Molecular characterization and expression analysis of the transferrin gene in Amur ide (Leuciscus waleckii) in response to high alkaline stress

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ABSTRACT

Transferrin plays an important role in iron metabolism and has been reported to be involved in the immune response to environmental stress. In this study, the transferrin gene (LwTf) from Amur ide (Leuciscus waleckii) was cloned and characterized. The cDNA was observed to contain an open reading frame (ORF) of 1998bp and encode a 665-amino-acid-long protein that shares 73.04% identity with the transferrin from silver carp (Hypophthalmichthys molitrix). Amino acid alignment analysis showed that LwTf contained an initial peptide and two lobes (N- and C-lobes). Quantitative real-time PCR (qPCR) analyses showed that the expression levels of LwTf were abundant in the liver of the fish but were also significant in the brain and spleen, with lower expression observed in six other tissues. The temporal expression profiles were detected during the alkaline challenge experiment in alkali-water and freshwater populations of healthy adult Amur ide. LwTf expression was significantly upregulated in the main immune organs (spleen, kidney, and head kidney) of the freshwater Amur ide population compared to that in the alkali-water Amur ide population. Taken together, these results indicate that in Amur ide, LwTf might play an important role in the innate immune response to a high alkaline stress.

INTRODUCTION

Transferrin (Tf) is a multi-functional protein that plays a central role in iron metabolism, and is also thought to be involved in the innate immune system response (Liu et al. 2010, 2012). Through the binding and transport of iron, transferrin participates in a wide variety of metabolic processes, including immune regulation, antimicrobial and antioxidant activity, DNA synthesis, cytoprotection, and electron transport (Stafford and Belosevic 2003; Ong et al. 2006). Transferrin has been characterized in a variety of vertebrates, including mammals, reptiles, and birds (Graham and Williams 1975; Schreiber et al. 1979; Yang et al. 1984; Ciuraszkiewicz et al. 2007). Cloning, structural, and functional studies have also been extended in various fish species (Denovan-Wright et al. 1996; Tange et al. 1997; Lee et al. 1998; Sahoo et al. 2009). The complete sequence of transferrin in Nile tilapia (Oreochromis niloticus) and functional studies in relation to saltwater resistance have been reported (Rengmark and Lingaas 2007). Structure and expression of the transferrin gene in channel catfish (Ictalurus punctatus) have also been reported (Liu et al. 2010). Neves et al. (2009) reported the response of transferrin from sea bass to bacterial infection. Transferrin gene expression in response to LPS challenge and heavy metal exposure in roughskin sculpin (Trachidermus fasciatus) has also been described (Liu et al. 2012). As an acute-phase protein in fish, the concentration of transferrin closely mirrors the condition of infection or stress, although its rise or fall varies with infectious microorganisms or tissue where the injury has occurred (Neves et al. 2009; Liu et al. 2010). Similar to the results obtained in mammals, transferrin is most abundantly expressed in the liver of fish, and its expression in the brain is species-specific (Tu et al. 1991; Denovan-Wright et al. 1996; Sahoo et al. 2009). However, the expression and function of transferrin in the fish innate immune response to high alkaline stress have not been reported.

Amur ide (Leuciscus waleckii) is a cold freshwater fish (Chang et al. 2014) that usually inhabits freshwater environments but can also survive in saline and alkaline lakes (Xu et al. 2017); it is primarily distributed in water regions around the Amur river, a few areas in the Liao and Yellow rivers, and inland lakes in Inner Mongolia around China (Chang et al. 2014). As an extreme example, Amur ide can survive in the highly alkaline (pH up to 9.6) water of Dali-Nor lake (116°25′−CO32−/CO3′-concentration over 53.57 mmol/L (Chang et al. 2013). Amur ide is an important source of income for local Mongolians who live around Dali-Nor lake, and it is an important food source for birds migrating from Siberia to the South (Xu et al. 2017). Despite the economic and ecological importance of Amur ide in Dali-Nor lake, the mechanisms underlying its high tolerance to alkalinity are still largely unknown (Xu et al. 2017). Recently, Chinese scholars have carried out a series of exploratory studies on the molecular mechanism of the adaptation of this fish to high alkalinity in terms of its physiology and biochemistry.
population genetics, transcriptome, and genomic adaptation (Cui et al. 2015; Chen et al. 2019). In particular, the expression of genes related to osmotic pressure regulation, acid–base balance, ion transport, and immunity was found to have significantly increased under high alkaline stress using second-generation sequencing technology (RNA-Seq) (Xu, Ji, et al. 2013; Xu, Li, et al. 2013).

