



## Short communication

Identification and expression analysis of suppressors of cytokine signaling (SOCS) from soiny mullet (*Liza haematocheila*)Yunjie Song<sup>a,b,c</sup>, Xingxing Cheng<sup>a,b,c</sup>, Xinyu Jiang<sup>a,b,c</sup>, Jingduo Gao<sup>a,b,c</sup>, Yujie Xue<sup>a,b,c</sup>, Jiayin Tian<sup>a,b,c</sup>, Chang Zhang<sup>a,b,c</sup>, Sisi Wang<sup>d</sup>, Jie Zhou<sup>e</sup>, Jun Zou<sup>a,b,c</sup>, Zhitao Qi<sup>d,\*</sup>, Qian Gao<sup>a,b,c,\*\*</sup><sup>a</sup> Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai, 201306, China<sup>b</sup> International Research Center for Marine Biosciences at Shanghai Ocean University, Ministry of Science and Technology, China<sup>c</sup> National Demonstration Center for Experimental Fisheries Science Education, Shanghai Ocean University, Shanghai, China<sup>d</sup> Key Laboratory of Biochemistry and Biotechnology of Marine Wetland of Jiangsu Province, Yancheng Institute of Technology, Yancheng, Jiangsu Province, 224051, China<sup>e</sup> State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei Province, 430072, China

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## ABSTRACT

The suppressor of cytokine signaling (SOCS) family members play crucial roles in regulating immune signal pathways by acting as inhibitors of cytokine receptor signaling. In this study, 10 SOCS genes were identified in soiny mullet (*Liza haematocheila*), an economically important aquaculture mugilid species in China and other Asian countries. Sequence comparison showed that the sequence identity between mullet SOCSs and their counterparts from other vertebrates ranged from 38.2% to 92.5%. All mullet SOCS genes were constitutively expressed in tissues examined, but their expression patterns were different. Further, following *Streptococcus dysgalactiae* infection, all mullet SOCS genes exhibited distinct expression patterns in tissues. These results suggest that SOCSs are involved in immune response to bacterial infection and provide the basis for understanding the complex cytokine regulatory network of teleosts.

## 1. Introduction

Cytokines are pleiotropic molecules that have crucial roles in growth, development, differentiation and immune responses [1,2]. Once cytokines bind to their receptors on the cell surface, a series of signal transduction pathways are activated to elicit downstream effects [3]. However, excessive cytokine signaling dis-regulates the normal homeostasis and cellular functions [4]. The suppressor of cytokine signaling (SOCS) family members are such negative regulators inhibiting activation of cytokine receptor signaling.

To date, eight members of SOCS family members have been identified in mammals, including SOCS1–7 and cytokine-inducible SH2 containing protein (CISH). Structurally, all SOCS family members contain a central SH2 domain and a conserved C-terminal SOCS box [3,5]. However, there are some structural differences between different members of SOCS family. The N-terminal regions of SOCS proteins are variable in length, ranging from 50 to 380 amino acids [6,7]. In addition, SOCS1 and SOCS3 possess a kinase-inhibitory region (KIR), which

is located at the upstream of SH2 domain. Moreover, SOCS2 and CISH possess an N-terminal extended SH2 sub-domain (N-ESS) instead of KIR [8]. These domain structures are central for the functions of SOCS family members. For example, the extended SH2 and KIR of SOCS1 bind to the activation and catalytic region of Janus tyrosine kinase (JAK) 2 and inhibit its function [9]. The SOCS box can bind to elongins B and C, leading to the proteasomal degradation [10]. In addition to acting as negative regulators of cytokine signaling [5,11], the SOCS molecules are also involved in other immune processes, such as directing macrophage polarization [12] and mediating immunomodulation of natural killer cells [13].

To date, several SOCS family members have been identified in 11 species of fish, including zebrafish (*Danio rerio*), tetraodon (*Tetraodon nigroviridis*), fugu (*Fugu rubripes*), stickleback (*Gasterosteus aculeatus*) [14–16], rainbow trout (*Oncorhynchus mykiss*) [17], Nile tilapia (*Oreochromis niloticus*) [18], channel catfish (*Ictalurus punctatus*) [19], Atlantic salmon (*Salmo salar*) [20], and miyu croaker (*Miichthys miyu*) [21], tongue sole (*Cynoglossus semilaevis*) [22], Japanese flounder

\* Corresponding author.

