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Microarray analysis of gene expression in the blue catfish liver reveals early activation of the MHC class I pathway after infection with *Edwardsiella ictaluri*

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Abstract

The acute nature of disease outbreaks in aquaculture settings has served to emphasize the importance of the innate immune response of fish for survival and led to the recent identification and characterization of many of its components. Catfish, the predominant aquaculture species in the United States, is an important model for the study of the teleost immune system. However, transcriptomic-level studies of disease-related gene expression in catfish have only recently been initiated, and understanding of immune responses to pathogen infections is limited. Here, we have developed and utilized a 28K *in situ* oligonucleotide microarray composed of blue catfish (*Ictalurus furcatus*) and channel catfish (*Ictalurus punctatus*) transcripts. While channel catfish accounts for the majority of commercial production, the closely related blue catfish possesses several economically important phenotypic traits. Microarray analysis of gene expression changes in blue catfish liver after infection with Gram-negative bacterium *Edwardsiella ictaluri* indicated the strong upregulation of several pathways involved in the inflammatory immune response and potentially in innate disease resistance. A multifaceted response to infection could be observed, encompassing the complement cascade, iron regulation, inflammatory cell signaling, and antigen processing and presentation. The induction of several components of the MHC class I-related pathway following infection with an intracellular bacterium is reported here for the first time in fish. A comparison with previously published expression profiles in the channel catfish liver was also made and the microarray results extended by use of quantitative RT-PCR. Our results add to the understanding of the teleost immune responses and provide a solid foundation for future functional characterization, genetic mapping, and QTL analysis of immunity-related genes from catfish.

Keywords: Catfish; Genome; Microarray; Immunity; Infection; Disease; Transcriptome

1. Introduction

Studies of acute inflammation in mammals have often focused on the liver, a major target for proinflammatory cytokines and the center of the acute phase response (APR) component of innate immunity (Olivier et al., 1999; Gabay and Kushner, 1999). The APR in mammals is characterized by rapid and dramatic changes in the concentrations of a set of plasma proteins termed the acute phase proteins (APP). Acute phase proteins are an established diagnostic tool as early indicators of inflammation and disease (Schillaci and Pirro, 2006), and many are now known to play beneficial roles in mediating the complex inflammatory response and seeking to restore homeostasis following infection or injury (Gabay and Kushner, 1999).

Recent studies using genomic approaches in teleost fish have indicated that the liver is an important source of immune transcripts (Martin et al., 2006; Ewart et al., 2005) and mediates a powerful, conserved APR (Bayne et al., 2001; Bayne and Gerwick, 2001; Gerwick et al., 2007; Lin et al., 2007). Research on the APR and innate immune response of teleost fish has taken on new importance as a growing worldwide aquaculture industry faces disease outbreaks resulting in devastating losses (Meyer, 1991). The outcome of these acute infections in fish appears to depend heavily on non-specific immune responses (Camp et al.,

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2000). Characterization of the gene components and pathways of the teleost innate immune system, therefore, has become an area of particular focus in fish immunology, and has resulted in the identification of large numbers of cytokines, complement components, pathogen recognition receptors (PRR), and antimicrobial peptides from several aquaculture species (reviewed by Magnadottir, 2006). Genome-wide comparative studies of immune components and their expression after infection provide basic assessment and understanding of disease resistance relevant to both basic research and practical applications.

The advent of microarray technology has allowed fish researchers to conduct simultaneous expression analysis on tens of thousands of genes in organisms subjected to a variety of diseases and environmental conditions (Rise et al., 2004; Ewart et al., 2005; Meijer et al., 2005; Martin et al., 2006; MacKenzie et al., 2006; Purcell et al., 2006; Morrison et al., 2006; Matsuyama et al., 2007; Li and Waldbieser, 2006; Roberge et al., 2007; Gerwick et al., 2007). Microarray studies provide important context for the study of the immune response, connecting known immune components to a broader set of genes with similar expression patterns. As more microarray studies have been conducted using a variety of pathogens, host response profiles have begun to highlight a number of genes with conserved expression patterns following infection (Roberge et al., 2007), many of which likely play important yet unknown roles in teleost immunity. These genes serve as natural targets for further functional characterization, development as molecular biomarkers for disease progression, and genetic mapping.

Channel catfish (*Ictalurus punctatus*), the predominant aquaculture species in the United States, serves as an important model for the study of the teleost immune system (Peatman et al., 2005; Bao et al., 2006; Bengten et al., 2006). While channel catfish accounts for the majority of commercial production, the closely related blue catfish (*Ictalurus furcatus*) possesses several superior performace traits including disease resistance that have led to the production of an interspecific hybrid (channel female × blue male) recently available for commercial use (He et al., 2003; Chatakondi et al., 2005). Catfish production suffers heavy losses due to enteric septicemia of catfish (ESC), caused by the Gram-negative, intracellular bacterium *Edwardsiella ictaluri* (USDA, 2003; Hawke et al., 1981). ESC in its acute form is characterized by gastroenteric septicemia and, under artificial challenge, often results in heavy mortalities as early as 4 days after infection (Newton et al., 1989; Wolters and Johnson, 1994). Under experimental conditions, blue catfish had significantly higher resistance to ESC than either channel catfish or hybrid catfish (Wolters et al., 1996). To move toward the goal of eventual identification of the molecular determinants to this increased resistance in blue catfish, we have previously developed extensive EST resources for both catfish species (Li et al., in press) and have developed interspecific mapping panels (Liu et al., 2003a). In order to study the transcriptomic responses of catfish following infection with *E. ictaluri* and develop important immune-related markers for characterization and genetic mapping, we developed a 28K *in situ* oligonucleotide microarray composed of blue catfish and channel catfish transcripts (Peatman et al., 2007). By adding 7159 additional transcripts from blue catfish, the new 28K microarray included all transcripts known to date from the two catfish species and represents a large proportion of the catfish transcriptome. Here we describe the microarray-based transcriptomic profiling of the livers of blue catfish following infection with *E. ictaluri* and compare expression patterns with those seen in channel catfish (Peatman et al., 2007).

2. Materials and methods

2.1. Disease challenge

All procedures involving the handling and treatment of fish used during this study were approved by the Auburn University Institutional Animal Care and Use Committee (AU-IACUC) prior to initiation. Blue catfish (D&B strain) and channel catfish (Kansas Random strain) fry were artificially spawned at the hatchery of the Auburn University Fish Genetics Research Unit. At 1 week post-hatch, they were transferred to troughs or aquaria at the USDA ARS Aquatic Animal Health Unit in Auburn, AL or the Auburn University Fish Pathology wet lab. In both locations, the use of recirculating systems and municipal or well water sources ensured that the catfish fingerlings remained naïve to *E. ictaluri* during grow-out.

