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Basal polarization of the mucosal compartment in *Flavobacterium columnare* susceptible and resistant channel catfish (*Ictalurus punctatus*)

Eric Peatman[a,*,1], Chao Li[a,1], Brian C. Peterson[b], David L. Straus[c], Bradley D. Farmer[c], Benjamin H. Beck[c]

[a] *Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL 36849, USA*
[b] *United States Department of Agriculture, Agricultural Research Service, Catfish Genetics Research Unit, Stoneville, MS, USA*
[c] *United States Department of Agriculture, Agricultural Research Service, Stuttgart National Aquaculture Research Center, Stuttgart, AR, USA*

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ABSTRACT

The freshwater bacterial pathogen, *Flavobacterium columnare*, infects a variety of ornamental and farmed fish species worldwide through mucosal attachment points on the gill and skin. While previous studies have demonstrated a chemotactic response of *F. columnare* to fish mucus, little is known about how host gill mucosal molecular and cellular constituents may impact rates of adhesion, tissue invasion, and ultimately, mortality. Here, we describe the use of RNA-seq to profile gill expression differences between channel catfish (*Ictalurus punctatus*) differing in their susceptibility to *F. columnare* both basally (before infection) and at three early timepoints post-infection (1 h, 2 h, and 8 h). After sequencing and *de novo* assembly of over 350 million 100 base-pair transcript reads, between group comparisons revealed 1714 unique genes differentially expressed greater than 1.5-fold at one or more timepoints. In the large dataset, we focused our analysis on basal differential expression between resistant and susceptible catfish as these genes could potentially reveal genetic and/or environmental factors linked with differential rates of infection. A number of critical innate immune components including iNOS2b, lysozyme C, IL-8, and TNF-alpha were constitutively higher in resistant catfish gill, while susceptible fish showed high expression levels of secreted mucin forms, a rhamnose-binding lectin previously linked to susceptibility, and mucosal immune factors such as CD103 and IL-17. Taken together, the immune and mucin profiles obtained by RNA-seq suggest a basal polarization in the gill mucosa, with susceptible fish possessing a putative mucosecretory, toleragenic phenotype which may predispose them to *F. columnare* infection.

1. Introduction

Aquaculture is the fastest growing sector of agriculture, accounting for close to half of the world's total food fish supply. Wild harvests for numerous species have reached or exceeded maximum sustainable yields, and aquaculture is expected to fill the void. Meanwhile, growing populations have increased demand for high-quality dietary protein sources. Improvements to aquaculture practices are urgently needed to meet world seafood demands. One of the highest priority areas for improvement is the development of effective strategies for decreasing disease mortality levels in aquaculture production, including implementation of better complete diets, vaccines and genetic selection programs. Towards this end, a better understanding of the components of the fish immune system and their functions in the context of pathogen invasion is needed (Esteban, 2012).

Mucosal surfaces in teleost fish (and their associated lymphoid tissue) form critical physical and immunological barriers between the organism and the external environment (Rombout et al., 2011; Salinas et al., 2011). While shared gross anatomy in the gut between terrestrial and aquatic vertebrates provides a starting context for functional studies in fish (Pérez et al., 2010), the skin and gill mucosa are comparatively unexplored and poorly understood. These interfaces, in constant, direct contact with water, must integrate signals based on environmental conditions, social cues, nutritional status, and interactions with commensal and pathogenic microorganisms. Understanding the molecular actors which govern this complex interplay and maintain homeostasis is critical in the development of improved rearing strategies, vaccines, and dietary formulations which provide for comprehensive mucosal health and protection. There are several short-term means by which such knowledge can be translated to real-world fish health solutions. Host mucosal receptors utilized by common

* Corresponding author at: Department of Fisheries and Allied Aquacultures, Auburn University, Auburn, AL 36849, USA. Tel.: +1 334 844 9319; fax: +1 334 844 4694.
E-mail address: peatmer@auburn.edu (E. Peatman).
[1] Both these authors contributed equally to the manuscript.

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bacterial pathogens can be identified and targeted for genetic selection or modulation through dietary or chemical supplements. Additionally, expression signatures indicative of high-health (resistant) fish can be utilized to evaluate the efficacy of different rearing systems, diets, and vaccines.

Columnaris disease, caused by the Gram-negative bacterium *Flavobacterium columnare*, is an opportunistic pathogen which impacts numerous freshwater cultured fish species worldwide (Plumb and Hanson, 2011). Indeed, no wild or cultured freshwater fish, including ornamental fish in aquaria, are completely resistant to columnaris disease (Plumb and Hanson, 2011). Members of the family Ictaluridae, particularly the economically important foodfish channel catfish (*Ictalurus punctatus*) are exceedingly susceptible to columnaris disease (Arias et al., 2004; National Animal Health Monitoring System (U.S.), 2010). In complete contrast to other bacterial pathogens of fish, experimental infection protocols with *F. columnare* are far more effective by contact exposure (*i.e.*, immersion protocols) than by injection (Decostere et al., 1999b; Pacha and Ordal, 1970). This disparity in challenge routes is likely linked to the preferential external pathogenesis of columnaris, as evidenced by observations in both naturally and experimentally infected fish demonstrating that columnaris causes few to no internal lesions, yet induces marked pathologic changes in ectopic tissues such as the skin and gill (Wakabayashi, 1991). Accordingly, the predilection of columnaris disease to adhere to and initiate disease on external surfaces makes it an excellent model for the study of surface mucosal immunity. Previously, we characterized gill mucosal responses in pond-run catfish (*I. punctatus*) to virulent *F. columnare* infection (Sun et al., 2012). We identified a putative host receptor of columnaris, a rhamnose-binding lectin (RBL) whose expression was dramatically induced early after infection. A more detailed subsequent study of RBL activity revealed that expression levels were correlated with columnaris susceptibility, and that saturation of the receptor with its putative ligands resulted in significantly decreased columnaris mortality (Beck et al., 2012). To provide a larger context for this finding, here we have carried out RNA-seq analysis of basal and early post-challenge expression differences in the gill mucosa between individuals from families with differing columnaris susceptibility. Our findings provide novel insights connecting teleost mucosal immune status with the subsequent ability to withstand pathogen infection.

