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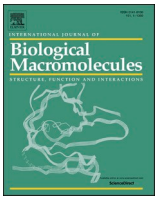

Identification, characterization, and transcription of serotonin receptors in rainbow trout (*Oncorhynchus mykiss*) in response to bacterial infection and salinity changes

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ABSTRACT

Serotonergic system is involved in the regulation of physiological functions and behavioral traits including cognition, memory, aggression, stress coping, appetite and immunomodulation. Serotonin exerts its functions via binding distinct serotonin receptors which are classified into 7 groups. Salmonid exhibits expanded functional gene copies due to salmonid-specific whole genome duplication. However, serotonin receptor (*htr*) repertoire is not fully identified in rainbow trout (*Oncorhynchus mykiss*). In this study, we identified 39 *htr* genes, including 14 *htr1*, 4 *htr2*, 4 *htr2* like, 3 *htr3*, 4 *htr4*, 2 htr5, 2 htr6, and 6 *htr7* subtypes. We investigated physiological functions of serotonin receptors in response to bacterial pathogens exposure and salinity changes. We showed *htr1*, *htr2*, *htr4* and *htr7* subtypes were associated with immunomodulation in response to *Vibrio anguillarum* or *Aeromonas salmonicida* infection. Saltwater (salinity of 15) transfer significantly altered *htr1*, *htr2*, *htr4*, and *htr7* subtypes, suggesting trout Htr was associated with osmoregulation. We further showed residues interacted with inverse agonist (methiothepin) and serotonin analogue (5-Carboxamidotryptamine) were conserved between trout and human, suggesting exogenous ligands targeting human HTRs might have a role in aquaculture. This study showed duplicated trout Htrs might be physiologically neofunctionalized and potentially exhibit pleiotropic effects in regulating immunomodulation and osmoregulation.

1. Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is an important monoamine neurotransmitter involved in regulating multiple physiological functions in the central nervous system (CNS) and peripheral systems [1]. Around 95 % of serotonin is secreted and stored in periphery tissues, especially the enterochromaffin (EC) cells in gastrointestinal (GI) tract [1,2]. In the CNS, serotonergic system regulates cognition, memory, appetite, and behaviors, while the peripheral serotonin is involved in vascular biology, cardiovascular function, and bowel motility [1,3]. Meanwhile, serotonin acts as immune modulators in CNS and peripheral tissues [1]. Serotonin regulates functions of immune cells via serotonin receptors which are widely expressed in immune cells, including monocytes, macrophages, dendritic cells, natural killer cells, T cells, and B cells [1]. For example, serotonin could recruit and exert chemoattractant properties on mammalian immune cells and stimulate pro-inflammatory cytokines production from macrophages (Reviewed in [1]).

Fish is an important high-quality protein source and statement of the Food and Agriculture Organization (FAO) of the United Nations showed fish provides ~16 % of animal protein consummation worldwide (FAO, 1997; [4]). Recently, a *Nature* paper published in 2021 showed global aquaculture production increased significantly from 34 million tonnes in 1997 to 112 million tonnes in 2017 [5]. Rainbow trout (*Oncorhynchus mykiss*) is an important aquaculture salmonid species with global production of ~1000 thousand tonnes in 2022 (The State of World Fisheries and Aquaculture, 2022). Infectious diseases outbreaks, such as vibriosis and furunculosis, result in significant setbacks and financial losses to trout culture. The *Aeromonas salmonicida* (*A. salmonicida*) and *Vibrio anguillarum* (*V. anguillarum*) are two bacterial pathogens result in severe

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fatal diseases of vibriosis and furunculosis, respectively [6,7]. However, effects of serotonin system on trout immune responses in response to vibriosis and furunculosis need to be further investigated.

Global climate change resulting from anthropogenic activities causes high levels of threat to freshwater ecosystem and water security [8,9]. Therefore, the freshwater allocation for aquaculture is limited. Marine aquaculture presents advantages for the growing demand for fishery protein with limited freshwater resources [10]. Offshore aquaculture in deep and open marine environment is an emerging approach to mariculture that provides new opportunities for large volumes of salmonid production [5]. However, the current marine trout production is only ~one third when compared to inland trout production (The State of World Fisheries and Aquaculture, 2022). After seawater transfer, ~15 % of the transferred fish failing to adapt the salinity conditions and eventually dying [11–13]. The L-tryptophan is the precursor of the serotonin and dietary supplementation with L-tryptophan has been reported to produce Super Smolt for salinity acclimation [14]. These evidences suggested serotonin system is involved in salinity acclimation in salmonid.

Serotonin receptors exhibit large members when comparing to other neurotransmitter receptors [15]. The 5-HT_{1A} receptor (gene symbol: *HTR1A*) is the first identified serotonin receptor, and after that, around 18 serotonin receptors have been identified and characterized into 7 families, including 5-HT_{1A} - 5-HT_{1F} except for 5-HT_{1C} (5-HT_{1C} is renamed to 5-HT_{2C}), 5-HT_{2A} - 5-HT_{2C}, 5-HT_{3A} - 5-HT_{3E}, 5-HT_4, 5-HT_{5A}, 5-HT_{5B}, 5-HT_6 and 5-HT_7 receptors. Most of serotonin receptors are G protein-coupled receptors (GPCRs), while 5-HT_3 receptor subtypes act as ligand-gated ion channels. Serotonin receptors are coupled with diverse downstream signaling and widely distributed in multiple tissues, leading to physiology and pharmacology diversity [15]. The 5-HT_1 and 5-HT_5 receptors coupled with $G\alpha_i$ subunit lead to reduced cyclic adenosine monophosphate (cAMP), and 5-HT_4, 5-HT_6 and 5-HT_7 receptors coupled with $G\alpha_s$ subunit cause increased intercellular cAMP levels after activation. The 5-HT_2 receptors interact with $G\alpha_q$ subunit and stimulation increase inositol triphosphate (IP3), calcium, and diacylglycerol (DAG) levels.

Two rounds (2R) of whole genome duplication (WGD) results in increased complexity and genome size in vertebrate [16,17]. Three rounds (3R) of WGD occurred in common teleost fish ancestor and four rounds (4R) of WGD occurred in Salmonids ancestor (also termed as salmonid-specific WGD, ssWGD) [18]. Gene duplication leads to novel gene functions and increases the biological complexity [19,20]. For example, ssWGD results in genetic expansions of GHRH-SST-GH-IGF system in salmonids, with duplicated paralogs exerting functional diversity in immunomodulation and osmoregulation [21–23]. Therefore, we investigated the serotonin receptor gene repertoire in rainbow trout and evaluated the physiology of the serotonin receptors in response to bacterial pathogens exposure and salinity change. Our study may provide insight into molecular mechanisms of serotonin systems in regulating immunomodulation and osmoregulation.

2. Materials and methods

2.1. Genome-wide identification of htr genes

The *htr* genes in rainbow trout were identified by searching the whole genomic sequence database (GCF_013265735.2). The *HTR* (*htr*) genes from the following species including human (*Homo sapiens*), zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*) were retrieved from NCBI (https://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) database as query sequences. TBLASTN and ClustalW software were used to remove the redundant sequence(s) and to generate the initial *htr* candidate sequences pool in rainbow trout for further analysis. The molecular weight (MW) and theoretical isoelectric point (pI) of Htr proteins were predicted by online ProtParam tool (https://web.expasy.org/protparam/). The *htr* genes were named (or renamed) based on the similarity to the vertebrate orthologs.

