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Early mucosal responses in blue catfish (*Ictalurus furcatus*) skin to *Aeromonas hydrophila* infection

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ABSTRACT

Bacterial pathogens are well-equipped to detect, adhere to, and initiate infection in their finfish hosts. The mucosal surfaces of fish, such as the skin, function as the front line of defense against such bacterial insults that are routinely encountered in the aquatic environment. While recent progress has been made, and despite the obvious importance of mucosal surfaces, the precise molecular events that occur soon after encountering bacterial pathogens remain unclear. Indeed, these early events are critical in mounting appropriate responses that ultimately determine host survival or death. In the present study, we investigated the transcriptional consequences of a virulent *Aeromonas hydrophila* challenge in the skin of blue catfish, *Ictalurus furcatus*. We utilized an 8×60K Agilent microarray to examine gene expression profiles at key early timepoints following challenge (2 h, 12 h, and 24 h). A total of 1155 unique genes were significantly altered during at least one timepoint. We observed dysregulation in a number of genes involved in diverse pathways including those involved in antioxidant responses, apoptosis, cytoskeletal rearrangement, immunity, and extracellular matrix protein diversity and regulation. Taken together, *A. hydrophila* coordinately modulates mucosal factors across numerous cellular pathways in a manner predicted to enhance its ability to adhere to and infect the blue catfish host.

1. Introduction

Fish skin is a critical regulatory organ, serving not only as a physical barrier to pathogen entry, but also as a sophisticated integrator of environmental, social and nutritional cues through roles in immunity, osmoregulation, and endocrine signaling. Integral to the complexity of teleost skin is the mucus layer secreted by epidermal goblet cells [1]. Epidermal mucus in fish is a primary biological barrier, known to change composition following infection [2], seasonal temperature changes [3], and dietary manipulations [4]. Key components of mucus have been well characterized including the high molecular mass mucin glycoproteins, immunoglobulins, lysozymes, proteases, lectins, complement factors and antimicrobial peptides [5–9]. Transcriptomic approaches to examining fish skin/mucosal samples have the benefit of capturing a more comprehensive picture of physiological responses to changes in stress or disease states compared to protein assays measuring known mucosal components. Recently, several studies in Atlantic salmon have utilized microarray technology and/or quantitative PCR (qPCR) to examine gene expression changes in skin after parasitic infection [10–12], while a broader characterization of the salmon skin transcriptome was carried out using 454-sequencing [7]. Smaller-scale studies of the channel catfish skin transcriptome have also been conducted [13]. However, our knowledge of the skin transcriptome of other cultured fish species is still lacking.

Catfish (*Ictalurus sp.*) are an important aquaculture organism and a long-standing research model for teleost immunology [14]. From an industry long dominated by the culture of channel catfish (*Ictalurus punctatus*), producers are rapidly adopting the use of a hybrid catfish (*Ictalurus punctatus* × blue catfish *Ictalurus furcatus*) with improved disease resistance to several common bacterial pathogens. There is a need, therefore, to understand the immune response of both parental species to guide selection of improved hybrid catfish. Disease research in catfish has traditionally centered on other bacterial pathogens including *Edwardsiella ictaluri* and *Flavobacterium columnare* [15–17]. However, severe outbreaks of a motile aeromonad septicemia (MAS), whose etiological agent is *Aeromonas hydrophila*, have in recent years devastated sectors of the US catfish industry. *A. hydrophila*, a Gram-negative bacterium, is

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usually considered a secondary pathogen in disease outbreaks among cultured fish species. However, in these cases, *A. hydrophila* appears to have emerged as a primary pathogen, causing the loss of more than 3 million pounds of channel catfish (*Ictalurus punctatus*) in 2009 alone [18]. Under experimental conditions, blue catfish were recently observed to have significantly higher resistance to virulent *A. hydrophila* than either channel catfish or hybrid catfish (Jeffery Terhune, *pers. communication*). To move toward the goal of identification of the molecular determinants of this increased resistance in blue catfish, we utilized a new 8×60K Agilent microarray for Ictalurid catfish to examine global mucosal immune responses in the blue catfish skin following experimental challenge with virulent *A. hydrophila*.

2. Methods

2.1. Experimental animals, tissue collection, and bacteriology

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 (23 ± 0.4 g) were reared at the Auburn University Fish Genetics Research Unit prior to challenge.

Fish were maintained in 30 L tanks (20 L water) and acclimatized for 5 days before immersion bath prior to experiment. Experimental fish were confirmed to be culture negative for bacterial infection by culturing posterior kidney tissues from representative groups of fish on tryptic soy agar (TSA) plates. A 12:12 h light:dark period was maintained and supplemental aeration was supplied by an air stone. The water temperature during the challenge was controlled at 28 °C.

Fish were challenged in 3 control and 3 treatment groups per timepoint. Aquaria were randomly divided into sampling timepoints – 2 h treatment, 12 h treatment, 24 h treatment, 2 h control, 12 h control, and 24 h control with thirty fish in each aquarium. *A. hydrophila* bacteria were cultured from a single isolate (AL09-71), used in a trial challenge, re-isolated from a single symptomatic fish and biochemically confirmed to be *A. hydrophila*, before being inoculated into tryptic soy broth (TSB) and incubated in a shaker incubator at 28 °C overnight. Isolate AL09-71 was first isolated from a farmed catfish epizootic and has been previously demonstrated to be highly virulent; at least 200-fold more virulent than other isolates of *A. hydrophila* [18]. The concentration of the bacteria was determined using colony forming unit (CFU) per mL by plating 10 μl of 10-fold serial dilutions onto TSA agar plates.

To aid in infection, skin mucus was removed by gentle scraping with a dull spatula from an approximately 4 cm^2 area below the dorsal fin immediately prior to immersion challenge. Immersion experiments were performed in a 25 L bucket with aeration. Briefly, 50 ml of bacterial cells (1.5×10^9) were added to water to give a final volume of 5 L (1.5×10^7 final exposure concentration). Thirty blue catfish were immersed in each bucket for 2 h in 28 °C water with aeration provided by air stone (~4 mg/L DO). After the 2 h immersion, the catfish were distributed to 30 L glass aquaria. Control fish were treated in the same manner as the infected fish with mucus scraped and were held in buckets with the addition of sterilized TSB prior to transfer to aquaria. The experiment was run in duplicate, with one set of fish used for sample collection and one set used for observation of mortality rates.

At 2 h, 12 h and 24 h after challenge, 30 fish were collected from each of the appropriate control and treatment aquaria at each timepoint and euthanized with MS-222 (300 mg/L). The skin from 8 fish/replicate pool (24 fish/treatment/timepoint) were pooled together in equal amounts and flash frozen in liquid nitrogen during collection and stored at −80 °C until RNA extraction. During the challenge, symptomatic treatment fish and control fish were collected and confirmed to be infected with *A. hydrophila* and pathogen-free, respectively, at the Fish Disease Diagnostic Laboratory, Auburn University.