2. Methods

2.1. Experimental animals and water quality parameters

The two families of channel catfish utilized in this study were previously revealed to have differing susceptibilities to columnaris disease (Beck et al., 2012; LaFrentz et al., 2012). Channel catfish fingerlings (13.9 ± 0.44 g) from the two families were stocked into eight 20-L aquaria at a density of 20 fish/aquaria. Aquaria contained 10 L of aerated flow-through well water using the "Ultra Low-Flow System" described by Mitchell and Farmer (2010). The flow rate was set to 29 ± 1 mL/min and monitored daily; this rate allows for a natural progression of the disease after challenge in a flow-through environment (Mitchell and Farmer, 2010). Water temperatures averaged 26.5 ± 0.02 °C and dissolved oxygen averaged 5.81 ± 0.03 mg/L. Total Ammonia Nitrogen (TAN) concentrations were determined in each tank with a Hach DR/4000 V spectrophotometer using the Nessler Method 8038 (Hach Company, Loveland, Colorado). An Accumet Basic AB15 pH meter (Fisher Scientific, Waltham, Massachusetts) was used to measure pH (7.5–8.2) during the study. Standard titration methods (APHA 2005) were used to measure total alkalinity (213 mg/L) and total hardness (112 mg/L).

2.2. Experimental challenge

There were four tanks for each family, three of which were challenged with *F. columnare* and the remaining tank for each family served as a negative (unchallenged) control. Other replicates of challenged and control tanks were present during this study and were used to determine the differences in survival between the two families and to examine bacterial adhesion kinetics, which was published previously (Beck et al., 2012). Tanks receiving a bacterial challenge were exposed to *F. columnare* isolate LV-359-01 (a genomovar II isolate as determined by using the methods of Arias et al., 2004) which was previously demonstrated to produce mortality from columnaris disease in the described system (Farmer et al., 2011). The isolate was retrieved from a −80 °C freezer and streaked on Ordals' medium (Anacker and Ordal, 1959); after 48 h the isolate was dislodged from the agar using a sterile cotton swab and inoculated into 5 mL of *F. columnare* Growth Medium (FCGM; (Farmer, 2004)). This suspension was incubated at 28 °C for 24 h and was used to inoculate 1 L of FCGM. The inoculated 1 L broth was incubated for up to 24 h at 28 °C in an orbital shaker incubator set at 200 rpm; when the bacterial growth reached an absorbance of 0.70 at 550 nm (approximately $4.0E^{10}$ bacteria/mL) the flask was removed and placed on a stir plate at room temperature. For the challenge, 150 mL of bacterial suspension was added to the appropriate tanks. Animal care and experimental protocols were approved by the Stuttgart National Aquaculture Research Center Institutional Animal Care and Use Committee and conformed to Agricultural Research Service Policies and Procedures 130.4 and 635.1 (Beck et al., 2012).

2.3. Sample collection, RNA extraction, library construction and sequencing

Gill tissues from 4 fish per replicate tank (3 replicates tanks/family) were collected at 1 h, 2 h, and 8 h after challenge. Equal amounts of tissue (approximately 50 mg) were collected from each fish within the three pools (3 pools of 4 fish each). The fish were euthanized with tricaine methanesulfonate (MS 222) at 300 mg/L (buffered with sodium bicarbonate) before tissues were collected. Gill tissues in the replicate pools were placed into 5 mL RNA later™ (Ambion, Austin, TX, USA) and stored at −80 °C until extractions could be completed at the end of the study. Samples were homogenized with mortar and pestle in the presence of liquid nitrogen.

Total RNA was extracted from tissues using the RNeasy Plus Universal Mini Kit (Qiagen) following manufacturer's instructions. RNA concentration and integrity of each sample was measured on an Agilent 2100 Bioanalyzer using a RNA Nano Bioanalysis chip. For each timepoint, equal amounts of RNA from the three replicates were pooled for RNA-seq library construction while preserving the replicate pools for replicated QPCR validation.

RNA-seq library preparation and sequencing was carried out by HudsonAlpha Genomic Services Lab (Huntsville, AL, USA). cDNA libraries were prepared with 2.14–3.25 μg of starting total RNA and using the Illumina TruSeq RNA Sample Preparation Kit (Illumina), as dictated by the TruSeq protocol. The libraries were amplified with 15 cycles of PCR and contained TruSeq indexes within the adaptors, specifically indexes 1–4. Finally, amplified library yields were 30 uL of 19.8–21.4 ng/uL with an average length of ~270 bp, indicating a concentration of 110–140 nM. After KAPA quantitation and dilution, the libraries were clustered 4 per lane and sequenced on an Illumina HiSeq 2000 instrument with 100 bp PE reads.

2.4. De novo assembly of sequencing reads

Before assembly, raw reads were trimmed by removing adaptor sequences and ambiguous nucleotides. Reads with quality scores

less than 20 and length below 30 bp were all trimmed. The resulting high-quality sequences were used in the subsequent assembly (Miller et al., 2010). The *de novo* assembly was performed by de Brujin graph assembler ABySS (version 1.3.2) (Simpson et al., 2009). Briefly, the clean reads were first hashed according to a predefined k-mer length, the 'k-mers'. After capturing overlaps of length k-1 between these k-mers, the short reads were assembled into contigs. The k-mer size was set from 50 to 96, assemblies from all k-mers were merged into one assembly by Trans-ABySS. In order to reduce redundancy, the assembly results from different assemblers were passed to CD-Hit version 4.5.4 (Li and Godzik, 2006) and CAP3 (Huang and Madan, 1999) for multiple alignments and consensus building after trimming contigs less than 200 bp. The threshold was set as identity equal to 1 in CD-Hit, the minimal overlap length and identity equal to 100 bp and 99% in CAP3.

2.5. Gene annotation and ontology

The assembly contigs were used as queries against the NCBI *zebrafish* protein database, the UniProtKB/SwissProt database and the non-redundant (nr) protein database using the BLASTX program. The cutoff *E*-value was set at 1e-5 and only the top gene id and name were initially assigned to each contig. Gene ontology (GO) annotation analysis was performed using the zebrafish BLAST results in Blast2GO version 2.6.4 (Gotz et al., 2008), which is an automated tool for the assignment of gene ontology terms. The zebrafish BLAST result or the nr BLAST result (when a "hypothetical" result was returned in the zebrafish database), was imported to BLAST2GO. 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.

2.6. Identification of differentially expressed contigs

The high quality reads from each sample were mapped onto the TransABySS reference assembly using CLC Genomics Workbench software. During mapping, at least 95% of the bases were required to align to the reference and a maximum of two mismatches were allowed. The total mapped reads number for each transcript was determined and then normalized to detect RPKM (Reads Per Kilobase of exon model per Million mapped reads). The proportions-based test was used to identify the differently expressed genes between resistant and susceptible families at 0 h, 1 h, 2 h, and 8 h with corrected p-value < 0.05 (Kal et al., 1999). After scaling normalization of the RPKM values, fold changes were calculated. Analysis was performed using the RNA-seq module and the expression analysis module in CLC Genomics Workbench (Robinson and Oshlack, 2010). Transcripts with absolute fold change values of larger than 1.5 were included in analysis as differently expressed genes.