2.2. Phylogenetic of HTRs (Htrs) and syntenic analyses

In order to investigate the evolutionary relationships of the *htr* gene family, the phylogenetic tree was conducted via amino acid sequences of HTR (Htr) proteins from human, mouse (*Mus musculus*), chicken (*Gallus gallus*), zebrafish, channel catfish (*Ictalurus punctatus*), fugu (*Takifugu rubripes*), Japanese medaka (*Oryzias latipes*), Nile tilapia (*Oreochromis niloticus*), Japanese flounder (*Paralichthys olivaceus*), Atlantic salmon and others. Multiple protein sequences were aligned by ClustalW and phylogenetic tree was built by MEGA 11 with Neighbor-joining method and Jones-Taylor-Thornton model with 1000 bootstrap replications. Additionally, the phylogenetic tree was further prettified by online website iTOL (https://itol.embl.de/). The rainbow trout genomic sequence database was used to obtain information about collinear block via TBtools. The position of each *htr* gene on the chromosome was displayed according to the coordinates on the genome.

2.3. Animal study

Animal studies were conducted in accordance with guidelines of Animal Research and Ethics Committee of Ocean University of China (Permit Number: 2014201), the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications NO. 8023, revised 1987). Endangered or protected species were not involved in this study. Trout were immature, and the influence of gender was not considered. This manuscript used RNA-Seq samples previously described in our papers to investigate the immunomodulation in response to bacterial infection (*A. salmonicida* and *V. anguillarum*) and endocrine regulation in response to salinity change [23–25]. The methods and protocols were briefly described below.

2.3.1. V. anguillarum infection

Our previous papers detail protocols of animal acclimation, animal stocking and bacterial challenge. We showed *V. anguillarum* of 10^7 CFU/mL exhibits mild vibriosis symptoms and a relatively lower mortality [26]. Briefly, trout were equally divided into control (Ctrl) group and infected group. Each group had 3 replications and each replication had 30 individual trout. Trout of infected group was intraperitoneally infected with 0.2 mL of *V. anguillarum* at 1×10^7 CFU/mL. In control group, trout were intraperitoneally infected with 0.2 mL of physiological saline (0.9 % NaCl). In infected group, the first three moribund trout (erratically swimming with severe vibriosis symptoms) in each replication were selected as symptomatic trout (ST). After 120 h of *V. anguillarum* challenge, the three surviving trout in each replication of infected group were selected as asymptomatic trout (AT). Brain, spleen and kidney samples were collected for RNA-Seq analysis. Three samples were pooled to reduce the individual variations.

2.3.2. A. salmonicida infection

Trout were divided into two groups as control (Ctrl) group and infected group. Based on our previous studies, infected group were intraperitoneally infected with 0.2 mL of *A. salmonicida* at 1×10^8 CFU/mL [25]. Trout in control group were intraperitoneally injected with 0.2 mL of phosphate buffer solution (PBS). Trout were sacrificed at 48 h post infection because furunculosis symptoms were observed. Brain and kidney samples were collected and two samples in infected group were pooled to reduce the individual variations for RNA-Seq analysis.

2.3.3. Saltwater exposure

Diploid and triploid trout (~9.7 g) were divided into four groups of diploid freshwater exposure (DF), diploid saltwater exposure (DS), triploid freshwater exposure (TF), and triploid saltwater exposure (TS) [23]. Each group had three replications and each replication had 18

trout. DF and TF were stocked with fresh water while DS and TS were stocked with saltwater at salinity of 15 (15 ppt). On day 7, the survival rates were over 95 % in all groups. The brain, liver, and kidney samples of DS, TS and TF were collected on day 7 for RNA-Seq analysis. Two samples from one replication were pooled into one sample for RNA-Seq analysis to reduce the individual variations.

2.4. RNA-Seq library and data analysis

A total of 78 libraries were constructed by the commercial provider (OEbiotech, Shanghai, China) for RNA-Seq analysis, including (1) 27 libraries for *V. anguillarum* infection (3 tissues × 3 replicated samples × 3 groups); (2) 12 libraries for *A. salmonicida* infection (2 tissues × 3 replicated samples × 2 groups); (3) 27 libraries for saltwater exposure (3 tissues × 3 replicated samples × 3 groups); (4) 6 libraries of trout intestine and 6 libraries of trout liver. Details of the library preparation and bioinformatics are shown in published papers [23–25,27]. The sequence reads are available in the Sequence Read Archive Database (SRA) with accession number PRJNA667799 (*V. anguillarum* infection), PRJNA753277 (*A. salmonicida* infection), PRJNA844477 (saltwater exposure), PRJNA866872 and PRJNA867038 (intestine and liver *htr* genes evaluation, not released).

According to previous studies in biomedical and fishery studies, we investigated the overall gene expression profiles of *htr* paralogs via multivariate analysis module in MetaboAnalyst (www.xialab.ca/tools.xATml) [28–30]. Count of *htr* genes were normalized by DESeq2 package in the R software [31] and then the normalized counts were uploaded for principal component analysis (PCA) and loading plot analysis. The 27 RNA-Seq libraries for *V. anguillarum* infection and 12 RNA-Seq libraries for *A. salmonicida* infection were conducted in two batches. Therefore, *htr* gene expression in *V. anguillarum* or *A. salmonicida* exposure group was normalized by the cognate control group (fold change to control group) to reduce the batch effect of RNA-Seq analysis. The 27 RNA-Seq libraries for saltwater exposure were conducted in same batch.

Univariate analysis was used to investigate the transcriptional signature of *htr* genes in response to bacterial infection and saltwater exposure. The count (normalized by DESeq2 package in the R software [31]) was used to investigate tissue *htr* gene expressions and generate the heatmap of in brain, intestine, kidney and liver. The following RNA-seq libraries from trout with similar body sizes (~10 g) were used for tissue *htr* gene expressions: brain and kidney from PRJNA753277, intestine from PRJNA866872 and liver from PRJNA867038. Effects of body size on tissue *htr* gene expressions were evaluated by comparing the large trout group (~108 g, PRJNA667799) and small trout group (~10 g, PRJNA753277). Univariate analysis of the individual *htr* gene expression was conducted via one-way analysis of variance with Tukey's test or Student's *t*-test via GraphPad Prism 8.0 ($p < 0.05$ as the significance threshold). Pearson correlation coefficient was used to evaluate the correlation analysis of gene expressions by GraphPad Prism 8.0 ($p < 0.05$ as the significance threshold).

2.5. Ligand binding pocket identification

The RNA-Seq data showed trout *htr1b3* and *htr7c2* were significantly changed in response to pathogen infection and salinity change. Therefore, we compared the binding pockets of exogenous ligands of trout Htr1b3 and Htr7c2 with human serotonin receptors (HTR1B and HTR7C). Based on crystal structures of human 5-HT1_B and 5-HT$_7$-(G_s) [32,33], comparisons between trout Htr1b3 (Htr7c2) and human receptors were generated via SEISS-MODEL. The Protein Data Bank (PDB) ID of human HTR1B and HTR7 were 5 V54 and 7XTC, respectively. The inverse agonist methiothepin (MT) and serotonin analogue 5-Carboxamidotryptamine (5-CT) were used to evaluate the binding pockets [32,33]. The PyMOL software was used to visualize the residues in the binding pocket.