Catfish fingerlings were grown out for 4 months to approximately 15 cm before artificial bacterial challenges. Challenges followed established detailed protocols for ESC (Dunham et al., 1993; Baoprasertkul et al., 2004) with modifications. Water temperature before challenge was gradually brought to 27 °C over the course of 1 week by mixing in heated water. Fish were challenged in 30-l aquaria with 6 control and 8 treatment aquaria used. Sixty fish were placed in each aquaria, 30 channel and 30 blue catfish each. Aquaria were divided randomly into replicates of sampling timepoints—24 h control (3 aquaria), 24 h treatment (3 aquaria), 3 day control (3 aquaria), 3 day treatment (3 aquaria), and moribund (2 aquaria). *E. ictaluri* bacteria were cultured from a single isolate (MS-S97-773) and used in a small test infection of several catfish. Bacteria were re-isolated from a single symptomatic fish and biochemically confirmed to be *E. ictaluri*, before being inoculated into brain heart infusion (BHI) medium and incubated in a shaker incubator at 28 °C overnight. The bacterial concentration was determined using colony forming unit (CFU) per millilitre by plating 10 μl of 10-fold serial dilutions onto BHI agar plates. At the time of challenge, the bacterial culture was added to the aquaria to a concentration of 4×10^8 CFU/ml. Water was turned off in the aquaria for 2 h of immersion exposure, and then continuous water flow-through resumed for the duration of the challenge experiment. Control aquaria were treated similarly with an identical volume of sterile BHI. Fish were fed lightly during challenge. At 24 h and 3 day post-infection, 25 fish from each species were collected from each of the appropriate control and treatment aquaria, euthanized with MS-222 (300 mg/l), and their tissues and organs were collected and pooled. Pooling was carried out for several reasons: (1) Due to budgetary limitations, analysis of a large number of individuals was not possible. In order to provide a meaningful alternative, we feel that analysis of three pools would reduce variability between arrays to allow assessment of

global expression changes, providing a better picture than analysis of three individuals; (2) the sizes of the juvenile fish were relatively small and their tissue samples were needed for both microarray analysis, RT-PCR and real time RT-PCR validation for a large number of anticipated differentially expressed genes; (3) for the similar reasons, gene expression analysis was previously conducted using pooled samples in channel catfish, and the same experimental design would allow us to compare the results between the two species that differ greatly in resistance to ESC. For the studies described here, liver tissues were collected. Samples were flash frozen in liquid nitrogen during collection and stored at −80 °C until RNA extraction. Procedures were the same for moribund fish except that they were collected over the course of the challenge as they lost equilibrium in the water. During the challenge, symptomatic treatment fish and control fish were collected and confirmed to be infected with *E. ictaluri* and pathogen-free, respectively, at the Fish Disease Diagnostic Laboratory, Auburn University.

2.2. Oligonucleotide microarray construction

A high-density *in situ* oligonucleotide microarray was constructed as previously described (Peatman et al., 2007). A total of 28,518 sequences from channel catfish and blue catfish were used to construct the microarray. A record of all sequences contained on the 28K catfish microarray, their putative identities, expression values on each slide, and other experimental data have been deposited in the NCBI Gene Expression Omnibus (GE0; http://www.ncbi.nlm.nih.gov/geo/) accessible through the GEO series accession number GSE6350.

Nimblegen Systems produced the physical microarrays utilizing an *in situ* maskless array synthesis technology to synthesize 24 base pair (24mer) oligos on the surface of the microarray slides (Singh-Gasson et al., 1999; Nuwaysir et al., 2002). At least twelve 24-mer oligonucleotides were designed for each EST present on the microarray. Half of these were perfect-match (PM) oligos selected along the length of the sequence, while the other half were duplicates of the first but with two mismatched (MM) bases at the #6 and #12 positions.

2.3. RNA extraction and labeling

Blue catfish liver control and treatment replicates at the 3 day timepoint were used for initial microarray analysis, and the 24 h and moribund timepoints were utilized for additional QRT-PCR analysis. Accordingly, the pooled livers ($n = 25$) from each replicate (three control replicates, three treatment replicates) were ground in liquid nitrogen by mortar and pestle to a fine powder and thoroughly mixed. Approximately 30 mg of tissue powder was homogenized in Buffer RLT Plus by passing the lysate several times through a 20-gauge needle fitted to a syringe according to the protocol of the RNeasy Plus Mini Kit (Qiagen, Valencia, CA). Samples were filtered through a genomic DNA elimination column included in the RNeasy Plus kit. Following the manufacturer's instructions, approximately 35 μg of total RNA was obtained from each extraction. RNA quality and concentration was checked by spectrophotometer analysis and gel electrophoresis. All extracted samples had an A260/280 ratio of greater than 1.8, and were diluted to 1 μg/μl. RNA labeling, array hybridization, washing, and scanning were carried out by NimbleGen Systems, Inc. (Madison, WI).

Briefly, total RNA was converted to double-stranded cDNA using a SuperScript II cDNA synthesis kit (Invitrogen) and an oligo-dT primer containing the T7 RNA polymerase promoter. *In vitro* transcription (IVT) was carried out to produce biotin-labeled cRNA from cDNA using the MEGAscript T7 kit (Ambion, Austin, TX). Briefly, 3 μl double-stranded cDNA was incubated with 7.5 mM ATP and GTP, 5.6 mM UTP and CTP, 1.875 mM bio-11-CTP and bio-16 UTP (Enzo) and 1× T7 enzyme mix in 1× reaction buffer for 16 h at 37 °C. The cRNA was then purified using an RNeasy mini kit (Qiagen, Valencia, CA). Before hybridization, cRNA was fragmented to an average size of 50–200 bp by incubation in a buffer of 100 mM potassium acetate, 30 mM magnesium acetate, and 40 mM tris-acetate for 35 min at 94 °C. Fragmentation was measured using a Bioanalyzer 1000 (Agilent Technologies, Palo Alto, CA).

2.4. Hybridization and image acquisition

The oligonucleotide microarrays were prehybridized with a solution of 2× MES hybridization buffer (100 mM 2-morpholinoethanesulfonic acid, 1.0 M Na^+, 20 mM EDTA, 0.01% Tween 20), 50 μg of herring sperm DNA, and 250 μg of acetylated bovine serum albumin (BSA) at 45 °C for 15 min followed by hybridization with 10 μg of denatured and fragmented cRNA per microarray, 3.5 μl of CPK6 control oligo, 35 μg of herring sperm DNA, 175 μg of acetylated BSA, and 2× MES buffer at 45 °C for 16–20 h with constant rotation. After hybridization, the microarrays were washed twice with nonstringent buffer (6× SSPE, 0.01% Tween 20) at room temperature followed by two stringent washes (100 mM MES salt and free acid solution, 0.1 M Na^+, 0.01% Tween 20) at 45 °C for 15 min each. After a final 1 min rinse with nonstringent buffer, the arrays were placed into a 1× stain solution (100 mM MES, 1 M Na^+, 0.05% Tween 20, 50 mg/ml BSA, and 1 μg/μl Cy3-streptavidin) at room temperature for 15 min, agitating every few minutes. The microarrays were removed from the stain solution and placed in fresh nonstringent wash buffer for 1 min. They were then placed into Nimblegen's proprietary final wash buffer for 30 s, and then immediately dried under a stream of argon gas and scanned using an Axon GenePix 4000B scanner (Molecular Devices, Union City, CA) at 5-μm resolution. Six microarrays were used in the experiment, corresponding to the three control pools and three treatment pools of RNA.

