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Molecular characterization, phylogenetic analysis and expression profiling of myoglobin and cytoglobin genes in response to heat stress in channel catfish *Ictalurus punctatus*

J. B. Feng*†, S. K. Liu*, R. J. Wang*, J. R. Zhang*, X. L. Wang*‡, L. Kaltenboeck*, J. L. Li† and Z. J. Liu*§

**Fish Molecular Genetics and Biotechnology Laboratory, School of Fisheries, Aquaculture and Aquatic Sciences, and Program of Cell and Molecular Biosciences, Aquatic Genomics Unit, Auburn University, Auburn, AL 36849, U.S.A., †Key Laboratory of Freshwater Fishery Germplasm Resources, Ministry of Agriculture, Shanghai Ocean University, Shanghai 201306, China and ‡College of Fisheries and Life Science, Dalian Ocean University, Dalian 116023, China*

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To understand the function of myoglobin (Mb) and cytoglobin (Cygb) in channel catfish *Ictalurus punctatus* in response to heat stress, *mb* and *cygb* genes were identified and characterized in this study. These genes were widely expressed in all the tested tissues, but strong tissue preferences were observed, with the *mb* gene being expressed most highly in the heart, *cygb1* most highly expressed in the intestine and *cygb2* most highly expressed in the brain. After heat-stress challenge, *mb* and *cygb* genes were up-regulated in almost all tested tissues. In general, such up-regulation was more dramatic in the tolerant group than in the intolerant group, suggesting that higher expression of *mb* and *cygb* genes contributed to greater tolerance of *I. punctatus* to heat stress.

Key words: evolution; fish; gene; genome; heat tolerance.

INTRODUCTION

Fishes are ectothermic with their body temperature changing with water temperature. Elevated water temperature has a direct effect on fish physiology, and also has an effect on fish physiology indirectly through decreased oxygen levels. A number of mechanisms have been evolved for fishes to cope with temperature alterations including increases in cardiac output, the number of red cells and the cellular haemoglobin (Hb) content. Fishes have the ability to adjust the globin-oxygen affinity to facilitate oxygen binding and releasing into tissues, and to change the gill morphology to increase the gill ventilation and lamellar perfusion in response to increased water temperature or reduced oxygen levels (Portner *et al.*, 2004; Sollid & Nilsson, 2006). In addition, the structure and functional properties of globins and metabolic enzymes may change to cope with alterations in water temperature and dissolved oxygen (Holbert *et al.*, 1978). Globin proteins have a markedly advanced ability to handle oxygen molecules

§Author to whom correspondence should be addressed. Tel.: +1 334 8444784; email: liuzhan@auburn.edu

according to surrounding biochemical conditions, thereby sustaining the aerobic metabolism of the respiratory chain (Nishi *et al.*, 2011).

Teleost globins, such as Hb, myoglobin (Mb) and cytoglobin (Cygb), have been demonstrated to be responsible for oxygen circulation *in vivo* and waste gas expiration (Roesner *et al.*, 2006; Olianas *et al.*, 2011; Tiedke *et al.*, 2011). The *hb* gene is ubiquitously expressed in all tissues with especially high levels of expression in blood, spleen and kidney; it is mainly in charge of oxygen and waste gas transportation (Jensen, 2007; Weber *et al.*, 2010). The *mb* gene is highly expressed in muscle, brain, gill, liver, intestine and kidney (Cossins *et al.*, 2009; Flogel *et al.*, 2010; Pedersen *et al.*, 2010); it is responsible for storing oxygen for metabolic respiration during periods of hypoxia or high level of oxygen demand, thereby facilitating diffusion of oxygen to the mitochondria of aerobic muscle, and to modulate oxygen and nitric oxide homeostasis (Wittenberg & Wittenberg, 2003; Fraser *et al.*, 2006; Cossins *et al.*, 2009; Kanatous & Mammen, 2010; Totzeck *et al.*, 2012). The *cygb* gene is predominantly expressed in connective tissues in various organs and tissues; it plays roles in oxygen sensing, storage and diffusion and in detoxification of reactive oxygen species, serving as a nitric oxide dioxygenase (Burge & Karlin, 1997; Liu, X., 2012). To date, globin genes have been identified in several teleost species (Fuchs *et al.*, 2005; Vlecken *et al.*, 2009; Hoffmann *et al.*, 2012). It has been demonstrated that the elevated water temperature could affect the affinity of oxygen binding to globins. For instance, high temperature was reported to reduce the affinity of Mb for oxygen, accelerate the rate of Mb oxygen dissociation, and facilitate intracellular oxygen diffusion from Mb to tissues (Dowd *et al.*, 1991; Sidell, 1998).

