

## **$\beta$ -Glucan-supplemented diets increase poly(I:C)-induced gene expression of Mx, possibly via Tlr3-mediated recognition mechanism in common carp (*Cyprinus carpio*)**

Alberto Falco<sup>a, b</sup>, Joanna J. Miest<sup>a</sup>, Nicolas Pionnier<sup>a</sup>, Danilo Pietretti<sup>b</sup>, Maria Forlenza<sup>b</sup>, Geert F. Wiegertjes<sup>b</sup>, David Hoole<sup>a</sup>, ,

<sup>a</sup> School of Life Sciences, Keele University, Keele, Staffordshire ST5 5BG, United Kingdom

<sup>b</sup> Cell Biology and Immunology Group, Wageningen Institute of Animal Sciences, Wageningen University, P.O. Box 338, Wageningen 6700 AH, The Netherlands

### Highlights

- mx expression was stable or slightly up-regulated in carp after  $\beta$ -glucan treatment.
- Poly(I:C) markedly increased mx expression in samples from  $\beta$ -glucan fed fish.
- Two sequences for carp tlr3 were retrieved (tlr3.1 and tlr3.2) and characterized.
- $\beta$ -Glucan-supplemented diets increased the transcript levels of both tlr3 genes.

### Abstract

We have previously observed that in common carp (*Cyprinus carpio*), administration of  $\beta$ -glucan (MacroGard®) as feed additive leads to a lower expression of pro-inflammatory cytokines suggesting that this immunostimulant may be preventing an acute and potentially dangerous response to infection, particularly in the gut. However, in general, mechanisms to detect and eliminate pathogens must also be induced in order to achieve an efficient clearance of the infection. Protection against viral diseases acquired through  $\beta$ -glucan-supplemented feed has been extensively reported for several experimental models in fish but the underlining mechanisms are still unknown. Thus, in order to better characterize the antiviral action induced by  $\beta$ -glucans in fish, MacroGard® was administered daily to common carp in the form of supplemented commercial food pellets. Carp were fed for a period of 25 days prior to intra-peritoneal injection with polyinosinic:polycytidylic acid (poly(I:C)), a well-known double-stranded RNA mimic that triggers a type-I interferon (IFN) response. Subsequently, a set of immune related genes, including mx, were analysed by real-time PCR on liver, spleen, head kidney and mid gut tissues. Results obtained confirmed that treatment with  $\beta$ -glucan alone generally down-regulated the mRNA expression of selected cytokines when compared to untreated fish, while mx gene expression remained stable or was slightly up-regulated. Injection with poly(I:C) induced a similar down-regulated gene expression pattern for cytokines in samples from  $\beta$ -glucan fed fish. In contrast, poly(I:C) injection markedly increased mx gene expression in samples from  $\beta$ -glucan fed fish but hardly in samples from

fish fed control feed. In an attempt to explain the high induction of mx, we studied Toll-like receptor 3 (TLR3) gene expression in these carp. TLR3 is a prototypical pattern recognition receptor considered important for the binding of viral double-stranded RNA and triggering of a type-I IFN response. Through genome data mining, two sequences for carp tlr3 were retrieved (tlr3.1 and tlr3.2) and characterized. Constitutive gene expression of both tlr3.1 and tlr3.2 was detected by real-time PCR in cDNA of all analysed carp organs. Strikingly, 25 days after  $\beta$ -glucan feeding, very high levels of tlr3.1 gene expression were observed in all analysed organs, with the exception of the liver. Our data suggest that  $\beta$ -glucan-mediated protection against viral diseases could be due to an increased Tlr3-mediated recognition of ligands, resulting in an increased antiviral activity of Mx.

#### Keywords

- $\beta$ -Glucan;
- TLR3;
- Type-I IFN response;
- Antiviral;
- *Cyprinus carpio*

## 1. Introduction

Additional strategies in disease prevention in cultured fish species are required, especially against viruses since diseases caused by these pathogens have a significant economic impact and are a major threat to the profitability and sustainability of the aquaculture industry ([www.oie.int/en/international-standard-setting/aquatic-code](http://www.oie.int/en/international-standard-setting/aquatic-code)). One important strategy is to increase the innate immune system using immunostimulants, such as  $\beta$ -glucans, as feed supplements [1].  $\beta$ -glucans are glucose polysaccharides that are connected by  $\beta$ -glycosidic bonds or d-glucose molecules which are repetitively linked at a specific position. In particular,  $\beta$ -1,3/1,6 glucans have been shown to be potent immunomodulators able to reduce the susceptibility to infection in fish against a wide array of pathogens, including bacteria [2], [3], [4], [5], [6] and [7], parasites [8] and viruses [9] and [10].

Although the protection conferred by  $\beta$ -glucans against viral infection has not been extensively reported in fish, recent studies have indicated that fungal derived compounds may induce significant protection against viral infections. For example, in Pacific herring (*Clupea pallasii*) fed  $\beta$ -glucan-supplemented diets approximately 50–80% protection against viral hemorrhagic septicaemia rhabdovirus (VHSV) was noted [10] and about 60% protection occurred against grass carp haemorrhage reovirus (GCHV) in grass carp (*Ctenopharyngodon idella*) [9]. In the latter study several immune parameters, i.e. superoxide dismutase and catalase enzyme activity were increased in the fish fed with the  $\beta$ -glucan-supplemented diet in comparison to control groups. Also, mx transcript levels, commonly used as reporter of type-I IFN induction, in the spleen, induced after poly(I:C) injection, were higher and persisted for longer in fish fed with  $\beta$ -glucan supplements.

The immunostimulatory and antiviral activity of  $\beta$ -glucans could be mediated by the modulation of pro-inflammatory cytokines and chemokines gene expression profile. However, in a previous study [11] we reported that the gene expression levels of several inflammation-related cytokines (i.e. *il1 $\beta$* , *m17*, *il10*, *tnf $\alpha$ 1* and *tnf $\alpha$ 2*) in common carp (*Cyprinus carpio*) were reduced when administering  $\beta$ -glucan (MacroGard®) as a feed additive.

To better understand the antiviral action induced by  $\beta$ -glucans in carp, MacroGard® was administered daily prior to intra-peritoneal injection with polyinosinic:polycytidylic acid (poly(I:C)), a well-known double-stranded RNA mimic that triggers a type-I interferon (IFN) response. Subsequently, a set of immune related genes including mx, commonly used as reporter of type-I IFN induction, were analysed. Poly(I:C) injection markedly increased mx gene expression in samples from  $\beta$ -glucan fed fish but hardly in samples from fish fed control feed. In an attempt to explain the high induction of Mx, we studied Toll-like receptor 3 (TLR3) gene expression in these carp. TLR3 is considered an important, although not the

only, pattern recognition receptor the recognition of viral double-stranded RNA and consequent triggering of a type-I IFN response leading to the expression of Mx, among other interferon stimulated genes (ISGs) (REF). Through genome data mining, two sequences for carp tlr3 were retrieved (tlr3.1 and tlr3.2) and further characterized at the molecular level and their expression levels analysed. The data in our study identify Tlr3 as an important mediator involved in the immunostimulatory effects triggered by  $\beta$ -glucan-supplemented diets. The implication for the aquaculture sector regarding increased protection against viral infections is discussed.

## 2. Materials and methods

### 2.1. Experimental design

European common carp (*C. carpio carpio*) (average weight of 40 g) were obtained from a commercial supplier (Fair Fisheries, UK) and kept in individual tanks with circulated dechlorinated aerated water at 22 °C under a 12 h/12 h light/dark cycle.

Initially, for the acclimatization period fish were divided into 2 groups and fed 1% bodyweight per day of a MacroGard® free control diet for two weeks. At this point, one group of fish was kept on the control diet (lacking MacroGard®) while the other one received a diet containing 0.1% MacroGard®, a daily dose of 10 mg/kg bodyweight MacroGard® recommended by the commercial supplier (Biorigin, Brazil). Both normal and supplemented diets were provided and formulated by Tetra GmbH (Germany) and the exact composition is described in our previous work [11].

After 25 days of feeding, 5 fish per group were sampled prior to further experimentation which comprised the injection of each feeding group with 50  $\mu$ l of either PBS or 5 mg/kg of poly(I:C) (Invivogen, UK) in PBS. Therefore the experiment included four treatment groups i.e. control feeding injected with PBS or poly(I:C) and  $\beta$ -glucan feeding injected with PBS or poly(I:C), each containing 4 fish. Animals were killed by immersion in a lethal dose (1:1000) of 2-Phenoxyethanol (Sigma Aldrich, UK) at 24 h post-injection and liver, spleen, head kidney and mid gut removed and stored in RNAlater (Invitrogen, UK) at -80 °C for analysis of gene expression. See reference [12] for further details about the experimental design.

### 2.2. RNA isolation and cDNA synthesis

RNA was isolated from organs using the RNeasy Mini Kit (Qiagen, UK) following the manufacturer's instructions. The RNA concentration was determined by nanodrop 1000 (Thermo Scientific, UK), normalized with DEPC-treated water (Invitrogen, UK) and stored at -80 °C before cDNA synthesis.

cDNA synthesis were carried out by using 0.5 µg of template RNA for a final 20 µl reaction volume. Random hexamers and the Moloney murine leukaemia virus reverse transcriptase (M-MLV) (both from Invitrogen, UK) were used according to manufacturer's instructions in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems) at 25 °C for 10 min followed by 30 min at 42 °C. Reactions were inactivated by incubation at 95 °C for 5 min. Samples were diluted 1:10 with DEPC-treated water and stored at -20 °C.

In order to determine the basal expression levels of the carp *tlr3* gene sequences, a cDNA library encompassing several tissues (gill, gut, head kidney, mid kidney, liver, muscle, peripheral blood leucocytes (PBL), skin, spleen and thymus) from five different healthy individuals was constructed as described earlier [13].