2.5. Microarray and bioinformatic data analysis

After extraction of data from raw images using the NimbleScan software (Nimblegen, Inc.), gene calls (a single expression intensity value based on the multiple probes for each gene) were generated using the Robust Multichip Average (RMA) algorithm (Irizarry et al., 2003) which takes into account only the perfect match oligos. RMA takes a background adjustment on the raw intensity scale, carries out quantile normalization

(Bolstad et al., 2003), takes the log 2 of the normalized background adjusted PM values, and then uses a linear model to estimate expression values on the log scale. Both programs are available in the affy package of the Bioconductor project (http://www.bioconductor.org). The normalized intensity values from the three control sample microarrays and the three ESC-infected sample microarrays were then analyzed using the Significance Analysis of Microarrays method (Tusher et al., 2001) in the two-class unpaired mode (SAM version 2.23A: http://www-stat.stanford.edu/~tibs/SAM/). SAM assigns each gene a relative difference score based on its change in gene expression relative to the standard deviation of replicate measurements for that gene. For genes falling above an adjustable threshold, permutations of repeated measurements are used to determine a percentage of genes identified by chance, the false discovery rate (FDR; Benjamini and Hochberg, 1995), which is presented as a *q*-value for each gene in the final list of significant genes (Tusher et al., 2001; Pawitan et al., 2005). The *q*-value, therefore, reflects the variability present in the data set for a given gene. A list of significant genes with at least two-fold expression changes between treatment and control and a global false discovery rate of <10% was produced, and sorted according to fold-change. BLASTX searches were conducted for each sequence on the list. In order to provide insight into the potential identities of the differentially expressed genes, a less stringent cutoff *E*-value (0.0001) was used, and the top informative hit was noted. Those sequences possessing no significant similarity to peptide sequences within the *nr* database were clustered to identify and remove any redundant (blue-channel) sequences. When a putative gene identity was shared by multiple sequences, further sequence analysis was carried out to remove redundancies. In cases where blue catfish and channel catfish orthologues of the same gene were differentially expressed, the blue catfish transcript was selected to represent this gene. If multiple blue catfish transcripts were found to be derived from the same gene, the transcript with the lowest *q*-value was chosen. In cases where two differentially expressed transcripts shared the same putative gene identity but likely represented paralogues, both transcripts were kept on the unique list.

Gene annotation was carried out using the BLAST2GO program (Conesa et al., 2005), a Java application which enables gene ontology (GO) based data mining on sequences for which no GO annotation is currently available. FASTA-formatted sequences representing the unique upregulated transcripts were uploaded to the program and BLASTX searches carried out. GO terms associated with the hits were retrieved by the program and queries were annotated based on hit similarity and GO evidence codes (EC). Some query sequences were not annotated by the BLAST2GO process due to uninformative top BLAST hits. These sequences were therefore searched against the UniProt database (http://www.pir.uniprot.org/) and manually annotated in the BLAST2GO program where appropriate.

2.6. QRT-PCR analysis

The RNA prepared for microarray analysis was also used for confirmation of the blue catfish microarray expression patterns of selected genes of interest by QRT-PCR. Additionally, RNA samples from the 24 h and moribund timepoints from both blue catfish and channel catfish were used to measure expression of selected genes. The three control pools and three treatment pools of RNA, each representing 25 fish, were utilized for each tested gene, both at the 3 days and 24 h timepoints. Only a single pooled sample was available for blue catfish and channel catfish moribund samples. One-step quantitative RT-PCR was carried out using a LightCycler 1.0 instrument (Roche Applied Science, Indianapolis, IN) and the Fast Start RNA Master SYBR Green I reagents kit (Roche Applied Science) following manufacturer's instructions with modifications. Briefly, all QRT-PCR reactions were performed in a 10 μl total reaction volume (9 μl master mix and 1 μl (100 ng) RNA template). The master mix contained 4.3 μl H_2O, 0.6 μl $Mn[OAc]_2$, 0.3 μl of each primer (0.1 μg/μl), and 3.5 μl of the SYBR Green mix. The same cycling parameters were used for all tested genes: (i) reverse transcription, 20 min at 61 °C; (ii) denaturation, 30 s at 95 °C; (iii) amplification repeated 50 times, 5 s at 95 °C, 5 s at 58 °C, 20 s at 72 °C; (iv) melting curve analysis, 5 s at 95 °C, 15 s at 65 °C, then up to 95 °C at a rate of 0.1 °C/s; (v) cooling, 30 s at 40 °C. Primers were designed using either the FastPCR program (http://www.biocenter.helsinki.fi/bi/Programs/fastpcr.htm) or the PriFi sequence alignment and primer design program (Fredslund et al., 2005; http://cgi-www.daimi.au.dk/cgi-chili/PriFi/main). Primer names, accession numbers, and sequences are listed in Table 1. The 18S ribosomal RNA gene was selected for normalization of expression levels due to its stable expression levels over a variety of tissues and treatment conditions in fish including catfish (Jorgensen et al., 2006; Murdock et al., 2006). The triplicate fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (C_t) values, were compared and converted to fold differences by the relative quantification method (Pfaffl, 2001) using the Relative Expression Software Tool 384 v. 1 (REST) and assuming 100% efficiencies. Expression differences between control and treatment groups were assessed for statistical significance using a randomization test in the REST software. The mRNA expression levels of all samples were normalized to the levels of 18S ribosomal RNA gene in the same samples. Expression levels of 18S were constant between all samples (<0.30 change in C_t). Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis.

3. Results

3.1. Microarray analysis of blue catfish expression following challenge

The artificial challenge with virulent *E. ictaluri* resulted in mortality of infected fish beginning at day 5 after exposure. No control fish manifested symptoms of ESC and randomly selected control fish were confirmed to be negative for *E. ictaluri* by standard diagnostic procedures. Dying fish manifested behavior and external signs associated with ESC infection including hanging in the water column with head up and tail down and petechial

Table 1
Primers used for QRT-PCR validation and additional expression profiling (5′–3′)

Gene	Accession	Forward	Reverse
SCYA106	AY555503	GTCTCTTGGAGAGCAAGCACTG	CATCAGCTCTCTGACCCAGTCG
Intelectin	CF970955	TCGGAGCTGCCGGGACATCAAGGAG	CCCTGCTCGCTTGACCAGCGATCAC
LAMP3	TC7925	TCTGAGGTGTTTCTGAACCAGG	CCATGCCGAACCTGGCCATCAC
Hemopexin	CK406564	TGACCGCTGTGAGGGCATCGAG	TGTGCATGCGGAAGGCTGCATCCA
MMP13	CF972078	GCTGGCATCGGTGGAGACGCTC	ACGTTGGAATGCTCAAGGCCTG
LY6E2	CK404046	GGACACGTCATGACGAGCTCTG	CCTTCAGGCACAGGCAGATGAC
SelH	CB940790	GGGGCAAAGCGCAAGGCAGACG	CGCACAGCATCGGCATTCCGCC
B2M	TC6716	CCGACCTGGCCTTTGAGAAGGG	CGCCAGCTGGAATGAAGCCCAG
CEBP	TC7043	CGCGCCGCTGACCGAGCGCG	GAGCGCATGTCGAAGCCCCCCG
PA28	CK409611	CAGCTGCAGATCCCCAGGATTG	GCCACAGCATCTCCTCTCTCGC
MHC1α	TC9859	GGAGAAGATTTCATCAGTCTGG	TCCTCTCCAGAGTCTCTCTGCC
Pentraxin	CK408173	TGTCTGCGTGCCTTCTCCGACC	CCTTCCCACGTAGCGCACACTG
18S rRNA	BE469353	TGCGCTTAATTTGACTCAACAC	CGATCGAGACTCACTAACATCG

SCYA106, CC chemokine SCYA106; LAMP3, lysosomal-associated membrane protein 3; MMP13, matrix metalloproteinase 13; LY6E2, lymphocyte antigen 6 complex, E2; Sel H, selenoprotein H; B2M, beta-2 microglobulin; CEBP, CCAAT/enhancer binding protein (C/EBP), beta; PA28, proteasome activator PA28 subunit, alpha; MHC1α, MHC class I alpha chain.

hemorrhages along their ventral surface. *E. ictaluri* bacteria were successfully isolated from randomly selected treatment fish.

