GENOMIC APPROACHES TO CHARACTERIZATION OF THE INNATE IMMUNE

RESPONSE OF CATFISH TO BACTERIAL INFECTION

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DISSERTATION ABSTRACT

GENOMIC APPROACHES TO CHARACTERIZATION OF THE INNATE IMMUNE RESPONSE OF CATFISH TO BACTERIAL INFECTION

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Genetic selection for disease resistance encoded in the genomes of blue catfish and channel catfish continues to hold the greatest potential for long-term solutions to aquaculture-based disease outbreaks. Progress towards this goal requires the development of genomic resources for catfish, including expressed sequence tags (ESTs). In the context of catfish immune research, ESTs provide a foundation for both research on individual immune-related genes and microarray-based transcriptome analysis following infection. Both approaches are needed to advance our knowledge of teleost immunity and move closer to identification of genetic sources of disease resistance. My research, as presented here, encompasses these two complementary approaches to EST research with in-depth studies of the catfish CC chemokine family and development and

utilization of a high-density oligonucleotide microarray for expression analysis following *E. ictaluri* infection.

Twenty-six CC chemokines from catfish were mapped to BAC clones. Through a combination of hybridization and fluorescent fingerprinting, 18 fingerprinted contigs were assembled from BACs containing catfish CC chemokine genes. The catfish CC chemokine genes were found to be not only highly clustered in the catfish genome, but also extensively duplicated at various levels. The catfish CC chemokine family is the largest characterized CC chemokine family to-date, and it serves as a reference for chemokine studies in teleost fish as well as for studies of gene duplication patterns in catfish.

ESTs were also utilized in the development of a 28K *in situ* oligonucleotide microarray composed of blue catfish (*Ictalurus furcatus*) and channel catfish (*Ictalurus punctatus*) transcripts. Initial microarray analyses in channel catfish and blue catfish liver following an infection with *E. ictaluri* captured a high number of unique, differentially expressed transcripts and indicated the strong upregulation of several pathways involved in the inflammatory immune response. The construction and utilization of high-density oligonucleotide microarrays from channel catfish and blue catfish ESTs represent a strong foundation for future, widespread use of microarrays in catfish research.

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I. INTRODUCTION

Overview

Channel catfish (*Ictalurus punctatus*) is the most important freshwater aquacultured species in the United States. In 2003, 660 million pounds of pond-reared catfish, representing two billion dollars in economic value, were harvested in the U.S. (USDA, 2003). Aquaculture has grown rapidly in the last three decades to become an important alternative food source to collapsing natural fisheries. Catfish producers, like those of more traditional livestock, desire improvement in important production and performance traits such as disease resistance, growth rate, feed conversion efficiency, body conformation and fillet yield. Of those traits, disease resistance remains the most important and the most elusive. Fast-acting bacterial pathogens (predominantly *Edwardsiella ictaluri* and *Flavobacterium columnare*) continue to cause widespread losses to the industry. While several vaccines have been recently developed, implementation and rigorous field testing have been slow, and their long term efficacy is still in question. Antibiotic use is limited by regulation, cost, and growing bacterial resistance. Direct selection for disease resistance has proven to be very difficult and is beyond the capacity of most catfish breeding companies (Dunham et al. 1993; Dunham and Liu 2003).

While channel catfish has been the species traditionally cultured in the U.S., small numbers of the closely-related (~98% nucleotide similarity) species blue catfish (*Ictalurus furcatus*) have been raised. Blue catfish exhibit markedly higher resistance to enteric septicemia of catfish (ESC, caused by *E. ictaluri*), but are inferior to channel catfish in resistance to columnaris disease (caused by *F. columnare*, Dunham et al. 1993; Wolters et al. 1994; 1996). Under artificial challenge, heavy mortalities occur as early as four days after onset of infection with either disease, before the adaptive immune response can be established (Wolters et al. 1994).

Genetic selection for disease resistance encoded in the genomes of blue catfish and channel catfish continues to hold the greatest potential for long-term solutions to aquaculture-based disease outbreaks. Efficient identification of genes contributing to resistance and susceptibility to disease, however, is a multi-stage process necessitating the implemenation of a genome-based research program. Genome research requires the development of a number of resources that facilitate the organization of large amounts of genetic information into units that can be easily captured, mapped, and characterized. These resources include linkage maps, physical maps, bacterial artificial chromosome (BAC) libraries, and expressed sequence tags (ESTs). The integration of these tools produces a genome framework upon which the researcher can pinpoint the genotypic origins of phenotypic trait differences.

Expressed sequence tags, as a component of genome research, serve several functions. They are effective tools for gene discovery, rich sources of molecular markers for mapping, and the raw material for the development of microarrays (Liu 2006). In the context of catfish immune research, ESTs provide a foundation for both

research on individual immune-related genes and microarray-based transcriptome analysis following infection. Both approaches are needed to advance our knowledge of teleost immunity and move closer to identification of genetic sources of disease resistance. The two approaches are complementary, in that gene-based research makes microarray results more informative while microarray research provides a broader context for the study of immune genes.

Historically, studies of the catfish immune system have focused on antibody-based defenses (see Bengten et al. 2006 for a summary). More recently, a consensus has grown that the teleost innate immune response is crucial in determining survival to acute pathogen infections (Ellis 2001). However, few of the innate immune components known from mammalian systems had been identified in fish until recently. Utilizing new catfish EST resources produced in part from the RNA of catfish infected with *E. ictaluri* (Liu et al. 2007) we have, therefore, isolated and characterized several components of the innate immune response in blue and channel catfish to search for the molecular underpinnings of these differences in disease resistance (He et al. 2004; Xu et al. 2005; Bao et al. 2005; 2006a; Wang et al. 2006a; 2006b; 2006c; Peatman et al. 2005; Peatman et al. 2006; Bao et al. 2006b). One objective of my research focused particularly on CC chemokines, a superfamily of chemotactic cytokines with over 26 members in catfish (He et al. 2004; Peatman et al. 2005; Peatman et al. 2006; Bao et al. 2006a).

Expressed sequence tags can also be utilized for microarray-based research. Microarray experiments, when properly conducted, offer an accurate, global assessment of gene expression under a given treatment condition. The high-density capacity of

microarrays allows the analysis of expression patterns under infection of a large set of all existing, unique blue catfish and channel catfish ESTs, rather than only the handful of genes previously identified as being involved in the teleost innate immune response. Transcriptome analysis is essential to identify catfish genes involved in immune responses which may not play similar roles in mammalian systems. Additionally, microarray technology allows faster functional screening of large EST datasets than is possible by traditional methods. This allows the researcher to narrow his focus from a large set of genes to a manageable subset of expression candidates for mapping and further functional characterization.

My research, as presented here, encompasses the two complementary approaches to EST research described above with in-depth studies of the catfish CC chemokine family and development and utilization of a high-density oligonucleotide microarray for expression analysis following *E. ictaluri* infection. Separate literature reviews of CC chemokines and microarray technology, below, are followed by a publication-based presentation of my findings. Following the three chapters of results, an overall conclusion serves to summarize the work, suggests implications of the research, and provides directions for future research in the field.

Chemokines

Inflammation, the attempt to localize cellular injury caused by an infectious agent, is an important part of inducible innate immunity and can be seen within 1-2 days of infection. Chemokines are a family of structurally related chemotactic cytokines that regulate the migration of leukocytes, under both physiological and inflammatory

conditions (Neville et al. 1997; Laing and Secombes 2004a; Liu and Peatman 2006). They are structurally-related small peptides, with the majority containing four conserved cysteine residues. Based on the arrangement of these conserved cysteine residues (Ahuja and Murphy 1996; Murphy et al. 2000), chemokines were divided into four subfamilies: CXC (α), CC (β), C, and CX3C. Corresponding to these subfamilies of chemokine proteins, their encoding genes were designated by SCY (for small inducible cytokines) followed by a letter A, B, C, or D (for CC, CXC, C, and CX3C, respectively). CXC and CC are the two major subfamilies. To date, 16 CXC chemokines, 28 CC chemokines, two C chemokines, and one CX3C chemokine have been identified from mammalian species (Bacon et al. 2003).

The identification of members of the largest family of chemokines, CC chemokines, in non-mammalian species has been slow, due in part to the rapid divergence rate of CC chemokines. In addition, most CC chemokines are small in size with fewer than 100 amino acids. As a result, molecular cloning based on hybridization or PCR using heterologous probes or primers designed from sequences of different species are not very effective. Similarly, low sequence conservation hinders sequence analysis using bioinformatic approaches at the nucleotide level. Nevertheless, a number of important single gene discoveries have aided our understanding of fish CC chemokines (Table 1; He et al. 2004).

The CK1 gene of rainbow trout (*Oncorhynchus mykiss*) was the first fish CC chemokine described (Dixon et al. 1998). It contains six cysteine residues and has the highest similarity to mammalian CCL20. The second fish CC chemokine, CC chemokine 1, was identified from common carp (*Cyprinus carpio)* (Fujiki et al. 1999).

Carp CC chemokine 1 shares highest similarity to the allergenic/MCP subgroup of mammalian CC chemokines. The third fish CC chemokine, CK2, was also identified from trout (Liu et al. 2002). These early efforts provided the first indication of the difficulty of establishing orthologies with mammalian sequences. All these proteins share only low levels of identity (<40%) with any potential mammalian counterparts.

The early scarcity of fish CC chemokine discoveries led to speculation that fish may have few orthologues for the chemokines existing in mammals (Laing and Secombes 2004a; Huising et al. 2003a). With a foundation of small discoveries, the expectation was that only a handful of distinct CC chemokines would be found in a given species. The high numbers of mammalian CC chemokines appeared unique in the phylogenetic spectrum. Therefore, the recent rapid discovery of fish chemokines using genomic approaches has come as a surprise to some. Seven new chemokines from two cichlid fish, *Paralabidochromis chilotes* and *Melanochromis auratus*, and catshark *Scyliorhinus canicula* were reported recently (Kuroda et al. 2003), followed by sequencing and analysis of 14 CC chemokines from catfish (He et al. 2004), and 15 trout CC chemokines (Laing and Secombes 2004b) (Table 1). The *in silico* identification of these last two large sets of CC chemokines, in particular, challenged notions of the fish immune system and prompted the further investigation of the catfish CC chemokine family. Analysis and further sequencing of new EST resources from catfish identified 12 additional CC chemokine cDNAs (Peatman et al. 2005), representing the largest CC chemokine family identified to-date. This work was followed up with isolation and sequencing of 23 of the 26 catfish CC chemokine genes corresponding to the identified transcripts (Bao et al. 2006a). Tissue expression

analysis was also carried out at this time, in an attempt to classify the chemokines by expression patterns. However, both gene structure and tissue expression provided few clues as the orthologies between catfish CC chemokines and their mammalian counterparts. It was known that chemokines were tightly clustered within the genomes of mammals and chicken (Nomiyama et al. 2001; Wang et al. 2005) and that correlations existed between their genomic architecture and the inducibility of their expression. Therefore, analysis of genomic clustering of the CC chemokines in catfish could reveal important information regarding their identities and help to explain the modes of duplication and divergence that resulted in the present repertoire of vertebrate CC chemokines. Chapter II describes the methodology used and results obtained in research on catfish CC chemokine genomic clustering, duplications, and expression after bacterial infection.

Table 1 Previously reported fish CC chemokines

Species	CC chemokine	Accession Number	References
Rainbow trout	CK1	AF093802	Dixon et al. 1998
	CK2	AF418561	Liu et al. 2002
	CK3	AJ315149	Sangrador-Vegas et al. unpublished
	CK4A	CA371157	Laing and Secombes, 2004b
	CK4B	CA352593	
	CK5A	CA383670	
	CK5B	CA374135	
	CK6	CA355962	
	CK7A	CA355962	
	CK7B	CA346976	
	CK8A	CB494647	
	CK8B	CA353159	
	CK9	CA378686	
	CK10	CA361535	
	CK11	BX072681	
	CK12A	CA358073	
	CK12B	CA346383	
Carp	CC chemokine 1	AB010469	Fujiki et al. 1999
Japanese flounder	CC chemokine	AU090535	Kono et al. 2003
	Paol-SCYA104	AB117523	Khattiya et al. 2004
Cichlids	Meau-SCYA101	AY178962	Kuroda et al. 2003
	Meau-SCYA102	AY178963	
	Pach-SCYA101	AY178964	
	Pach-SCYA103	AY178965	
	Pach-SCYA104	AY178966	
	Pach-SCYA105	AY178967	
	Pach-SCYA106	AY178968	
Dogfish	Trsc-SCYA107	AB174767	Inoue et al. 2005
	MIP3α1	AB174768	
	MIP3α2	AB174766	
Catshark	Scca-SCYA107	AY178970	Kuroda et al. 2003
Catfish	Icfu-SCYA101	AY555498	He et al. 2004
	Icpu-SCYA102	AY555499	
	Icfu-SCYA103	AY555500	
	Icfu-SCYA104	AY555501	
	Icpu-SCYA105	AY555502	
	Icfu-SCYA106	AY555503	
	Icfu-SCYA107	AY555504	
	Icpu-SCYA108	AY555505	
	Icfu-SCYA109	AY555506	
	Icfu-SCYA110	AY555507	
	Icpu-SCYA111	AY555508	
	Icpu-SCYA112	AY555509	
	Icpu-SCYA113	AY555510	
	Icfu-SCYA114	AY555511	

Microarrays

Researchers have long harnessed the basic molecular principle of nucleic acid hybridization to study the expression patterns of cell transcripts. Transcript studies allow a valuable assessment of the genetic response to environmental changes (i.e. infection, temperature, feeding rates). Incremental progress over the last two decades has been made from radioactively-labeled probing of one gene to tens of genes to nylon-filter-based macro-arrays containing hundreds of genes. In early years, progress in transcript detection techniques largely corresponded to strides in gene sequencing and discovery. However, as gene sequencing grew exponentially in the early 1990s and genomic approaches such as PCR revolutionized molecular biology, a similarly radical leap forward was needed to bring transcript studies into the "–omics" era. This leap was provided by microarrays. While microarrays utilize several recent technological innovations, they are, at their core, simply a high density dot blot. In both cases, DNA is anchored or spotted onto a surface and then probed with labeled molecules. Hybridization and subsequent signal detection depends on the presence of complementary nucleotide sequences between the probes and the spotted sample. Microarrays achieve higher gene feature densities and, therefore, greater power for expression analysis by applying new tools to this old process. High-density spotting robots and photolithography allow each feature to be placed accurately within nanometers of the next feature on a glass slide, clearly an impossible task with the human hand. Furthermore, fluorescence-based probe labeling provides a cleaner and clearer signal than the radiation traditionally used in blotting. Finally, laser scanners

facilitate the resolution of such tremendous feature densities and provide accurate fluorescent signal quantification (Peatman and Liu 2007).

There are two primary approaches to microarrays, differing in both their construction and their sample labeling. Spotted arrays are constructed by spotting long oligos or cDNAs using a printing robot (Schena et al. 1995), whereas *in situ* arrays are constructed by synthesizing short or long oligos directly onto the slide by photolithography (Fodor et al. 1991; see Table 2 for a comparison of the two platforms).

A decade of refinements of both spotted and *in situ* microarray technologies have resulted in further capacity increases and widened array applications without altering the fundamentals of either approach. Microarray technology is now widely accessible in biomedical and agricultural genetics research.

Table 2 A comparison of several important aspects of *in situ* and spotted array platforms. *Cost/slide can vary significantly from these figures depending on design, quantities ordered, core facility discounts, etc.

	***In situ* arrays**	**Spotted arrays**
Starting material	DNA sequences	DNA sequences or cDNA
Array fabrication	*In situ* synthesis by photolithography	Robotic spotting
Features	>400,000	<50,000
Spot quality	High	Variable
Oligo length	23-25mer, 60-70mer	Usually 70mer
Labeling	Single dye label- *e.g.,* biotin-streptavidin- phycoerithryn	Two dye label-Cy3, Cy5
Cost/slide	>$500*	<$100*
Probe/slide	One	Two
Dye swapping?	No	Yes
Controls	PM/MM, +/-	Duplicates, +/-
Providers	Affymetrix, Nimblegen, *etc.*	Species groups, core facilities, biotech

Only within the last several years, however, have researchers in aquaculture species generated sufficient expressed sequence tags (EST) to justify using transcriptomic approaches for expression analysis. The field is still in its infancy and distribution of resources remains uneven. Concerted effort by researchers working on salmonid

species has resulted in the generation of several arrays that are now available to the general research community. These arrays have been rapidly integrated into salmonid research, as seen in Table 3. The largest salmonid microarray generated to-date contains 16,006 cDNAs with 13,421 coming from Atlantic salmon and 2,576 from rainbow trout (von Schalburg et al. 2005a). Table 3 lists additional microarray studies conducted on aquaculture species or aquaculture-associated pathogens. With the exception of salmonids, other microarray studies have, for the most part, been small-scale, non-collaborative efforts. A forthcoming microarray from oyster should also be widely distributed. To-date, the vast majority of published microarray studies has used PCR-amplified spotted cDNA clones to fabricate the array. However, as microarray research typically takes several years from its inception to reach publication, the recent trends toward spotted oligos and *in situ* microarrays may not be reflected in the aquaculture literature for several years. A well-designed microarray can be a valuable asset to an aquaculture species group, especially if the cost per slide can be minimized to the extent that researchers can integrate transcriptomic approaches into their already established research. Microarray studies are most successful when they are just one of several approaches used to answer biological questions. For example, salmonid researchers have implemented array technology in their study of reproductive development, toxicology, physiology, and repeat structures (von Schalburg et al. 2006; Tilton et al. 2005; Vornanen et al. 2005; Krasnov et al. 2005a). While species with completed genome sequences have expanded microarray research into such fields as comparative genomic hybridization (CGH), SNP analysis, methylation analysis,

proteomics, and metabolomics in recent years, research on aquaculture species has been confined to expression analysis.

Table 3 Microarray studies in aquaculture species and their pathogens

Species	Common name	References
Cyprinus carpio	Common carp	Gracey et al. 2004
Ictalurus punctatus	Channel catfish	Ju et al. 2002 Li and Waldbieser 2006
Ameiurus catus	White catfish	Kocabas et al. 2004
Paralicthys olivaceous	Japanese flounder	Kurobe et al. 2005; Byon et al. 2005; 2006
Platichthys flesus	European flounder	Williams et al. 2003
Salmo salar	Atlantic salmon	Morrison et al. 2006; Martin et al. 2006 ; Jordal et al. 2005; von Schalburg et al. 2005a; Aubin-Horth et al. 2005; Ewart et al. 2005; Rise et al. 2004a; 2004b
Oncorhynchus mykiss	Rainbow trout	Purcell et al. 2006; MacKenzie et al. 2006; von Schalburg et al. 2006;2005b Tilton et al. 2005; Krasnov et al. 2005a;2005b;2005c Vornanen et al. 2005; Koskinen et al. 2004a;2004b
Oncorhynchus keta	Chum salmon	Moriya et al. 2004
Astatotilapia burtoni	African cichlid	Renn et al. 2004
WSSV and Penaeus sp.	White spot syndrome virus and shrimp species	Lan et al. 2006; Marks et al. 2005; Tsai et al. 2004; Dhar et al. 2003; Khadijah et al. 2003
Sparus auratus	Gilthead seabream	Sarropoulou et al. 2005
Aeromonas salmonicida	Furunculosis	Nash et al. 2006
Crassostrea sp.	Oyster	Submitted

In catfish, a priority was placed on establishing a high quality EST resource with a large number of unique genes before constructing microarrays. An initial *in situ* oligonucleotide microarray was constructed utilizing only ESTs from channel catfish and validated with LPS-injected fish (Li and Waldbieser 2006). However, this array did

not include ESTs from blue catfish, important for additional, unique genes contained in their sequences as well as for analysis of differential expression between the two species. Additionally, the original array design did not contain several hundred immune-related genes recently generated in our lab from both blue and channel catfish. Construction of a more comprehensive catfish oligonucleotide microarray and its validation for capturing the expression profiles of channel catfish and blue catfish after *E. ictaluri* infection are described in Chapters III and IV of the dissertation.

II. CATFISH CC CHEMOKINES: GENOMIC CLUSTERING, DUPLICATIONS, AND EXPRESSION AFTER BACTERIAL INFECTION WITH *EDWARDSIELLA ICTALURI*

Abstract Chemokines are a family of structurally related chemotactic cytokines that regulate the migration of leukocytes under both physiological and inflammatory conditions. CC chemokines represent the largest subfamily of chemokines with 28 genes in mammals. Sequence conservation of chemokines between teleost fish and higher vertebrates is low and duplication and divergence may have occurred at a significantly faster rate than in other genes. One feature of CC chemokine genes known to be conserved is genomic clustering. CC chemokines are highly clustered within the genomes of human, mouse, and chicken. To exploit knowledge from comparative genome analysis between catfish and higher vertebrates, here we mapped to BAC clones 26 previously identified catfish (*Ictalurus sp.*) chemokine cDNAs. Through a combination of hybridization and fluorescent fingerprinting, 18 fingerprinted contigs were assembled from BACs containing catfish CC chemokine genes. The catfish CC chemokine genes were found to be not only highly clustered in the catfish genome, but also extensively duplicated at various levels. Comparisons of the syntenic relationships of CC chemokines may help to explain the modes of duplication and divergence that resulted in the present repertoire of vertebrate CC chemokines. Here we have also analyzed the expression of the transcripts of the 26 catfish CC chemokines in head kidney and spleen in response to bacterial infection of *Edwardsiella ictaluri*, an economically devastating catfish pathogen. Such information should pinpoint research efforts on the CC chemokines most likely involved in inflammatory responses.

Introduction

Chemokines are a superfamily of chemotactic cytokines in mammals and a crucial part of the innate immune response of higher vertebrates. They play roles in immunosurveillance under homeostasis as well as stimulating the recruitment, activation, and adhesion of cells to sites of infection or injury (Neville et al.1997; Moser and Loetscher 2001; Laing and Secombes 2004a). Recent research has found that some chemokine genes have important roles during normal development and growth (e.g., David et al. 2002; Molyneaux et al. 2003; Baoprasertkul et al. 2005). Chemokines are structurally related small peptides, with the majority containing four conserved cysteine residues. Based on the arrangement of these conserved cysteine residues (Murphy et al. 2000), chemokines were divided into four subfamilies:CXC (α), CC (β), C, and CX3C. CC chemokines constitute the largest subfamily of chemokines with 28 CC chemokines identified from mammalian species (Bacon et al. 2003). The largest number of CC chemokines found in a single species is 24 from humans, missing orthologues to the murine CCL6, CCL9/CCL10, and CCL12.

The majority of human, murine, and chicken CC chemokine genes are organized in gene clusters within their genomes. The largest clusters are found on human chromosome 17, mouse chromosome 11, and chicken chromosome 19 (Nomiyama et al. 2001; Wang et al. 2005). There are correlations between genomic architecture and the inducibility of their expression, with inflammatory CC chemokines constituting the large clusters, and a few homeostatic CC chemokines distributed among several chromosomes. Additionally, orthologies across species are relatively high between the

non-clustered CC chemokines, but low when comparing the clustered CC chemokines of several species (Wang et al. 2005; Peatman et al. 2005).

Establishing orthology between fish and mammalian CC chemokines has been problematic. Sequence conservation of chemokines is low and duplication and divergence may have occurred at a significantly faster rate than in other genes. Concrete orthologues cannot be identified for the majority of CC chemokine transcripts found from catfish or trout based on either sequence identities or phylogenetics (Laing and Secombes 2004b; He et al. 2004; Peatman et al. 2005). Even gene organization (exon/intron) has been found to differ between evident orthologous chemokines in human, chicken and catfish (Wang et al. 2005; Bao et al. 2006a). Genomic location of CC chemokines is important, therefore, in attempting to trace the origins of CC chemokines in teleosts and higher vertebrates. Comparisons of syntenic relationships of CC chemokines may help to explain the modes of duplication and divergence that resulted in the present repertoire of vertebrate CC chemokines.

Progress on identifying immune molecules in teleost fish has not traditionally come from the genome-enabled model species (*Danio rerio, Takifugu rubripes*). Rather it has been generated more slowly in several aquaculture species (catfish, salmonids, carps, flounders) where disease problems are a serious economic issue. The lack of even a draft genomic sequence in catfish makes cross-species comparisons of genomic neighborhoods much more difficult. We have used, therefore, a novel approach of overgo and cDNA hybridizations and bacterial artificial chromosome (BAC) fingerprinting and clustering to determine the architecture of the catfish CC chemokines without a draft genome sequence. Here we report the genomic architecture of

previously sequenced catfish CC chemokine genes as well as their expression patterns after bacterial infection. Comparisons of CC chemokine arrangements and duplication between catfish, chickens, and humans reveal rapid multiplication of some chemokine genes.

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Materials and methods

BAC library screening and BAC isolation

High-density filters of the channel catfish BAC library were purchased from Children's Hospital of the Oakland Research Institute (CHORI, Oakland, CA), and screened using overgo hybridization probes (Cai et al.1998; Bao et al. 2005; Xu et al. 2005). Each set of filters contained a 10X genome coverage of the channel catfish BAC clones from BAC library CHORI 212 (http://bacpac.chori.org/library.php?id=103). The catfish BAC library was screened using a two-step procedure. First, pooled overgo probes of catfish CC chemokines were used to identify BAC clones with inserts likely containing chemokine genes. These positive BACs were then manually re-arrayed onto nylon filters and screened individually using labeled cDNA probes.

Overgo primers were designed based on the coding sequence of the 26 chemokine cDNAs (Table 1). The overgo hybridization method was adapted from a web protocol (http://www.tree.caltech.edu/) with modifications (Bao et al. 2005; Xu et al. 2005). Briefly, overgos were selected following a BLAST search against GenBank to screen out repeated sequences and then purchased from Sigma Genosys (Woodlands,

Texas). Twenty-six overgos were pooled together, initially. Overgos were labeled with 32P-dATP and 32P-dCTP (Amersham, Piscataway, NJ) in overgo labeling buffer (Ross et al. 1999) at room temperature for 1 h. After removal of unincorporated nucleotides using a Sephadex G50 spin column, probes were denatured at 95°C for 10 min and added to the hybridization tubes. Hybridization was performed at 54°C for 18 hr in hybridization solution (50 ml of 1% BSA, 1 mM EDTA at pH 8.0, 7% SDS, 0.5 mM sodium phosphate, pH 7.2). Filters were washed and exposed to X-ray film at -80°C for two days.

