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Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a Gram-negative bacterium

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Abstract

The acute phase response (APR) is a set of metabolic and physiological reactions occurring in the host in response to tissue infection or injury and is a crucial component of the larger innate immune response. The APR is best characterized by dramatic changes in the concentration of a group of plasma proteins known as acute phase proteins (APPs) which are synthesized in the liver and function in a wide range of immunity-related activities. Utilizing a new high-density *in situ* oligonucleotide microarray, we have evaluated the APR in channel catfish liver following infection with *Edwardsiella ictaluri*, a bacterial pathogen that causes enteric septicemia of catfish. Our catfish microarray design (28K) builds upon a previous 19K channel catfish array by adding recently sequenced immune transcripts from channel catfish along with 7159 unique sequences from closely related blue catfish. The analysis of microarray results using a traditional 2-fold change in gene expression cutoff and a 10% false-discovery rate revealed a well-developed APR in catfish, with particularly high upregulation (>50-fold) of genes involved in iron homeostasis (i.e. intelectin, hemopexin, haptoglobin, ferritin, and transferrin). Other classical APP genes upregulated greater than 2-fold included coagulation factors, proteinase inhibitors, transport proteins, and complement components. Upregulation of the majority of the complement cascade was observed including the membrane attack complex components and complement inhibitors. A number of pathogen recognition receptors (PRRs) and chemokines were also differentially expressed in the liver following infection. Independent testing of a selection of differentially expressed genes with real-time RT-PCR confirmed microarray results.

Keywords: Fish; Catfish; Microarray; Immunity; Infection; Acute phase response

1. Introduction

The acute phase response (APR) is a group of rapid physiological responses to infection or injury [1,2] and is one of the several components which

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comprise the innate immune system. The molecular signals leading to the induction of the APR following infection, best characterized in mammals, can now be traced from initial recognition of pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) by host Toll-like receptors (TLRs), through a resulting signaling cascade, to the ultimate activation of target genes encoding pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α [3]. The release of these cytokines has long been known to stimulate the APR and rapidly alter rates of synthesis of a group of plasma proteins known as acute phase proteins (APPs) [4]. As the site of synthesis of the majority of plasma proteins, the liver is commonly considered the center of the APR. APPs are an established diagnostic tool as early indicators of inflammation and disease [5], but many are now known to play beneficial roles in mediating the complex inflammatory response and seeking to restore homeostasis [2].

Research on the APR and the larger innate immune response of teleost fish has received more attention only recently as a growing worldwide aquaculture industry faces disease outbreaks resulting in devastating losses [6]. The acute nature of these infections has drawn attention to the importance of the innate immune response in fish. The APR has been best characterized previously in rainbow trout, *Oncorhynchus mykiss*, using gene and protein-based techniques [7–9] and recently using a small oligo-based microarray [10]. Expression of a number of acute phase reactants has also recently been measured in zebrafish *Danio rerio* using real-time polymerase chain reaction (PCR) [11].

No information is known, however, about the nature of the APR in channel catfish (*Ictalurus punctatus*), the predominant aquaculture species in the United States and one of the best characterized teleost immune models to-date [12]. Catfish production suffers heavy losses due to enteric septicemia of catfish (ESC), caused by the Gram-negative, intracellular bacterium *Edwardsiella ictaluri* [13,14]. ESC in its acute form is characterized by gastroenteric septicemia and, under artificial challenge, often results in heavy mortalities as early as four days after onset of infection [15,16]. To better understand the crucial innate immune response of catfish in the context of ESC, we have previously identified and characterized a large number of cytokines, chemokines, antimicrobial peptides, and Toll-like receptors from catfish [17–31] and identified additional immune-related genes through EST sequencing. To study the expression of these important immune components in the larger context of the catfish transcriptome following ESC infection, we have developed a high-density *in situ* oligonucleotide microarray for catfish based upon a previous 19K channel catfish array [32]. By adding 7159 additional transcripts from blue catfish (*Ictalurus furcatus*), a closely related species to channel catfish sharing greater than 98% nucleotide similarity within cDNA transcripts [33], along with additional immune and non-immune transcripts from channel catfish, the new 28K microarray design should capture a large proportion of the catfish transcriptome. Here we describe the utilization of this 28K microarray for gene profiling of the APR of channel catfish following infection with *E. ictaluri*.