RT-qPCR assays were performed as in our previous studies [11], [12] and [14], and all genes analysed and primers used (Eurofins MWG Operon, London, UK) are listed in Table 1 (primers for carp *mx* gene were designed based on sequences retrieved from the carp genome using as template a partial carp *mx* sequence available in GenBank, accession number: EF635410). Thermal cycling and melting curve conditions were conducted as previously described [11] and [12]. Gene expression results were analysed according to the  $2^{-\Delta\Delta Ct}$  method [15], and data for all treatment groups were compared after the feeding period to the control feeding group and after the treatment with poly(I:C) to the control feeding group injected with PBS. The data obtained for each gene were normalized against those obtained for the expression of the S11 protein of the 40S subunit reference gene (*40s*) which did not differ significantly at each time point. Data are represented as the mean fold changes  $\pm$  standard deviation.

Table 1.  
Oligonucleotide primers used in this study.

Gene	Primer sequences (5'-3')	GenBank Accession No.	Reference
<i>40s</i>	FW: CCGTGGGTGACATCGTTACA	AB012087	[43]
	RV: TCAGGACATTGAACCTCACTGTCT		
<i>il1<math>\beta</math></i>	FW: AAGGAGGCCAGTGGCTCTGT	CCA245635	[43]
	RV: CCTGAAGAAGAGGAGGCTGTCA		
<i>il10</i>	FW: CGCCAGCATAAAGA AACTCGT	AB110780	[44]
	RV: TGCCAAATACTGCTCGATGT		
<i>tnfa1</i>	FW: GAGCTTCACGAGGACTAATAGACAGT	AJ311800	[45]
	RV: CTGCGGTAAGGGCAGCAATC		
<i>tnfa2</i>	FW: CGGCACGAGGAGAAACCGAGC	AJ311801	[45]
	RV: CATCGTTGTGTCTGTTAGTAAGTTC		
<i>mx</i>	FW: TGGATAAGGGA AACTGAAGAG	EF635410	
	RV: AGTGGCTGTCAAAGGTCA		

Gene	Primer sequences (5'–3')	GenBank Accession No.	Reference
<i>cxca</i>	FW: GGGTGTAGATCCACGCTGTC	AJ550164	[46]
	RV: CTTTACAGTGTGGGCTTGGAG		
<i>cxcb</i>	FW: GCTGCCTGCTTGTGTAGAG	AB082985	[46]
	RV: ATCTGTTTTGGAGGAACCA		
<i>cctlr3.1</i>	FW: GTTATCCCTGGCGCATAATA	KF387571	Present study
	RV: TCTTCAATAATTGGTAAGGATGATG		
<i>cctlr3.2</i>	FW: GTTTATCCCTGGAGCATAACT	KF387572	Present study
	RV: CTCAATAACTGGTAAAGACGAAC		

### 2.3. Statistical analysis

Gene expression data were statistically analysed by using two-way ANOVA and Bonferroni's multiple comparisons to determine significant differences between the different treatments and their respective control groups. GraphPad Prism v5 software was used for creating the graphs and statistical analysis.

### 2.4. Searching for tlr3 orthologues in the genome of carp

Using the complete coding sequence of a previously reported carp tlr3 mRNA sequence (GenBank accession number: DQ885910) [16] as template, the common carp genome [17] was screened with the Basic Local Alignment Search Tool (BLAST) (Default algorithm parameters) from the CLC bio Genomics Workbench v4.9 software (CLC bio, Aarhus, Denmark). Predicted open reading frames (ORF) were translated to protein sequence by using the expasy 'Translate' online software (<http://web.expasy.org/translate/>). Specific primers for each of the two carp tlr3 isoforms were manually designed for gene expression analysis.

### 2.5. In silico analysis of Tlr3 of carp

Tlr3 protein sequences from various fish and non-fish species were gathered from UniProtKB database (Table 2). SignalP v4.1 ([www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)) was used to predict signal peptide sequences [18]. The multiple alignments of mature protein sequences were performed with ClustalW2 (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>) [19] and a phylogenetic tree was constructed using the MEGA v4.0 software [20]. Carp Tlr3 TIR domain, transmembrane region or leucine-rich repeats were predicted by using PROSITE (<http://prosite.expasy.org/>) and manually refined. The molecular weight of the proteins was calculated using the ProtParam tool (<http://web.expasy.org/protparam/>).

Table 2.

TLR3 protein sequences gathered from UniProtKB database.

Organism – <i>Scientific name</i> (common name)	Abbreviation	Accession number
<i>Bos taurus</i> (Cattle)	btTlr3	Q5TJ59
<i>Sus scrofa</i> (Wild boar)	ssTlr3	A0ENH4
<i>Homo sapiens</i> (Human)	hsTLR3	O15455
<i>Mus musculus</i> (Mouse)	mmTlr3	Q99MB1
<i>Monodelphis domestica</i> (Gray short-tailed opossum)	mdTlr3	F6TNZ8
<i>Meleagris gallopavo</i> (Wild turkey)	mgTlr3 <sup>f</sup>	G1NFY1
<i>Gallus gallus</i> (Chicken)	ggTlr3	A1YV56
<i>Cairina moschata</i> (Muscovy duck)	cmTlr3	J9Q6B3
<i>Taeniopygia guttata</i> (Zebra finch)	tgTlr3 <sup>f</sup>	H0Z936
<i>Anolis carolinensis</i> (Carolina anole)	acTlr3 <sup>f</sup>	G1K9T8
<i>Xenopus tropicalis</i> (Western clawed frog)	xtTlr3 <sup>f</sup>	F6YQH2
<i>Ictalurus punctatus</i> (Channel catfish)	ipTlr3	Q00L02
<i>Danio rerio</i> (Zebrafish)	drTlr3	B8JIL3
<i>Cyprinus carpio</i> (Common carp)	ccTlr3	B5G4V9
<i>Ctenopharyngodon idella</i> (Grass carp)	ciTlr3	B4UUN2
<i>Carassius auratus</i> (Goldfish)	caTlr3	Q2I686
<i>Oncorhynchus mykiss</i> (Rainbow trout)	omTlr3	Q3KUR8
<i>Oryzias latipes</i> (Medaka)	olTlr3	H2LVY8
<i>Tetraodon nigroviridis</i> (Green spotted puffer)	tnTlr3	H3CNU2
<i>Takifugu rubripes</i> (Torafugu)	trTlr3 <sup>f</sup>	H2SIG3
<i>Paralichthys olivaceus</i> (Olive flounder)	poTlr3	I0J0U2
<i>Oreochromis niloticus</i> (Nile tilapia)	onTlr3	I3JV98
<i>Gasterosteus aculeatus</i> (Three-spined stickleback)	gaTlr3	G3PXE9
<i>Epinephelus coioides</i> (Orange-spotted grouper)	ecTlr3	K9L2J1
<i>Larimichthys crocea</i> (Yellow croaker)	icTlr3	F8S833

f, fragment.

Genomic sequences, used for comparing the intron–exon organization of the *tlr3* genes of carp with other species, were retrieved from corresponding genome databases (zebrafish and human) in ensembl (<http://www.ensembl.org>) by performing BLAST with corresponding protein sequences listed in Table 2 as template. Intron–exon gene organization scheme was first drafted by using the online tool at [http://wormweb.org/exonintron\\_old](http://wormweb.org/exonintron_old) and manually refined afterwards.

### 3. Results

#### 3.1. Effect of $\beta$ -glucan-supplemented diets on the expression of selected immune genes

The expression of selected inflammatory-related genes i.e. *il1 $\beta$* , *il10*, *tnf $\alpha$ 1*, *tnf $\alpha$ 2*, *cxca* and *cxcb*, and *mx* ( Fig. 1) in carp fed control and  $\beta$ -glucan-supplemented diets changed significantly in the spleen ( $F = 19.64$ ;  $p < 0.0001$ ), head kidney ( $F = 25.26$ ;  $p < 0.0001$ ) and mid gut ( $F = 60.24$ ;  $p < 0.0001$ ) but not in the liver ( $F = 0.19$ ;  $p = 0.6635$ ) after 25 days. With the exception of *mx*, a down-regulation was generally observed for all genes analysed in spleen, head kidney and, particularly, in mid gut tissues when feeding with  $\beta$ -glucan additives.

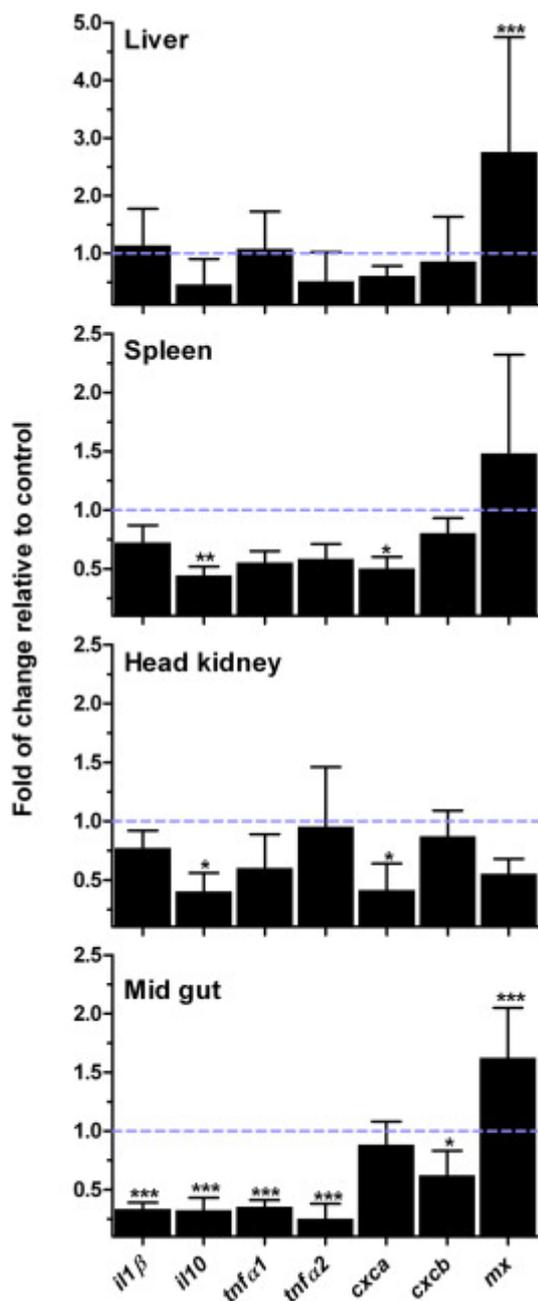


Fig. 1.