The expression profile of blue catfish liver 3 days after infection with *E. ictaluri* were compared with the levels seen in uninfected blue catfish. After data normalization and gene expression calculation in RMA (Irizarry et al., 2003), the resulting expression intensity values were analyzed in SAM (Tusher et al., 2001). The criteria of a two-fold or greater change in expression and a global false discovery rate (FDR) of 10% were chosen to determine upregulated or downregulated genes in the infected replicates. Using these criteria, 126 transcripts were significantly upregulated, and 5 were significantly downregulated (Supplemental Tables 1 and 2). Of the 126 upregulated catfish transcripts, 98 of these are believed to represent unique genes. The redundant transcripts resulted either from blue and channel orthologues of the same gene or multiple transcripts from non-overlapping regions of a large gene being included on the microarray.

Of the 98 unique, significantly upregulated transcripts after infection, putative identities could be determined for 76 based on sequence similarity by BLASTX searches while 22 had no significant similarity to protein sequences in the *nr* database (cut-off *E*-value = 0.0001; Table 2 and Supplemental Table 3). Gene ontology annotation was carried out using the BLAST2GO program (Conesa et al., 2005) and by searches against the UniProt database. Annotation results are summarized in Fig. 1. GO terms were ultimately assigned to 70 sequences. Analysis of specific (>level 6) GO terms for biological processes assigned to the upregulated transcripts revealed that many shared putative functions related to ion homeostasis and immune responses. Other large categories included those related to protein modification, folding, and transport (Fig. 2). The 76 sequences with significant BLASTX hits were divided into similar broad functional categories in Table 2. The majority of the upregulated transcripts were grouped into six categories each with at least five members—acute phase response; complement activation; metal ion binding/transport; immune/defense response; protein processing, localization, folding; and protein degradation.

3.2. Conserved acute phase response in blue catfish and induction of defense/immune related transcripts

A conserved acute phase response was evident in the significantly upregulated catfish transcripts following infection. At least 20 of the 98 unique, annotated transcripts represented acute phase proteins (APP; Bayne et al., 2001), divided among the acute phase response, complement activation and metal ion binding/transport categories in Table 2 (bold names). Transcripts falling within these categories were among the most highly upregulated following ESC infection. An active complement response to infection was observed, with three forms of complement C3 upregulated along with C4 and members of the membrane attack complex (C7, C9). The complement regulatory protein factor H was also strongly upregulated (>14-fold). Genes involved in iron binding and transport were strongly induced following infection. These included intelectin, haptoglobin, hemopexin/warm-temperature-acclimation-related 65 kDa protein, ceruloplasmin, and transferrin. Other upregulated APP included pentraxin (serum amyloid P-like), fibrinogen, and angiotensinogen (Table 2).

Upregulated transcripts with established roles in immune responses comprised another large functional category, indicating that active immunosurveillance, immune signaling, and immune cell activation were occurring in the infected blue cat-

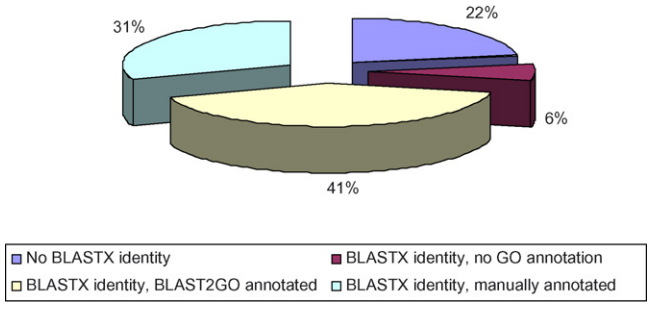

Fig. 1. Analysis and gene ontology (GO) annotation of 98 unique, significantly upregulated transcripts in blue catfish.

Table 2
Catfish transcripts upregulated in the blue catfish liver following ESC infection

Functional classification	Accession	Putative Id	Fold change	*q*-Value (%)
Acute phase response[a]	CK407841	**Fibrinogen gamma polypeptide**	5.7	5.63
	CF971953	**Fibrinogen, beta chain isoform 4**	5.6	9.82
	TC8490	**Fibrinogen, B beta polypeptide**	4.2	7.00
	CK408173	**Pentraxin (serum amyloid P-like)**	4.1	8.70
	CF971852	**Fibrinogen alpha chain**	3.8	9.82
	BM438634	**Angiotensinogen**	2.4	9.82
Complement activation	EE993177	**Complement factor H precursor**	14.5	0.00
	EE993354	**Complement component 7 precursor**	10.1	9.18
	CV997126	**Complement C3**	6.1	8.97
	TC7660	**Complement component C9**	5.2	8.70
	EE993343	**Complement C4**	3.9	5.63
	CV987901	**Complement C3-H1**	3.2	5.63
	CK406493	**Complement C3-Q1**	3.1	5.63
Metal ion binding/transport	CF970955	**Intelectin**	48.6	9.82
	CF971897	**Intelectin 2**	37.7	5.63
	CK408483	**Haptoglobin precursor**	20.4	9.82
	CF971550	**Warm-temperature-acclimation-related-65 kDa (Hemopexin-like)**	12.3	8.70
	CK406564	**Hemopexin precursor**	9.7	5.63
	CK408512	Solute carrier family 31 (copper transporters), member 1	7.1	8.70
	CF971219	**Ceruloplasmin**	4.7	9.18
	CK408666	**Transferrin**	4.1	9.82
	CK418197	Cytochrome P450 3A	3.6	7.00
Immune/defense response	AY555503	CC chemokine SCYA106	105.1	9.18
	TC7475	CC chemokine SCYA113	12.5	8.97
	TC7925	Lysosomal-associated membrane protein 3 (CD208)	5.7	9.82
	TC9859	MHC class I alpha chain	4.7	8.70
	CF972295	Thioredoxin	3.8	9.82
	BM438717	Tumor necrosis factor, alpha-induced protein 9 isoform 2	3.4	7.00
	CF971576	Tumor necrosis factor, alpha-induced protein 9	3.1	9.18
	EE993326	CD63	2.6	9.82
	TC7043	CCAAT/enhancer binding protein (C/EBP), beta	2.6	7.00
	CK404046	Lymphocyte antigen 6 complex, locus E ligand isoform 2	2.5	9.82
	CK401855	MHC class I alpha chain	2.5	5.63
	TC6716	Beta-2 microglobulin precursor	2.3	9.82
	TC8645	Lectin, galactoside-binding, soluble, 9 (galectin 9)-like 1	2.3	9.82
	CV989503	CXCL14	2.2	9.82
Protein processing, localization, folding	TC9330	ER-resident chaperone calreticulin	4.8	5.63
	CK407547	Protein disulfide isomerase associated 4 (Erp72)	4.7	5.63
	TC7345	Fetuin-B precursor (IRL685)	3.3	9.18
	CK411755	Integral membrane protein 1 (STT3)	3.0	5.63
	CK405569	Translocon-associated protein beta (SSR2)	2.8	9.82
	BM438439	Signal sequence receptor, alpha (SSR1)	2.6	8.70
	TC8981	FK506 binding protein 2 (PPIase)	2.6	5.63
	CK415655	Endoplasmin (GRP94)	2.4	9.82
	TC9170	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase (48 kDa)	2.3	9.82
	CK406459	Dnajb11 protein (ERdj3)	2.1	9.18
Protein degradation	CK409611	Proteasome activator PA28 subunit, alpha	3.2	9.82
	TC9755	Proteasome (prosome, macropain) subunit, alpha type, 3	3.1	9.18
	CF972078	Matrix metalloproteinase 13	2.8	5.63
	TC6963	Proteasome activator PA28 subunit, beta	2.3	9.82
	TC7388	Proteasome (prosome, macropain) subunit, beta type, 6	2.2	9.82
Miscellaneous	CK406362	Microfibrillar-associated protein 4	27.1	5.63
	CK405246	Methionine adenosyltransferase II alpha subunit	8.3	9.82
	CK407588	Catechol-*O*-methyltransferase domain containing 1	7.9	8.70
	BM438689	Microfibrillar-associated protein 4	7.5	8.97
	CK408412	Apolipoprotein Apoa4 protein	4.1	8.70
	CK408535	Microfibrillar-associated protein 4	4.1	5.63
	CK405317	Beta-actin	4.0	5.63
	TC6790	Atpase H+ transporting lysosomal vacuolar proton pump	3.0	5.63
	TC7903	Armet protein	2.9	9.18