Channel catfish *Ictalurus punctatus* (Rafinesque 1818), the primary aquaculture species in the U.S.A., is extremely adaptable to a wide range of temperatures. A previous study has indicated that the expression of several globin genes was significantly altered in response to heat stress (Liu *et al.*, 2013). *I. punctatus hb* and *mb* genes have been identified (Chen *et al.*, 2010; Feng *et al.*, 2014), and the expression of *hb* genes has been analysed in response to heat stress (Feng *et al.*, 2014). The *I. punctatus cygb* gene, however, has not been identified, and expression profiles of *mb* and *cygb* genes under elevated temperature conditions remain unknown. In this study, the identification, phylogenetic analysis and expression profiling of *mb* and *cygb* genes under heat-stress conditions in *I. punctatus* groups that are sensitive and tolerant to high water temperature are reported. The information provided should be valuable to assist selective breeding of *I. punctatus* strains that are tolerant to high temperature for aquaculture.

MATERIALS AND METHODS

SEQUENCE ANALYSIS OF *MB* AND *CYGB* GENES

To identify *cygb* genes, RNA sequencing (RNA-Seq) and the whole genome sequence databases of *I. punctatus* were searched using *cygb* genes of zebrafish *Danio rerio* (Hamilton 1822) and goldfish *Carassius auratus* (L.1758) as queries. The RNA-Seq database was generated from the transcriptome assembly of expressed short reads (Liu, *et al.*, 2012) of a doubled haploid *I. punctatus* (Waldbieser *et al.*, 2010). The quality of RNA sequences obtained from RNA-Seq database was confirmed by comparison with the preliminary *I. punctatus* whole genome assembly, which also originated from sequencing a doubled haploid *I. punctatus* (Waldbieser *et al.*, 2010). The retrieved reconstructed transcripts were translated using open

TABLE I. Primers used for this study

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
mb	GGCAGCGTGGAAGCCAACTAT	ATTTCCAGCCGCATCAGCAGCG
cygb1	AAGAGGCAGCAGGAGTCGCAG	CGCTGTCTTCTCTCCATCGTCG
cygb2	TCTCATCTGTGCTGGAGGTG	CAGTAGAGCAGTGCCATCAG
18S rRNA	GAGAAACGGCTACCACATCC	GATACGCTCATTCCGATTACAG

reading frame (ORF) finder (www.ncbi.nlm.nih.gov) and Fgenesh (Salamov & Solovyev, 2000). The predicted ORFs were verified by basic local-alignment search tool p (BLASTp) against National Center for Biotechnology Information (NCBI) non-redundant protein sequence database. Functional domains were annotated using SMART 7 (Aursnes *et al.*, 2011).

PHYLOGENETIC ANALYSIS

The protein sequences of *mb* and *cygb* genes from various vertebrates including a number of teleost species were retrieved from NCBI or Ensembl database and aligned by using Muscle 3.8 (Edgar, 2004) with E-INS-i, G-INS-i and L-INS-i methods from Mafft 6.8 (Katoh *et al.*, 2005). The best alignments were selected using MUMSA (Lassmann & Sonnhammer, 2005, 2006) for constructing the phylogenetic trees.

Phylogenetic trees of *mb* and *cygb* genes were constructed using both maximum likelihood (ML) and Bayesian methods. The Le and Gascuel (LG) model for *mb* and the Jones-Taylor-Thornton (JTT) model for *cygb* both with gamma distribution of rates and invariant site categories were determined to be the best-fitting amino acid substitution models according to the Akaike information criterion (AIC) with the correction for small sample size (AICc) and Bayesian information criterion using the Prottest 3.0 (Darriba *et al.*, 2011). The ML tree was constructed with 1000 bootstraps in Treefinder software (Jobb *et al.*, 2004). Bayesian phylogenies were conducted in MrBayes 3.2.1 (Ronquist *et al.*, 2012), running four simultaneous chains for 2 000 000 generations runs of four chains, sampling every 200 generations of amino acid substitution and using default priors. Because MrBayes does not support the LG model of evolution and no other models received an AICc weight $> 0{\cdot}0001$, the Whelan and Goldman (WAG) model was used to substitute the LG model. Support for the nodes and parameter estimates were derived from a majority rule consensus of the last 2500 trees. MrBayes analyses were run using the Cyberinfrastructure for Phylogenic Research (CIPRES; www.phylo.org) portal (Miller *et al.*, 2009).