2. Materials and methods

2.1. Experimental fish, disease challenge and sampling

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 (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 one 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 four months to approximately 15 cm before artificial bacterial challenges. Challenges followed established detailed protocols for ESC [18,34] with modifications. Water temperature before challenge was gradually brought from 18 to 27 °C by mixing in heated water. Fish were kept at the elevated temperature for several weeks before challenge. Fish were challenged in 30-l aquaria with six control and eight 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 (three aquaria),

24 h treatment (three aquaria), 3 d control (three aquaria), 3 d treatment (three aquaria), and moribund (two aquaria). *E. ictaluri* bacteria were cultured from a single isolate (MS-S97-773) and used in a small test infection of several channel 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 ml 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 d 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 due to tissue constraints in the juvenile fish and to reduce variability between arrays to allow assessment of broad expression changes (see Discussion). Collected tissues and organs included head kidney, spleen, trunk kidney, liver, gill, and skin. 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 of each species were collected and confirmed to be infected with *E. ictaluri* and pathogen-free, respectively, at the Fish Disease Diagnostic Laboratory, Auburn University.

2.2. RNA extraction and labeling

Due to financial constraints, only channel catfish liver control and treatment replicates at the 3 d time point were used for initial microarray 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-gage 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 were checked by spectrophotometer analysis and gel electrophoresis. All extracted samples had an *A*260/280 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).

2.3. Microarray fabrication, hybridization and image acquisition

A high-density *in situ* oligonucleotide microarray was constructed, building on a previously published 19K catfish design [32]. Newly sequenced transcripts including many ESTs related to immune functions from channel catfish were added bringing the number of sequences from that species to 21,359. Additionally, 7159 unique ESTs from the closely related species blue catfish (*I. furcatus*) were added to the microarray to increase the number of informative genes on the array in cases where blue catfish ESTs contained a gene not present in the channel catfish ESTs or to allow better eventual comparisons between the species in cases where putative orthologs are present. To obtain a unique

set of blue catfish ESTs, all sequences available in the NCBI GenBank for the species as of March 2005 were downloaded in FASTA format, added into the ContigExpress program of the Vector NTI software suite (Invitrogen, Carlsbad, CA) and assembled. Singletons (non-clustering sequences) and representative clones from contigs were selected and reassembled in ContigExpress to ensure a unique gene set as described previously by Peatman et al. [35]. A total of 28,518 sequences were used, therefore, to construct the new catfish microarray. The added channel catfish and blue catfish sequences were compared by BLASTX against the non-redundant (*nr*) protein database at NCBI, with a cutoff *E*-value = 0.00001 for annotation. 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 GSE6105.

Nimblegen Systems produced the physical microarrays utilizing an *in situ* maskless array synthesis technology to synthesize 24 base pair (24-mer) oligos on the surface of the microarray slides [36,37]. At least 12 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.

The 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 h with constant rotation in a hybridization oven. After hybridization, the microarrays were washed twice with non-stringent buffer (6× SSPE, 0.01% Tween 20) at room temperature followed by two stringent washes (0.1 M Na^+, 0.01% Tween 20) at 45 °C for 15 min each. After a final 1-min rinse with non-stringent 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 non-stringent 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 isolated three days after infection.