Effect of  $\beta$ -glucan-supplemented diet on the transcript levels of selected inflammatory-related genes in immune relevant organs of carp 25 days after experimental feeding. *ill1 $\beta$* , *ill10*, *tnfa1*, *tnfa2*, *cxca*, *cxcb* and *mx* transcript levels were analysed by RT-qPCR. Expression levels in the  $\beta$ -glucan-supplemented group are reported as fold change relative to the control feed group (dashed line at y-value equal to 1). Significant differences are represented by \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). Mean  $\pm$  SD;  $n = 5$ .

Mx expression levels however, were slightly up-regulated in liver, spleen and mid gut in the  $\beta$ -glucan fed group ( Fig. 1), although significant changes occurred in the liver (2.77 fold,  $p < 0.001$ ) and the mid gut (1.63 fold,  $p < 0.001$ ) only.

### 3.2. Effect of $\beta$ -glucan-supplemented diets on the response to poly(I:C) injection

Poly(I:C) injection after 25 days of feeding (MacroGard® or control diet) significantly up-regulated the expression of selected inflammatory-related genes in all the tissues analysed (Fig. 2), i.e. liver ( $F = 169.1$ ;  $p < 0.0001$ ), spleen ( $F = 16.41$ ;  $p < 0.0001$ ), head kidney ( $F = 686.2$ ;  $p < 0.0001$ ) and mid gut ( $F = 77.94$ ;  $p < 0.0001$ ). In  $\beta$ -glucan-supplemented groups all selected genes apart from *mx* were generally down-regulated in head kidney and mid gut, and to a lesser extent in the spleen. Differences in the expression levels of these genes were not observed in liver except for a moderate, but non-significant, up-regulation in the  $\beta$ -glucan fed/poly(I:C)-injected group. In contrast, a significant increase in *mx* expression levels were detected for all tissues examined from the  $\beta$ -glucan fed/poly(I:C) injected group when compared to feeding (normal fed/poly(I:C) injection) and injection ( $\beta$ -glucan fed/PBS) control groups. In particular, *mx* expression levels for the  $\beta$ -glucan fed/poly(I:C) injected group were increased by  $1451.9 \pm 189.6$  fold in the liver,  $466.1 \pm 226.5$  in the spleen,  $1007.9 \pm 75.6$  in the head kidney and  $1299.3 \pm 292.4$  in the gut, therefore about 4, 30, 33 and 121 times more respectively than in the normal fed/poly(I:C) injection group.

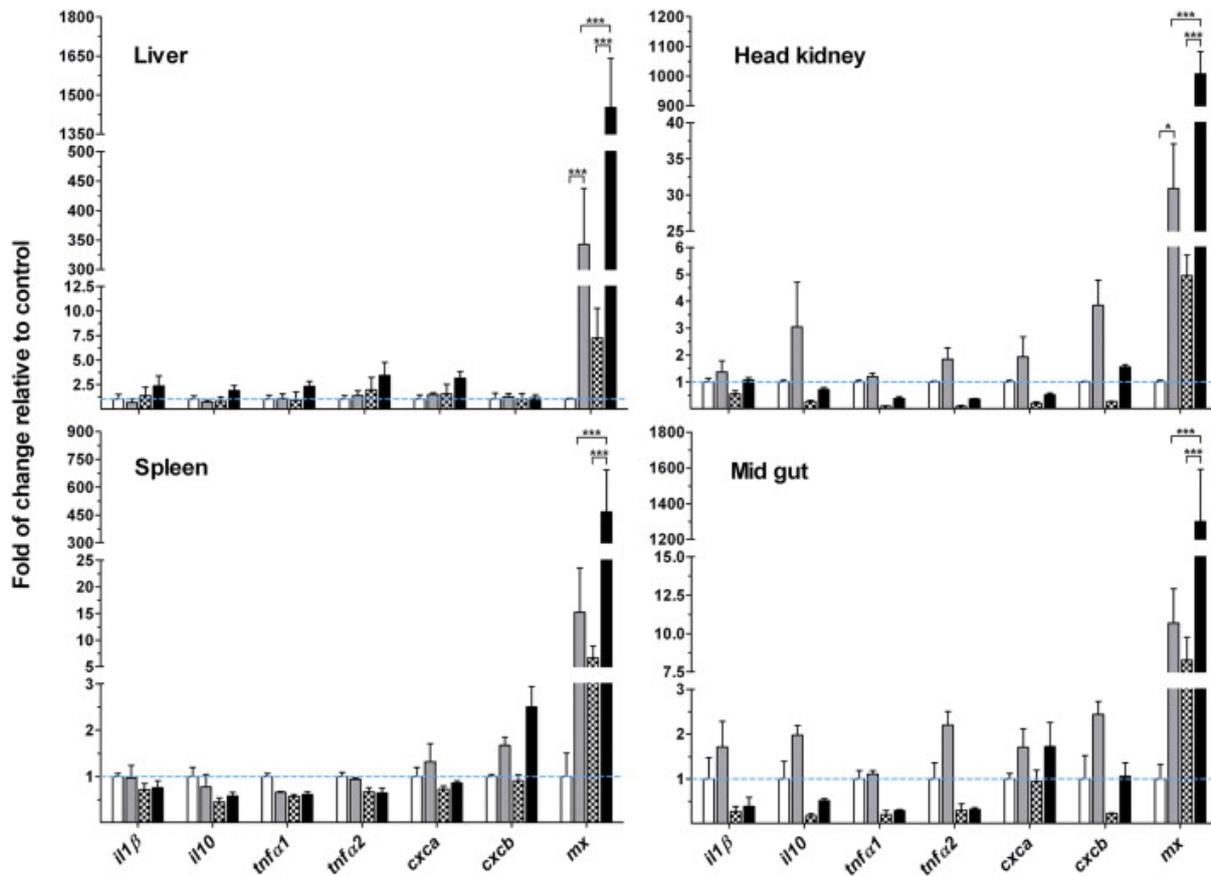


Fig. 2.

Expression of *il1β*, *il10*, *tnfa1*, *tnfa2*, *cxca*, *cxcb* and *mx* in the liver, spleen, head kidney and mid gut of 25-days carp fed with experimental diets after 1 day from intra-peritoneal injection with poly(I:C) (5 mg/kg). Expression was determined by RT-qPCR and normalized to *40s* gene expression. The gene expression data for all treatment groups were compared to the control group (control diet, non-injected) and represented as the mean fold changes  $\pm$  SD ( $n = 4$ ). Significant differences when comparing  $\beta$ -glucan-supplemented diet groups with their associated control diet groups are represented by \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). Groups:  $\square$ , control diet, non-infected;  $\blacksquare$ ,  $\beta$ -glucan-supplemented diet, non-injected;  $\blacksquare$ , control diet, infected;  $\blacksquare$ ,  $\beta$ -glucan-supplemented diet, infected.

### 3.3. Identification of carp *tlr3* genes

Since poly(I:C) injection further increased the expression of *mx* in all the tissues analysed in carp fed diets with  $\beta$ -glucan supplements in comparison to fish injected with poly(I:C) and fed with a normal diet, it might indicate that  $\beta$ -glucans may be synergizing or converging with the signalling pathways leading to expression of type-I interferon (*ifn*), which in turn will induce the expression of IFN stimulated genes (ISGs), including *mx*. In an attempt to explain this fact, we identified carp *tlr3* genes and studied their expression in these carp.

A carp tlr3 sequence already available in the database (GenBank accession number: DQ885910) [16] was used as template to search the carp genome for additional tlr3 paralogs. Two tlr3 sequences were retrieved, none of which corresponded to that obtained by Yang [16]. The newly identified carp tlr3 sequences were named tlr3.1 (GenBank accession number: KF387571) and tlr3.2 (GenBank accession number: KF387572) and shared 88% and 90% identity, respectively, in protein sequence with the previous reported carp tlr3 sequence. Tlr3.1 was more similar (82% identity in protein sequence) than Tlr3.2 (81%) to their unique orthologue in zebrafish (*Danio rerio*). Percentage of identity between Tlr3.1 and Tlr3.2 protein sequences was 90%. In addition, several annotated tlr3 orthologues in fish, and particularly cyprinids, were found in the database by BLAST by using carp tlr3.1 and tlr3.2 predicted ORF nucleotide sequences as templates, which confirmed the reliability of the sequences retrieved.

ORFs for both carp tlr3.1 and tlr3.2 comprised 2715 base pairs (bp) ( Fig. 4A), coding for 904-amino acids (aa) proteins whose sequences were predicted to have a signal peptide of 21 aa for Tlr3.1 and 22 aa for Tlr3.2 (Fig. 3). Therefore, the mature protein and estimated molecular weight is corresponding to 883 aa and 100.3 kDa for Tlr3.1 and 882 aa and 100.0 kDa for Tlr3.2 (Fig. 3).