HEAT-STRESS CHALLENGE AND TISSUE SAMPLING

All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of 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 of 24·0° C, range $\pm$ 0·5° C and oxygen level (8·9–9·2 mg l^{-1}). A total of 300 fish were transferred to a treatment tank (length: 3·2 m, width: 50 cm and depth: 1 m). After acclimation for 72 h, 45 fish were removed to form the control group and reared at ambient temperature. Water temperature in the treatment tank was elevated at a rate of 4° C h^{-1} to 32° C, and then 1° C h^{-1} to 36° C, and then held constant at 36° C. Dissolved oxygen concentration was allowed to fluctuate naturally between 6·8 to 8·5 mg l^{-1} during the treatment. The fish were closely monitored for the signs of stress, and the first 45 fish (intolerant group) and last 45 fish (tolerant group), both of which showed loss of balance, were quickly removed and euthanized with MS-222 at 300 mg l^{-1}. Sampled fish were weighed and their total lengths (L_T) were measured. Tissues including brain, gill, heart, liver, head kidney, trunk kidney, spleen, intestine, muscle and skin were dissected. For each group, each 15 individuals of the same group were

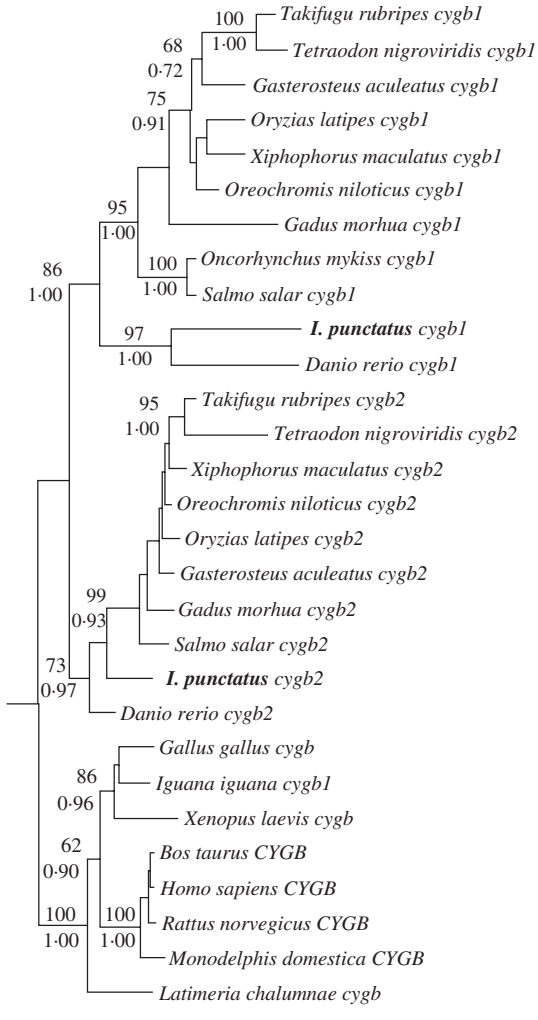

FIG. 1. Phylogenetic analysis of *Ictalurus punctatus* cytoglobin (*cygb*) genes. The topological stability of the tree was evaluated by bootstrapping with bootstrap values (above) and Bayesian posterior probabilities (below) indicated by numbers at the nodes.

randomly picked and pooled together into RNA*later* (Invitrogen; www.invitrogen.com). All samples were stored at room temperature for 24 h to allow RNA*later* to penetrate the tissues, and then transferred to −80° C until RNA extraction.