Contigs with previously identified gene matches were carried forward for further analysis. Functional groups and pathways encompassing the differently expressed genes were identified based on GO analysis, pathway analysis based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, and manual literature review.

2.7. Gene ontology and enrichment analysis

In order to identify overrepresented GO annotations in the differentially expressed gene set compared to the broader reference assembly, GO analysis and enrichment analysis of significantly expressed GO terms was performed using Ontologizer 2.0 using the Parent–Child-Intersection method with a Benjamini–Hochberg multiple testing correction (Bauer et al., 2008; Grossmann et al., 2007). GO terms for each gene were obtained by utilizing zebrafish annotations for the unigene set. The difference of the frequency of assignment of gene ontology terms in the differentially expressed genes sets were compared to the overall catfish reference assembly. The threshold was set as FDR value < 0.1.

2.8. Experimental validation: QPCR

Thirteen significantly expressed genes with different expression patterns were selected for validation using real time QPCR with gene specific primers designed using Primer3 software. Primers were designed based on contig sequences (Supplementary Table 1). Total RNA was extracted using the RNeasy Plus 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). The thermal cycling profile consisted of an initial denaturation at 95 °C (for 30 s), followed by 40 cycles of denaturation at 94 °C (5 s), and an appropriate annealing/extension temperature (58 °C, 5 s). An additional temperature ramping step was utilized to produce melting curves of the reaction from 65 °C to 95 °C. Results were expressed relative to the expression levels of 18S rRNA in each sample using the Relative Expression Software Tool (REST) version 2009 (Pfaffl et al., 2002). The biological replicate 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. 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 to 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. F. columnare challenge

As previously reported, the two catfish families exhibited different susceptibilities to columnaris disease (Beck et al., 2012). No mortality was observed in the resistant family in the 7 day study period while 11 out of 60 (18.3%) fish died in the susceptible family. This rate of mortality was consistent with that previously published with the Ultra-Low-Flow system; a challenge system designed to reliably reproduce columnaris disease with the kinetics and severity of mortality approximating columnaris disease epizootics. The mortality observed in the susceptible family of fish occurred on days 2–5 post-challenge (Beck et al., 2012).

3.2. Sequencing of short expressed reads from catfish gill

Illumina-based RNA-sequencing (RNA-seq) was carried out on gill samples from the two catfish families. Reads from timepoint-specific samples were distinguished through the use of multiple identifier (MID) tags. A total of 350 million 100 bp high quality reads were generated on an Illumina HiSeq 2000 instrument in a single lane. Greater than 39 million reads were sequenced for each of the eight libraries. After removing ambiguous nucleotides, low-quality sequences (quality scores < 20) and short reads (length < 30 bp), the

Table 1
Summary of *de novo* assembly results of Illumina sequence data from catfish gill using Trans-ABySS.

	Trans-ABySS
Contigs(≥100 bp)	1,117,006
Large contigs (≥1000 bp)	197,119
Maximum length (bp)	26,890
Average length (bp)	617.3
N50 (bp)	1418
Contigs after length filtering(≥200 bp)	657,211
Percentage contigs kept after length filtering (%)	58.84
Average contig length after length filtering (bp)	950.7
Contigs (After CD-HIT-EST + CAP3)	444,715
Average length (bp) (After CD-HIT-EST + CAP3)	805.6
Reads mapped to final reference (%)	81.3

remaining high-quality reads were carried forward for assembly and analysis. Raw read data are archived at the NCBI Sequence Read Archive (SRA) under Accession SRP017689.

3.3. De novo assembly of catfish gill transcriptome with ABySS and Trans-ABySS

ABySS and Trans-ABySS were used to generate an optimized reference for mapping of high quality reads and accurate determination of differentially expressed genes after challenge, based on previously demonstrated superior assemblies when compared with Velvet and CLCbio (Li et al., 2012b). Use of Trans-ABySS to merge ABySS multi-k-assembled contigs, resulted in approximately 1.1 million contigs with average length of 617.3 bp and N50 size of 1418 bp. A total of 657,211 contigs with lengths greater than 200 bp were carried forward for additional analysis. Approximately 0.2 million contigs were removed during the length and redundancy filtration steps (CD-Hit and CAP-3), resulting in a final average contig size and contig number of 805.6 bp and 444,715, respectively (Table 1). This non-redundant, length-filtered assembly was used as the reference catfish gill transcriptome in the following steps of analysis including transcriptome annotation and gene expression profiling.

3.4. Gene identification and annotation

BLAST-based gene identification was performed to annotate the channel catfish transcriptome and inform downstream differential expression analysis. After gene annotation, 140,210 of the Trans-ABySS contigs had a significant BLAST hit against 17,481 unique zebrafish genes (unigenes; Table 2). In order to further evaluate the quality of the assembled genes, 15,873 unigenes were identified based on hits to the zebrafish database with the more stringent criteria of a BLAST score ≥ 100 and *E*-value ≤ 1e-20 (quality matches). The same BLAST criteria were used in comparison of the Trans-ABySS reference contigs with the UniProt and nr databases. The largest number of matches was to the nr database with 148,313 contigs with putative gene matches to nr and 24,442 quality unigene matches (Table 2).

Table 3
Statistics of differentially expressed genes at early timepoints following *F. columnare* challenge in resistant and susceptible fish relative to their 0 h control samples. *Shared* category indicates the number of genes significantly differentially expressed in the same direction in both groups at a given timepoint, while percentage is the number of shared genes/number of potentially shared genes. Values indicate contigs/genes passing cutoff values of fold change ≥1.5 ($p < 0.05$).

	1 h	2 h	8 h
Up-regulated			
Resistant	272	261	791
Susceptible	197	229	905
Shared (%)	78 (39.6%)	67 (29.3%)	526 (66.5%)
Down-regulated			
Resistant	84	209	407
Susceptible	135	228	447
Shared (%)	14 (16.7%)	40 (19.1%)	184 (45.2%)

3.5. Identification and analysis of differentially expressed genes: Within group

Differential expression in comparison to 0 h control samples was carried out for the resistant and susceptible fish groups, respectively (Table 3). Similar numbers of genes were differentially expressed at 1 h, 2 h, and 8 h, post-challenge in both groups, with the number of dysregulated genes rising with time. For example, at 1 h, 356 and 332 genes were differentially expressed in resistant and susceptible samples, respectively while these numbers rose to 1198 and 1352 genes in the same respective groups at 8 h. We also determined the number of genes that were significantly differentially expressed in the same direction in both groups at a given timepoint (magnitude differed). Greater numbers of upregulated genes were shared between groups than downregulated genes. By 8 h, a majority of upregulated (66.5%) and close to a majority of downregulated (45.2%) genes were shared between the two groups, indicating that many of the same response mechanisms and pathways were being initiated in both groups, albeit at different levels (Table 3). Differentially expressed genes from the separate resistant and susceptible expression analyses are compiled in Supplementary Tables 2 and 3.