3. Results

3.1. The whole repertoire of trout serotonin receptors

Totally, 39 *htr* genes were identified in rainbow trout, with 14 *htr1* subtypes, 4 *htr2* subtypes, 4 *htr2* like subtypes, 3 *htr3* subtypes, 4 *htr4* subtypes, 2 *htr5* subtypes, 2 *htr6* subtypes, and 6 *htr7* like subtypes (Fig. 1 and Table S1). Phylogenetic analysis showed 7 generated clades of Htrs, which was consistent with mammalian subgroup classifications (Fig. 1A). The collinear map of trout Htr subtypes showed *htr* genes were dispersed among 20 chromosomes (Fig. 1B). The collinear map showed the HTR (Htr) subgroups were paired with each other. The genomic distribution of *htr* genes was separated into two types: individual (mostly) and tandem arrangements (*htr3a1* and *htr3b*). The *htr3b* appeared to be a duplicate of *htr3a1* because it was localized on the same chromosome as tandem duplicated genes (Fig. 1B). Multiple protein sequence alignments of HTR (Htr) subtypes between human and trout are shown in Fig. 2 and Figs. S1 - S7.

3.2. Tissue expression of serotonin receptors

Tissue expression of *htr* genes in brain, intestine, kidney and liver are clustered by heatmaps (Fig. 3A - D). In brain, the top 10 highly expressed genes were *htr1aa1*, *htr1aa2*, *htr2cl1a*, *htr5ab2*, *htr2aa2*, *htr1ab2*, *htr1b1*, *htr2b2*, *htr7c2*, and *htr1ab1* (Fig. 3E). The top 10 highly expressed intestine *htr* genes included *htr4b*, *htr4a*, *htr2b2*, *htr2b1*, *htr2cl2a*, *htr4d*, *htr4c*, *htr7c2*, *htr2aa1*, and *htr7c1* (Fig. 3F). The *htr2b1*, *htr4a*, *htr1b4*, *htr4c*, *htr4b*, *htr4d*, *htr2cl2a*, *htr5ab2*, *htr7c1* and *htr2cl2b* exhibited higher expression in kidney (Fig. 3G), while *htr2b1*, *htr2b2*, *htr4b*, *htr2cl1b*, *htr6b* and *htr4d* were highly expressed in liver in basal conditions (Fig. 3H). Most of brain and kidney *htr* genes exhibited similar transcriptional profiles between larger and smaller trout (Fig. 3I - J), excepting for brain *htr3a1* and kidney *htr4b* (Fig. 3I - J).

3.3. Transcriptional profiles of serotonin receptors in response to bacterial infection

3.3.1. Brain transcriptional profiles

The overall transcriptional profiles of brain serotonin receptors in response to bacterial infection were clustered by heatmap (Fig. 4A). The data were further analyzed by unsupervised PCA for dimension reduction and discriminative gene selection. PCA exerted obvious discrimination between *V. anguillarum* and *A. salmonicida* infection groups (Fig. 4B). The loading plot showed brain *htr1b3*, *htr3b*, *htr4b*, *htr4d* and *htr6a* were genes for PCA discrimination (Fig. 4C). Compared to Ctrl group, symptomatic trout showed significantly up-regulated brain *htr1b3* and *htr2cl1b*, while asymptomatic trout showed significantly up-regulation of brain *htr1ab1*, *htr1b4*, *htr1fa1* and *htr2cl1a* after *V. anguillarum* infection (Fig. 4D and E). Symptomatic trout showed significantly up-regulated brain *htr1b3* when compared to asymptomatic group (Fig. 4F). Compared to Ctrl group, the brain *htr1b4*, *htr4d*, and *htr7c2* were significantly up-regulated after *A. salmonicida* infection (Fig. 4G). Brain *htr1aa2* exhibited positive correlations with *htr1aa1* and *htr1ab1* after *V. anguillarum* or *A. salmonicida* infection (exception between *htr1aa2* and *htr1ab1* after *A. salmonicida* infection with $p = 0.0996$, Fig. 4H and I). Brain *htr1ab1* showed positive correlations with *htr1fa1* after *V. anguillarum* or *A. salmonicida* infection (Fig. 4J), while brain *htr7a1* and *htr7a2* exerted positive correlation after *V. anguillarum* infection (Fig. 4K).

3.3.2. Kidney and spleen transcriptional profiles

Symptomatic trout showed a significantly up-regulated spleen *htr2cl1a* expression (Fig. 5A) and an increase trend of *htr7a4* expression ($p \sim 0.078$–0.095, Fig. 5B) after *V. anguillarum* infection. In kidney, symptomatic trout showed significantly down-regulated *htr2cl1a* expression when compared to Ctrl group after *V. anguillarum* infection