Table 1 Overgo probes for BAC hybridization

Gene	Upper primer (5'-3')	Lower primer (5'-3')
SCYA101	GCGTTGCTATTTCGCTGGCAAATC	CACACAGTCTCTCTCTGATTTGCC
SCYA102	GTGCTGCTTGCACTTTTTGGATGC	CAGGTGCAGTAGTGATGCATCCAA
SCYA103	GTCCTCTGTTTTCTCCTGCTTCTG	TTGGGTACATGCATGCCAGAAGCA
SCYA104	CCTGTCTTCAGTCCTTCACAATGG	CCGTTTGCATTCTGTGCCATTGTG
SCYA105	ACAAACGTCGTGTGTGTGCAAACC	ACCCACTCATCCTTGGGGTTTGCA
SCYA106	AACAGCGGCATCTGATATTGGCAC	CACACGTCCTGTTTCTGTGCCAAT
SCYA107	AGGCTTCCACCAAAGAAATCACCG	AATCCTGTGATGGGCACGGTGATT
SCYA108	GTAAACACCAGTGTGGAAACGCTG	AGAGGAAAGACCTGAGCAGCGTTT
SCYA109	CAACCGTAATGGCAAGAGCAAAGG	GGTCTTTCACTGAGCTCCTTTGCT
SCYA110	GAAACAGCACTGTGTGGATCCAAC	GTTGACCCAAACAGCTGTTGGATC
SCYA111	GCTCATGTTGTTCCTCCTACTTCC	GGGGGAATTTTCCCATGGAAGTAG
SCYA112	CCTCCACAAATGTGTGAACACCTC	GACATAGCCACGTGAAGAGGTGTT
SCYA113	CAAAGCCTGGTGGAATCCTACTAC	TCTCTGGAGTCTGAACGTAGTAGG
SCYA114	CCATCTGGACTGTAACAGATGCAG	CAGGGGCTCACTTTTTCTGCATCT
SCYA115	TTCACTGAAGGGATGCGTTTCACG	AGACGTTTTTGGTGCCCGTGAAAC
SCYA116	CATGGCCTTTTTGGACCACAGAGG	ATTCCCTGGTGGCATGCCTCTGTT
SCYA117	TCTACTCAGACGCTCAGCCTTTTG	TCAGGATGTGCAGGAGCAAAAGGC
SCYA118	TCCTAAGCAAGTCCGTGTGACAAG	CCAGTAGCTCACAATGCTTGTCAC
SCYA119	CTGCTCTATCCACTCTTCTTCTGC	AGAGGCAGAACACCATGCAGAAGA
SCYA120	cDNA probe	cDNA probe
SCYA121	AGATGAATCGTGTGGTTTTGGTCC	ATCAGGAAGAAGCCCAGGACCAAA
SCYA122	CAGCAAGGCTTCATTGTTACGACG	GGTTAGGGAACTTAGGCGTCGTAA
SCYA123	AACGTAGTGTGTGTGCAAACCCCA	TGCACCCACTTATCCTTGGGGTTT
SCYA124	CTCGACCTAACCTCAAACGTGTGT	TGGCCAGAGGATTTAAACACACGT
SCYA125	TTGACTCAGAGAGACCTCACCTTG	ACTGAATCGCATGGCTCAAGGTGA
SCYA126	CTCGTGCTGCTTATTCGTGGAAAG	TTGGTGCGCACAATCTCTTTCCAC

Positive clones were identified according to the clone distribution instructions from CHORI and picked from the channel catfish BAC library. Approximately 200 positive BAC clones were identified through the hybridization of overgos for the 26 catfish CC chemokines. These 200 BAC clones were picked, cultured in 2X YT media overnight, and manually arrayed on Immobilon nylon membranes (Millipore, Bedford, MA). Briefly, 4 μL of each overnight culture well was spotted in duplicate on the

membrane and allowed to dry. The membranes were placed in a dish containing 3M Whatman paper saturated with 10% SDS for 3 min, transferred to a second tray containing 0.5 N NaOH, 1.5 M NaCl for eight minutes without agitation, before being transferred to another dish containing 1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM EDTA and immersed for 3 minutes with agitation. This second wash was repeated in a new dish with fresh solution. The membrane was air-dried at room temperature and DNA was fixed to the membrane by UV cross linking using a UV Stratalinker 2400 (Stratagene, La Jolla, CA) with the auto crosslink function.

Probes based on catfish CC chemokine cDNAs were prepared from previously cloned plasmids. Probes were prepared using the random primer labeling method (Sambrook et al. 1989) with a labeling kit from Roche Diagnostics (Indianapolis, Indiana). The membranes were pre-hybridized in 50% formamide, 5X SSC, 0.1% SDS (w/v), 5X Denhardt's and 100 μg/ml sonicated and denatured Atlantic salmon sperm DNA for 2 h. Hybridization was conducted for at least 16 h at 42°C in 50% formamide, 5X SSC, 0.1% SDS (w/v), and 100 μg/ml sonicated and denatured Atlantic salmon sperm DNA with probes added. The nylon membranes were washed first in 500 ml of 2X SSC for 10 min, followed by three washes in 0.2X SSC with SDS at 0.2% (w/v) at 45°C for 15 min each. The membranes were then wrapped in Saran wrap and exposed to Kodak BioMax MS film for autoradiography. Positive BAC clones were identified for each catfish CC chemokine and BAC DNA was isolated with the Qiagen R.E.A.L Prep 96 BAC DNA isolation kit (Qiagen, Valencia, CA) following the manufacturer's protocol.

Fluorescent fingerprinting and BAC contig construction

Positive BAC clones were fingerprinted and assembled, where possible, using the protocol described by Luo et al. (2003) with modifications. Briefly, BAC DNA was simultaneously digested with four 6-base pair (bp) recognizing restriction endonucleases (*Eco*RI, *Bam*HI, *Xba*I, *Xho*I) generating 3' recessed ends and one 4-bp recognizing restriction endonuclease (*Hae*III) generating a blunt end. Each of the four recessed 3' ends of restriction fragments was filled in using DNA polymerase with different fluorescent dyes using the SNaPshot kit (Applied Biosystems). Such labeling reactions allowed labeling of four sets of restriction fragments, providing a high level of confidence for contig assembly. Restriction fragments ranging from 50 bp to 500 bp were sized by an ABI PRISM 3130 XL automated sequencer producing *.fsa files. Genoprofiler (You et al. 2003) converts *.fsa files to *.sizes files which can be utilized by FPC (Soderlund, et al. 1997; 2000) for contig assembly. A 0.2 to 0.4 bp tolerance range was used in FPC, keeping the probability of coincidence (Sulston score, Sulston et al. 1988; 1989) low to avoid false assembly. A p-value of 10^{-10} was used for contig assembly. The results of BAC fingerprinting from FPC are image files of each BAC contig. Data from contig assembly were used with previous hybridization data to obtain the patterns of CC chemokine genes. Fingerprinting does not allow the user to discern the order of genes within each contig, therefore the order of genes was arbitrarily assigned. Each fingerprint contig or singleton should represent a different genomic region based on its restriction pattern. In order to assess the reliability of the BAC contigs, we conducted two types of analyses. First, overgo hybridization and cDNA

hybridization data was carefully analyzed to match the contigs assembled from fingerprinting; second, cut off p-values were varied, using a range of p-values from 10^{-10}, 10^{-8}, 10^{-6}, and 10^{-2}, in order to see how that would affect the contig assembly. Only by increasing the p-value for assembly to 10^{-2}, an unacceptably low standard for assembly, are any of the contigs or singletons merged together. In cases where the combination of contig assembly and hybridization suggested the presence of multiple gene copies, the letters of A-F were assigned to differentiate between distinct genomic copies of the catfish CC chemokines.

Fish rearing, bacterial challenge and sampling

Channel catfish were reared at the hatchery of the Auburn University Fish Genetics Research Unit. Challenge experiments were conducted as previously described (Dunham et al. 1993) with modifications. Briefly, catfish were challenged in a rectangular tank by immersion exposure for 2 h with freshly prepared culture of ESC bacteria, *E. ictaluri*. One single colony of *E. ictaluri* was isolated from a natural outbreak in Alabama (outbreak number ALG-02-414) and 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 tank to a concentration of 3 x10^7 CFU/ml. During challenge, an oxygen tank was used to ensure a dissolved oxygen concentration above 5 mg/L. After 2 h of immersion exposure, 15 fish were randomly taken and placed into a

rectangular trough containing pond water with constant water flow through. Replicates of troughs were used to provide one trough for each sampling time point. For the control fish, 15 fish were incubated in a separate rectangular tank with the same fish density as the challenge tanks. The only difference was that ESC bacteria were not added. After 2 h, these control fish were incubated in a separate trough at the same density as the challenged fish.

After challenge, head kidney and spleen samples were collected at 4 h, 24 h, and 3 days. At each time point, 10 fish were sacrificed for sampling. The fish were euthanized with tricaine methanesulfonate (MS 222) at 300 mg/L before tissues were collected. Tissues were kept in a –80 °C ultra-low freezer until preparation of RNA. Samples of each tissue from 10 fish were pooled. The pooled tissues were rapidly frozen with liquid nitrogen. In order to obtain samples representing the average of the 10 fish, the pooled tissue samples were ground with a mortar/pestle to fine powders and were thoroughly mixed. A fraction of the mixed tissue samples was used for RNA isolation.

RNA isolation and RT-PCR

RNA was isolated following the guanidium thiocyanate method (Chomczynski and Sacchi 1987) using the Trizol reagents kit from Invitrogen (Carlsbad, CA) following manufacturer's instructions. Extracted RNA was stored in a -70°C freezer until used as template for reverse transcriptase PCR (RT-PCR). The RT-PCR reaction was conducted using a two-step approach with M-MuLV reverse transcriptase (New

England Biolabs, Ipswich, MA) with the primers listed in Table 2. Detailed procedures followed the manufacturer's instructions (Wang et al. 2006a). Briefly, 1 μg of total RNA was used in each first-strand reaction. PCR reactions were carried out as described above with two modifications. The primers of β-actin (Table 2) were added to serve as an internal control. Challenge tissue RNA samples were amplified for 32 cycles. RT-PCR reactions were conducted for one gene at a time, and the images of agarose gels were compiled together into a single figure and, therefore, expression levels can only be analyzed separately for each gene.

Table 2 Primers for PCR and RT-PCR

Gene	Upper primer (5’ to 3’)	Lower primer (5’ to 3’)
SCYA101	TGTGTGCTGTAAGGAGGTTTCC	TTCTGTGGCACGATTGTGGTCG
SCYA102	CTGCACCTGGTAACTACCGTCG	GTTTCTTTGGGATCCAGCGTGC
SCYA103	TGCATGTACCCAAGTTTGGCAC	TTCATCAGTTCTTGCACCCAGG
SCYA104	TCTCTCCTGCTGGTTCTGCTGG	TAATTTGTCGCCGGAGTCTTGG
SCYA105	AGATACCAGACACAACCGATCC	GCTGATCAGTTGTTTGCTTGCT
SCYA106	GTCTCTTGGAGAGCAAGCACTG	CATCAGCTCTCTGACCCAGTCG
SCYA107	CAGCCAGAAGATCCGAAGCCTC	TGGAAGTGGAGCCGGTTGTCTG
SCYA108	TGCAAACGAACCAGAACCATGC	TCGGTTGAGGTTGGATCACGTC
SCYA109	ACCAGCGACACTTTCGTTCCAC	GCTCTTGCCATTACGGTTGTCC
SCYA110	ATGAGGAACCTGACGGCTCTGC	AGCTGTTGGATCCACACAGTGC
SCYA111	AGACGCTACCTATCAAGCGCTC	CAGTTGCGTGAAAGCTGCAGTG
SCYA112	TCGCTGGATGCTGGCTTCTGTG	TGACCTTGTTATGAGGTTGCTG
SCYA113	TCCACAAAGCCTGGTGGAATCC	AGTTGTTCTTTGTCGCACGAGG
SCYA114	ATGAGGAGCCTGGCTGCCATAG	GATGCAGGGAGGCAGTGGTTGG
SCYA115	TGGTGCTGCTGAGTGCAGTCAC	ACCCAGGCGTCAGTGGGTTTGG
SCYA116	ACTCCACTTCTCAGCTGCCCTG	CAAGGTGAGGACGGGTCCAAGC
SCYA117	TCCTGCACATCCTGAGGATTGC	TCTCAGTAGCCGGGACTTCACG
SCYA118	CACCACTGCAGTGTTCTCCAGC	TCTCCTTTGGAGCATCTGGTGC
SCYA119	TGGTGTTCTGCCTCTGTGCCAG	TGTTCTGTGGAATGGTCACCTC
SCYA120	CTGCTGGTTCTGCTGGGTCTCG	TGCGGTCTGCACACGCCTTACG
SCYA121	TCTGCATCCATCTGCTGAGAAC	GTGCGTACGTGTTGCGTCTCAG
SCYA122	TGAGCTTCACACACCTGCTGAG	AGCCTTGCTGTTCACACTGTGC
SCYA123	TCCTTCACAGCGGCTCAGAGTG	TGGGGTTTGCACACACACTACG
SCYA124	GCCTTCAGTCCTTCACAACAGC	TGACATCAGGGTCTGCACACAC
SCYA125	CTTCAGCCTGGCACAAGGTTCG	CTAGCGCAAATGAGCCGACCTC
SCYA126	TTCTACAGCGCCACTGAGTCGA	AGTTAGGTCTCAGAAACGTTGC
Actin	AGAGAGAAATTGTCCGTGACATC	CTCCGATCCAGACAGAGTATTTG

Phylogenetic analyses and comparative genomics

Phylogenetic trees were drawn from ClustalW (Thompson et al. 1994) generated multiple sequence alignments of amino acid sequences using the neighbor-joining method (Saitou and Nei 1987) within the Molecular Evolutionary Genetics Analysis

[MEGA (3.0)] package (Kumar et al. 2004). Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor joining trees was evaluated by 1000 bootstrapping replications.

Comparisons of genomic organization and architecture of the CC chemokines among catfish, chicken, and humans were made with the aid of BLAST searches, phylogenetic sequence comparisons, and searches against the Ensembl genome browsers for human, and chicken.

Results

Mapping catfish CC chemokine genes to BACs

We previously identified a total of 26 CC chemokines in catfish through the analysis of ESTs (He et al. 2004; Peatman et al. 2005) named SCYA101-SCYA126. In order to map these chemokine genes to BACs, overgo probes were designed based on the cDNA sequences and used to screen high-density BAC filters. Initially, pooled overgo probes for the 26 CC chemokines were used in the first screening that resulted in the identification of a pool of potential BACs positive to CC chemokine probes. The positive pool of BAC clones was picked from the arrayed BAC library and re-arrayed to nylon membranes for confirmation using individual cDNA probes. cDNA probes for each CC chemokine were used to screen the positive BACs.

As shown in Table 3, use of 26 cDNA probes in separate hybridizations resulted in 232 cumulative positive BAC hits for the catfish chemokine genes. The

hybridization pattern, however, indicated that many of the chemokine probes had positive results on the same BAC clones. Considering these overlaps, only 92 distinct BAC clones were represented in the positive set. This pattern of distinct cDNA probes hybridizing to the same BAC clones strongly suggested the presence of clusters of catfish CC chemokine genes in the genome context.

Table 3 Mapping of catfish CC chemokine genes to BACs through cDNA hybridization. A total of 92 unique BACs are represented in a cumulative total of 232 positive clones

Genes	Positive BAC clones
SCYA101	105_D15**,** 006_I13, 028_G4, 025_A20, 067_J3, 026_K13, 050_J5, 051_D5, 115_I22, 167_G22, 090_M4, 007_C11, 003_N13
SCYA102	104_A3,088_M10, 164_N20, 044_A24, 069_N2,108_I9, 126_K10, 117_D24, 149_M19, 011_N1, 125_O17
SCYA103	039_K13
SCYA104	152_F2, 122_C9, 147_M12, 062_A9, 141_G12, 015_J14, 069_A16, 066_B19, 082_A13, 091_H12, 159_B7
SCYA105	50_J5
SCYA106	029_L5, 080_O10, 143_I8, 097_I13, 161_K1, 103_L4, 189_G23, 129_N10, 098_H1
SCYA107	006_I13, 067_J3, 050_J5, 090_M4, 007_C11, 003_N13, 184_M14, 071_C6
SCYA108	042_A8, 052_C23, 149_I8
SCYA109	029_L5, 080_O10, 143_I8, 097_I13, 161_K1, 103_L4, 189_G23, 129_N10, 098_H1
SCYA110	030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 142_A8, 045_L17, 105_B8, 144_H14, 119_E18, 179_H22
SCYA111	030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 142_A8, 045_L17, 105_B8, 144_H14, 121_I22, 129_P14, 119_E18
SCYA112	105_D15, 006_I13, 067_J3, 026_K13, 167_G22, 090_M4, 007_C11
SCYA113	037_D15, 052_F9
SCYA114	090_M4, 164_N20, 044_A24, 069_N2, 126_K10, 117_D24, 009_P8
SCYA115	105_D15, 099_C4, 179_H22, 006_I13, 028_G4, 025_A20, 067_J3, 026_K13, 050_J5, 051_D5, 115_I22, 167_G22, 090_M4, 007_C11, 003_N13
SCYA116	006_I13, 067_J3, 050_J5, 003_N13, 184_M14
SCYA117	105_D15, 030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 045_L17, 105_B8, 144_H14, 107_K11, 102_J7, 153_I24, 154_F9, 163_F4, 072_K10, 125_D4, 073_P7, 121_I22, 065_H1, 061_G20, 129_P14, 059_H18, 119_E18, 062_I3, 167_E6, 045_09
SCYA118	067_J3, 090_M4, 003_N13, 184_M14
SCYA119	104_A3, 088_M10, 164_N20, 044_A24, 069_N2, 108_I9, 126_K10, 117_D24, 149_M19, 009_P8, 049_P12
SCYA120	152_F2, 122_C9
SCYA121	105_D15, 030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 142_A8, 045_L17, 105_B8, 144_H14, 107_K11, 102_J7, 153_I24, 154_F9, 163_F4, 072_K10, 125_D4, 073_P7, 121_I22, 065_H1, 061_G20, 129_P14, 059_H18
SCYA122	030_D8, 099_C4, 149_D11, 003_P23, 041_O13, 142_A8, 045_L17, 105_B8, 144_H14, 102_J7, 129_P14, 119_E18, 062_I3
SCYA123	042_A8, 152_F2, 122_C9, 029_N24, 147_M12, 052_C23, 149_I8, 143_P9, 139_D5
SCYA124	152_F2
SCYA125	031_N17, 042_B8, 188_D18, 192_L24, 163_G22, 158_L9
SCYA126	099_C4, 142_A8, 045_L17, 105_B8, 144_H14, 102_J7, 121_I22, 129_P14, 119_E18, 062_I3 047_K12

Genomic clustering and duplication of catfish CC chemokine genes

Given the likelihood of genomic clustering of CC chemokine genes within the channel catfish genome, we utilized our pool of positive BAC clones for analysis using fluorescent fingerprinting to determine genomic copy numbers and cluster membership.

The fingerprinted contigs and singletons (those BAC clones that did not assemble with others) are listed in Table 4. A total of 18 contigs were constructed after BAC fingerprinting, and an example of the contigs is shown in Fig. 1. Eight BAC clones for which we had hybridization data were not assembled into contigs and are listed as singletons at the bottom of the table. A pattern of gene duplication and clustering was immediately obvious from the merged data from fingerprinting and hybridization. Only five CC chemokines, SCYA103, SCYA105, SCYA108, SCYA113, and SCYA124, were present in a single copy. Five CC chemokines have at least two copies in the catfish genome—SCYA110, SCYA111, SCYA116, SCYA118, and SCYA125. Three genomic copies were found for eight of the catfish CC chemokines including SCYA102, SCYA104, SCYA106, SCYA109, SCYA119, SCYA120, SCYA122, and SCYA126. Four copies were found for five of the catfish CC chemokines including SCYA101, SCYA112, SCYA114, SCYA115, and SCYA126. Two CC chemokines, SCYA121 and SCYA123, had five genomic copies. Lastly, six distinct genomic copies were found for SCYA117 (Table 4).

Table 4 Contigs and singletons produced by fluorescent fingerprinting of catfish BAC clones. BAC contigs were constructed using fluorescent fingerprinting with a cut off p-value of 10^{-10}. BAC clones containing CC chemokine genes were initially selected for fingerprinting by pooled overgo probes, and, in most cases, also confirmed by using individual cDNA probes. Assignment of letters A-F to chemokine genes was arbitrary to differentiate between distinct copies of chemokines in different genomic regions. "*" indicates two distinct copies as determined by direct sequencing

Contigs	Chemokines together based on fingerprinting and/or cDNA hybridization	BAC clones in each contig/singleton
1	125A	163_G22, 055_F15, 135_J15, 045_I14, 003_B23, 015_L09, 013_M08, 190_O24, 186_O14, 103_I22, 163_G22, 136_B14, 080_J22, 139_A20, 082_G20, 176_O19
2	103	039_K13, 183_K11, 151_B13, 064_C5, 068_K21, 127_B21, 182_I19, I82_K19, 120_O3, 071_O4, 110_J16, 176_K21
3	113	037_D15, 052_F9, 146_N23, 093_C8, 025_C1
4	102A-114C-119A	104_A3, 088_M10, 009_P8
5	102B-114A,B*-119B-107A	164_N20, 044_A24, 069_N2,108_I9, 126_K10, 117_D24, 149_M19, 011_N1, 071_C6
6	107B-101A-112A-115A-116A-118A	006_I13, 028_G4, 067_J3, 003_N13, 184_M14
7	107C-101B-112B-115B-116B-118B-114D-105	050_J5, 090_M4
8	101C-112C-115C	025_A20, 026_K13, 051_D5, 115_I22, 167_G22, 007_C11
9	101D-112D-115D-117A-121A	105_D15, 154_F9, 073_P7, 065_H1
10	117B-121B	153_I24, 072_K10, 125_D4, 061_G20, 059_H18, 167_E6, 045_09
11	117C-121C-110A-111A-122A-126A	030_D8, 003_P23, 041_O13, 142_A8, 121_I22, 129_P14, 102_J7, 163_F4
12	117D-121D-110B-111B-122B-126B-115E	099_C4, 149_D11, 045_L17, 105_B8, 144_H14, 119_E18, 179_H22
13	126C	047_K12, 108_F9, 103_G10
14	106A-109A	097_I13, 161_K1, 129_N10
15	106B-109B	029_L5, 080_O10, 143_I8, 103_L4
16	106C-109C	189_G23, 182_C23, 050_E23
17	108-123A	042_A8, 052_C23, 149_I8, 029_N24, 143_P9, 139_C13
18	104A-123B	147_M12, 062_A9, 141_G12, 015_J14, 069_A16, 066_B19, 082_A13, 091_H12, 159_B7
Singletons		
1	104B-120A,B*-123C	122_C9
2	104C-120C-123D-124	152_F2
3	123E	139_D5
4	117E-121E	107_K11
5	117F-122C-126D	062_I3

6	119C	049_P12
7	125B	158_L9
8	102C	125_O17

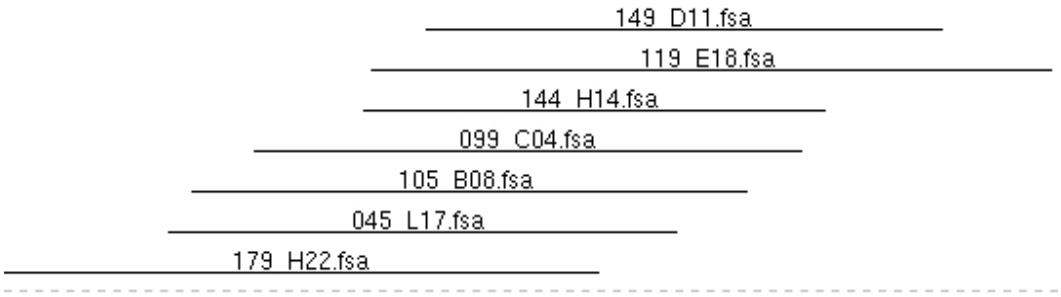

Fig. 1 Example of fingerprinted BAC contigs--contig 12 containing 117D-121D-110B-111B-122B-126B-115E. Identifiers on each line are BAC clone names. Note that the contigs do not allow the determination of CC chemokine gene arrangement order.

Eighteen of the fingerprinted BAC contigs or singletons contained more than one catfish CC chemokine gene, illustrating extensive and repetitive genomic clustering. Clusters of genes ranged in size from containing eight genes (Contig 7) to containing only two (numerous contigs). Membership within the different contigs was often highly similar or identical, suggesting that segmental gene duplication was likely responsible for the genesis of many of these clusters. For example, there are three contigs containing SCYA106 and SCYA109, each in a different contig. Likewise, contigs 11 and 12 share identical members with the exception of SCYA115. Several of the catfish CC chemokines, such as SCYA117 and SCYA121, are present in both the smaller contigs (i.e. Contig 10) and the larger clusters (i.e. Contigs 9,11), indicating possible genomic rearrangements.

Genomic architecture and phylogenetic analysis

Genomic sequencing allowed us to previously obtain the encoding genes for 23 of the 26 catfish CC chemokine cDNAs (Bao et al. 2006a). When the deduced amino acid sequences of the coding regions of the 23 genes and the three cDNAs were subjected to phylogenetic analysis, and sequence similarity compared with genomic location, several interesting patterns emerged (Fig. 2). In several instances there was a high correlation between sequence similarity and genomic architecture. For instance, SCYA123, SCYA108, SCYA124, SCYA120A, and SCYA120B are located together in the genome, and they also share a branch of the phylogenetic tree as analyzed by sequence similarities. A very strong clade containing SCYA111, SCYA121, SCYA117, and SCYA122 is present on the tree, and the four CC chemokines also are found together in Contigs 11-12. Two additional and similar correlations between tree position and genomic architecture can be seen for SCYA106-SCYA109 and for SCYA116-SCYA118-SCYA102-SCYA101-SCYA107-SCYA114A-SCYA114B (Fig. 2). Such correlations provide additional support for the theory that tandem and/or segmental gene duplications were involved in the evolution of the catfish CC chemokine genes.

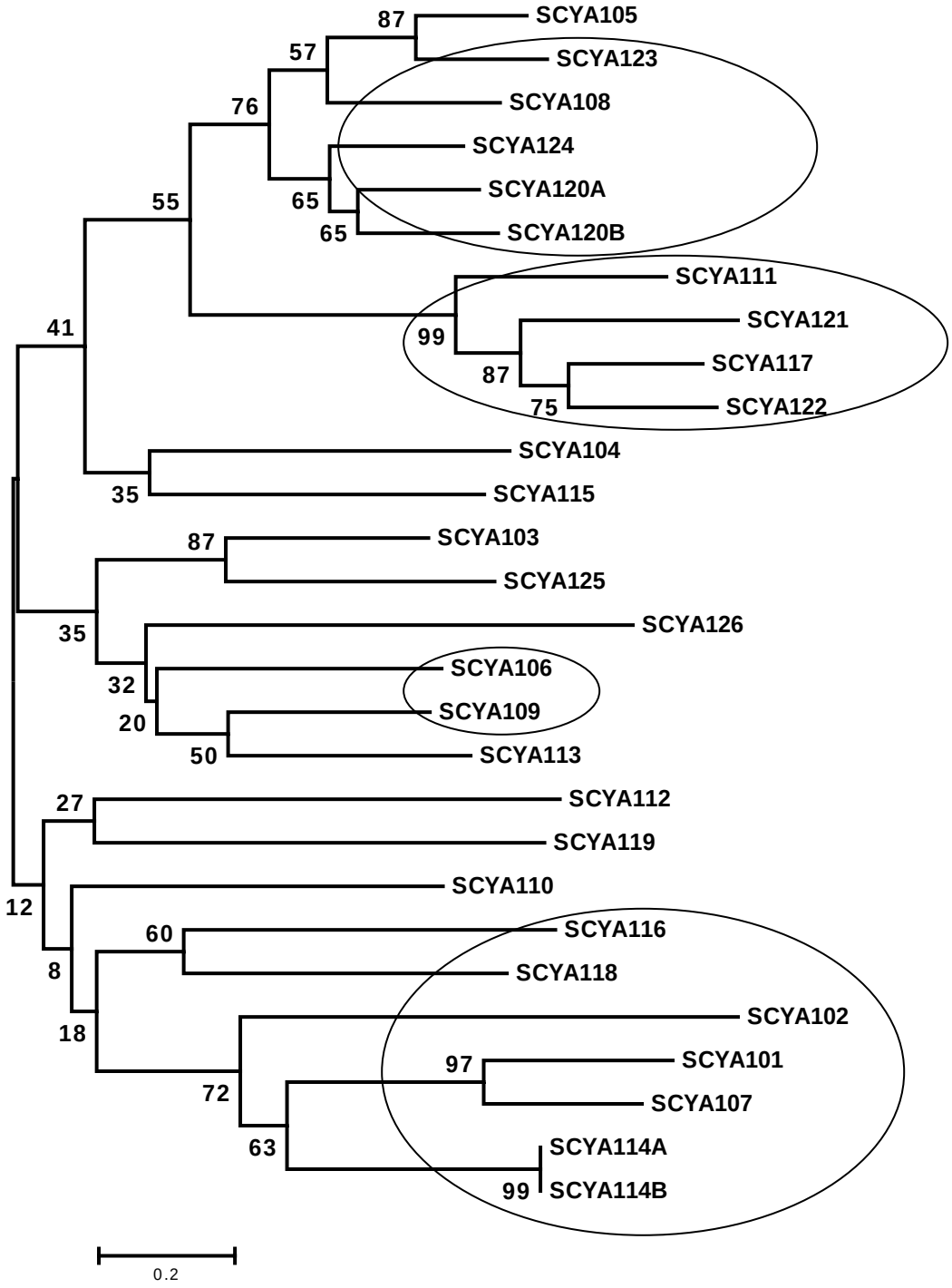

Fig. 2 The phylogenetic tree was drawn from ClustalW generated multiple sequence alignment of amino acid sequences using the neighbor-joining method within the MEGA

(3.0) package. Data were analyzed using Poisson correction and gaps were removed by complete deletion. The topological stability of the neighbor joining trees was evaluated by 1000 bootstrapping replications, and the bootstrapping values are indicated by numbers at the nodes. Circles indicate chemokines sharing both sequence similarity and genomic architecture as described in the text. GenBank accession numbers of the sequences used are: DQ173276 (SCYA101), DQ173277 (SCYA102), DQ173278(SCYA103), DQ173279(SCYA104), AY555502(SCYA105), DQ173280(SCYA106), DQ173281(SCYA107), DQ173282(SCYA108), DQ173283(SCYA109), DQ173284(SCYA110), DQ173285(SCYA111), DQ173286(SCYA112), DQ173287(SCYA113), DQ173288(SCYA114A), DQ173289(SCYA115), DQ173290(SCYA116), DQ173291(SCYA117), DQ173292(SCYA118), DQ173293(SCYA119), DQ173294(SCYA120A), DQ173295(SCYA121), DQ173296(SCYA122), CB937548(SCYA123), DQ173297(SCYA124), BM028237(SCYA125), and DQ173298(SCYA126).