Table 2 (*Continued*)

Functional classification	Accession	Putative Id	Fold change	*q*-Value (%)
	CK406132	Alpha-1-tubulin	2.9	9.82
	CK404348	H2A histone family, member V, isoform 1	2.7	8.97
	CK401686	WW domain binding protein 2	2.5	9.82
	CK424035	Neuronal myosin light chain kinase 1	2.5	8.97
	TC9648	Amyloid beta (A4) precursor protein-binding, family B, member 2	2.4	9.82
	CV987949	Fructose-1,6-bisphosphatase 1, like	2.3	8.70
	CV990995	Coactosin-like 1	2.3	9.82
	CV995433	Sterol regulatory element-binding protein 2 (SREBP-2)	2.2	9.82
	CV995162	Alcohol dehydrogenase 5	2.2	9.82
	CK407421	Glutaredoxin (thioltransferase)	2.0	9.82
Unknown	CF971597	Hypothetical protein XP_683888	20.6	9.82
	CK401799	MGC68649 protein	3.8	5.63
	CK407596	LOC407646 protein	3.7	9.82
	TC6930	LR8 protein	2.3	9.18
	CK406492	Similar to family with sequence similarity 46, member A isoform 1	2.3	0.00
	TC9161	Hypothetical protein LOC641319	2.0	5.63

Accession, GenBank accession number or TIGR consensus number of the sequence on the microarray; Putative Id, top informative BLASTX hit; *q*-value, false-discovery rate for the particular gene; Functional classification, putative functions assigned based on gene ontology annotations and Uniprot entries of top BLAST hits.

[a] Acute phase response encompasses bold transcripts included in other categories. Transcripts were grouped into broad functional categories of at least five unique transcripts. Some transcripts could be classified into multiple categories but are listed under the most specific category. Gene names appearing more than once should represent paralogues. Genes were sorted by fold change within functional categories.

fish liver. These included the most highly upregulated transcript observed, CC chemokine SCYA106, at 105-fold. Other induced immune genes included two types of MHC class I alpha chain, CD63, CC chemokine SCYA113, CXCL14, and galectin 9, among others (Table 2).

3.3. Induction of transcripts for protein processing, localization, folding and degradation after ESC infection

A large number of transcripts with functions in protein modifications and degradation were upregulated in the liver following

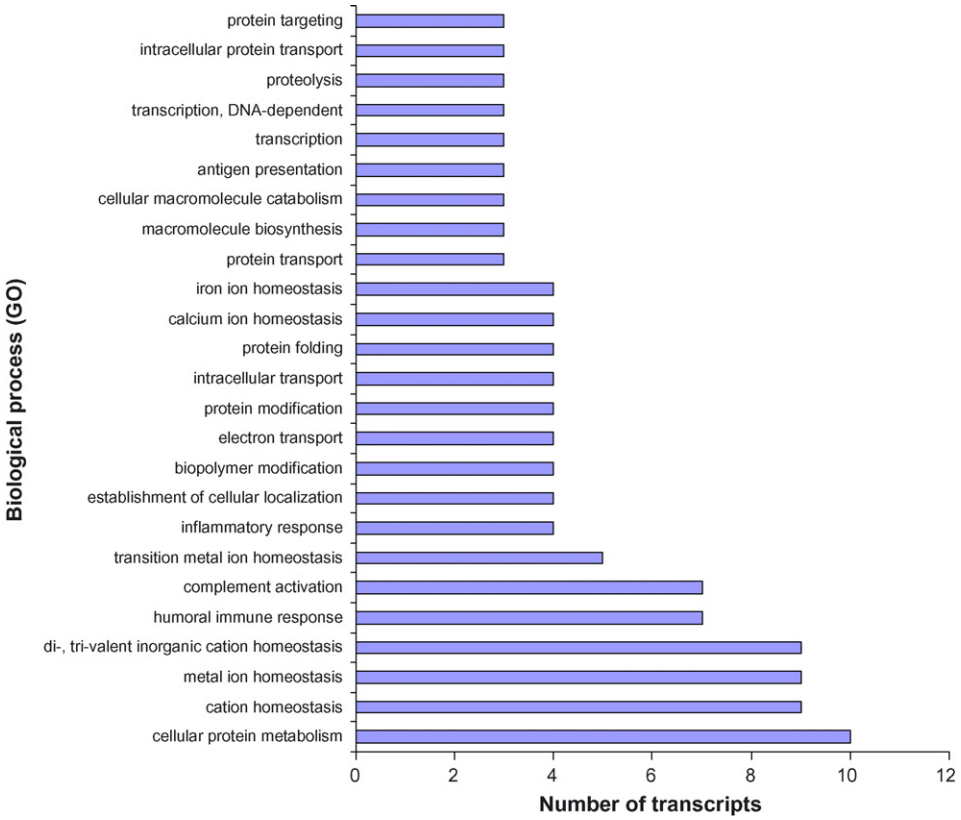

Fig. 2. Significantly upregulated transcripts assigned to lower level (>6) GO biological process categories. Fifty three sequences had a biological process GO term.

Table 3
Unique, significantly downregulated catfish transcripts in blue catfish liver after ESC infection

Accession	Putative identity	Fold change	*q*-Value	Function
CK417600	No significant similarity	−2.5	9.57	NA
TC9079	Anaphase promoting complex subunit 13	−2.0	9.57	Cell cycle
CB940790	Selenoprotein H	−2.0	9.57	Stress or defense response
TC9060	No significant similarity	−2.0	9.57	NA
CF971521	Selenoprotein P, plasma, 1b	−2.0	9.57	Stress or defense response

Accession refers to the GenBank accession number or TIGR consensus number of the sequence on the microarray. Putative Id is the top informative BLASTX hit. *q*-Value is the false-discovery rate for the particular gene. Function is putative function of top BLAST hit.

infection. Members of these two groups of genes were likely connected to the endoplasmic reticulum's (ER) unfolded protein response (UPR) which upregulates chaperones and genes for protein degradation upon the accumulation of unfolded proteins during stress (Szegezdi et al., 2006), or to the degradation and processing of antigens for the MHC class I molecule. At least 15 unique transcripts were upregulated in these two categories including chaperones, proteasome activators, and proteasome subunits (Table 2).