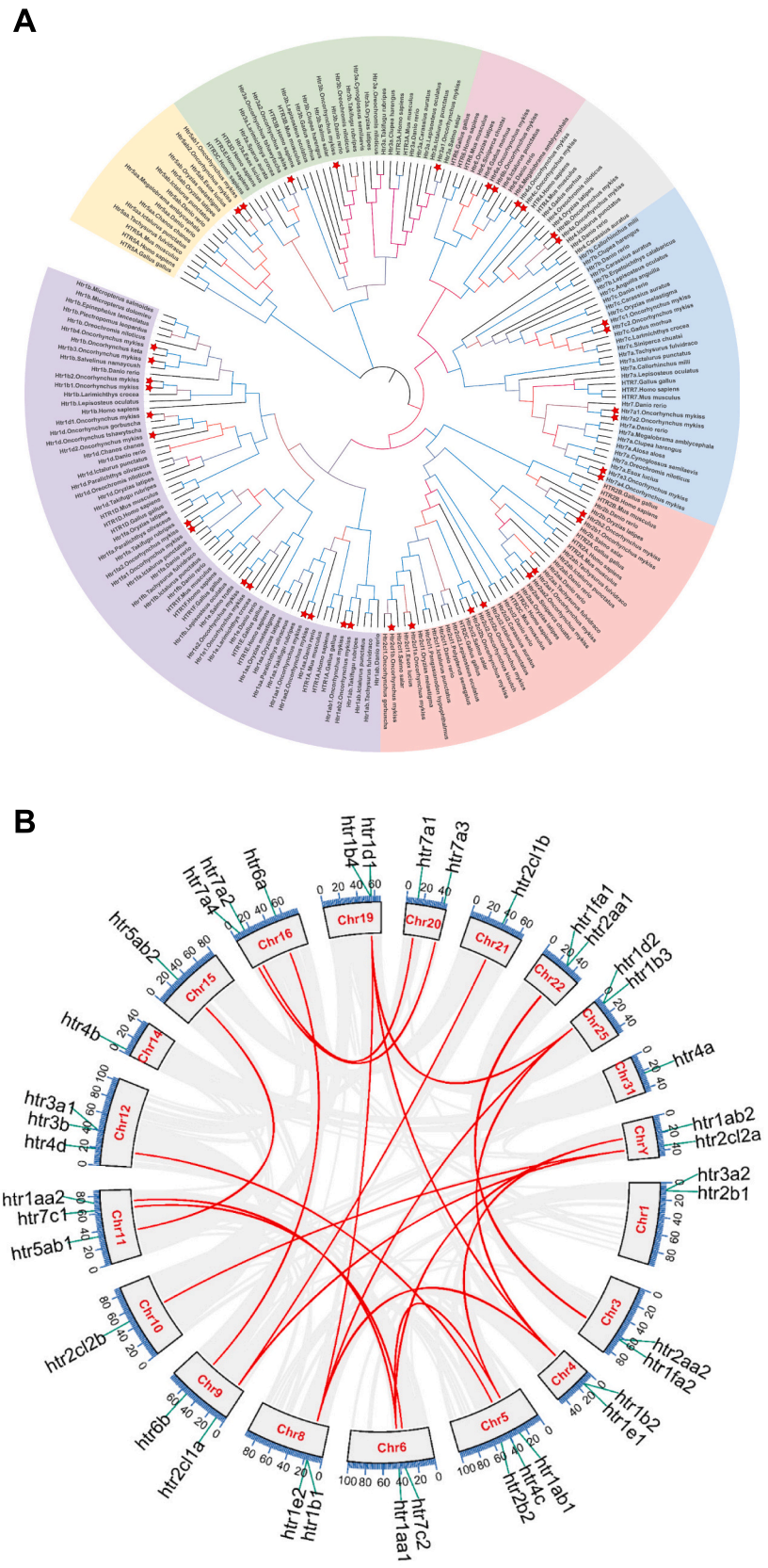

Fig. 1. Phylogenetic tree of HTR (Htr) and collinear map of trout *htr*.

A: Amino acid sequences of HTRs (Htrs) were obtained from NCBI and/or Ensemble databases. The red star labelled out Htrs of trout.

B: The interchromosomal relationships of *htr* genes. Gray lines represented all synteny blocks in the trout genome and the red lines indicated duplicated *htr* gene pairs.

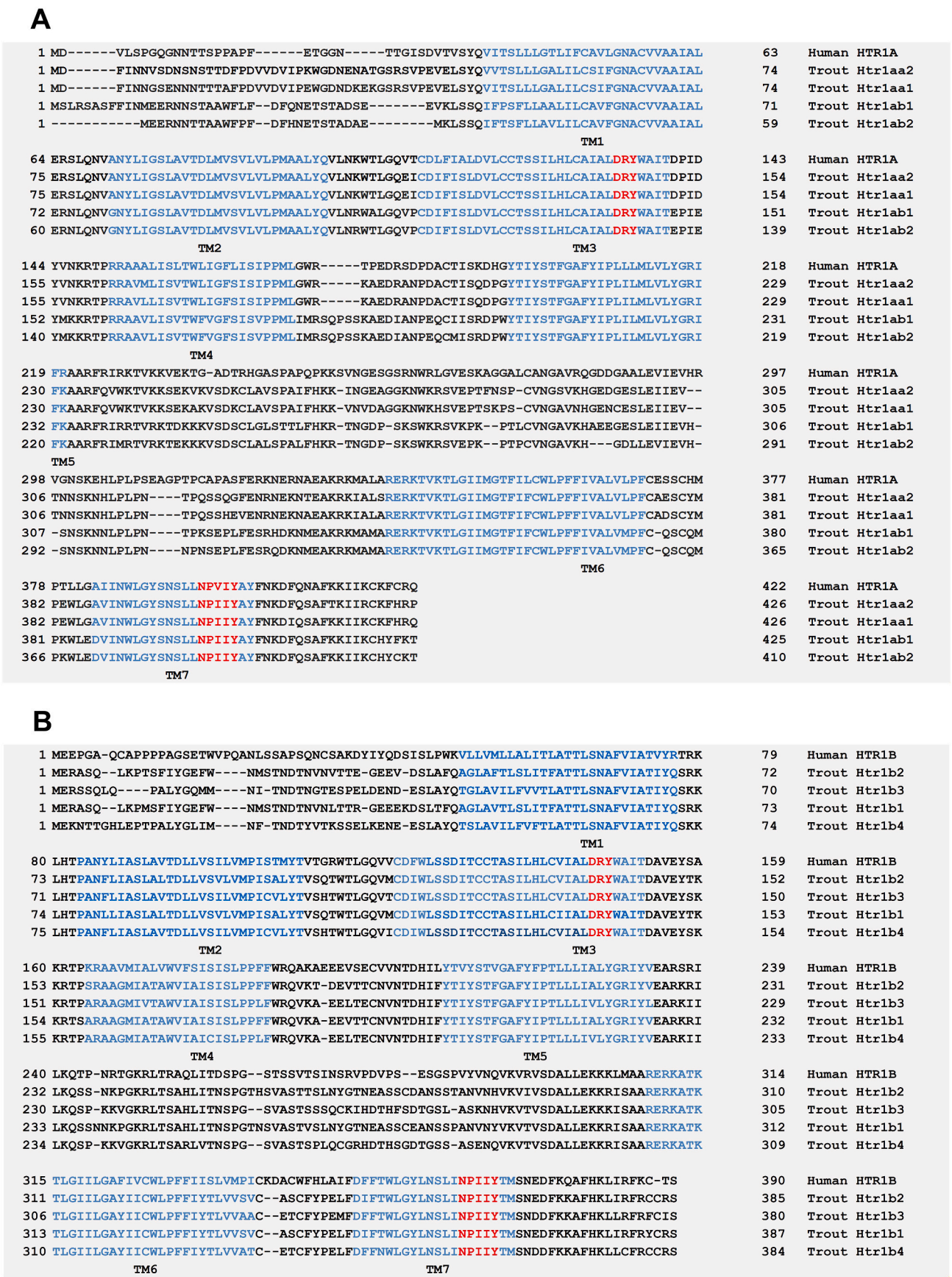

Fig. 2. Multiple protein sequence alignments of HTR1A (Htr1a) and HTR1B (Htr1b) subtypes between human and trout.
The seven transmembrane domains and conserved motifs (DRY, and NPxxY) are highlighted in HTR1A (Htr1a) (A) and HTR1B (Htr1b) (B) subtypes. Multiple protein sequence alignments of other HTR (*Htr*) subtypes are shown in Figs. S1 – S7 in the supplementary materials.