3.6. Identification and analysis of differentially expressed genes: Between groups

Additional levels of analysis were conducted on the comparisons of greatest interest, differences in gene expression profiles between resistant and susceptible fish at 0 h, and 1 h, 2 h, and 8 h post-*F. columnare* challenge. Designating the resistant family as the control group, a comparison of global transcription levels was made between resistant and susceptible families at each timepoint. A total of 8584 of the 444,175 final reference contigs showed significant differential expression between groups for at least one timepoint following infection. The identified contigs represented 1714 unigenes, including 1581 unique genes with more stringent criteria of a BLAST score ≥ 100 and *E*-value ≤ 1e-20, and 133 unique genes with BLAST *E*-value from 1e-20 to 1e-5 (Table 4; Supplementary Table 4). The greatest degree of differential expression between groups was observed at 1 h (972 genes), followed by 0 h (795 genes).

Table 2
Summary of gene identification and annotation of assembled catfish contigs based on BLAST homology searches against various protein databases (Zebrafish, UniProt, nr). Putative gene matches were at *E*-value ≤ 1e-5. Hypothetical gene matches denote those BLAST hits with uninformative annotation. Quality unigene hits denote more stringent parameters, including score ≥ 100, *E*-value ≤ 1e-20.

	Contigs with putative gene matches	Annotated contigs ≥500 bp	Annotated contigs ≥1000 bp	Unigene matches	Hypothetical gene matches	Quality Unigene matches
Zebrafish	140,210	95,705	60,930	17,481	1104	15,873
UniProt	116,739	86,381	57,546	23,403	0	18,602
NR	148,313	101,596	64,279	33,303	2473	24,442

Table 4
Statistics of differently expressed genes pre-challenge (0 h) and at 1 h, 2 h, and 8 h following *F. columnare* challenge. *Resistant < Susceptible* indicates numbers of genes with significantly higher expression (read numbers) in susceptible samples relative to resistant samples. These genes elsewhere are indicated with positive values. *Resistant > Susceptible* indicates numbers of genes with significantly lower expression (read numbers) in susceptible samples relative to resistant samples. These genes elsewhere are indicated with negative values. *Reads per contig* indicates average number of reads in differentially expressed contigs/genes being compared at a given timepoint. Values indicate contigs/genes passing cutoff values of fold change ≥1.5 ($p < 0.05$).

Resistant *vs* susceptible	0 h	1 h	2 h	8 h
Resistant < susceptible (+)	388	382	385	319
Resistant > susceptible (−)	407	590	362	276
Total	795	972	747	595
Reads per contig	125	245	140	166
Total unigenes		1714		

No clear trend toward overall higher expression levels in either the resistant or susceptible group was observed (Table 4).

3.7. Enrichment and pathway analysis

Differently expressed genes between the two groups were then used as inputs to perform gene ontology (GO) annotation by Blast2GO. A total of 3033 GO terms including 795 (26.21%) cellular component terms, 816 (26.9%) molecular functions terms and 1422 (42.88%) biological process terms were assigned to 1478 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 1465 unigenes to detect significantly overrepresented GO terms. A total of 68 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 (Supplementary Table 5). The GO terms include functions and processes including cellular nitrogen compound biosynthetic process, regulation of cell death, regulation of cell adhesion and epithelial tube formation.

Based on enrichment analysis and manual annotation and literature searches, representative key genes were arranged into 4 broad functional categories believed to best reflect the polarization between resistant and susceptible fish particularly prior to challenge (0 h). These included differences in putative mucosal immune factors and in mucin secretion and modification (Table 5; Fig. 1) as well as cytoskeletal/junctional regulation and cell survival and proliferation (Supplementary Table 6). Putative functional roles of immune and mucin-related genes, judged to be the most critical in modulating differential disease resistance, are covered in-depth in Section 4.

3.8. Validation of RNA-seq profiles by QPCR

In order to validate the differentially expressed genes identified by RNA-Seq, we selected 10 genes for QPCR confirmation, selecting from those with differing expression patterns and from genes of interest based on functional enrichment and pathway results. We also selected three genes among those with read counts of "0" in more than one sample in order to ascertain the reliability of this portion of the dataset. Samples from 0 h control, and 1 h, 2 h and 8 h following challenge (with three replicate sample pools per timepoint) were used for QPCR. Primers were designed based on contig sequences (Supplementary Table 1). Melting-curve analysis revealed a single product for all tested genes. Fold changes from QPCR were compared with the RNA-seq expression analysis results. As shown in Fig. 2, QPCR results from non-zero read genes were significantly correlated with the RNA-seq results at each timepoint (average correlation coefficient 0.87, p-value < 0.001; Fig. 2). Correlations were weaker among strongly dysregulated genes (>10-fold change), although no clear relationship between technology (RNA-seq or QPCR) and higher expression levels was observed. In the three "0" level genes tested, QPCR indicated that while this portion of the RNA-seq results likely does indicate differentially expressed genes, fold-change levels cannot be accurately estimated from the assumption of no expression (0 reads captured) in a given sample. For example for the chemokine SCYA105, QPCR indicated 1746-fold higher expression at 0 h in susceptible fish than resistant fish, with 81 and 0 reads in the two groups, respectively. However, TNFRSF14, with 79 and 0 reads in susceptible and resistant fish, respectively, showed 11.76-fold higher expression in susceptible fish (Supplementary Table 7). The discrepancy between the two techniques on this subset of genes may be due to expression of alternatively spliced allelic forms or incorrect assignment of RNA-seq reads to paralogous genes. Given the lower reliability of "0" read genes, we separated key genes in this category into separate subheadings in Table 5 and Supplementary Table 6. In general, however, the RNA-seq results were confirmed by the QPCR results, indicating the reliability and accuracy of the Trans-ABySS reference assembly and RNA-seq expression analysis. The QPCR validation also was an important indication that the master pooled samples (3 pools of 4 fish each) used for RNA-seq analysis reflected expression levels in the individual pools.