3.4. Downregulated transcripts following ESC infection

A much smaller number of catfish transcripts were significantly downregulated following infection with *E. ictaluri* (Table 3). Interestingly, two of the three transcripts with known identities were catfish selenoproteins P1b and selenoprotein H which may possess antioxidant properties (Steinbrenner et al., 2006). A cell cycle gene, anaphase promoting complex subunit 13, was also downregulated. Identities of the remaining two down-regulated genes after infection cannot be determined at present.

3.5. QRT-PCR confirmation of microarray results

Expression patterns of six genes, upregulated ranging from 2.5-fold to 105-fold in the microarray experiment, were selected for validation using QRT-PCR. QRT-PCR results (Table 4) generally confirmed the microarray results, with all tested genes except intelectin showing statistically significant upregulation ($p \leq 0.05$). A strong upregulation of intelectin following infection was confirmed, despite the *p*-value falling slightly above the set threshold due to greater variations among the biological replicates. Fold changes measured by QRT-PCR were larger than those measured by microarray likely due to the more specific binding conditions of the PCR reaction, and perhaps also due to the greater accuracy in quantitation by QRT-PCR than by microarrays.

3.6. Comparison of expression profiles between blue catfish and channel catfish after bacterial infection

Channel catfish is generally susceptible whereas blue catfish is generally resistant to *E. ictaluri* infection. A comparison of gene expression of blue catfish with that of channel catfish (Peatman et al., 2007) after bacterial infection may provide insights into the molecular determinants for the resistance in blue catfish. Both species shared a wide spectrum of similarities in gene expression profiles after infection including an acute phase response, and strong induction of complement components and iron regulatory genes at day 3 after infection (Table 2 and Supplemental Table 1). However, significant differences were also observed between the expression profiles of the two species. A total of 58 genes were differentially expressed in blue catfish liver but not in channel catfish liver at day 3 after infection (Table 5). While more differences in the expression patterns were evident at lower fold changes (<4×; Table 5), CC chemokine SCYA106, the most highly induced transcript in blue catfish, was not differentially expressed in channel catfish. It was also noted in the analysis that several MHC class I-related components, as well as several other immune-related genes, were upregulated in blue catfish but not in channel catfish (see Section 4).

Ten genes (from the 58) were selected and primers were designed to amplify both blue catfish and channel catfish gene orthologues. Expression of these 10 genes was further measured by QRT-PCR at 24 h after infection and in moribund fish from both blue catfish and channel catfish in order to analyze temporal differences in gene expression in relation to infection

Table 4
Validation of microarray results by QRT-PCR

Gene	Accession	Microarray fold change	QRT-PCR fold change
SCYA106	AY555503	+105	+741 ($p = 0.047$)
Intelectin	CF970955	+48	+455 ($p = 0.055$)
LAMP3	TC7925	+5.7	+90.9 ($p = 0.047$)
Hemopexin	CK406564	+9.7	+39 ($p = 0.047$)
MMP13	CF972078	+2.8	+2.6 ($p = 0.047$)
LY6E2	CK404046	+2.5	+4.3 ($p = 0.047$)

SCYA106, CC chemokine SCYA106; LAMP3, Lysosomal-associated membrane protein 3; MMP13, matrix metalloproteinase 13; LY6E2, lymphocyte antigen 6 complex, E2.

Table 5
Transcripts significantly differentially expressed in blue catfish but not in channel catfish at day 3 after infection

Accession	Putative Id	Fold change
AY555503	**CC chemokine SCYA106**	105.1
CK405246	Methionine adenosyltransferase II alpha subunit	8.3
TC7925	**Lysosomal-associated membrane protein 3**	5.7
TC9859	**MHC class I alpha chain**	4.7
BQ096774	No significant similarity	4.1
CK408173	**Pentraxin**	4.1
CK405317	Beta-actin	4.0
CK403482	No significant similarity	3.9
CK401799	MGC68649 protein	3.8
CK418197	Cytochrome P450 3A	3.6
CF972133	No significant similarity	3.6
CK414572	No significant similarity	3.5
TC7345	Fetuin-B precursor (IRL685)	3.3
CK409611	**Proteasome activator PA28 subunit**	3.2
CF972140	No significant similarity	3.1
TC9755	Proteasome (prosome, macropain) subunit, alpha type, 3	3.1
TC6790	Atpase H+ transporting lysosomal vacuolar proton pump	3.0
CK406132	Alpha-1-tubulin	2.9
CK405386	No significant similarity	2.9
CF971092	No significant similarity	2.9
CF972078	**Matrix metalloproteinase 13**	2.8
CK404348	H2A histone family, member V, isoform 1	2.7
TC7398	CC chemokine SCYA106	2.6
TC7043	**CCAAT/enhancer binding protein (C/EBP), beta**	2.6
EE993326	CD63	2.6
CF972066	No significant similarity	2.6
CK404046	**Lymphocyte antigen 6 complex, locus E ligand isoform 2**	2.5
CK401855	MHC class I alpha chain	2.5
CK424035	Neuronal myosin light chain kinase 1	2.5
TC9648	Amyloid beta (A4) precursor protein-binding, family B, member 2, partial	2.4
TC8526	Proteasome activator subunit 1	2.4
TC6716	**Beta-2 microglobulin precursor**	2.3
CV990995	Coactosin-like 1	2.3
TC9170	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase	2.3
CV987949	Fructose-1,6-bisphosphatase 1, like	2.3
TC8645	Lectin, galactoside-binding, soluble, 9 (galectin 9)-like 1	2.3
TC6930	LR8 protein	2.3
CK423344	No significant similarity	2.3
TC6963	Proteasome activator subunit 2	2.3
CK406492	Similar to family with sequence similarity 46, member A isoform 1	2.3
TC8465	WW domain binding protein 2	2.3
TC7388	Proteasome (prosome, macropain) subunit, beta type, 6	2.2
CV995433	Sterol regulatory element-binding protein 2 (SREBP-2)	2.2
CK404782	Methionine adenosyltransferase II alpha subunit	2.1
CK410925	No significant similarity	2.1
CV994277	No significant similarity	2.1
CK408989	No significant similarity	2.1
CK424843	No significant similarity	2.1
BM425334	No significant similarity	2.1
CK407421	Glutaredoxin (thioltransferase)	2.0
CV997128	No significant similarity	2.0
CK403934	No significant similarity	2.0
TC9161	Hypothetical protein LOC641319	2.0
CK417600	No significant similarity	−2.5
TC9079	Anaphase promoting complex subunit 13	−2.0
CB940790	**Selenoprotein H**	−2.0
TC9060	No significant similarity	−2.0
CF971521	Selenoprotein P, plasma, 1b	−2.0

Expression of bold transcripts was tested at additional timepoints in blue catfish and channel catfish using QRT-PCR (Table 6).

