Molecular characterization of four innate immune genes in *Tor putitora* and their comparative transcriptional abundance during wild- and captive-bred ontogenetic developmental stages

Priyanka H. Tripathi\(^a, b\), Anupam Pandey\(^a, b\), Alexander Ciji\(^a\), Veena Pande\(^b\), Manchi Rajesh\(^a\), B.S. Kamalam\(^a\), M.S. Akhtar\(^a, b\)

\(^a\) Nutritional Physiology Laboratory, ICAR-Directorate of Coldwater Fisheries Research, Bhimtal, Uttarakhand 263136, India  
\(^b\) Department of Biotechnology, Sir J C Bose Campus, Kumaun University, Bhimtal Campus, Nainital, Uttarakhand 263136, India

**Abstract**

The current study was designed to characterize immune genes and compare their expression during ontogenetic developmental stages in progenies of wild-collected and captive-matured *T. putitora*. The full-length cDNA sequences of *Tplt2*, *Tpmld88*, *Tpcd36*, and *Tpill8* were 2814, 1545, 1807, and 653 bp in length, with ORFs of 2379 bp, 855 bp, 1410 bp, and 297 bp, encoding for putative peptides of 793, 284, 469 and 98 amino acids, respectively. The predicted peptide sequences of the genes had high sequence homology and structural conservation with other teleost fishes, especially cyprinids. The expression of *Tplt2* was relatively low in both wild- and captive-bred offsprings during the early embryonic stages, but significantly increased later in development. The mRNA abundance of the *Tpmld88* gene was significantly low until the blastula stage, then increased notably from the gastrula stage to the advanced fry stage. The *Tpcd36* expression elevated during later developmental stages, peaking at advanced fry stage in both. On the other hand, expression of *Tpill8* was relatively low until the blastula stage and showed a moderate increase from the organogenesis stage onwards in wild-bred offsprings, whereas a significant upregulation was seen in the captive-bred offsprings from the organogenesis stage until the advanced fry stage, with its maximum expression at the pre-metamorphosis stage. Overall, the findings suggest the crucial role of *Tpmld88*, *Tplt2*, *Tpcd36*, and *Tpill8* in inducing innate immunity in embryonic and larval stages of *T. putitora*. Further, the considerably higher expression of the immune genes in the embryonic and larval stages of captive-bred offsprings may indicate a stronger immune system.

**Keywords:** TLR2, MYD88, CD36, Ontogeny, Gene expression, *Tor putitora*

**1. Introduction**

Golden mahseer, *Tor putitora* (Hamilton, 1822) is an internationally sought-after sport fish and is being promoted as the flagship species of the Indian subcontinent [1–3]. Besides food values, golden mahseer has great aquaculture perspectives for eco-tourism and provides economic opportunities to native communities [4,5]. Unfortunately, due to over-exploitation, environmental degradation, pollution and various other anthropogenic factors, the mahseer population has reduced to an alarming level, and it has been categorized as ‘endangered’ species [6]. Thus, its protection and conservation is a paramount concern for scientific communities, conservationists, ecologists, and policymakers [7].

Large-scale seed production using captive broodstock of *T. putitora* and subsequent stock enhancement through release of hatchery produced healthy fingerlings/juveniles has been suggested as an effective strategy for its conservation and rehabilitation in their natural environment [4,8]. In this line, consistent efforts have been made to achieve artificial propagation and mass scale seed production in captivity [1]. Nevertheless, the success of stock enhancement/ranching programs strongly depend upon the quality and overall fitness of the seed/juvenile released [9]. Substantial evidences suggest lower fitness and survival of hatchery reared fish when released to their natural conditions [10–13]. Like in several teleost, the egg quality, larval fitness and survival of hatchery reared fish was released to their natural conditions [10–13]. In this context, understanding the innate immune response during the early developmental stages of *T. putitora* may help in devising management strategies.
for improving larval fitness and survival during nursery rearing.

The higher mortality noticed during early developmental stages are often related to infectious diseases, and fish rely heavily on innate immunity as adaptive immune functions are undeveloped or underdeveloped in embryonic stages [15,16]. The innate immune system in fish identifies the pathogens/microbes associated molecular patterns (PAMPs/MAMPs) through pattern recognition receptors (PRRs) that are present on their cell surface or secreted into blood circulation and tissue fluids [17]. Toll-like receptors (TLRs) are one of the major classes of PRRs [18], and are reported to induce innate immunity in fish embryos [19]. Following pathogenic ligand detection by corresponding TLRs, a signalling cascade is activated via MyD88-dependent or MyD88-independent pathways, culminating in the stimulation of an array of innate immune genes. Among them, interleukin 8 (ILB) is a proinflammatory cytokine, belonging to chemokine family that recruits neutrophils to the site of injury or infection [20]. Expression of IL8 during early developmental stages were reported in Labeo rohita and Oncorhynchus mykiss in response to peptidoglycan and formalin killed A. hydrophila stimulation, as well as pathogenic infections suggesting their vital role in early stage immunity [19,21,22]. On the other hand, cluster of differentiation 36 (CD36) is a member of scavenger receptor subfamily of PRRs, and are reported to be involved in antigen presentation, phagocytosis, and apoptotic cell clearance [23-25]. Moreover, CD36 mRNA expression was reported at different developmental stages in zebrafish possibly suggesting their role in embryo development [26].

So far, very limited information is available on the onset and progression of the immune system in wild and captive/hatchery produced golden mahseer, knowledge of which is necessary to understand the differences in their performance during early life stages. Therefore, in this study, we aimed to characterize thr2 (toll-like receptor 2) and its downstream signalling molecules such as myd88 (myeloid differentiation primary response protein 88), il8 (interleukin 8) and cd36 (cluster of differentiation 36) in T. putitora, and examined their comparative expression in wild- and captive-bred ontogenetic developmental stages.

2. Materials and methods

2.1. Sample collection

To analyze the ontogenetic developmental pattern of immune genes in captive- and wild-bred T. putitora, the eggs/embryos and larvae samples were obtained from the Mahseer Hatchery Complex of ICAR-Directorate of Coldwater Fisheries Research Nainital, Uttarakhand, India. Standardized protocols were followed for breeding, egg incubation, and nursery rearing of wild-collected [7,27] and captive-reared brooders [28]. Ontogeny samples from wild- and captive-bred brooders were collected at various hours of development throughout the embryonic and larval stages, consisting of (i) Unfertilized egg (collected quickly after stripping of eggs and washing with PBS); (ii) Just fertilized egg (0 h post-fertilization; hp); (iii) Morula (4.25 hp), (iv) Blastula (8.25 hp), (v) Gastrula (32.25 hp), (vi) Organogenesis (56 hp), (vii) Just hatched (100 hp; time point when 50% of the embryos were hatched), (viii) Pre-metamorphosis (3 days post-hatching, dph), (ix) Post metamorphosis (11 dph), and (x) Advanced fry (31 dph) stage. The collection of wild origin ontogenic samples was based on our earlier publication [29], while captive origin developmental stages were sampled based on microscopical observations. Six samples (n = 6) were collected and stored in RNA later solution (Invitrogen, Carlsbad, CA, USA) at every developmental time point (a pool of six eggs/embryos constituted one sample of the first six stages, while a pool of three whole larvae/fry represented a sample of the last four stages). T. putitora juveniles were euthanized using clove oil (100 μL/L) before dissection following which liver and head kidney tissues of sub-adults and the pooled embryos/eggs and whole-larvae samples from different time points, respectively. RNA was quantified by measuring the OD ratio at 260 and 280 nm in a spectrophotometer (Eon3, Biotek, VT, USA) and RNA integrity was examined by performing 1% agarose gel electrophoresis. The absorbance ratio (A260:280) for all the RNA samples was approximately 2 and the RNA integrity on agarose gel showed intact 2:1 ratio for 28S:18S rRNA suggesting good quality yield of RNA with no degradation. Later, cDNA synthesis was carried out with 1μg of the isolated RNA (as the template) and PrimeScript™ 1-strand cDNA Synthesis Kit (Clontech, Takara, CA, USA) according to the manufacturer’s instructions.