	Signal peptide	▼ LRR1	▼ LRR1																																																
ccTlr3.1	MRLMKLLLLPLFCMCPFHGHCA	TAYPHKSTCTIENAKADCS	MNLD	DAVPTDLPKNIITLID 60																																															
ccTlr3.2	MELMKLLLLPLFCMCPFHGHCA	TAYPHKSKCTIENAKADCS	MNLD	DAVPTDLPKNIITLID 60																																															
ccTlr3	MELMKLLLLPLFYTCFHABCA	GSAYPHRSTCTIENAKADCS	MNLD	VVPTNLPHNIITLID 60																																															
drTlr3	MRLMKLLLLPLFYACFSABCA	GTTNARKSACMIKNAKADCS	MNLD	DAIPTDLPNIITLID 60																																															
hsTLR3	--NRQQLTLCYFPGWGLLPFGML	CASSTTKCTVSHREVADCS	MLKE	TQVFDLPTNIITLID 57																																															
ccTlr3.1	VSENRKLTLSLHLYSN--	LVTIDASYNSLTAIEKDL	CLSVSHLQILAVC	HEVYLISEK 118																																															
ccTlr3.2	VSENRKLTLSLHLYSN--	LVMIDASYNSLTVIEKDL	CLSLPHLQSLNQC	HEVYLISEK 118																																															
ccTlr3	VSENRKLTLSLHLYSN--	LVMVDASYNSLTAIEKDL	CLSLPHLQILAVC	HEVYLMNEK 118																																															
drTlr3	VSENRKLTLSLHMYTM--	LVMIDASYNSLAGIEKDL	CLSLPHLQFLAVC	HEVYLISEK 118																																															
hsTLR3	LTHNQRLRLPAANPTRYSLT	SLWVGFMTISKLEPELQ	KLPLMKVLANLQ	HELSQLSDK 117																																															
ccTlr3.1	DLKNCFLRLDLSNRL--	KLNGEPPSVLKS	LTWLDVSRNKLKSAKLG	TQPQLPNIITLV 177																																															
ccTlr3.2	DLKNCFLRLDLSNRL--	KLKGEPPSVLKS	LTWLDVSRNKLKSAKLG	TQPQLPNIITLV 177																																															
ccTlr3	VLKNCFLRLDLSNRL--	KLQGEPPSVLKNL	TWLDVSRNKLKSAKLG	TQPQLPNIITLV 177																																															
drTlr3	NLKNCFLRLDLSNRL--	KLQGEPPSLKLNL	TWLDVSRNKLKSAKLG	TQPQLPNIITLV 177																																															
hsTlr3	TFAPCTNLDELHMSNSIQKI	KNNPFVKQNLITL	DLSHNGLSSTKLG	TQVQLENLQELL 177																																															
ccTlr3.1	LSGNEISVLQKNDPFLSNSSA	FRVILLSLSLKKVENG	CFQAIARLSDLV	LDVYSKISLQ 237																																															
ccTlr3.2	LSGNEISVLQKNDPFLSNSSA	FRVILLSLSLKKVENG	CFQAIARLSDLV	LDVYSKISLQ 237																																															
ccTlr3	LSGNEISALQKNDPFLSNSSA	FRVILLSLSLKKVENG	CFQAIARLSDLV	DWSRLSQ 237																																															
drTlr3	LSGNNIILQENDPFLSNSSS	FRVILLSLILKKVENG	CFKAITLSDLV	LDSSKLSLQ 237																																															
hsTLR3	LSNNKIQALKSELDIFAN--	SSLKLELSSNQIKFSP	QCFHAI	IGRLPGLFLNNVQLGPS 236																																															
ccTlr3.1	FTTSLWEELAATAARLKL	SLKNTQVTL	SNITTFQGLEKTNIT	VLDELSSNTSKIADGAFQ 297																																															
ccTlr3.2	FIPSLCEELAGTALRNLS	SLKNTQVTL	SNITTFQGLDKTNIT	VLDELSSNTSKIADGAFQ 297																																															
ccTlr3	FTTMCEELAGTALRNLS	SLKNTQVTL	SDITTFQGLDKTNIT	VLDELSSNTSKIADGAFQ 297																																															
drTlr3	FTTSLFLEADTALRNLS	SLKSTEQVTL	SNITTFQGLEKTKIT	VLDELSENRIKIVDGFQ 297																																															
hsTLR3	LTEKLCLELANTSIENLS	SLMSQLSTT	SNITTFGLKNTNL	TMLDLEYNINLVGNDG 296																																															
ccTlr3.1	FRLEMLSLAHNTLRHLTK	DTFRGLGNLRQLNLQKAL	IK--SHTSSLPI	IEDPSPHHLVQ 355																																															
ccTlr3.2	FRLEBSLSLEHNSLQKLT	KDTPLGLGNLRQLNLQKAL	IK--SHSSSLP	IVIEDPSPHHLVQ 355																																															
ccTlr3	LPRLEPLSLEHNSLRHLTK	DTFRGLGNLRQLNLQKAL	IK--SHTSSLPI	IEDPSPHHLVQ 355																																															
drTlr3	LPOLEPLSLEHNTLRHLT	NDSPSGLGNLRQLNLKAL	IK--SHAS--	LPVDDPSPHHLVQ 354																																															
hsTLR3	LPOLEYFPLEYNNIQHL	FSHSLHGLFNRYLN	LKRSFTKQGISL	SLSPKIDDPSPHHLVQ 356																																															
ccTlr3.1	LEHLCMANTA	FRREITEHIFSGLR	KLKTL	DLSSWSTIGLKT	VNTRTF	FAALQESPL	QTLNLT 415																																												
ccTlr3.2	LEHLCMANTA	FRREITEHIFSGLL	HLKTL	DLSSWSTIGLKT	VNTRTF	FAALQESPL	QTLNLT 415																																												
ccTlr3	LEHLCMANTA	FRREITEHIFSGLL	HLKTL	DLSSWSTIGLKT	VNTRTF	FAALQESPL	QTLNLT 415																																												
drTlr3	LEYLCMANTA	FRREITEHIFSGLR	KLKTL	DLSSWSTIGI	KTVNTR	TFAGLQESPL	QTLNLT 414																																												
hsTLR3	LEHLMMEENDIPG	IKSNMFG	LINLKYLSLNS	SFTSLR	LTNET	TFVSLA	HSP--LHILNLT 415																																												
ccTlr3.1	AMGINKLAPGAPSSLGNL	TLLDLSHNF	INQQLKGD	DELGLSNIK	IEDMS	INQGISL	TLNT 475																																												
ccTlr3.2	AMGINKLAPGAPSSLGNL	TLLDLSHNF	INQQLKGD	DELGLSNIK	IEDMS	INQGISL	TLNT 475																																												
ccTlr3	AMGINKLAPGAPSSLGNL	TLLDLSHNF	INQQLKGD	DELGLSNIK	IEDMS	INQGISL	TLNT 475																																												