2.2. RNA extraction and probe labeling

Samples were homogenized with mortar and pestle in the presence of liquid nitrogen prior to RNA extraction by the RNeasy Plus Universal Mini Kit (Qiagen) following manufacturer's instructions. RNA concentration and integrity of each sample was measured using a NanoDrop ND-1000 UV–VIS Spectrophotometer version 3.2.1. Fluorescently labeled complementary RNA (cRNA) probes were generated using the Two Color Microarray Quick Labeling kit (Agilent Technologies, Palo Alto, CA, USA) and following the manufacturer's instructions. Briefly, cDNA was generated from 500 ng of each isolated RNA sample; cRNA was then made using Cy3-CTP or Cy5-CTP incorporation for labeling purposes. The fluorescently labeled cRNA probes were purified using the Qiagen RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA), and the concentration, fluorescent intensities, and quality of labeled cRNA probes were determined using a Nano-drop spectrophotometer. At each timepoint, 3 Cy3 labeled cRNA and 3 Cy5 labeled cRNA were generated with randomized dye assignments between treatment and control samples.

2.3. Microarray design and probe hybridization

Microarray interrogations were performed using a custom-designed, Agilent-based microarray platform with 8×60K probes per slide layout. Catfish cDNA contigs were collected from previous catfish EST and RNA-seq studies [19–21]. Annotation was conducted based on the NCBI zebrafish database using the BLASTX program, a cutoff E-value of e^{-10}, and selection of the top informative hit. In total, 29,732 contigs from channel catfish and 21,208 contigs from blue catfish were selected, and 38 genes were selected as controls and repeated 10 times across the array (Supplementary Table 1). The rest of the array was populated with Agilent positive and negative controls. Specific probes were designed using Agilent's eArray online probe design tool with X-hyb potential less than 2 and a Base Composition (BC) content score below BC3. Hybridization, washing and scanning were performed according to the Agilent two-color microarray-based gene expression analysis protocol (version 5.5, February 2007) by University of Florida.

2.4. Microarray data analysis

Following hybridization, the slides were scanned using a GenePix personal 4100A Scanner (Axon Instruments) and initial analysis was performed with Feature Extraction software v9.5.3 (Agilent). Background correction of feature intensities was performed within this software. After lowess normalization of background-corrected data, normalized data was imported to ArrayStar software 5 (DNASTAR Inc., Madison, WI), and then the Moderated *t*-test was performed to detect the differently expressed genes [22]. At each timepoint, the expression values of the three replicates of *A. hydrophila* infected fish (8 pooled samples/replicate) were compared to that of the three replicates of the control fish and used to calculate fold changes and *p*-values. The genes with fold change greater than 2.0 and $p \leq 0.05$ were considered as differently expressed. Only blue catfish features (known and unknown) were used for expression analysis in this experiment. Functional groups of the differently expressed genes were identified based on GO analysis and manual literature review.

2.5. Gene ontology analysis

Gene ontology (GO) annotation analysis was performed using the zebrafish BLAST results in Blast2GO version 2.5.0 (http://www.blast2go.org/), which is an automated tool for the assignment of gene ontology terms. The final annotation file was produced after gene-ID mapping, GO term assignment, annotation augmentation and generic GO-Slim process. The annotation result was categorized with respect to Biological Process, Molecular Function, and Cellular Component at level 2.

In order to identify overrepresented GO annotations in the differentially expressed gene set compared to the whole blue microarray gene set, GO analysis and enrichment analysis of significantly expressed GO terms was performed using Ontologizer 2.0 [23] using the Parent–Child-Intersection method with a Benjamini–Hochberg multiple testing correction [24]. GO terms for each gene were obtained by utilizing zebrafish annotations for the unigene set. The frequency of assignment of gene ontology terms in the differentially expressed genes sets were compared to frequency within the overall blue catfish reference transcriptome. The threshold was set as FDR value <0.1.

2.6. Experimental validation—QPCR

Ten significantly expressed genes with different expression patterns were selected for validation using real time QPCR with gene specific primers designed using Primer3 software. Total RNA was extracted using the RNeasy Plus Universal Mini Kit (Qiagen) following manufacturer's instructions. First strand cDNA was synthesized by iScript™ cDNA Synthesis Kit (Bio-Rad) according to manufacturer's protocol. The iScript chemistry uses a blend of oligo-dT and random hexamer primers. All the cDNA products were diluted to 250 ng/μl and utilized for the quantitative real-time PCR reaction using the SsoFast™ EvaGreen® Supermix on a CFX96 real-time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). Real-time PCR was performed in a total volume of 20 μl with cycling conditions: 94 °C for 5 s, followed by 40 cycles of 94 °C for 5 s, 60 °C for 5 s (fluorescence measured), and a disassociation curve profile of 65 °C–95 °C for 5 s/0.5 °C increment. Results were expressed relative to the expression levels of 18S rRNA in each sample using the Relative Expression Software Tool (REST) version 2009 [25]. The biological replicate fluorescence intensities of the control and treatment products for each gene, as measured by crossing-point (*C*t) values, were compared and converted to fold differences by the relative quantification method. Expression differences between 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. Test amplifications were conducted with ensure that 18S and target genes were within an acceptable range. A no-template control was run on all plates. QPCR analysis was repeated in triplicate runs (technical replicates) to confirm expression patterns.

3. Results

3.1. A. hydrophila challenge

Pre-challenge experiments examined the ability of virulent *A. hydrophila* to infect catfish following bath challenge to allow natural routes of infection vs. i.p. injection [26]. Bath challenges alone, regardless of exposure concentration or conditions, were unsuccessful in initiating *A. hydrophila* infection. However, disrupting the mucus covering of the skin by gentle scraping (see Methods), followed by immediate challenge produced an effective, reproducible challenge model. We, therefore, adopted this model here to study infection-based changes in skin following infection.

The artificial challenge with virulent *A. hydrophila* showed initial mortality of infected fish beginning at 24 h after exposure and resulting in a final cumulative mortality of 32% at 48 h in a simultaneous trial run alongside the sampled fish. No control fish manifested symptoms of *A. hydrophila*, and randomly selected control fish were confirmed to be negative for *A. hydrophila* by standard diagnosis procedures. Dying fish manifested external signs associated with *A. hydrophila* infection including redness in the eyes, petechial hemorrhaging and exophthalmia. *A. hydrophila* bacteria were successfully isolated from randomly selected challenge fish.