2.2. Isolation of RNA and first-strand cDNA synthesis

For molecular cloning and gene expression studies, RNA was extracted by the TRIzol method [30] from the freshly dissected liver and head kidney tissue of T. putitora sub-adults and the pooled embryos/eggs and whole-larvae samples from different time points, respectively. RNA was quantified by measuring the OD ratio at 260 and 280 nm in a spectrophotometer (Eon3, Biotek, VT, USA) and RNA integrity was examined by performing 1% agarose gel electrophoresis. The absorbance ratio (A260:280) for all the RNA samples was approximately 2 and the RNA integrity on agarose gel showed intact 2:1 ratio for 28S:18S rRNA suggesting good quality yield of RNA with no degradation. Later, cDNA synthesis was carried out with 1μg of the isolated RNA (as the template) and PrimeScript™ 1-strand cDNA Synthesis Kit (Clontech, Takara, CA, USA) according to the manufacturer’s instructions.

2.3. PCR amplification and cloning full-length target immune genes of T. putitora

Reverse Transcriptase-PCR and Rapid Amplification of cDNA Ends (RACE) were done for amplification of the partial cDNA sequence and to obtain 3’ and 5’ ends of the genes, namely Tpmyd88, Tpthr2, TpCd36 and TpIl8. Degenerate primers were designed for RT-PCR amplification based on conserved homologous regions of target immune genes in phylogenetically related fish species (Table 1). The PCR amplification was performed using 50 μL PCR master mix which contained high fidelity Q5 polymerase, buffer (New England Biolabs, MA, USA), 0.5 μM of forward and reverse primer, 0.2 mM dNTP and 2.5 μL of template cDNA. The thermo-cycling condition included a cycle of initial denaturation at 98 °C for 30 sec followed by 35 cycles of denaturation at 98 °C for 10 sec, annealing at gradient temperature (58 - 72 °C, depending on the primer annealing temperature) for 20 sec, extension at 72 °C for 25 sec and a final extension at 72 °C for 2 min. After optimization, the PCR amplicons were analyzed on 1% agarose gel, and single bands, specific to target immune genes, were eluted using gel extraction kit (QIAGEN, Germany). The purified products were then cloned in zero blunt vector (Invitrogen, Carlsbad, CA, USA), sequenced bidirectionally using Sanger sequencing method, and analyzed using NCBI- BLAST search. Subsequently, 3’RACE and 5’RACE PCR were performed to obtain 3’ and 5’ ends of the target immune genes using RACE cDNA kit (Clontech-Takara)

Table 1

| Primers | Sequence (5’-3’) |
|---------|----------------|
| PCR primers | RACE primers |
| Tpthr2 | F1-GCCTTGAGTGTCCTTGAATCA |
| Tpmyd88 | F-GAGTGGGAACACTGTCAG |
| TpCd36 | F-GGAGCCATCTTTGAGGCT |
| TpIl8 | F-GGAGGACATCTTGACAGA |
| RACE primers | Tpthr2 5’ RACE1 | AGGGTGGGAGACACTCATGAGAGC |
| Tpthr2 3’ RACE 2 | GAGCCATCGGAGCGCGG |
| Tpmyd88 5’ RACE1 | TGGTCAAGAGGACACACATGCTAGG |
| Tpmyd88 3’ RACE2 | AGCACGAGAAGCAATGCTAGG |
| TpCd36 5’ RACE2 | TCGAGTCAACAGTCCCATACACAGC |
| TpCd36 3’ RACE2 | TCTAGGAGGACATGCTACACAG |
| TpIl8 3’ RACE1 | AGCCGACACCTGTCAGAGG |
| TpIl8 3’ RACE2 | AGCCGACACCTGTCAGAGG |
| UPM long, Clontech | UPM short, Clontech |
| CTAATACGACTCACTATAGGG | CTAATACGACTCACTATAGGG |
Bio, CA, USA) as per manufacturer’s protocol. The 3’ and 5’ RACE products were then cloned into a zero blunt vector and sequenced using Sanger sequencing method.

2.4. Bioinformatic analysis of target immune genes

The sequences of all the target genes were assessed (ABI sequence scanner v1.0) and aligned (BioEdit version 7.0.5.2) separately before submitting to NCBI GenBank. The deduced protein sequence of each target immune gene was obtained using ExPASy translate online software (https://web.expasy.org/translate/). The translated immune gene sequences were aligned with other species using the Clustal Omega program. The alignment was used to construct phylogenetic trees using neighbor-joining (NJ) statistical method with number of bootstrap replicates set to 1000. The phylogenetic tree was visualized using the MEGA-X program and the bootstrap values were represented as percentage next to the branch nodes. Domain architectures for the deduced protein sequences were analyzed in SMART program (http://smart.embl-heidelberg.de). Biochemical properties like molecular mass and theoretical pI (isoelectric point) were determined using the ProtParam tool (https://web.expasy.org/protparam/). SignalP 4.1 software was used to predict the signal peptide where the presence of cleavage sites and a signal peptide/non-signal peptide were predicted based on the D-score (Discrimination score). Distinct N-glycosylation and phosphorylation sites were predicted in NetNGlyc 1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/NetNGlyc1.0Server/) and NetPhos 3.1 (http://www.cbs.dtu.dk/services/NetPhos/), respectively. The NetNGlyc 1.0 server predicts the N-glycosylation sites based on artificial neural networks that examines the Asn-Xaa-Ser/Thr sequons. Likewise, ensembles of neural network are applied by NetPhos 3.1 server to predict potential protein phosphorylation sites. The N-glycosylation and phosphorylation potential ranges from 0 to 1 with 0.5 defined as threshold value. Higher potential indicates increased confidence of the site prediction. CELLO2GO web server (http://cello.life.nctu.edu.tw/cello2go/) and STRING (https://string-db.org/) biological database was used for predicting subcellular localization and possible protein-protein interactions of the deduced proteins, respectively. CELLO2GO predicts protein subcellular localization in eukaryotes depending upon SVM interactions of the deduced proteins, respectively. CELLO2GO predicts potential protein phosphorylation sites. The STRING software predicts the possible protein-protein interactions using algorithm based on the available literature, gene fusion, neighborhood, cooccurrence, experimental outputs and text mining [31]. Homology-based multiple protein sequence alignments were generated with CLC genomic workbench (CLC Bio, Aarhus, Denmark).