2.4. Microarray 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 [38] which takes into account only the perfect match oligos. RMA takes a background adjustment on the raw intensity scale, carries out quantile normalization [39], 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 *E. ictaluri*-infected sample microarrays were then analyzed using the Significance Analysis of Microarrays method [40] in the two-class unpaired mode (SAM version 2.23A:http://www-stat.stanford.edu/~tibs/SAM/). SAM assigned each gene a relative difference score and carried out permutations of repeated measurements to determine a percentage of genes identified by chance, the false-discovery rate (FDR) [41], presented as a *q*-value for each gene in the final list of significant genes [40,42,43]. A list of differentially expressed genes with at least 2-fold expression changes between treatment and control and a global FDR 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 hit with the most negative *E*-value was noted. Those sequences possessing no significant similarity to peptide sequences within the *nr* database were assembled in ContigExpress to identify and remove any redundant

sequences. When a putative gene identity was shared by multiple sequences, further sequence analysis was carried out to remove redundancies. In cases where features representing both channel catfish and blue catfish putative orthologs of the same gene showed significant fold changes, the channel catfish transcript was selected to represent this gene. If multiple channel catfish transcripts were determined 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 paralogs, both transcripts were kept on the unique list.

2.5. Real-time RT-PCR analysis

The RNA prepared for microarray analysis was also used for confirmation of the expression pattern of selected genes of interest by quantitative real-time reverse-transcription polymerase chain reaction (qRT-PCR). The three control pools and three treatment pools of RNA, each representing 25 fish, were utilized for each tested gene. One-step qRT-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 [44] (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 catfish [45]. The triplicate (biological) fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (Ct) values, were compared and converted to fold differences by the relative quantification method [46] 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 (<0.35 change in Ct). 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. Bacterial challenge, microarray sample selection and hybridization

The artificial challenge with virulent *E. ictaluri* resulted in widespread mortality of infected fish at day 5 after exposure. No control fish manifested symptoms of ESC, and randomly selected control

Table 1
Primers used for qRT-PCR confirmation (5′–3′)

Gene	Accession	Forward	Reverse
TLR5	CV993724	ATTAGCACGCCTTCCACAGC	AGAGGTTCTGCAAGCCGGTC
Intelectin	TC6845	TCGGAGCTGCCGGGACATCAAGGAG	CCCTGCTCGCTTGACCAGCGATCAC
Hemopexin	TC8425	TGACCGCTGTGAGGGCATCGAG	TGTGCATGCGGAAGGCTGCATCCA
SCYA113	AY555510	TCCACAAAGCCTGGTGGAATCC	AGTTGTTCTTTGTCGCACGAGG
Ferritin	CK404798	CAGAGCGTGACGAGTGGGGCAG	AGGCGCTCCCATACGGCGCAGG
CD59	TC9110	TGGTGGCCATAATGACGGCTGC	GCTCACAGATGCACCGTTACAC
SelX	EE993544	TGGCTATGAGCTGTTCTCCAGC	TCAGAATCGGGACAGTCCATGC
LEAP-2	AY845143	GTACTCGCCCAACAGGTAGCTC	AAGGAGCAGTGTCCTTTCCTGC
18S	BE469353	TGCGCTTAATTTGACTCAACAC	CGATCGAGACTCACTAACATCG

fish were confirmed to be negative for *E. ictaluri* by standard diagnosis 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 hemorrhages along their ventral surface. *E. ictaluri* bacteria were successfully isolated from randomly selected treatment fish. While two timepoints (24 h and 3 d) were selected for sampling, only the 3 d time point was chosen for microarray analysis, due to financial restraints and a desire to include sufficient biological replicates to allow robust statistical analysis. As liver is central to the APR and is an important organ to innate immunity, it was selected for microarray analysis. Six RNA samples were successfully extracted from the livers of the three control replicate pools ($n = 25$) and the three treatment replicate pools ($n = 25$), labeled, and hybridized to six high-density *in situ* oligonucleotide microarrays for catfish. The catfish microarray contains 28,518 expressed sequences from channel catfish and blue catfish, each represented by at least six probe pairs of 24 oligonucleotides each.