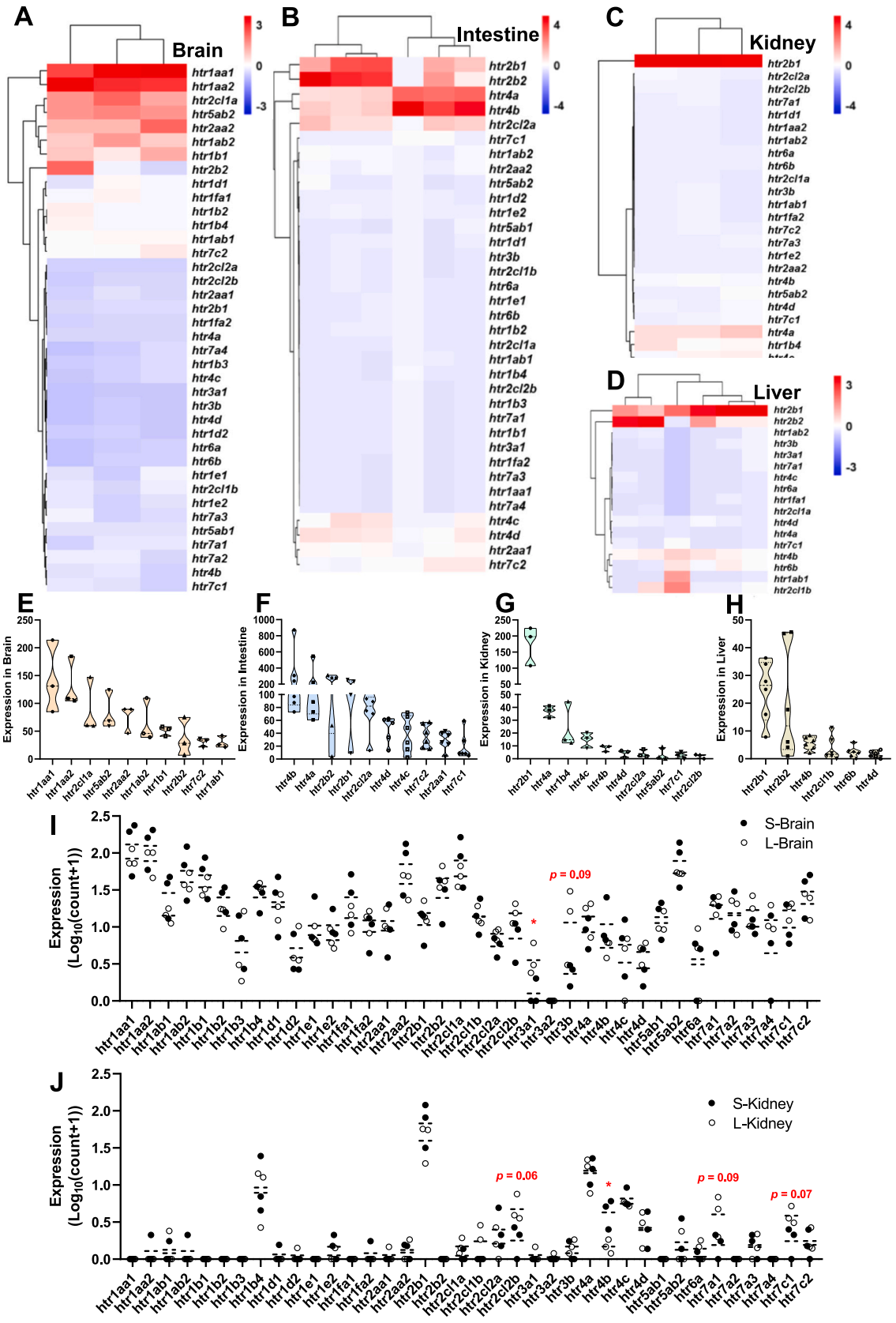

Fig. 3. Tissue expression of *htr* genes in trout.

A-D: Heatmap of *htr* expressions in brain (A), intestine (B), kidney (C) and liver (D).

E-H: The top 10 high expression *htr* genes in brain (E), intestine (F), kidney (G) and the top 6 high expression *htr* genes in liver (H).

I and J: Comparison of the *htr* gene expressions in brain (I) and kidney (J) between large and small trout. L indicates large trout and S indicates small trout. Normalized count by DESeq2 package was used to investigate *htr* gene expressions. Individual *htr* gene expression was conducted via Student's *t*-test. The * indicates $p < 0.05$.

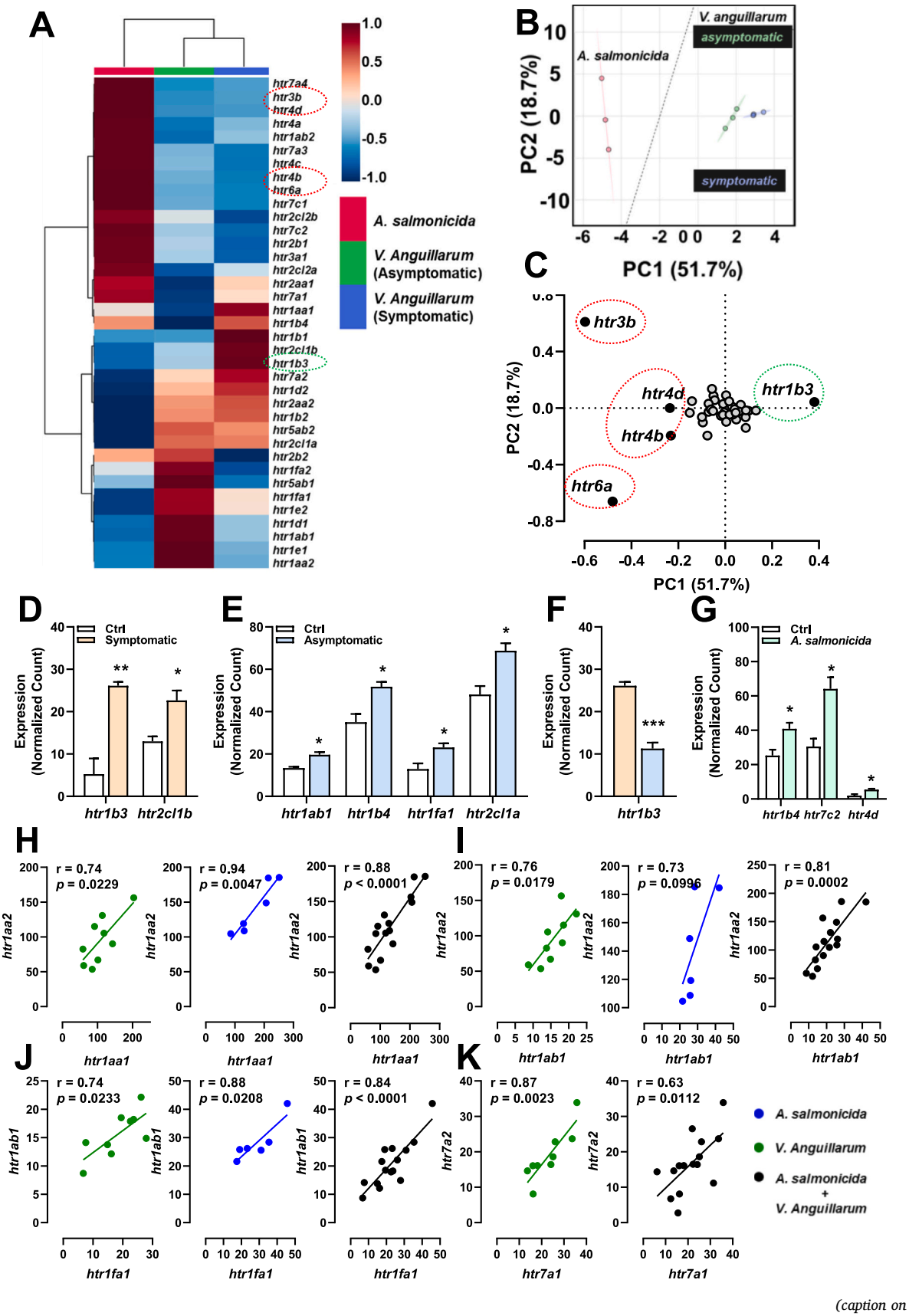

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Fig. 4. Transcriptional profiles of brain *htr* genes in response to bacterial infection.
A. The heatmap of brain *htr* genes.
B and C. PCA (B) and loading plot (C) of brain *htr* genes. In loading plot, gene(s) further away from center point (0, 0) exerts significant effects on PCA.
D - F: Key brain *htr* genes in response to *V. anguillarum* infection between Ctrl and ST (D), Ctrl and AT (E) and AT and ST (F).
G: Key brain *htr* genes in response to *A. salmonicida* infection between Ctrl and IT.
The *htr* gene expression was conducted via Student's *t*-test. The * and ** indicates $p < 0.05$ and $p < 0.01$, respectively.
H - K: Correlation analysis of brain *htr* genes between *htr1aa1* and *htr1aa2* (H), *htr1ab1* and *htr1aa2* (I), *htr1fa1* and *htr1ab1* (J), *htr7a1* and *htr7a2* (K). Pearson correlation coefficient was used to evaluate the correlation analysis of brain *htr* genes. The left panel shows correlation analysis in trout infected with *V. anguillarum*, the middle panel shows correlation analysis in trout infected with *A. salmonicida*, and the right panel shows the combined data of *V. anguillarum* infection and *A. salmonicida* infection.