QUANTITATIVE REAL-TIME REVERSE-TRANSCRIPTION PCR ANALYSIS

Total RNA was extracted using the Trizol Reagent (Qiagen; www.qiagen.com), and then quantified using UV-spectrophotometer. First strand cDNA synthesized using iScript cDNA Synthesis Kit (Bio-Rad Laboratories; www.bio-rad.com) was used for determination of gene expression by quantitative real-time reverse-transcription PCR (qRT-PCR). qRT-PCR was performed using EvaGreen Supermix (Bio-Rad) on the CFX Real-Time PCR Detection System (Bio-Rad). The primers used in qRT-PCR were designed using primer 3 plus (Boomker, 1979) and their sequences are listed in Table I. The *18s* rRNA gene was used as an internal control for

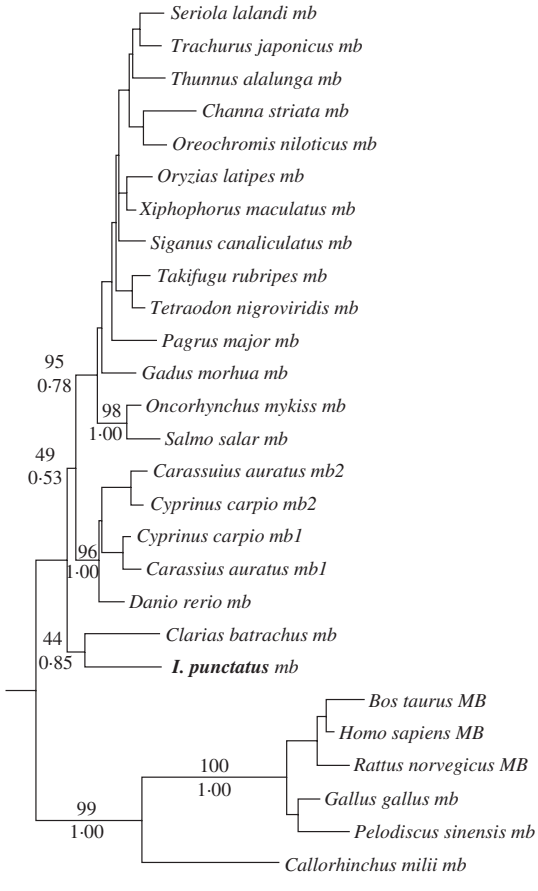

FIG. 2. Phylogenetic analysis of *Ictalurus punctatus* myoglobin (*mb*) gene. The topological stability of the tree was evaluated by bootstrapping with bootstrap values (above) and Bayesian posterior probabilities (below) indicated by numbers at the nodes.

normalization of expression levels (Small *et al.*, 2008; Qin *et al.*, 2012; Zhou *et al.*, 2012). To assess the relative expression of globin genes in various *I. punctatus* tissues, the trunk kidney RNA sample was arbitrarily chosen as the calibrator.

Expression data were transferred and analysed in linear regression software LinReg PCR (Ramakers *et al.*, 2003; Ruijter *et al.*, 2009) with quantification cycle values (Cq) generated by qRT-PCR. Expression difference between control and treatment groups was assessed for statistical significance using a pair-wise fixed reallocation randomization test within the Relative Expression Software Tool 384 1 (Pfaffl *et al.*, 2002). The fold change of the *I. punctatus mb* and *cygb* after treatments was made into a graphical presentation with statistical significance level of $P < 0{\cdot}05$.

RESULTS

SEQUENCE AND PHYLOGENETIC ANALYSIS OF *MB* AND *CYGB* GENES

The complete cDNA sequence of the *I. punctatus mb* gene was previously reported (Chen *et al.*, 2010). Two distinct *cygb* genes (*cygb1* and *cygb2*) were identified from