3.2. Analysis of catfish gene expression profiles after ESC infection

The expression levels of the 28,518 catfish transcripts in liver three days after infection with *E. ictaluri* were compared with the levels seen in uninfected catfish. After data normalization and gene expression calculation in the RMA program [38], the resulting expression intensity values were analyzed in Significance Analysis of Microarrays (SAM) [40]. The criteria of a 2-fold or greater change in expression and a global FDR of 10% were chosen to determine upregulated or downregulated genes in the infected replicates. Using these criteria, 301 transcripts were significantly upregulated (Supplemental Table S1), and six were significantly downregulated. Of the 301 upregulated catfish transcripts, 207 of these are believed to represent unique genes, and five of the six significantly downregulated transcripts were unique. The redundant transcripts resulted either from blue and channel putative orthologs of the same gene or multiple transcripts from non-overlapping regions of a large cDNA being included on the microarray. A wide range of levels of gene upregulation was observed. Fourteen genes were upregulated from 10 to 85 fold following infection; 16 genes were upregulated from 5 to 10 fold; 27 genes were upregulated from 3 to 5 fold; and 150 genes were upregulated from 2 to 3 fold.

3.3. Putative identities of differentially expressed genes after infection with E. ictaluri

Of the 207 unique, significantly upregulated transcripts after infection, 127 could be annotated based on sequence similarity by BLASTX searches while 80 had no significant similarity to protein sequences in the *nr* database (cutoff *E*-value = 0.0001; see Supplemental Tables S2 and S3 for unique upregulated transcripts with and without annotation). Thirty catfish genes were upregulated 5-fold or greater, and their putative functions, as obtained by PubMed and UniProt (http://www.pir.uniprot.org/) searches, are listed in Table 2.

3.4. Profiling of the APR in catfish

A conserved APR was evident in the significantly upregulated catfish transcripts following infection. At least 35 of the 127 unique, annotated transcripts (Supplemental Table S2) represented APPs [7], including coagulation factors, proteinase inhibitors, transport proteins, and complement components. Many of the APPs were upregulated greater than 5-fold (Table 2). Several APPs included on the microarray were not upregulated at the studied timepoint. These included mannose-binding lectin 2, serum amyloid P, and heparin cofactor II.

Two subgroups of APP, iron transport/homeostasis proteins and complement components, were represented by particularly high numbers of upregulated transcripts. Transcripts representing at least 15 unique complement components or inhibitors were upregulated 2-fold or greater following infection. These included: a short transcript likely representing C1q (CV996365) upregulated 15.3-fold (Supplemental Table S1); ficolin-like genes upregulated as much as 32-fold (BM438750); complement C2/Bf; several C3 isoforms; complement component C4; complement component C5; complement components C7, C8, and C9 active in the membrane attack complex; and several complement regulatory proteins including MAC inhibitor CD59, C1 inhibitor, and Factor H.

The most highly upregulated group of functionally related catfish genes was composed of genes involved in iron homeostasis. These included intelectin, the most highly upregulated gene observed at >85-fold, haptoglobin (>34-fold),

Table 2
Catfish genes upregulated 5-fold or greater in the liver following *E. ictaluri* infection