Expression analysis of catfish CC chemokine genes

We previously reported the expression of 12 catfish CC chemokine (SCYA115-SCYA126) after challenge with *Edwardsiella ictaluri* (Peatman et al. 2005). To determine expression patterns of all known catfish CC chemokines, here we conducted expression analysis of the remaining 14 known catfish CC chemokines (SCYA101-SCYA114) using RT-PCR in the head kidney and spleen tissues from both healthy fish and fish challenged with the bacterial pathogen *Edwardsiella ictaluri*. In order to be

able to compare information on expression of all 26 known catfish CC chemokines, we present here the novel expression data combined with previously published expression data on SCYA115-126. As shown in Fig. 3, and summarized in Tables 5 and 6, four main expression patterns were observed. The majority (16) of the 26 CC chemokines were, on the whole, constitutively expressed with no effect observed after bacterial infection (Fig. 3). These included SCYA102, SCYA104, SCYA107, SCYA110, SCYA111, SCYA119, SCYA120, SCYA122, SCYA123, SCYA124, SCYA126, SCYA101, SCYA106, SCYA108, SCYA114, and SCYA103. Of these, SCYA101, SCYA106, SCYA108, and SCYA114 may be slightly up-regulated, and SCYA103 may be slightly down-regulated, but the extent of up- or down-regulation was minor, and for the purpose of discussion here, we categorized them into the constitutively expressed group.

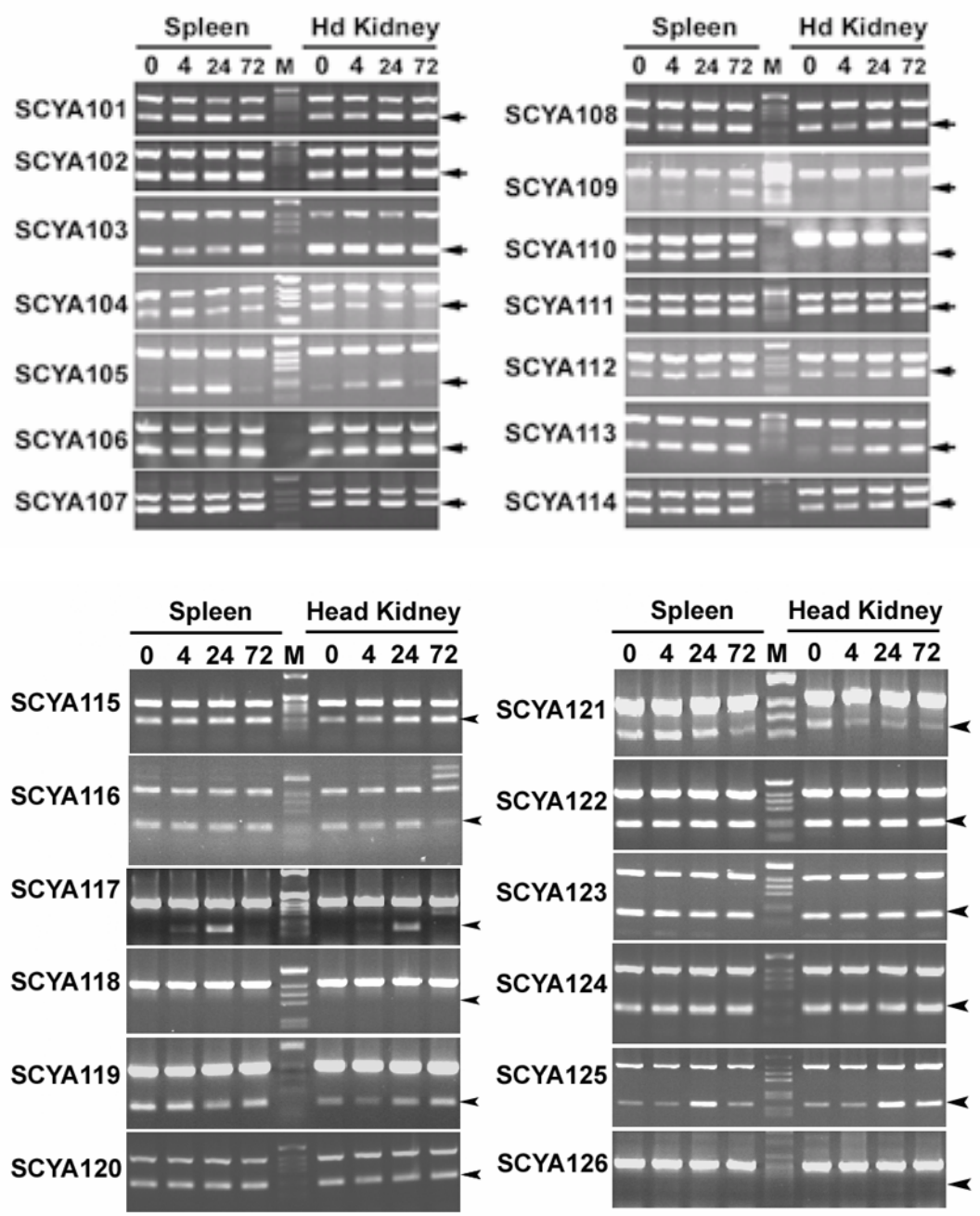

Fig. 3. Expression analysis of the 26 catfish CC chemokines using RT-PCR. RT-PCR reactions were conducted as described in the Materials and Methods. RT-PCR products were analyzed by agarose gel electrophoresis. Two tissues, spleen and head kidney (Hd kidney), were used in the study, as indicated at the top of the figure. The names of the

catfish CC chemokines were indicated on the left margins of each panel of the gels. Samples from healthy fish (0) and infected fish at 4h (4), 24h (24), and 72h (72) were used. Molecular marker (M) was 1-kb ladder purchased from Invitrogen. Arrows indicate the expected positions of the catfish CC chemokine RT-PCR products. The RT-PCR product of the internal control, beta-actin, was not indicated, but in all cases, it was the upper band on the gel. Note that RT-PCR reactions were conducted for one gene at a time, and the images of agarose gels were compiled together into a single figure and, therefore, expression levels can only be analyzed separately for each gene. Note also that 32 PCR cycles were used for SCYA119 and SCYA121, whereas 29 cycles were used for the remaining chemokines.

Table 5 Up-regulated CC chemokines. NC denotes no change in expression; 0 indicated no expression detected; "+" indicates slightly up, "++" indicates intermediately up; and "+++" indicates greatly up. All comparisons of expression levels are within each individual gene and not among the other genes

	Spleen			Head kidney		
	4h	**24h**	**72h**	**4h**	**24h**	**72h**
SCYA105	++	+++	NC	++	+++	NC
SCYA117	++	+++	+	++	+++	+
SCYA109	+	+	+++	0	0	0
SCYA112	NC	NC	++	NC	NC	++
SCYA113	NC	NC	NC	+	++	+++
SCYA115	NC	NC	NC	NC	+	++
SCYA125	NC	++	+	NC	++	+

Table 6 Down-regulated CC chemokines. The asterisk (*) indicated the presence of additional PCR bands for SCYA116. All comparisons of expression levels are within each individual gene and not among the other genes

	Spleen			Head kidney		
	4h	**24h**	**72h**	**4h**	**24h**	**72h**
SCYA116	NC	NC	NC	NC	NC	-*
SCYA121	NC	NC	-	-	-	-

Seven of the 26 CC chemokines were up-regulated upon bacterial infection (Fig. 3 and Table 5). These included SCYA105, SCYA109, SCYA112, SCYA113, SCYA115, SCYA117, and SCYA125. Of these up-regulated CC chemokines, the most interesting were SCYA105, SCYA109, and SCYA117, which were expressed at very low levels before infection, but their expression was dramatically induced after challenge (Fig. 3, Peatman et al. 2005).

SCYA116 and SCYA121 were down-regulated upon bacterial infection (Fig. 3 and Table 6). The down-regulation was more evident with SCYA121, which showed a significant reduction of RT-PCR products in both the head kidney and spleen, but the response was more rapid in head kidney than in spleen (Fig. 3). SCYA116 expression was lower three days after infection; extra bands were detected using RT-PCR, possibly from unspliced products. With SCYA118 and SCYA126, no expression was detected at any time point of the analysis. In order to confirm the lack of expression for SCYA118 and SCYA126, PCR amplification was repeated with extended cycles, but no products were observed.

Differences were observed in the time points and tissues involving up-regulation and down-regulation. For instance, SCYA105, SCYA117 and SCYA125 were rapidly and highly induced after bacterial infection in both the spleen and the head kidney tissues, whereas SCYA109 was only induced in spleen, but no expression was detected from head kidney. In contrast, SCYA115 was moderately upregulated only in the head kidney, but not in the spleen. SCYA121 expression was down-regulated in both spleen and head kidney tissues, but more rapidly in head kidney (Fig. 3).

Discussion

In this study, all 26 previously identified catfish CC chemokine cDNAs were mapped to BAC clones, setting the foundation for comparative genome analysis in the genomic regions containing chemokine genes. Through a combination of cDNA probe hybridizations and fluorescent fingerprinting, 18 fingerprinted contigs were assembled from BACs containing catfish CC chemokine genes. The catfish CC chemokine genes were found to be not only extensively clustered in the catfish genome, but also highly duplicated at various levels. As many as six copies of a single catfish chemokine were found from separate genomic regions. Although a draft genome is not available for catfish, our approach allowed us to study the local genomic architecture of the catfish CC chemokines in order to better understand the origins and orthologies of these important immune molecules. With genome-enablement still years away in many economically important species, our methods may serve as an important model for researchers working with other similar species who want to harness genome information on a limited budget. Here we have also analyzed the expression of the transcripts of the 26 catfish CC chemokines in head kidney and spleen in response to bacterial infection of *Edwardsiella ictaluri*, an economically devastating catfish pathogen. Such analysis will allow us to concentrate research efforts on the CC chemokines most likely involved in inflammatory responses.

The clustering of CC chemokine genes on chromosomes was previously revealed in human, mouse, and chicken (Nomiyama et al. 2001, Wang et al. 2005). In humans, the largest group of CC chemokine genes is located on chromosome 17, and

several clusters of CC chemokines genes are also found on chromosomes 7, 9, and 16. Chicken has a large cluster of CC chemokines on chromosome 19, with member genes orthologous to CC chemokines on human chromosome 17. A segment of mouse chromosome 11 additionally corresponds to human chromosome 17. In the case of both chicken and mouse, however, syntenies are only partially conserved with humans. For example, chicken has three genes on chromosome 19 corresponding to a single gene, CCL13, on human chromosome 17. Likewise, mouse has two genes CCL9 and CCL6 on Chromosome 11 that lack orthologues in human despite conservation of the genomic neighborhood. This phenomenon, coupled with the high sequence similarity between the non-orthologous CC chemokines of a given species, is highly suggestive of a pattern of species-specific gene duplications and changes after species divergence. An expectation of distinct expansions of the CC chemokine family within each species means that the identification of orthologues by phylogenetic analysis will be largely unsuccessful. Identification of a smaller ancestral set of CC chemokines and comparisons of genomic organization and architecture across species, therefore, may be more realistic aims for those describing novel sets of chemokines in lower vertebrates.

Duplication of CC chemokines within the human genome, before largely unanalyzed or ignored, has become an important matter for research only lately. The discovery that CC chemokine receptor 5 (CCR5) is an entry point for infection of cells by HIV-1 (Alkhatib et al. 1996), and that CCL3 and CCL4, by binding to CCR5, limit infection by HIV-1 (Nibbs et al. 1999), increased interest in understanding the chemokine repertoire and their functions. More recently, researchers have focused on CCL3 and CCL4 duplications and their correlation in disease severity (Townson et al.

2002). CCL3L1 and CCL4L1 have been discovered in segmental duplications on chromosome 17 (Modi 2004). Of greatest note has been a recent study strongly correlating copy number of a segmental duplication encompassing the gene encoding CCL3L1 with HIV/AIDS susceptibility. Possession of a copy number of CCL3L1 lower than the population average markedly enhances susceptibility (Gonzalez et al. 2005; reviewed by Julg and Goebel, 2005). Additional correlations have been made between copy numbers or chemokine loci and other diseases such as tuberculosis (Jamieson et al. 2004). Chemokine architecture and duplication, therefore, is an important matter for investigation in studies of the innate immune components of lower vertebrates. Comparison of the major CC chemokine clusters across vertebrate species may reveal important patterns of divergence or conservation and help to pinpoint similar disease quantitative trait loci (QTL) in agricultural species.

Sequence and phylogenetic analyses are currently not capable of establishing orthologies between the majority of mammalian CC chemokines and fish CC chemokines (He et al. 2004; Laing and Secombes 2004b; Peatman et al. 2005), probably because of the pattern of duplication and divergence described above. Nonetheless, we attempted to match the genomic segments containing catfish CC chemokine genes we obtained through fingerprinting with the largest of the human chromosome cluster of CC chemokines (Fig. 4). It must be noted that we are still missing the larger genomic context of these chemokine-containing contigs. Since only BAC clones positive for CC probes were included in the fingerprinting, we lack the surrounding genomic regions. A physical map of the catfish genome, currently under construction, when linked with available linkage maps, will tell us whether these contigs are distinct contigs on the

same chromosome or on entirely different chromosomes. Based on our current knowledge, therefore, we aligned the larger of the catfish clusters with the human chromosomal segments using their top BLASTX identities. Some of the genomic segments of catfish appeared loosely conserved, in that all chemokine gene members shared highest identities with CC chemokines on the same chromosomal stretch in humans (i.e. SCYA108, SCYA123, SCYA120, SCYA124, and SCYA104). Other segments, such as the one containing SCYA107, SCYA101, SCYA112, SCYA105, SCYA116, SCYA118 and SCYA115, showed no discernible pattern of conservation. From this contig, SCYA101 and SCYA107 appear to be fish specific CC chemokines (He et al. 2004) while SCYA112 shares clear orthology with human CCL20 which is localized on Chromosome 2. Other chemokines from this contig share highest identities with CC chemokines on human chromosome 17 (Fig. 4). One notable feature of this "comparative map" was that many of the catfish CC chemokines share highest identity with CCL3 or CCL14. These CC chemokines may represent part of the ancestral repertoire before species-specific duplications and divergence.

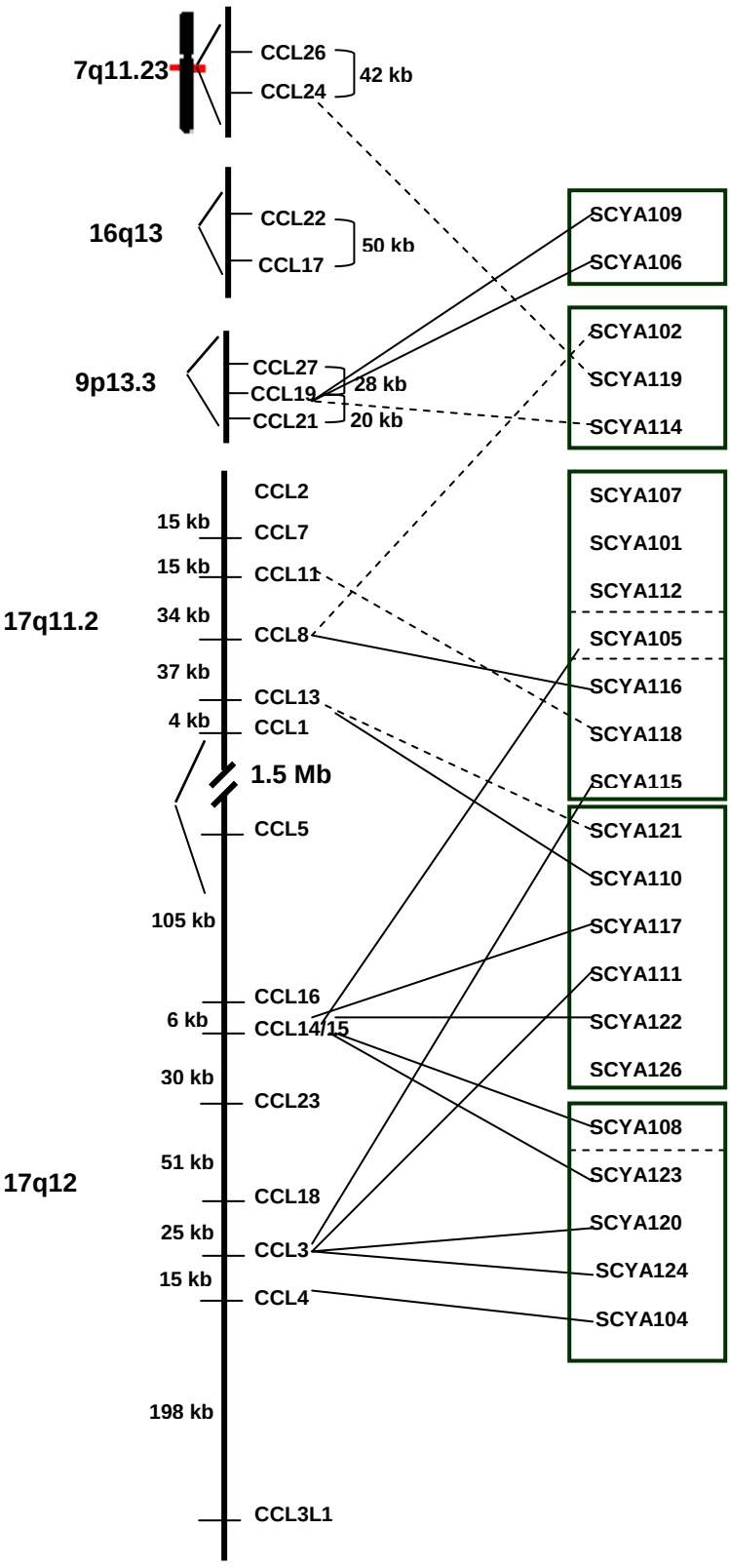
7q11.23
CCL26
CCL24
42 kb
16q13
CCL22
CCL17
50 kb
9p13.3
CCL27
CCL19
CCL21
28 kb
20 kb
17q11.2
CCL2
15 kb
CCL7
15 kb
CCL11
34 kb
CCL8
37 kb
CCL13
4 kb
CCL1
1.5 Mb
CCL5
105 kb
CCL16
6 kb
CCL14/15
30 kb
CCL23
17q12
51 kb
CCL18
25 kb
CCL3
15 kb
CCL4
198 kb
CCL3L1
SCYA109
SCYA106
SCYA102
SCYA119
SCYA114
SCYA107
SCYA101
SCYA112
SCYA105
SCYA116
SCYA118
SCYA115
SCYA121
SCYA110
SCYA117
SCYA111
SCYA122
SCYA126
SCYA108
SCYA123
SCYA120
SCYA124
SCYA104

Fig. 4. A comparison of genomic contigs containing catfish CC chemokines with clusters of human CC chemokines on several human chromosomal segments. Chromosomal segment names for human are given on the left. Distances between the human CC chemokines are noted in kilo-bases (kb) or mega-bases (Mb). Orientation of the catfish contigs is unknown, and was arranged based on BLASTX identities as described in the text. Dashed lines between the catfish and human CC chemokines indicate especially low BLASTX identity. Boxes indicate catfish genomic contigs. Dashed lines within the catfish contigs surround SCYA105 and SCYA108, whose genomic contigs were merged with those shown to avoid showing duplicate copies in the figure. Note that the order and orientation of individual catfish CC chemokine genes were not determined and their relative positions shown in the contig (box) were arbitrary.

Sequence similarities between the catfish CC chemokines correlated strongly with genomic architecture (Table 4, Fig. 2) strongly suggesting tandem and segmental gene duplications as the evolutionary mechanism responsible for the diversity of these molecules presently in catfish. The 26 CC chemokine cDNAs with the additional genomic copies revealed by fingerprinting leaves us with a tentative total of at least 75 genes. However, due to the relatively small pool of catfish ESTs in the GenBank, it is not possible yet to provide solid EST evidence for these genes. Using the 26 genes as queries against catfish ESTs allowed the identification of 186 catfish CC chemokine-related ESTs. Cluster analysis using relatively stringent overlapping (90 bp) allowed us

to identify 18 additional CC chemokines with protein sequences different from the queries. Thus, at the molecular level, it appeared that EST evidence as available now supports the presence of additional genes in channel catfish, consistent with our conclusions made from the BAC-based contig analysis. It is noteworthy that BAC-based physical analysis is accurate in answering whether sequences similar to CC chemokine genes physically exist in the catfish genome. Whether such sequences are transcribed requires further analysis using transcriptome approaches. Future work, including BAC sequencing and FISH, will help resolve questions related to the ontogeny of this large catfish gene family.

Most of the inducible/inflammatory human CC chemokines are highly clustered on chromosome 17, while the constitutive/homeostatic CC chemokines are on other chromosomes (Moser et al. 2004). Despite the extensive clustering of the catfish CC chemokines, a similar correlation between inducible expression and genomic architecture was not observed after infection with *Edwardsiella ictaluri* in head kidney and spleen tissues. Patterns of expression of genes within the same genomic clusters often differed (i.e. SCYA117, SCYA121, SCYA122, SCYA126). It is possible that the highly duplicated nature of the catfish CC chemokine genes has allowed division of roles that may be manifested in spatial, temporal, or functional differences. Further functional studies are needed to pinpoint the catfish CC chemokines integral to successful innate immune responses against bacterial and viral pathogens, particularly with regard to their function in directing leukocyte traffic after infection.

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III. MICROARRAY-BASED GENE PROFILING OF THE ACUTE PHASE RESPONSE IN CHANNEL CATFISH (*ICTALURUS PUNCTATUS*) AFTER INFECTION WITH A GRAM NEGATIVE BACTERIUM

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 (APP) 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. Analysis of microarray results using a traditional two-fold change in gene expression cutoff and a 10% false discovery rate revealed a well-developed APR in catfish, with particularly high up-regulation (>50-fold) of genes involved in iron homeostasis (i.e. intelectin, hemopexin, haptoglobin, ferritin, and transferrin). Other classical APP genes upregulated greater than two-fold included coagulation factors, proteinase inhibitors, transport proteins, and complement components. Up-regulation 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 up-regulated genes with real-time RT-PCR confirmed microarray results.

1. Introduction

The acute phase response (APR) is a group of rapid physiological responses to infection or injury (Bayne and Gerwick et al. 2001; Gabay and Kushner 1999) and is one of several components which 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 (Pandey and Agrawal 2006). 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 (APP) Fey and Gauldie 1990). 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 (Schillaci and Pirro 2006), but many are now known to play beneficial roles in mediating the complex inflammatory response and seeking to restore homeostasis(Gabay and Kushner 1999).

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 (Meyer 1991). 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 (Bayne et al. 2001; Gerwick et al. 2002; Russell et al. 2006) and recently using a small oligo-based microarray (Gerwick et al. 2007). Expression of a number of acute phase reactants has also recently been measured in zebrafish *Danio rerio* using real-time PCR (Lin et al. 2007).

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 (Bengten et al. 2006). 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 four days after onset of infection (Newton et al. 1989; Wolters et al. 1994). 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 (He et al. 2004; Baoprasertkul et al. 2004; Chen et al. 2005a; Baoprasertkul et al. 2005; Peatman et al. 2005; Bao et al. 2006a; 2006b; Peatman et al. 2006; Wang et al. 2006a; 2006b; 2006c; Bao et al. 2005; Xu et al. 2005; Baoprasertkul et al. 2006; 2007) and identified additional immune-related genes through EST sequencing (Ju et al. 2000; Cao et al. 2001; Karsi et al. 2002; Kocabas et al. 2002). 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 (Li and Waldbieser 2006). By adding 7,159 additional transcripts from blue catfish (*Ictalurus furcatus*), a closely-related species to channel catfish sharing greater than 98% nucleotide similarity within cDNA transcripts (He et al. 2003), 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 acute phase response 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 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 (over the course of 1 week) brought from 18°C to 27°C 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 d control (3 aquaria), 3 d 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 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 $4X10^8$ 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 (3 control replicates, 3 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 1x T7 enzyme mix in 1x 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 to 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 (Li and Waldbieser 2006). 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, 7,159 unique ESTs from the closely-related species blue catfish (*Ictalurus 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 orthologues 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. (2004). 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 (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.

The microarrays were prehybridized with a solution of 2x 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 2X MES buffer at 45°C for 16 h with constant rotation in a hybridization oven. After hybridization, the microarrays were washed twice with nonstringent buffer (6x 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 one minute rinse with nonstringent buffer, the arrays were placed into a 1x 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 one minute. 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 3 control pools and 3 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 (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 log2 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 (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; Larsson et al. 2005). The *q*-value, therefore, reflects the variability present in the data set for a given gene. A list of differentially expressed genes with at least 2-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 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 channel catfish and blue catfish putative orthologues of the same gene were differentially expressed, 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 paralogues, both transcripts were kept on the unique list. A list of all sequences (including redundancies) meeting the threshold parameters can be found as Supplementary Tables S1 and S2.

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 per second; (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 catfish (Murdock et al. 2006). 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 (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 (<0.35 change in Ct). Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis.

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
18S	BE469353	TGCGCTTAATTTGACTCAACAC	CGATCGAGACTCACTAACATCG

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 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 Robust Multichip Average program (Irizarry et al. 2003), the resulting expression intensity values were analyzed in SAM (Significance Analysis of Microarrays) (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, 301 transcripts were significantly upregulated, and 6 were significantly downregulated (Supplemental Tables 1 and 2—see appendices). Of the 301 upregulated catfish transcripts, 207 of these are believed to represent unique genes, and 5 of the 6 significantly downregulated transcripts were unique. The redundant transcripts resulted either from blue and channel putative

orthologues 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 10-85 fold following infection; 16 genes were upregulated from 5-10 fold; 27 genes were upregulated from 3-5 fold; and 150 genes were upregulated from 2-3 fold (Table 2).

Table 2 Profile of significant, differentially-expressed genes in catfish following *E. ictaluri* infection.

Transcripts on the array	28,518
Number of upregulated transcripts	301
Number of unique upregulated genes	207
Number of unique genes upregulated >10 fold	14
Number of unique genes upregulated 5-10 fold	16
Number of unique genes upregulated 3-5 fold	27
Number of unique genes upregulated 2-3 fold	150
Number of downregulated transcripts	6
Number of unique downregulated genes	5

3.3. Putative identities of differentially expressed genes after infection with Edwardsiella 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 3 and 4 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 3.

Table 3 Catfish genes upregulated 5-fold or greater in the liver following *E. ictaluri* infection. *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

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 paralogues
BM438689	Microfibrillar-associated protein 4	25.6	0.00	Same as above—putative paralogues
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 orthologue 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 paralogues
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 paralogues
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

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 3) represented APPs (Bayne et al. 2001), including coagulation factors, proteinase inhibitors, transport proteins, and complement components. Many of the APPs were upregulated greater than 5-fold (Table 3).

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 1);

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), 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 3; Supplementary table S3).

3.6. Downregulated genes

A much smaller number of catfish transcripts were significantly downregulated following infection with a narrow range of suppression (Table 4). These included liver-expressed antimicrobial peptide-2, which is believed to be involved in the defense

response to bacteria (Bao et al. 2006b), and thioredoxin-interacting protein which functions in the oxidative stress response in mammals.