3.2. Identification and analysis of differentially expressed genes

A total of 1155 unigenes showed significant differential expression in blue catfish skin during at least one timepoint following infection. In detail, there were 607 differentially expressed genes at 2 h after challenge relative to control, 306 genes differently expressed at 8 h after challenge relative to control, and 486 genes differently expressed at 24 h after challenge relative to control. At 2 h and 8 h, there were greater numbers of up-regulated genes than down-regulated genes, whereas by 24 h similar numbers of genes were up- and downregulated (Table 1). Details of the 1155 differentially expressed genes are given in Supplementary Table 2. Feature values and metadata of the experiment are archived at the NCBI Gene Expression Omnibus (GEO) under Accession GSE42491.

3.3. Gene ontology and enrichment analysis

Differently expressed genes were then used as inputs to perform gene ontology (GO) annotation by Blast2GO. A total of 2713 GO terms including 624 (23.00%) cellular component terms, 620 (22.85%) molecular functions terms and 1469 (54.15%) biological process terms were assigned to 1155 unique gene matches. The percentages of annotated catfish sequences assigned to GO terms are shown in Supplementary Fig. 1. Analysis of level 2 GO term distribution showed that metabolic process (GO:0008152), cellular process (GO:0009987), binding (GO:0005488) and cell (GO:0005623) were the most common annotation terms in the three GO categories.

The differently expressed unique genes were then used as inputs to perform enrichment analysis using Ontologizer. Parent–child GO term enrichment analysis was performed for the 1155 unigenes to detect significantly overrepresented GO terms. A total of 33 terms with *p*-value (FDR-corrected) <0.1 were considered significantly overrepresented. Ten higher level GO terms were retained as informative for further pathway analysis (Table 2). The GO terms include functions and processes including response to chemical stimulus, regulation of cell death, regulation of response to stimulus and hemoglobin biosynthetic process. Based on enrichment analysis and manual annotation and literature searches, representative key genes were arranged into 5 broad categories, including oxidative stress/apoptosis, cytoskeletal rearrangement,

Table 1
Statistics of differently expressed genes at different timepoints following *Aeromonas hydrophila* challenge. Values indicate cutoff values of fold change ≥ 2 ($p < 0.05$) at a least one timepoint following challenge.

	2 h	12 h	24 h
Up-regulated	356	224	247
Down-regulated	251	82	239
Total	607	306	486
Total unigenes	1155		

Table 2
Summary of GO term enrichment result of significantly expressed genes in blue catfish following *A. hydrophila* challenge. The 1155 differentially expressed genes were analyzed as the study set in comparison to all the catfish unigenes. *p*-value ≤0.1 was considered significant. Population count is the number of genes associated with the term in the population set. Study count is the number of genes associated with the term in the study set. GO names were retained only from GO terms of levels >2.

GO ID	GO name	*p*-value (FDR)	Population count	Study count
GO:0016265	Death	0.0056	234	29
GO:0042221	Response to chemical stimulus	0.0280	399	40
GO:0008219	Cell death	0.0434	234	30
GO:0007229	Integrin-mediated signaling pathway	0.0434	37	7
GO:0023051	Regulation of signaling	0.0842	474	42
GO:0010941	Regulation of cell death	0.0932	155	19
GO:0048583	Regulation of response to stimulus	0.0970	499	45
GO:0042541	Hemoglobin biosynthetic process	0.0970	8	4
GO:0050661	NADP binding	0.0985	25	7
GO:0042180	Cellular ketone metabolic process	0.0985	253	31

protease-related, extracellular matrix, and immune response (Table 3).

3.4. Validation of microarray profiles by QPCR

In order to validate the differentially expressed genes identified by microarray, 10 genes were selected for QPCR confirmation, selecting from those with differing expression patterns and from genes of interest based on functional enrichment and pathway results. Samples from 2 h control, 12 h control, 24 h control, and 2 h, 12 h and 24 h following challenge (with three replicate sample pools per timepoint) were used for QPCR. Primers were designed based on blue catfish contig sequences (Supplementary Table 3). Melting-curve analysis revealed a single product for all tested genes. Fold changes from QPCR were compared with the microarray expression analysis results. As shown in Table 4, QPCR results were significantly correlated with the microarray results at each timepoint (correlation coefficient $R = 0.74$, p-value <0.001). With the exception of TLR5b at the 2 h timepoint and noelin at the 12 h timepoint, all examined genes had the same direction of differential expression by both methods. No consistent bias toward higher expression levels was observed by either method. We observed that concordance between QPCR values and microarray expression levels decreased at higher fold changes. For example, in the cases of galactoside-binding soluble lectin 9 and syncollin-like expression changes were much higher in the microarray rather than QPCR. These differences likely represented differential hybridization kinetics and/or the presence of paralogues contributing to expression levels which we were unable to predict without a fully annotated genome. In spite of these discordant results in regards to some fold change levels, the microarray platform consistently indicated differentially expressed genes for further analysis.

4. Discussion

The availability of transcriptomic tools including microarrays and RNA-Seq has recently opened a window through which we can observe the complexity of fish host–pathogen interactions during infection. Transcriptomic studies of catfish responses to *E. ictaluri* infection [16,17,21] and *F. columnare* [27] have identified shared molecular signatures of inflammation and apoptosis, but have also highlighted non-classical immune candidates which may be critical in pathogen entry and replication within the host. Follow-up studies have served to further characterize roles of these candidates and examine their utility as potential biomarkers in genetic selection programs [28–30]. In this vein, here, we utilized a new 8×60K Agilent microarray platform to examine gene expression in blue catfish skin at early timepoints following *A. hydrophila* infection.

We chose here to use pooled biological replicates to study broader transcriptional patterns. While this approach does not capture the full extent of individual biological variability, we believe it was the best approach given the observed dynamics of the *A. hydrophila* infection. The virulent isolate we utilized, while generating an acute infection, typically lead to 40–60% mortality within 24 h. Thereafter, mortality rates dropped precipitously, leaving a group of surviving fish which rarely manifested clinical signs of *A. hydrophila*. Collection of tissues from challenged fish at early timepoints, therefore, of necessity included individuals which would have ultimately evaded acute infection and death. We utilized pooled samples for biological replicates to increase the likelihood of capturing expression signatures tied to pathogen attachment and entry. Candidate genes identified here may be valuable as markers for future identification of individuals belonging to these phenotypic groups prior to pathogen exposure.

A total of 13,377 unique, annotated blue catfish features were present in the utilized 8×60K Agilent microarray. Of these, expression of 1155 was significantly perturbed during at least one early timepoint following infection. We categorized differentially expressed genes into five broad categories based on GO analysis and manual literature and pathway analyses. These included oxidative stress/apoptosis, cytoskeletal rearrangement, protease-related, extracellular matrix, and immune responses (Table 3). Below we highlight key constituents of these categories and their potential functions in the context of host responses to virulent *A. hydrophila*.