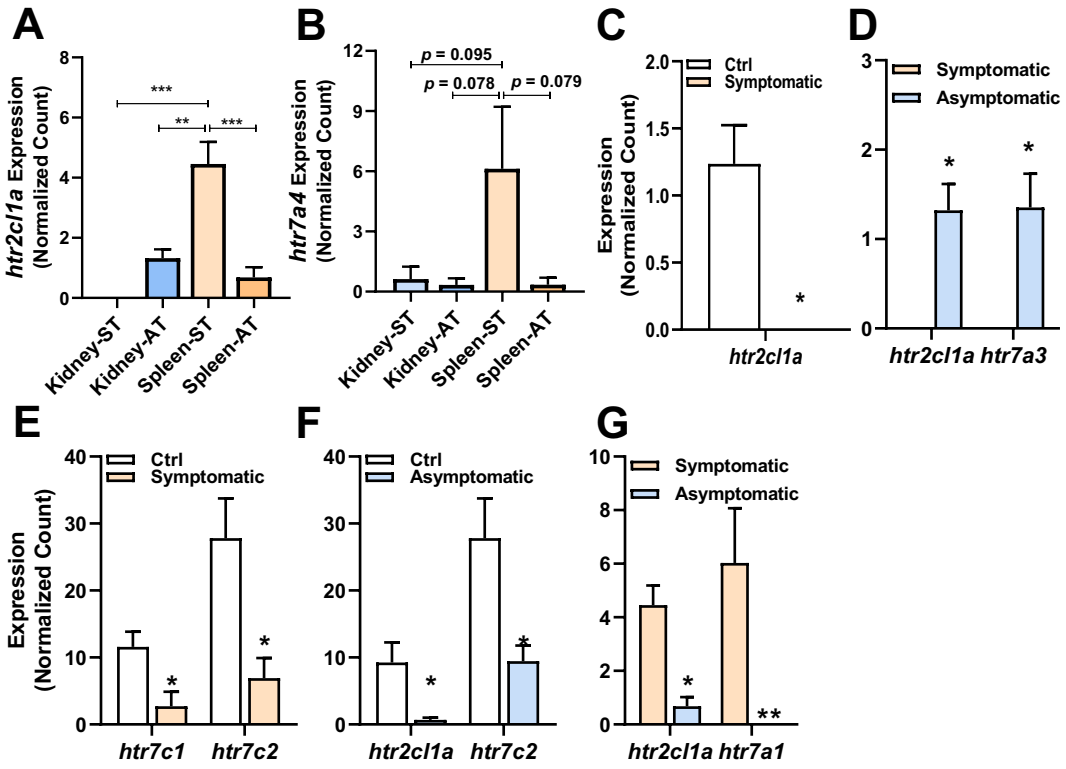

Fig. 5. Transcriptional profiles of kidney and spleen *htr* genes in response to *V. anguillarum* infection.
A and B: Transcriptional profiles of *htr2cl1*a (A) and *htr7a4* (B) in kidney and spleen.
The *htr* gene expression was conducted via one-way analysis of variance with Tukey's test. The *, ** and *** indicates $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.
C and D: Transcriptional profiles of kidney *htr2cl1*a between Ctrl and ST (C), kidney *htr2cl1*a and *htr7a3* between AT and ST (D).
E - G: Transcriptional profile of spleen *htr7c1* and *htr7c2* between Ctrl and ST (E), spleen *htr2cl1*a and *htr7c2* between Ctrl and AT (F), spleen *htr2cl1*a and *htr7a1* between AT and ST (G).
The *htr* gene expression was conducted via Student's *t*-test. The * and ** indicates $p < 0.05$ and $p < 0.01$, respectively.

(Fig. 5C). Asymptomatic trout exhibited up-regulated kidney *htr2cl1a* and *htr7a3* after *V. anguillarum* infection (Fig. 5D). In spleen, symptomatic trout showed significant down-regulation of *htr7c1* and *htr7c2* (Fig. 5E), while asymptomatic trout showed significant down-regulation of *htr2cl1a* and *htr7c2* after *V. anguillarum* infection (Fig. 5F). Asymptomatic trout exhibited down-regulated spleen *htr2cl1a* and *htr7c1* after *V. anguillarum* infection (Fig. 5G). Expressional profiles of kidney and spleen serotonin receptors were not significantly changed by *A. salmonicida* infection.

3.4. Transcriptional profiles of serotonin receptors in response to salinity change

After saltwater exposure, the transcriptional profiles of serotonin receptors in brain, kidney and liver were clustered by heatmap (Fig. 6A – C). Unsupervised PCA showed tissue-specific expressions of serotonin receptors when comparing TS to DS (or TF) (Fig. 6D and E). Saltwater transfer significantly decreased brain *htr1aa1*, *htr2aa2*, *htr1b2* and *htr4c* (Fig. 6F), and increased kidney *htr7c1* and liver *htr7c2* in triploid trout (Fig. 6G and H). Compared to diploid trout, triploid trout showed significant down-regulation of kidney *htr4a* and liver *htr2b2,* and up-regulation of liver *htr7c2* (Fig. 6I and J). Positive correlations were observed between *htr1aa2* and *htr1ab2*, *htr1fa1* and *htr1ab2*, *htr1fa1* and *htr6b*, *htr2cl2a* and *htr4d*, and *htr4d* and *htr6b* (Fig. 6K - O).

3.5. Identification of the exogenous ligand binding pocket

The SWISS-MODEL showed conserved motifs between human HTR1B and trout Htr1b3 and human HYR7 and trout Htr7c2. Based on the alignment of amino acid sequences, the conserved seven transmembrane domains, DRY motif and NPxxY motif were observed between human HTR1B and trout Htr1b3 (Fig. 2B). Human HTR1B and trout Htr1b3 showed conserved residues in the orthosteric binding pocket and the extended binding pocket of methiothepin (Fig. 7A). Meanwhile, the amino acid sequences showed conserved seven transmembrane domains, DRY and NPxxY motifs, and binding pocket of 5-CT