\*\* Corresponding author. Key Laboratory of Exploration and Utilization of Aquatic Genetic Resources, Ministry of Education, Shanghai Ocean University, Shanghai, 201306, China.

E-mail addresses: [qizhitao@ycit.edu.cn](mailto:qizhitao@ycit.edu.cn) (Z. Qi), [qgao@shou.edu.cn](mailto:qgao@shou.edu.cn) (Q. Gao).<https://doi.org/10.1016/j.fsi.2019.04.299>

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(*Paralichthys olivaceus*) [23]. These studies indicated that teleost SOCS genes have distinct roles in different tissues or cells. Teleost SOCSs can be induced by several stimuli, such as pathogen-associated molecular patterns (PAMPs), bacteria and viruses. Fish SOCS1-3 and CISH can be up-regulated by lipopolysaccharide (LPS) and bacterial infection [18,19]. In salmon and miiuy croaker, infection of salmon alphavirus (SAV3) or stimulation leads to induction of SOCS1 expression respectively.

Soiny mullet (*Liza haematocheila*) has become an economically important aquaculture mugilid species in China and other Asian countries [24]. However, studies on its immune system are scarce, with limited immune genes identified [25,26]. The present work aimed to identify firstly the SOCS genes from fish species in Mugiliformes and determine their expression profiles after gram-positive bacterial infections by using the intraperitoneal injection with *Streptococcus dysgalactiae*.

## 2. Materials and methods

### 2.1. Fish and bacterial challenge

Soiny mullet (with average weight of  $8 \pm 2$  g) were purchased from a local fish farm in Yancheng (Jiangsu province, China) and maintained at  $28 \pm 2^\circ\text{C}$  in 300 L opaque polyethylene tanks in a recirculation system. Prior to the experiment, the fish were acclimatized for at least one week. Eight tissues including gills, skin, muscle, liver, spleen, head kidney (HK), intestine and brain from five healthy fish were collected under anesthesia with MS222 (0.1 g/L). The collected tissues were immediately frozen in liquid nitrogen, and then stored at  $-80^\circ\text{C}$  until further analysis.

Twenty healthy fish were randomly divided into two groups (ten fish each group). One group was intraperitoneally (i.p.) injected with  $4 \times 10^5$  CFU of *Streptococcus dysgalactiae* [24] and the other group with PBS (control). Eight tissues (gills, skin, muscle, liver, spleen, head kidney (HK), intestine and brain) of five fish in each group were collected at 12 h and 24 h post injection (hpi) and processed as described above.

### 2.2. RNA isolation and synthesis of cDNA

Total RNA of each tissue was extracted with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The cDNA was synthesized using a SMART™ RACE cDNA Amplification Kit (Clontech, USA) for gene cloning. The Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, USA) was used to synthesize cDNA for quantitative real-time PCR (qPCR).

### 2.3. Cloning of the full-length mullet SOCS sequences

To obtain the partial sequences of mullet SOCS family members, zebrafish SOCS family members were used to search the spleen transcriptome assembled sequences of mullet by local BLAST software [24]. Specific primers were designed and PCR was conducted to clone the SOCS sequences. Full cDNA sequence of mullet SOCSs was obtained by RACE PCR using a SMART™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's instructions. PCR products were separated on an agarose gel by electrophoresis, purified, ligated into the pMD18-T vector (TaKaRa, Japan), and sequenced on an automatic DNA sequencer (Gigascience, China).

### 2.4. Sequence analysis

The amino acid sequences were deduced based on cDNA sequences using the Translate program, and the molecular weight and theoretical isoelectric point were predicted using the ProParam (Protein Parameters) software [27]. Sequence identity of amino acids was calculated using the MatGAT (Matrix Global Alignment Tool) 2.02

software. A multiple sequence alignment was generated using the ClustalO and modified with the BoxShade software ([http://www.chi.embnet.org/software/BOX\\_form.html](http://www.chi.embnet.org/software/BOX_form.html)). The signal peptide was predicted with the SignalP 4.1 program (<http://www.cbs.dtu.dk/services/SignalP>) [28] and the protein domains were predicted using the Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>). A phylogenetic tree was constructed with the MEGA (Molecular Evolutionary Genetics Analysis) 7.0 software (Jones-Taylor-Thornton (JTT) model), with the bootstrap setting as 10,000 to test the reliability of branching [29].