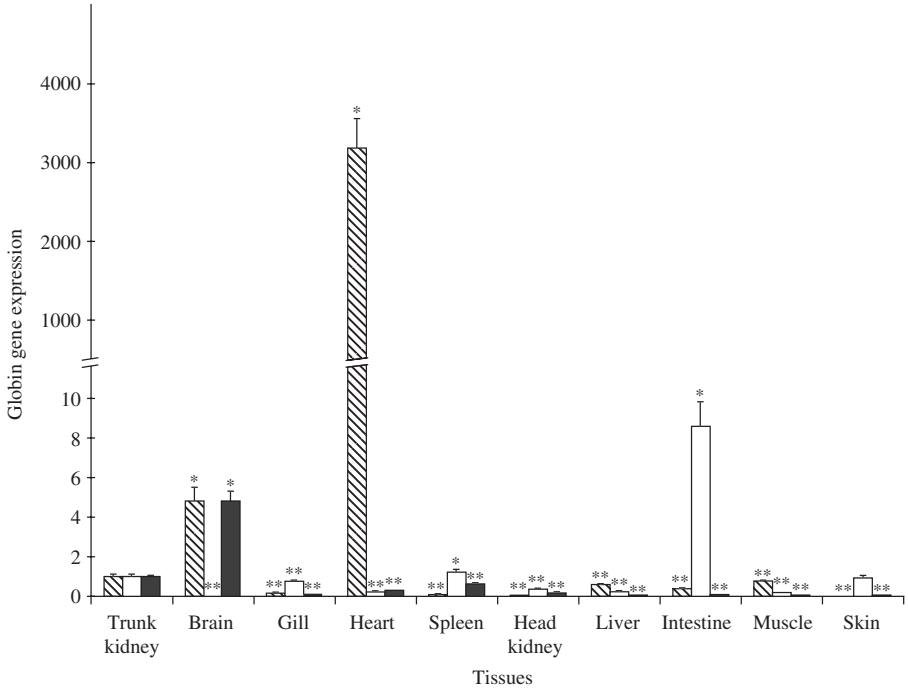

FIG. 3. Tissue expression of myoglobin (*mb*; ▨), cytoglobin (*cygb1*; □) and *cygb2* (■) genes in *Ictalurus punctatus* determined using real-time reverse-transcription PCR. The *y* axis represents *18s rRNA*-normalized expression values. Tissue RNA expression values are presented relative to that of trunk kidney tissue. Values are mean ± S.E. expression of three-tested pools (15 fish each). *, up- and **, down-regulation with statistical significance level of $P < 0{\cdot}05$.

RNA-Seq and the whole genome sequence database of *I. punctatus*, and their sequences were deposited to GenBank with accession numbers of KF471013 and KF471114. The genomic organization of the *I. punctatus mb*, *cygb1* and *cygb2* genes are well conserved with three exons interrupted at the conserved intron positions B12.2 (*i.e.* between codon positions 2 and 3 of the 12th amino acid of helix B) and G7.0 (*i.e.* between the codons for amino acids 6 and 7 of helix G). The deduced protein sequences of the *I. punctatus mb*, *cygb1* and *cygb2* genes consisted of 147, 173 and 179 amino acids, respectively. Their amino acid sequences are well conserved when compared with those of the carps (71·5 to 86%).

Phylogenetic trees supported the identities of both *cygb* (Fig. 1) and *mb* (Fig. 2) genes in *I punctatus*. Apparently, neither *cygb* nor *mb* of teleosts were monophyletic relative to their tetrapod counterparts. In case of *cygb* genes (Fig. 1), teleost *cygb1* and *cygb2* genes were separated into two different monophyletic clades. While the *I. punctatus cygb1* is placed in the same clade with the *D. rerio cygb1*, its *cygb2* gene was placed neighbouring the *D. rerio* counterpart, suggesting that *cygb1* exhibited a greater level of sequence similarity to the *D. rerio* gene than *cygb2*. Apparently, the *I. punctatus cygb1* and *cygb2*, like those in many fish species, were paralogues that were derived from gene duplication. Only one *mb* gene was identified from *I. punctatus*, but two *mb* genes were identified from carps *Cyprinus* sp., reflecting their nature as a tetraploid (Fig. 2).