4.1. Oxidative stress/apoptosis

A study of several *Aeromonas* species has highlighted that their infection pattern is characterized by a robust stimulation and/or intracellular accumulation of host reactive oxygen species (ROS) and nitric oxide radical (NO), leading to loss of mitochondrial membrane potential and induction of apoptosis [31]. The most potent virulence factor of *A. hydrophila* strains infecting mammalian species, cytotoxic enterotoxin Act, has been shown, upon binding, to stimulate monocyte/macrophage infiltration and to induce release of ROS [32,33]. A similar process was evident in the gene responses to *A. hydrophila* in blue catfish skin. Pro-apoptotic genes such as anamorsin and programmed cell death protein 2-like were upregulated (Table 3). Moreover, several selenoproteins involved in cell-protective antioxidant responses (Sel T1b, T2, and W2b) were downregulated, along with other genes involved in free radical scavenging pathways (peroxiredoxins and HSC70-like).

4.2. Cytoskeletal rearrangement

The host actin cytoskeleton is an early target of a myriad of pathogens. Manipulation of the cytoskeleton can aid attachment, cell entry, motility, and host evasion [34]. These disruptions themselves can often lead to host cell death at sites of infection, illustrating the overlapping nature of gene functional classifications [35]. Actin can polymerize into fine and dynamic fibrils or filaments which provide shape and mobility to epithelial cells. Adhesion of bacteria to the host cell surface triggers the accumulation of actin cytoskeletal components forming aggregates and promoting the development of membrane extensions. This bundling of actin

Table 3
Representative key functional categories and gene members differentially expressed in skin following *A. hydrophila* challenge. Bold values indicate significant fold change ($p \leq 0.05$).

Gene name	Probe_ID	2 h	12 h	24 h
Oxidative stress/apoptosis				
Anamorsin	UN11453	1.88	**1.39**	**5.16**
Fas (TNF receptor superfamily, member 6)	UN05087	**−2.13**	**−1.48**	−1.85
Fep15 selenoprotein	UN09717	**3.56**	1.13	2.86
Glutathione S-transferase theta 1b	UN07156	1.60	1.05	**4.40**
Heat shock cognate 70-like	UN04723	**−2.75**	3.02	2.34
Inhibitor of DNA binding 3	UN02479	**5.24**	1.06	**2.77**
Peroxiredoxin-1	UN03477	1.16	−1.11	**−11.51**
Peroxiredoxin-2	UN07748	**−2.43**	−2.00	**−3.30**
Programmed cell death protein 2-like	UN22376	1.34	1.16	**3.69**
Programmed cell death protein 4	UN32399	**2.54**	1.77	−1.43
Programmed cell death protein 6	UN00116	**−3.21**	**−1.56**	**−5.65**
Selenoprotein T1b precursor	UN05919	**−2.43**	**−1.32**	−1.19
Selenoprotein T2 precursor	UN18711	**−2.41**	**−1.43**	1.36
Selenoprotein W, 2b	UN23273	−1.79	1.22	**−7.99**
Cytoskeletal rearrangement				
Abl interactor 2	UN16097	**4.85**	1.87	2.80
AHNAK nucleoprotein	UN06664	3.25	2.08	**3.22**
Catenin beta-1	Contig_540925	−2.32	**−2.47**	1.30
Coronin-1A	UN06809	−1.02	−1.05	**−5.57**
Cytoplasmic dynein 2 heavy chain 1-like	Contig_538634	**2.94**	1.31	2.11
Ezrin like	UN07684	−1.27	1.08	**3.93**
Fgd3	UN02569	**4.93**	1.94	**4.63**
Integrin, beta 1a	UN31730	**2.14**	2.27	1.38
Integrin, beta 1b	UN07913	1.56	1.14	**3.37**
Rap guanine nucleotide exchange factor (GEF) 1	Contig_524842	**8.94**	1.84	1.43
Twinfilin, actin-binding protein, homolog 1b	UN20587	1.19	**2.20**	−3.82
Villin 1-like	UN50356	**2.43**	1.44	1.65
Protease-related				
ADAM8a	UN02972	1.35	1.99	**−19.38**
ADAM8b	UN07164	**−3.81**	−1.65	**−7.53**
ADAM9	Contig_523987	−1.62	−1.11	**−24**
Carboxyl ester lipase, like	UN07012	**−14.01**	1.68	−2.51
Carboxypeptidase B	UN06900	**−25.93**	2.47	1.42
Cationic trypsin-3	UN05715	**−29.28**	**3.59**	−3.37
Chymotrypsin B1	UN06108	**−17.26**	1.85	−7.45
Chymotrypsin-like	UN06897	**−7.48**	1.49	1.54
Granzyme-like	UN03917	**2.53**	−1.08	1.40
Matrix metalloproteinase 9-like	UN18543	1.80	1.34	**−23.24**
Matrix metalloproteinase 13 preproprotein-like	UN06623	1.08	−1.38	**−6.78**
Pancreatic secretory GP2-like	UN04138	**7.70**	−1.19	**−2.39**
Protease, serine, 35-like	UN10486	1.94	**2.68**	1.09
Serine protease inhibitor, Kunitz type 1 b	UN07517	**−3.04**	−1.33	−3.67
Syncollin-like	UN59514	**−38.88**	**3.00**	−2.80
Transmembrane protease serine 2	UN04563	**4.89**	−1.09	**−2.92**
Ubiquitin specific protease 18-like	Contig_522281	**−5.7**	1.12	−1.54
Extracellular matrix				
Collagen alpha-1(I) chain	UN06845	−2.182	**−1.616**	**10.618**
Collagen alpha-2(I) chain	UN11295	1.362	−1.618	**5.306**
Collagen, type VI, alpha 2	UN22719	−1.09	1.18	**17.72**
Dermatopontin	UN00581	**−1.74**	−1.05	**13.79**
Focal adhesion kinase 1	Contig_423967	−1.18	1.01	**3.29**
Periostin isoform 1	UN07163	−1.13	−1.21	**18.33**
Plakophilin 1-like	UN08649	1.10	−1.23	**2.49**
Thrombospondin 2-like	UN08307	**2.03**	1.15	**24.04**
Immune response				
CD74 molecule, MHC II invariant chain	UN06241	−1.09	−1.24	**4.00**
CD9 antigen	UN07656	**−2.11**	−1.10	−1.32
Chemokine (C–C motif)-like, member A3-like	UN45058	**3.35**	−1.40	−1.35
Complement C4-1-like	UN07060	**3.20**	**1.30**	**14.94**
Complement component 1, q subcomponent 4	UN14752	2.22	1.51	**3.17**
Complement component 6-like	UN02544	**−8.39**	3.87	−1.28
CXC chemokine-like	UN01082	1.59	**2.55**	**−15.71**
FinTRIM family, member 19	UN36859	**−4.92**	−1.01	−1.42
Fish virus induced TRIM protein-like	Contig_415668	**−5.06**	−1.15	−1.13
Galectin-9	UN00303	−1.80	1.18	**−63.43**
H2A histone family, member Y	UN35011	−1.23	−2.96	**−4.70**
Histone cluster 1, H2bc-like	UN39764	**2.63**	1.34	**6.50**
Interferon regulatory factor 3	UN16976	**−5.54**	−1.19	−2.14
Interferon regulatory factor 9	UN46763	**−2.83**	−1.12	−1.90
Interleukin 1, beta	UN05665	2.50	1.60	**−39.00**
Interleukin 17d	Contig_552591	1.95	1.12	**12.04**
Interleukin 2 receptor, gamma b	UN28913	1.81	1.10	**−6.99**