drTlr3	MGINKLAPGAPSSLGNL	SLNLSRNF	INQQLQGN	EPKLSN	IKIEDMS	INQGISL	TNA 474																																												
hsTLR3	KNKISKIESDAP	SWLGHLE	VDLGLNE	IGQELT	QENR	GLNIF	EIYLSYNKY--LQLTRN 474																																												
ccTlr3.1	SFIHVPTLR	TLKLRALK	KTLDME	PSFRPL	VNLT	ILDL	SNNTIANINAGL	LKGLYHLKV 535																																											
ccTlr3.2	SFIHVPTLR	TLKLRALK	KTLDME	PSFRPL	VNLT	ILDL	SNNTIANINAGL	LKGLYHLKV 535																																											
ccTlr3	SFIHVPTLR	TLKLRALK	KTLDME	PSFRPL	VNLT	ILDL	SNNTIANINAGL	LKGLYHLKV 535																																											
drTlr3	SFIHVPTLR	TLKLRALK	KTLDME	PSFRPL	VNLT	ILDL	SNNTIANINADL	LEGLYHLKV 534																																											
hsTLR3	SFALVPSLQRLM	LRVALKN	VDS	SPFP	PLNLT	ILDL	SNNTIANINADL	LEGLKLEI 534																																											
ccTlr3.1	LKMQHNL	LARL	NKTAN	PGGP	VFL	QDARK	LSFL	LDLYNGL	DEIPL	NALRGL	SEL	SLH 595																																							
ccTlr3.2	LKMQHNL	LARL	NKTAN	PGGP	VFL	QDARK	LSFL	LDLYNGL	DEIPL	NALRGL	SEL	SLH 595																																							
ccTlr3	LKMQHNL	LARL	NKTAN	PGGP	VFL	QDARK	LSFL	LDLYNGL	DEIPL	NALRGL	SEL	SLH 595																																							
drTlr3	VMMQHNL	LARL	NKTAN	PGGP	VFL	QDARK	LSFL	LDLYNGL	DEI	PPNA	FRG	SEL	SLH 594																																						
hsTLR3	LDLQHNH	LARL	NKHAN	PGGP	IY	FLKGL	SHLH	ILNLS	NGDEI	PVEV	PKDL	FLK	IDLG 594																																						
ccTlr3.1	GNLDDQLH	ASV	FDLQ	SLKYL	YLQK	NLITS	VQR	VTF	GVPL	SNL	TDL	YMDH	NP	DCT	CE	SI 655																																			
ccTlr3.2	GNLDDQLH	ASV	FDLQ	SLKYL	YLQK	NLITS	VQR	VTF	GVPL	SNL	TDL	YMDH	NP	DCT	CE	SI 655																																			
ccTlr3	GNLDDQLH	ASV	FDLQ	SLKYL	YLQK	NLITS	VQR	VTF	GVPL	SNL	TDL	YMDH	NP	DCT	CE	SI 655																																			
drTlr3	GNLDDQLH	ASV	FDLQ	SLKYL	YLQK	NLITS	VQR	VTF	GVPL	SNL	TDL	YMDH	NP	DCT	CE	SI 654																																			
hsTLR3	LNNLNTL	PASV	FMQV	SLKSL	NLQK	NLITS	VEK	VFG	PA	FR	NL	TEL	DM	FR	NP	DCT	CE	SI 654																																	
ccTlr3.1	LMPSEWLN	STNAS	IPG	FP	QSY	IC	NT	PNAY	PNS	VMD	FD	PL	S	CK	DM	TP	PKA	Y	IL	SS	TAVL 715																														
ccTlr3.2	LMPSEWLN	STNAS	IPG	FP	QSY	IC	NT	PNAY	PNS	VMD	FD	PL	S	CK	DM	TP	PKA	Y	IL	SS	TAVL 715																														
ccTlr3	LMPSEWLN	STNAS	IPG	FP	QSY	IC	NT	PNAY	PNS	VMD	FD	PL	S	CK	DM	TP	PKA	Y	IL	SS	TAVL 715																														
drTlr3	LMPSEWLN	STNAS	IPG	FP	QSY	IC	NT	PNAY	PNS	VMD	FD	PL	S	CK	DM	TP	PKA	Y	IL	SS	TAVL 714																														
hsTLR3	AMFVWV	NRET	HTNI	PEL	SSH	YL	CNT	PHY	HGF	IV	RL	FD	T	S	CK	D	S	APP	EL	FF	M	INT	S	ILL 714																											
ccTlr3.1	NLLFSA	FLVH	FQGR	I	QFF	N	M	VNR	M	L	S	L	K	D	E	S	I	T	E	G	R	Y	A	Y	A	I	H	S	A	D	R	E	K	S	V	E	R	S 775													
ccTlr3.2	NLLFSA	FLVH	FQGR	I	QFF	N	M	VNR	M	L	S	L	K	D	E	S	I	T	E	G	R	Y	A	Y	A	I	H	S	A	D	R	E	K	S	V	E	R	S 775													
ccTlr3	NLLFSA	FLVH	FQGR	I	QFF	N	M	VNR	M	L	S	L	K	D	E	S	I	T	E	G	R	Y	A	Y	A	I	H	S	A	D	R	E	K	S	V	E	R	S 775													
drTlr3	NLLFIS	FLVH	FQGR	I	QFF	N	M	VNR	M	L	S	L	K	D	A	R	V	T	E	G	R	F	O	F	D	A	I	H	A	G	E	D	K	S	V	E	R	S 774													
hsTLR3	IFIFIV	LLIH	FG	WR	IS	F	Y	N	N	V	S	V	H	R	V	L	G	-	F	K	E	I	D	R	Q	T	B	Q	F	E	A	A	I	I	H	A	Y	K	D	K	D	V	M	H 773							
ccTlr3.1	LPLR	--	DEK	F	N	F	P	L	E	R	D	A	I	P	G	F	S	O	L	D	T	I	V	E	N	M	G	S	R	K	I	L	F	V	I	T	E	M	L	K	D	P	N	C	R	F	K	A	H 833		
ccTlr3.2	LPLR	--	DEK	F	N	F	P	L	E	R	D	A	I	P	G	F	S	O	L	D	T	I	V	E	N	M	G	S	R	K	I	L	F	V	I	T	E	M	L	K	D	P	N	C	R	F	K	A	H 833		
ccTlr3	LPLR	--	DEK	F	N	F	P	L	E	R	D	A	I	P	G	F	S	O	L	D	T	I	V	E	N	M	G	S	R	K	I	L	F	V	I	T	E	M	L	K	D	P	N	C	R	F	K	A	H 833		
drTlr3	LPLR	--	DEK	F	N	F	P	L	E	R	D	A	I	P	G	F	S	O	L	D	T	I	V	E	N	M	G	S	R	K	I	L	F	V	I	T	E	M	L	K	D	P	N	C	R	F	K	A	H 832		
hsTLR3	FSSME	KD	Q	L	K	P	C	L	E	R	D	F	E	A	C	V	F	E	L	A	I	V	N	S	I	K	R	S	R	K	I	L	F	V	I	T	H	L	K	D	P	N	C	R	F	K	V	H 833			
ccTlr3.1	HALHVM	EN	DR	SL	IL	I	F	L	Q	D	V	T	D	Y	N	L	N	S	L	I	L	R	G	M	L	K	P	H	C	I	L	Y	W	P	L	H	R	R	I	P	A	F	H	O	R	L 893					
ccTlr3.2	HALHVM	EN	DR	SL	IL	I	F	L	Q	D	V	T	D	Y	N	L	N	S	L	I	L	R	G	M	L	K	P	H	C	I	L	Y	W	P	L	H	R	R	I	P	A	F	H	O	R	L 893					
ccTlr3	HALHVM	EN	DR	SL	IL	I	F	L	Q	D	V	T	D	Y	N	L	N	S	L	I	L	R	G	M	L	K	P	H	C	I	L	Y	W	P	L	H	R	R	I	P	A	F	H	O	R	L 893					
drTlr3	HALHVM	EN	DR	SL	IL	I	F	L	Q	D	V	T	D	Y	N	L	N	S	L	I	L	R	G	M	L	K	P	H	C	I	L	Y	W	P	L	H	R	R	I	P	A	F	H	O	R	L 892					
hsTLR3	HAVQ	A	T	E	N	L	D	S	I	L	A	V	F	L	E	R	I	P	D	Y	K	L	N	H	A	L	C	L	R	G	M	P	K	S	H	C	I	L	N	W	P	O	K	R	I	G	A	P	H	R	L 893
ccTlr3.1	CSAL	ST	N	R	V	N	904																																												
ccTlr3.2	CSAL	ST	N	R	V	N	904																																												
ccTlr3	CSAL	ST	N	R	V	N	904																																												
drTlr3	CSAL	ST	N	R	V	N	903																																												
hsTLR3	CSAL	ST	N	R	V	N	904																																												