### 2.5. qPCR

The expression levels of mullet SOCS genes were assessed by qPCR using SYBR green master mix (Yeasen shanghai, China) on the LightCycler 96 Real Time PCR System (Roche, Switzerland). 10  $\mu\text{L}$  of PCR reaction volume contained 4.6  $\mu\text{L}$  of cDNA template, 5  $\mu\text{L}$  of SYBR Premix, 0.2  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ). The PCR program was as follows: 1 cycle of  $95^\circ\text{C}$  for 30 s, 40 cycles of  $95^\circ\text{C}$  for 5 s,  $60^\circ\text{C}$  for 60 s, and 1 cycle of  $72^\circ\text{C}$  for 10 s. A melting curve analysis of amplified products was performed at the end of each PCR reaction to confirm primer specificity. The relative expression levels of mullet SOCS genes were calculated using  $2^{-\Delta\Delta\text{Ct}}$  method [30]. Results were expressed as mean  $\pm$  standard error. The SPSS statistics package 24 (SPSS Inc., Chicago, Illinois) was used for statistical analysis. The data from the infection experiments were analyzed using one way-analysis of variance (ANOVA) and LSD post hoc test.  $P$  value ( $< 0.05$ ) was considered to be statistically significant. All primers used for qPCR analysis were listed in annex 1.

## 3. Results and discussion

### 3.1. Gene clone and sequence analysis of mullet SOCS

In the present study, ten SOCS genes, SOCS1 (GenBank accession No. MK361108), SOCS2 (GenBank accession No. MK361109), SOCS3a (GenBank accession No. MK361110), SOCS3b (GenBank accession No. MK361111), SOCS4 (GenBank accession No. MK361112), SOCS5a (GenBank accession No. MK361113), SOCS6 (GenBank accession No. MK361114), SOCS7 (GenBank accession No. MK361115), SOCS8 (GenBank accession No. MK361116), and SOCS9/SOCS5b (GenBank accession No. MK361117), were sequenced from soiny mullet. The sequence features of mullet SOCS were summarized in Table 1. Each cDNA sequence contained a polyadenylation signal (AATAAA motif/ATTAATA motif) preceding the poly A tail, indicating complete ORFs were obtained (Annex 2).

Sequence comparison showed that the sequence identity between mullet SOCSs and their counterparts from other vertebrates ranged from 38.2% to 92.5% (Table 2). For example, mullet SOCS1 shared 48–71.4% with teleost SOCS1, 38.3% with mouse SOCS1 and 38.2% with human SOCS1. Mullet SOCS2 shared 74.6–84.6% with teleost SOCS2, which was higher than with that of high vertebrates (48.2–57.2%). Two SOCS3 were identified from mullet, termed as SOCS3a and SOCS3b and contained similar domains including the kinase-inhibitory region (KIR) domain, extended SH2 subdomain (ESS), the SH2-domain and the SOCS box domain [19]. The two SOCS3 genes might be duplicated from a common ancestor gene.

### 3.2. Phylogenetic tree analysis

To evaluate the evolutionary relationships among different SOCS genes, a phylogenetic tree was constructed using the MEGA software. As shown in Fig. 1, our results were in agreement with previous studies [17,31], showing mullet SOCS genes were clustered well with their counterparts from teleost species. Fish SOCS1 and SOCS3 were grouped into a single clade which was supported by high bootstrap value,

**Table 1**  
The sequence and physiochemical features of mullet SOCSs.

Feature	SOCS1	SOCS2	SOCS3a	SOCS3b	SOCS4	SOCS5a	SOCS6	SOCS7	SOCS8	SOCS9/SOCS5b
GenBank accession numbers	MK 361108	MK 361109	MK 361110	MK 361111	MK 361112	MK 361113	MK 361114	MK 361115	MK 361116	MK 361117
cDNA length (bp)	1568	2155	1991	2997	2390	2600	5287	3476	2165	2133
ORF (bp)	636	609	618	687	1188	1695	1611	2553	642	1638
5'-UTR (bp)	309	249	201	30	234	108	411	189	1110	219
3'-UTR (bp)	623	1297	1172	2280	968	797	3265	734	413	276
ATTTA motif	2	2	4	3	1	2	6	1	1	3
AATAAA/ATTA motif	1	1	1	1	1	1	1	1	1	1
PolyA tail	yes									
Signal peptide	No									
SH <sub>2</sub> -Domain	yes									
SOCS-box	yes									
Pfam-SOCS-Domain	No	No	No	No	yes	yes	No	No	No	yes
Amino acids (aa)	211	202	205	228	395	564	536	850	213	545
Molecular weight (kDa)	24.03	22.62	23.42	25.51	44.37	62.29	91.66	88.69	23.96	60.65
pI	9.01	8.83	8.80	8.44	8.69	8.95	6.51	7.06	8.52	6.63