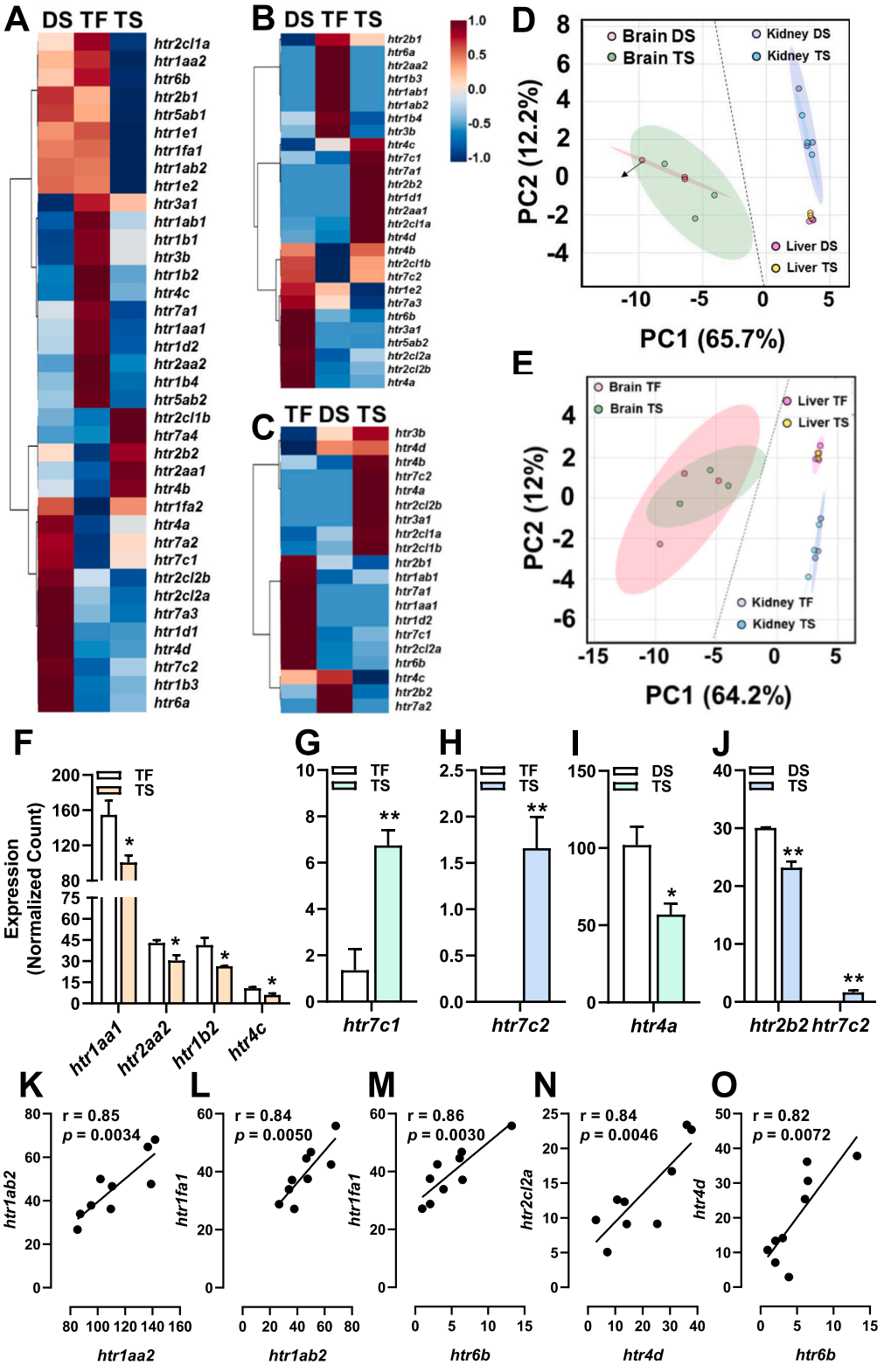

Fig. 6. Transcriptional profiles of brain *htr* genes in osmoregulation.
A - C. The heatmap of *htr* genes in brain, kidney and liver.
D and E. PCA of *htr* genes in brain, kidney and liver between DS and TS (D) and between TF and TS (E).
D - F: Key *htr* genes associated with osmoregulation between TF and TS in brain (F), kidney (G) and liver (H).
Key *htr* genes in osmoregulation between DS and TS in kidney (I) and liver (J).
The *htr* gene expression was conducted via Student's *t*-test. The * and ** indicates $p < 0.05$ and $p < 0.01$, respectively.
K - O: Correlation analysis of *htr* genes between *htr1aa2* and *htr1ab2* (K), *htr1ab2* and *htr1fa1* (L), *htr1fa1* and *htr6b* (M), *htr2cl2a* and *htr4d* (N), *htr6b* and *htr4d* (O).
Pearson correlation coefficient was used to evaluate the correlation analysis of *htr* genes in DS, TS and TF.

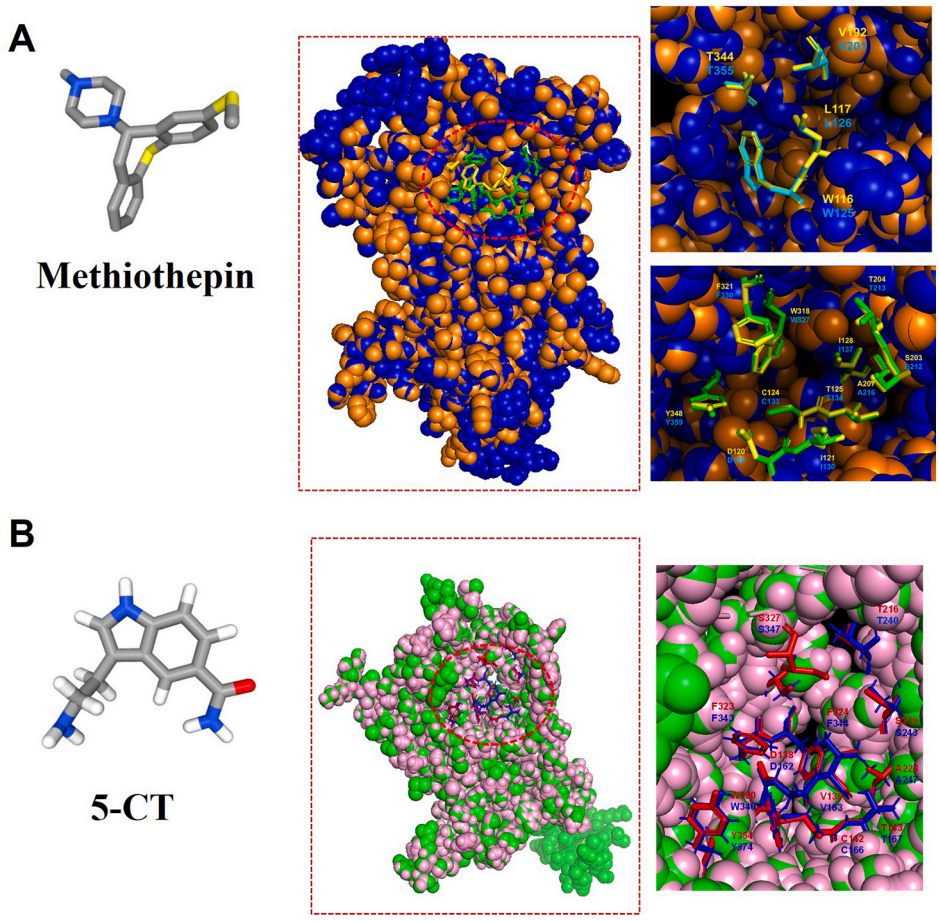

Fig. 7. Exogenous ligand binding pocket in trout serotonin receptors.
A. Comparison of the MT binding pocket between human HTR1B (blue) and trout Htr1b3 (orange). Blue (or green) sticks showed binding pocket in human HTR1B while yellow sticks showed binding pocket in trout Htr1b3. The W116 (W123), L117 (L126), V192 (V201) and T344 (T355) are extended binding pocket. The PDB ID of human HTR1B is 5V54.
B. Comparison of the 5-CT binding pocket between human HTR7 (pink) and trout HTR7C2 (green). Blue sticks showed binding pocket in human HTR7 while red sticks showed binding pocket in trout Htr7c2. The PDB ID of human HTR1B is 7XTC.

between human HTR7 and trout Htr7c2 (Figs. S7 and 7B).