4. Discussion

Knowledge of mucosal actors in the teleost gill and their impact on pathogen susceptibility has been limited. Recent studies have examined the gill transcriptome of several salmonid species in the context of stress (Jeffries et al., 2012; Sánchez et al., 2011) and parasite infection (Morrison et al., 2006a; Wynne et al., 2008), but no studies have examined the basal status of fish mucosa in the context of disease resistance. Here, we have profiled global transcriptional differences in the gill both before and at early timepoints following *F. columnare* challenge in fish from resistant and susceptible families of channel catfish (*I. punctatus*). Our results indicate that a polarization in mucosal status prior to infection may impact early adherence, entry, and host inflammatory processes in a manner which ultimately determines disease outcome.

A clear correlation between *F. columnare* virulence and adherence to the gill epithelium has previously been demonstrated (Decostere, 2002). Additionally, surface mucus from both catfish and salmon has been shown to benefit *F. columnare* growth and promote chemotaxis (Klesius et al., 2008; Staroscik and Nelson, 2008). Recently, Olivares-Fuster et al. (2011) observed cells of *F. columnare* aggregated within and surrounding mucus pores of the skin and capping tissue of the gill filament in channel catfish as soon as 30 min post-challenge. This mucosal host-pathogen association has previously been postulated to be lectin-mediated based on sugar-blocking studies in carp (Decostere et al., 1999a) and catfish (Klesius et al., 2010). Our recent work (Beck et al., 2012) identified a putative host lectin receptor in catfish gill, a rhamnose-binding lectin, with pre- and post-challenge expression levels inversely correlated with *F. columnare* resistance. We investigated here the larger context of differential RBL expression in resistant and susceptible catfish using RNA-seq profiling. Our greatest interest lay in capturing basal differences in expression prior to *F. columnare* challenge between resistant and susceptible fish, as these signatures could potentially

Table 5
Differentially expressed genes in the gill between catfish resistant and susceptible to *F. columnare* and with putative key functions in mucosal immunity. Timepoints of comparison are pre-challenge (0 h) and at 1 h, 2 h, and 8 h post-challenge. Positive values indicate higher expression in susceptible catfish, while negative values indicate higher expression in resistant catfish. Bold values indicate significant fold change ($p \leq 0.05$). When reads number equaled to 0 in resistant or susceptible group, the fold change is presented as normalized read number in resistant fish/normalized read number in susceptible fish.

Gene name	Contig ID	0 h	1 h	2 h	8 h
Immune component					
Antimicrobial peptide NK-lysin-like	Contig30003	**8.11**	**1.35**	**3.25**	1.12
B7-H3 protein precursor	k90_1004142	−4.57	**28.64**	2.31	**−4.50**
Beta-2 microglobulin precursor	Contig27654	−1.17	1.31	**2.79**	**4.55**
Catalase	k68_2467845	−3.33	−5.85	**−6.19**	**−17.51**
CC chemokine SCYA120	k82_1576433	8.34	**10.45**	3.11	−2.71
C–C motif chemokine 19-like precursor	k71_2254471	**−3.51**	**−3.14**	**−1.88**	4.66
C–C motif chemokine 20-like	Contig23454	**3.13**	**1.70**	**2.20**	**2.23**
CD2 antigen cytoplasmic tail-binding protein 2	Contig8122	**5.15**	1.84	**3.23**	2.58
CD40 antigen precursor	Contig17086	3.06	**12.06**	**10.96**	**2.91**
CD74 molecule, MHC II invariant	k86_1288168	**4.43**	**4.24**	**4.62**	**4.95**
CD8 antigen, alpha polypeptide precursor	k60_3009101	**8.82**	−1.03	**3.81**	1.39
CD83	k81_1706265	**2.94**	1.53	**4.76**	3.91
Chemokine C–X–C motif receptor 4a	Contig3928	**3.69**	**1.75**	1.86	1.53
COMM domain-containing protein 6	k77_1936198	**18.39**	**80.38**	**6.69**	**16.90**
Complement C1q subcomponent subunit A precursor	Contig9882	−1.13	**2.25**	**1.62**	**4.07**
Complement factor I	k60_3040837	3.36	**5.64**	**16.97**	−1.64
Diacylglycerol kinase zeta	Contig22646	**−9.91**	**−35.17**	**−12.64**	**−7.40**
E3 ubiquitin/ISG15 ligase TRIM25 isoform 1	k52_3538500	**8.44**	**7.27**	**5.22**	8.31
Eosinophil peroxidase precursor	Contig28132	**2.88**	**2.72**	1.49	**1.89**
Fc receptor-like protein 5-like	Contig18486	−2.42	−9.62	1.42	**−45.37**
H-2 class I histocompatibility antigen, Q10 alpha	k72_2189144	**−10.70**	**−2.19**	**−2.43**	**−3.61**
Hephaestin-like	k60_3040383	**−10.97**	**−39.81**	**−14.44**	**−12.33**
HLA class II histocompatibility antigen, DP alpha 1	k73_2144285	**−31.28**	**−24.00**	**−13.34**	**−20.30**
IgGFc-binding protein-like	Contig27011	**3.49**	**6.92**	**4.58**	**6.72**
Immunoglobulin-binding protein 1	Contig20110	**−10.86**	**−6.94**	**−5.11**	**−12.62**
Integrin alpha-E-like (CD103)	Contig9090	**2.40**	1.34	**61.31**	**2.24**
Integrin alpha-M-like (CD11b)	Contig28672	**−1.71**	**−1.78**	**−2.11**	−1.63
Interferon, gamma 1–1 precursor	k62_2847986	**−5.47**	−2.18	2.50	1.88
Interferon-induced very large GTPase 1-like	Contig12397	**4.38**	**3.47**	2.42	−1.43
Interleukin 1 receptor accessory protein	Contig9637	**31.32**	**17.75**	**12.21**	**7.77**
Interleukin-10 receptor subunit beta precursor	k81_1681887	**2.97**	**2.92**	**3.11**	**4.14**
Interleukin-13 receptor subunit alpha-2 precursor	Contig475	**2.48**	**1.70**	**3.04**	2.06
Interleukin-17 receptor A	Contig15946	**1.69**	−1.04	1.21	1.25
Interleukin-22 receptor subunit alpha-2 precursor	k53_3522444	**−4.50**	1.00	**−2.80**	−1.32
Interleukin-8 variant 3	Contig89	**−2.54**	**−13.93**	**−5.16**	**−2.62**
Lectin, mannose-binding 2-like b precursor	Contig14979	−3.08	**−86.59**	−1.31	**−15.29**
Lipopolysaccharide-induced tumor necrosis factor-alpha	Contig24393	**−2.25**	**−2.27**	**−8.83**	**−3.33**
L-Rhamnose-binding lectin CSL2	Contig1154	**3.17**	**−2.50**	−1.64	2.66
Lysozyme C	Contig6206	**−6.62**	**−7.48**	**−8.58**	**−2.71**
Macrophage mannose receptor 1-like	Contig12298	**3.94**	**3.45**	**6.39**	**3.62**
MHC I UDA precursor	Contig14838	**−7.64**	**4.60**	**−5.48**	**−2.12**
MHC I UXA2 precursor	Contig19729	**21.65**	**4.24**	−1.46	1.11
Matrix metalloproteinase 13a precursor	k96_511386	−2.77	−1.04	1.51	**5.34**
MHC class II beta chain	Contig15710	**8.59**	**−22.23**	**5.32**	**16.66**
Microfibril-associated glycoprotein 4-like	Contig17008	**6.12**	**16.05**	**20.74**	**12.55**
MALT lymphoma translocation gene 1	Contig5059	**−6.40**	**−4.94**	−1.09	−1.56
NACHT, LRR and PYD domains-containing protein 14	Contig27718	**2.91**	**3.03**	1.67	2.30
Natterin-like protein	k96_502354	**−4.23**	**−4.30**	**−2.94**	**−4.15**
Nitric oxide synthase 2b, inducible	Contig9783	**−5.20**	**−15.34**	**−4.21**	**−3.60**
Novel protein containing immunoglobulin domains	Contig29892	1.90	**13.90**	**3.63**	**21.07**
Protein LSM14 homolog A	k78_1881343	6.89	4.09	**24.59**	**93.27**
Protein NLRC3-like	k95_593007	**6.30**	−1.13	**−5.08**	2.15
Retinoic acid receptor alpha-B	Contig23851	**7.09**	1.63	2.20	2.82
Rhamnose binding lectin-like precursor	Contig2789	**10.08**	**11.05**	**16.72**	**14.83**
Serum amyloid P-component precursor	Contig1999	**3.40**	**1.48**	**1.93**	1.02
Similar to interferon-induced, hepatitis C-associated	k72_2228955	1.59	4.02	**9.41**	**13.43**
Stonustoxin subunit beta	Contig4216	**11.50**	**1.79**	**2.64**	1.50
Toll-like receptor 21 precursor	Contig3417	2.22	**1.98**	1.63	**4.35**
Toxin-1 precursor	Contig4333	**−11.91**	**−3.98**	−2.96	−1.91
Tumor necrosis factor ligand superfamily member 12	k67_2576854	1.99	**4.58**	**5.48**	3.80
Tumor necrosis factor receptor superfamily member 14	Contig14203	−1.23	**9.46**	−1.19	**102.40**
Immune component (reads number contain 0)					
CC chemokine SCYA105	Contig30409	**0/81**	**0/121**	**0/33**	**0/160**
Chemokine CCL-C25y precursor	k71_2311470	**22/0**	**−15.59**	**−9.52**	**−7.36**
Interleukin 17a/f2 precursor	k50_3550089	**12.43**	2.32	1.48	0/2
Interleukin 2 receptor, gamma b precursor	k55_3392983	**11.24**	**0.00**	**0/35**	**0/27**
Major histocompatibility complex class I UBA precursor	Contig19309	**379/0**	0/2	**244/0**	**67/0**
MHC I alpha chain, partial	k81_1660488	−1.75	**24/0**	**−25.08**	4/0
MHC II integral membrane protein alpha chain 3	k76_1957396	**0/199**	**0/319**	**0/295**	**0/51**
MHC non-classical class I heavy chain	k90_981934	**8/0**	**20/0**	**−25.82**	**10/0**
NACHT, LRR and PYD domains-containing protein 1	Contig27568	**42/0**	**−18.41**	**32/0**	**−25.89**

