infers the origin of the vertebrate globin clusters. BMC Evol. Biol. 10, 315.
Yeh, H.Y., Shoemaker, C.A., Klesius, P.H., 2006. Molecular cloning and sequencing of hemoglobin-beta gene of channel catfish, *Ictalurus punctatus* Rafinesque. Fish Physiol. Biochem. 32, 83–92.
Zhou, Z., Liu, H., Liu, S., Sun, F., Peatman, E., Kucuktas, H., Kaltenboeck, L., Feng, T., Zhang, H., Niu, D., Lu, J., Waldbieser, G., Liu, Z., 2012. Alternative complement pathway of channel catfish (*Ictalurus punctatus*): molecular characterization, mapping and expression analysis of factors Bf/C2 and Df. J. Shellfish Res. 32, 186–195.