In this study, we first cloned and characterized the cDNA sequence of the transferrin gene (LwTf) and then examined the expression of LwTf in different tissues of Amur ide originating from Dali-Nor lake and the Songhuajiang River under the same alkaline stress conditions. Simultaneously, we investigated the changes in the expression of LwTf in seven tissues associated with immunity after high alkaline challenge. This study will help in enhancing our understanding of the role of LwTf in the innate immune defense during the adaptation of Amur ide to high alkalinity.

Material and methods

Fish maintenance and sampling

The F3 offspring of alkali-water species (abbreviated hereafter as AW) produced in the laboratory using the broodstock collected from the Dali-Nor lake in 2009 and F1 progenies of freshwater species (abbreviated hereafter as FW) found in the Songhuajiang River in 2016 were collected for this experimentation. Approximately 1000 AW and FW individuals were reared in different outdoor ponds spanning one acre in the Hulan Experimental Station of the Heilongjiang River Fisheries Research Institute (126.63°E, 45.97°N) for three months. A total of 240 fish with an average body weight of 48.72 ± 16.89 g were distributed equally with 25 fish in eight 200 l experimental tanks. Each group consisted of three randomly assigned tanks.

To achieve 50 mmol/L bicarbonate alkalinity, a total of 839 g of NaCHO3 (Tianjin Kemioiu Chemical Reagent Co. Ltd., China) was dissolved and added into the system. The concentration of bicarbonate was monitored daily by using the titration method with 0.02 mmol/L HCl. The water quality was monitored using a YSI analyzer during the experiments with the temperature at 23.76 ± 0.87°C, salinity 2.16 ± 0.06 mg/L, dissolved oxygen 11.73 ± 0.39 mg/L, ammonia 0.53 ± 0.12 mg/L, and pH 9.61 ± 0.07. Sampling for the time course experiment started at 24 h (1 d) after the alkaline challenge and continued at 48 h intervals (e.g. 3, 5, and 7 d) with 3 fish sampled from each tank (9 fish for each group), while fish from the control group were collected at the end of the experiment and were recorded as 0 d sample. The sampled fish were anesthetized with 75 mg/L MS-222, and seven tissues associated with immunity, including liver, spleen, intestine, kidney, head kidney, brain, and heart tissues, were rapidly collected for RNA extraction. All tissue samples were immediately frozen in liquid nitrogen and then stored at −80°C until RNA extraction. All animal procedures in this study were conducted according to the guidelines for the care and use of laboratory animals of Heilongjiang River Fisheries Research Institute, CAFS.

DNA isolation and first-strand cDNA synthesis

Total RNA was extracted using Trizol® Reagent (Invitrogen, USA) according to the manufacturer’s protocol. The quality and quantity of RNA of each sample were measured using a NanoDrop™ 8000 spectrophotometer (Thermo Scientific, USA). All extracted samples had an A260/280 ratio greater than 1.8 and were diluted to 250 ng/μL. One microgram of total RNA from each sample was used as the template for reverse transcription using the PrimeScript® RT Reagent Kit With gDNA Eraser (TaKaRa, Dalian, China). Genomic DNA elimination reaction was performed in a total volume of 10 μL containing 2 μL 5xgDNA Eraser Buffer, 2 μL gDNA Eraser, 1 μg total RNA, and 5 μL RNase free dH2O. The reaction was carried out at 42°C for 2 min. Reverse-transcription reaction was performed in a 20 μL reaction volume containing 10 μL of upper reaction solution, 4 μL 5xPrimeScript Buffer 2, 1 μL PrimeScript RT Enzyme Mix 1, 1 μL RT PrimerMix, and 4 μL RNase free dH2O. The reactions were carried out at 37°C for 15 min followed by 85°C for 5 s. The reaction products were stored at −20°C till further analysis.