Table 3 (*continued*)

Gene name	Probe_ID	2 h	12 h	24 h
Interleukin-6 receptor subunit beta	UN11193	**1.84**	−1.27	**2.94**
Lectin, mannose-binding, 1	UN03883	**−2.49**	−1.06	−1.87
Lysozyme-like protein 2 (LysC)	UN03806	1.24	1.81	**−98.49**
Microfibrillar-associated protein 4-like	UN60825	−1.00	1.12	**−9.72**
Microfibrillar-associated protein 4-like isoform 1	UN60035	−2.30	**−1.80**	**−13.30**
Myxovirus (influenza virus) resistance G	UN06471	4.44	1.23	**−36.06**
NRAMP2	UN51887	**−2.36**	−1.28	−1.39
Neurotoxin/C59/Ly-6-like protein-like	UN07110	**−8.88**	**−1.34**	−1.02
Neutrophil cytosol factor 4	UN06986	**−1.88**	1.08	**−13.23**
NLR family, pyrin domain containing 1-like	Contig_389093	−1.20	1.60	**4.50**
Nucleotide-binding oligomerization domain 2	UN08194	1.84	**3.28**	1.60
Peroxisome proliferator-activated receptor alpha	UN05846	1.50	−1.42	**4.32**
Prostaglandin E synthase 2	Contig_532755	**−2.28**	−2.37	−1.11
Rhamnose binding lectin-like	UN04653	**4.54**	−3.72	−1.32
TNF receptor-associated factor 6	Contig_554384	**2.42**	−1.20	1.65
Toll-like receptor 5a	UN24516	1.23	3.11	**−12.86**
Toll-like receptor 5b	UN23482	−1.16	1.69	**−16.22**

filaments facilitates entry via a membrane-containing vacuole that protects the bacteria from lysosomal degradation [36]. Several genes associated with bacterially-induced creation of actin-rich structures were among the most strongly upregulated in blue catfish at the initial sampling timepoint, 2 h. These included RAP-GEF1 (8.94-fold), a guanine nucleotide exchange factor, which interacts with c-Abl in the cytoskeleton to facilitate cell death [37]. Abl-interactor 2, a target of Abl tyrosine kinases is implicated in Rac-dependent cytoskeletal reorganization and localized to the tips of lamellipodia and filopodia [38], was also upregulated (4.85-fold). Other cytoskeletal regulators, including AHNAK, twinfilin, villin1, and ezrin-like were upregulated both here, and observed previously following infection in other catfish mucosal tissues [21,27] indicating shared mechanisms/targets of pathogenesis. An overall pattern of induction of gene expression, primarily at 2 h and 24 h, was observed in this category.

4.3. Proteases and extracellular matrix

Microarray analyses also highlighted two groups of genes with potentially contrasting functional roles in wound repair as well as infection and inflammation, specifically extracellular proteases and components of the extracellular matrix (ECM; Table 3). Perturbance of these genes indicates changes in the blue catfish response to skin damage caused by *A. hydrophila* infection and resulting inflammation. A recent study by Krasnov et al. [11] studied this process in detail in Atlantic salmon skin following parasite infection and/or cortisol implantation. There, parasitic copepod infection alone led to relatively modest changes in protease and ECM expression. By contrast, *A. hydrophila* infection appeared to lead to systematic downregulation of protease expression, particularly at 2 h post-infection. This pattern could be interpreted as a protective mechanism of the host to minimize release of toxic enzymes capable of damaging the extracellular matrix, or as a temporary depletion of stored proteases following release upon bacterial contact [39]. For example, levels of trypsins, downregulated ~7–30 fold relative to control at 2 h, rose to basal levels or higher by 12 h. Alternatively, host blue catfish serine proteases may be targeted by inhibitors secreted by the pathogen to facilitate infection [40]. Secreted proteases have long been reported to have strong bactericidal activity, making them a high priority target for pathogen evasion strategies [3,41].

Two additional putative protease-related genes warrant comment here. A GP2-like gene was one of the highest upregulated genes at 2 h post infection (7.70-fold). GP2 is the major zymogen granule membrane protein, with roles in storage of inactive protease forms [42]. GP2 exists in secreted and membrane bound forms and interacts across the granule membrane with syncollin (downregulated here −38.88-fold at 2 h [43];). New roles for GP2 have been described in recent research. Yu and Lowe [44] described the mannose-mediated binding of GP2 to *Escherichia coli* Type I fimbria, followed by a report that GP2 takes up bacteria through M cells within the Peyer's patches and lymphoid follicles to initiate the mucosal immune response [45]. Most recently, GP2 has been shown to be ubiquitously expressed on mammalian mucosal cell types, with roles in immunomodulation of T cell activation and proliferation [46]. Further research is needed to determine the blue catfish skin cells contributing to the observed GP2 upregulation and whether they may be involved in antigen sampling. More broadly, the role of GP2 in teleost mucosal immune responses warrants

Table 4
QPCR validation details. Fold changes of selected genes are given either according to microarray or QPCR results.

Gene	2 h fold change after infection		12 h fold change after infection		24 h fold change after infection	
	QPCR	Microarray	QPCR	Microarray	QPCR	Microarray
Galactoside-binding soluble lectin 9	−2.07	−1.80	2.69	1.18	−10.39	−63.43
Leptin-like	4.27	2.35	2.57	1.40	−4.22	−6.10
Lysozyme-like protein 2	1.57	1.24	4.46	1.81	−83.67	−98.49
Matrix metalloproteinase 9-like	1.69	1.80	1.97	1.34	−5.11	−23.24
MyD88	2.00	1.36	1.18	2.05	−5.82	−1.80
Noelin-like	−6.12	−3.55	3.51	−1.08	−13.93	−19.22
Programmed cell death protein 6	−1.85	−3.21	−1.04	−1.56	−5.49	−5.65
Syncollin-like	−12.66	−38.88	2.61	3.00	−2.70	−2.80
TLR5a	1.68	1.23	2.87	3.11	−7.96	−12.86
TLR5b	2.00	−1.16	3.36	1.69	−5.39	−16.22

investigation, especially in light of our limited understanding of antigen collection mechanisms [1].