Accession	Putative identity	Fold change	*q*-Value	Function
CF970955	Intelectin	85.4	1.25	Pathogen recognition; iron metabolism
CK408483	Haptoglobin precursor	34.3	0.00	Binds hemoglobin; APP
BM438750	Microfibrillar-associated protein 4	32.9	1.25	Unknown; lectin similar to ficolin and tachylectin—initiates complement?
TC6845	Intelectin	28.0	2.36	Same as above—putative paralogs
BM438689	Microfibrillar-associated protein 4	25.6	0.00	Same as above—putative paralogs
TC8425	Warm-temperature-acclimation-related-65kda-protein-like-protein	23.4	0.00	Similar to hemopexin—sequesters heme
TC7475	CC chemokine SCYA113	21.5	3.27	Unknown; putative catfish ortholog of human CCL19/ MIP-3-beta
CK406396	Neurotoxin/C59/Ly 6-like protein	21.3	1.25	Unknown; possible phospholipase inhibitor or complement membrane attack complex inhibitor
CV994031	Catechol-*O*-methyltransferase domain containing 1	14.8	0.00	Unknown; putative *O*-methyltransferase
TC9205	Hypothetical protein XP_683888	14.4	1.25	Unknown
TC8426	Hemopexin precursor	13.6	2.36	Sequesters heme to liver
CV996638	Apolipoprotein Apoa4 protein	13.0	2.36	Lipid binding and transport
CV993724	Toll-like receptor 5	11.8	0.00	Pathogen recognition receptor—flagellin
CV987901	Complement C3-H1	10.0	1.71	Complement pathway; inflammation
EE993362	Complement protein component C7-1	9.7	0.00	Membrane attack complex component
TC9637	Fibrinogen alpha chain	9.6	0.00	Coagulation factor; APP
TC9194	Complement regulatory plasma protein	8.9	3.27	Factor H; complement inhibition
CV992853	Ceruloplasmin	8.5	0.00	Iron transport; APP
TC9833	Microfibrillar-associated protein 4	8.4	1.25	Same as above—putative paralogs
TC8765	Transferrin	7.7	0.00	Transports iron—APP
TC8306	Fibrinogen gamma polypeptide	6.1	0.00	Coagulation factor; APP
CV989503	CXCL14	5.7	0.00	Chemokine—stimulates monocytes, NK cells
CV997126	Complement C3	5.6	0.00	Complement pathway; inflammation
TC7892	Ceruloplasmin	5.4	0.00	Same as above—putative paralogs
CV992447	Complement component C8 beta	5.4	3.27	Membrane attack complex component
TC7741	Complement factor B/C2-A3	5.4	3.74	Complement pathway
BM494620	Serum/glucocorticoid regulated kinase	5.3	1.25	Cellular stress response
CV995884	Solute carrier family 31 (copper transporters), member 1	5.3	0.00	Copper ion transport
TC8490	Fibrinogen, B beta polypeptide	5.2	1.71	Coagulation factor; APP
EE993545	Erythroblast membrane-associated protein	5.0	3.74	Cell adhesion or receptor molecule of erythroid cells; Ig superfamily member

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

hemopexin (>25-fold), ceruloplasmin (8.5-fold), transferrin (>7-fold), and ferritin (>2-fold).

3.5. Additional upregulated genes with putative immune functions

A number of additional genes believed to play important roles in the innate immune response, inflammation, and/or cellular responses to infection were upregulated after infection. These included Toll-like receptor 5, CC chemokine SCYA113, CXC chemokine CXCL14, selenoprotein Pa, selenoprotein X, selenium-binding protein, chemotaxin, and several lectins (Table 2; Supplemental Table S2).

3.6. Downregulated genes

A much smaller number of catfish transcripts were significantly downregulated following infection with a narrow range of suppression (Table 3). These included liver-expressed antimicrobial peptide-2, which is believed to be involved in the defense

Table 3
Significantly downregulated catfish transcripts in liver after *E. ictaluri* infection

Accession	Putative identity	Fold change	*q*-Value	Function
TC7457	Eukaryotic translation initiation factor 3, subunit 6 interacting protein	0.42	6.5	Translation regulation
CK404061	No significant similarity	0.44	5.2	NA
AY845143	Liver-expressed antimicrobial peptide 2	0.45	6.5	Defense response to bacteria
CK403219	No significant similarity	0.47	7.5	NA
TC6758	Thioredoxin interacting protein	0.49	1.7	Oxidative stress mediator
TC6756	Thioredoxin interacting protein	0.50	1.7	Oxidative stress mediator

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

response to bacteria [23], and thioredoxin-interacting protein which functions in the oxidative stress response in mammals.

3.7. Real-time RT-PCR confirmation of microarray results

Expression patterns of eight genes identified by microarray analysis as differentially expressed following infection were selected for confirmation using qRT-PCR. Genes differentially expressed ranging from 2-fold to 85-fold in the microarray experiment were selected and primers designed (Table 1). qRT-PCR results (Table 4) generally confirmed the trend of the microarray results, with all tested genes showing statistically significant upregulation or downregulation greater than 2-fold ($p < 0.05$). Fold change levels obtained by microarray analysis were not necessarily predictive of those obtained by qRT-PCR, with gene orders sometimes differing between the two techniques. 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 (Table 4), and perhaps also due to the greater accuracy in quantitation by qRT-PCR than by microarrays.

