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Short communication

# *Ichthyophthirius multifiliis* infection induces massive up-regulation of serum amyloid A in carp (*Cyprinus carpio*)

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**Abstract**

A real time quantitative PCR (RQ-PCR) assay was developed for measurement of differential expression of the genes encoding the acute phase reactant serum amyloid A (SAA), transferrin (TF) and a C-type lectin molecule (CL) in skin, blood and liver from *Cyprinus carpio* following infection with the ectoparasite *Ichthyophthirius multifiliis.* Serum amyloid A and CL were constitutively expressed in all organs evaluated while TF transcripts were only detected in the liver. A dramatic up-regulation (1600 times) in the expression levels of SAA was observed in skin 36 h after the parasite infection. A similar increase in the number of RNA molecules encoding for SAA was observed in the liver. The CL expression was significantly down regulated in all the organs and no significant change was observed in the expression levels of the TF in the liver. These results indicate that SAA plays a major role in the acute phase response in fish infected with *I. multifiliis* and emphasize the importance of the fish skin as an active organ in response to an ectoparasite infection.


*Keywords:* Real-time PCR; Gene expression; Acute phase protein; Serum amyloid A; C-lectin; Transferrin; *Ichthyophthirius multifiliis*; Fish; Skin; Parasite

## 1. Introduction

The skin parasitic ciliate *Ichthyophthirius multifiliis* is able to induce immunity in fish following infection and although both antibody production and cellular factors may play a role in protection, other factors such acute phase reactants, may be of importance (Cross et al., 1994; Buchmann et al., 2001). The acute phase response (APR) is a systemic reaction to tissue injury and/or infection and involves the increased production of certain plasma proteins known as acute phase proteins (APPs). The great majority of these proteins are synthesized in the liver although sites of extra-hepatic synthesis have been described (Ramadori et al., 1985; Wang et al., 1998; Saeij et al., 2003; Larson et al., 2005). Serum amyloid A (SAA) is a highly conserved apolipoprotein identified in all vertebrate groups and is one of the major APPs in mammals (Uhlar and Whitehead, 1999). The serum concentration of this protein can increase by as much as 1000-fold during the APR (Steel and Whitehead, 1994). Serum amyloid A is likely to be a multifunctional molecule, involved in the modulation of lipid metabolism (Hoffman and Benditt, 1982; Banka et al., 1995), in the inflammatory process activating several immunological active cell types (Badolato et al., 2000) and acting as chemoattractant (Badolato et al., 1994, 1995; Su et al.,

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1999). Moreover, Hari-Dass et al. (2005) have recently demonstrated that SAA bind to a range of Gram-negative bacteria, which suggest that this molecule may be a pattern recognition protein. Serum amyloid A induction has been described in liver from arctic char following infection with *Aeromonas salmonicida* (Jensen et al., 1997), in carp after tissue injury (Fujiki et al., 2000; Huising et al., 2003) and after infection with the blood flagellate *Trypanoplasma borreli* (Saeij et al., 2003).

Transferrin (TF) is a monomeric glycoprotein responsible for the transport and delivery of iron to cells (Aisen and Listowsky, 1980) and it plays a key role in the activation and nitric oxide production of goldfish macrophages (Stafford and Belosevic, 2003; Stafford et al., 2004). Some studies have indicated that TF may be an APP in fish (Bayne and Gerwick, 2001). RT-PCR analysis has revealed that TF mRNA is constitutively expressed by goldfish liver cells and is induced following the activation of cultured goldfish macrophages (Stafford and Belosevic, 2003).

Lectins, a key component of the innate immune system, have broad carbohydrate specificity and the ability to bind to surfaces of various infectious antigens functioning directly as effectors to neutralize or clear pathogens (Russell and Lumsden, 2005). Buchmann (2001) suggested that host responses towards parasite infections in fish could involve carbohydrate-lectin binding. Most lectins appear to be constitutively produced but a few may be induced as part of the APR (Russell and Lumsden, 2005). Many C-type lectins have been cloned in fish, including carp (Fujiki et al., 2001; Savan et al., 2004) and a prominent constitutive expression of a C-lectin in heads kidney, intestine, liver and gills together with a moderate induction in haematopoietic organs of carp after LPS stimulation has been described (Savan et al., 2004).

The objective of the present study was the development of a real-time quantitative PCR (RQ-PCR) assay to measure differential real-time expression of SAA, CL and TF in skin, whole blood and liver from *Cyprinus carpio* during a period of 48 h after the infection with the ectoparasite *I. multifiliis*.

## 2. Materials and methods

### 2.1. Experimental infection and sampling collection

Common carp (*Cyprinus carpio* L.) were obtained from the Wageningen University fish culture facility ''De Haar Viseen'', Wageningen, The Netherlands. Fish (mean body weight 40 g) were acclimatized for 6 months before the infection procedure and were reared under pathogen free conditions at 23 °C in aerated recirculated local tap water. *I. multifiliis* was harvested from the skin of rainbow trout following the procedure described by Sigh et al. (2004). A total of 25 carps were transferred to a 100 l aquarium with a concentration of 10,000 parasites per fish. Parasite concentration was determined by counting the number of parasites by microscopy. Samples from blood, liver and skin were collected at 3, 12, 24, 36 and 48 h after the infection (three fish per time point). Sampling points were limited to 48 h in order to avoid opportunistic bacterial infections. Blood (30 μl) was collected from the caudal vein in heparinized syringes and immediately snap-frozen in liquid nitrogen in order to prevent RNA degradation. Skin samples (0.5 $cm^2$), aseptically dissected from the dorsal part of the fish, and liver samples (0.03 g) were immediately used for RNA extraction. Samples from four uninfected fish (time 0) were collected in a similar manner and used as negative controls (0 h).

### 2.2. RNA extraction and cDNA generation

Total RNA was extracted from the sampled organs using RNeasy Mini Kit columns (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. A total of 0.03 g of skin, liver and 25 μl of whole blood were disrupted in 600 μl lysis buffer (RLT) by sonication on ice (Sonicator Ultrasonic Liquid Processor Model XL 2020, Heat Systems, New York, USA). The concentrations and the quality of the isolated RNA were determined by measuring their absorbance at 260 nm (GeneQuant II RNA/DNA Calculator, Pharmacia Biotech, Hørsholm, Denmark). The integrity of the RNA samples was examined visually by electrophoresis in 1% agarose gel. First strand cDNA was synthesized using 1 μg of total RNA. Quantitec reverse transcription kit (QIAGEN, Hilden, Germany) was performed according to the manufacturer's instructions. The kit includes a DNAse treatment to eliminate genomic DNA contaminations and a RT primer mix consisting of an optimized blend of oligo-dT and random primers, which allows high cDNA yields from all regions of RNA transcripts. The entire reaction was placed at 42 °C for 30 min and subsequently inactivated at 95 °C for 3 min. Finally, the cDNA was diluted 20 times in ultrapure water to a final volume of 100 μl.

### 2.3. Real-time quantitative PCR

Two PCR primers sets specific for the molecules TF and CL were designed by using the Primer3 program