Changes in extracellular matrix genes are also subject to alternative interpretations, depending on whether the captured expression profiles signal early tissue repair or further pathogen manipulation and infection. In blue catfish skin following *A. hydrophila* infection, we observed a consistent pattern of non-significant or minor fold-changes in ECM genes at 2 h and 12 h, followed by strong, concerted upregulation of these genes at 24 h. Successful infection of many bacterial pathogens requires deeper penetration and adherence beyond the mucosal surface to avoid removal by physical forces. Indeed, pathogenic bacteria (including *A. hydrophila*) encode ECM binding proteins [47] to bind collagen and other matrix components [48,49]. Infection by the human parasite *Trypanosoma cruzi* depends in part on binding to ECM component thrombospondin, upregulated here 24.04-fold [50]. Given the early post-infection timepoints under study, we speculate that expression of host ECM may be manipulated by *A. hydrophila* to enhance adherence into deeper subendothelial layers. Further study is needed to connect ECM gene expression with disease outcome in catfish.

4.4. Immune response

An array of classical and fish-specific immune-related genes were perturbed by infection with *A. hydrophila* in blue catfish skin. While the overall pattern was suggestive of suppression of the host response, there were notable exceptions. Few immune genes showed significant changes at the 12 h timepoint, with smaller changes at 2 h, followed by larger responses at 24 h. Interestingly, genes showing differential expression at 2 h seldom manifested large significant changes in expression at 24 h, and vice versa, suggesting two early waves of the host innate immune response. At 2 h, a number of immune factors were downregulated significantly, including finTRIM family members [51], a mannose binding lectin [52], and NRAMP2 [53]. Complement component 6-like (responsible in part for assembly of the membrane attack complex) was downregulated −8.39-fold at 2 h, matching a similar report of downregulation in grass carp skin following *A. hydrophila* infection [54]. Neurotoxin/C59/Ly6 (or differentially regulated trout protein 1) is a fish-specific immune regulator often perturbed soon after infection [21,27,55,56], but whose function is still poorly understood. Here it was downregulated −8.88-fold at 2 h.

Among a smaller set of immune genes induced to higher expression at 2 h was a rhamnose-binding lectin (RBL; +4.54-fold). Previously, we observed strong upregulation of catfish RBL in gill at 4 h following *F. columnare* infection [27] and demonstrated that the lectin had a potential role in pathogen binding that was modulated by feeding status [30]. Interestingly, a recent microarray study of early Atlantic salmon skin responses to salmon louse, also found higher RBL levels at 1 d post-infection [10]. These studies suggest that rhamnose binding lectins may be important fish mucosal pathogen recognition receptors which are exploited by a variety of aquatic pathogens [57].

At 24 h post-infection, we observed the downregulation of several genes often reported to be induced by infection in other contexts. Toll-like receptor 5a (TLR5a) and TLR5b, known to recognize bacterial flagellin, were significantly downregulated at this timepoint (Table 3). This was in contrast to other reports of upregulation following bacterial infection in catfish [16,21,58,59]. Also suppressed were myxovirus resistance G (−36.06 fold), and two MFAP4-like genes [29]. C-type lysozyme (lysozyme-like protein 2), an important secreted mucus component (along with serine proteases), was downregulated close to 100-fold (Table 3) at 24 h, potentially opening up the catfish host to continued infection. Galectin-9, another potential skin mucus component [6,10], was also downregulated over 60-fold. Interleukin 1 beta (IL1b), a primary inflammatory mediator [60], was downregulated 39-fold at 24 h, differing from a report of strong induction at earlier timepoints in the head kidney of channel catfish injected with *A. hydrophila* [26]. A smaller set of immune-related genes were upregulated at 24 h. These included cytokine interleukin 17d (+12.04-fold), important in defense mechanisms against extracellular pathogens at mucosal surfaces and recently described in Atlantic salmon skin [11,61]. Also induced were two components of the complement system, C1q and C4, which have been demonstrated to part of the complement classical pathway response to *A. hydrophila* in mammalian hosts [62]. However, while the conventional view of the complement cascade is principally antimicrobial, some Aeromonads are resistant to complement mediated lysis as evidenced by in vitro killing assays employing either human or fish sera [63,64]. Taken together, the broad suppression of key innate immune factors at 24 h is likely part of pathogen-directed immune-sculpting to facilitate successful infection [65].

5. Conclusions

Utilizing a high density Agilent microarray, here we examined gene expression profiles at key early timepoints following *A. hydrophila* challenge. A total of 1155 unique genes were significantly altered during at least one timepoint. We observed dysregulation in a number of genes involved in diverse pathways including those involved in antioxidant responses, apoptosis, cytoskeletal rearrangement, immunity, and extracellular matrix protein diversity and regulation. A growing understanding of catfish mucosal responses to primary bacterial pathogens should lead to the identification of shared and pathogen/tissue specific signatures with utility as disease biomarkers and/or targets for improved vaccination strategies.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2013.01.002.

References

[1] Salinas I, Zhang YA, Sunyer JO. Mucosal immunoglobulins and B cells of teleost fish. Dev Comp Immunol 2011;35:1346–65.
[2] Easy RH, Ross NW. Changes in Atlantic salmon (*Salmo salar*) epidermal mucus protein composition profiles following infection with sea lice (*Lepeophtheirus salmonis*). Comp Biochem Physiol Part D Genomics Proteomics 2009;4:159–67.
[3] Jung TS, Del Castillo CS, Javaregowda PK, Dalvi RS, Nho SW, Park SB, et al. Seasonal variation and comparative analysis of non-specific humoral immune substances in the skin mucus of olive flounder (*Paralichthys olivaceus*). Dev Comp Immunol 2012;38:295–301.
[4] Sheikhzadeh N, Panchah IK, Asadpour R, Tayefi-Nasrabadi H, Mahmoudi H. Effects of *Haematococcus pluvialis* in maternal diet on reproductive performance and egg quality in rainbow trout (*Oncorhynchus mykiss*). Anim Reprod Sci 2012;130:119–23.
[5] Ellis AE. Innate host defense mechanisms of fish against viruses and bacteria. Dev Comp Immunol 2001;25:827–39.
[6] Rajan B, Fernandes JM, Caipang CM, Kiron V, Rombout JH, Brinchmann MF. Proteome reference map of the skin mucus of Atlantic cod (*Gadus morhua*) revealing immune competent molecules. Fish Shellfish Immunol 2011;31:224–31.