4. Discussion

Utilization of a new catfish microarray led to the identification of 212 unique, differentially expressed transcripts in the liver of channel catfish following infection with Gram-negative bacterium *E. ictaluri*. Our aims in the present experiment were to: (a) validate a new catfish *in situ* oligonucleotide microarray design which included larger numbers of immune transcripts; (b) capture and quantify the APR of catfish and compare it with previously described classical mammalian and fish APRs; and (c) identify further immune-relevant transcripts from catfish as potential biomarkers for stress and disease [47–50] and for future functional characterization, genetic mapping, and QTL analysis for potential linkage to loci contributing to disease resistance or susceptibility [51]. Our results will allow us to fulfill these aims and move toward the long-term goal of improving disease resistance in catfish broodstocks.

Microarray-based transcriptomic profiling of the liver in teleost fish has been utilized for measuring gene responses to a wide range of stimulants, in addition to disease, including environmental toxicants, growth hormone transgenesis, and hypoxia [52–56] making it an ideal tissue for comparison of conserved expression patterns. The catfish APR as measured in liver three days after infection included many of the genetic components of the classical mammalian APR and also had overlapping results with a recent APR study in rainbow trout liver [10] and other previous salmonid and carp microarray experiments measuring expression in liver after application of a variety of stressors [57–60]. A number of informative transcripts were shared between the compared experiments and a potentially conserved set of both mammalian and teleost acute phase reactants could be identified. Among the mammalian APP [2] upregulated greater than 2-fold in catfish were haptoglobin, hemopexin, ferritin, transferrin, ceruloplasmin, fibrinogen,

Table 4
Confirmation of microarray results by qRT-PCR

Gene	Accession	Microarray fold change	qRT-PCR fold change
Intelectin	TC6845	+85.4	+545 ($p = 0.001$)
Hemopexin	TC8425	+23.4	+65 ($p = 0.001$)
SCYA113	AY555510	+21.5	+235 ($p = 0.001$)
TLR5	CV993724	+11.8	+71 ($p = 0.013$)
Ferritin	CK404798	+2.3	+10 ($p = 0.03$)
CD59	TC9110	+2.2	+3.5 ($p = 0.025$)
SelX	EE993544	+2.03	+7.4 ($p = 0.014$)
LEAP-2	AY845143	−2.3	−8 ($p = 0.047$)

thrombin, alpha-2-macroglobulin, trypsin inhibitor, plasmin inhibitor, plasminogen, and angiotensinogen, and a large number of complement components and inhibitors (Fig. 1). Smaller subsets of APP were reported to be differentially expressed in rainbow trout [10] and as measured by real-time PCR in zebrafish [11], indicating the likely conservation of function of the vast majority of APP between mammals and teleost fish.

A number of genes not classically considered acute phase reactants were also observed to be shared between our results from catfish and several of the other microarray experiments involving teleost liver, and some of these warrant further comment as they may represent novel immunoregulators in fish. They included intelectin (also reported by [10,60]), microfibrillar-associated protein 4 [60], Toll-like receptor 5 [11,58], neurotoxin/differentially regulated trout protein [10,57–59], SEC31/high affinity copper uptake protein [59], and SEC61 [10].

Intelectin was the most highly upregulated catfish transcript in liver following infection (Table 2). Five transcripts representing intelectin on the catfish microarray were upregulated greater than 5-fold (Supplemental Table S1). qRT-PCR showed a 545-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 [61] and may function as a receptor for lactoferrin, an iron sequestering homolog of transferrin [62]. We are currently investigating the function of catfish intelectins in the context of iron and disease.

Microfibrillar-associated protein 4 (*mfap4*) was also highly upregulated in catfish following ESC infection and was also identified to be highly upregulated in carp liver after cadmium exposure [60]. Multiple transcripts representing several *mfap4* genes are on the catfish microarray. *Mfap4* is represented by a multi-gene family in zebrafish and has strong similarity to ficolin and tachylectin. It may be functioning in pathogen recognition and initiation of the lectin complement pathway [63].