(Table 6). Few large expression differences between blue and channel catfish were observed at the 24 h timepoint. There was little evidence of an earlier induction of these genes in channel catfish. However, matrix metalloproteinase 13 (MMP-13), was upregulated more than 20-fold in channel catfish at 24 h with only a slight induction of expression relative to controls in blue catfish. This gene has also been reported to be induced following an *Edwardsiella tarda* infection in Japanese flounder (Matsuyama et al., 2007). Induction of expression of the studied genes was generally higher in moribund fish. Expression of SCYA106 rose sharply in both dying blue catfish and channel catfish relative to controls, but showed greater upregulation in blue catfish. A similar pattern of expression was observed in lysosomal-associated membrane protein 3 (LAMP3). MMP-13 induction in moribund channel catfish was markedly higher than that observed in moribund blue catfish (Table 6).

4. Discussion

We have developed and utilized a high-density oligonucleotide microarray for catfish to study the transcriptomic responses of blue catfish following infection with *E. ictaluri*. Microarray analysis of the transcriptome profile of the blue catfish liver following infection with the Gram-negative bacterium led to the identification of 103 differentially expressed transcripts. Among the differentially expressed genes, the most notable category was acute phase response genes. The blue catfish APR as measured 3 days after infection included many of the components of the typical mammalian APR and also contained commonalities with APR in salmonids and carp (Gerwick et al., 2007; Tilton et al., 2005; Ewart et al., 2005; Martin et al., 2006; Reynders et al., 2006). Acute phase proteins accounted for a significant percentage of upregulated transcripts in blue catfish, which included haptoglobin, hemopexin, transferrin, ceruloplasmin, fibrinogen, angiotensinogen, pentraxin and several complement components (Table 2). Similar subsets of APP were reported to be differentially expressed in rainbow trout (Gerwick et al., 2007) and as measured by real-time PCR in zebrafish (Lin et al., 2007), indicating the likely conservation

Table 6
Additional QRT-PCR analysis of representative genes significantly differentially expressed in blue catfish but not in channel catfish at 3 day following infection (see Table 5)

Gene	24 h after infection QRT-PCR		3 days after infection Microarray		Moribund fish QRT-PCR	
	Blue catfish	Channel catfish	Blue catfish	Channel catfish	Blue catfish	Channel catfish
SCYA106	+1.2	+1.2	+105.1	+1.1	+396	+74.4
LAMP3	+1.4	+2.6	+5.7	+1.2	+59.9	+14.8
MHC1α	+1.3	+1.2	+4.7	−1.1	+7.1	+5.7
Pentraxin	+2.0	+2.3	+4.1	+1.7	+1.5	−2.6
PA28	+1.9	+1.7	+3.2	−1.3	+12.8	+6.8
MMP13	+1.6	+21.8	+2.8	−5.0*	+2.0	+123.1
CEBP	+1.2	+1.3	+2.6	+1.7	+1.6	3.1
LY6E2	+1.2	+2.1	+2.5	+1.4	−1.3	−1.1
B2M	+2.8	+1.9	+2.3	+1.1	+18.9	+10.5
SelH	+1.5	+1.7	−2.0	−2.0*	−1.4	−2.5

Expression of these genes at 24 h, 3 days (microarray), and in moribund fish is tabulated to compare temporal differences in expression patterns. Genes with expression changes marked with an *, while two-fold or higher induced or repressed, were not significantly differentially expressed in channel catfish due to high variability between replicates.

of function of the vast majority of APP between mammals and teleost fish.

Many of the APP observed to be upregulated in blue catfish liver were likely serving important functions in host defense. Pentraxin, upregulated 4.1-fold in the current study, has recently been shown to be capable of initiating the complement cascade and possesses opsonizing activity in the snapper *Pagrus auratus* (Cook et al., 2003, 2005). The complement system of teleost fish plays conserved roles in sensing and clearing pathogens (Boshra et al., 2006). C3, as the central component of the complement system, is present in multiple forms in fish, possibly serving as an expanded pathogen recognition mechanism (Sunyer et al., 1998). We detected three upregulated forms of C3 in blue catfish liver, emphasizing its importance in the teleost innate immune response. Complement C4, important for the activation of the lectin and classical complement pathways, was also upregulated strongly. Two components of the membrane attack complex which carries out cell lysis, C7 and C9, were both upregulated greater than five-fold. Interestingly, the highest upregulation among complement-related factors (14.5-fold) was seen for complement factor H which may inactivate C3b in the alternative complement pathway (Boshra et al., 2006), suggesting that the host fish were attempting to modulate the complement response (Table 2).

Intelectin was the most highly upregulated gene among several likely involved in iron homeostasis, binding, and transport (Table 2). Induction of intelectin was previously reported in carp (Reynders et al., 2006) and rainbow trout (Gerwick et al., 2007). Four transcripts representing intelectin on the catfish microarray were highly upregulated (Supplemental Table 1) QRT-PCR showed a 455-fold upregulation in gene expression following infection (Table 4). In mammals, intelectin is believed to be involved in pathogen defense mechanisms, recognizing galactofuranose in carbohydrate chains of bacterial cell walls (Tsuji et al., 2001) and may function as a receptor for lactoferrin, an iron sequestering homologue of transferrin (Suzuki et al., 2001). We are currently investigating the function of catfish intelectins in the context of iron and disease.

A large group of transcripts with putative roles in immune responses to infection were upregulated (Table 2). Two CC chemokines, SCYA106 and SCYA113, previously identified from catfish were highly induced (Peatman et al., 2006; Bao et al., 2006). SCYA106 was the most highly upregulated transcript in this study (>105-fold). Both SCYA106 and SCYA113 are most similar to mammalian CCL19 (MIP-3β), a regulator of dendritic cell trafficking to secondary lymphoid organs (Humrich et al., 2006). Upregulation of CCL19-like genes after infection has also been recently reported in rainbow trout and Atlantic salmon (Morrison et al., 2006; Martin et al., 2006). A catfish orthologue of CXCL14 chemokine (Baoprasertkul et al., 2005) also exhibited elevated expression in the liver after infection. In mammals, CXCL14 is known as a chemoattractant for activated monocytes, immature dendritic cells, and NK cells (Starnes et al., 2006).

Two lesser known immune transcripts observed in catfish were also induced in zebrafish following infection with *Mycobacterium marinum* (Meijer et al., 2005). Lysosomal-associated membrane protein 3 (LAMP-3) was upregulated strongly both in the microarray analysis and in QRT-PCR confirmation and is associated with the endosomal/lysosomal MHC II compartments of dendritic cells in humans (de Saint-Vis et al., 1998; Arruda et al., 2006). Galectin-9 has recently been reported to play roles in both innate and adaptive immunity—it possesses eosinophil chemoattractant activity, induces superoxide production, induces dendritic cell maturation, and promotes Th1 immune responses (Dai et al., 2005).

Thioredoxin, upregulated 3.8-fold in this study, has been reported previously to have important roles in the activation and proliferation of catfish B cells (Khayat et al., 2001), and may be also protecting the catfish liver against oxidative stress-induced damage (Isoda et al., 2006). A catfish transcript with highest similarity to lymphocyte antigen 6 complex, locus E (LY6E) was also induced. Interestingly, this gene in chicken has been identified as a putative disease resistance gene for Marek's disease virus by protein binding assays, linkage analysis, and microarrays (Liu et al., 2003b). The upregulation of CCAAT/enhancer

binding protein beta (C/EBP) was likely linked to the active acute phase response observed (Table 2). This transcription factor is induced by pro-inflammatory cytokines, and in turn regulates the expression of many acute phase reactants (Poli, 1998).