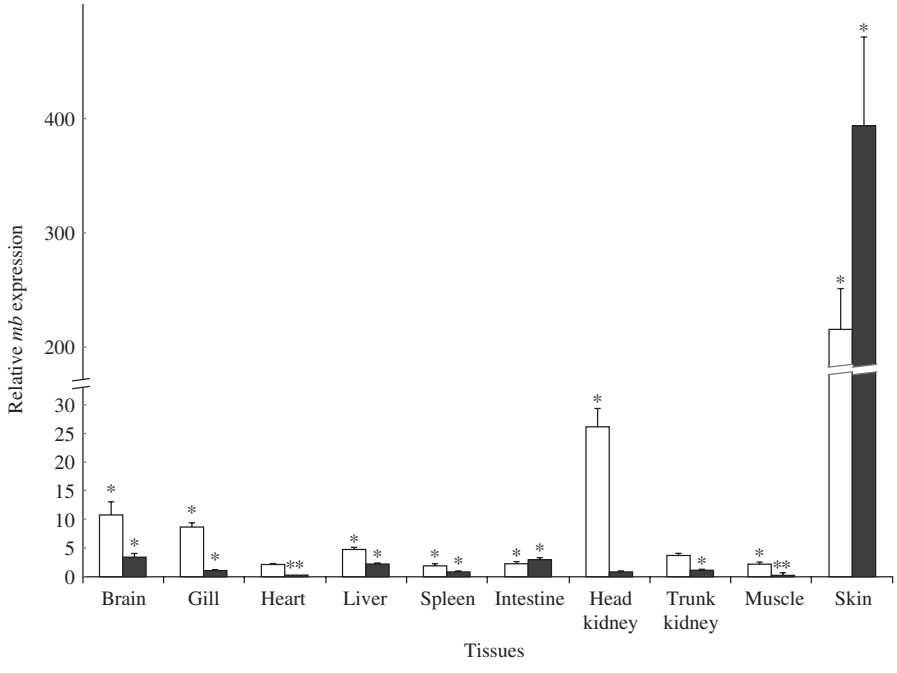

FIG. 4. Expression of *Ictalurus punctatus* myoglobin (*mb*) gene after heat-stress challenge (□, tolerant; ■, intolerant). Relative gene expression was presented as fold change over control samples taken at the same time points and normalized to change in expression in the *18s rRNA* internal reference gene. Values are mean ± S.E. expression of three-tested pools (15 fish each). ∗, up- and ∗∗, down-regulation with statistical significance level of $P < 0{\cdot}05$.

TISSUE EXPRESSION ANALYSIS OF *MB* AND *CYGB* GENES

As shown in Fig. 3, the *mb* gene was widely expressed in all 10 tissues tested, but expressed at the highest level in the heart, at least hundreds to thousands times more than its expression in any other tissues. Other than the heart, it was expressed at a relatively high level in the brain. The *cygb* genes were expressed in all 10 tissues tested as well. The highest expression of *cygb1* was observed in the intestine, while the high expression of *cygb2* was observed in the brain. Expression of *cygb1* and *cygb2* was relatively low in all other tested tissues (Fig. 3).

EXPRESSION OF *MB* AND *CYGB* GENES AFTER HEAT-STRESS TREATMENT

As shown in Fig. 4, *mb* expression was generally up-regulated in most tissues after heat-stress treatment in both the tolerant and intolerant groups except in the heart and muscle, where *mb* expression was suppressed after heat-stress treatment in the intolerant group. The induced expression was much dramatic in tolerant fish than in intolerant fish with the exception of skin and intestine where induction was more dramatic in the intolerant fish (Fig. 4).

Similar to the situation of the *mb* gene, expression of *cygb1* (Fig. 5) and *cygb2* (Fig. 6) was induced with heat-stress treatment, and the induction was generally more with tolerant fish than with intolerant fish. Overall, heat-stress-induced gene expression was