2.5. Real-time PCR to analyze the transcriptional abundance of non-specific immune genes

The mRNA transcripts of targeted immune genes were analyzed using real-time PCR in early ontogenetic developmental stages of *T. putitora* grown under wild and captive conditions. The real-time qPCR was done on a 96 well StepOnePlus PCR thermocycler (Applied Biosystems, WLM, MA, USA). Every 15 µL qPCR master mixture had 7.5 µL of 2X SYBR Premix Ex Taq II (Th RNase H Plus) (Takara, USA), 5.0 µL of the cDNA template (diluted 1:50), 0.3 µL of ROX reference dye, 1.0 µL of PCR grade water and 0.6 µL each of gene-specific primers (400mM) (Table 2). The thermal profile was adopted as Akhtar et al. [27]. The real-time qPCR reaction was run in technical duplicates for each sample, and PCR amplification with 90 to 110% efficiencies was accepted. A unimodal (single) peak in the melting curve was obtained for all the qPCR products confirming the specificity of genes. The 18S rRNA was employed as a reference gene for data normalization. Relative expression of the target innate immune genes viz. *Tpcd36*, *Tpmyd88*, *TpTlr2* and *TpIl8* and reference gene at each time-point was calculated according to Pfaffi (2001) method [32].

2.6. Statistical analysis

GraphPad Prism (La Jolla, CA, USA) was employed for performing all the statistical analyses. The relative expression data was analyzed using two-way ANOVA, followed by Tukey’s post-hoc test among different developmental stages and brooders source (wild and captive). Data were considered significantly different when the probability (p) value was ≤ 0.05. The relative expression data are presented as means ± SD, n = 6.

2.7. Ethical statement

The animal handlings were done conforming the guidelines approved by the Animal Ethics Committee of the institute.

3. Results

3.1. Cloning, characterization and bioinformatics analysis of immune genes in *T. putitora*

The complete cDNA sequences obtained for the immune genes of *T. putitora* were subjected to bioinformatics analysis. The results and observations of the same are given below:

3.1.1. *Tor putitora* toll-like receptor 2 (*TpTlr2*)

The full-length sequence of *TpTlr2* (MW322911) was 2814 bp, with a 5′ UTR of 187 bp, an ORF of 2379 bp encoding a putative peptide of 793 amino acids (AAs) and a 3′ UTR of 248 bp (Fig. 1). Homology analysis using protein BLAST indicated high sequence similarity of *TpTlr2* protein with the TLR2 protein of different fish species. The *T. putitora* TLR2 protein exhibited 90.77% similarity with *Cyprinus carpio*, 89.77% with *Labeo rohita*, 87.53% with *Ctenopharyngodon idella* and 79.42% with *Danio rerio* (Table 3). The phylogenetic tree of *TpTlr2* showed two

| Table 2 |
| List of real-time qPCR primers (self-designed). |

| Primers | Sequence (5′-3′) | Ta (°C) | Amplification efficiency (%) | Amplicon size (bp) | Accession no. |
|---------|-----------------|--------|-----------------------------|-------------------|---------------|
| TpTlr2  | F-TGGCACCACCAGACCTGCT R-TAGTGTGCGCTCTTCTGCCC | 60     | 104.7                       | 94                | MW322911      |
| Tpmyd88 | F-GAGTCGAAAGCCACCTGTGGCAGC R-AGAAAAATGCCACCACTGCTTTT | 60     | 101.5                       | 121               | MW322908      |
| TpIl8   | F-AGACACAGAGATCATGGCCACCC R-TCACAGATGACGCACCACCTCC | 60     | 96.6                        | 133               | MW322909      |
| TpCd36  | F-GTGACGACGGACGGAGAACG R-CTCAGACGGACGTTCCTTTGTAAC | 60     | 106.6                       | 174               | MW322905      |
| TpIl8   | F-ATTTAGCGAAAGCCACACCC R-CAGACAAAATGCCACCAACT | 60     | 102.4                       | 166               | SRX2442156    |
major clades, clade I included teleosts and clade II higher vertebrates. TpTLR2 was seen closely clustered with TLR2 of other cyprinids such as Carassius auratus, C. carpio and L. rohita showing common ancestry (Fig. 2A). The TpTLR2 protein was predicted to have a molecular weight and isoelectric point (pI) of 90.8 kDa and 6.38, respectively (Table 3).

Analysis of primary TpTLR2 protein structure using SMART tool displayed a signal peptide of 30 AAs (Figs. 1 and S1A), leucine-rich repeat (LRR) regions, transmembrane region (590-612 AAs) and TIR domain (645-790 AAs). TpTLR2 protein consisted of one LRR at N-terminal (32 to 58 AAs), one at C-terminal (537 to 590 AAs), and six LRRs (77-100, 113-145, 148-180, 183-215, 218-249, 252-283).
102-124, 150-173, 361-386, 416-439 and 480-503 AAs) were located in between C- and N-terminal of the protein (Fig. 1). Several N-glycosylation motifs and phosphorylation sites were detected in the protein structure, suggesting post-translation modification (Figs. S2A and S3A). Further, CELLO2GO predicted TpTLR2 protein to be principally localized in the plasma membrane (35.2%) and nuclear (29.2%) regions at the subcellular level (Fig. S4A). STRING analysis revealed protein-protein interaction networks of TpTLR2, a few of which were myeloid differentiation primary response protein 88 (MYD88; 0.999), toll-interleukin 1 receptor domain (TIR) containing adaptor protein (TIRAP; 0.996), interleukin-1 receptor-associated kinase 1 (IRAK1; 0.974), TNF receptor-associated factor 6 (TRAF6; 0.961), and toll interacting protein (TOLLIP; 0.986) (Fig. S5A). Multiple sequence alignment of predicted TpTLR2 protein with other species indicated that TLR2 protein is evolutionarily conserved among vertebrates (Fig. 3).

### 3.2. Tor putitora myeloid differentiation factor 88 (Tpmyd88)

The complete cDNA sequence of the Tpmyd88 (MW322908) gene was 1545 bp in length, consisting of an ORF of 855 bp encoding for a 284-amino acid polypeptide and a 5’ and 3’ UTR region of 181 bp and 509 bp, respectively (Fig. 4). Sequence identity analysis using BLASTp revealed that TpMYD88 protein shared 94.72% sequence homology with MYD88 of C. carpio, 95% with C. idella, 92.9% with D. rerio and 90% with L. rohita (Table 3). The molecular mass and pl of TpMYD88 was predicted to be 32.95 kDa and 5.73, respectively. Phylogenetic tree analysis revealed two major clusters, where the deduced TpMYD88 peptide sequence grouped with other teleosts in cluster I, sharing a common ancestry, whereas the elasmobranchs and higher vertebrates were grouped in cluster II. Among teleosts, TpMYD88 was seen closely clustered with D. rerio MYD88 and L. rohita MYD88 (Fig. 2B). Domain architectural analysis revealed the presence of a DEATH domain (11 to 101 AAs) at the amino-terminus and a TIR domain (148–284 AAs) at the carboxy-terminal of the deduced TpMYD88 peptide sequence (Fig. 4). The post-translation modification was evidenced by the presence of two N-glycosylation sites at positions 18 and 30 and several phosphorylation (threonine, serine and tyrosine) sites in the polypeptide sequence (Figs. S2B and S3B). Multiple sequence alignment of predicted TpMYD88 protein with other species suggested the presence of three conserved amino acid residues in the DEATH domain: tryptophan at position 80, leucine at positions 93 and 97; and three conserved motifs in the TIR domain, namely motif 1: TADAFICIYCYQ, motif 2: LCVFVDRLPPTGC and motif 3: a conserved tryptophan residue surrounded by a basic amino acid (FW) residue (Fig. 5). Furthermore, TpMYD88 at the subcellular level was localized predominantly in the nucleus (35.5%) and cytoplasm (25.5%) (Fig. S4B). Protein-protein interaction analysis of TpMYD88 identified TNF receptor-associated factor 6 (TRAF6; 0.999), TIR domain containing adaptor protein (TIRAP; 0.999), interleukin-1 receptor-associated kinase 4 (IRAK4; 0.999), interleukin-1 receptor-associated kinase 1 (IRAK1; 0.999), and toll-like receptor 2 (TLR2; 0.999) as its functional partners (Fig. S5B).