Table 5 (*Continued*)

Gene name	Contig ID	0 h	1 h	2 h	8 h
Signaling lymphocytic activation molecule-like	Contig19933	**46/0**	**20/0**	**69/0**	0/0
Similar to Poly [ADP-ribose] polymerase 14	Contig27481	**21.45**	**7.88**	**11.88**	**0/16**
Tumor necrosis factor alpha-induced protein 8-like protein 3	Contig18314	0/26	**14.07**	6.10	2.03
Tumor necrosis factor receptor superfamily member 14	k85_1384186	**0/79**	0/23	0/25	0/12
Vitelline membrane outer layer protein 1 homolog	k90_954683	0/589	**75.12**	**55.40**	**44.97**
Mucin secretion and modification					
Alpha-1,3-fucosyltransferase 9B	Contig23983	−**10.54**	−2.22	1.01	−**12.58**
Beta-1,3-galactosyltransferase 2-like	Contig2355	**5.16**	**9.23**	3.50	3.83
Mgat1a	Contig2190	−**12.40**	−**9.11**	−**5.18**	−**7.79**
Mucin-19-like, partial	Contig30403	**634.29**	**15.73**	**10.41**	**13.83**
Mucin-2	Contig28752	**30.39**	**7.10**	**1.95**	**2.51**
Mucin-2-like	Contig25990	**57.46**	**9.04**	**7.96**	**5.52**
Mucin-5AC	k96_156852	**2.92**	**1.56**	−**4.77**	1.14
Mucin-5AC-like	Contig28993	**157.30**	**2.04**	**3.43**	**6.29**
Mucolipin-3-like	Contig9791	2.16	**3.93**	**2.95**	1.47
Mucolipin-3-like	Contig30888	**4.05**	**3.33**	**3.16**	**2.62**
Ubiquitin carboxyl-terminal hydrolase 19	Contig18301	1.31	**9.92**	−2.09	5.58
Mucin secretion and modification (reads number contain 0)					
Beta-1,4 N-acetylgalactosaminyltransferase 1-like	k60_2977720	5.45	3.16	**0/32**	1.47
CMP-N-acetylneuraminate-beta-galactosamide-alpha-2	Contig22040	**13.13**	**15.67**	1.25	**0/54**
Glycogenin 1a	k64_2782278	**9.70**	14.74	**9.84**	0/6
ST8SIA3	k52_715050	−**48.95**	13/0	−24.40	−15.78
Tissue specific transplantation antigen P35B	k85_1375453	**377.58**	**805.33**	0/0	1/0

serve as expression QTL and/or biomarkers for selection of catfish resistant to *F. columnare* without the necessity of repeated trial infections (Liu et al., 2011).