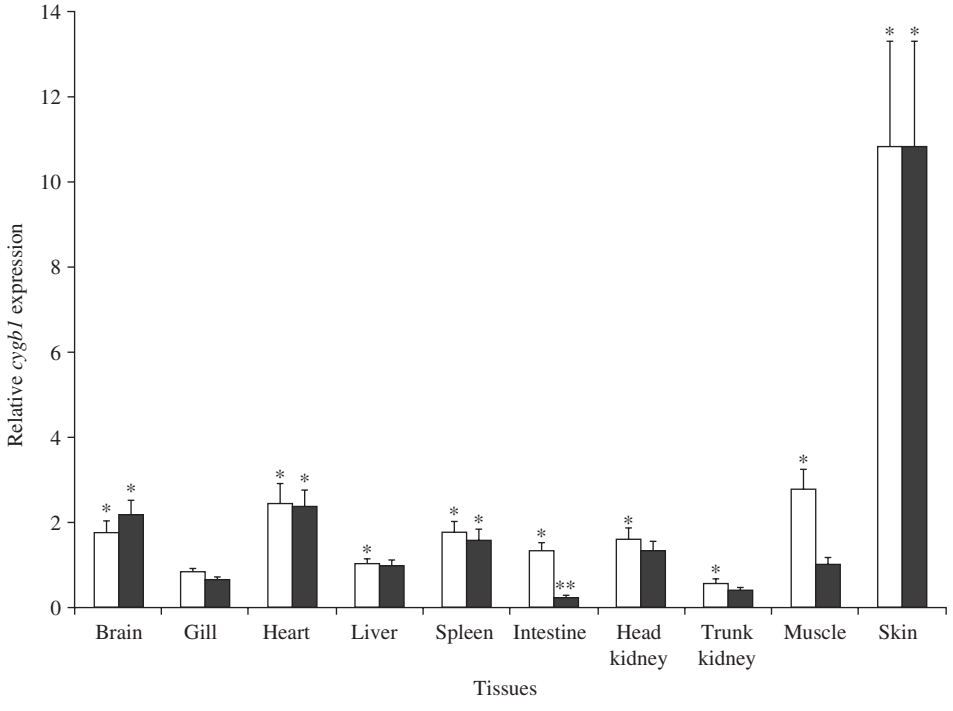

FIG. 5. Expression of *Ictalurus punctatus* cytoglobin 1 (*cygb1*) gene after heat-stress challenge (□, tolerant; ■, intolerant). Relative gene expression was presented as fold change over control samples taken at the same time points and normalized to change in expression in the *18s rRNA* internal reference gene. Values are mean ± S.E. expression of three-tested pools (15 fish each). *, up- and **, down-regulation with statistical significance level of $P < 0{\cdot}05$.

much more modest with *cygb1* and *cygb2* than with *mb*. In the skin, *mb* expression was induced over 300-fold after heat-stress treatment (Fig. 4), whereas *cygb1* and *cygb2* were induced *c*. 10-fold after heat-stress treatment (Figs 5 and 6).

DISCUSSION

In this study, two *cygb* genes were identified, *mb* gene and *cygb* genes characterized and their expression analysed in normal tissues and after heat-stress treatment. *Ictalurus punctatus* had only one *mb* gene, similar to the situation in several other fish species as reported for tuna *Auxis rochei* (Risso 1810), tilapia *Oreochromis niloticus* (L. 1758), cod *Gadus morhua* L. 1758, Atlantic salmon *Salmo salar* L.1758 and carangids (Brown *et al.*, 1994; Taylor *et al.*, 2001; Ueki *et al.*, 2005; Lurman *et al.*, 2007; Hasan *et al.*, 2012). Two *mb* genes were reported in common carp *Cyprinus carpio* L.1758 and goldfish *Carassius auratus* (L. 1758) (Fraser *et al.*, 2006; Roesner *et al.*, 2008), but they are generally regarded as tetraploid fishes (Larhammar & Risinger, 1994). In this regard, it is interesting that only one *mb* gene was reported in *S. salar* and rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) that are also believed to be tetraploidy. The *I. punctatus* possessed two *cygb* genes. Phylogenetic analysis suggested the two