### 3.3. Tor putitora cluster of differentiation 36 (Tpcd36)

The full-length Tpcd36 (MW322905) sequence was 1807 bp in length, consisting of a 5’ UTR region of 165 bp, a 3’ UTR region of 232 bp and an ORF of 1410 bp encoding a putative protein of 469 AAs with a predicted molecular mass and pl of 52.43 kDa and 4.95, respectively (Fig. 6 and Table 3). Sequence homology of TpCD36 revealed high sequence similarity with CD36 protein of Cyprinidae such as C. carpio (91.85%), C. idella (82.66%), L. rohita (78.43%) and D. rerio (79.14%) (Table 3). Evolutionary relationship of TpCD36 was investigated through phylogenetic tree (Fig. 7A), which showed two major clades that were further branched. The tetrapods (mammalian, amphibians, aves and reptilians) were grouped in one cluster while teleost fishes branched out into another cluster highlighting the close relationship among all teleost CD36 protein. TpCD36 was seen closely clustered with C. idella, C. auratus and C. carpio due to high sequence identity. Further analysis of post-translation modifications revealed several N-glycosylation sites and phosphorylation (threonine, serine and tyrosine) sites in the predicted protein sequence (Figs. S6A and S7A). The full-length TpCD36 revealed high sequence similarity with CD36 protein of Cyprinidae such as C. carpio (91.85%), C. idella (82.66%), L. rohita (78.43%) and D. rerio (79.14%) (Table 3). Evolutionary relationship of TpCD36 was investigated through phylogenetic tree (Fig. 7A), which showed two major clades that were further branched. The tetrapods (mammalian, amphibians, aves and reptilians) were grouped in one cluster while teleost fishes branched out into another cluster highlighting the close relationship among all teleost CD36 protein. TpCD36 was seen closely clustered with C. idella, C. auratus and C. carpio due to high sequence identity. Further analysis of post-translation modifications revealed several N-glycosylation sites and phosphorylation (threonine, serine and tyrosine) sites in the predicted protein sequence (Figs. S6A and S7A).
Fig. 2. Phylogenetic tree constructed based on the deduced amino acid sequences of TLR 2 (A) and MYD88 (B), using MEGA X software. Numbers next to the branches represent bootstrap values and scale bars indicate evolutionary distance.
Fig. 3. Multiple alignment of *T. putitora* with other TLR2 protein. Alignment was done using CLC genomic workbench (version 11.0.1) software program. The conserved alignment position were represented as bar graph at the bottom (on a scale of 0 to 100%). The LLRNT, LRR and LRRCT domains were marked in grey, green and red box, respectively. Cysteine residues of LRRNT and LRRCT region are marked with black arrows. The TIR domain is marked in a blue box with its three highly conserved motif boxed in yellow.
thrombospondin 2b (THBS2b; 0.908) as functional partners of TpCD36 (Fig. S8A).

3.4. Tor putitora interleukin 8 (TpIl8)

The complete cDNA of the TpIl8 (MW322909) gene was of 653 bp in length. The 5'UTR and 3'UTR regions were of 113 bp and 28 bp,
respectively. While the ORF region was 297 bp in length and encoded for a putative peptide of 98 amino acid residues with an estimated theoretical molecular mass of 10.92 kDa and pI value of 8.33 (Fig. 9, Table 3). Sequence homology comparison of TpIL8 protein with IL8 of different species revealed high similarity with C. carpio (91.84%), L. rohita (89.80%), C. idella (76.53) and D. rerio (73.96) (Table 3). The phylogenetic tree showed TpIL8 protein (Fig. 7B) closely clustering on a branch with C. carpio followed by C. auratus and then grouped together.
Fig. 4. Full-length cDNA sequence and domain topology of Tpmyd88 of T. putitora. The 5’ UTR (small letters), the ORF region (deduced amino acid (upper row) and nucleotide sequence (lower row)) and 3’ UTR (small letters) are shown and numbered on the left. The initiation codon (ATG) and termination codon (TGA) are colored in green and red, respectively. The instability motif (atta) and polyadenylation signal (aataaa) in the 3’ UTR region is highlighted in pink and yellow, respectively. The death domain is marked in red box and TIR (Toll/IL-1 receptor) domain is marked in green box. The mature peptide is shown in bold amino acid sequence.
Fig. 5. Multiple alignment of TpMYD88 with other MYD88 protein. Alignment was done using CLC genomic workbench (version 11.0.1) software program. The conserved alignment position were represented as bar graph at the bottom (on a scale of 0 to 100%). The Death domain and TIR domain were marked in red and black box, respectively. The conserved tryptophan and leucine residues in the death domain are marked with black arrows. Three highly conserved motifs in the TIR domain are boxed in yellow along with highlighted BB and DD loop (in blue box) region.
Fig. 6. Full-length cDNA sequence and domain topology of Tpcd36 of T. putitora. The 5’ UTR (small letters), the ORF region (deduced amino acid (upper row) and nucleotide sequence (lower row)) and 3’ UTR (small letters) are shown and numbered on the left. The initiation codon (ATG) and termination codon (TGA) are colored in green and red, respectively. The CD36 domain is highlighted in grey and trans-membrane domain present at N- and C- terminal is marked in red box. The mature peptide is shown in bold amino acid sequence.
Fig. 7. Phylogenetic tree constructed based on the deduced amino acid sequences of CD36 (A) and IL8 (B), using MEGA X software. Numbers next to the branches represent bootstrap values and scale bars indicate evolutionary distance.
Fig. 8. Multiple alignment of TpCD36 with other CD36 protein. Alignment was done using CLC genomic workbench (version 11.0.1) software program. The conserved alignment position were represented as bar graph at the bottom (on a scale of 0 to 100%). The transmembrane domain at N- and C- terminal were marked in red box. The conserved cysteine residues in the protein are marked with black arrows and conserved N-glycosylation sites are marked with blue dots. The amino acids in the black and yellow box are important amino acid residues present at the C- terminal.
Fig. 8. (continued).