indicating they are closely related. It has been shown that fish SOCS1 and SOCS3 shared high sequence homology, similar gene structures, and similar domain structures [31]. SOCS4 and SOCS5/SOCS9 formed a separate clade from other SOCS groups. SOCS9 were firstly termed by Jin et al. [16], however, later analysis by Wang et al. reclassified the fish SOCS5 as SOCS5a and fish SOCS9 as SOCS5b [31]. This nomenclature is well supported by the present study [31]. Thus, we termed these two mullet SOCS genes as SOCS5a and SOCS5b. SOCS4, SOCS5/9, SOCS6 and SOCS7 belong to type I SOCS subfamily [16]. However, SOCS6 and SOCS7 formed a distinct clade from other SOCS groups, indicating that type I SOCS might have undergone divergence during evolution. SOCS8 was first identified from four model fish species [16], and was thought to be the paralogue of CISH in mammals [32].

### 3.3. Tissue distribution of mullet SOCS genes

The expression levels of ten mullet SOCS genes were examined in eight tissues using qPCR method. The results showed that all mullet SOCS genes were constitutively expressed with different expression patterns. Relatively high levels of individual mullet SOCSs were detected in various tissues: SOCS1 in spleen (Fig. 2A); SOCS2 in liver (Fig. 2B); SOCS3a in skin (Fig. 2C); SOCS4 in liver, HK and skin (Fig. 2E); SOCS5a and SOCS7 in brain (Fig. 2F and H); SOCS6 in liver, spleen, muscle and brain (Fig. 2G); SOCS8 in gills (Fig. 2I). The expression levels of SOCS3b in tissues were similar except HK with the lowest expression (Fig. 2D). SOCS5b/9 had higher levels of expression in liver and brain than that in intestine, spleen, head, kidney and gill (Fig. 2J). The results suggest that SOCSs may have tissue specificity for actions. It has been well documented that SOCS1 had important roles in IFN- $\gamma$  signaling, T cell activation, some cytokines production by JAK-STAT, insulin and Toll-like receptor signaling pathways [33–35]. Spleen is an important lymphoid organ of fish [24]. High expression of

mullet SOCS1 in spleen was perhaps not surprising. SOCS2 had dual roles in regulating growth hormone (GH) signaling; low level SOCS2 moderately inhibited GH signaling whilst higher levels had opposite effects [36,37]. In agreement with this notion, mullet SOCS2 was found to be highly expressed in liver, an important organ for metabolism. Mullet SOCS3a and SOCS3b had different expression patterns, which were also observed for SOCS5a and SOCS5b, indicating that functions of these genes had diverged during evolution.

### 3.4. Expression analysis of mullet SOCS after *S. dysgalactiae* infection

Expressions of SOCS genes were analyzed by qPCR following *S. dysgalactiae* infection. At 12 hpi, up-regulation was detected for SOCS1 in intestine (Fig. 3A), SOCS1 and SOCS5b in head kidney (Fig. 3A and J), SOCS1 in skin (Fig. 3A), SOCS1 and SOCS5a in muscle and gills (Fig. 3A and F). Down-regulated genes included SOCS2 and SOCS3b in head kidney (Fig. 3B and D); SOCS3a and SOCS5a in skin (Fig. 3C and F); SOCS6, SOCS7 and SOCS5b in muscle (Fig. 3G, 3H and 3J); SOCS6 and SOCS7 in gills (Fig. 3G and H).

At 24 hpi, expression of SOCS3b and SOCS4 increased in intestine (Fig. 3D and E). Similarly, SOCS2, SOCS5a and SOCS5b was up-regulated in spleen (Fig. 3B, F and 3J), SOCS1, SOCS5a, and SOCS5b in head kidney (Fig. 3A, F and 3J), SOCS1, SOCS3a, SOCS3b, SOCS5a, SOCS6, SOCS8 and SOCS5b in skin (Fig. 3A, C, 3D, 3F, 3G, 3I and 3J), SOCS1 and SOCS3b in gills (Fig. 3A and D).