The upregulation of two different MHC class I alpha chains and beta-2-microglobulin (β_2m) indicated active antigen processing and presentation were likely occurring in the blue catfish liver 3 days after infection as part of a cell-mediated immune response. *E. ictaluri*, as an intracellular bacterium, has been observed by electron microscopy in vacuoles within liver macrophages 48 h post infection and within the vacuoles of hepatocytes 72 h post infection. The bacterium was also observed to survive and replicate within phagocytic cells (Baldwin and Newton, 1993). A MHC class I and $CD8^+$ cytotoxic T lymphocyte (CTL)-mediated response, therefore, would be an expected response to *E. ictaluri*-infected cell types in the liver. The MHC class I genes from catfish have been extensively characterized (Antao et al., 1999, 2001), but little is known about their expression patterns following pathogen infections. Similarly, minimal expression analysis of MHC class I-related genes has been conducted in teleost species following infection with intracellular bacteria. In mammalian systems, *Listeria monocytogenes* is an intracellular bacterial pathogen that has been well characterized as a model organism for the study of cell-mediated immunity. Several recently described characteristics of the host response to *L. monocytogenes* may help to explain the expression patterns observed in blue catfish. After exposure to *L. monocytogenes*, hepatocytes upregulate MHC class I heavy chain and β_2m, producing a rapid influx of newly generated peptides into the endoplasmic reticulum (Chen et al., 2005b). $CD8^+$ T cells have been found to serve an important role in the innate immune response 3 days after infection by *L. monocytogenes* by rapidly secreting IFN-γ in response to IL-12 and IL-18 (Berg et al., 2003). This rapid $CD8^+$ T cell IFN-γ response has been associated with lower bacterial burdens in the liver 3 days post infection and is correlated with host resistance in mice (D'Orazio et al., 2006).

In mammals, antigenic peptides presented on MHC class I molecules to CTLs are generated in the cytosol by degradation in the proteasome, translocated into the endoplasmic reticulum, and loaded onto the MHC molecule with the help of several protein components. Genes associated with the generation of peptides and peptide-loading for the MHC class I molecules, PA28α and PA28β, were also observed to be upregulated in blue catfish liver (Table 2). Studies of intracellular bacterium *L. monocytogenes* again provide insights into these expression patterns. Khan et al. (2001) reported the replacement of constitutive proteasomes with immunoproteasomes in mice livers starting 2 days after infection with *L. monocytogenes*. Immunoproteasomes support the generation of MHC class I epiptopes and shape immunodominance hierarchies of $CD8^+$ T cells (Chen et al., 2001). This switch in mice is marked by the upregulation of proteasome activator PA28α and PA28β subunits (Khan et al., 2001), which alter the fragmentation of polypeptides through the proteasome and are inducible by IFN-γ (Ahn et al., 1995; Groettrup et al., 1996). Both PA28α and PA28β proteasome activator subunits were observed to be upregulated in blue catfish (Table 2), suggesting a shift toward MHC class I antigen processing. This pathway has recently been reported to be particularly important for protection against *L. monocytogenes* in hepatocytes, where infection triggers expression of immunoproteasomes and eventual generation of $CD8^+$ T-cell epitopes needed for bacterial clearance (Strehl et al., 2006).

Two ER chaperones, calreticulin and endoplasmin (GRP94), were also induced in the blue catfish liver, providing further evidence of an active MHC class I-mediated response (Table 2). Among ER chaperones, GRP94 and calreticulin are apparently unique in their ability to bind peptides suitable for assembly on to MHC class I molecules (Nicchitta and Reed, 2000). We also noted that tapasin, another molecule involved in MHC class I antigen loading, was upregulated 2.3-fold on the microarray, but, with a *q*-value of 11%, was excluded from the set of genes declared significantly upregulated. Recently, the coordinated upregulation of MHC class I alpha chain, β_2m, and PA28β was reported in large yellow croaker (*Pseudosciana crocea*) following poly I:C injection (Liu et al., 2007). Our findings represent the first report of the coordinated upregulation of these and several other MHC class I-related components following a bacterial infection in fish. The induction of genes involved in MHC class I cascades in blue catfish was one major difference observed in the comparison of blue catfish and channel catfish expression profiles (Table 5). An earlier, or more efficient, MHC class I/CTL response to the intracellular bacteria could potentially account for some of the phenotypic differences in resistance in the two species. Further gene and cellular-based studies are clearly needed in catfish to understand the importance of MHC class I/CTL-mediated responses to *E. ictaluri* infection and to unravel the molecular basis for superior disease resistance in blue catfish to ESC.

A thorough comparison of dynamic expression profiles from blue catfish and channel catfish is difficult utilizing a single timepoint. Differences in gene expression could arise from different rates of disease progression between the two species and are better studied over several timepoints. Due to the expense of microarray technology for research on non-model organisms such as catfish, however, we were able to utilize microarrays only for analysis of expression 3 days after infection. To better understand the larger expression patterns of some of the 58 transcripts differentially expressed in blue catfish but not in channel catfish, we extended our analysis using QRT-PCR to measure expression in the 24 h and moribund sampling points of each species (Table 6). We were particularly interested in the expression of MHC class I-related components including MHC class I alpha chain, beta-2-microglobulin, and proteasome activator PA28α. Interestingly, these genes showed little induction in channel catfish or blue catfish at 24 h but were strongly upregulated in moribund fish of both species. This indicates that the MHC class I components were also upregulated in channel catfish, but at a later timepoint than in blue catfish. Similarly, CC chemokine SCYA106 expression, while little changed in either species at 24 h, was drastically induced in moribund fish (Table 6). Further expression-based studies are needed to determine whether

earlier induction of these and other immune components in blue catfish contributes to higher survival rates for ESC. Additionally, linkage mapping of these differentially expressed genes utilizing the channel catfish × blue catfish interspecific hybrid mapping panel will allow us to further characterize potential associations with resistance.

A far smaller number of transcripts were declared significantly downregulated than significantly upregulated, a seemingly characteristic result of transcriptomic analyses of bacterial infections (Ewart et al., 2005). This may reflect the nature of the inflammatory response in liver and/or be the result of the more transitory downregulation of genes being masked in the pooled samples. Genes that are differentially expressed in a sustained manner were more likely to be identified as significant, given that the pooled fish were potentially at different stages of the infection. The larger set of non-significant downregulated genes may still yield candidates for further mapping and analysis.

In conclusion, microarray analysis of gene expression changes in blue catfish liver after infection with Gram-negative bacterium *E. ictaluri* indicated the upregulation of several pathways likely involved in the inflammatory immune response, similar to those observed in channel catfish. However, significant differences were noted between the two species at day 3 after infection including a set of 58 genes that were significantly differentially expressed in blue catfish but not in channel catfish. Among these, a notable number of genes have functions in the MHC class I pathway. While the same set of genes was eventually also upregulated in moribund channel catfish, they appeared to be activated earlier in blue catfish, perhaps providing an immune advantage. Taken together, the microarray results add to our understanding of the teleost immune responses, provide potential insights into the molecular basis for blue catfish resistance, and lay a solid foundation for future functional characterization, genetic mapping, and QTL analysis of immunity-related genes from catfish.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molimm.2007.05.012.

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