Fig. 9. Full-length cDNA sequence and domain topology of TpIIl8 of *T. putitora*. The 5'UTR (small letters), the ORF region (deduced amino acid (upper row) and nucleotide sequence (lower row)) and 3'UTR (small letters) are shown and numbered on the left. The initiation codon (ATG) and termination codon (TGA) are colored in green and red, respectively. The predicted signal peptide and IL8 domain are marked as ▲▼; the SCY domain is boxed in black. The instability motif and polyadenylation signal is highlighted in pink and yellow, respectively. The mature peptide is shown in bold amino acid sequence.
with IL8 proteins of other teleosts forming a fish specific clade. Whereas bird, human, amphibian, and reptile’s IL8 protein were clustered separately and formed other clades. Domain annotation using SMART identified a signal peptide of 22 amino acids (1 to 22 amino acids) at the N-terminal and a characteristic SCY (small cytokine C-X-C (Intercrine alpha family)) (at position 31 to 92) domain of IL8 protein (Figs. 9, S9B). The same result was confirmed through multiple sequence alignment of TpIL8 with other teleosts and higher vertebrates (Fig. 10). Further, four conserved cysteine residues and CXC motif were also present in the protein sequence, and the ELR signature was absent, displaying the typical teleost chemokine (CXC) structure. Protein-protein interaction analysis revealed various chemokine receptors as functional partners (Fig. S8B). Post-translational modifications in the TpIL8 protein structure were predicted by the presence of a few serine and threonine phosphorylation sites (Figs. S6B and S7B). Subcellular localization of TpIL8 protein was predicted mainly in the extracellular region (22.4%) (Fig. S4D).

3.5. mRNA expression of target genes during wild- and captive-bred embryonic and larval stages

To analyze the effect of different environmental conditions/brooder sources on the immunity of T. putitora, we compared the transcriptional abundance of selected genes, namely tlr2, cd36, myd88 and il8 in embryonic and larval stages of T. putitora obtained from wild- and captive-bred brooders. The expression of the Tpmyd88 gene was relatively low during early embryonic stages, from the unfertilised egg to the blastula stage (Fig. 11A). However, the expression suddenly increased at the gastrula stage and slightly decreased at the organogenesis stage in captive/wild origin individuals. Afterwards, the expression of Tpmyd88 exhibited a significant increase from the just-hatched stage, which peaked at the advanced fry stage in captive origin individuals, whereas no significant change was seen in its expression in wild origin individuals until the advanced fry stage. Similar expression patterns were observed for Tpcd36 and Tpil8 gene (Figs. 11B and 12B), where their expression was found to be relatively low during early embryonic stages in captive/wild origin individuals. However, Tpil8 showed a higher expression level from the organogenesis to the advanced fry stage with a peak at the pre-metamorphosis stage in captive origin individuals, whereas no significant changes in its expression was observed in wild origin individuals. On the other hand, the expression abundance of Tpcd36 transcript in wild origin individuals was consistently low until hatching, and it was observed to increase from the post-metamorphosis stage and peaked at the advanced fry stage, whereas in captive origin individuals, its expression increased post-hatching and peaked at the advanced fry stage. The expression of Tpilr2 in captive origin individuals was observed to be relatively low before hatching except for the just fertilized and the organogenesis stages, where its expression increased significantly (Fig. 12A). Afterwards, the expression of Tpilr2 mRNA exhibited a slight decrease and remained almost consistent till the post metamorphosis stage and later peaked at the advanced fry stage. On the other hand, expression of Tpilr2 mRNA in wild origin individuals remained relatively lower until the blastula stage except for the morula stage, where its expression further decreased but was non-significant. The expression of Tpilr2 mRNA further decreased at the gastrula stage and was consistently low until the post-metamorphosis and the advanced fry stage, where a slight increase in its expression was noted.

4. Discussion

In this study, we have cloned, sequenced, and characterized the full-

Fig. 10. Multiple alignment of TpIL8 with other IL8 protein. Alignment was done using CLC genomic workbench (version 11.0.1) software program. The conserved alignment position were represented as bar graph at the bottom (on a scale of 0 to 100%). The SCY domain is delineated by blue line arrows and the CXC motif is marked in green box. The structurally essential cysteine residues in the IL-8 protein are marked with ▾.
length cDNA of four immune genes of T. putitora, namely toll-like receptor 2 (tlr2), cluster of differentiation 36 (cd36), myeloid differentiation primary response protein 88 (myd88), and interleukin 8 (il8).

Further, we analyzed the mRNA expression of these genes during ontogenetic and larval stages of T. putitora obtained from wild-collected and captive-matured brooders.

The immune system of a fish plays a central role in regulating the internal environment by combating the external risk factors. Just like other vertebrates, innate immunity in fish is the primary line of defense against pathogenic organisms [33]. It triggers the release of inflammatory cytokines and other stimulatory molecules once the pathogen recognition receptors (PRRs) detect the pathogen-associated molecular patterns (PAMPs) [34]. Toll-like receptors (TLRs) are dedicated pathogen-recognizing receptors that identify PAMPs and induce downstream signalling pathways by associating with suitable adaptor proteins to induce an innate immune response [35,36]. TLRs have been identified in several fishes to understand their role in innate immunity towards pathogen defense [37]. Nevertheless, they (particularly TLR2) have not been explored in golden mahseer so far. In this study, the Tptlr2 gene in golden mahseer was cloned, sequenced, and characterized. We found that the Tptlr2 gene encodes for a putative peptide of 792 AAs and display a high sequence homology with that of C. carpio (791 AAs), L. rohita (792 AAs) and C. idella (786 AAs), suggesting that tlr2 gene is highly conserved. The presence of a signal peptide (30 AAs) towards

Fig. 11. Relative fold-expression of myd88 (A) and cd36 (B) genes during embryonic and larval developmental stages in T. putitora obtained from wild- and captive-bred brooders. Expression values are normalized to 18S ribosomal RNA (18S) transcripts. Data are presented as mean ± standard deviation, n = 6. Different superscripts (a–f) above the bars in each series indicate significant difference (p < 0.05).
amino terminal of TpTLR2 protein suggests its translocation to the cellular membrane, which is also in agreement with the presence of the transmembrane domain. The LRR (leucine-rich repeat) domain of TLRs plays a vital role in recognizing pathogenic components. LRRs are 20-30 amino acid long and fold into a U-shaped structure; they provide the structural framework for protein-protein interactions [38]. The domain architecture analysis of mature TpTLR2 peptide also showed the presence of typical LRR domains that are associated with ligand recognition. TpTLR2 contained 6 LRR domains and two other LRR domains, one each at N- and C-terminal of the protein. The number of LRR domains in TLR2 protein varies in different species, suggesting a change in their TLR2 ligand binding; like, Danio rerio TLR2 contained 7 LRRs, Cyprinus carpio, Oncorhynchus mykiss, and Ictalurus punctatus TLR2 contained 8 LRRs, Takifugu rubripes and Homo sapiens TLR2 contained 9