[7] Micallef G, Bickerdike R, Reiff C, Fernandes JM, Bowman AS, Martin SA. Exploring the transcriptome of Atlantic salmon (*Salmo salar*) skin, a major defense organ. Mar Biotechnol (NY) 2012;14:559–69.
[8] Palaksha KJ, Shin GW, Kim YR, Jung TS. Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*). Fish Shellfish Immunol 2008;24:479–88.
[9] Rombout JH, Abelli L, Picchietti S, Scapigliati G, Kiron V. Teleost intestinal immunology. Fish Shellfish Immunol 2011;31:616–26.
[10] Tadiso TM, Krasnov A, Skugor S, Afanasyev S, Hordvik I, Nilsen F. Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (*Lepeophtheirus salmonis*) revealed bi-phasic responses coinciding with the copepod-chalimus transition. BMC Genomics 2011;12:141.
[11] Krasnov A, Skugor S, Todorcevic M, Glover KA, Nilsen F. Gene expression in Atlantic salmon skin in response to infection with the parasitic copepod *Lepeophtheirus salmonis*, cortisol implant, and their combination. BMC Genomics 2012;13:130.
[12] Braden LM, Barker DE, Koop BF, Jones SRM. Comparative defense-associated responses in salmon skin elicited by the ectoparasite Lepeophtheirus salmonis. Comp Biochem Physiol Part D Genomics Proteomics 2012;7:100–9.
[13] Karsi A, Cao D, Li P, Patterson A, Kocabas A, Feng J, et al. Transcriptome analysis of channel catfish (*Ictalurus punctatus*): initial analysis of gene expression and microsatellite-containing cDNAs in the skin. Gene 2002;285: 157–68.
[14] Bengten E, Clem LW, Miller NW, Warr GW, Wilson M. Channel catfish immunoglobulins: repertoire and expression. Dev Comp Immunol 2006;30: 77–92.
[15] Arias CR, Cai WL, Peatman E, Bullard SA. Catfish hybrid *Ictalurus punctatus* × *I. furcatus* exhibits higher resistance to columnaris disease than the parental species. Dis Aquat Organ 2012;100:77–81.
[16] Peatman E, Baoprasertkul P, Terhune J, Xu P, Nandi S, Kucuktas H, et al. Expression analysis of the acute phase response in channel catfish (*Ictalurus punctatus*) after infection with a gram-negative bacterium. Dev Comp Immunol 2007;31:1183–96.
[17] Peatman E, Terhune J, Baoprasertkul P, Xu P, Nandi S, Wang S, et al. Microarray analysis of gene expression in the blue catfish liver reveals early activation of the MHC class I pathway after infection with *Edwardsiella ictaluri*. Mol Immunol 2008;45:553–66.
[18] Pridgeon JW, Klesius PH. Molecular identification and virulence of three *Aeromonas hydrophila* isolates cultured from infected channel catfish during a disease outbreak in west Alabama (USA) in 2009. Dis Aquat Organ 2011;94: 249–53.
[19] Wang SL, Peatman E, Abernathy J, Waldbieser G, Lindquist E, Richardson P, et al. Assembly of 500,000 inter-specific catfish expressed sequence tags and large scale gene-associated marker development for whole genome association studies. Genome Biol 2010;11:R8.
[20] Liu SK, Zhou ZC, Lu JG, Sun FY, Wang SL, Liu H, et al. Generation of genome-scale gene-associated SNPs in catfish for the construction of a high-density SNP array. BMC Genomics 2011;12:53.
[21] Li C, Zhang Y, Wang R, Lu J, Nandi S, Mohanty S, et al. RNA-seq analysis of mucosal immune responses reveals signatures of intestinal barrier disruption and pathogen entry following *Edwardsiella ictaluri* infection in channel catfish, *Ictalurus punctatus*. Fish Shellfish Immunol 2012;32:816–27.
[22] Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3. Article3.
[23] Bauer S, Grossmann S, Vingron M, Robinson PN. Ontologizer 2.0-a multifunctional tool for GO term enrichment analysis and data exploration. Bioinformatics 2008;24:1650–1.
[24] Grossmann S, Bauer S, Robinson PN, Vingron M. Improved detection of overrepresentation of Gene-Ontology annotations with parent child analysis. Bioinformatics 2007;23:3024–31.
[25] Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res 2002;30:e36.
[26] Mu X, Pridgeon JW, Klesius PH. Transcriptional profiles of multiple genes in the anterior kidney of channel catfish vaccinated with an attenuated *Aeromonas hydrophila*. Fish Shellfish Immunol 2011;31:1162–72.
[27] Sun F, Peatman E, Li C, Liu S, Jiang Y, Zhou Z, et al. Transcriptomic signatures of attachment, NF-kappaB suppression and IFN stimulation in the catfish gill following columnaris bacterial infection. Dev Comp Immunol 2012;38: 169–80.
[28] Takano T, Sha Z, Peatman E, Terhune J, Liu H, Kucuktas H, et al. The two channel catfish intelectin genes exhibit highly differential patterns of tissue expression and regulation after infection with *Edwardsiella ictaluri*. Dev Comp Immunol 2008;32:693–705.
[29] Niu D, Peatman E, Liu H, Lu J, Kucuktas H, Liu S, et al. Microfibrillar-associated protein 4 (MFAP4) genes in catfish play a novel role in innate immune responses. Dev Comp Immunol 2011;35:568–79.
[30] Beck BH, Farmer BD, Straus DL, Li C, Peatman E. Putative roles for a rhamnose binding lectin in *Flavobacterium columnare* pathogenesis in channel catfish *Ictalurus punctatus*. Fish Shellfish Immunol 2012;33:1008–15.
[31] Krzyminska S, Tanska A, Kaznowski A. *Aeromonas spp.* induce apoptosis of epithelial cells through an oxidant-dependent activation of the mitochondrial pathway. J Med Microbiol 2011;60:889–98.
[32] Chopra AK, Xu XJ, Ribardo D, Gonzalez M, Kuhl K, Peterson JW, et al. The cytotoxic enterotoxin of *Aeromonas hydrophila* induces proinflammatory cytokine production and activates arachidonic acid metabolism in macrophages. Infect Immun 2000;68:2808–18.
[33] Sha J, Kozlova EV, Chopra AK. Role of various enterotoxins in *Aeromonas hydrophila*-induced gastroenteritis: generation of enterotoxin gene-deficient mutants and evaluation of their enterotoxic activity. Infect Immun 2002;70: 1924–35.
[34] Gruenheid S, Finlay BB. Microbial pathogenesis and cytoskeletal function. Nature 2003;422:775–81.
[35] Suarez G, Khajanchi BK, Sierra JC, Erova TE, Sha J, Chopra AK. Actin cross-linking domain of *Aeromonas hydrophila* repeat in toxin A (RtxA) induces host cell rounding and apoptosis. Gene 2012;506:369–76.
[36] Guttman JA, Finlay BB. Tight junctions as targets of infectious agents. Biochim Biophys Acta 2009;1788:832–41.
[37] Mitra A, Radha V. F-actin-binding domain of c-Abl regulates localized phosphorylation of C3G: role of C3G in c-Abl-mediated cell death. Oncogene 2010; 29:4528–42.
[38] Stradal T, Courtney KD, Rottner K, Hahne P, Small JV, Pendergast AM. The Abl interactor proteins localize to sites of actin polymerization at the tips of lamellipodia and filopodia. Curr Biol 2001;11:891–5.
[39] Aranishi F, Mano N, Nakane M, Hirose H. Epidermal response of the Japanese eel to environmental stress. Fish Physiol Biochem 1998;3:197–203.
[40] Eschenlauer SC, Faria MS, Morrison LS, Bland N, Ribeiro-Gomes FL, DosReis GA, et al. Influence of parasite encoded inhibitors of serine peptidases in early infection of macrophages with Leishmania major. Cell Microbiol 2009; 11:106–20.
[41] Ross NW, Firth KJ, Wang A, Burka JF, Johnson SC. Changes in hydrolytic enzyme activities of naive Atlantic salmon *Salmo salar* skin mucus due to infection with the salmon louse *Lepeophtheirus salmonis* and cortisol implantation. Dis Aquat Organ 2000;41:43–51.
[42] Fukuoka S, Freedman SD, Yu H, Sukhatme VP, Scheele GA. GP-2/THP gene family encodes self-binding glycosylphosphatidylinositol-anchored proteins in apical secretory compartments of pancreas and kidney. Proc Natl Acad Sci U S A 1992;89:1189–93.
[43] Kalus I, Hodel A, Koch A, Kleene R, Edwardson JM, Schrader M. Interaction of syncollin with GP-2, the major membrane protein of pancreatic zymogen granules, and association with lipid microdomains. Biochem J 2002;362: 433–42.
[44] Yu S, Lowe AW. The pancreatic zymogen granule membrane protein, GP2, binds *Escherichia coli* Type 1 fimbriae. BMC Gastroenterol 2009;9:58.
[45] Hase K, Kawano K, Nochi T, Pontes GS, Fukuda S, Ebisawa M, et al. Uptake through glycoprotein 2 of FimH(+) bacteria by M cells initiates mucosal immune response. Nature 2009;462. 226-U101.
[46] Werner L, Paclik D, Fritz C, Reinhold D, Roggenbuck D, Sturm A. Identification of pancreatic glycoprotein 2 as an endogenous immunomodulator of innate and adaptive immune responses. J Immunol 2012;189:2774–83.
[47] Flock JI. Extracellular-matrix-binding proteins as targets for the prevention of Staphylococcus aureus infections. Mol Med Today 1999;5:532–7.
[48] Ascencio F, Wadstrom T. A collagen-binding protein produced by *Aeromonas hydrophila*. J Med Microbiol 1998;47:417–25.
[49] Dubreuil JD, Del Giudice G, Rappuoli R. *Helicobacter pylori* interactions with host serum and extracellular matrix proteins: potential role in the infectious process. Microbiol Mol Biol Rev 2002;66:617.
[50] Simmons KJ, Nde PN, Kleshchenko YY, Lima MF, Villalta F. Stable RNA interference of host thrombospondin-1 blocks *Trypanosoma cruzi* infection. FEBS Lett 2006;580:2365–70.
[51] van der Aa LM, Jouneau L, Laplantine E, Bouchez O, Van Kemenade L, Boudinot P. FinTRIMs, fish virus-inducible proteins with E3 ubiquitin ligase activity. Dev Comp Immunol 2012;36:433–41.
[52] Zhang H, Peatman E, Liu H, Niu DH, Feng TT, Kucuktas H, et al. Characterization of a mannose-binding lectin from channel catfish (*Ictalurus punctatus*). Res Vet Sci 2012;92:408–13.
[53] Chen H, Waldbieser GC, Rice CD, Elibol B, Wolters WR, Hanson LA. Isolation and characterization of channel catfish natural resistance associated macrophage protein gene. Dev Comp Immunol 2002;26:517–31.
[54] Shen YB, Zhang JB, Xu XY, Li JL. Molecular cloning, characterization and expression analysis of the complement component C6 gene in grass carp. Vet Immunol Immunopathol 2011;141:139–43.
[55] Talbot AT, Pottinger TG, Smith TJ, Cairns MT. Acute phase gene expression in rainbow trout (*Oncorhynchus mykiss*) after exposure to a confinement stressor: a comparison of pooled and individual data. Fish Shellfish Immunol 2009;27:309–17.
[56] Talbot AT, Smith TJ, Cairns MT. Characterisation of the differentially regulated trout protein 1 (DRTP1) gene in rainbow trout (*Oncorhynchus mykiss*). Fish Shellfish Immunol 2009;26:589–98.
[57] Ogawa T, Watanabe M, Naganuma T, Muramoto K. Diversified carbohydrate-binding lectins from marine resources. J Amino Acids 2011; 2011:838914.
[58] Bilodeau AL, Waldbieser GC. Activation of TLR3 and TLR5 in channel catfish exposed to virulent *Edwardsiella Ictaluri*. Dev Comp Immunol 2005;29: 713–21.
[59] Russo R. An attenuated strain of *Edwardsiella ictaluri* is killed by channel catfish (*Ictalurus punctatus*) macrophages and confers protection in few days. Dyn Biochem Process Biotech Mol Biol 2011;5:76–82.