Toll-like receptor 5 is a well-characterized pathogen recognition receptor in both mammals and fish [64,65]. It was also well-characterized in catfish previously and was shown to be upregulated in the liver following ESC infection [66,67] and was upregulated in the spleen after LPS exposure [32]. In this study, TLR5 was observed to be upregulated greater than 11-fold by microarray analysis.

A catfish transcript similar to neurotoxin/C59/Ly-6-like protein from grass carp and differentially regulated trout protein was upregulated greater than 20-fold following infection. The upregulation of this gene has been reported in several salmonid microarray experiments on liver to-date and has been tentatively suggested to be an APP [7]. While its function is unknown, it shares some similarity with membrane attack complex inhibitor CD59.

The absence of the iron regulatory hormone hepcidin [68] from the transcriptomic profile of catfish liver was notable. This result, though dissimilar to what was found in several other teleost expression studies in liver [10,11,57–59], confirmed our previous expression studies which showed that hepcidin was not upregulated three days after infection [28]. In mammals, an inflammatory stimulus (IL-6) induces production of hepcidin in the liver. Hepcidin then blocks the release of iron from macrophages, hepatocytes, and enterocytes by internalizing and degrading ferroportin, the site of cellular iron export [69]. This leads to drastically decreased plasma iron levels during infection, a potential host defense mechanism to deny bacteria access to the critical metal [70]. As plasma iron

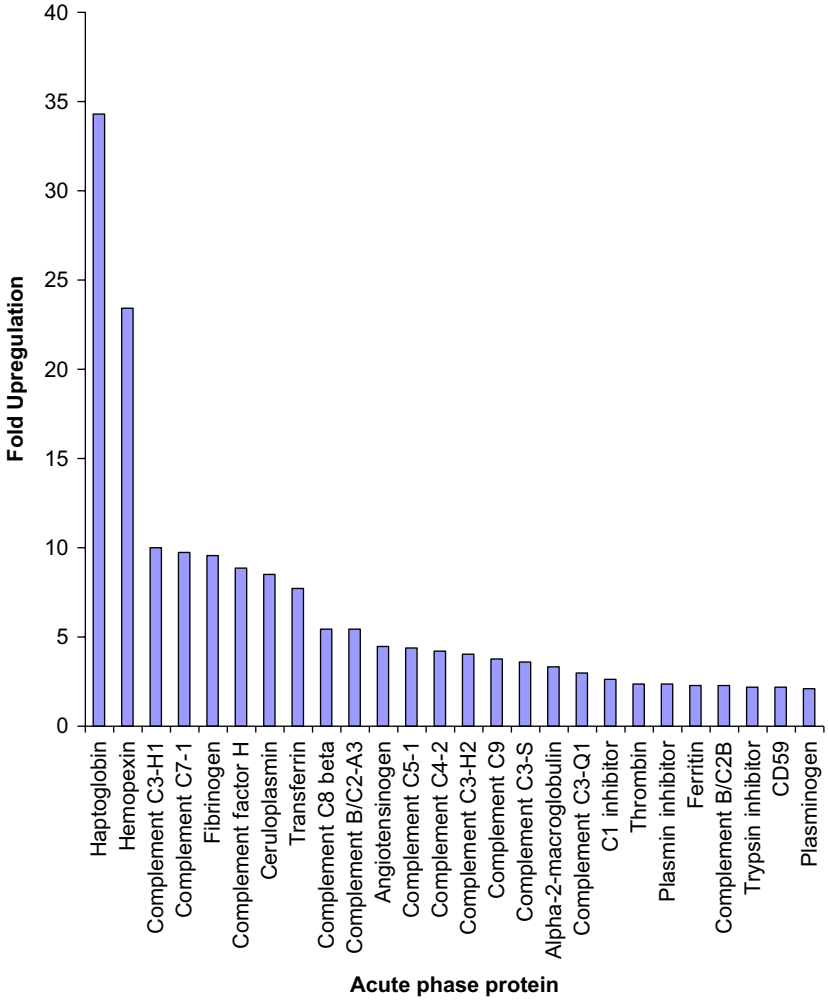

Fig. 1. APP genes upregulated 2-fold or greater in channel catfish following infection with *E. ictaluri*.

levels decrease, a feedback mechanism is believed to downregulate hepcidin production in the liver [71].