Cloning of full-length cDNA of LwTf

To amplify the core sequence of LwTf gene, gene-specific primer pairs (TF-F1/TF-R1) were designed using Primer Premier 5.0 based on the previous transcriptome data of Amur ide (Chang et al. 2013). PCR was performed in a 25 μL reaction volume containing 18.3 μL of double-distilled water, 2.5 μL of 10x Ex Taq buffer, 1.0 μL of dNTP mix
Bioinformatics analysis

The LwTf cDNA sequence was analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast), and then deposited to GenBank under accession number MN379464. The ORF of LwTf was predicted using ORF finder (http://www.ncbi.nlm.nih.gov/orffinder/cgi). Protein domain features were predicted by SMART (http://smart.embl-heidelberg.de/). Signal peptides were predicted using SignalP5.0 (http://www.cbs.dtu.dk/services/SignalP/). The protein families, domains, and functional sites were determined using InterPro software (http://www.ebi.ac.uk/interpro/). The physical and chemical properties of the proteins were predicted by ProtParam (http://web.expasy.org/protparam/). Transferrin coding sequences from Amur ide and various species were retrieved from GenBank for multiple sequence alignment using Clustal Omega multiple alignment program (http://www.ebi.ac.uk/Tools/msa/clustalo/). A phylogenetic tree was reconstructed by the neighbor-joining method implemented in MEGA version 7.0 based on the sequence alignment using Clustal W and DNAMAN software. The branch supports were assessed with 10,000 bootstrap replications.

Quantitative real-time PCR (qPCR) and statistical analysis

Quantitative real-time PCR (qPCR) was used to determine the expression of LwTf in nine different tissues as well as its expression pattern in seven tissues associated with immunity after high alkaline challenge. First-strand cDNA was synthesized using PrimeScript® RT Reagent Kit with gDNA Eraser (TaKaRa). The cDNA was diluted to 1:100 and stored at −20°C. Primers used for qPCR are given in Table 1. Two LwTf gene-specific primers were used to amplify a 142 bp target product. In addition, primers for 18S ribosome RNA gene were used to amplify a 183 bp product as an internal control (Brown et al. 1983). qPCR was performed in triplicates for each sample using an ABI 7500 (Applied Biosystems, USA). The reaction was performed in a 20 μL reaction volume containing 10 μL of 2××TB Green Premix Ex Taq (TaKaRa), 0.4 μL of 50××ROX Reference Dyel, 0.8 μL each of 10 μM forward and reverse primers, 2.0 μL of cDNA, and 6.0 μL of RNase-free water. qPCR was performed as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and a melt curve step (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s). All samples were analyzed in triplicates. The variance of each replicate was tested in one group in order to make sure there were no tank effects. The relative levels of LwTf were calculated using the 2^ΔΔCT method (Livak and Schmittgen 2001). Statistical analysis was performed with one-way ANOVA and Duncan’s variance significance tests for pairwise comparison, using SPSS 13.0 software. Differences were considered significant at P < 0.05.