Comparison of expression levels of resistant and susceptible catfish by RPKM analysis of RNA-seq read numbers generated 1714 unique genes differing in expression by 1.5-fold or greater at at least one timepoint. We categorized key genes within this set into four broad functional categories: immune-related, mucin secretion and modification, junction/cytoskeletal regulation, and cell survival and proliferation (Table 5 and Supplementary Table 6, Fig. 1). As noted

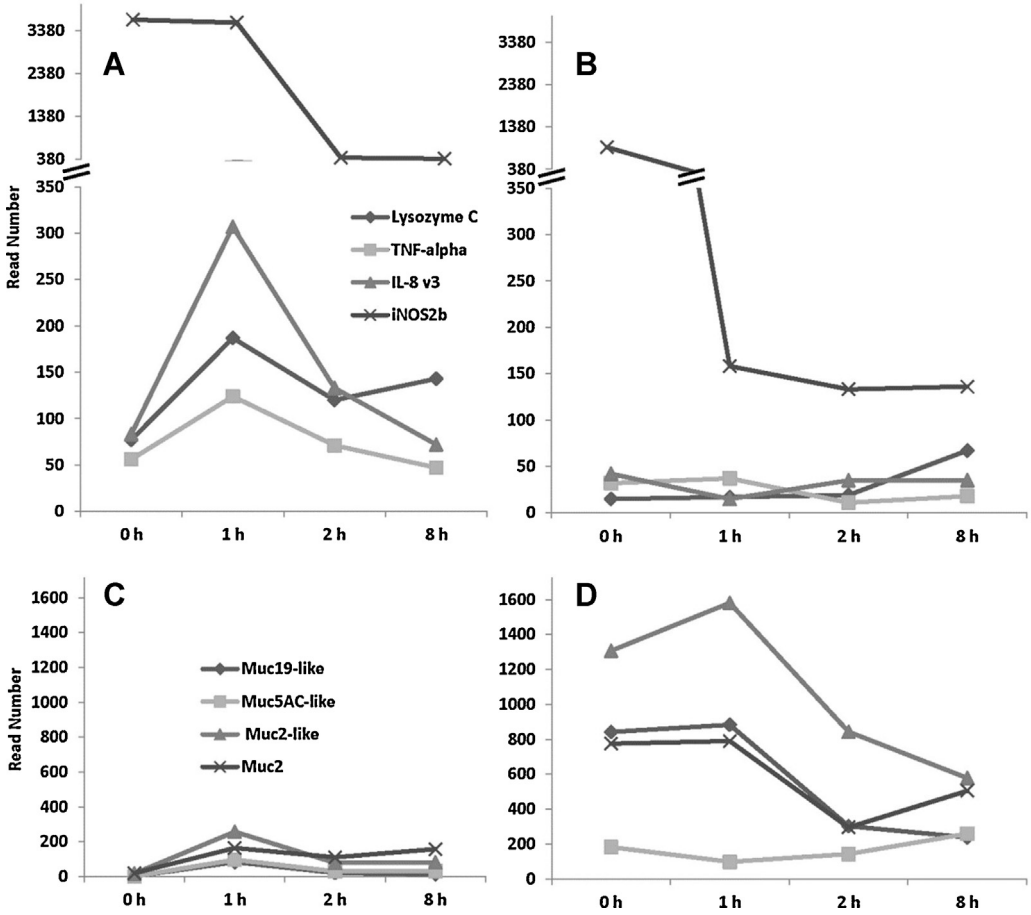

Fig. 1. Representative signatures of polarization between resistant (left) and susceptible (right) catfish to *F. columnare*. (A and B) Mucin (Muc) read numbers at 0 h, 1 h, 2 h, and 8 h post-infection in resistant and susceptible catfish, respectively. (C and D) Key immune gene read numbers for lysozyme C, TNF-alpha, IL-8 v3, and iNOS2b in resistant and susceptible catfish, respectively.