Regulation of iron homeostasis was a key aspect of the APR observed in catfish. Several physiological explanations can be suggested to explain the high upregulation of a large group of iron regulatory genes following infection. Liver iron stores are known to be significantly increased by hepcidin, even as plasma iron concentrations decline [72]. One would expect, therefore, an increase in iron storage, binding, and transport proteins such as haptoglobin, hemopexin, transferrin, ceruloplasmin, and ferritin as seen in the results. Hepatocytes, which account for 80% of the liver mass, are the primary site of synthesis for all these genes [73]. In mammals many of these genes are active in sequestering iron to restrict its availability to invading bacteria, and several are now known to possess immunoregulatory and antioxidant properties under pathological conditions which may supersede the importance of their roles in normal iron metabolism [73]. Although the exact mechanisms of gene upregulation in catfish are unknown at present, upregulation of a large number of genes involved in iron homeostasis suggests conservation of the Fe homeostasis pathway in dealing with infectious bacteria.

Two chemokines from catfish previously characterized in our laboratory, SCYA113 and CXCL14, were upregulated greater than 5-fold after infection in liver [17,20,22,24]. SCYA113 is a catfish CC chemokine most similar to mammalian CCL19, while catfish CXCL14 is the putative ortholog of mammalian CXCL14 [20]. Upregulation of CCL19-like genes after infection has also been recently

reported in rainbow trout and Atlantic salmon [63,74]. Interestingly, both chemokines are among a small handful of CC and CXC chemokines traditionally considered to have homeostatic rather than inflammatory functions [75]. Their roles in the catfish immune response are still unclear.

The desire to capture genes that are involved in disease response at the species level rather than inter-individual variations, financial cost, and small tissue sample sizes were all factors that entered into our decision to create replicate pools for initial microarray analysis. The debate over pooling of biological samples for microarray analysis has been contentious [76,77]. However, a consensus has emerged recently recognizing the advantages of pooling for decreasing variability between arrays and cost, *if* multiple pools are analyzed per group [78]. We utilized RNA samples from three distinct treatment pools and three distinct control pools for microarray analysis and were able to identify a large, reproducible set of differentially expressed transcripts. We did observe that variability between pools was larger among downregulated transcripts, resulting in only a small number of transcripts being declared significantly downregulated using a 10% FDR cutoff. A similarly small number of transcripts were reported to be significantly downregulated in the head kidney, spleen, and liver of Atlantic salmon following a Gram-negative bacterial infection [58]. This may reflect the nature of the late-stage inflammatory response 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 [58]. Also, the lower levels of fold changes observed for downregulated genes meant that even small degrees of variation between replicates could mask significance. Analysis of the 3 d rather than 24 h timepoint likely favored identification of upregulated genes, as the fish were likely in the process of terminal infection. We hope to conduct more comprehensive expression analysis in the future to better understand this phenomenon. A larger set of less than 2-fold downregulated genes, while not allowing global conclusions, will still yield candidates for further genome mapping and analysis (Supplemental Table S4).

In summary, microarray analysis of transcriptomic changes in channel catfish liver following an infection with Gram-negative bacterium *E. ictaluri* indicated that a conserved APR occurs as part of the innate immune response of primitive teleost fish. The majority of classical APPs were strongly upregulated in catfish along with a set of putative "teleost" acute phase reactants. Several transcripts involved in iron homeostasis were highly induced, suggesting that physiological mechanisms within catfish may attempt to limit free iron availability to inhibit bacterial growth and avoid metal-induced cellular damage. Strong upregulation of the complement cascade, pathogen recognition receptors (PRRs) and chemokines indicated that the catfish liver likely plays an important role in pathogen recognition and defense as well as inflammatory signaling.

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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.dci.2007.03.003.

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