Fig. 3.

Multiple alignment of amino acid sequences of carp, zebrafish and human Tlr3. Alignment was performed using the Tlr3 complete sequences of carp characterized in this work (ccTlr3.1 and ccTlr3.2), the East-Asian common carp sequence (ccTlr3) and the zebrafish (drTlr3) and human (hsTLR3) ones (Table 2). The putative signal peptide is in bold. Conserved cysteine residues important for LRRNT and LRRCT domains are indicated by an arrow above the alignment. Important residues for the recognition of the TLR3 ligand are boxed. LRRs are numbered and highlighted in grey. The transmembrane region is highlighted in black and TIR domain is underlined by a bold line. All mentioned domains are annotated above the alignment. Sequences are numbered on the right side

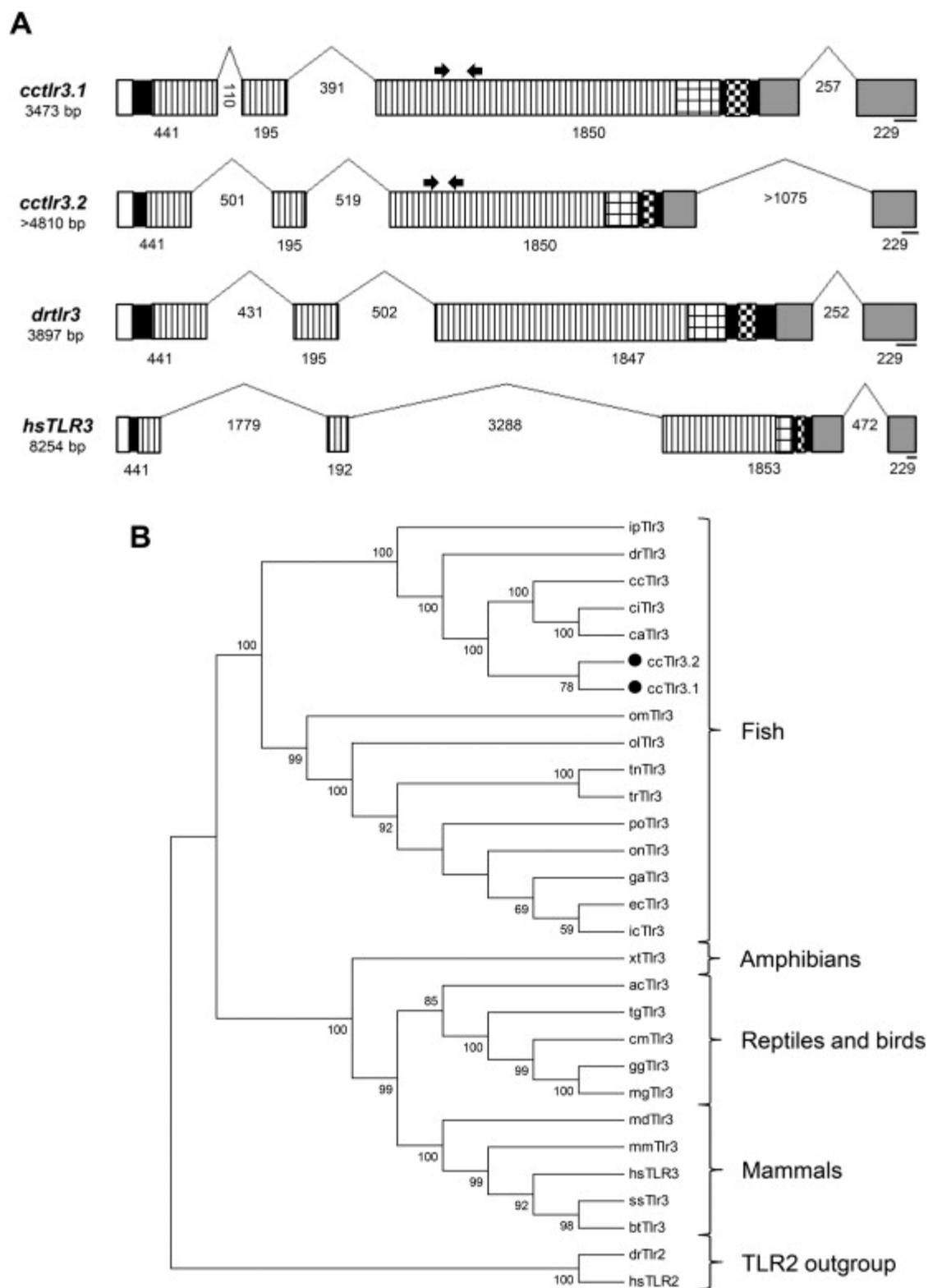


Fig. 4. Gene organization (A) and phylogenetic analysis (B) of carp Tlr3.1 and Tlr3.2. A) Diagram of carp (*cctlr3.1* and *cctlr3.2*), zebrafish (*drtlr3*) and human (*hsTLR3*) TLR3 genes. Exons and introns are represented by boxes and lines, respectively. For each gene, inside the boxes corresponding to exons, the signal peptides are indicated by a white box, the LRR regions by stripes, the LRRCTs by white squares pattern, the transmembrane regions by black and white

squares patterns and the TIR domains by a grey box. Lengths are scaled and represented in base pair numbers at the bottom of the corresponding introns and exons. B) Phylogenetic tree constructed using amino acid sequences of TLR3 from several organisms. The tree was constructed using the Neighbour-Joining algorithm in MEGA version 5 and bootstrapped 10,000 times. Bootstrap values above 50 are shown next to the branches. Mature protein sequences used for the construction of the phylogenetic tree are provided in Table 2. Zebrafish and human TLR2 sequences (Q9QUN7 and O60603 UniProtKB accession numbers, respectively) were used as an outgroup. Positions corresponding to carp Tlr3.1 and Tlr3.2 are indicated with (●).

### 3.4. Molecular characterization of carp tlr3 genes

Multiple sequence alignments of the deduced protein sequences (Fig. 3), including the one previously described for carp and those available from zebrafish and human, indicated that both carp Tlr3 molecules show typical features of Tlr3 family members such as one N-terminal leucine rich repeat (LRR) (LRRNT), 25 LRRs (as for human and catfish (*Ictalurus punctatus*) [21]), a C-terminal LRR (LRRCT), a transmembrane and a TIR domain. Amino acids in the extracellular domain of TLR3 noted as important for binding dsRNA ligands, i.e. Hys-17, -28, -86 and Asn-519 [22] and [23], were localized in the corresponding positions when compared to the Tlr3 sequences of other species (Fig. 3, boxed residues).

tlr3 genomic organization was also preserved in carp since predicted ORFs for both tlr3.1 and tlr3.2 were divided into 4 exons in both tlr3 genomic sequences, as occurs in human and zebrafish ( Fig. 4A). Nucleotide sequences corresponding to each Tlr3 domain were distributed within the exon in a distribution similar among all organisms compared. Moreover, such conservation was not only noted in the organization of the splicing sites but also in their location since the first two splicing sites were in frame but not the last one (-1) in all the organisms compared.

Phylogenetic analysis was performed based on amino acid sequences (excluding the signal peptide) of known Tlr3 molecules (Table 2). Bootstrap values were high in the entire phylogenetic tree, with the exception of the split corresponding to olive flounder and Nile tilapia Tlr3 molecules. Tlr3 sequences from fish and tetrapods clustered together with a bootstrap value of 100. Reptilian and avian TLR3 sequences branched separately to the mammalian group (99). The Tlr3 from the Western clawed frog diverged from the rest of tetrapods with a bootstrap value of 100, and zebrafish and human Tlr2 formed a distinct clade, proving to be suitable comparative outgroups.

### 3.5. tlr3 constitutive expression in carp

Tissue distribution of *tlr3.1* and *tlr3.2* mRNA basal levels in naive fish was investigated by RT-qPCR with specific primers that were designed for both genes in a region of the cDNA corresponding to the beginning of the third exon (see primers location in Fig. 3A). *tlr3.1* and *tlr3.2* basal expression levels for each sample were normalised relative to the *40s* gene. There were similar expression levels for *tlr3.1* and *tlr3.2* genes in gill, muscle, peripheral blood leucocytes (PBLs) and skin ( Fig. 5). *tlr3.1* was primarily expressed in gut, head and mid kidney, spleen and thymus, whilst *tlr3.2* was more expressed in gut, liver and PBLs. Lowest expression values were found in the muscle for both genes and in the liver for *tlr3.1*. In fact, liver is the only tissue in which *tlr3.2* is much more expressed than *tlr3.1*. None of these values were statistically significant since variability within individuals was high primarily due to *tlr3.1* ( Fig. 6).

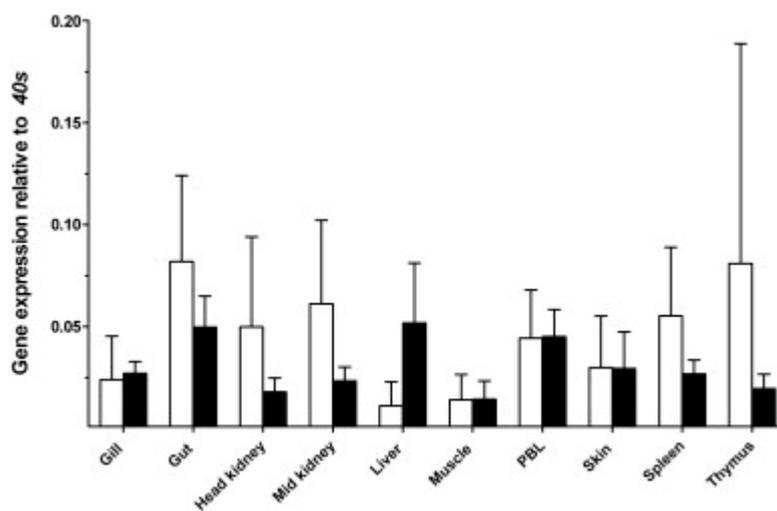


Fig. 5.

Expression analysis of carp *tlr3.1* and *tlr3.2* in various tissues of healthy carp by RT-qPCR. Total RNA from different organ tissues was isolated from five individuals. Expression was determined by RT-qPCR and expressed relative to *40s* gene expression. Data are represented as the mean changes  $\pm$  SD ( $n = 5$ ). □, *tlr3.1*; ■, *tlr3.2*

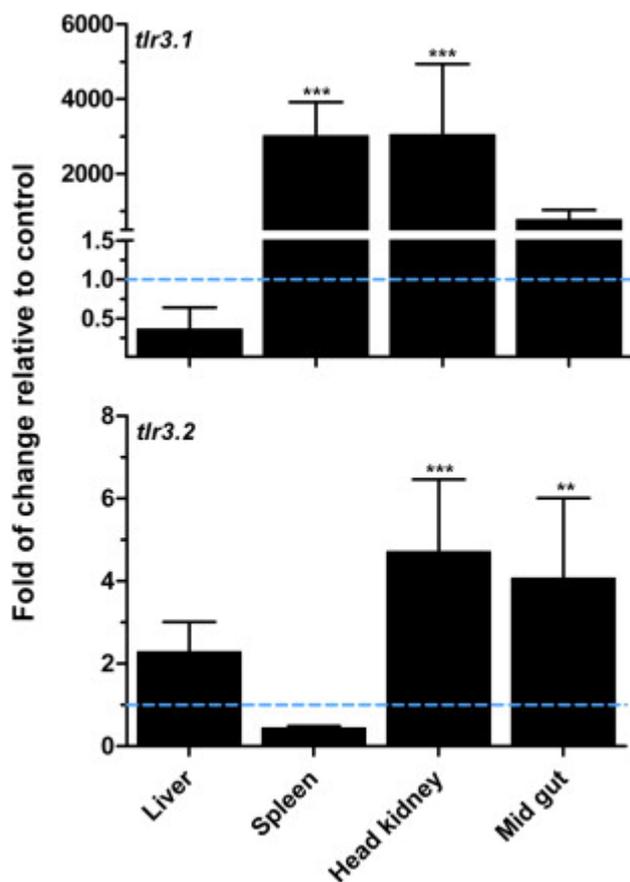


Fig. 6.

Effect of  $\beta$ -glucan-supplemented diet on the transcript levels of carp *tlr3.1* and *tlr3.2* in liver, spleen, head kidney and mid gut of carp 25 days after experimental feeding. Both *tlr3* genes transcript levels were analysed by RT-qPCR. Expression levels of  $\beta$ -glucan-supplemented feeding samples are reported as fold change relative to the control feeding samples (dashed line at y-value equal to 1). Significant differences when comparing  $\beta$ -glucan-supplemented diet groups with their associated control diet groups are represented by \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ) and \*\*\* ( $p < 0.001$ ). Mean  $\pm$  SD;  $n = 5$ .