Table 4 Unique, significantly downregulated catfish transcripts in liver after *E. ictaluri* infection. *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

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

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

Expression patterns of five genes identified by microarray analysis as differentially expressed following infection were selected for confirmation using qRT-PCR. Genes upregulated ranging from 2-fold to 85-fold in the microarray experiment were selected and primers designed (Table 1). qRT-PCR results (Table 5) generally confirmed the microarray results, with all tested genes showing statistically significant upregulation greater than 2-fold ($P<0.05$). 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 5), and perhaps also due to the greater accuracy in quantitation by qRT-PCR than by microarrays.

Table 5 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)

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*. The challenge inherent to microarray expression analysis is to move inward from large sets of raw data to a smaller set of significant results and, finally, to answers to biological questions. 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 (Rise et al. 2004b; Meijer et al. 2005; Kurobe et al. 2005; Mancia et al. 2006) and for future functional characterization, genetic mapping, and QTL analysis (Xu et al. 2006). Our results will allow us to fulfill these aims and move towards 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 (Lam et al. 2006; Krasnov et al. 2005c; Williams et al. 2006; Rise et al. 2006; Ju et al. 2006) 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 (Gerwick et al. 2007) and other previous salmonid and carp microarray experiments measuring expression in liver after application of a variety of stressors (Tilton et al. 2005; Ewart et al. 2005; Martin et al. 2006; Reynders et al. 2006). 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 (Gabay and Kushner 1999) upregulated greater than 2-fold in catfish were haptoglobin, hemopexin, ferritin, transferrin, ceruloplasmin, fibrinogen, 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 (Gerwick et al. 2007) and as measured by real-time PCR in zebrafish (Lin et al. 2006), indicating the likely conservation of function of the vast majority of APP between mammals and teleost fish. Comparative expression analysis under specific conditions could provide fish biologists with important shortcuts towards a better understanding of function of genes related to immunity and inflammatory responses.

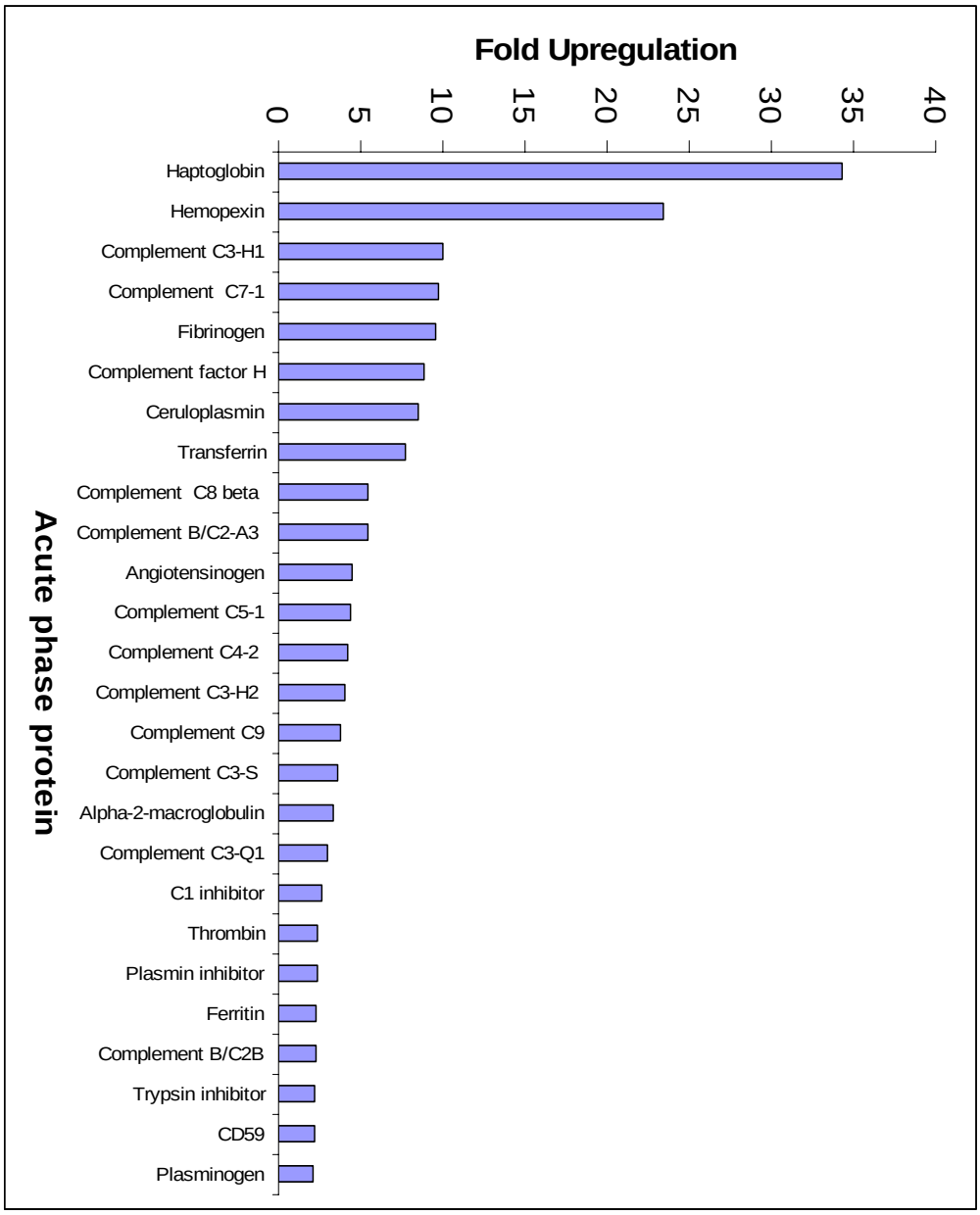

Fig. 1 APP genes upregulated two-fold or greater in channel catfish following infection with *E. ictaluri*.

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 Reynders et al. 2006; Gerwick et al. 2007), microfibrillar-associated protein 4 (Reynders et al. 2006), Toll-like receptor 5 (Ewart et al. 2005; Lin et al. 2007), neurotoxin/differentially regulated trout protein (Tilton et al. 2005; Gerwick et al. 2007; Martin et al. 2006; Ewart et al. 2005), SEC31/high affinity copper uptake protein (Martin et al. 2006), and SEC61 (Gerwick et al. 2007).

Intelectin was the most highly upregulated catfish transcript in liver following infection (Table 3). Five transcripts representing intelectin on the catfish microarray were upregulated greater than 5-fold (Supplemental Table 1). qRT-PCR showed a 545-fold upregulation in gene expression following infection (Table 5). 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.

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 (Reynders et al. 2006). 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 (Boshra et al. 2006).

Toll-like receptor 5 is a well-characterized pathogen recognition receptor in both mammals and fish (Gewirtz et al. 2001; Tsujita et al. 2004). TLR5 has been well-characterized in catfish previously and was shown to be upregulated in the liver following ESC infection (Bilodeau and Waldbieser 2005; Bilodeau et al. 2006) and was upregulated in the spleen after LPS exposure (Li and Waldbieser et al. 2006). 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 (Bayne et al. 2001). While its function is unknown, it shares some similarity with membrane attack complex inhibitor CD59.

The absence of the iron regulatory hormone hepcidin (Park et al. 2001) 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 (Gerwick et al. 2007; Tilton et al. 2005; Ewart et al. 2005; Martin et al. 2006; Lin et al. 2007), confirmed our previous expression studies which showed that hepcidin was not upregulated three days after infection (Bao et al. 2005). 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 (Nemeth et al. 2004a). This leads to drastically decreased plasma iron levels during infection, a potential host defense mechanism to deny bacteria

access to the critical metal (Schaible et al. 2002). As plasma iron levels decrease, a feedback mechanism is believed to downregulate hepcidin production in the liver (Nemeth et al. 2004b).

Regulation of iron homeostasis was a key aspect of the APR observed in catfish (Fig. 2). 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 (Rivera et al. 2005). 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 (Anderson and Frazer 2005). 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 (Melamed-Frank et al. 2001; Gueye et al. 2006; Tolosano et al. 2002; Legrand et al. 2005; Giurgea et al. 2005; Anderson and Frazer 2005). 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. Further investigation of this important group of upregulated catfish transcripts is currently underway in our lab to elucidate their functions and place them on catfish physical and linkage maps.

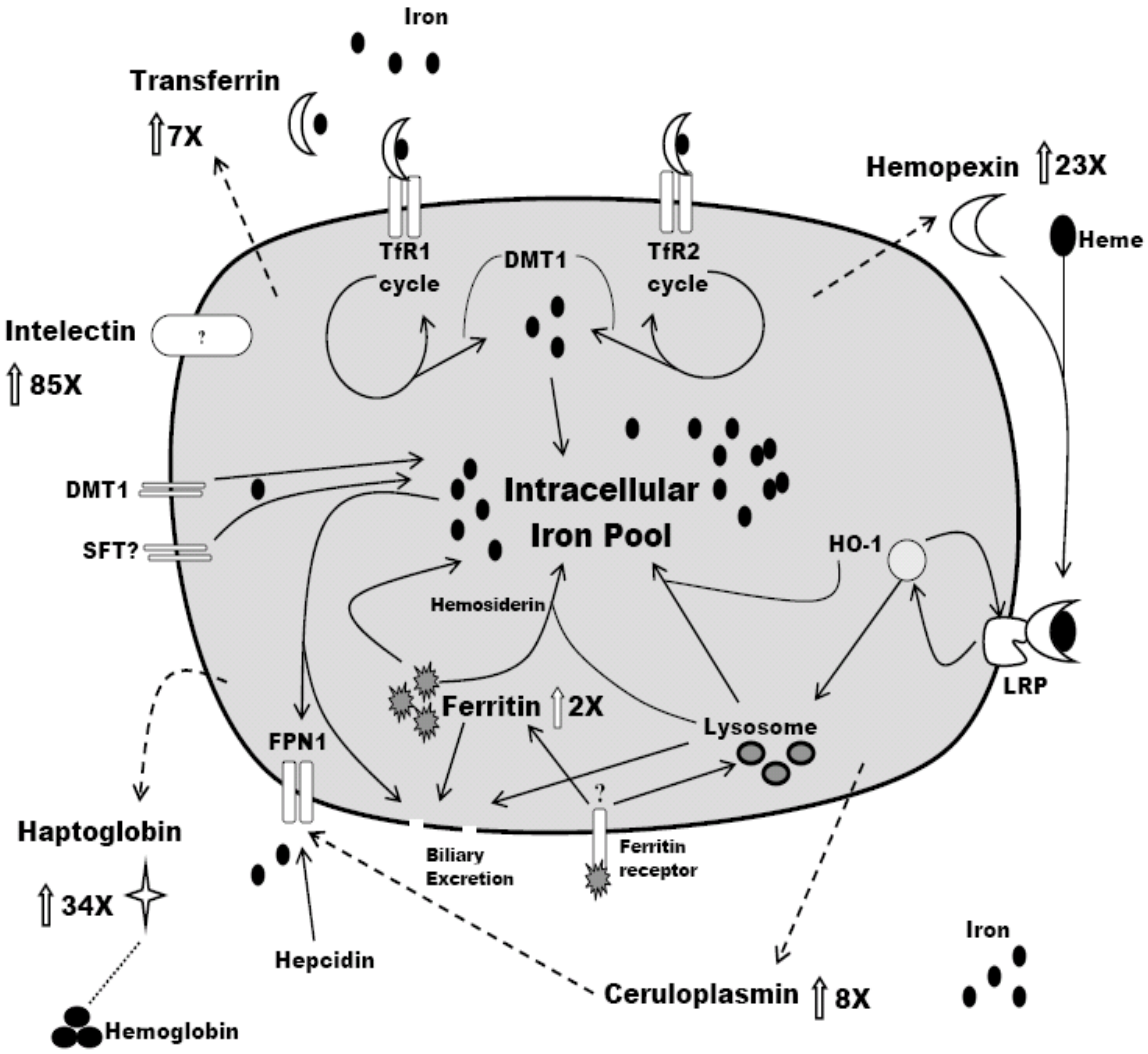

Fig. 2 Putative relationships of a set of upregulated catfish genes involved in iron regulation highlighted on a diagram of pathways of iron homeostasis in mammalian hepatocytes [adapted from Anderson and Frazer (2005)]. Upregulated catfish genes are indicated in larger text with arrows and fold-upregulation nearby. The conservation of these pathways in catfish is currently unknown. DMT1, divalent metal transporter 1; FPN1, ferriportin 1; SFT, stimulator of Fe transport; TfR1, transferrin receptor 1; TfR2, transferrin receptor 2; HO-1, heme oxygenase 1; LRP, low density lipoprotein receptor-related protein.

Two chemokines from catfish previously characterized in our laboratory, SCYA113 and CXCL14, were upregulated greater than 5-fold after infection in liver (He et al. 2004; Peatman et al. 2006; Bao et al. 2006a; Baoprasertkul et al. 2005). SCYA113 is a catfish CC chemokine most similar to mammalian CCL19, while catfish CXCL14 is the putative orthologue of mammalian CXCL14 (Baoprasertkul et al. 2005). 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). Interestingly, both chemokines are among a small handful of CC and CXC chemokines traditionally considered to have homeostatic rather than inflammatory functions (Moser et al. 2004). 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 (Kendziorski et al. 2005; Jolly et al. 2005). 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 (Allison et al. 2006). 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 noticeably 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 (Ewart et al. 2005). 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 (Ewart et al. 2005). 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. The larger set of non-significant downregulated genes, while not allowing global conclusions, will still yield candidates for further genome mapping and analysis.

In summary, microarray analysis of transcriptomic changes in channel catfish liver following an infection with Gram negative bacterium *E. ictaluri* indicated 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 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 and chemokines indicated that the catfish liver plays an integral role in pathogen recognition and defense as well as inflammatory signaling. Ongoing functional characterization,

genetic mapping, and QTL analysis of many of these immune-related genes from catfish will add to our understanding of the teleost immune system.

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IV. TRANSCRIPTOMIC PROFILING OF THE LIVERS OF BLUE CATFISH (*ICTALURUS FURCATUS*) FOLLOWING INFECTION WITH *EDWARDSIELLA ICTALURI*

Abstract

The acute nature of disease outbreaks in aquaculture settings has emphasized 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, serves as 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. 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.

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, 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 the larger 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. 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 gene transcripts in organisms subjected to a variety of diseases and environmental conditions (Rise et al. 2004b; 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. 2006; 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 like 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.

Catfish (*Ictalurus spp.*), the predominant aquaculture species in the United States, serves as an important model for the study of the teleost immune system (Bao et al. 2006a; Bengten et al. 2006). While channel catfish (*I. punctatus*) accounts for the majority of commercial production, the closely related blue catfish (*I. furcatus*) possesses several economically important phenotypic traits that have led to the production of an interspecific hybrid (channel female x 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 four days after infection (Newton et al. 1989; Wolters and Johnson 1994). In trials, 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 eventually identifying molecular contributors to this increased resistance in blue catfish, we have previously developed extensive EST resources for both catfish species and have developed interspecific mapping panels (Liu et al. 2003b, He et al. 2003). In order to study the transcriptomic responses of blue catfish following infection with *E. ictaluri* and develop important immune-related markers for characterization and genetic mapping, we have developed a 28K *in situ* oligonucleotide microarray composed of blue catfish and channel catfish transcripts based upon a previous 19K channel catfish array (Li and Waldbieser 2006). By adding 7,159 additional transcripts from blue catfish, along with additional immune and non-immune transcripts from channel catfish, the new 28K microarray design 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.*

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 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 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 (over the course of 1 week) brought to 27°C 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 hr control (3 aquaria), 24 hr treatment (3 aquaria), 3 d control (3 aquaria), 3 d 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 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 $4X10^8$ 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 hr 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 global expression changes. For the studies described here, liver tissues were collected at day 3 after infection. 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, building on a previously-published 19K catfish design (Li and Waldbieser, 2006). 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, 7,159 unique ESTs from blue catfish (*Ictalurus 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 orthologues 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 clustered. Singletons (non-clustering sequences) and representative clones from contigs were selected and reclustered to ensure a unique gene set as described previously by Peatman et al. (2004). 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 **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 d time point were used for initial microarray analysis. Accordingly, the pooled livers (n=25) from each replicate (3 control replicates, 3 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). 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 1x T7 enzyme mix in 1x 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 to 200 bp by incubation in a buffer of 100 mM potassium acetate, 30 mM magnesium acetate, and 40mM 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 2x 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 2X MES buffer at 45°C for 16–20 h with constant rotation. After hybridization, the microarrays were washed twice with nonstringent buffer (6x 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 one minute rinse with nonstringent buffer, the arrays were placed into a 1x 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 one minute. 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 3 control pools and 3 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 log2 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; Larsson 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 2-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. Real-time RT-PCR analysis

The RNA prepared for microarray analysis was also used for validation of selected genes of interest by real-time RT-PCR. The three control pools and three treatment pools of RNA, each representing 25 fish, were utilized for each tested gene. 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 real time RT-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 per second; (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 catfish (Murdock et al. 2006). The triplicate 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 (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 Ct). Each primer set amplified a single product as indicated by a single peak present for each gene during melting curve analysis.

Table 1 Primers used for real-time RT-PCR validation (5'-3'). *SCYA106*, CC chemokine SCYA106; *LAMP3*, Lysosomal-associated membrane protein 3; *MMP13*, matrix metalloproteinase 13; *LY6E2*, lymphocyte antigen 6 complex, E2

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
18S	BE469353	TGCGCTTAATTTGACTCAACAC	CGATCGAGACTCACTAACATCG

3. Results

3.1. Bacterial challenge and microarray hybridization

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 hemorrhages along their ventral surface. *E. ictaluri* bacteria were successfully isolated from randomly-selected treatment fish. While two time points (24 hr 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 an important organ to innate immunity, it was selected for microarray analysis. Six RNA samples were 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. Microarray analysis of blue catfish expression following challenge

The expression profile of blue catfish liver three 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 5 and 6—see appendices). 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.

3.3. Bioinformatic analysis of induced transcripts following infection

Of the 98 unique, significantly upregulated transcripts after infection, 76 could be annotated based on sequence similarity by BLASTX searches while 22 had no significant similarity to protein sequences in the *nr* database (cutoff E-value=0.0001; Table 2 and Supplemental Table 7). 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 5 members—acute phase response; complement activation; metal ion binding/transport; immune/defense response; protein processing, localization, folding; and protein degradation.

Table 2 Catfish transcripts upregulated in the blue catfish liver following ESC infection. *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. *Acute phase response encompasses ***bold*** transcripts included in other categories. Transcripts were grouped into broad functional categories of at least 5 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

Functional Classification	Accession	Putative Id	Fold Change	q-value (%)
Acute phase response*	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-65kDa (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 (48kDa)	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	3.0	5.63

		vacuolar proton pump		
	TC7903	Armet protein	2.9	9.18
	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

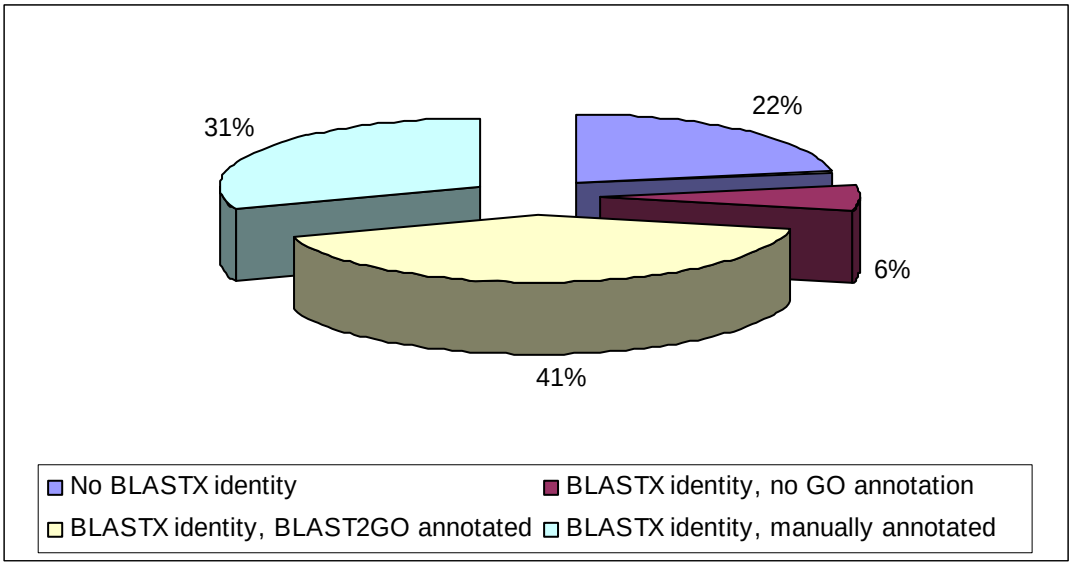

Fig. 1. Analysis and Gene Ontology (GO) annotation of 98 unique, significantly upregulated transcripts in blue catfish.

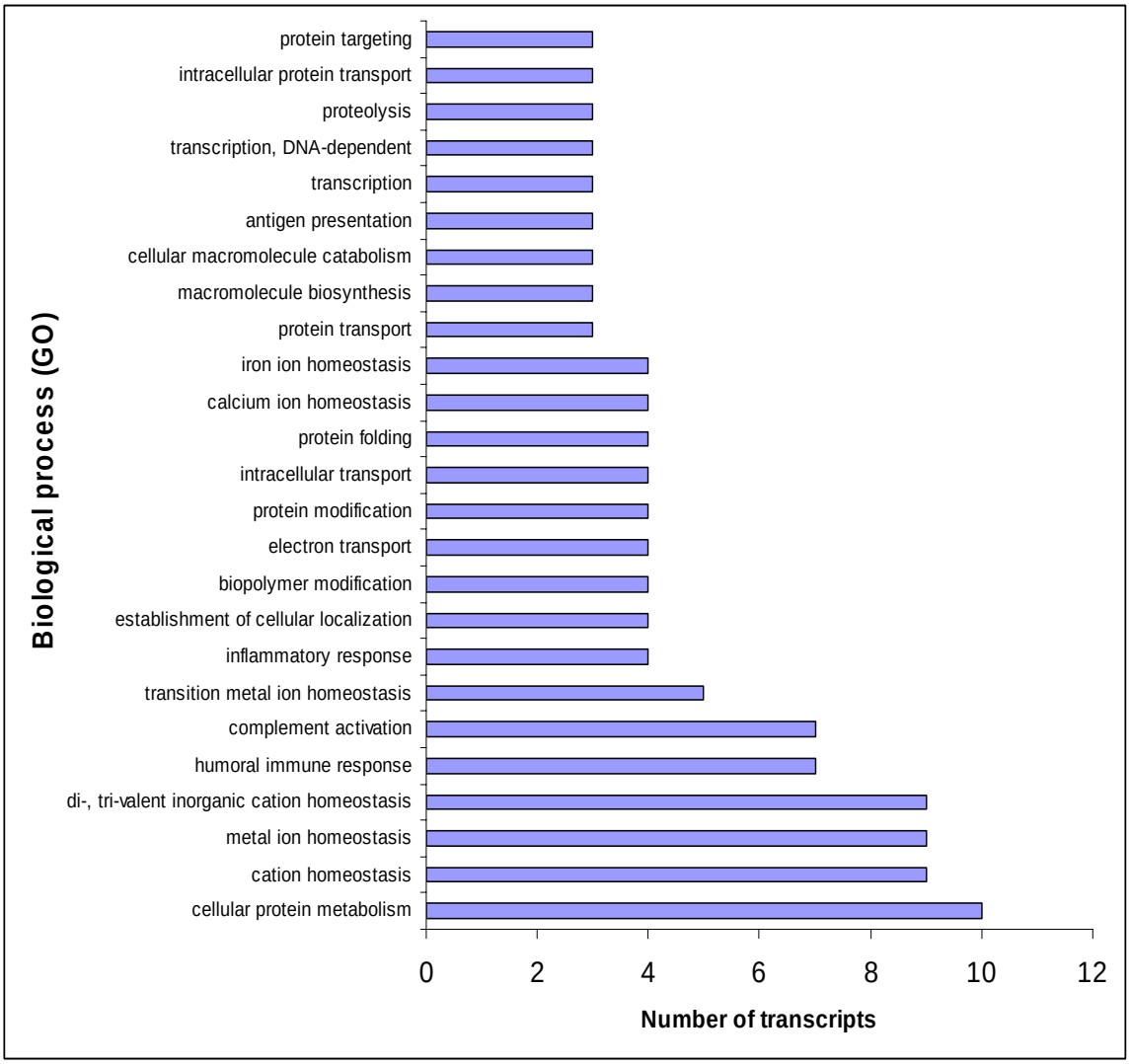

Fig. 2. Significantly upregulated transcripts assigned to lower level (>6) GO biological process categories. 53 sequences had a biological process GO term.

3.4. Conserved acute phase response in blue catfish

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 likely 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 in mammals were strongly induced following infection. These included intelectin, haptoglobin, hemopexin/warm-temperature-acclimation- related, ceruloplasmin, and transferrin. Other upregulated APP included pentraxin (serum amyloid P-like), fibrinogen, and angiotensinogen (Table 2).

3.5. Protein processing, localization, folding and degradation after ESC infection

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

3.6. Induction of immune/defense response related transcripts

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 catfish 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).

3.7. Downregulated transcripts following ESC infection

A 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.

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

Accession	Putative identity	Fold Change	q-value	Function
CK417600	No significant similarity	0.4	9.57	NA
TC9079	Anaphase promoting complex subunit 13	0.5	9.57	Cell cycle
CB940790	Selenoprotein H	0.5	9.57	Stress or defense response
TC9060	No significant similarity	0.5	9.57	NA
CF971521	Selenoprotein P, plasma, 1b	0.5	9.57	Stress or defense response

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

Expression patterns of six genes identified by microarray analysis as differentially expressed following infection were selected for validation using real-time RT-PCR. Genes upregulated ranging from 2.5-fold to 105-fold in the microarray experiment were selected and primers designed (Table 1). Real-time RT-PCR results (Table 4) generally confirmed the microarray results, with all tested genes except intelectin showing statistically significant upregulation greater than 2-fold ($P<0.05$). 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 real time RT-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 real-time PCR than by microarrays.

Table 4 Validation of microarray results by QRT-PCR. *SCYA106*, CC chemokine SCYA106; *LAMP3*, Lysosomal-associated membrane protein 3; *MMP13*, matrix metalloproteinase 13; *LY6E2*, lymphocyte antigen 6 complex, E2

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)

4. Discussion

We have utilized a high-density oligonucleotide microarray for catfish in order to study the transcriptomic responses of blue catfish following infection with *E. ictaluri* and to identify and develop important immune-related markers for future characterization and genetic mapping. 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.

The generation of a large set of catfish ESTs has aided the rapid identification and characterization of many innate immune components including cytokines and chemokines (He et al. 2004; Baoprasertkul et al. 2004; Chen et al. 2005a; Baoprasertkul et al. 2005; Peatman et al. 2005; Bao et al. 2006a; Peatman et al. 2006;Wang et al.

2006c), antimicrobial peptides (Bao et al. 2005; 2006b; Wang et al. 2006a; 2006b; Xu et al. 2005), and Toll-like receptors (Baoprasertkul et al. 2006; 2007). To better utilize catfish EST resources and to analyze the expression of these important immune components in the larger context of the catfish transcriptome following ESC infection, a 28K *in situ* oligonucleotide microarray was designed. The 28K catfish array provides a reasonably comprehensive platform from which to study expression in important tissues and organs of catfish species.

The liver was targeted as the center of the acute phase response and as a likely contributor to the acute inflammatory reaction observed in the catfish response to virulent *E. ictaluri*. The catfish APR as measured three days after infection included many of the components of the mammalian APR and also contained commonalities with a recent APR study in rainbow trout liver (Gerwick et al. 2007) and other previous salmonid and carp microarray experiments measuring expression in liver after application of a variety of stressors (Tilton et al. 2005; Ewart et al. 2005; Martin et al. 2006; Reynders et al. 2006). Acute phase proteins composed a significant percentage of upregulated transcripts in blue catfish. Among the mammalian APP (Gabay and Kushner, 1999) upregulated greater than 2-fold in blue catfish were 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 of function of the vast majority of APP between mammals and teleost fish.