Results

Identification and sequence alignment of LwTf

The full-length LwTf cDNA sequence (accession no. MN379464) contained an open reading frame (ORF) of 199bp encoding 665 amino acids (aa), a 5’- untranslated region (UTR) of 29 bp, and a 3’-UTR of 279 bp with a stop codon (TAA) as well as a stop polyadenylation signal (AATAAA). The deduced protein possessed 665 residues with a putative 20-amino-acid N-terminal signal peptide. The mature transferrin protein had a theoretical molecular mass of 72.9 kDa and an isoelectric point of 6.36. In contrast to the mammalian transferrin gene, LwTf did not contain potential N-glycosylation sites, similar to that observed in cyprinid fish (Lin et al. 2007; Sahoo et al. 2009), but not in salmonid fish (Lee et al. 1998). LwTf contained two lobes (N-lobe: residues 24–331 and C-lobe: residues 335–658) of approximately 300–330 amino acids each (Figure 1). There was a 31.34% homology between the two lobes of LwTf. Inter-Pro software analysis showed two structural domains (N-lobe and C-lobe) of LwTf contained four iron-binding sites, two anion sites, and the signature features of the transferrin family, namely, transferrin signatures 1, 2, and 3, which suggested that LwTf also had the necessary structural properties to serve as an iron transport protein (Tu et al. 1991). The LwTf iron-binding sites at Asp73/Asp382, Tyr301/Tyr416, Cys396/Tyr511, and Arg252/His581 corresponded to those of the end of the N-lobe was lower than that in the C-terminal region DyeII, 0.8 μL of 50××ROX Reference Dyel, 0.8 μL each of 10 μM forward and reverse primers, 2.0 μL of cDNA, and 6.0 μL of RNase-free water. qPCR was performed as follows: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s, 60°C for 30 s, and a melt curve step (95°C for 15 s, 60°C for 1 min, and 95°C for 15 s). All samples were analyzed in triplicates. The variance of each replicate was tested in one group in order to make sure there were no tank effects. The relative levels of LwTf were calculated using the 2^ΔΔCT method (Livak and Schmittgen 2001). Statistical analysis was performed with one-way ANOVA and Duncan’s variance significance tests for pairwise comparison, using SPSS 13.0 software. Differences were considered significant at P < 0.05.

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Homology and phylogenetic analysis

Analysis of the deduced amino acid sequence by multiple sequence alignment indicated that LwTf had the highest, viz. 73.04%, identity match with the transferrin protein sequence from silver carp (Hypophthalmichthys molitrix), LwTf shared 64–74% identity with transferrin protein sequences from cyprinid fish, including grass carp (Ctenopharyngodon idella), goldfish (Carassius auratus), common carp (Cyprinus carpio), bighead carp (Hypophthalmichthys nobilis), and zebrafish (Danio rerio), and 43–45% identity with mammalian (human and mouse) serum transferrin and lactoferrin (Table 2). BLAST homology search indicated that the transferrin gene is moderately conserved through evolution. Multiple sequence alignment showed more similarities in the C-lobe when aligned with seven other homologous sequences (Figure 2). The number of the conserved amino acid sequences that are shaded at the end of the N-lobe was lower than that in the C-terminal
Table 1. Primers used for transferrin (Tf) gene cloning and expression analysis in Amur ide.

| Primer name | Sequence (5′–3′) | Ta (°C) | Target size (bp) | Application |
|-------------|-----------------|--------|-----------------|-------------|
| Tf-F1       | AAGACTTCTCGCCACAGTTGATAT | 55     | 1648            | Cloning of Tf gene |
| Tf-R1       | TAGGAACACTAATCCACAAAGTGC | 55     | 500             | 3′RACE (1st round PCR) |
| Tf-3        | CATGTAACGTCAGTGACAGCGG | 55     | 600             | 5′RACE (1st round PCR) |
| Tf-3′       | CATTGGGAGGATTGATGAGA | 55     | 600             | 5′RACE (2nd round PCR) |
| Tf-5′       | TTGATATCCAGGAACACAAGTGC | 55     | 600             | 5′RACE (2nd round PCR) |
| Tf-QF       | ATGGGACCCCTTTGCCTGCTT | 60     | 142             | qRT-PCR of TF |
| Tf-QR       | TTTCTAGCTGCCGCTCTGC | 60     |                 | qRT-PCR of 18S |
| Oligo(dT)17 | GACTCGAGTCGACATCGA(T) 17 |         |                 | Universal primer for 3′ RACE |
| Linker adapter | GACCTCGAGCTGGACATCG |         |                 | Universal primer for 5′ RACE |
| A′ oligo    | AAGGACGTTGATCAGCAGAGTACGGGG |         |                 |                                    |
| 5′CDS        | T25′VN           |         |                 |                                    |
| UPM         | CTATACGACACTATAAGGGCGAGCAGTATCAACGAGGAGACGAGTACGGGG |         |                 |                                    |
| NUP         | AAGGACGTTGATCAGCAGAGTACGGGG |         |                 |                                    |
| 18S-QF      | GGAGGTTCAAGAGGATACG | 60     | 183             | qRT-PCR of 18S |
| 18S-QR      | GTGAGGTTTCCCGTGTGAGG | 60     |                 | qRT-PCR of 18S |

Notes: V = A/C/G; N = A/C/G/T; Ta: annealing temperature; RACE: rapid amplification of cDNA ends.