Fig. 12. Relative fold-expression of tlr2 (A) and il8 (B) genes during embryonic and larval development stages in T. putitora under wild and captive conditions. Expression values are normalized to 18S ribosomal RNA (18S) transcripts. Data are presented as mean ± standard deviation, n = 6. Different superscripts (a, b, c, d, e, f) above the bars in each series indicate significant difference (p < 0.05).
The TIR domain in TpTLR2 contained three conserved motifs: motif-1: Y/FDA; motif-2: DXFG and motif-3: a conserved tryptophan (W) residue surrounded by a basic amino acid residue; and it also shared high sequence similarity with other teleost species and higher vertebrates, suggesting a common mechanism in signal transduction [41, 42]. Motif-1 and motif-2 played roles in mediating downstream inflammation signalling pathways, whereas motif-3 was reported to be involved in cellular localization of the receptor by interacting with cytoskeletal elements [43]. Additionally, the TIR domain present at the C-terminal of TpTLR2 protein shared homology with the cytoplasmic region of interleukin-1 receptor family highlighting the fact that members of TLRs along with interleukin-1 receptors (IL-1Rs) belong to the TLR/IL-1R superfamily and are crucial signalling receptors of innate immune system [44]. The TIR domain of TLRs and IL-1Rs is a highly conserved functional motif present in the cytoplasmic region [45]. Furthermore, potential phosphorylation and N-glycosylation sites were identified in the peptide sequence suggesting post-translational modifications in the TpTLR2 protein sequence. In general, phosphorylation and glycosylation are thought to be key mechanisms regulating the TLR signalling pathway. Several studies have reported tyrosine phosphorylation of TLR2 in response to agonist stimulation [46, 47]. Tyrosine phosphorylation of TLR2 is also a prerequisite for PI3K (Phosphatidylinositol 3-kinase) and Rac-1 (a member of the Rho family of GTPases) recruitment for the downstream activation of NF-kB [46]. Consequently, tyrosine phosphorylation may regulate TLR signalling by providing docking sites for other proteins. Furthermore, TLR2 phosphorylation is also crucial for host defense [47]. Concerning glycosylation, it was shown that in case of TLR2, 4 N-linked glycosylation sites present within the extracellular region are essential for biosynthesis and secretion and influenced trafficking, surface representation and pattern recognition [48].

The TLR2 protein is primarily responsible for recognising and responding to pathogenic molecular ligands such as lipoteichoic acid and peptidoglycan from gram-positive bacteria in mammals as well as in teleosts [49, 50]. However, it was reported that fish cells require high concentrations of pathogenic ligands for activation and subsequent signal transduction [51]. The binding of ligand to the extracellular LRR regions of TLR2 results in its dimerization, thereby inducing conformational changes. This, in turn, stimulates the downstream signalling cascade by activating cytosolic adaptor protein molecules containing TIR domain such as MYD88 and TIRAP (MYD88 adaptor like protein), which further activates TOLLIP, IRAKs and TRAF6 molecules and transduces signal from cell membrane to cytosol and finally to nucleus, thereby resulting in production of inflammatory cytokines and anti-infection molecules [52]. This correlates with the protein-protein interaction analysis of TpTLR2 that showed its interaction with MYD88, TIRAP (TIR domain containing adaptor protein), TOLLIP (Toll interacting protein), IRAK1 (IL-1 receptor associated kinase 1) and TRAF6 (TNF receptor associated factor 6) molecules.

Myeloid differentiation factor 88 (MYD88) is a key adaptor protein that activates downstream signal transmission in the TLR signalling pathway. All the TLR signalling pathways are MYD88 dependent except for TLR3 and TLR22 [54]. The myd88 gene has been identified and characterised in several birds, mammals, fishes and amphibians [35, 53]. In the current study, the myd88 gene was characterized in T. putitora for the first time. TmpMyd88 encoded for a putative peptide of 284 AA that displayed high sequence identity with C. carpio (288 AA), C. idella (284 AA), D. rerio (284 AA) and O. mykiss (283 AA). Phylogenetic analysis showed TmpMyd88 was ordered in a common group with other teleost species and closely related to D. rerio and L. rohita. Domain analysis revealed DEATH domain (DD) at N-terminal and TIR domain at the C-terminal of deduced TmpMYD88 sequence. The death domain acts as a mediator and facilitates the interaction of MYD88 protein with the molecules downstream of the TLR signalling pathway involved in inflammatory response and apoptosis [54, 55]. On the other hand, the TIR domain is highly conserved from fishes to higher vertebrates suggesting a similar function of MYD88 protein in regulating downstream elements in the TLR signalling pathway. The TIR domain in the TmpMYD88 protein possessed three conserved motifs that are FADAFICYCQ, LCVFDRDVLPGT and FW, respectively. Based on the previous studies, the former two motifs are suggested to play an important role in associating MYD88 with other TIR domain-containing proteins [56], while the third motif is involved in receptor localization [43]. MYD88 plays a crucial role in innate immunity and is a key adaptor molecule in the TLR signalling pathway. Purcell et al. [57] identified conserved key proteins that are involved in TLR downstream signalling. The study showed MYD88, TIRAP, TRAF, and IRFs as a part of the TLR signalling network. Another study showed that after activation of MYD88 protein, its death domain (DD) interacts with IRAKs, which leads to its activation. IRAKs, after their activation, recruit and activate another adaptor molecule, TRAF6, which further transduces signal downstream and leads to the transcription of genes involved in initiating an inflammation response [58]. In agreement with the above findings, our protein-protein interaction analysis also predicted TLR2, IRAKs, TRAFs and TIRAPs as functional partners of TpMYD88. Moreover, several potential phosphorylation sites were identified in the TpMYD88 protein sequence suggesting post-translational modifications. Reports suggest that MYD88 is modified post transcriptionally via its tyrosine residue phosphorylation by Syk (Spleen tyrosine kinase) resulting in downregulation of TLR signalling pathway [59].

Cluster of differentiation 36 (CD36) belongs to the scavenger receptor (class B) family of proteins that play multifunctional roles and regulates lipid metabolism, angiogenesis, atherosclerosis, platelet functions and innate immunity [60, 61]. CD36 is identified and characterized only in a few teleosts. This is the first study identifying CD36 protein in T. putitora. Characterization of the Tpcd36 (MW322905) gene identified a putative peptide of 469 AAs with a predicted molecular mass of 52.43 kDa and pl of 4.95. The TpCd36 protein displayed high sequence homology with that of C. carpio (468), C. idella (468) and D. rerio (465). Cyprinid CD36 protein is reported to be an integral membrane glycoprotein, consisting of two transmembrane domains, one big extracellular loop and two small intracellular tail regions, one each at N- and C-terminal [60]. Likewise, domain analysis in TpCd36 showed the presence of two transmembrane domains, one big extracellular loop and two small intracellular tails. Sequence alignment analysis predicted several N-glycosylation sites in the extracellular region and conserved cysteine residues in both the extracellular loop and the intracellular tail region similar to the mammalian CD36 [62], common carp CD36 and zebrafish CD36 protein [60]. Glycosylation of CD36 is essential for its proper folding, stability, trafficking and protein function [63]. Further, phosphorylation of CD36 takes place on the extracellular threonine (putative protein kinase C site) residue which may modulate ligand recognition, effect internalization and downstream signalling [64, 65]. Unlike other cyprinids, TpCd36 was found lacking the tyrosine and cysteine residue and had only one cysteine present at the C-terminal. This could affect their role in phagocytosis and subsequent signalling. Moreover, CD36 is a multifunctional protein known to play crucial roles in lipid metabolism, innate immunity, platelet functions, and athero-sclerosis [60], which is evidenced in our findings that predicts its interaction with various thrombospondin and apolipoprotein molecules.