Many of 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 5-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 5) and these transcripts appear to represent at least two genes. Primers were designed for real-time RT-PCR confirmation that could amplify all the differentially expressed transcripts.

Real-time RT-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.

Regulation of iron homeostasis was a key component of the acute phase response observed in blue catfish. Iron regulation also plays an important role in the mammalian host response to pathogens. In mammals, interleukin-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 (Nemeth et al. 2004a). This leads to drastically decreased plasma iron levels during infection, a potential host defense mechanism to deny bacteria access to the critical metal (Schaible et al. 2002). Liver iron stores are known to be significantly increased by hepcidin, even as plasma iron concentrations decline (Rivera et al. 2005). The increase in expression of iron storage, binding, and transport proteins seen in the results (Table 2) may be the result of increasing iron concentrations in the liver. Hepatocytes, which account for 80% of the liver mass, are the primary site of synthesis for haptoglobin, hemopexin, transferrin, and ceruloplasmin (Anderson and Frazer, 2005). In mammals many of these genes are active in sequestering iron to restrict its availability to invading bacteria, and several are known to possess immunoregulatory and antioxidant properties under pathological conditions which may supersede the importance of their roles in normal iron metabolism

(Melamed-Frank et al. 2001; Gueye et al. 2006; Tolosano and Altruda, 2002; Legrand et al. 2005; Giurgea et al. 2005; Anderson and Frazer, 2005). Further efforts are needed to elucidate the role iron regulation plays in the catfish defense response.

The absence of the iron regulatory hormone hepcidin (Park et al. 2001) from the set of upregulated genes in blue catfish liver was notable given that it was observed to be highly upregulated in other teleost expression studies in liver (Gerwick et al. 2007; Tilton et al. 2005; Ewart et al. 2005; Martin et al. 2006; Lin et al. 2007). Hepcidin is represented by at least three transcripts on the microarray, none of which showed significant upregulation. This result, however, agrees with our previous investigation of hepcidin expression in channel catfish (Bao et al. 2005) which showed that hepcidin expression was minimally upregulated in liver at 3 d post ESC infection. We speculate that by the 3 d time point after infection in blue catfish, hepcidin may have returned to basal levels after an earlier induction.

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 (He et al. 2004; Peatman et al. 2006; Bao et al. 2006a). 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 showed heightened 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 (DC-LAMP) was upregulated strongly both in the microarray analysis and in real-time RT-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. 2003a). 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 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 hr post infection and within the vacuoles of hepatocytes 72 hr 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 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 two 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 PA28 α and β 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). The author's data supported the view that, during infection, hepatocytes act as effective antigen presenting cells.

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 PA28-β 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. Further gene and cellular-based studies are needed in catfish to understand the importance of MHC class I/CTL-mediated responses to ESC infection.

Pooling and variation, in relation in microarray analysis, have been subjects of vigorous debate (Kendziorski et al. 2005; Jolly et al. 2005). In the present experiment, 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. Our interest in primary transcriptome analysis lies more with global expression patterns rather than inter-individual variations. In a recent review article in Nature Genetics, Allison et al. (2006) recognized the advantages of pooling for decreasing both variability between arrays and cost, if multiple pools were analyzed per group. Most of the catfish transcripts differentially expressed greater than

two-fold had q-values of 5% or greater, indicating a certain level of variation existed even between the replicate pools. Real time RT-PCR, however, confirmed that transcripts approaching the 10% FDR cutoff were still significantly upregulated (Tables 2 and 4). In addition, the induction of multiple components belonging to the same pathways and biological processes (described above) provided validation of the new catfish microarray as a powerful tool for immune-related transcriptomic analysis. A far smaller number of transcripts were declared significantly downregulated than significantly upregulated, a seemingly characteristic result of transcriptomic analyses of bacterial infections. A similarly small number of transcripts were reported to be significantly downregulated in Atlantic salmon following a Gram negative bacterial infection (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. 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. Taken together, the microarray results add to our understanding of the teleost immune responses and will provide a solid foundation for future functional characterization, genetic mapping, and QTL analysis of immunity-related genes from catfish.

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V. CONCLUSIONS

The development of genetically-superior catfish brood stocks resistant to major diseases would represent a significant, lasting economic stimulus to the U.S. catfish industry. Progress toward this goal will be made more rapidly as genomic tools and reagents are developed and implemented for catfish. These tools will automate and simplify once overwhelming tasks and serve to connect phenotypic differences with their genotypic origins. Expressed sequence tags (ESTs) are one such genomic tool. Additional work is required, however, to utilize effectively the short transcript sequences for genome research. This may take the form of: development of markers from microsatellites contained within ESTs; sequencing, expression analysis, and mapping of important genes or gene families identified from ESTs; or construction of microarrays for transcriptome analysis based on the EST sequences.

In this work, 26 CC chemokines from catfish were mapped to BAC clones. Through a combination of hybridization and fluorescent fingerprinting, 18 fingerprinted contigs were assembled from BACs containing catfish CC chemokine genes. The catfish CC chemokine genes were found to be not only highly clustered in the catfish genome, but also extensively duplicated at various levels. Additionally, the expression patterns of the transcripts of the 26 catfish CC chemokines were analyzed in head kidney and spleen in response to bacterial infection of *E. ictaluri*.

ESTs were also utilized in the development of a 28K *in situ* oligonucleotide microarray composed of blue catfish (*Ictalurus furcatus*) and channel catfish (*Ictalurus punctatus*) transcripts. Initial microarray analysis in channel catfish liver following an infection with *E. ictaluri* captured 212 unique, differentially expressed transcripts, and indicated a conserved acute phase response occurs as part of the innate immune response of primitive teleost fish. The majority of classical acute phase proteins 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 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 and chemokines indicated that the catfish liver plays an integral role in pathogen recognition and defense as well as inflammatory signaling.

In a parallel study to validate the microarray for blue catfish, analysis of gene expression changes in blue catfish liver after *E. ictaluri* infection captured 103 unique, differentially expressed transcripts, and indicated the strong upregulation of several pathways involved in the inflammatory immune response. 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 was reported for the first time in fish.

The CC chemokine family from catfish, as the largest chemokine family characterized from fish to-date, has served as a reference source for description of

chemokines from other fish species (i.e. Gonzalez et al. 2007; Peatman and Liu 2006). More concrete comparisons can be made between catfish and other fish chemokines than with mammalian chemokines. Additional functional information will be gained as orthologues of catfish CC chemokines are identified in other fish species during the course of infection studies. The mapping of the CC chemokine gene family provides a useful dataset for the study of gene duplication and divergence in catfish. A database-searchable, BAC-based physical map for catfish will soon be available (Xu et al. 2007). This will allow further analysis of the extent of clustering of the CC chemokines in catfish. Additionally, microsatellites identified from sequencing of the 26 CC chemokine genes (Bao et al. 2006) are currently being utilized for mapping this family to the catfish linkage map. This will provide information as to the chromosomal arrangement of the chemokines and will serve to integrate the two maps as well as testing for correlations with resistance or susceptibility to ESC. The investigation of the catfish CC chemokines also led to the subsequent identification of a large number of unannotated CC chemokine genes in zebrafish (Peatman and Liu 2006). Analysis of the CC chemokines of catfish and zebrafish confirmed that lower teleost fish appear to have a large number of species-specific tandem and segmental duplications of CC chemokine loci. Further investigation of this phenomenon is needed given that similar genomic regions in humans are increasingly recognized as hot spots for gene copy number variation and are often linked to disease (Redon et al. 2006).

The construction and utilization of high-density oligonucleotide microarrays from channel catfish and blue catfish ESTs represent a strong foundation for future, widespread use of microarrays in catfish research. As technological breakthroughs

continue to increase gene spotting densities and detection sensitivity and decrease cost/array, microarrays will become a more practical tool for agricultural research. The initial studies presented here produced informative, reproducible results. They also helped to identify areas for improvement for future array designs and studies in catfish.

The microarray results demonstrated the importance of a multifaceted, integrated approach to genome research. A number of chemokines, Toll-like receptors, and antimicrobial peptides previously characterized in our lab were differentially expressed on the microarrays. This made results more informative and facilitates integrating expression candidates into mapping and QTL analysis. Additionally, the current studies revealed a number of genes which may play important, but previously undescribed, roles in catfish innate immune responses. A number of these genes are currently being mapped to the BAC-based physical map, analyzed for expression patterns in additional tissues, and sequenced to identify microsatellites for linkage mapping. Besides making the catfish genome maps more informative, this will allow QTL analysis of these genes in regards to ESC resistance. The entire set of differentially expressed transcripts is also currently being searched for SNPs that would allow them to be genetically mapped. These projects will lend greater biological relevance to the data obtained by microarray analysis.

Comparison of sets of differentially expressed transcripts from channel catfish and blue catfish revealed more similiarities than differences. Both species manifested high induced expression of acute phase proteins, complement components, and genes involved in iron homeostasis. A MHC class I-related response was evident in blue catfish but not in channel catfish. Overall, however, expression patterns may differ

more between the two species in their timing than in the presence or absence of certain gene components. Studies are underway to examine and compare expression patterns of a subset of genes at the 24 hr timepoint in both species using quantitative real time RT-PCR. Obviously, future studies could improve on the ones described here by conducting microarray analysis over several timepoints. This would likely capture a larger set of genes, and earlier timepoints would probably include more downregulated genes than were seen at the 3 d timepoint. In the same vein, studies of additional tissues would capture different sections of the transcriptome that are contributing to the innate immune response. While liver is fairly homogenous with regards to cell types it contains, isolation of individual cell populations from various tissues may be more informative than whole tissue analysis.

Additional changes in the future may lend greater power to microarray-based expression profiling in catfish. An ongoing Joint Genome Institute project to sequence 300,000 ESTs from blue catfish and channel catfish will provide a significantly larger set of unique transcripts. This will necessitate construction of a new microarray incorporating these transcripts. Since the vast majority of catfish genes should then be included in the transcript set, it may prove feasible to construct spotted oligo arrays for catfish. While initial start-up costs for these arrays will be significantly higher than the *in situ* arrays used here, long-term cost/array will be less, allowing more in-depth microarray studies in catfish. Further work is also needed on developing infection challenge models that more closely imitate disease conditions catfish experience in the pond. Challenges utilizing lower bacterial concentrations and cohabitation challenges may provide more realistic expression profiles. Finally, the ability to correlate gene

expression patterns with the ultimate survival or death of a fish is crucial in ultimately identifying the genetic origins of phenotypic differences in disease resistance and susceptibility.

CUMULATIVE BIBLIOGRAPHY

Ahn JY, Tanahashi N, Akiyama K, Hisamatsu H, Noda C, Tanaka K, Chung CH, Shibmara N, Willy PJ, Mott JD, et al. (1995) Primary structures of two homologous subunits of PA28, a gamma-interferon-inducible protein activator of the 20S proteasome. FEBS Lett 366:37-42

Ahuja SK, Murphy PM (1996) The CXC chemokines growth-regulated oncogene (GRO) alpha, GRObeta, GROgamma, neutrophil-activating peptide-2, and epithelial cell-derived neutrophil-activating peptide-78 are potent agonists for the type B, but not the type A, human interleukin-8 receptor. J Biol Chem 271:20545–20550

Alkhatib G, Combadiere C, Broder CC, Feng Y, Kennedy PE, Murphy PM, Berger EA (1996) CC CKR5: A RANTES, MIP 1a, MIP-1b receptor as a fusion cofactor for macrophage-tropic HIV1. Science 272:1955–1958

Allison DB, Cui X, Page GP, Sabripour M (2006) Microarray data analysis: from disarray to consolidation and consensus. Nat Rev Genet 7:55-65

Anderson GJ, Frazer DM (2005) Hepatic iron metabolism. Semin Liver Dis 25:420-432

Antao AB, Chinchar VG, McConnell TJ, Miller NW, Clem LW, Wilson MR (1999) MHC class I genes of the channel catfish: sequence analysis and expression. Immunogenetics 49:303-311

Antao AB, Wilson M, Wang J, Bengten E, Miller NW, Clem LW, Chinchar VG (2001) Genomic organization and differential expression of channel catfish MHC class I genes. Dev Comp Immunol 25:579-595

Arruda LB, Sim D, Chikhlikar PR, Maciel M Jr, Akasaki K, August JT, Marques ET (2006) Dendritic cell-lysosomal-associated membrane protein (LAMP) and LAMP-1-HIV-1 gag chimeras have distinct cellular trafficking pathways and prime T and B cell responses to a diverse repertoire of epitopes. J Immunol 177:2265-2275

Aubin-Horth N, Landry CR, Letcher BH, Hofmann HA (2005) Alternative life histories shape brain gene expression profiles in males of the same population. Proc Biol Sci 272:1655-1662

Bacon K, Baggiolini M, Broxmeyer H, Horuk R, Lindley I, Mantovani A, Matsushima K, Murphy P, Nomiyama H, Oppenheim J, Rot A, Schall T, Tsang M, Thorpe R, Van Damme J, Wadhwa M, Yoshie O, Zlotnik A, Zoon K (2003) Chemokine/chemokine receptor nomenclature. Cytokine 21:48–49

Baldwin TJ, Newton JC (1993) Pathogenesis of enteric septicemia of channel catfish, caused by *Edwardsiella ictaluri*: Bacteriologic and light and electron microscopic findings. J Aquat Anim Health 5:189–198

Bao B, Peatman E, Li P, He C, Liu ZJ (2005) Catfish hepcidin gene is expressed in a wide range of tissues and exhibits tissue-specific upregulation after bacterial infection. Dev Comp Immunol 29:939-950

Bao B, Peatman E, Peng X, Baoprasertkul P, Wang G, Liu Z (2006a) Characterization of 23 CC chemokine genes and analysis of their expression in channel catfish (*Ictalurus punctatus*). Dev Comp Immunol 30:783-796

Bao B, Peatman E, Xu P, Li P, Zeng H, He C, Liu ZJ (2006b) The catfish liver-expressed antimicrobial peptide 2 (LEAP-2) gene is expressed in a wide range of tissues and developmentally regulated. Mol Immunol 43:367-377

Baoprasertkul P, He C, Peatman E, Zhang S, Li P, Liu Z (2005) Constitutive expression of three novel catfish CXC chemokines; homeostatic chemokines in teleost fish. Mol Immunol 42:1355-1366

Baoprasertkul P, Peatman E, Abernathy J, Liu Z (2007) Structural characterization and expression analysis of Toll-like receptor 2 gene from catfish. Fish Shellfish Immunol, in press

Baoprasertkul P, Peatman E, He C, Kucuktas H, Li P, Chen L, Simmons M, Liu Z (2004) Sequence analysis and expression of a CXC chemokine in resistant and susceptible catfish after infection of *Edwardsiella ictaluri*. Dev Comp Immunol 28:769-780

Baoprasertkul P, Peatman E, Somridhivej B, Liu Z (2006) Toll-like receptor 3 and TICAM genes in catfish (*Ictalurus sp.*): species-specific expression profiles following infection with *Edwardsiella ictaluri*. Immunogenetics 58:817-830

Baxi MK, Baxi S, Clavijo A, Burton KM, Deregt D (2005) Microarray-based detection and typing of foot-and-mouth disease virus. Vet J 172:473-481

Bayne CJ, Gerwick L, Fujiki K, Nakao M, Yano T (2001) Immune-relevant (including acute phase) genes identified in the livers of rainbow trout, *Oncorhynchus mykiss*, by means of suppression subtractive hybridization. Dev Comp Immunol 25:205-217

Bayne CJ, Gerwick L (2001) The acute phase response and innate immunity of fish. Dev Comp Immunol 25:725-743

Bengten E, Clem LW, Miller NW, Warr GW, Wilson M (2006) Channel catfish immunoglobulins: repertoire and expression. Dev Comp Immunol 30:77-92

Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate—a practical and powerful approach to multiple testing. J R Stat Soc Ser B 57:289-300

Berg RE, Crossley E, Murray S, Forman J (2003) Memory CD8+ T cells provide innate immune protection against *Listeria monocytogenes* in the absence of cognate antigen. J Exp Med 198:1583-1593

Bilodeau AL, Peterson BC, Bosworth BG (2006) Response of toll-like receptors, lysozyme, and IGF-I in back-cross hybrid (F1 male (blue x channel) x female channel) catfish challenged with virulent *Edwardsiella ictaluri*. Fish Shellfish Immunol 20:29-39

Bilodeau AL, Waldbieser GC (2005) Activation of TLR3 and TLR5 in channel catfish exposed to virulent *Edwardsiella ictaluri*. Dev Comp Immunol29:713-721

Bolstad BM, Irizarry RA, Astrand M, Speed TP (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 19:185-193

Boshra H, Li J, Sunyer JO (2006) Recent advances on the complement system of teleost fish. Fish Shellfish Immunol 20:239-262

Byon JY, Ohira T, Hirono I, Aoki T (2005) Use of a cDNA microarray to study immunity against viral hemorrhagic septicemia (VHS) in Japanese flounder (*Paralichthys olivaceus*) following DNA vaccination. Fish Shellfish Immunol 18:135-147

Byon JY, Ohira T, Hirono I, Aoki T (2006) Comparative immune responses in Japanese flounder, *Paralichthys olivaceus* after vaccination with viral hemorrhagic septicemia virus (VHSV) recombinant glycoprotein and DNA vaccine using a microarray analysis. Vaccine 24:921-930

Cai WW, Reneker J, Chow CW, Vaishnav M, Bradley A (1998) An anchored framework BAC map of mouse chromosome 11 assembled using multiplex oligonucleotide hybridization. Genomics 54:387–397

Camp KL, Wolters WR, Rice CD (2000) Survivability and immune responses after challenge with *Edwardsiella ictaluri* in susceptible and resistant families of channel catfish, *Ictalurus punctatus.* Fish Shellfish Immunol 10:475-487

Cao D, Kocabas A, Ju Z, Karsi A, Li P, Patterson A, Liu Z (2001) Transcriptome of channel catfish (*Ictalurus punctatus*): initial analysis of genes and expression profiles of the head kidney. Anim Genet 32:169-188

Chatakondi NG, Yant DR, Dunham RA (2005) Commercial production and performance evaluation of channel catfish, *Ictalurus punctatus* female X blue catfish, *Ictalurus furcatus* male F-1 hybrids. Aquaculture 247:1-4

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

Chen M, Tabaczewski P, Truscott SM, Van Kaer L, Stroynowski I (2005b) Hepatocytes express abundant surface class I MHC and efficiently use transporter associated with antigen processing, tapasin, and low molecular weight polypeptide

proteasome subunit components of antigen processing and presentation pathway. J Immunol 175:1047-1055

Chen W, Norbury CC, Cho Y, Yewdell JW, Bennink JR (2001) Immunoproteasomes shape immunodominance hierarchies of antiviral CD8(+) T cells at the levels of T cell repertoire and presentation of viral antigens. J. Exp. Med. 193:1319-1326

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156-159

Conesa A, Gotz S, Garcia-Gomez JM, Terol J, Talon M, Robles M (2005) Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674-3676

Cook MT, Hayball PJ, Birdseye L, Bagley C, Nowak BF, Hayball JD (2003) Isolation and partial characterization of a pentraxin-like protein with complement-fixing activity from snapper (*Pagrus auratus*, Sparidae) serum. Dev Comp Immunol 27:579-588

Cook MT, Hayball PJ, Nowak BF, Hayball JD (2005) The opsonising activity of a pentraxin-like protein isolated from snapper (*Pagrus auratus*, Sparidae) serum. Dev Comp Immunol 29:703-712

Cretich M, Damin F, Pirri G, Chiari M (2006) Protein and peptide arrays: Recent trends and new directions. Biomol Eng 23:77-88

Dai SY, Nakagawa R, Itoh A, Murakami H, Kashio Y, Abe H, Katoh S, Kontani K, Kihara M, Zhang SL, Hata T, Nakamura T, Yamauchi A, Hirashima M (2005) Galectin-9 induces maturation of human monocyte-derived dendritic cells. J Immunol 175:2974-2981

David NB, Sapede D, Saint-Etienne L, Thisse C, Thisse B, Dambly-Chaudiere C, Rosa FM, Ghysen A (2002) Molecular basis of cell migration in the fish lateral line: role of the chemokine receptor CXCR4 and of its ligand, SDF1. Proc Natl Acad Sci U S A 99:16297-16302

de Saint-Vis B, Vincent J, Vandenabeele S, Vanbervliet B, Pin JJ, Ait-Yahia S, Patel S, Mattei MG, Banchereau J, Zurawski S, Davoust J, Caux C, Lebecque S (1998) A novel lysosome-associated membrane glycoprotein, DC-LAMP, induced upon DC maturation, is transiently expressed in MHC class II compartment. Immunity 9:325-336

Dhar AK, Dettori A, Roux MM, Klimpel KR, Read B (2003) Identification of differentially expressed genes in shrimp (*Penaeus stylirostris*) infected with White spot syndrome virus by cDNA microarrays. Arch Virol 148:2381-2396

Dixon B, Shum B, Adams EJ, Magor KE, Hedrick RP, Muir DG, Parham P (1998) CK-1, a putative chemokine of rainbow trout (*Oncorhynchus mykiss*). Immunol Rev 166:341–348

D'Orazio SE, Troese MJ, Starnbach MN (2006) Cytosolic localization of *Listeria monocytogenes* triggers an early IFN-{gamma}response by CD8+ T Cells that correlates with innate resistance to infection. J Immunol 177:7146-7154

Dunham RA, Brady Y, Vinitnantharat S (1993) Response to challenge with *Edwardsiella ictaluri* by channel catfish, *Ictalurus punctatus*, selected for resistance to *E. ictaluri*. J Appl Aquaculture 3:211-222

Dunham RA, Liu Z (2003) Gene mapping, isolation and genetic improvement in catfish. In Aoki (ed) Aquacuture Genomics, Springer-Verlag, New York pp. 45-60

Ellis AE (2001) Innate host defense mechanisms of fish against viruses and bacteria. Dev Comp Immunol 25:827-839

Ewart KV, Belanger JC, Williams J, Karakach T, Penny S, Tsoi SC, Richards RC, Douglas SE (2005) Identification of genes differentially expressed in Atlantic salmon (*Salmo salar*) in response to infection by *Aeromonas salmonicida* using cDNA microarray technology. Dev Comp Immunol 29:333-347

Fey GH, Gauldie J (1990) The acute phase response of the liver in inflammation. Prog Liver Dis 9:89-116

Fodor SP, Read JL, Pirrung MC, Stryer L, Lu AT, Solas D (1991) Light-directed, spatially addressable parallel chemical synthesis. Science 251:767-773

Fredslund J, Schauser L, Madsen LH, Sandal N, Stougaard J (2005) PriFi: using a multiple alignment of related sequences to find primers for amplification of homologs. Nucleic Acids Res 33:W516-520

Fujiki K, Shin DH, Nakao M, Yano T (1999) Molecular cloning of carp (*Cyprinus carpio*) CC chemokine, CXC chemokine receptors, allograft inflammatory factor-1, and natural killer cell enhancing factor by use of suppression subtractive hybridization. Immunogenetics 49:909–914

Gabay C, Kushner I (1999) Acute-phase proteins and other systemic responses to inflammation. N Engl J Med 340:448-454

Gerwick L, Corley-Smith G, Bayne CJ (2007) Gene transcript changes in individual rainbow trout livers following an inflammatory stimulus. Fish Shellfish Immunol in press

Gerwick L, Steinhauer R, Lapatra S, Sandell T, Ortuno J, Hajiseyedjavadi N, Bayne CJ (2002) The acute phase response of rainbow trout (*Oncorhynchus mykiss*) plasma proteins to viral, bacterial and fungal inflammatory agents. Fish Shellfish Immunol 12:229-242

Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL (2001) Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. J Immunol 167:1882-1885

Giurgea N, Constantinescu MI, Stanciu R, Suciu S, Muresan A (2005) Ceruloplasmin - acute-phase reactant or endogenous antioxidant? The case of cardiovascular disease. Med Sci Monit 11:RA48-51

Gonzalez SF, Chatziandreou N, Nielsen ME, Li W, Rogers J, Taylor R, Santos Y, Cossins A (2007) Cutaneous immune responses in the common carp detected using transcript analysis. Mol Immunol 44:1675-1690

Gonzalez E, Kulkarni H, Bolivar H, Mangano A, Sanchez R, Catano G, Nibbs RJ, Freedman BI, Quinones MP, Bamshad MJ, Murthy KK, Rovin BH, Bradley W, Clark RA, Anderson SA, O'connell RJ, Agan BK, Ahuja SS, Bologna R, Sen L, Dolan MJ, Ahuja SK (2005) The influence of CCL3L1 gene-containing segmental duplications on HIV-1/AIDS susceptibility. Science 307:1434-1440

Gracey AY, Fraser EJ, Li W, Fang Y, Taylor RR, Rogers J, Brass A, Cossins AR (2004) Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. Proc Natl Acad Sci U S A 101:16970-16975

Groettrup M, Soza A, Eggers M, Kuehn L, Dick TP, Schild H, Rammensee HG, Koszinowski UH, Kloetzel PM (1996) A role for the proteasome regulator PA28alpha in antigen presentation. Nature 381:166-168

Gueye PM, Glasser N, Ferard G, Lessinger JM (2006) Influence of human haptoglobin polymorphism on oxidative stress induced by free hemoglobin on red blood cells. Clin Chem Lab Med 44:542-547

Hawke JP, McWhorter AC, Steigerwalt AG, Brenner DJ (1981) *Edwardsiella ictaluri* sp. nov., the causative agent of enteric septicemia of catfish. Int J Syst Bacteriol 31:396–400

He C, Chen L, Simmons M, Li P, Kim S, Liu ZJ (2003) Putative SNP discovery in interspecific hybrids of catfish by comparative EST analysis. Anim Genet 34:445-448

He C, Peatman E, Baoprasertkul P, Kucuktas H, Liu Z (2004) Multiple CC chemokines in channel catfish and blue catfish as revealed by analysis of expressed sequence tags. Immunogenetics 56:379-387

Humrich JY, Humrich JH, Averbeck M, Thumann P, Termeer C, Kampgen E, Schuler G, Jenne L (2006) Mature monocyte-derived dendritic cells respond more strongly to CCL19 than to CXCL12: consequences for directional migration. Immunology 117:238-247

Inoue Y, Saito T, Endo M, Haruta C, Nakai T, Moritomo T, Nakanishi T (2005) Molecular cloning and preliminary expression analysis of banded dogfish (*Triakis scyllia*) CC chemokine cDNAs by use of suppression subtractive hybridization. Immunogenetics 56:722-734

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249-264

Isoda K, Arita E, Kojima M, Ikkaku M, Tashiro F, Yamato E, Miyazaki J, Kawase M, Kondoh M, Yagi K (2006) Protection against CCl4-induced injury in liver by adenovirally introduced thioredoxin gene. Biochem Biophys Res Commun 350:157-161

Jamieson SE, Miller EN, Black GF, Peacock CS, Cordell HJ, Howson JM, Shaw MA, Burgner D, Xu W, Lins-Lainson Z, Shaw JJ, Ramos F, Silveira F, Blackwell JM (2004) Evidence for a cluster of genes on chromosome 17q11-q21 controlling susceptibility to tuberculosis and leprosy in Brazilians. Genes Immun 5:46-57

Jolly RA, Goldstein KM, Wei T, Gao H, Chen P, Huang S, Colet JM, Ryan TP, Thomas CE, Estrem ST (2005) Pooling samples within microarray studies: a comparative analysis of rat liver transcription response to prototypical toxicants. Physiol Genomics 22:346-355

Jordal AE, Torstensen BE, Tsoi S, Tocher DR, Lall SP, Douglas SE (2005) Dietary rapeseed oil affects the expression of genes involved in hepatic lipid metabolism in Atlantic salmon (*Salmo salar* L.). J Nutr 135:2355-2361

Ju Z, Dunham RA, Liu Z (2002) Differential gene expression in the brain of channel catfish (*Ictalurus punctatus*) in response to cold acclimation. Mol Genet Genomics 268:87-95

Ju Z, Karsi A, Kocabas A, Patterson A, Li P, Cao D, Dunham R, Liu Z (2000) Transcriptome analysis of channel catfish (*Ictalurus punctatus*): genes and expression profile from the brain. Gene 261:373-382

Ju Z, Wells MC, Heater SJ, Walter RB (2006) Multiple tissue gene expression analyses in Japanese medaka (*Oryzias latipes*) exposed to hypoxia. Comp Biochem Physiol C Toxicol Pharmacol, in press