Expression analysis of Tf in different tissues

In AW, Tf was expressed in all examined tissues and was particularly highly expressed in the liver, followed by spleen, heart, and intestine (Figure 4, P < 0.05). Moderate expression levels were found in the muscle, brain, head kidney, and kidney. The lowest expression level was found in the gill. In FW as well, Tf was expressed in all examined tissues and was found to be highly expressed in the liver, followed by the brain, spleen, and intestine (Figure 4, P < 0.05). Moderate expression levels were found in the head kidney, kidney, heart, and muscle. A low expression level was also observed in the gill. Taken together, Tf was highly expressed in classical immune-related organs (liver and spleen).

Expression profiles of Tf in AW after high alkaline stress

Transferrin modulation in seven tissues associated with immunity was explored to determine its involvement in the immune response of AW against high alkaline stress. In the liver, Tf expression was significantly downregulated from day 1 to day 3 post-challenge compared with that in the control group (P < 0.01). Afterward, Tf expression gradually recovered to the level observed on day 1. During the whole process of high alkaline stress, the expression level of Tf was significantly downregulated (Figure 5(a)). In the spleen, Tf expression was decreased on day 1 post-challenge compared with that in the control group (P > 0.05). However, at 3 d to 5 d post-challenge, Tf expression was significantly upregulated. The expression levels then returned to normal on day 7 post-challenge (Figure 5(b)). During the whole process of high alkaline stress (from day 1–7), the expression level of Tf was significantly downregulated in the intestine and kidney, as compared with that in the control group (Figure 5(c,d)). In the head kidney, Tf expression was significantly upregulated from day 1 to day 3 post-challenge as compared with that in the control group. However, on 5–7 days post-challenge, Tf was significantly downregulated compared with that in the control group (Figure 5(e)). In the brain, Tf expression was significantly upregulated on day 3 post-challenge compared with that in the control group. However, on 5–7 days post-challenge, Tf was significantly downregulated throughout the whole duration of high alkaline stress, although there was a slow recovery on day 3 and day 5 (Figure 5(g)).

Expression profiles of Tf in FW after high alkaline stress

The expression profiles of Tf in seven tissues associated with immunity in FW were determined to show the response of transferrin after high alkaline stress. In the liver, Tf expression was significantly upregulated at day 3 post-challenge compared with that in the control group (P < 0.01). The expression levels then returned to normal on day 7 post-challenge (Figure 6(a)). In the spleen, Tf expression was found to have increased on day 1 post-challenge compared with that in the control group (P > 0.05). Furthermore, on days 3–5 post-challenge, Tf expression was significantly upregulated. The expression levels then returned to normal on day 7 post-challenge (Figure 6(b)). In the intestine and brain, during the whole duration of high alkaline stress (from days 1–7), the expression level of Tf was significantly downregulated compared with that in the control group (Figure 6(c,f)). In the kidney, Tf expression was significantly downregulated on...
Figure 1. The mRNA and predicted amino acid sequence of *Leuciscus waleckii* transferrin (abbreviated as *LwTf*). The nucleotides and amino acids are indicated in the upper and lower row, respectively. The signal peptide sequence is in italics. The regions of the N-lobe and the C-lobe are indicated by an arrow. The four iron-binding sites in each lobe of transferrin are indicated by the panes, and the two anion sites in each lobe are shaded. The characteristic transferrin motifs are underlined as follows: transferrin signature 1, transferrin signature 2, and transferrin signature 3.
Table 2. Percent identity and similarity of transferrin amino acid sequences of *Leuciscus waleckii* to those of other organisms.