The alteration of SOCS expression detected in the present study was in line with previous studies in fish. Tongue sole SOCS1-6 were significantly induced at different time following *Edwardsiella tarda* and *Vibrio harveyi* infection, SOCS7 were also induced by *E. tarda* but dropped by *V. harveyi* infection at 12hpi [22]. Among 12 SOCSs of channel catfish, only SOCS1a, SOCS3a and CISH were up-regulated at the early stage of *Flavobacterium columnare* or *Edwardsiella ictaluri*

**Table 2**  
The sequence identity between mullet SOCSs genes and these of other vertebrates.

<i>L. haematocheila</i>	SOCS1	SOCS2	SOCS3a	SOCS3b	SOCS4	SOCS5a	SOCS5b(9)	SOCS6	SOCS7	SOCS8
<i>H. sapiens</i>	38.2%	48.2%	63.7%	45.6%	50.9%	69.1%	50.4%	73.3%	55.5%	45.5%
<i>M. musculus</i>	38.3%	57.2%	63.7%	45.6%	50.7%	69.7%	50%	69.1%	55.2%	45.4%
<i>D. rerio</i>	48%	80.2%	67.8%	54.8%	75.3%	69.2%	74.4%	81.3%	61.5%	48.8%
<i>O. mykiss</i>	55.9%	80.6%	77.1%	50.0%	74.7%	47.3%	80.1%	66.1%	43.9%	51.4%
<i>O. latipes</i>	71.4%	74.6%	62%	67.3%	85%	89.3%	85.8%	90.3%	84.1%	73.7%
<i>T. nigroviridis</i>	60.6%	81.6%	81.1%	78.3%	79.8%	88.7%	81.3%	90.6%	87.5%	63.8%
<i>T. rubripes</i>	62.1%	80.1%	79.6%	48.3%	84.8%	88%	79.3%	91.3%	87.4%	62.4%
<i>G. aculeatus</i>	64%	84.6%	82.4%	78.1%	82.4%	91.4%	85.5%	92.5%	46.5%	77.5%

Note: because of there is no SOCS8 or SOCS9 in human and mouse, and the homology of SOCS8 and SOCS9 in fish is relatively high with CISH and SOCS5 in human and mouse, SOCS8 and SOCS9 in mullet are compared with CISH and SOCS5 in human and mouse, respectively.



Fig. 1. Phylogenetic tree analysis of Soiny mullet SOCSs with SOCSs from different vertebrate species using MEGA software with the Neighbor-Joining method. Bootstrap was calculated with 10,000 repeats. Accession numbers for sequences were listed following the species names.

infection [19]. Japanese flounder SOCS1 and SOCS3 were up-regulated by formalin-killed *E. tarda* from 3 hpi to 24 hpi, SOCS 6 and SOCS9 were up-regulated at 12 hpi, while SOCS5 and SOCS6 were down-regulated at 3 hpi [23]. These results revealed that the expression of teleost SOCSs might be species-specific and time-dependent. The alteration of SOCS expression could be a host response to limit excessive inflammation triggered by bacterial infection [4]. In addition to bacterial infection, some PAMPs could also induce the expression of teleost SOCSs. Nile tilapia SOCS1 and SOCS3 were up-regulated in gill and liver at 12 hpi post LPS stimulation [18]. PolyI:C, a mimic of viral double-stranded RNA, could increase the expression of SOCS1a, SOCS1b and SOCS3a of Japanese flounder at different temperature

[23]. LPS and polyI:C are ligands of Toll-like receptors (TLRs), important patterns recognizing receptors (PRRs) of innate immunity [38]. These findings indicated that teleost SOCSs might involve in the regulation of innate immunity of teleost.

In conclusion, ten members of SOCS family were identified in soiny mullet and their expression profiles analyzed after *S. dysgalactiae* infection. The results suggest that SOCSs may have distinct roles in regulating homeostasis in tissues and immune response to bacterial infection. The SOCS sequences identified provide useful information for analysis of evolution of SOCS family and further understanding of the roles of SOCS in regulating the complex cytokine network in fish.