Julg B, Goebel FD (2005) Susceptibility to HIV/AIDS: an individual characteristic we can measure? Infection 33:160-162

Karsi A, Cao D, Li P, Patterson A, Kocabas A, Feng J, Ju Z, Mickett KD, Liu Z (2002) Transcriptome analysis of channel catfish (*Ictalurus punctatus*): initial analysis of gene expression and microsatellite-containing cDNAs in the skin. Gene 285:157-168

Kendziorski C, Irizarry RA, Chen KS, Haag JD, Gould MN (2005) On the utility of pooling biological samples in microarray experiments. Proc Natl Acad Sci USA 102:4252-4257

Khadijah S, Neo SY, Hossain MS, Miller LD, Mathavan S, Kwang J (2003) Identification of white spot syndrome virus latency-related genes in specific-pathogen-free shrimps by use of a microarray. J Virol 77:10162-10167

Khan S, van den Broek M, Schwarz K, de Giuli R, Diener PA, Groettrup M (2001) Immunoproteasomes largely replace constitutive proteasomes during an antiviral and antibacterial immune response in the liver. J Immunol 167:6859-6868

Khattiya R, Ohira T, Hirono I, Aoki T (2004) Identification of a novel Japanese flounder (*Paralichthys olivaceus*) CC chemokine gene and an analysis of its function. Immunogenetics 55:763–769

Khayat M, Stuge TB, Wilson M, Bengten E, Miller NW, Clem LW (2001) Thioredoxin acts as a B cell growth factor in channel catfish. J Immunol 166:2937-2943

Kocabas A, Dunham R, Liu ZJ (2004) Alterations in gene expression in the brain of white catfish (*Ameirus catus*) in response to cold acclimation. Marine Biotechnology 6:S431-S438

Kocabas AM, Li P, Cao D, Karsi A, He C, Patterson A, Ju Z, Dunham RA, Liu Z (2002) Expression profile of the channel catfish spleen: analysis of genes involved in immune functions. Mar Biotechnol 4:526-536

Kono T, Kusada R, Kawahara E, Sakai M (2003) The analysis of immune responses of a novel CC-chemokine gene from Japanese flounder *Paralichthys olivaceus*. Vaccine, 21:446–457

Koskinen H, Krasnov A, Rexroad C, Gorodilov Y, Afanasyev S, Molsa H (2004a) The 14-3-3 proteins in the teleost fish rainbow trout (*Oncorhynchus mykiss*). J Exp Biol 207:3361-3368

Koskinen H, Pehkonen P, Vehniainen E, Krasnov A, Rexroad C, Afanasyev S, Molsa H, Oikari A (2004b) Response of rainbow trout transcriptome to model chemical contaminants. Biochem Biophys Res Commun 320:745-753

Krasnov A, Koskinen H, Afanasyev S, Molsa H (2005a) Transcribed Tc1-like transposons in salmonid fish. BMC Genomics 6:107

Krasnov A, Koskinen H, Pehkonen P, Rexroad CE 3rd, Afanasyev S, Molsa H (2005b) Gene expression in the brain and kidney of rainbow trout in response to handling stress. BMC Genomics 6:3

Krasnov A, Koskinen H, Rexroad C, Afanasyev S, Molsa H, Oikari A (2005c) Transcriptome responses to carbon tetrachloride and pyrene in the kidney and liver of juvenile rainbow trout (*Oncorhynchus mykiss*). Aquat Toxicol 74:70-81

Kumar S, Tamura K, Nei M (2004) MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. Briefings in Bioinformatics 5:150-163

Kurobe T, Yasuike M, Kimura T, Hirono I, Aoki T (2005) Expression profiling of immune-related genes from Japanese flounder *Paralichthys olivaceus* kidney cells using cDNA microarrays. Dev Comp Immunol 29:515-523

Kuroda N, Uinuk-ool TS, Sato A, Samonte IE, Figueroa F, Mayer WE, Klein J (2003) Identification of chemokines and a chemokine receptor in cichlid fish, shark, and lamprey. Immunogenetics 54:884-895

Laing KJ, Secombes CJ (2004b) Trout CC chemokines: comparison of their sequences and expression patterns. Mol Immunol 41:793-808

Laing KJ, Secombes CJ (2004a) Chemokines. Dev Comp Immunol 28:443–460

Lam SH, Winata CL, Tong Y, Korzh S, Lim WS, Korzh V, Spitsbergen J, Mathavan S, Miller LD, Liu ET, Gong Z (2006) Transcriptome kinetics of arsenic-induced adaptive response in zebrafish liver. Physiol Genomics 27:351-361

Lan Y, Xu X, Yang F, Zhang X (2006) Transcriptional profile of shrimp white spot syndrome virus (WSSV) genes with DNA microarray. Arch Virol 151:1723-1733

Larsson O, Wahlestedt C, Timmons JA (2005) Considerations when using the significance analysis of microarrays (SAM) algorithm. BMC Bioinformatics 6:129

Legrand D, Elass E, Carpentier M, Mazurier J (2005) Lactoferrin: a modulator of immune and inflammatory responses. Cell Mol Life Sci 62:2549-2559

Li J, Burmeister M (2005) Genetical genomics: combining genetics with gene expression analysis. Hum Mol Genet 2:R163-R169

Li RW, Waldbieser GC (2006) Production and utilization of a high-density oligonucleotide microarray in channel catfish, *Ictalurus punctatus*. BMC Genomics 7:134

Li P, Peatman E, Wang S, Feng J, He C, Baoprasertkul P, Xu P, Kucuktas H, Nandi S, Somridhivej B, Serapion J, Simmons M, Turan C, Liu L, Muir W, Dunham R, Brady Y, Grizzle J, Liu Z (2007) Towards the catfish transcriptome: development of molecular tools from 31,215 catfish ESTs. BMC Genomics, under review

Lin B, Chen S, Cao Z, Lin Y, Mo D, Zhang H, Gu J, Dong M, Liu Z, Xu A (2007) Acute phase response in zebrafish upon *Aeromonas salmonicida* and *Staphylococcus aureus* infection: Striking similarities and obvious differences with mammals. Mol Immunol 44:295-301

Liu ZJ (2006) Transcriptome characterization through the generation and analysis of expressed sequence tags: Factors to consider for a successful EST project. Israel J Aquaculture 58:328-341

Liu Z, Peatman E (2006) Chemokines in fish: a rapidly expanding repertoire. Chapter in: Focus on Immunology Research, B.A. Veskler ed., Nova Science Publishers, NY. pp. 121-144

Liu G, Zheng W, Chen X (2007) Molecular cloning of proteasome activator PA28-beta subunit of large yellow croaker (*Pseudosciana crocea*) and its coordinated up-regulation with MHC class I alpha-chain and beta(2)-microglobulin in poly I:C-treated fish. Mol Immunol 44:1190-1197

Liu HC, Niikura M, Fulton JE, Cheng HH (2003a) Identification of chicken lymphocyte antigen 6 complex, locus E (LY6E, alias SCA2) as a putative Marek's disease resistance gene via a virus-host protein interaction screen. Cytogenet Genome Res 102:304-308

Liu L, Fujiki K, Dixon B, Sundick RS (2002) Cloning of a novel rainbow trout (*Oncorhynchus mykiss*) CC chemokine with a fractalkine-like stalk and a TNF decoy receptor using cDNA fragments containing Au-rich elements. Cytokine 17:71–81

Liu Z, Karsi A, Li P, Cao D, Dunham R (2003b) An AFLP-based genetic linkage map of channel catfish (*Ictalurus punctatus*) constructed by using an interspecific hybrid resource family. Genetics 165:687-694

Luo, MC, Thomas C, You FM, Hsiao J, Ouyang S, Buell CR, Malandro M, McGuire PE, Anderson OD, Dvorak J (2003) High-throughput fingerprinting of bacterial artificial chromosomes using the SNaPshot labeling kit and sizing of restriction fragments by capillary electrophoresis. Genomics 82:378-389

MacKenzie S, Iliev D, Liarte C, Koskinen H, Planas JV, Goetz FW, Molsa H, Krasnov A, Tort L (2006) Transcriptional analysis of LPS-stimulated activation of trout (*Oncorhynchus mykiss*) monocyte/macrophage cells in primary culture treated with cortisol. Mol Immunol 43:1340-1348

Magnadottir B (2006) Innate immunity of fish (overview). Fish Shellfish Immunol 20:137-151

Mancia A, Lundqvist ML, Romano TA, Peden-Adams MM, Fair PA, Kindy MS, Ellis BC, Gattoni-Celli S, McKillen DJ, Trent HF, Ann Chen Y, Almeida JS, Gross PS, Chapman RW, Warr GW (2006) A dolphin peripheral blood leukocyte cDNA microarray for studies of immune function and stress reactions. Dev Comp Immunol 2006, in press

Marks H, Vorst O, van Houwelingen AM, van Hulten MC, Vlak JM (2005) Gene-expression profiling of White spot syndrome virus in vivo. J Gen Virol 86:2081-2100

Martin SA, Blaney SC, Houlihan DF, Secombes CJ (2006) Transcriptome response following administration of a live bacterial vaccine in Atlantic salmon (*Salmo salar*). Mol Immunol 43:1900-1911

Matsuyama T, Fujiwara A, Nakayasu C, Kamaishi T, Oseko N, Hirono I, Aoki T (2006) Gene expression of leucocytes in vaccinated Japanese flounder (*Paralichthys olivaceus*) during the course of experimental infection with *Edwardsiella tarda*. Fish Shellfish Immunol, in press

Meijer AH, Verbeek FJ, Salas-Vidal E, Corredor-Adamez M, Bussman J, van der Sar AM, Otto GW, Geisler R, Spaink HP (2005) Transcriptome profiling of adult zebrafish at the late stage of chronic tuberculosis due to *Mycobacterium marinum* infection. Mol Immunol 42:1185-1203

Melamed-Frank M, Lache O, Enav BI, Szafranek T, Levy NS, Ricklis RM, Levy AP (2001) Structure function analysis of the antioxidant properties of haptoglobin. Blood 98:3693–3698

Meyer FP (1991) Aquaculture disease and health management. J Anim Sci 69:4201-4208

Modi WS (2004) CCL3L1 and CCL4L1 chemokine genes are located in a segmental duplication at chromosome17q12. Genomics 83:735-738

Molyneaux KA, Zinszner H, Kunwar PS, Schaible K, Stebler J, Sunshine MJ, O'Brien W, Raz E, Littman D, Wylie C, Lehmann R (2003) The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival. Development 130:4279-4286

Moriya S, Urawa S, Suzuki O, Urano A, Abe S (2004) DNA microarray for rapid detection of mitochondrial DNA haplotypes of chum salmon. Mar Biotechnol (NY) 6:430-434

Morrison RN, Cooper GA, Koop BF, Rise ML, Bridle AR, Adams MB, Nowak BF (2006) Transcriptome profiling the gills of amoebic gill disease (AGD)-affected Atlantic salmon (*Salmo salar* L.) - A role for tumor suppressor p53 in AGD-pathogenesis? Physiol Genomics 26:15-34

Moser B, Loetscher P (2001) Lymphocyte traffic control by chemokines. Nat Immunol 2:123-128

Moser B, Wolf M, Walz A, Loetscher P (2004) Chemokines: multiple levels of leukocyte migration control. Trends Immunol 25:75-85

Murdock CA, Small BC, Bilodeau AL, Peterson BC, Waldbieser GC (2006) Comparative analysis of the expression of reference genes in response to different physiological conditions in channel catfish. Abstract, Aquaculture America 2006. Las Vegas, NV 2006; p. 232.

Murphy PM, Baggiolini M, Charo IF, Hebert CA, Horuk R, Matsushima K, Miller LH, Oppenheim JJ, Power CA (2000) International union of pharmacology. XXII. Nomenclature for chemokine receptors. Pharmacol Rev 52:145–176

Nash JH, Findlay WA, Luebbert CC, Mykytczuk OL, Foote SJ, Taboada EN, Carrillo CD, Boyd JM, Colquhoun DJ, Reith ME, Brown LL (2006) Comparative genomics profiling of clinical isolates of Aeromonas salmonicida using DNA microarrays. BMC Genomics 7:43

Nemeth E, Rivera S, Gabayan V, Keller C, Taudorf S, Pedersen BK, Ganz T (2004b) IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin. J Clin Invest 113:1271-1276

Nemeth E, Tuttle MS, Powelson J, et al (2004a) Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science 306:2090–2093

Neville LF, Mathiak G, Bagasra O (1997) The immunobiology of interferon-gamma inducible protein 10 kD (IP-10): a novel, pleiotropic member of the C-X-C chemokine superfamily. Cytokine Growth Factor Rev 8:207–219

Newton JC, Wolfe LG, Grizzle JM, Plumb JA (1989) Pathology of experimental enteric septicaemia in channel catfish, *Ictalurus punctatus* (Rafinesque), following immersion-exposure to *Edwardsiella ictaluri*. J Fish Dis 12:335–347

Nibbs RJ, Yang J, Landau NR, Mao JH, Graham GJ (1999) LD78beta, a non-allelic variant of human MIP-1alpha (LD78alpha),has enhanced receptor interactions and potent HIV suppressive activity. J Biol Chem 274:17478-17483

Nicchitta CV, Reed RC (2000) The immunological properties of endoplasmic reticulum chaperones: a conflict of interest? Essays Biochem 36:15-25

Nomiyama H, Mera A, Ohneda O, Miura R, Suda T, Yoshie O (2001) Organization of the chemokine genes in the human and mouse major clusters of CC and CXC chemokines: diversification between the two species. 2:110-113

Nuwaysir EF, Huang W, Albert TJ, Singh J, Nuwaysir K, Pitas A, Richmond T, Gorski T, Berg JP, Ballin J, McCormick M, Norton J, Pollock T, Sumwalt T, Butcher L, Porter D, Molla M, Hall C, Blattner F, Sussman MR, Wallace RL, Cerrina F, Green RD (2002) Gene expression analysis using oligonucleotide arrays produced by maskless photolithography. Genome Res 12:1749-1755

Olivier E, Soury E, Risler JL, Smih F, Schneider K, Lochner K, Jouzeau JY, Fey GH, Salier JP (1999) A novel set of hepatic mRNAs preferentially expressed during an acute inflammation in rat represents mostly intracellular proteins. Genomics 57:352-364

Pandey S, Agrawal DK (2006) Immunobiology of Toll-like receptors: emerging trends. Immunol Cell Biol 84:333-341

Park CH, Valore EV, Waring AJ, et al (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 276:7806–7810

Pawitan Y, Michiels S, Koscielny S, Gusnanto A, Ploner A (2005) False discovery rate, sensitivity and sample size for microarray studies. Bioinformatics 21:3017-3024

Peatman E and Liu Z (2006) CC chemokines in zebrafish: evidence of extensive intrachromosomal gene duplications. Genomics 88:381-385

Peatman E and Liu Z (2007) Microarray fundamentals: Basic principles and applications in aquaculture. Chapter in Aquaculture Genome Technology, Z. Liu ed., Blackwell Scientific (in press)

Peatman E, Bao B, Xu P, Baoprasertkul P, Brady Y, Liu Z (2006) Catfish CC chemokines: genomic clustering, duplications, and expression after bacterial infection with *Edwardsiella ictaluri*. Mol Genet Genom 275:297-309

Peatman E, Baoprasertkul P, He C, Li P, Kucuktas H, Liu Z (2005) *In silico* identification and expression analysis of 12 novel CC chemokines in catfish. Immunogenetics 57:409-419

Peatman EJ, Wei X, Feng J, Liu L, Kucuktas H, Li P, He C, Rouse D, Wallace R, Dunham R, Liu Z (2004) Development of expressed sequence tags from Eastern oyster (*Crassostrea virginica*): lessons learned from previous efforts. Proc Mar Biotech 6:S491-496

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29:e45

Poli V (1998) The role of C/EBP isoforms in the control of inflammatory and native immunity functions. J Biol Chem 273:29279-29282

Purcell MK, Nichols KM, Winton JR, Kurath G, Thorgaard GH, Wheeler P, Hansen JD, Herwig RP, Park LK (2006) Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Mol Immunol 43:2089-2106

Redon R, Ishikawa S, Fitch KR, Feuk L, Perry GH, Andrews TD, Fiegler H, Shapero MH, Carson AR, Chen W, Cho EK, Dallaire S, Freeman JL, Gonzalez JR, Gratacos M, Huang J, et al. (2006) Global variation in copy number in the human genome. Nature 444:444-454

Renn SC, Aubin-Horth N, Hofmann HA (2004) Biologically meaningful expression profiling across species using heterologous hybridization to a cDNA microarray. BMC Genomics 5:42

Reynders H, van der Ven K, Moens LN, van Remortel P, De Coen WM, Blust R (2006) Patterns of gene expression in carp liver after exposure to a mixture of waterborne and dietary cadmium using a custom-made microarray. Aquat Toxicol, in press

Rise ML, Douglas SE, Sakhrani D, Williams J, Ewart KV, Rise M, Davidson WS, Koop BF, Devlin RH (2006) Multiple microarray platforms utilized for hepatic gene expression profiling of GH transgenic coho salmon with and without ration restriction. J Mol Endocrinol 37:259-282

Rise ML, Jones SR, Brown GD, von Schalburg KR, Davidson WS, Koop BF (2004b) Microarray analyses identify molecular biomarkers of Atlantic salmon macrophage and hematopoietic kidney response to *Piscirickettsia salmonis* infection. Physiol Genomics 20:21-35

Rise ML, von Schalburg KR, Brown GD, Mawer MA, Devlin RH, Kuipers N, Busby M, Beetz-Sargent M, Alberto R, Gibbs AR, Hunt P, Shukin R, Zeznik JA, Nelson C, Jones SR, Smailus DE, Jones SJ, Schein JE, Marra MA, Butterfield YS, Stott JM, Ng SH, Davidson WS, Koop BF (2004a) Development and application of a

salmonid EST database and cDNA microarray: data mining and interspecific hybridization characteristics. Genome Res 14:478-490

Rivera S, Liu L, Nemeth E, Gabayan V, Sorensen OE, Ganz T (2005) Hepcidin excess induces the sequestration of iron and exacerbates tumor-associated anemia. Blood 105:1797-1802

Roberge C, Paez DJ, Rossignol O, Guderley H, Dodson J, Bernatchez L (2007) Genome-wide survey of the gene expression response to saprolegniasis in Atlantic salmon. Mol. Immunol 44:1374-1383

Ross MT, LaBrie S, McPherson J, Stanton VP (1999) Screening large-insert libraries by hybridization. In: Boyl A, ed Current protocols in human genetics. Wiley, New York, pp 5.6.1–52

Russell S, Hayes MA, Simko E, Lumsden JS (2006) Plasma proteomic analysis of the acute phase response of rainbow trout (*Oncorhynchus mykiss*) to intraperitoneal inflammation and LPS injection. Dev Comp Immunol 30:393-406

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406–425

Sambrook J, Frisch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor

Sarropoulou E, Kotoulas G, Power DM, Geisler R (2005) Gene expression profiling of gilthead sea bream during early development and detection of stress-related genes by the application of cDNA microarray technology. Physiol Genomics 23:182-191

Schaible UE, Collins HL, Priem F, Kaufmann SH (2002) Correction of the iron overload defect in beta-2-microglobulin knockout mice by lactoferrin abolishes their increased susceptibility to tuberculosis. J Exp Med 196:1507–1513

Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270:467-470

Schillaci G, Pirro M (2006) C-reactive protein in hypertension: clinical significance and predictive value. Nutr Metab Cardiovasc Dis 16:500-508

Schmitt B, Henderson L (2005) Diagnostic tools for animal diseases. Rev Sci Tech. 24:243-250

Singh-Gasson S, Green RD, Yue Y, Nelson C, Blattner F, Sussman MR, Cerrina F (1999) Maskless fabrication of light-directed oligonucleotide microarrays using a digital micromirror array. Nat Biotechnol 17: 974-978

Soderlund, C, Humphray S, Dunham A, French L (2000) Contigs built with fingerprints, markers, and FPC V4.7. Genome Res 10:1772-1787

Soderlund, C, Longden I, Mott R (1997) FPC: a system for building contigs from restriction fingerprinted clones. Comput Appl Biosci 13:523-535

Starnes T, Rasila KK, Robertson MJ, Brahmi Z, Dahl R, Christopherson K, Hromas R (2006) The chemokine CXCL14 (BRAK) stimulates activated NK cell migration: implications for the downregulation of CXCL14 in malignancy. Exp Hematol 34:1101-1105

Steinbrenner H, Bilgic E, Alili L, Sies H, Brenneisen P (2006) Selenoprotein P protects endothelial cells from oxidative damage by stimulation of glutathione peroxidase expression and activity. Free Radic Res 40:936-943

Strehl B, Joeris T, Rieger M, Visekruna A, Textoris-Taube K, Kaufmann SH, Kloetzel PM, Kuckelkorn U, Steinhoff U (2006) Immunoproteasomes are essential for clearance of *Listeria monocytogenes* in nonlymphoid tissues but not for induction of bacteria-specific CD8+ T cells. J Immunol 177:6238-6244

Sulston, et al. (1988) Software for genome mapping by fingerprint techniques. Comput Appl Biosci 4:125-132

Sulston, J, Mallett F, Durbin R, Horsnell T (1989) Image analysis of restriction enzyme fingerprint autoradiograms. Comput Appl Biosci 5:101-106

Sunyer JO, Zarkadis IK, Lambris JD (1998) Complement diversity: a mechanism for generating immune diversity? Immunol Today 19:519-523

Suzuki YA, Shin K, Lonnerdal B (2001) Molecular cloning and functional expression of a human intestinal lactoferrin receptor. Biochemistry 40:15771-15779

Szegezdi E, Logue SE, Gorman AM, Samali A (2006) Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep 7:880-885

Thompson JD, Higgins DG, Gibson TJ (1994) ClustalW: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680

Tilton SC, Gerwick LG, Hendricks JD, Rosato CS, Corley-Smith G, Givan SA, Bailey GS, Bayne CJ, Williams DE (2005) Use of a rainbow trout oligonucleotide microarray to determine transcriptional patterns in aflatoxin B1-induced hepatocellular carcinoma compared to adjacent liver. Toxicol Sci 88:319-330

Tolosano E, Altruda F (2002) Hemopexin: structure, function, and regulation. DNA Cell Biol 21:297-306

Townson JR, Barcellos LF, Nibbs RJ (2002) Gene copy number regulates the production of the human chemokine CCL3-L1. Eur J Immunol 32:3016-3026

Tsai JM, Wang HC, Leu JH, Hsiao HH, Wang AH, Kou GH, Lo CF (2004) Genomic and proteomic analysis of thirty-nine structural proteins of shrimp white spot syndrome virus. J Virol 78:11360-11370

Tsuji S, Uehori J, Matsumoto M, Suzuki Y, Matsuhisa A, Toyoshima K, Seya T (2001) Human intelectin is a novel soluble lectin that recognizes galactofuranose in carbohydrate chains of bacterial cell wall. J Biol Chem 276:23456-23463

Tsujita T, Tsukada H, Nakao M, Oshiumi H, Matsumoto M, Seya T (2004) Sensing bacterial flagellin by membrane and soluble orthologs of Toll-like receptor 5 in rainbow trout (*Onchorhynchus mikiss*). J Biol Chem 279:48588-48597

Tusher VG, Tibshirani R, Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98:5116-5121

USDA, 2000. Aquaculture Outlook, March 13, 2000. USDA Economic Research Service Publication #LDP-AQS-11

USDA, 2003. Part II: Reference of Foodsize Catfish Health and Production Practices in the United States, 2003, USDA:APHIS:VS,CEAH, National Animal Health Monitoring System, Fort Collins, CO, pp. 56–70.

von Schalburg KR, McCarthy SP, Rise ML, Hutson JC, Davidson WS, Koop BF (2006) Expression of morphogenic genes in mature ovarian and testicular tissues: potential stem-cell niche markers and patterning factors. Mol Reprod Dev 73:142-152

von Schalburg KR, Rise ML, Brown GD, Davidson WS, Koop BF (2005b) A comprehensive survey of the genes involved in maturation and development of the rainbow trout ovary. Biol Reprod 72:687-699

von Schalburg KR, Rise ML, Cooper GA, Brown GD, Gibbs AR, Nelson CC, Davidson WS, Koop BF (2005a) Fish and chips: various methodologies demonstrate utility of a 16,006-gene salmonid microarray. BMC Genomics 6:126

Vornanen M, Hassinen M, Koskinen H, Krasnov A (2005) Steady-state effects of temperature acclimation on the transcriptome of the rainbow trout heart. Am J Physiol Regul Integr Comp Physiol 289:R1177-1184

Walsh B, Henderson D (2004) Microarrays and beyond: what potential do current and future genomics tools have for breeders? J Anim Sci E-Suppl:E292-299

Wang J, Adelson DL, Yilmaz A, Sze SH, Jin Y, Zhu JJ (2005) Genomic organization, annotation, and ligand-receptor inferences of chicken chemokines and chemokine receptor genes based on comparative genomics. BMC Genomics 6:45

Wang Q, Bao B, Wang Y, Peatman E, Liu Z (2006a) Characterization of a NK-lysin antimicrobial peptide gene from channel catfish. Fish Shellfish Immunol 20:419-426

Wang Q, Wang Y, Xu P, Liu Z (2006b) NK-lysin of channel catfish: gene triplication, sequence variation, and expression analysis. Mol Immunol 43:1676-1686

Wang Y, Wang Q, Baoprasertkul P, Peatman E, Liu Z (2006c) Genomic organization, gene duplication, and expression analysis of interleukin-1beta in channel catfish (*Ictalurus punctatus*). Mol Immunol 43:1653-1664

Williams TD, Diab AM, George SG, Godfrey RE, Sabine V, Conesa A, Minchin SD, Watts PC, Chipman JK (2006) Development of the GENIPOL European flounder (*Platichthys flesus*) microarray and determination of temporal transcriptional responses to cadmium at low dose. Environ Sci Technol 40:6479-6488

Williams TD, Gensberg K, Minchin SD, Chipman JK (2003) A DNA expression array to detect toxic stress response in European flounder (*Platichthys flesus*). Aquat Toxicol 65:141-157

Wolters WR, Johnson MR (1994) Enteric septicemia resistance in blue catfish and three channel catfish strains. J Aquatic Animal Health 6:329-334

Wolters WR, Wise DJ, Klesius PH (1996) Survival and antibody response of channel catfish, blue catfish, and catfish hybrids after exposure to *E. ictaluri*. J Aquatic Animal Health 8:249-254

Xu P, Bao B, Zhang S, Peatman E, He C, Liu ZJ (2005) Characterization and expression analysis of bactericidal permeability-increasing protein (BPI) antimicrobial peptide gene from channel catfish *Ictalurus punctatus.* Dev Comp Immunol 29:865-878

Xu P, Wang S, Liu L, Peatman E, Somridhivej B, Thimmapuram J, Gong G, Liu Z (2006) Channel catfish BAC-end sequences for marker development and assessment of syntenic conservation with other fish species. Anim Genet 37:321-326

Xu P, Wang S, Liu L, Thorsen J, Liu ZJ (2007) A BAC-based physical map of the channel catfish genome. Genetics, in press

You FM, Luo MC (2003) GenoProfiler User's Manual. http://wheat.pw.usda.gov/PhysicalMapping/tools/genoprofiler/manual/.