Interleukin 8 (IL8/CXCL8), a pro-inflammatory cytokine, belongs to the family of CXC chemokines that regulates inflammation and immune response in an organism [66, 67]. IL8 plays a pivotal role in recruitment of neutrophils and other immune cells to the inflammatory site, initiating respiratory burst and inducing cell adhesion [68–71]. It has been extensively studied in mammals and several teleost fishes [71–75]. However, IL8 has not been studied and characterized in T. putitora. In this study, we characterized Il8 gene in golden mahseer and reported it to be 653 bp in length encoding a 98 AAs peptide. The BLASTP search showed that TpIL8 protein has a high sequence identity (%) with C. carpio (98), C. idella (98), L. rohita (97) and D. rerio (97). Domain architectural analysis showed a signal peptide (22 amino acids) and a characteristic SCY domain spanning the entire TpIL8 protein, suggesting...
its role as an extracellular secretory cytokine, as similar domains have been identified in other secretory chemokines [72,76]. Like other tetraons, the 3’UTR region of TpIL8 gene has two RNA instability motifs (ATTTA), suggesting that TpIL8 protein may encounter rapid turnovers [72,73]. In agreement with the role of IL8 as a chemokine, protein-protein interaction analysis revealed various chemokine receptors as its functional partners. Furthermore, multiple sequence analysis of the deduced TpIL8 protein with other species identified four conserved cysteine residues at positions 34, 36, 60 and 77 (T. putitora numbering) that is typical structure of CXC chemokine function and confirming the role in tertiary structure formation. The ELR motif (located immediately upstream of CXC motif) responsible for receptor binding, angiogenic and chemotactic activity was not present in TpIL8, like the majority of teleost such as rainbow trout, zebrafish, common carp, etc do not possess ELR motif [77]. Instead, a DPR motif was present in TpIL8. However, several studies have shown that modification of ELR motifs in different fish species does not modify the biological activity of IL8 [78-80].

Conservation breeding and restocking the natural reserves through large-scale seed production is the need of the hour for sustainable management of golden mahseer. The reduced fitness and survival of captive origin T. putitora in comparison to wild origin larvae [14], may limit the success of mahseer stock rehabilitation initiatives. In line with the current information available, we investigated the comparative expression of target innate immune genes (Tptlr2, Tpmyd88, Tpdc36 and Tpil8) during the ontogenetic developmental stages of T. putitora obtained from the wild and captive broodstocks.

Several studies in tetraons have reported the development of innate immune response involving MYD88 dependent TLR signalling during early embryonic stages [81,19]. In this study, constitutive expression of Tptlr2, Tpmyd88, Tpdc36 and Tpil8 gene was reported across all the developmental stages right from the unfertilized egg stage and onwards (in both wild and captive origin offspring’s), indicating the likelihood of maternal contribution. Samanta et al. [82] reported tlr2 expression in rohu (L. rohita) at 7 hph (hours post fertilization) and thereafter detected stable expression in all embryonic, hatchlings and larval stages. Likewise, constitutive expression of tlr2 gene was observed in all embryonic and larval stages of mirgal carp by Basu et al. [19] suggesting their protective role in early life stages. In the current study, the expression of Tptlr2 transcripts was relatively low during the early stages of embryogenesis irrespective of the brooders source (wild or captive), except for the just hatched and the organogenesis stage in captive origin individuals where the Tptlr2 expression was significantly higher and remained high in comparison to wild throughout while peaking at the advanced fry stage. On the other hand, Tpmyd88 expression level was found to be similar until blastula stages in both wild and captive origin embryos, with sudden increase in its expression from gastrula stage to advanced fry stage. The expression of Tpmyd88 was found to be significantly greater in post-hatchings of captive-matured brooders in comparison to wild origin progeny. Similar results were seen in zebrafish where the expression of maternal myd88 mRNA levels were relatively low during blastula and gastrula stages and increased significantly thereafter during segmental and later developmental stages [81]. Likewise, Basu et al. [19] reported low but detectable expression of myd88 mRNA in all the developmental stages of mirgal carp with highest expression of myd88 reported 10 h post-fertilization. The above results suggest that MYD88 dependent TLR2 signalling may play a protective role in immune surveillance system of embryonic and larval stages of T. putitora.

Interleukin-8 is a pro-inflammatory cytokine that is induced and released through activation of NF-kB by Myd88 dependent TLR2 signalling pathway. Activation of IL8 further activates other cytokines (such as IL-1β), thereby generating an inflammatory response [19]. On the other hand, CD36 is a membrane glycoprotein known to play highly diverse roles in homeostatic and immunological processes [62]. It is a scavenger receptor that interacts with pathogenic ligands or lipoproteins and recruits them to preformed TLR2-TR6 heterodimers, following which activation of intracellular signalling and initiation of phagocytosis takes place [83]. Studies on teleosts have suggested the expression of il8 and cd36 transcripts during early development stages as in mirgal carp, zebrafish and common carp [60,21,26]. In the present study, Tpil8 and Tpdc36 mRNA expression was found to be low during the initial stages of embryogenesis, however the expression significantly increased post-hatching for Tpil8 and post metamorphosis stage for Tpdc36 gene in both wild and captive origin offspring’s. Kim et al. [84] reported similar results in Olive flounder (Paralichthys olivaceus) where expression of il8 remained low during early developmental stages and increased after day 15. Moreover, constitutive expression of il8 gene was also reported by Oehlerls et al. [75] in the developmental stages of zebrafish.

Overall, the comparative ontogenetic expression analyses of the studied immune genes indicated that the onset of non-specific immunity occurred in both wild and captive origin progeny at early stages of embryonic development. This innate immunity may protect embryos and larvae from harmful pathogens and diseases. The significantly higher transcript abundance of all the examined immune genes (Tpmyd88, Tptlr2, Tpdc36 and Tpil8) in captive origin golden mahseer larvae may indicate their stronger immune function. Another plausible explanation for the increased mRNA expression of pro-inflammatory genes could be the prevalence of stressful and inflammatory conditions in captivity, where the brooders were raised. However, these assumptions are to be further substantiated through bacterial or other pathogenic challenge experiments and detailed functional analysis of the genes.

5. Conclusion

In the full-length cDNA sequences of Tpmyd88, Tptlr2, Tpdc36 and Tpil8 showed high sequence homology with evolutionarily conserved protein structures of C. carpio, C. idella, C. auratus and D. rerio. The comparative ontogenetic mRNA expression analyses of the above genes indicated the onset of early innate immune function in both wild and captive origin individuals, with greater expression in captive progeny. Besides, constitutive expression of Tptlr2, Tpmyd88, Tpdc36 and Tpil8 genes in the early developmental stages, right from the unfertilized egg, indicated their possible maternal contribution (the transfer of the immunological components from parents, particularly mothers, to their offspring through gametes). However, the immune response has to be validated through further challenge studies employing pathogens.

Declaration of Competing Interest

None.

Acknowledgments

We are grateful to the Director, ICAR- DCFR, Bhimtal, for all the logistic and infrastructural support. The Department of Biotechnology, Government of India is greatly acknowledged for funding (BT/PR26920/AAQ/3/884/2017) the study.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2022.100058.

References

[1] M.S. Akhtar, M. Rajesh, A. Ciji, P. Sharma, B.S. Kamalam, R.S. Patiyal, D. Sarma, Photo-thermal manipulations induce captive maturation and spawning in endangered golden mahseer (T. putitora): a silver-lining in the strangled conservation efforts of decades, Aquaculture 497 (2018) 336-347.
[58] T. Horng, R. Medzhitov, Drosophila MyD88 is an adapter in the toll signaling pathway, Proc. Natl. Acad. Sci. 98 (2001) 12654–12658.