| Organism                          | Identity (%) | Similarity (%) |
|----------------------------------|-------------|----------------|
| *Leuciscus waleckii*             | 100         | 96.87          |
| *Mus musculus*                   | 45.20       | 47.32          |
| *Danio rerio*                    | 41.36       | 47.32          |
| *Cyprinus carpio*                | 45.95       | 47.32          |
| *Carassius auratus*              | 45.95       | 47.32          |
| *Ctenopharyngodon idella*        | 45.95       | 47.32          |
| *Hypophthalmichthys molitrix*    | 45.95       | 47.32          |
| *Hypophthalmichthys nobilis*     | 45.95       | 47.32          |
| *Ictalurus punctatus*             | 45.95       | 47.32          |
| *Paralichthys olivaceus*         | 45.95       | 47.32          |
| *Oncorhynchus nerka*             | 45.95       | 47.32          |
| *Salmo salar*                    | 45.95       | 47.32          |
| *Salmo trutta*                   | 45.95       | 47.32          |

Notes: The MegAlign program of the DNAstar software package was utilized to calculate percentage identities of amino acid sequences of aligned transferrin (LwTf) and other transferrins (Tfs) from multispecies. Data in table represent the percentage identities between two amino acid sequences. GenBank accession numbers of the Tfs in this table: *Leuciscus waleckii* MN379464, *Mus musculus* AAH92046.1, *Danio rerio* AAH64001.2, *Cyprinus carpio* ACD99639.1, *Carassius auratus* AAM90972.1, *Ctenopharyngodon idella* ACF18029.1, *Hypophthalmichthys molitrix* ADI67810.1, *Hypophthalmichthys nobilis* ADF97634.1, *Ictalurus punctatus* ACD26711.1, *Paralichthys olivaceus* BAA28944.1, *Oncorhynchus nerka* BAA40961.1, *Salmo salar* AA418338.1, *Salmo trutta* BAA4102.1, *Acanthopagrus schlegeli* AAQ63949.1, *Lates calcarifer* BAF81983.

Discussion

In this study, the transferrin gene of Amur ide was identified and characterized. The results of the similarity study indicated that the transferrin gene has been moderately conserved through evolution to enhance the transport of iron ions alone and have been thought to be formed by gene duplication during evolution to enhance the transport of iron ions. The transferrin gene has been moderately conserved through evolution to enhance the transport of iron ions alone and have been thought to be formed by gene duplication during evolution to enhance the transport of iron ions. The transferrin gene has been moderately conserved through evolution to enhance the transport of iron ions alone and have been thought to be formed by gene duplication during evolution to enhance the transport of iron ions. The transferrin gene has been moderately conserved through evolution to enhance the transport of iron ions alone and have been thought to be formed by gene duplication during evolution to enhance the transport of iron ions.
with that in the spleen, intestine, and brain in FW ($P < 0.05$). Previous studies have reported that transferrin expression is species-specific in the brains of teleost fish and mammals (Tu et al. 1991; Denovan-Wright et al. 1996; Sahoo et al. 2009). Different results for transferrin expression were observed in the brain tissues among various species (Kvingedal et al. 1993; Gao et al. 2013). In this study, the LwTF expression patterns in the brain were found to be opposite in AW and FW. Due to their long-term geographical isolation and environmental adaptability, Amur ide have formed two different geographic
populations (AW and FW), which may be a reason for the different expression levels of \( LwTf \) in the brain. In vertebrates, with a relatively independent environment owing to the blood–brain barrier, transferrin is likely synthesized locally (Liu et al. 2012; Chen et al. 2014). However, delineating the underlying mechanism could contribute to a better understanding of why transferrin expression is observed in the brain of some fish species but not in other closely related species.

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**Figure 3.** Phylogenetic analysis of *Leuciscus waleckii* and other vertebrate transferrin genes. The phylogenetic tree was constructed based on ClustalW-generated multiple sequence alignment of amino acid sequences using the neighbor-joining method within the MEGA 7.0 package. The topological stability of the neighbor-joining trees was evaluated by 1000 bootstrapping replications, and the bootstrapping percentage values are indicated by numbers at the nodes. The GenBank accession number for each sequence is given after the species name.