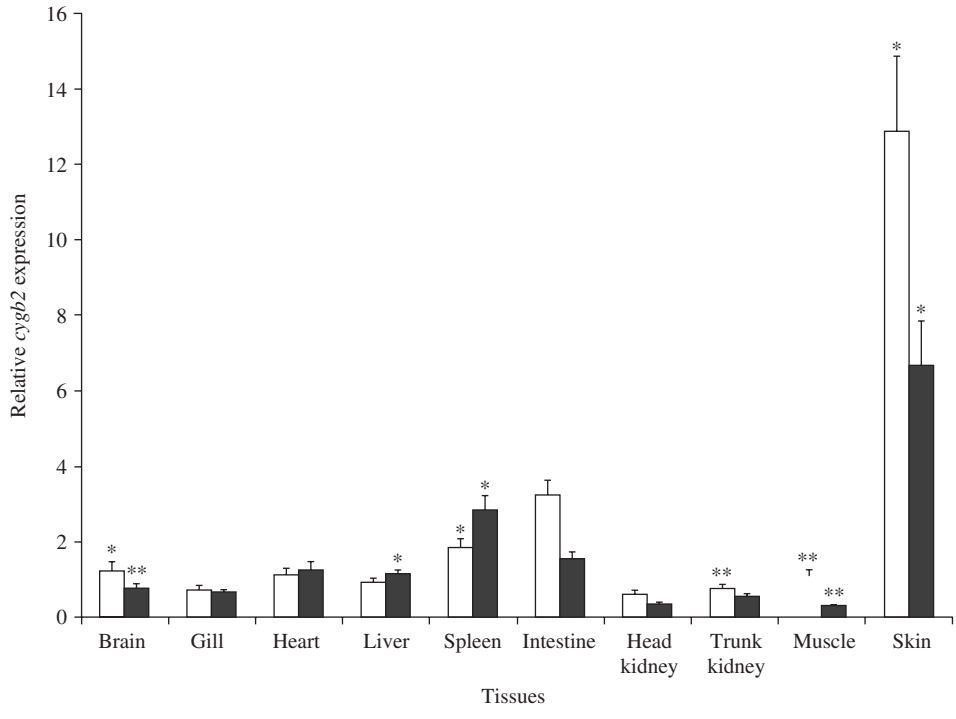

FIG. 6. Expression of *Ictalurus punctatus* cytoglobin 2 (*cygb2*) gene after heat-stress challenge (□, tolerant; ■, intolerant). Relative gene expression was presented as fold change over control samples taken at the same time points and normalized to change in expression in the *18s rRNA* internal reference gene. Values are mean ± S.E. expression of three-tested pools (15 fish each). *, up- and **, down-regulation with statistical significance level of $P < 0{\cdot}05$.

genes were paralogous, similar to the situations in other teleost species (Hoffmann *et al.*, 2011).

Fish *mb* has been usually considered to be a typical muscle protein expressed in skeletal and heart muscle tissues, but recent reports have demonstrated that *mb* was also expressed in brain, gill, liver and kidney (Fraser *et al.*, 2006; Cossins *et al.*, 2009; Liu *et al.*, 2009; Helbo *et al.*, 2012). In this study, it was found that the *I. punctatus mb* and *cygb* genes were broadly expressed in all tested tissues, although it was apparent that these genes exhibited their tissue preference in expression. For instance, *mb* gene was expressed most highly in the heart, while *cygb1* was expressed most highly in the intestine, and *cygb2* was expressed most highly in the brain (Fig. 3). *Ictalurus punctatus* is white-muscled fish, which have a lower amount of *mb* in contrast to the red-muscled fish such as *D. rerio* and *C. carpio* (Verde *et al.*, 2006). This could be the reason that low expression of *mb* was observed in the muscle of *I. punctatus* in this study. The differential expression of *cygb* genes was dramatic between intestine and brain, where *cygb1* was expressed 100 fold higher than that of *cygb2* in intestine, but 1000 fold lower in brain. Similar expression profiles of *cygb* genes were also observed in other teleosts such as *D. rerio*, Japanese rice fish *Oryzias latipes* (Temminck & Schlegel 1846) and spotted green pufferfish *Tetraodon nigroviridis* Marion de Procé 1822 (Fuchs *et al.*, 2005).

After heat-stress treatment, *mb* and *cygb* genes were generally induced although the extent of induction was highly differential depending on the tissues. Induction was the highest in the skin for all three genes, but it was most dramatic with *mb*. When the phenotypes were considered, it is generally true that the induction was more significant in the tolerant fish than in the intolerant fish, suggesting the correlation of expression of *mb* gene and the *cygb* genes with the tolerance of *I. punctatus* to heat stress. Although such correlation was also true with *hb* genes (Feng *et al.*, 2014), only a subset of *hb* genes were up-regulated by heat-stress treatment. Of the 14 *hb* genes of *I. punctatus*, only six were significantly up-regulated after heat stress, *i.e*. MN *hbα4*, MN *hbα5*, MN *hbα6*, MN *hbβ4*, MN *hbβ5* and MN *hbβ6*. This was believed to be caused by developmental stage-specific expression of *hb* genes (Feng *et al.*, 2014). Taken together, the globin genes, including *hb* genes, *mb* gene and *cygb* genes are all important players under heat stress. Although no physiological data were recorded in this study, it is speculated that the elevated expression of globin genes under heat stress would allow more efficient transport and storage of oxygen when dissolved oxygen becomes low under elevated temperature. The exact mechanism of their contribution to heat stress other than providing more oxygen availability is unknown at present and requires future studies.

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