[60] Wang YP, Wang Q, Baoprasertkul P, Peatman E, Liu ZJ. Genomic organization, gene duplication, and expression analysis of interleukin-1 beta in channel catfish (*Ictalurus punctatus*). Mol Immunol 2006;43: 1653–64.
[61] Kumari J, Larsen AN, Bogwald J, Dalmo RA. Interleukin-17D in Atlantic salmon (*Salmo salar*): molecular characterization, 3D modelling and promoter analysis. Fish Shellfish Immunol 2009;27:647–59.
[62] Merino S, Nogueras MM, Aguilar A, Rubires X, Alberti S, Benedi VJ, et al. Activation of the complement classical pathway (C1q binding) by mesophilic *Aeromonas hydrophila* outer membrane protein. Infect Immun 1998;66: 3825–31.
[63] Janda JM, Kokka RP, Guthertz LS. The susceptibility of S-layer-positive and S-layer-negative *Aeromonas* strains to complement-mediated lysis. Microbiology 1994;140(Pt 10):2899–905.
[64] Cipriano RC. *Aeromonas hydrophila* and motile aeromonad septicemias of fish. Revision fish disease leaflet 68; 2001.
[65] Hertzog PJ, Mansell A, van Driel IR, Hartland EL. Sculpting the immune response to infection. Nat Immunol 2011;12:579–82.