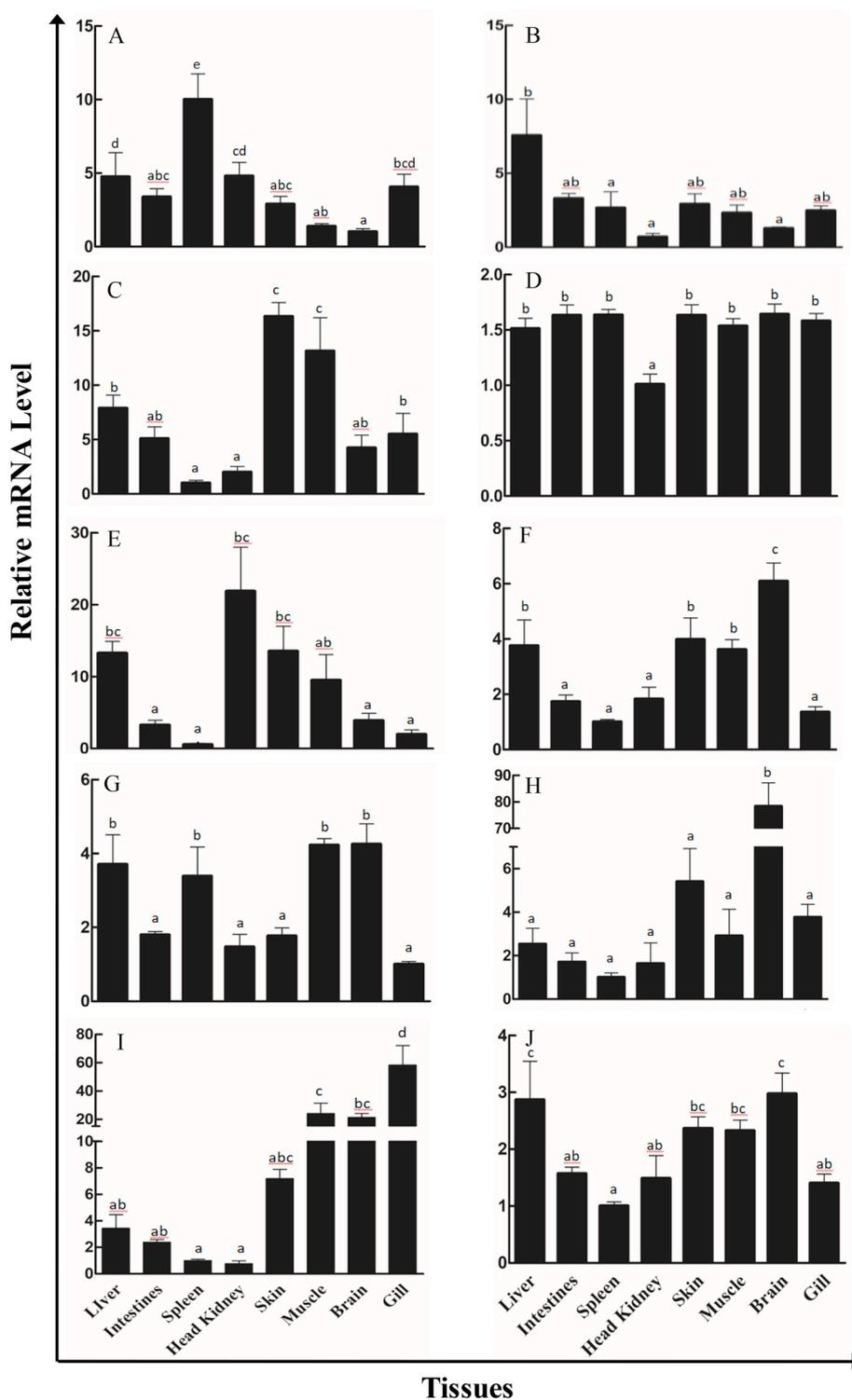


Fig. 2. Expression analysis of SOCSs in normal tissues of Soiny mullet. The expression levels of the SOCSs in brain, gills, liver, skin, spleen, head kidney, intestine, and muscle were determined by qPCR. For expression comparison, the expression level of each gene in the tissue with the lowest expression was set as 1. Vertical bars represent means + SE (N = 4). No significant difference was indicated with the same letters (P > 0.05) and significant differences were indicated with different letters (P < 0.05).