APPENDICES

Supplemental Table 1
All significantly upregulated catfish transcripts in liver after *E. ictaluri* infection. *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 along with the E-value of that hit. *q-value* is the false-discovery rate for the particular gene

Accession	Putative Id	E-value	Fold Change	q-value(%)
CF970955	Intelectin [Ctenopharyngodon idella]	7.00E-32	85.4	1.25
CF971897	Intelectin 2 [Danio rerio]	3.00E-117	40.0	3.27
CK407451	Intelectin 2 [Danio rerio]	8.00E-120	36.0	1.25
BM439193	No significant similarity		35.5	3.27
CK408483	Haptoglobin precursor [Danio rerio]	1.00E-56	34.3	0.00
CK409144	No significant similarity		33.3	3.27
BM438750	Microfibrillar-associated protein 4 [Danio rerio]	1.00E-35	32.9	1.25
CF970863	Microfibrillar-associated protein 4 [Danio rerio]	1.00E-57	30.0	1.25
TC6845	Intelectin [Ctenopharyngodon idella]	4.00E-128	28.0	2.36
BM438689	Microfibrillar-associated protein 4 [Rattus norvegicus]	3.00E-68	25.6	0.00
TC8425	Warm-temperature-acclimation-related-65kda- protein-like-protein [Oryzias latipes]	2.00E-118	23.4	0.00
CK406362	Microfibrillar-associated protein 4 [Danio rerio]	2.00E-75	23.2	0.00
AY555510	CC chemokine SCYA113 [Ictalurus punctatus]	4.00E-37	22.7	3.27
TC7475	CC chemokine SCYA113 [Ictalurus punctatus]	5.00E-43	21.5	3.27
CK406396	Neurotoxin/C59/Ly-6-like protein [Ctenopharyngodon idella]	6.00E-21	21.3	1.25
CV995517	CC chemokine SCYA113 [Ictalurus punctatus]	2.00E-39	17.7	3.27
CV996365	No significant similarity		15.3	1.25
CV994031	Catechol-O-methyltransferase domain containing 1 [Danio rerio]	3.00E-76	14.8	0.00
TC9205	Hypothetical protein XP_683888 [Danio rerio]	7.00E-48	14.4	1.25
CK406832	No significant similarity		14.2	0.00
CF971597	Hypothetical protein XP_683888 [Danio rerio]	4.00E-44	14.0	1.25
TC8426	Hemopexin precursor [Danio rerio]	5.00E-75	13.6	2.36
CV996638	Apoa4 protein [Danio rerio]	1.00E-93	13.0	2.36
CK406564	Hemopexin precursor [Danio rerio]	1.00E-90	12.9	6.47
CF971550	Warm-temperature-acclimation-related-65 [Oryzias latipes]	7.00E-104	12.4	0.00
CV993724	Toll-like receptor 5 [Ictalurus punctatus]	2.00E-118	11.8	0.00
EE993358	Complement C3 [Ctenopharyngodon	8.00E-85	11.3	1.25

	idella]			
EE993177	Complement factor H precursor [Danio rerio]	2.00E-26	10.9	1.25
CF971448	Catechol-O-methyltransferase domain containing 1 [Danio rerio]	9.00E-48	10.5	0.00
EE993360	Toll-like receptor 5 [Ictalurus punctatus]	1.00E-84	10.0	0.00
CV987901	Complement C3-H1 [Cyprinus carpio]	3.00E-59	10.0	1.71
EE993362	Complement protein component C7-1[Danio rerio]	6.00E-104	9.7	0.00
TC9637	Fibrinogen alpha chain [Danio rerio]	2.00E-95	9.6	0.00
CK407984	Complement regulatory plasma protein [Paralabrax nebulifer]	1.00E-14	8.9	3.27
TC9194	Complement regulatory plasma protein [Paralabrax nebulifer]	7.00E-27	8.9	3.27
CV992853	Ceruloplasmin [Danio rerio]	8.00E-98	8.5	0.00
TC9833	Microfibrillar-associated protein 4 [Danio rerio]	1.00E-57	8.4	1.25
TC8307	Fibrinogen gamma polypeptide [Danio rerio]	6.00E-162	8.4	1.25
CK407355	Complement regulatory plasma protein [Paralabrax nebulifer]	2.00E-41	8.1	3.27
CF970899	No significant similarity		7.9	3.27
BM439040	No significant similarity		7.8	3.27
TC8765	Transferrin [Salvelinus fontinalis]	1.00E-160	7.7	0.00
CK407588	Catechol-O-methyltransferase domain containing 1 [Danio rerio]	2.00E-87	7.5	0.00
CK407841	Fibrinogen gamma polypeptide [Danio rerio]	5.00E-109	7.3	3.27
CV996644	Complement component factor H [Rattus norvegicus]	7.00E-05	7.3	3.27
CK406972	No significant similarity		7.2	1.71
BM438893	No significant similarity		6.9	0.00
CK406644	Apoa4 protein [Danio rerio]	2.00E-19	6.9	6.47
CF971378	Fibrinogen, B beta polypeptide [Danio rerio]	1.00E-21	6.4	1.71
CV989967	Complement C3 [Ctenopharyngodon idella]	4.00E-100	6.3	1.25
CK406609	Fibrinogen gamma polypeptide [Ictalurus punctatus]	2.00E-63	6.2	6.47
TC8306	Fibrinogen gamma polypeptide [Danio rerio]	6.00E-162	6.1	0.00
CF971612	No significant similarity		6.0	3.74
CK408142	No significant similarity		5.9	1.71
CV997096	Apoa4 protein [Danio rerio]	5.00E-55	5.9	5.24
CV996287	No significant similarity		5.7	3.27
CV989503	CXCL14 [Ictalurus punctatus]	2.00E-52	5.7	0.00
BM439121	No significant similarity		5.6	5.24
CV997126	Complement C3 [Ctenopharyngodon idella]	3.00E-44	5.6	0.00

CF971852	Fibrinogen alpha chain [Danio rerio]	7.00E-68	5.5	0.00
BM438459	Fibrinogen gamma polypeptide [Danio rerio]	5.00E-28	5.4	1.71
TC7741	Complement factor B/C2-A3 [Cyprinus carpio]	2.00E-114	5.4	3.74
CV992447	Complement component C8 beta [Oncorhynchus mykiss]	4.00E-38	5.4	3.27
BM425349	Complement C4 [Oncorhynchus mykiss]	2.00E-18	5.4	3.27
TC7892	Ceruloplasmin [Danio rerio]	2.00E-104	5.4	0.00
CV995884	Solute carrier family 31 (copper transporters), member 1 [Danio rerio]	4.00E-67	5.3	0.00
BM494620	Serum/glucocorticoid regulated kinase [Danio rerio]	5.00E-73	5.3	1.25
CK407596	LOC407646 protein [Danio rerio]	3.00E-67	5.3	3.74
EE993354	Complement component 7 precursor [Danio rerio]	5.00E-105	5.3	0.00
CF971561	Transferrin [Oncorhynchus tshawytscha]	3.00E-35	5.2	5.24
TC8490	Fibrinogen, B beta polypeptide [Danio rerio]	0	5.2	1.71
BM438919	Intelectin [Ctenopharyngodon idella]	2.00E-11	5.0	0.00
EE993545	Erythroblast membrane-associated protein [Danio rerio]	8.00E-16	5.0	3.74
TC7498	No significant similarity		4.9	1.25
TC7249	LOC407646 protein [Danio rerio]	2.00E-84	4.9	0.00
BM438712	Tryptophan 2,3-dioxygenase [Danio rerio]	1.00E-13	4.8	0.00
CK408512	Solute carrier family 31 (copper transporters), member 1 [Danio rerio]	8.00E-77	4.8	1.25
CK406912	Complement C4 [Oncorhynchus mykiss]	6.00E-57	4.8	3.27
CK408638	Transferrin [Salvelinus fontinalis]	8.00E-83	4.7	1.71
AY836756	CXCL14 [Ictalurus punctatus]	2.00E-52	4.7	3.27
CK406849	Angiotensinogen [Danio rerio]	4.00E-90	4.7	3.74
TC9712	No significant similarity		4.6	3.27
BM438818	No significant similarity		4.5	0.00
CF971802	LOC407646 protein [Danio rerio]	1.00E-24	4.5	0.00
BM438634	Angiotensinogen [Danio rerio]	6.00E-57	4.5	1.25
CK420078	Selenoprotein P, plasma, 1a [Danio rerio]	5.00E-67	4.4	7.53
BM027942	Selenoprotein P, plasma, 1a [Danio rerio]	4.00E-55	4.4	6.47
CF971024	No significant similarity		4.4	3.27
EE993162	Complement component C8 beta [Oncorhynchus mykiss]	9.00E-67	4.4	3.27
BM438200	Complement component C5-1 [Cyprinus carpio]	1.00E-43	4.4	1.25
CF971526	No significant similarity		4.2	3.74
TC9358	Complement C4-2 [Cyprinus carpio]	1.00E-87	4.2	0.00
BM438717	TNFAIP9 protein isoform 2 [Danio rerio]	2.00E-12	4.1	1.25
CK407254	Complement component 9 [Danio rerio]	7.00E-75	4.1	3.27

BM438848	No significant similarity		4.0	1.25
BQ097411	No significant similarity		4.0	3.27
CF971664	Fibrinogen alpha chain [Danio rerio]	5.00E-46	4.0	1.25
BM438654	Complement factor H precursor [Oncorhynchus mykiss]	3.00E-21	4.0	3.27
EE993174	Complement C3-H2 [Cyprinus carpio]	5.00E-84	4.0	1.25
CK408253	Uridine phosphorylase, like [Danio rerio]	2.00E-63	3.9	3.74
CB939682	Protein disulfide isomerase-associated 4 [Danio rerio]	8.00E-76	3.9	1.71
BM438858	No significant similarity		3.9	3.27
CK407775	Fibrinogen, B beta polypeptide [Danio rerio]	2.00E-25	3.9	1.71
CK409245	Fibrinogen alpha chain [Danio rerio]	2.00E-32	3.8	3.27
CK407193	Complement factor H-related 1 [Homo sapiens]	9.00E-23	3.8	3.27
TC7660	Complement component C9 [Ctenopharyngodon idella]	1.00E-19	3.8	1.71
CK407328	Coagulation factor XIII B chain precursor [Canis familiaris]	1.00E-13	3.8	6.47
TC7576	Tumor necrosis factor, alpha-induced protein 9	4.00E-74	3.7	0.00
BM439116	No significant similarity		3.7	3.27
CK417270	Cytochrome b5 [Danio rerio]	1.00E-36	3.7	1.71
TC10030	Protein disulfide-isomerase [Cricetulus griseus]	0	3.6	7.53
CF971645	No significant similarity		3.6	3.27
BM438615	Complement C3-S [Danio rerio]	4.00E-69	3.6	3.74
TC8237	Protein disulfide isomerase associated 4 [Danio rerio]	6.00E-108	3.5	1.71
CK407075	No significant similarity		3.5	3.27
TC9113	No significant similarity		3.5	3.74
BQ097150	SSR alpha subunit [Oncorhynchus mykiss]	1.00E-83	3.4	0.00
CK405481	Solute carrier family 38, member 2 [Xenopus tropicalis]	4.00E-12	3.4	3.27
CF970857	Protein disulfide isomerase associated 4 [Danio rerio]	1.00E-57	3.4	6.47
CK406974	No significant similarity		3.4	1.71
BM438944	No significant similarity		3.4	3.27
CK423432	No significant similarity		3.4	3.27
EE993343	Complement C4 [Oncorhynchus mykiss]	3.00E-80	3.4	1.71
TC8567	Chemotaxin [Oncorhynchus mykiss]	2.00E-46	3.4	1.71
TC7903	Armet protein [Xenopus laevis]	3.00E-60	3.4	7.53
CK407547	Protein disulfide isomerase associated 4 [Danio rerio]	1.00E-122	3.3	6.47
CF972223	No significant similarity		3.3	1.71
TC6684	Jun B proto-oncogene, like [Danio rerio]	3.00E-14	3.3	3.27
CV988633	Apoa4 protein [Danio rerio]	2.00E-65	3.3	5.24

BM438229	Alpha-2-macroglobulin-2 [Cyprinus carpio]	3.00E-63	3.3	3.74
CV988413	Alpha-2-macroglobulin-2 [Cyprinus carpio]	1.00E-92	3.3	1.71
BM438317	Tryptophan 2,3-dioxygenase [Danio rerio]	4.00E-77	3.2	2.36
CK406955	No significant similarity		3.2	3.27
CV996636	No significant similarity		3.2	5.24
CK407024	Complement C3-S [Cyprinus carpio]	7.00E-05	3.2	3.74
BM438664	Coagulation factor 10 [Danio rerio]	4.00E-92	3.2	2.36
CK421169	Canopy1 [Danio rerio]	1.00E-69	3.2	3.27
CK414114	Solute carrier family 38, member 2 SLC38A2 [Danio rerio]	5.00E-112	3.1	0.00
CV989134	Calumenin isoform 2 isoform 3 [Canis familiaris]	3.00E-75	3.1	3.74
CF970862	No significant similarity		3.1	7.53
CK407645	No significant similarity		3.1	2.36
CK409512	No significant similarity		3.1	3.74
CV992516	Complement factor B/C2B [Danio rerio]	3.00E-25	3.1	6.47
TC7895	SSR alpha subunit/Translocon-associated protein subunit alpha precursor (TRAP-alpha) [Oncorhynchus mykiss]	3.00E-75	3.0	3.74
TC7025	No significant similarity		3.0	0.00
CF971953	Fibrinogen, beta chain isoform 4 [Macaca mulatta]	5.00E-88	3.0	3.27
CK406847	Fibrinogen alpha chain [Danio rerio]	9.00E-11	3.0	3.74
CV994441	Complement C3-Q1, partial [Danio rerio]	4.00E-09	3.0	0.00
CK407507	Type III iodothyronine deiodinase [Oreochromis niloticus]	2.00E-90	2.9	3.74
CK408666	Transferrin [Salmo trutta]	2.00E-68	2.9	3.74
CF971394	No significant similarity		2.9	3.27
EE993209	No significant similarity		2.9	3.27
TC8545	No significant similarity		2.9	1.71
CF971799	No significant similarity		2.9	3.74
CK409240	Coagulation factor 10 [Danio rerio]	1.00E-73	2.9	3.27
CF971219	Ceruloplasmin [Danio rerio]	7.00E-82	2.9	5.24
CV987559	SEC61, alpha subunit [Danio rerio]	5.00E-100	2.8	3.27
TC7371	No significant similarity		2.8	5.24
CK407648	No significant similarity		2.8	5.24
CK406724	No significant similarity		2.8	7.53
CK413362	No significant similarity		2.8	3.27
CV994570	No significant similarity		2.8	1.71
CK425818	No significant similarity		2.8	1.71
CK408535	Microfibrillar-associated protein 4 [Danio rerio]	2.00E-17	2.8	1.71
CV995270	Apolipoprotein B-100 precursor [Danio rerio]	1.00E-76	2.8	6.47

CK406836	Alpha-2-macroglobulin [Ctenopharyngodon idella]	1.00E-76	2.8	3.27
CK406859	Transmembrane emp24 domain-containing protein 10 [Danio rerio]	3.00E-91	2.7	0.00
CK410283	Transmembrane emp24 domain-containing protein 10 [Danio rerio]	9.00E-101	2.7	1.71
CV996617	Transferrin [Salmo trutta]	1.00E-76	2.7	3.27
TC12946	Transferrin [Salmo trutta]	3.00E-141	2.7	3.74
EE993484	Selenium binding protein 1 isoform 4 [Danio rerio]	2.00E-76	2.7	7.53
CK406562	No significant similarity		2.7	3.74
CK406432	No significant similarity		2.7	1.71
CV989409	Dnajb11 protein [Danio rerio]	2.00E-65	2.7	1.71
TC9511	Calreticulin [Ictalurus punctatus]	0	2.7	0.00
CK407198	Alpha-2-antiplasmin precursor [Danio rerio]	7.00E-19	2.7	3.27
TC8542	SEC11-like 1/SPC18 [Danio rerio]	2.00E-87	2.6	3.74
CV990480	Isocitrate dehydrogenase 1 (NADP+), soluble [Danio rerio]	7.00E-20	2.6	1.25
TC8074	Deleted in malignant brain tumors 1 [Danio rerio]	9.00E-28	2.6	3.74
BM438622	C1 inhibitor [Oncorhynchus mykiss]	1.00E-43	2.6	3.74
TC6883	Thioredoxin [Ictalurus punctatus]	2.00E-51	2.6	3.74
CV993716	SAR1a-like protein 2 [Ictalurus punctatus]	4.00E-27	2.5	0.00
CK407814	Plasminogen [Danio rerio]	1.00E-149	2.5	5.24
CB939893	No significant similarity		2.5	6.47
BM029249	No significant similarity		2.5	0.00
TC8843	No significant similarity		2.5	3.74
CK409289	No significant similarity		2.5	3.27
CV994205	No significant similarity		2.5	5.24
CV993853	No significant similarity		2.5	5.24
CK409109	No significant similarity		2.5	3.27
CK404043	No significant similarity		2.5	1.71
TC8981	FK506 binding protein 2 [Mus musculus]	9.00E-56	2.5	3.74
CB937094	Eif4e1a protein [Danio rerio]	8.00E-99	2.5	1.25
TC7047	DC2 protein [Xenopus tropicalis]	3.00E-76	2.5	0.00
CV990348	Calreticulin, like 2 [Danio rerio]	5.00E-106	2.5	3.74
CV995732	Asparagine synthetase [Danio rerio]	3.00E-79	2.5	0.00
CF971399	Transferrin [Paralichthys olivaceus]	4.00E-44	2.4	7.53
CK410712	Sorting nexin-17 isoform 7 [Canis familiaris]	1.00E-21	2.4	1.71
TC9317	Solute carrier family 21, member 14/Oatp [Danio rerio]	4.00E-121	2.4	3.27
CK408024	No significant similarity		2.4	5.24
CK422256	No significant similarity		2.4	3.74

CK407036	No significant similarity		2.4	0.00
CV992084	No significant similarity		2.4	1.25
BM424895	No significant similarity		2.4	2.36
CV990387	No significant similarity		2.4	5.24
CV995916	No significant similarity		2.4	3.74
CF970874	No significant similarity		2.4	3.74
CK407673	Leucine-rich alpha-2-glycoprotein [Ctenopharyngodon idella]	3.00E-91	2.4	1.71
CV993838	Isocitrate dehydrogenase 1 (NADP+), soluble [Danio rerio]	1e-109	2.4	6.47
TC8308	Fibrinogen gamma polypeptide [Danio rerio]	6.00E-162	2.4	1.71
EE993353	Complement C3 precursor alpha chain [Danio rerio]	6.00E-27	2.4	1.25
BM438581	Colipase preproprotein [Homo sapiens]	2.00E-23	2.4	6.47
CK409467	Coagulation factor II (thrombin) [Danio rerio]	4.00E-12	2.4	7.53
TC8712	Bsg protein [Danio rerio]	3.00E-06	2.4	3.74
CV994347	Alpha-2-antiplasmin precursor [Danio rerio]	2.00E-17	2.4	3.27
CV987909	Unnamed protein product [Tetraodon nigroviridis]	3.00E-12	2.3	3.27
TC7791	Asparagine-linked glycosylation 5 homolog [Xenopus tropicalis]	6.00E-46	2.3	7.53
CF971576	Tumor necrosis factor, alpha-induced protein 9 [Homo sapiens]	5.00E-53	2.3	1.25
CV992736	Integral membrane protein 1 [Danio rerio]	8.00E-140	2.3	0.00
CK405052	SEC11-like 1 (S. Cerevisiae) [Danio rerio]	5.00E-52	2.3	1.71
CK409154	Ras homolog gene family, member E [Danio rerio]	5.00E-43	2.3	5.24
CV991869	Protein disulfide isomerase-related protein P5 precursor [Danio rerio]	9.00E-64	2.3	0.00
CK411358	Phosphoethanolamine N-methyltransferase [Strongylocentrotus purpuratus]	3.00E-78	2.3	1.25
CK418743	No significant similarity		2.3	2.36
CK421480	No significant similarity		2.3	0.00
CK407038	No significant similarity		2.3	1.25
CK410384	No significant similarity		2.3	5.24
TC9282	No significant similarity		2.3	3.74
CK402366	No significant similarity		2.3	3.27
CK412705	No significant similarity		2.3	1.71
CK425589	No significant similarity		2.3	5.24
TC6827	NADH dehydrogenase subunit 1 [Ictalurus punctatus]	7.00E-124	2.3	0.00
CK401667	Isocitrate dehydrogenase 1 (NADP+), soluble [Danio rerio]	8.00E-146	2.3	1.25
BM027884	Glucose transporter 1A [Danio rerio]	4.00E-77	2.3	5.24

CK404798	Ferritin heavy subunit [Ictalurus punctatus]	1.00E-95	2.3	6.47
CK407169	Cytochrome P450, family 8, subfamily B [Gallus gallus]	4.00E-71	2.3	2.36
CV991514	Complement factor B/C2B [Cyprinus carpio]	6.00E-76	2.3	3.74
CF971843	Colipase preproprotein [Homo sapiens]	3.00E-21	2.3	7.53
CV988651	Coated vesicle membrane protein [Mus musculus]	3.00E-91	2.3	0.00
CF971415	Canopy1 [Danio rerio]	2.00E-23	2.3	3.27
CV994492	Apoa4 protein [Danio rerio]	1.00E-54	2.3	3.74
TC9203	Inter-alpha trypsin inhibitor [Fundulus heteroclitus]	1.00E-54	2.2	5.24
TC8465	WW domain binding protein 2 [Ictalurus punctatus]	4.00E-95	2.2	3.74
CK425032	Translocon-associated protein beta/SSR beta [Danio rerio]	2.00E-89	2.2	0.00
CF972062	Translocon-associated protein beta/SSR beta [Danio rerio]	1.00E-40	2.2	3.27
TC7124	Stress-associated endoplasmic reticulum protein 1 [Homo sapiens]	5.00E-27	2.2	5.24
TC7513	Sec61 gamma subunit [Homo sapiens]	1.00E-13	2.2	5.24
CK407263	No significant similarity		2.2	6.47
CV994712	No significant similarity		2.2	3.74
CK404612	No significant similarity		2.2	3.74
CK418449	No significant similarity		2.2	7.53
CK403577	No significant similarity		2.2	3.27
CK425361	No significant similarity		2.2	3.74
BM424296	No significant similarity		2.2	3.74
CB939641	No significant similarity		2.2	3.27
TC8290	NADH dehydrogenase subunit 5 [Ictalurus punctatus]	0.00	2.2	1.71
TC6955	Jun B proto-oncogene, like [Danio rerio]	1.00E-54	2.2	0.00
CK415655	Endoplasmin [Aedes aegypti]	3.00E-42	2.2	7.53
TC9110	CD59 [Ictalurus punctatus]	6.00E-58	2.2	0.00
TC8069	C type lectin receptor A isoform 3 [Danio rerio]	2.00E-48	2.2	5.24
CV992060	Transposase [Bacillus cereus]	2.00E-64	2.1	6.47
TC6893	Signal sequence receptor delta [Ictalurus punctatus]	4.00E-82	2.1	3.74
TC7064	Signal sequence receptor, gamma (translocon-associated protein gamma)	2e-86	2.1	6.47
CK411755	Integral membrane protein 1 [Danio rerio]	4.00E-24	2.1	1.71
BM438221	SEC61, beta subunit [Danio rerio]	4.00E-98	2.1	0.00
TC7429	SEC22 vesicle trafficking protein homolog B [Danio rerio]	9.00E-98	2.1	3.74
TC8540	Ribosomal protein L23a [Ictalurus punctatus]	2.00E-69	2.1	6.47
CV987930	Plasminogen [Danio rerio]	3.00E-111	2.1	7.53

CV994121	Nucleobindin 2a [Danio rerio]	2.00E-75	2.1	0.00
BM438887	No significant similarity		2.1	3.74
CK417505	No significant similarity		2.1	3.27
CK407464	No significant similarity		2.1	3.27
CF972234	No significant similarity		2.1	3.74
CB936516	Low density lipoprotein receptor-related protein associated protein 1 [Danio rerio]	7.00E-98	2.1	0.00
CK408501	Intraflagellar transport protein 20 [Danio rerio]	9.00E-55	2.1	1.71
TC8637	Hypothetical protein [Rattus norvegicus]	9.00E-16	2.1	1.25
TC10083	Glucose regulated protein 58kd [Bos taurus]	0	2.1	6.47
TC9827	Cytochrome c oxidase subunit 1 [Ictalurus furcatus]	0	2.1	0.00
TC8986	Cytochrome b5 [Danio rerio]	2.00E-42	2.1	3.27
BM438646	Ceruloplasmin [Danio rerio]	1.00E-22	2.1	3.74
EE993184	C type lectin receptor A isoform 3 [Danio rerio]	1.00E-72	2.1	3.27
CK406535	Cathepsin L preproprotein [Cyprinus carpio]	1.00E-144	2.1	1.71
BM438153	Transducer of ERBB2, 1a [Danio rerio]	2.00E-32	2.0	6.47
TC9714	Integral membrane protein 1 [Danio rerio]	3.00E-171	2.0	0.00
EE993544	Selenoprotein X, 1 [Danio rerio]	6.00E-48	2.0	3.74
CF971017	SEC13-like 1 (S. Cerevisiae) [Danio rerio]	5.00E-08	2.0	3.74
CK402358	Retinol dehydrogenase 1, like [Danio rerio]	9.00E-96	2.0	7.53
TC8057	Profilin 2 like [Danio rerio]	2.00E-51	2.0	3.74
CF971814	Plasminogen [Oncorhynchus mykiss]	2.00E-11	2.0	6.47
TC9540	Peptidylprolyl isomerase B/cyclophilin B [Ictalurus punctatus]	9.00E-113	2.0	3.74
CK421111	No significant similarity		2.0	7.53
CV991696	No significant similarity		2.0	3.27
CK406865	No significant similarity		2.0	3.74
CV991348	No significant similarity		2.0	3.27
CK406938	No significant similarity		2.0	3.27
CK407170	No significant similarity		2.0	5.24
CK405156	No significant similarity		2.0	1.71
BQ096912	Glucose regulated protein 58kd [Bos taurus]	8.00E-45	2.0	3.74
CK404833	Ferritin heavy subunit [Ictalurus punctatus]	6.00E-54	2.0	7.53
CK408710	Cytochrome P450, family 3, subfamily A, polypeptide 65 [Danio rerio]	4.00E-90	2.0	5.24
CK422124	Calpain-like protease [Gallus gallus]	1.00E-100	2.0	3.74

Supplemental Table 2
All significantly downregulated catfish transcripts in liver after *E. ictaluri* infection. *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 along with the E-value of that hit. *q-value* is the false-discovery rate for the particular gene

Accession	Putative identity	E-value	Fold Change	q-value
TC7457	Eukaryotic translation initiation factor 3, subunit 6 interacting protein [*Danio rerio*]	1.00e-74	0.42	6.5
CK404061	No significant similarity		0.44	5.2
AY845143	Liver-expressed antimicrobial peptide 2 [*Ictalurus punctatus*]	5.00e-29	0.45	6.5
CK403219	No significant similarity		0.47	7.5
TC6758	Thioredoxin interacting protein [*Danio rerio*]	0.0	0.49	1.7
TC6756	Thioredoxin interacting protein [*Danio rerio*]	0.0	0.50	1.7

Supplemental Table 3
Unique, significantly up-regulated catfish transcripts in liver after *E. ictaluri* infection that could be annotated by sequence similarity. *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

Accession	Putative identity	Fold Change	q-value
CF970955	Intelectin [*Ctenopharyngodon idella*]	85.4	1.25
CK408483	Haptoglobin precursor [*Danio rerio*]	34.3	0.00
BM438750	Microfibrillar-associated protein 4 [*Danio rerio*]	32.9	1.25
TC6845	Intelectin [*Ctenopharyngodon idella]*	28.0	2.36
BM438689	Microfibrillar-associated protein 4 [*Rattus norvegicus*]	25.6	0.00
TC8425	Warm-temperature-acclimation-related-65kda- protein-like-protein [*Oryzias latipes*]	23.4	0.00
TC7475	CC chemokine SCYA113 [*Ictalurus punctatus*]	21.5	3.27
CK406396	Neurotoxin/C59/Ly-6-like protein [*Ctenopharyngodon idella*]	21.3	1.25
CV994031	Catechol-O-methyltransferase domain containing 1 [*Danio rerio*]	14.8	0.00
TC9205	Hypothetical protein XP_683888 [*Danio rerio*]	14.4	1.25
TC8426	Hemopexin precursor [*Danio rerio*]	13.6	2.36
CV996638	Apolipoprotein Apoa4 protein [*Danio rerio*]	13.0	2.36
CV993724	Toll-like receptor 5 [*Ictalurus punctatus*]	11.8	0.00
CV987901	Complement C3-H1 [*Cyprinus carpio*]	10.0	1.71
EE993362	Complement protein component C7-1[*Danio rerio*]	9.7	0.00
TC9637	Fibrinogen alpha chain [*Danio rerio*]	9.6	0.00
TC9194	Complement regulatory plasma protein [*Paralabrax nebulifer*]	8.9	3.27
CV992853	Ceruloplasmin [*Danio rerio*]	8.5	0.00
TC9833	Microfibrillar-associated protein 4 [*Danio rerio*]	8.4	1.25
TC8765	Transferrin [*Salvelinus fontinalis*]	7.7	0.00
TC8306	Fibrinogen gamma polypeptide [*Danio rerio*]	6.1	0.00
CV989503	CXCL14 [*Ictalurus punctatus*]	5.7	0.00
CV997126	Complement C3 [*Ctenopharyngodon idella*]	5.6	0.00
TC7892	Ceruloplasmin [*Danio rerio*]	5.4	0.00
CV992447	Complement component C8 beta [*Oncorhynchus mykiss*]	5.4	3.27
TC7741	Complement factor B/C2-A3 [*Cyprinus carpio*]	5.4	3.74
BM494620	Serum/glucocorticoid regulated kinase [*Danio rerio*]	5.3	1.25
CV995884	Solute carrier family 31 (copper transporters), member 1 [*Danio rerio*]	5.3	0.00
TC8490	Fibrinogen, B beta polypeptide [*Danio rerio*]	5.2	1.71
EE993545	Erythroblast membrane-associated protein [*Danio rerio*]	5.0	3.74
TC7249	LOC407646 protein [*Danio rerio*]	4.9	0.00