### 3.6. Effect of $\beta$ -glucan-supplemented diets on the gene expression of carp *tlr3* genes

The expression of both *tlr3.1* and *tlr3.2* was significantly influenced after 25 days of feeding with  $\beta$ -glucan-supplemented diets ( $F = 52.29$ ;  $p < 0.0001$  and  $F = 23.86$ ;  $p < 0.0001$ , respectively). *tlr3.1*, was significantly up-regulated in spleen ( $3014.6 \pm 900.1$ ) and head kidney ( $3043.5 \pm 1893.3$ ) of  $\beta$ -glucan fed fish when compared to fish fed the control diet. *tlr3.2*, was also significantly up-regulated in  $\beta$ -glucan fed fish, although to a lesser extent than *tlr3.1*, up-regulation was found in head kidney ( $4.7 \pm 1.7$ ) and mid gut ( $4.1 \pm 1.9$ ) from  $\beta$ -glucan fed fish compared to their corresponding control groups ( $p < 0.001$ ;  $p < 0.01$ , respectively).

#### 4. Discussion

This work has shown that feeding a  $\beta$ -glucan-supplemented diet to carp affects the modulation of inflammation-related genes and genes involved in the interferon response, such as *mx*. After 25 days of feeding a  $\beta$ -glucan-supplemented diet, a general down-regulation of the expression of selected pro-inflammatory genes was observed. Down-regulation was significant in spleen, head kidney and, in particular, in the mid gut, confirming our previous results [11] and supporting our suggestion that  $\beta$ -glucans may prevent an acute and potential dangerous response in gut. Specific leucocyte types may however respond differently to  $\beta$ -glucan although, as it was shown recently by Pietretti et al. [24] when glucans from different sources increased the transcript levels of *il1 $\beta$* , *il6* and *il10* in head kidney derived macrophages. Conversely, the analysis of *mx* expression showed moderate up-regulation in liver and gut in the  $\beta$ -glucan fed group.

Poly(I:C) did not significantly affect the expression of *il1 $\beta$* , *il10*, *tnfa1*, *tnfa2*, *cxca* and *cxcb* genes in any of the tissues analysed 25 days after feeding whereas *mx* expression was significantly up-regulated in the poly(I:C)-injected group, and even greater in the  $\beta$ -glucan fed and poly(I:C)-injected group. These results are consistent with the few studies carried out on fish treated with  $\beta$ -glucan, where *mx* expression was increased and conferred protection to virus infection [9], [10] and [25]. For example, Kim et al. [9] observed that grass carp challenged with grass carp haemorrhage virus (GCHV) presented higher and prolonged levels of *mx* expression together with 3 times higher fish survival rates when injected with  $\beta$ -glucan derived from *Poria cocos* 85 mycelia 15 days prior to infection. In a more recent study [10], groups of Pacific herring (*C. pallasii*) that were fed with  $\beta$ -glucan containing diets for 40 days had approximately double the survival rates of the control groups after challenge with viral haemorrhagic septicaemia rhabdovirus (VHSV). In Atlantic salmon (*Salmo salar*) [25], gene expression of *mx* was determined in response to administration of PAMPs including  $\beta$ -glucan and it was shown that the latter had no effect on *mx* expression. This supports the observations made by Kim et al. [9] and the work described herein, which revealed that another stimuli such as virus [9] or poly(I:C), respectively, are needed to induce the expression of *mx*. In mammals, it was also shown that bone marrow derived-dendritic cells from mice were synergistically induced to produce *Ifn $\beta$*  when co-stimulated with zymosan (a cell wall extract from *Saccharomyces cerevisiae*) and poly(I:C) in vitro [26].

Since feed supplemented  $\beta$ -glucan was facilitating the induction of *mx* when treating with poly(I:C), the pathways that are activated by double-stranded RNA (dsRNA) or its chemical analogue, such as poly(I:C), might be promising candidates to investigate further as it is known from studies in mammals that these specific pathways have low convergence with other pathways that generally lead to transcription of pro-inflammatory cytokines such as those that were not regulated in our investigation [17] and [18]. Unfortunately the complexity of these pathways and the fact that many of their components have not been fully characterized in fish make selection of the molecule to study a difficult task. However, the fact that in our studies the *mx* expression levels were increased, it is likely that the molecule of interest would be at the beginning of that signalling cascade, and therefore, it could be a receptor. There are a number of possible receptors which bind exogenous RNA, for example

RIG-I [27], MDA5 [28], TLR3 [29], TLR7 [30], TLR22 [31], and the one that was selected to investigate in this study, TLR3.

TLR3 constitutes by itself a different subfamily of TLRs [32]. Together with TLR7, TLR3 is expressed in the endosomal compartment and complements TLR7 function by detecting dsRNA [29], however, localization of human TLR3 on the cell surface has also been reported [33]. TLR3 is one of the most studied TLRs and several homologues have been found and characterized in different fish species, i.e. zebrafish [34] and [35], rare minnow (*Gobiocypris rarus*) [36], grass carp [37], common carp [16], rohu (*Labeo rohita*) [38] and catfish [21]. TLR3 seems to play an important role in the antiviral defense in all vertebrates studies including fish where it has been reported that polymorphisms/haplotypes of *Tlr3* in grass carp have significant association with resistance/susceptibility to GCRV [39]. The previously reported common carp *tlr3* sequence [16] was found to be a different gene to the ones described here and despite further search within the current version of the carp genome, no more *tlr3*-like sequences were discovered. The presence of two *tlr3* genes in common carp is not surprising though since the genome of this organism is duplicated. In addition, efforts to amplify and sequence the previous carp *tlr3* sequence using specific primers in samples obtained from our fish failed (data not shown). Apart from the fact that the carp genome available is still incomplete, another explanation could be that different strains of carp were used in the studies carried out by Yang [16] and described here, i.e. European and East-Asian common carp (*C. carpio carpio* and *C. carpio haematopterus*, respectively). Although these diverged more than 500,000 years ago [40] both are commonly used in aquaculture and laboratory research.

*tlr3* gene expression has been detected in a wide range of tissues of different cyprinid species, with high levels of expression being recorded in skin, liver, gut, gills and spleen [16], [35], [36], [38] and [41]. Studies on the *tlr3* genes reported here have confirmed this differential gene expression in organs, for example carp *tlr3.1* is highly expressed in mid gut, head kidney and mid kidney, spleen and thymus whilst carp *tlr3.2* is predominantly expressed in mid gut, liver and PBLs. In addition, we have shown for the first time that this differential expression appears to be affected by diet as  $\beta$ -glucan-supplemented diets induced different gene expression levels of both carp *tlr3* paralogs in different tissues with the exception of the liver. *tlr3.1* showed the highest levels of expression after feeding with  $\beta$ -glucans. Interestingly, in a recent publication [42] it has been shown that in murine macrophage-derived RAW264.7 cells a significant increase in the transcript levels of other receptors involved in sensing viral components, i.e. RIG-I and MDA5, occurred 5 h after stimulation with  $\beta$ -glucan purified from *Aureobasidium pullulans*. Moreover, pre-treatment with this  $\beta$ -glucan compound conferred protection against the A/Puerto Rico/8/34 (PR8; H1N1) strain of influenza virus in vitro (RAW264.7 cells) and in vivo in orally administered mice [42]. Altogether, these results suggest an evolutionary conserved protective activity of  $\beta$ -glucans by means of increasing the sensitivity of treated cells to viral components.

In conclusion, we have found and described two *tlr3* genes present in European common carp and have shown that  $\beta$ -glucan-supplemented diets orally administered increased the transcript levels of both. We have also shown that there is a differential expression profile where for example *tlr3.1* was primarily up-regulated in the spleen, head kidney and mid gut whilst *tlr3.2* was up-regulated in the head kidney and mid gut. We suggest that the up-regulation of *tlr3* genes may explain why the injection of poly(I:C) specifically increased the expression levels of *mx* among other cytokines analysed, which is in concordance with reported increases of protection against viral pathogens conferred by  $\beta$ -glucan compounds in fish [9] and [10] and mammals [42]. Further assays on the expression levels of other virus sensing receptors in response to  $\beta$ -glucan and/or the functional groups from  $\beta$ -glucan with this activity are required to better understand the immunological activities of this immunostimulant and its role in virus defense.

### Acknowledgements

The research leading to these results has received funding from the European Community's Seventh Framework Programme [FP7/2007-2013] under grant agreement n° PITN-GA-2008-214505. Dr. Alberto Falco acknowledges the support for a postdoctoral research contract from the European Community's Seventh Framework Programme (SP3-People IEF under grant agreement n° PIEF-GA-2010-273391).

- [1] Dalmo RA, Bogwald J. Beta-glucans as conductors of immune symphonies. *Fish Shellfish Immunol* 2008;25(4):384e96.
- [2] Rodriguez I, Chamorro R, Novoa B, Figueras A. beta-Glucan administration enhances disease resistance and some innate immune responses in zebrafish (*Danio rerio*). *Fish Shellfish Immunol* 2009;27(2):369e73.
- [3] El-Boshy ME, El-Ashram AM, Abdelhamid FM, Gadalla HA. Immunomodulatory effect of dietary *Saccharomyces cerevisiae*, beta-glucan and laminaran in mercuric chloride treated Nile tilapia (*Oreochromis niloticus*) and experimentally infected with *Aeromonas hydrophila*. *Fish Shellfish Immunol* 2010;28(5e6):802e8.
- [4] Kumari J, Sahoo PK. Dietary immunostimulants influence specific immune response and resistance of healthy and immunocompromised Asian catfish *Clarias batrachus* to *Aeromonas hydrophila* infection. *Dis Aquat Org* 2006;70(1e2):63e70.
- [5] Kumari J, Sahoo PK. Dietary beta-1,3 glucan potentiates innate immunity and disease resistance of Asian catfish, *Clarias batrachus* (L.). *J Fish Dis* 2006;29(2):95e101.
- [6] Selvaraj V, Sampath K, Sekar V. Adjuvant and immunostimulatory effects of beta-glucan administration in combination with lipopolysaccharide enhances survival and some immune parameters in carp challenged with *Aeromonas hydrophila*. *Vet Immunol Immunopathol* 2006;114(1e2):15e24.
- [7] Sahoo PK, Mukherjee SC. The effect of dietary immunomodulation upon *Edwardsiella tarda* vaccination in healthy and immunocompromised Indian major carp (*Labeo rohita*). *Fish Shellfish Immunol* 2002;12(1):1e16.
- [8] Xueqin J, Kania PW, Buchmann K. Comparative effects of four feed types on white spot disease susceptibility and skin immune parameters in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis* 2012;35(2):127e35.
- [9] Kim YS, Ke F, Zhang QY. Effect of b-glucan on activity of antioxidant enzymes and Mx gene expression in virus infected grass carp. *Fish Shellfish Immunol* 2009;27(2):336e40.