4. Discussion

4.1. Repertoire of serotonin receptors

Compared with human and zebrafish, which showed ~17 and 22 *HTR (htr)* genes due to 2R and 3R, rainbow trout showed expanded gene copies of *htr* genes [34,35]. A total of 39 *htr* genes were identified from trout genome due to 4R. The expanded *htr* paralogs, such as *htr1aa1*, *htr1aa2*, *htr1ab1* and *htr1ab2*, were localized on different chromosomes rather than arranged on the same chromosomes as tandem duplication, suggesting they were derived from 3R or 4R [36,37]. Gene duplication was the key mechanism for the generation of novel functional genes with biological processes, thus contributing to the potential function diversity of trout Htrs [38,39].

4.2. Tissue transcriptional profile of serotonin receptors

Serotonin and its receptors exert diverse physiology effects in regulating homeostasis [3]. HTR1A exhibits broad brain expression in multiple mammalian species including zebrafish, mouse, pig and human [40]. Due to salmonid specific whole genome duplication, four *htr1* subtypes exert high expressions in trout brain, suggesting *htr1* paralogs exert conserved tissue distribution and physiology in regulating brain neurotransmitter activities. Two *htr4* paralogs showed high expressions in intestine. In mammals, HTR4s were expressed in nerve terminals and activation of presynaptic HTR4s regulate intestine motor function and food intake [41]. Physiological studies showed HTR4s activation increases feeding in obesity mice and pharmacologic evidence showed antagonist of 5-HT4 receptors decrease satiety [42–44]. Intestine act as an important regulator in food intake and our study showed trout exerted high expressions of two *htr*4 subtypes, suggesting Htr4 might trigger a higher orexigenic signal in trout.

4.3. Transcriptional profiles of serotonin receptors in immunomodulation

Our studies showed trout brain *htr1* subtypes were significantly upregulated in response to both *V. anguillarum* and *A. salmonicida* infections. In mammals, altered serotonin system triggers physiological and behavioral changes response to inflammatory events and pathogen infections, including neurobehavioral disorders and sickness behavior [45,46]. In teleost, fish infected by pathogens (e.g., *A. salmonicida*) exerts sickness behaviors with neuroinflammation [25,47]. Our results concluded *htr1* subtypes might act as general immune modulators in response to pathogen infection. The *htr7c2* was significantly upregulated due to *A. salmonicida* infection. Previous studies showed HTR7 plays a key role in CNS inflammation and repair, and receptor agonism could rescue neuronal apoptosis and synaptic dysfunction [48,49]. Meanwhile, HTR7 acts as a paracrine and/or autocrine signal that stimulates T cell activation and *htr1* upregulation (reviewed in [1]). Therefore, we observed upregulated *htr1b4* and *htr7c2* due to *A. salmonicida* infection. Our results showed Htr1 and Htr7 were key neuroimmune regulators in trout.

In the periphery, serotonin and receptors are widely present in the immune system, thus coordinating cellular immune cascades including inflammation, chemotaxis, and phagocytosis [50]. Symptomatic trout showed significantly altered *htr2 and htr7 subtypes when* comparing to Ctrl or asymptomatic group. In mammals, HTR2 subtypes are widely expressed in immune cells including monocytes, macrophages, T cells

and B cells. Serotonin exerts chemoattractant properties via HTR2 subtypes and stimulates proinflammatory cytokine secretion, which is important for defense mechanism against *V. anguillarum* infection [1,24,51,52]. Target blocking of 5-HT activation via HTR2 antagonists has also been shown to reduce inflammatory responses [1]. Meanwhile, serotonin-stimulated DC triggers a more inflammatory responses via HTR7 and inhibition of the HTR7 signaling results in reduced inflammatory responses [53,54]. We showed activation of the proinflammatory cytokine cascade is a common immune response due to *V. anguillarum* infection [24]. Our results suggested trout Htr2 and Htr7 might be involved in regulation of *V. anguillarum*-induced inflammatory responses.

4.4. Transcriptional profiles of serotonin receptors in smoltification

The total loss of salmonids in seawater stage is around 15 %, and a major percentage of this loss occurs just after seawater transfer due to salinity change [12,13]. The "failed smolts or growth-stunted fish" fail to feed and exerted progressive body weight loss and a behaviorally inhibited profile [55]. Our results showed down-regulated brain *htr1* subtypes and *htr4c* in response to salinity change. Neuroendocrinology studies showed serotonin is involved in dynamic changes of the osmoregulatory response in rat [56,57]. In teleosts, selective serotonin reuptake inhibitor dysregulates osmoregulation in gulf toadfish (*Opsanus beta*), while dietary L-tryptophan supplementation has been reported to produce "Super Smolt" for salinity acclimation [14]. Meanwhile, triploid trout showed downregulated *htr2*, *htr4* and *htr7* subtypes during osmoregulation when comparing to diploid groups. Triploid exerts different smolt physiology when comparing to the diploid counterparts [58,59]. These results indicated targeting serotonin or other monoamine neurotransmitters are important for osmoregulation in salmonids in response to salinity change.

4.5. Application of the exogenous ligands targeting serotonin receptors in aquaculture

Several non-selective and selective ligands targeting serotonin receptors have been developed into drugs for disease treatment [32,33]. 5-CT is a serotonin analogue with high affinities to 5-HT_1 and 5-HT_7 receptor subtypes, while MT is an inverse agonist of 5-HT_{1B} receptor. Binding pocket of 5-CT and MT were conserved between human (HTR1B and HTR7) and trout (Htr1b3 and Htr7c2) serotonin receptors. Considering HTR1B3 was involved in immunomodulation in response to disease and HTR7C2 was associated with osmoregulation, we proposed exogenous ligands targeting human serotonin receptors might regulate trout physiology via serotonin receptors. Future studies might focus on physiological functions and pharmacological characteristics of exogenous ligands targeting serotonin receptors in rainbow trout.

5. Conclusion

In this study, we showed rainbow trout exerted expanded copies of serotonin receptors due to 4 rounds of whole genome duplication. Totally, 39 serotonin receptor genes were identified. Transcriptional profiles of trout *htr* genes were significantly altered by pathogen infection and salinity changes, suggesting serotonin receptors might be involved in immunomodulation and osmoregulation. Trout serotonin receptors (Htr1b3 and Htr7c2) exhibited conserved residues in MT or 5-CT binding pocket with human serotonin receptors (HTR1 and HTR7C), potentially implying exogenous ligands targeting human serotonin receptors might be used to modulate trout immunomodulation and osmoregulation via serotonin receptors. Our future studies will evaluate the pharmacology of trout serotonin receptors.

CRediT authorship contribution statement

Conceptualization: Z.H., J.L., H.W., and Q.G.; methodology: Z.H., M.L., X.D., Q.Y., H.W., and Q.G.; validation, Z.H. and M.L.; formal analysis: Z.H., M.L., Z.L., K.D., Y.T., X.H., and M.Q.; data curation: Z.H., M.L., H.W., and Q.G.; writing-original draft preparation: Z.H., M.L., H.W., and Q.G.; writing-review and editing: Z.H., M.L., H.W., and Q.G.; supervision: Z.H. Q.G., and H.W.; project administration: Z.H. Q.G., and H.W.; funding acquisition: Z.H. Q.G., and H.W.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2023.125930.

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