[59] C. Han, J. Jin, S. Xu, H. Liu, N. Li, X. Cao, Integrin CD11b negatively regulates TLR-triggered inflammatory responses by activating Syk and promoting degradation of MyD88 and TRIF via Chb-b, Nat. Immunol. 11 (2010) 734–742.

[60] J.R. Fink, E.L. Benard, T. Herman, A.H. Meijer, M. Forelzena, G.F. Wiegertjes, Molecular and functional characterization of the scavenger receptor CD36 in zebrafish and common carp, Mol. Immunol. 63 (2015) 381–393.

[61] Z. Ding, N. Luo, Y. Kong, J. Li, Y. Zhang, F. Cao, J. Ye, Scavenger receptor class B, type I, a CD36 related protein in Macrophagocytosis: characterization, RNA interference, and expression analysis with different dietary lipid sources, Int. J. Genom. 2016 (2016), 6325927.

[62] M. Febbraio, D.P. Hajjar, R.L. Silverstein, CD36: a class B scavenger receptor scavenger receptor CD36: glycosylation status and its role in trafficking and function, J. Biol. Chem. 284 (2009) 16277–16288.

[63] A.S. Asch, I. Liu, F.M. Briccetti, J.W. Barnwell, F. Kwakye-Berko, A. Dokun, S.J. Hoosdally, E.J. Andress, C. Wooding, C.A. Martin, K.J. Linton, The human interleukins of fish, Dev. Comp. Immunol. 35 (2011) 133–142.

[64] M. Ho, H.L. Hoang, K.M. Lee, N. Liu, T. MacRae, L. Montes, C.L. Flatt, B.G. Yipp, J. Oppenheim, E.J. Leonard, Purification of a human monocyte-derived neutrophil chemotactic factor that has peptide sequence similarity to other host defense cytokines, Proc. Natl. Acad. Sci. 84 (1987) 9233.

[65] T. Horng, R. Medzhitov, Drosophila MyD88 is an adapter in the toll signaling pathway, Proc. Natl. Acad. Sci. 98 (2001) 12654–12658.

[66] C.J. Secombes, T. Wang, S. Bird, The interleukins of fish, Dev. Comp. Immunol. 35 (2011) 133–142.

[67] A.M. Van der Sar, O.W. Stockhammer, C. van der Laan, H.P. Spaink, W. Bitter, A. Meijer, MyD88 innate immune function in a zebrafish embryo infection model, Infect. Immun. 74 (2006) 8179–8187.

[68] C.J. Secombes, T. Wang, S. Bird, The interleukins of fish, Dev. Comp. Immunol. 35 (2011) 1336–1345.

[69] P.P. Sordillo, D.C. Sordillo, L. Helson, The prolonged QT Interval: role of pro-inflammatory cytokines, reactive oxygen species and the ceramide and sphingosine-1 phosphate pathways, In Vivo 29 (2015) 619–636.

[70] C. Li, C.L. Yao, Molecular and expression characterizations of interleukin-8 gene in large yellow croaker (Larimichthys crocea), Fish Shellfish Immunol. 34 (2013) 799–809.

[71] S.J. Allen, S.E. Crown, J.W. Barnwell, F. Kwakye-Berko, A. Dokun, S.J. Hoosdally, E.J. Andress, C. Wooding, C.A. Martin, K.J. Linton, The human interleukins of fish, Dev. Comp. Immunol. 35 (2011) 133–142.

[72] J.K. Laing, J.J. Zou, T. Wang, N. Bolo, I. Hiroko, T. Aoki, C.J. Secombes, Identification and analysis of an interleukin 8-like molecule in rainbow trout Oncorhyncus mykiss, Dev. Comp. Immunol. 26 (2002) 433–444.

[73] L. Chen, C. He, P. Basprasetulk, P. Xu, P. Li, J. Serapion, G. Waldbieser, W. Wolters, Z. Liu, Analysis of a catfish gene resembling interleukin-8: cDNA cloning, gene structure, and expression after infection with Edwardsiella ictaluri, Dev. Comp. Immunol. 29 (2005) 135–142.

[74] M. Seppola, A.N. Larsen, K. Steiro, I. Jensen, Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (Gadus morhua L.), Mol. Immunol. 45 (2008) 887–897.

[75] S.H. Oethlers, M.V. Flores, C.J. Hall, R. O’Todde, S. Swift, K.E. Crosier, P.S. Crosier, Expression of zebrafish ccl8 (interleukin-8) and its receptors during development and in response to immune stimulation, Dev. Comp. Immunol. 34 (2010) 352–359.

[76] K. Vaddi, M. Keller, R. Newton, The Chemokine Factsbook: Ligands and Receptors, Elsevier, 1997.

[77] C. Bizzarri, A.R. Becari, R. Bertini, M.R. Cavicchia, S. Giorgini, M. Allegretti, ELR + CXC chemokines and their receptors (CXC chemokine receptor 1 and CXC chemokine receptor 2) as new therapeutic targets, Pharmacol. Ther. 112 (2006) 139–149.

[78] Z. Cai, C. Gao, Y. Zhang, X. Xing, Functional characterization of the ELR motif in piscine ELR + CXC chemokine, Mar. Biotechnol. 11 (2009) 505–512.

[79] Y.H. Hu, L. Chen, L. Sun, CXCL8 of Scophthalmus maximus: expression, biological activity and immunoregulatory effect, Dev. Comp. Immunol. 35 (2011) 1032–1039.

[80] O. Kurata, S. Wada, T. Matsuyama, T. Sakai, T. Takano, N-terminal region is responsible for chemotaxis-inducing activity of flounder IL-8, Fish Shellfish Immunol. 38 (2014) 361–366.

[81] A.M. Van der Sar, O.W. Stockhammer, C. van der Laan, H.P. Spaink, W. Bitter, A. Meijer, MyD88 innate immune function in a zebrafish embryo infection model, Infect. Immun. 74 (2006) 2436–2441.

[82] M. Samanta, B. Swain, M. Basu, P. Panda, G.B. Mohapatra, B.R. Sahoo, N.K. Maiti, Molecular characterization of toll-like receptor 2 (TLR2), analysis of its inductive expression and associated down-stream signaling molecules following ligands exposure and bacterial infection in the Indian major carp, rohu (Labeo rohita), Fish Shellfish Immunol. 32 (2012) 411–425.

[83] M. Triantafilou, F.G. Gamper, R.M. Haston, M.A. Mouratis, S. Morath, T. Hartung, M. Triantafilou, Membrane sorting of toll-like receptor (TLR)-2/6 and TLR2/1 heterodimers at the cell surface determines heterotypic associations with CD36 and in response to immune stimulation, Dev. Comp. Immunol. 34 (2010) 352–359.

[84] K.J. Laing, J.J. Zou, T. Wang, N. Bolo, I. Hiroko, T. Aoki, C.J. Secombes, Identification and analysis of an interleukin 8-like molecule in rainbow trout Oncorhyncus mykiss, Dev. Comp. Immunol. 26 (2002) 433–444.