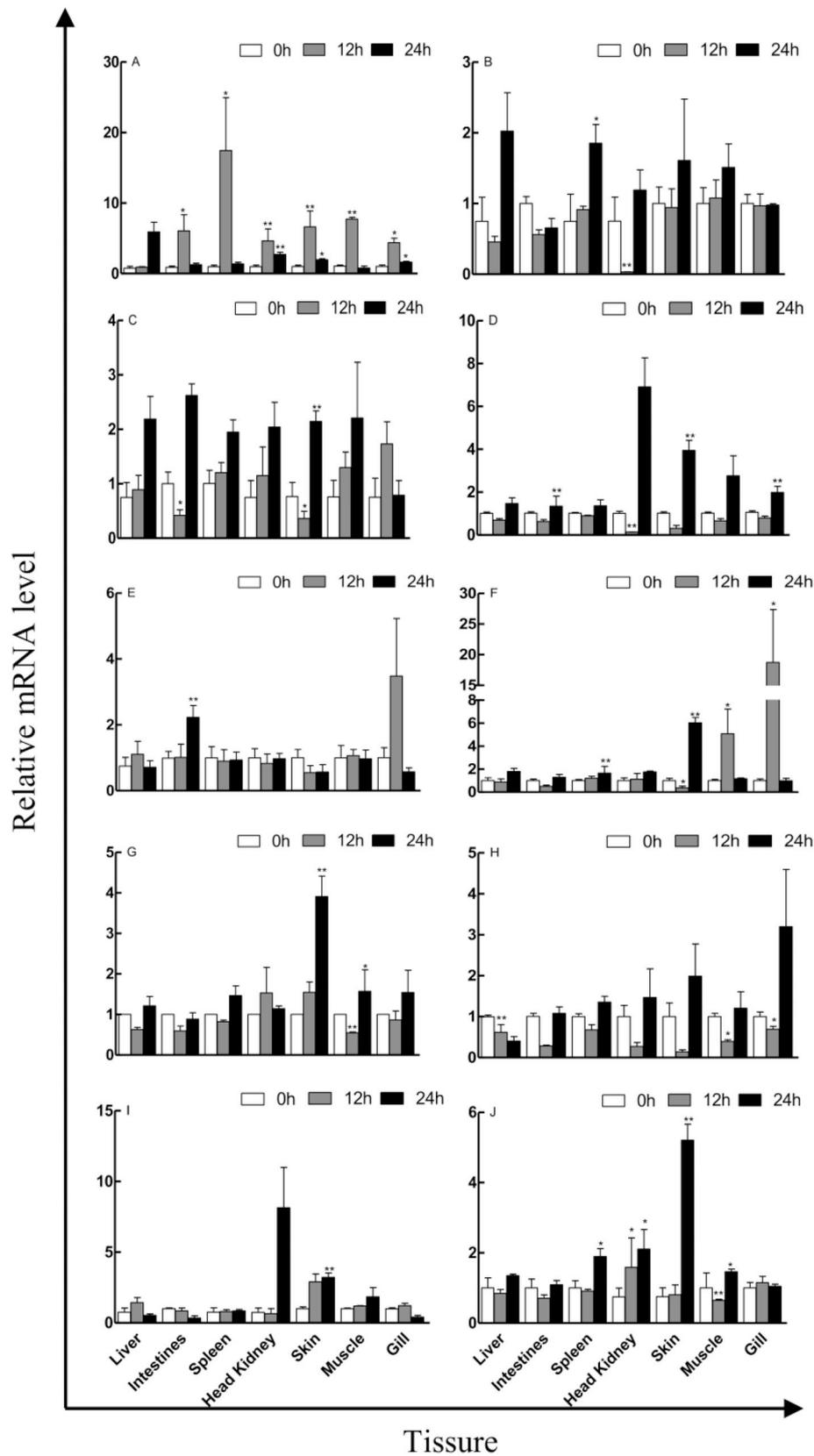


Fig. 3. Expression of Soiny mullet SOCSs following *S. dysgalactiae* infection. Soiny mullet were infected with or without (control) *S. dysgalactiae*, and SOCS expression was determined in liver, intestines spleen, head kidney, skin, muscle and gills by qPCR at 12 h and 24 h post infection. At each time point, the expression level of the control fish was set as 1, which was represented by a dotted horizontal line. Values are shown as means + SE (N = 4). \*\* $P < 0.01$ , \* $P < 0.05$ .

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fsi.2019.04.299>.

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