- [10] Beaulaurier J, Bickford N, Gregg JL, Grady CA, Gannam AL, Winton JR, et al. Susceptibility of Pacific herring to viral hemorrhagic septicemia is influenced by diet. *J Aquat Anim Health* 2012;24(1):43e8.
- [11] Falco A, Frost P, Miest J, Pionnier N, Irnazarow I, Hoole D. Reduced inflammatory response to *Aeromonas salmonicida* infection in common carp (*Cyprinus carpio* L.) fed with beta-glucan supplements. *Fish Shellfish Immunol* 2012;32(6):1051e7.
- [12] Miest JJ, Falco A, Pionnier NP, Frost P, Irnazarow I, Williams GT, et al. The influence of dietary beta-glucan, PAMP exposure and *Aeromonas salmonicida* on apoptosis modulation in common carp (*Cyprinus carpio*). *Fish Shellfish Immunol* 2012;33(4):846e56.
- [13] Stolte EH, Savelkoul HF, Wiegertjes G, Flik G, Lidy Verburg-van Kemenade B. Differential expression of two interferon-g genes in common carp (*Cyprinus carpio* L.). *Dev Comp Immunol* 2008;32(12):1467e81.
- [14] Pionnier N, Falco A, Miest J, Frost P, Irnazarow I, Shrive A, et al. Dietary betaglucan stimulate complement and C-reactive protein acute phase responses in common carp (*Cyprinus carpio*) during an *Aeromonas salmonicida* infection. *Fish Shellfish Immunol* 2013;34(3):819e31.
- [15] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using realtime quantitative PCR and the 2(DDC(T)) method. *Methods* 2001;25(4):402e8.
- [16] Yang C, Su J. Molecular identification and expression analysis of Toll-like receptor 3 in common carp *Cyprinus carpio*. *J Fish Biol* 2010;76(8):1926e39.
- [17] Henkel CV, Dirks RP, Jansen HJ, Forlenza M, Wiegertjes GF, Howe K, et al. Comparison of the exomes of common carp (*Cyprinus carpio*) and zebrafish (*Danio rerio*). *Zebrafish* 2012;9(2):59e67.
- [18] Emanuelsson O, Brunak S, von Heijne G, Nielsen H. Locating proteins in the cell using TargetP, SignalP and related tools. *Nat Protoc* 2007;2(4):953e71.
- [19] Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, et al. Clustal W and Clustal X version 2.0. *Bioinformatics* 2007;23(21):2947e8.

- [20] Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007;24(8):1596e9.
- [21] Quiniou SM, Boudinot P, Bengten E. Comprehensive survey and genomic characterization of Toll-like receptors (TLRs) in channel catfish, *Ictalurus punctatus*: identification of novel fish TLRs. *Immunogenetics* 2013;65(7):511e30.
- [22] Alexopoulou L, Holt AC, Medzhitov R, Flavell RA. Recognition of doublestranded RNA and activation of NF- $\kappa$ B by Toll-like receptor 3. *Nature* 2001;413(6857):732e8.
- [23] Botos I, Liu L, Wang Y, Segal DM, Davies DR. The toll-like receptor 3: dsRNA signaling complex. *Biochim Biophys Acta (BBA) e Gene Regul Mech* 2009;1789(9):667e74.
- [24] Pietretti D, Vera-Jimenez NI, Hoole D, Wiegertjes GF. Oxidative burst and nitric oxide responses in carp macrophages induced by zymosan, MacroGard and selective dectin-1 agonists suggest recognition by multiple pattern recognition receptors. *Fish Shellfish Immunol* 2013;35(3):847e57.
- [25] Salinas I, Lockhart K, Bowden TJ, Collet B, Secombes CJ, Ellis AE. An assessment of immunostimulants as Mx inducers in Atlantic salmon (*Salmo salar* L.) parr and the effect of temperature on the kinetics of Mx responses. *Fish Shellfish Immunol* 2004;17(2):159e70.
- [26] Ainai A, Ichinohe T, Tamura SI, Kurata T, Sata T, Tashiro M, et al. Zymosan enhances the mucosal adjuvant activity of poly (I:C) in a nasal influenza vaccine. *J Med Virol* 2010;82(3):476e84.
- [27] Sarkar D, DeSalle R, Fisher PB. Evolution of MDA-5/RIG-I-dependent innate immunity: independent evolution by domain grafting. *Proc Natl Acad Sci U S A* 2008;105(44):17040e5.
- [28] Kato H, Takeuchi O, Mikamo-Satoh E, Hirai R, Kawai T, Matsushita K, et al. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med* 2008;205(7):1601e10.
- [29] Nishiya T, Kajita E, Miwa S, DeFranco AL. TLR3 and TLR7 are targeted to the

same intracellular compartments by distinct regulatory elements. *J Biol Chem* 2005;280(44):37107e17.

[30] Diebold SS. Recognition of viral single-stranded RNA by Toll-like receptors. *Adv Drug Deliv Rev* 2008;60(7):813e23.

[31] Matsuo A, Oshiumi H, Tsujita T, Mitani H, Kasai H, Yoshimizu M, et al. Teleost TLR22 recognizes RNA duplex to induce IFN and protect cells from birnaviruses. *J Immunol* 2008;181(5):3474e85.

[32] Pietretti D, Wiegertjes GF. Ligand specificities of Toll-like receptors in fish: indications from infection studies. *Dev Comp Immunol* 2013.

[33] Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, et al. Subcellular localization of Toll-like receptor 3 in human dendritic cells. *J Immunol* 2003;171(6):3154e62.

[34] Meijer AH, Gabby KSF, Medina RIA, He S, Bitter W, Ewa S-JB, et al. Expression analysis of the Toll-like receptor and TIR domain adaptor families of zebrafish. *Mol Immunol* 2004;40(11):773.

[35] Jault C, Pichon L, Chluba J. Toll-like receptor gene family and TIR-domain adapters in *Danio rerio*. *Mol Immunol* 2004;40(11):759e71.

[36] Su J, Zhu Z, Wang Y, Zou J, Hu W. Toll-like receptor 3 regulates Mx expression in rare minnow *Gobiocypris rarus* after viral infection. *Immunogenetics* 2008;60(3):195e205.

[37] Su J, Jang S, Yang C, Wang Y, Zhu Z. Genomic organization and expression analysis of Toll-like receptor 3 in grass carp (*Ctenopharyngodon idella*). *Fish Shellfish Immunol* 2009;27(3):433e9.

[38] Samanta M, Basu M, Swain B, Panda P, Jayasankar P. Molecular cloning and characterization of toll-like receptor 3, and inductive expression analysis of type I IFN, Mx and pro-inflammatory cytokines in the Indian carp, rohu (*Labeo rohita*). *Mol Biol Rep* 2013;40(1):225e35.

[39] Heng J, Su J, Huang T, Dong J, Chen L. The polymorphism and haplotype of TLR3 gene in grass carp (*Ctenopharyngodon idella*) and their associations with susceptibility/resistance to grass carp reovirus. *Fish Shellfish Immunol* 2011;30(1):45e50.

- [40] Zhou JF, Wu QJ, Ye YZ, Tong JG. Genetic divergence between *Cyprinus carpio* *carpio* and *Cyprinus carpio* *haematopterus* as assessed by mitochondrial DNA analysis, with emphasis on origin of European domestic carp. *Genetica* 2003;119(1):93e7.
- [41] Phelan PE, Mellon MT, Kim CH. Functional characterization of full-length TLR3, IRAK-4, and TRAF6 in zebrafish (*Danio rerio*). *Mol Immunol* 2005;42(9):1057e71.
- [42] Muramatsu D, Iwai A, Aoki S, Uchiyama H, Kawata K, Nakayama Y, et al.  $\beta$ Glucan derived from *Aureobasidium pullulans* is effective for the prevention of influenza in mice. *PLoS One* 2012;7(7):e41399.
- [43] Huttenhuis HB, Taverne-Thiele AJ, Grou CP, Bergsma J, Saeij JP, Nakayasu C, et al. Ontogeny of the common carp (*Cyprinus carpio* L.) innate immune system. *Dev Comp Immunol* 2006;30(6):557e74.
- [44] Forlenza M, Walker PD, de Vries BJ, Wendelaar Bonga SE, Wiegertjes GF. Transcriptional analysis of the common carp (*Cyprinus carpio* L.) immune response to the fish louse *Argulus japonicus* Thiele (Crustacea: Branchiura). *Fish Shellfish Immunol* 2008;25(1e2):76e83.
- [45] Forlenza M, Magez S, Scharsack JP, Westphal A, Savelkoul HF, Wiegertjes GF. Receptor-mediated and lectin-like activities of carp (*Cyprinus carpio*) TNF $\alpha$ . *J Immunol* 2009;183(8):5319e32.
- [46] Gonzalez SF, Buchmann K, Nielsen ME. Real-time gene expression analysis in carp (*Cyprinus carpio* L.) skin: inflammatory responses caused by the ectoparasite *Ichthyophthirius multifiliis*. *Fish Shellfish Immunol* 2007;22(6):641e50.