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**Figure 4.** Gene expression analysis of \( LwTf \) in different tissues of alkali-adapted species (abbreviated as AW) and freshwater species (abbreviated as FW) in Amur ide. Expression levels were calculated against the tissue that had the lowest expression level, and the 18S rRNA housekeeping gene was used as a reference gene. HK is the abbreviation for head kidney. Significant differences are indicated by different letters \((n = 3, P < 0.05)\).
Figure 5. Time-course analysis of the LwTf expression patterns in alkali-adapted species (abbreviated as AW) after high alkaline stress treatment in the liver, spleen, intestine, kidney, head kidney, brain and heart using qRT-PCR. The samples were analyzed at 1, 3, 5, and 7 d post-treatment. Fold change was calculated by the change in expression at a given time point relative to the untreated control and normalized by the change in the 18S rRNA housekeeping gene. The results are presented as the mean ± SE of fold changes, and significant differences in different time points compared to the control (0 d) are indicated by asterisks (*P < 0.05, **P < 0.01).
Figure 6. Time-course analysis of the \textit{LwTf} transferrin expression patterns in freshwater species (abbreviated as FW) after high alkaline stress treatment in the liver, spleen, intestine, kidney, head kidney, brain and heart using qRT-PCR. The samples were analyzed at 1, 3, 5, and 7 d post-treatment. Fold change was calculated by the change in expression at a given time point relative to the untreated control and normalized by change in the 18S rRNA housekeeping gene. The results are presented as the mean ± SE of fold changes, and significant differences in different time points compared to the control (0 d) are indicated by asterisks (*$P$ < 0.05, **$P$ < 0.01).
Amur ide usually inhabits freshwater environments but can also survive in the highly alkaline (up to pH 9.6) water of Dali-Nor lake, an extremely alkaline lake with \( \text{HCO}_3^-/\text{CO}_2^- \) concentration over 53.57 mmol/L. The fish has evolved special mechanisms to adapt to changes in alkalinity, although the mechanisms underlying its high tolerance to alkalinity are still largely unknown. Recently, a series of exploratory studies on the molecular mechanism of Amur ide’s adaptation to high alkalinity have been carried out in the context of several aspects of physiology and biochemistry, population genetics, and transcriptome (Xu, Li, et al. 2013; Cui et al. 2015). Many of these studies found that the expression of genes related to osmotic pressure regulation, acid–base balance, ion transport, and immunity was significantly increased under high alkaline stress (Xu et al. 2017; Chen et al. 2019). Interestingly, \( LwTf \) expression was closely related to the osmotic pressure. The FW Amur ide needs more \( LwTf \) expression to resist high alkalinity compared to the AW. Hence, the \( LwTf \) expression in FW was significantly upregulated from days 1–3 post-challenge. This finding suggested that although transferrin is constitutively expressed in the liver, its expression can be induced throughout the body under high alkaline stimulation, which is related to the extensive involvement of transferrin in body’s immune defense function (Liu et al. 2012; Yin et al. 2018). As described above, the patterns of \( LwTf \) expression in seven tissues associated with the immunity of AW and FW were complicated. Consequently, further studies and functional analyses of the gene regulatory network of \( LwTf \) will help to provide important insights into the mechanisms that regulate \( LwTf \) expression upon high alkaline stress.

In conclusion, the full-length cDNA of \( LwTf \) was cloned and characterized. The results showed a high degree of conservation of gene and protein structure when compared with other fish species. The expression analyses demonstrated that \( LwTf \) was ubiquitously present in seven tested tissues associated with immunity and was highly expressed in classical immune organs (liver and spleen). Finally, it was interesting to find a significant upregulation of \( LwTf \) in the liver, spleen, intestine, kidney, head kidney, and heart in FW after high alkaline challenge. Thus, this protein might play a role in the immune system and help fish adapt to high alkalinity. The mechanisms involved in the function of \( LwTf \) during the adaptation of \( L. waleckii \) to high alkalinity need further investigation.

Disclosure statement
No potential conflict of interest was reported by the author(s).

Funding
This study was supported by the National Key R & D Program of China [2020YFD0900402, 2019YFD0900405], the Natural Science Foundation of Heilongjiang Province [QC2018038], the Natural Science Foundation of China [31602136], the Basic scientific research business expenses project of Chinese Academy of Fishery Sciences [2020TD56, 2020TD22] and the Key Laboratory of Freshwater Aquatic Biotechnology and Breeding of Ministry of Agriculture and Rural Affairs [FBB2017-04].

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