BM438712	Tryptophan 2,3-dioxygenase [*Danio rerio*]	4.8	0.00
BM438634	Angiotensinogen [*Danio rerio*]	4.5	1.25
BM438200	Complement component C5-1 [*Cyprinus carpio*]	4.4	1.25
BM027942	Selenoprotein P, plasma, 1a [*Danio rerio*]	4.4	6.47
TC9358	Complement C4-2 [*Cyprinus carpio*]	4.2	0.00
BM438717	Tumor necrosis factor, alpha-induced protein 9 isoform 2 [*Danio rerio*]	4.1	1.25
EE993174	Complement C3-H2 [*Cyprinus carpio*]	4.0	1.25
CB939682	Protein disulfide isomerase-associated 4 [*Danio rerio*]	3.9	1.71
CK408253	Uridine phosphorylase, like [*Danio rerio*]	3.9	3.74
CK407328	Coagulation factor XIII B chain precursor [*Canis familiaris*]	3.8	6.47
TC7660	Complement component C9 [*Ctenopharyngodon idella*]	3.8	1.71
CK417270	Cytochrome b5 [*Danio rerio*]	3.7	1.71
TC7576	Tumor necrosis factor, alpha-induced protein 9 [*Homo sapiens*]	3.7	0.00
BM438615	Complement C3-S [*Danio rerio]*	3.6	3.74
TC10030	Protein disulfide-isomerase [*Cricetulus griseus*]	3.6	7.53
TC7903	Armet protein [*Xenopus laevis*]	3.4	7.53
TC8567	Chemotaxin [*Oncorhynchus mykiss*]	3.4	1.71
CV988413	Alpha-2-macroglobulin-2 [*Cyprinus carpio*]	3.3	1.71
CV988633	Apolipoprotein Apoa4 protein [*Danio rerio*]	3.3	5.24
CK421169	Canopy1[*Danio rerio*]	3.2	3.27
BM438664	Coagulation factor 10 [*Danio rerio*]	3.2	2.36
CV989134	Calumenin isoform 2 isoform 3 [*Canis familiaris*]	3.1	3.74
CK414114	Solute carrier family 38, member 2 SLC38A2 [*Danio rerio*]	3.1	0.00
CV994441	Complement C3-Q1, partial [*Danio rerio*]	3.0	0.00
CF971953	Fibrinogen, beta chain isoform 4 [*Macaca mulatta*]	3.0	3.27
TC7895	SSR alpha subunit/Translocon-associated protein subunit alpha precursor (TRAP-alpha) [*Oncorhynchus mykiss*]	3.0	3.74
CK407507	Type III iodothyronine deiodinase [*Oreochromis niloticus*]	2.9	3.74
CV995270	Apolipoprotein B-100 precursor [*Danio rerio*]	2.8	6.47
CV987559	SEC61, alpha subunit [*Danio rerio*]	2.8	3.27
TC9511	Calreticulin [*Ictalurus punctatus*]	2.7	0.00
CV989409	Dnajb11 protein [*Danio rerio*]	2.7	1.71
EE993484	Selenium binding protein 1 isoform 4 [*Danio rerio*]	2.7	7.53
TC12946	Transferrin [*Salmo trutta*]	2.7	3.74
CK410283	Transmembrane emp24 domain containing protein 10 [*Danio rerio*]	2.7	1.71
BM438622	C1 inhibitor [*Oncorhynchus mykiss*]	2.6	3.74
TC8074	Deleted in malignant brain tumors 1 [*Danio rerio*]	2.6	3.74
CV990480	Isocitrate dehydrogenase 1 (NADP+), soluble [*Danio*	2.6	1.25

	rerio]		
TC8542	SEC11-like 1/SPC18 [*Danio rerio*]	2.6	3.74
TC6883	Thioredoxin [*Ictalurus punctatus*]	2.6	3.74
CV995732	Asparagine synthetase [*Danio rerio*]	2.5	0.00
CV990348	Calreticulin, like 2 [*Danio rerio*]	2.5	3.74
TC7047	DC2 protein [*Xenopus tropicalis*]	2.5	0.00
CB937094	Eif4e1a protein [*Danio rerio*]	2.5	1.25
TC8981	FK506 binding protein 2 [*Mus musculus*]	2.5	3.74
CV993716	SAR1a-like protein 2 [*Ictalurus punctatus*]	2.5	0.00
CV994347	Alpha-2-antiplasmin precursor [*Danio rerio]*	2.4	3.27
TC8712	Bsg protein [*Danio rerio*]	2.4	3.74
CK409467	Coagulation factor II (thrombin) [*Danio rerio*]	2.4	7.53
BM438581	Colipase preproprotein [*Homo sapiens*]	2.4	6.47
CK407673	Leucine-rich alpha-2-glycoprotein [*Ctenopharyngodon idella*]	2.4	1.71
TC9317	Solute carrier family 21, member 14/Oatp [*Danio rerio*]	2.4	3.27
CK410712	Sorting nexin-17 isoform 7 [*Canis familiaris*]	2.4	1.71
TC7791	Asparagine-linked glycosylation 5 homolog [*Xenopus tropicalis*]	2.3	7.53
CV988651	Coated vesicle membrane protein [*Mus musculus*]	2.3	0.00
CV991514	Complement factor B/C2B [*Cyprinus carpio*]	2.3	3.74
CK407169	Cytochrome P450, family 8, subfamily B [*Gallus gallus*]	2.3	2.36
CK404798	Ferritin heavy subunit [*Ictalurus punctatus*]	2.3	6.47
BM027884	Glucose transporter 1A [*Danio rerio*]	2.3	5.24
TC6827	NADH dehydrogenase subunit 1 [*Ictalurus punctatus*]	2.3	0.00
CK411358	Phosphoethanolamine N-methyltransferase [*Strongylocentrotus purpuratus*]	2.3	1.25
CV991869	Protein disulfide isomerase-related protein P5 precursor [*Danio rerio*]	2.3	0.00
CK409154	Ras homolog gene family, member E [*Danio rerio*]	2.3	5.24
CV987909	Unnamed protein product [*Tetraodon nigroviridis*]	2.3	3.27
TC8069	C type lectin receptor A isoform 3 [*Danio rerio*]	2.2	5.24
TC9110	CD59 [*Ictalurus punctatus*]	2.2	0.00
CK415655	Endoplasmin [*Aedes aegypti*]	2.2	7.53
TC9203	Inter-alpha trypsin inhibitor [*Fundulus heteroclitus*]	2.2	5.24
TC6955	Jun B proto-oncogene, like [*Danio rerio*]	2.2	0.00
TC8290	NADH dehydrogenase subunit 5 [*Ictalurus punctatus*]	2.2	1.71
TC7513	SEC61, gamma subunit [*Homo sapiens*]	2.2	5.24
TC7124	Stress-associated endoplasmic reticulum protein 1 [*Homo sapiens*]	2.2	5.24
CK425032	Translocon-associated protein beta/SSR beta [*Danio rerio*]	2.2	0.00
TC8465	WW domain binding protein 2 [*Ictalurus punctatus*]	2.2	3.74
CK406535	Cathepsin L preproprotein [*Cyprinus carpio*]	2.1	1.71

TC9827	Cytochrome c oxidase subunit 1 [*Ictalurus furcatus*]	2.1	0.00
TC10083	Glucose regulated protein 58kd [*Bos taurus*]	2.1	6.47
TC8637	Hypothetical protein [*Rattus norvegicus*]	2.1	1.25
CK408501	Intraflagellar transport protein 20 [*Danio rerio*]	2.1	1.71
CB936516	Low density lipoprotein receptor-related protein associated protein 1 [*Danio rerio*]	2.1	0.00
CV994121	Nucleobindin 2a [*Danio rerio*]	2.1	0.00
CV987930	Plasminogen [*Danio rerio*]	2.1	7.53
TC8540	Ribosomal protein L23a [*Ictalurus punctatus*]	2.1	6.47
TC7429	SEC22 vesicle trafficking protein homolog B [*Danio rerio*]	2.1	3.74
BM438221	SEC61, beta subunit [*Danio rerio*]	2.1	0.00
TC6893	Signal sequence receptor delta [*Ictalurus punctatus*]	2.1	3.74
TC7064	Signal sequence receptor, gamma (translocon-associated protein gamma) [*Danio rerio*]	2.1	6.47
CV992060	Transposase [*Bacillus cereus*]	2.1	6.47
CK422124	Calpain-like protease [*Gallus gallus*]	2.0	3.74
CK408710	Cytochrome P450, family 3, subfamily A, polypeptide 65 [*Danio rerio*]	2.0	5.24
TC9714	Integral membrane protein 1 [*Danio rerio*]	2.0	0.00
TC9540	Peptidylprolyl isomerase B/cyclophilin B [*Ictalurus punctatus*]	2.0	3.74
TC8057	Profilin 2 like [*Danio rerio*]	2.0	3.74
CK402358	Retinol dehydrogenase 1, like [*Danio rerio*]	2.0	7.53
CF971017	SEC13-like 1 (S. Cerevisiae) [*Danio rerio*]	2.0	3.74
EE993544	Selenoprotein X, 1 [*Danio rerio*]	2.0	3.74
BM438153	Transducer of ERBB2, 1a [*Danio rerio*]	2.0	6.47

Supplemental Table 4
Unique, significantly up-regulated catfish transcripts in liver after *E. ictaluri* infection that could not be annotated by sequence similarity. *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

Accession	Putative identity	Fold Change	q-value
BM439193	No significant similarity	35.5	3.27
CV996365	No significant similarity	15.3	1.25
CK406832	No significant similarity	14.2	0.00
CF970899	No significant similarity	7.9	3.27
BM439040	No significant similarity	7.8	3.27
BM438893	No significant similarity	6.9	0.00
CF971612	No significant similarity	6.0	3.74
CK408142	No significant similarity	5.9	1.71
CV996287	No significant similarity	5.7	3.27
BM439121	No significant similarity	5.6	5.24
TC7498	No significant similarity	4.9	1.25
TC9712	No significant similarity	4.6	3.27
BM438818	No significant similarity	4.5	0.00
CF971526	No significant similarity	4.2	3.74
BM438848	No significant similarity	4.0	1.25
BQ097411	No significant similarity	4.0	3.27
BM438858	No significant similarity	3.9	3.27
BM439116	No significant similarity	3.7	3.27
CF971645	No significant similarity	3.6	3.27
CK407075	No significant similarity	3.5	3.27
TC9113	No significant similarity	3.5	3.74
BM438944	No significant similarity	3.4	3.27
CK423432	No significant similarity	3.4	3.27
CF972223	No significant similarity	3.3	1.71
CK406955	No significant similarity	3.2	3.27
CV996636	No significant similarity	3.2	5.24
CF970862	No significant similarity	3.1	7.53
CK409512	No significant similarity	3.1	3.74
TC7025	No significant similarity	3.0	0.00
CF971394	No significant similarity	2.9	3.27
EE993209	No significant similarity	2.9	3.27
TC8545	No significant similarity	2.9	1.71
CF971799	No significant similarity	2.9	3.74
TC7371	No significant similarity	2.8	5.24
CK407648	No significant similarity	2.8	5.24

CK406724	No significant similarity	2.8	7.53
CK413362	No significant similarity	2.8	3.27
CV994570	No significant similarity	2.8	1.71
CK425818	No significant similarity	2.8	1.71
CK406432	No significant similarity	2.7	1.71
CB939893	No significant similarity	2.5	6.47
BM029249	No significant similarity	2.5	0.00
TC8843	No significant similarity	2.5	3.74
CK409289	No significant similarity	2.5	3.27
CV994205	No significant similarity	2.5	5.24
CV993853	No significant similarity	2.5	5.24
CK409109	No significant similarity	2.5	3.27
CK404043	No significant similarity	2.5	1.71
CK422256	No significant similarity	2.4	3.74
CK407036	No significant similarity	2.4	0.00
CV992084	No significant similarity	2.4	1.25
BM424895	No significant similarity	2.4	2.36
CV990387	No significant similarity	2.4	5.24
CV995916	No significant similarity	2.4	3.74
CF970874	No significant similarity	2.4	3.74
CK418743	No significant similarity	2.3	2.36
CK421480	No significant similarity	2.3	0.00
CK410384	No significant similarity	2.3	5.24
TC9282	No significant similarity	2.3	3.74
CK402366	No significant similarity	2.3	3.27
CK412705	No significant similarity	2.3	1.71
CK425589	No significant similarity	2.3	5.24
CV994712	No significant similarity	2.2	3.74
CK404612	No significant similarity	2.2	3.74
CK418449	No significant similarity	2.2	7.53
CK403577	No significant similarity	2.2	3.27
CK425361	No significant similarity	2.2	3.74
BM424296	No significant similarity	2.2	3.74
CB939641	No significant similarity	2.2	3.27
BM438887	No significant similarity	2.1	3.74
CK417505	No significant similarity	2.1	3.27
CK407464	No significant similarity	2.1	3.27
CF972234	No significant similarity	2.1	3.74
CK421111	No significant similarity	2.0	7.53
CV991696	No significant similarity	2.0	3.27
CK406865	No significant similarity	2.0	3.74
CV991348	No significant similarity	2.0	3.27

CK406938	No significant similarity	2.0	3.27
CK407170	No significant similarity	2.0	5.24
CK405156	No significant similarity	2.0	1.71

Supplemental Table 5 All significantly upregulated transcripts in blue catfish liver. *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

Accession	PutativeId	E-value	Fold Change	q-value (%)
AY555503	CC chemokine SCYA106 [Ictalurus furcatus]	2.00E-59	105.1	9.18
CF970955	Intelectin [Ctenopharyngodon idella]	7.00E-32	48.6	9.82
CF971897	Intelectin 2 [Danio rerio]	3.00E-117	37.7	5.63
TC9205	Hypothetical protein XP_683888 [Danio rerio]	7.00E-48	30.9	5.63
TC6845	Intelectin [Ctenopharyngodon idella]	4.00E-128	30.0	9.82
CK407451	Intelectin 2 [Danio rerio]	8.00E-120	28.4	9.18
CK406362	Microfibrillar-associated protein 4 [Danio rerio]	2.00E-75	27.1	5.63
CF970863	Microfibrillar-associated protein 4 [Danio rerio]	1.00E-57	22.9	5.63
CF971597	Hypothetical protein XP_683888 [Danio rerio]	4.00E-44	20.6	9.82
CK408483	Haptoglobin precursor [Danio rerio]	1.00E-56	20.4	9.82
EE993177	Complement factor H precursor [Danio rerio]	2.00E-26	14.5	0.00
TC7475	CC chemokine SCYA113 [Ictalurus punctatus]	5.00E-43	12.5	8.97
CF971550	Warm-temperature-acclimation-related-65 [Oryzias latipes]	7.00E-104	12.3	8.70
TC8425	Warm-temperature-acclimation-related-65kda-protein-like-protein [Oryzias latipes]	2.00E-118	11.9	9.82
TC7892	Ceruloplasmin [Danio rerio]	2.00E-104	11.4	5.63
CK406832	No significant similarity		11.2	5.63
EE993354	Complement component 7 precursor [Danio rerio]	5.00E-105	10.1	9.18
CK406564	Hemopexin precursor [Danio rerio]	1.00E-90	9.7	5.63
CK405246	Methionine adenosyltransferase II alpha subunit [Mus musculus]	8.00E-66	8.3	9.82
CK407588	Catechol-O-methyltransferase domain containing 1 [Danio rerio]	2.00E-87	7.9	8.70
TC8426	Hemopexin precursor [Danio rerio]	5.00E-75	7.5	5.63
BM438689	Microfibrillar-associated protein 4 [Rattus norvegicus]	3.00E-68	7.5	8.97
CK407645	No significant similarity		7.4	9.82
CK408512	Solute carrier family 31 (copper transporters), member 1 [Danio rerio]	8.00E-77	7.1	8.70

TC9194	Complement regulatory plasma protein [Paralabrax nebulifer]	7.00E-27	6.8	8.97
CV994031	Catechol-O-methyltransferase domain containing 1 [Danio rerio]	3.00E-76	6.7	8.70
CV992853	Ceruloplasmin [Danio rerio]	8.00E-98	6.2	5.63
CV997126	Complement C3 [Ctenopharyngodon idella]	3.00E-44	6.1	8.97
CK407841	Fibrinogen gamma polypeptide [Danio rerio]	5.00E-109	5.7	5.63
TC7925	Lysosomal-associated membrane protein 3 [Canis familiaris]	5.00E-24	5.7	9.82
CF971953	Fibrinogen, beta chain isoform 4 [Macaca mulatta]	5.00E-88	5.6	9.82
CF972223	No significant similarity		5.5	7.00
TC7660	Complement component C9 [Ctenopharyngodon idella]	1.00E-19	5.2	8.70
CK407260	Complement inhibitory factor H [Rattus norvegicus]	3.00E-24	5.2	9.18
CK407984	Complement regulatory plasma protein [Paralabrax nebulifer]	1.00E-14	5.0	9.82
TC9330	ER-resident chaperone calreticulin [Ictalurus punctatus]	0	4.8	5.63
TC8545	No significant similarity		4.8	0.00
CF971219	Ceruloplasmin [Danio rerio]	7.00E-82	4.7	9.18
TC9859	MHC class I alpha chain [Ictalurus punctatus]	1.00E-130	4.7	8.70
CK407547	Protein disulfide isomerase associated 4 [Danio rerio]	1.00E-122	4.7	5.63
CV995884	Solute carrier family 31 (copper transporters), member 1 [Danio rerio]	4.00E-67	4.7	7.00
CF971394	No significant similarity		4.5	8.97
CB939682	Protein disulfide isomerase-associated 4 [Danio rerio]	8.00E-76	4.5	5.63
TC8306	Fibrinogen gamma polypeptide [Danio rerio]	6.00E-162	4.4	7.00
CK406609	Fibrinogen gamma polypeptide [Ictalurus punctatus]	2.00E-63	4.4	9.82
BM439121	No significant similarity		4.3	9.18
TC8490	Fibrinogen, B beta polypeptide [Danio rerio]	0	4.2	7.00
CK408412	Apoa4 protein [Danio rerio]	9.00E-61	4.1	8.70
CK408535	Microfibrillar-associated protein 4 [Danio rerio]	2.00E-17	4.1	5.63
BQ096774	No significant similarity		4.1	9.18
CK408173	Pentraxin [Salmo salar]	5.00E-42	4.1	8.70
CK408666	Transferrin [Salmo trutta]	2.00E-68	4.1	9.82
CK405317	Beta-actin [Tigriopus japonicus]	7.00E-90	4.0	5.63
EE993343	Complement C4 [Oncorhynchus mykiss]	3.00E-80	3.9	5.63
BM439116	No significant similarity		3.9	5.63
CK403482	No significant similarity		3.9	8.70
CF971852	Fibrinogen alpha chain [Danio rerio]	7.00E-68	3.8	9.82
CK401799	MGC68649 protein [Danio rerio]	7.00E-67	3.8	5.63
CF972295	Thioredoxin [Ictalurus punctatus]	1.00E-25	3.8	9.82

TC7576	Tumor necrosis factor, alpha-induced protein 9	4.00E-74	3.8	8.70
CK407596	LOC407646 protein [Danio rerio]	3.00E-67	3.7	9.82
CK418197	Cytochrome P450 3A [Ctenopharyngodon idella]	5.00E-79	3.6	7.00
CF972133	No significant similarity		3.6	5.63
CK414572	No significant similarity		3.5	9.82
TC6882	Thioredoxin [Ictalurus punctatus]	5.00E-51	3.4	9.18
BM438717	TNFAIP9 protein isoform 2 [Danio rerio]	2.00E-12	3.4	7.00
TC7345	Fetuin-B precursor (IRL685) [Danio rerio]	1.00E-50	3.3	9.18
CV993853	No significant similarity		3.3	9.82
CV987901	Complement C3-H1 [Cyprinus carpio]	3.00E-59	3.2	5.63
CK409611	Proteasome activator PA28 subunit [Cyprinus carpio]	7.00E-98	3.2	9.82
CK406493	Complement C3-Q1 [Cyprinus carpio]	6.00E-54	3.1	5.63
CF972140	No significant similarity		3.1	9.18
TC9755	Proteasome (prosome, macropain) subunit, alpha type, 3 [Xenopus tropicalis]	1.00E-126	3.1	9.18
CF971576	Tumor necrosis factor, alpha-induced protein 9 [Homo sapiens]	5.00E-53	3.1	9.18
TC6790	Atpase H+ transporting lysosomal vacuolar proton pump [Pagrus major]	5.00E-57	3.0	5.63
CK411755	Integral membrane protein 1 [Danio rerio]	4.00E-24	3.0	5.63
CK406132	Alpha-1-tubulin [Gecarcinus lateralis]	3.00E-24	2.9	9.82
TC7903	Armet protein [Xenopus laevis]	3.00E-60	2.9	9.18
CK405386	No significant similarity		2.9	9.82
CF971092	No significant similarity		2.9	9.82
CF972078	Matrix metalloproteinase 13 [Danio rerio]	5.00E-80	2.8	5.63
CK405569	Translocon-associated protein beta [Danio rerio]	3.00E-89	2.8	9.82
CK404348	H2A histone family, member V, isoform 1 [Homo sapiens]	7.00E-55	2.7	8.97
TC12946	Transferrin [Salmo trutta]	3.00E-141	2.7	9.82
TC7398	CC chemokine SCYA106 [Ictalurus furcatus]	1.00E-07	2.6	8.97
TC7043	CCAAT/enhancer binding protein (C/EBP), beta [Danio rerio]	2.00E-82	2.6	7.00
EE993326	CD63 [Oncorhynchus mykiss]	2.00E-62	2.6	9.82
TC8981	FK506 binding protein 2 [Mus musculus]	9.00E-56	2.6	5.63
CF972066	No significant similarity		2.6	9.82
BM438439	Signal sequence receptor, alpha [Danio rerio]	3.00E-68	2.6	8.70
CK404046	Lymphocyte antigen 6 complex, locus E ligand isoform 2 [Danio rerio]	1.00E-12	2.5	9.82
CK401855	MHC class I alpha chain [Ictalurus punctatus]	2.00E-27	2.5	5.63
CK424035	Neuronal myosin light chain kinase 1 [Danio rerio]	2.00E-77	2.5	8.97
CV995916	No significant similarity		2.5	9.82
CK401686	WW domain binding protein 2 [Ictalurus punctatus]	1.00E-93	2.5	9.82
TC9648	Amyloid beta (A4) precursor protein-binding,	7.00E-107	2.4	9.82

	family B, member 2, partial [Danio rerio]			
BM438634	Angiotensinogen [Danio rerio]	6.00E-57	2.4	9.82
CV989409	Dnajb11 protein [Danio rerio]	2.00E-65	2.4	9.18
CK415655	Endoplasmin [Aedes aegypti]	3.00E-42	2.4	9.82
TC8526	Proteasome activator subunit 1 [Danio rerio]	7.00E-100	2.4	5.63
TC6716	Beta-2 microglobulin precursor [Ictalurus punctatus]	3.00E-52	2.3	9.82
CV990995	Coactosin-like 1 [Danio rerio]	1.00E-59	2.3	9.82
TC9170	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase [Danio rerio]	2.00E-61	2.3	9.82
CV987949	Fructose-1,6-bisphosphatase 1, like [Danio rerio]	3.00E-85	2.3	8.70
TC8645	Lectin, galactoside-binding, soluble, 9 (galectin 9)-like 1 [Danio rerio]	1.00E-108	2.3	9.82
TC6930	LR8 protein [Danio rerio]	1.00E-52	2.3	9.18
CK423344	No significant similarity		2.3	9.82
TC6963	Proteasome activator subunit 2 [Danio rerio]	1.00E-87	2.3	9.82
CK406492	Similar to family with sequence similarity 46, member A isoform 1 [Danio rerio]	1.00E-92	2.3	0.00
TC8465	WW domain binding protein 2 [Ictalurus punctatus]	4.00E-95	2.3	9.82
CV995162	Alcohol dehydrogenase 5 [Danio rerio]	8.00E-93	2.2	9.82
CV989503	CXCL14 [Ictalurus punctatus]	2.00E-52	2.2	9.82
TC7388	Proteasome (prosome, macropain) subunit, beta type, 6 [Xenopus tropicalis]	2.00E-89	2.2	9.82
CV995433	Sterol regulatory element-binding protein 2 (SREBP-2) [Cricetulus griseus]	5.00E-48	2.2	9.82
CK406459	Dnajb11 protein [Danio rerio]	1.00E-26	2.1	9.18
CK404782	Methionine adenosyltransferase II alpha subunit [Mus musculus]	2.00E-80	2.1	5.63
CK410925	No significant similarity		2.1	9.18
CV994277	No significant similarity		2.1	9.82
CV996636	No significant similarity		2.1	8.70
CK408989	No significant similarity		2.1	9.18
CK424843	No significant similarity		2.1	8.70
BM425334	No significant similarity		2.1	9.82
CK407421	Glutaredoxin (thioltransferase) [Danio rerio]	2.00E-40	2.0	9.82
CV997128	No significant similarity		2.0	9.18
CK403934	No significant similarity		2.0	8.70
TC9161	hypothetical protein LOC641319 [Danio rerio]	4.00E-62	2.0	5.63

Supplemental Table 6 All significant, downregulated transcripts in blue catfish liver. *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

Accession	Putative Identity	E-value	Fold Change	q-value
CK417600	No significant similarity		0.4	9.57
TC9079	Anaphase promoting complex subunit 13 [Danio rerio]	2.00E-35	0.5	9.57
CB940790	Selenoprotein H [Danio rerio]	4.00E-29	0.5	9.57
TC9060	No significant similarity		0.5	9.57
CF971521	Selenoprotein P, plasma, 1b [Danio rerio]	2.00E-60	0.5	9.57

Supplemental Table 7 Unique, significantly up-regulated blue catfish transcripts in liver after ESC infection that could not be annotated by sequence similarity. *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

Accession	Putative Id	Fold Change	q-value (%)
CK406832	No significant similarity	11.2	5.63
CF972223	No significant similarity	5.5	7.00
CF971394	No significant similarity	4.5	8.97
BM439121	No significant similarity	4.3	9.18
BM439116	No significant similarity	3.9	5.63
CF972133	No significant similarity	3.6	5.63
CK414572	No significant similarity	3.5	9.82
CV993853	No significant similarity	3.3	9.82
CF972140	No significant similarity	3.1	9.18
CK405386	No significant similarity	2.9	9.82
CF971092	No significant similarity	2.9	9.82
CF972066	No significant similarity	2.6	9.82
CV995916	No significant similarity	2.5	9.82
CK423344	No significant similarity	2.3	9.82
CK410925	No significant similarity	2.1	9.18
CV994277	No significant similarity	2.1	9.82
CV996636	No significant similarity	2.1	8.70
CK408989	No significant similarity	2.1	9.18
CK424843	No significant similarity	2.1	8.70
BM425334	No significant similarity	2.1	9.82
CV997128	No significant similarity	2.0	9.18
CK403934	No significant similarity	2.0	8.70