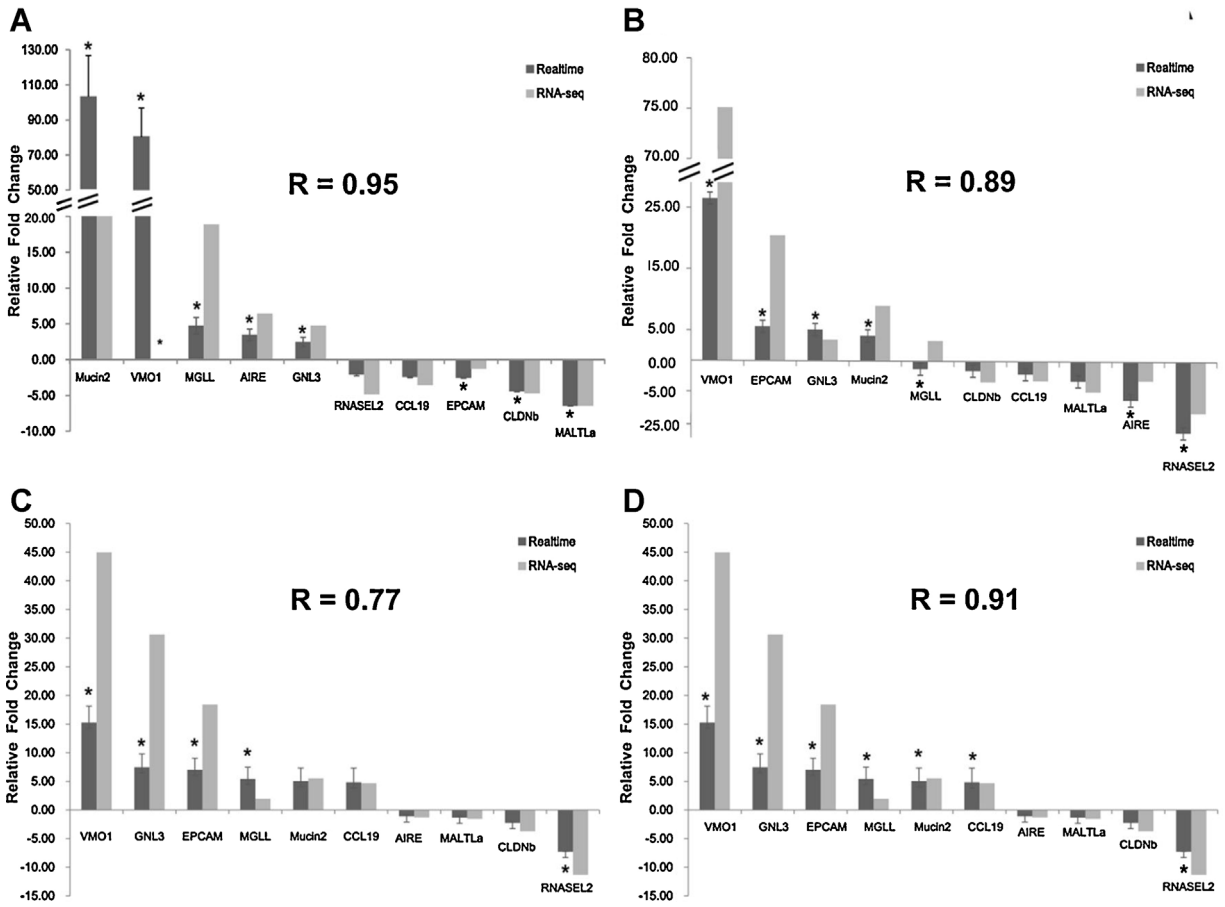

Fig. 2. Comparison of relative fold changes between RNA-seq and QPCR results in catfish gill at 0 h (A), 1 h (B), 2 h (C) and 8 h (D). Gene abbreviations are: Monoglyceride lipase, MGLL; Mucosa associated lymphoid tissue lymphoma translocation gene 1, MALTLa; C-C motif chemokine 19-like precursor, CCL19; Epithelial cell adhesion molecule precursor, EPCAM; Autoimmune regulator-like, AIRE; Ribonuclease like 2 precursor, RNASEL2; Guanine nucleotide-binding protein-like 3, GNL3; Claudin b, CLDNb; Vitelline membrane outer layer protein 1 homolog, VMO1 (*reads number equal to zero at 1 h).

previously (Li et al., 2012b; Sun et al., 2012), automated GO annotation and pathway analyses, while valuable, often provide limited and/or out-of-date functional assignments for fish genes. Additionally, the extensive duplication and non-standardized nomenclature in zebrafish necessitate careful manual analyses using reciprocal BLAST, sequence alignments, phylogenetic tree construction, and current literature searches to provide a comprehensive understanding of gene identity and function. Below we highlight key aspects of two of these important categories likely mediating the catfish response to columnaris infection.

4.1. Immune component

We first observed that our RNA-seq results validated our previous finding of higher gill expression of a rhamnose-binding lectin in individual susceptible fish when compared with resistant individuals (Beck et al., 2012). By RNA-seq, expression levels in our pooled samples were ~10–17-fold higher at all timepoints including 0 h (Table 5). Beyond RBL, several key innate defense genes differed in their expression levels between the two groups. Notably, inducible nitric oxide synthase 2b (iNOS2b) was significantly higher in resistant fish gill at all timepoints. iNOS2b had the highest read counts of any differentially expressed gene captured in resistant samples (Supplementary Table 4, Fig. 1), with 3636 reads at 0 h compared with less than 1000 in susceptible samples. Interestingly, iNOS2b levels were not induced in either group following *F. columnare* infection. Rather, they declined roughly 10-fold by 8 h from their basal levels (Fig. 1). Inducible nitric oxide synthases (iNOS) generate nitric oxide (NO) from L-arginine. Often produced by macrophages, NO is a potent cytotoxic agent in immune defenses which can have beneficial antimicrobial activity, but which can also have far-reaching tissue-damaging effects (Aktan, 2004; Bogdan, 2001). iNOS2b in zebrafish has been reported to be orthologous to mammalian NOS2 (iNOS), constitutively expressed in all studied tissues, and inducible by LPS and Poly I:C (Lepiller et al., 2009). iNOS expression has been previously detected in the gills of bacterially-challenged rainbow trout (Campos-Perez et al., 2000; Laing et al., 1999) but no constitutive expression was observed in this tissue. Our finding of high levels of constitutive iNOS expression in catfish gill matches often overlooked reports of continuous high-level iNOS expression in healthy human respiratory epithelium (Guo et al., 1995). There, NO is believed to modulate mucociliary clearance and mediate cytotoxicity against a range of pathogens (Bogdan, 2001; Jain et al., 1993). Though further research is clearly needed, higher constitutive iNOS2b levels in resistant catfish may have far-ranging effects on mucosal health and explain, in part, other downstream differential expression.

Lysozyme C (chicken-type) also displayed consistently higher expression in resistant catfish gill than that observed in susceptible fish (Table 5). Plasma lysozyme levels have been studied for several decades in the context of fish immunity (Ellis, 2001), but relatively little attention has been given to the level and roles of lysozyme in mucosal surfaces (Bergsson et al., 2005; Nigam et al., 2012). In mammals, lysozymes are among the most abundant secreted mucosal enzymes from the epithelium as well as a major component of granules of professional phagocytes. They

help to kill bacterial pathogens through enzymatic and antimicrobial activity (Davis and Weiser, 2011). In previous studies in catfish challenged with *Edwardsiella ictaluri*, plasma lysozyme dynamics differed between resistant and susceptible fish, with a faster response (Bilodeau et al., 2005) and elevated lysozyme levels (Bilodeau-Bourgeois et al., 2008) characterizing resistant catfish strains. Also supporting the potential importance of high mucosal levels of lysozyme for disease resistance is research from zebrafish. Yazawa et al. (2006) established a transgenic zebrafish strain expressing a chicken lysozyme gene under the control of a keratin promoter which resulted in a 65% survival rate against *F. columnare* compared to 0% survival in wild-type fish. Future studies will characterize whether catfish mucosal lysozyme activity correlates with transcript levels observed in the current study.

Other important mediators of innate immunity, including an IL-8 variant (Chen et al., 2005) and TNF-alpha also were basally higher in resistant fish and showed a rapid induction upon infection relative to susceptible fish which failed to upregulate many proinflammatory cytokines immediately following challenge (Table 5, Fig. 1). In contrast, several important immune antimicrobial peptides, cytokines, and mucosal cell population markers were basally higher in susceptible fish. These included the antimicrobial peptide NK-lysin (Wang et al., 2006), CD8, and CD103, a marker for mucosal dendritic cells believed to regulate tolerance in the mammalian gut (Scott et al., 2011) but uncharacterized to-date in fish. Also higher at time 0 in susceptible fish were IL-17A/F2 and IL-17RA, important mucosal cytokines recently described in fish for the first time (Monte et al., 2013). A microfibril-associated glycoprotein 4 (MFAP4)-like molecule was more highly expressed at all timepoints in susceptible fish. We have previously observed marked differential expression of MFAP4 genes in several different bacterial challenges in catfish (Li et al., 2012a,b, 2013; Peatman et al., 2007, 2008; Sun et al., 2012). Previously, we characterized the ficolin-like nature of MFAP4 (Niu et al., 2011) and noted its use as macrophage-specific marker in zebrafish (Zakrzewska et al., 2010). While further cellular characterization is needed in catfish, MFAP4 genes have emerged as some of the most predictable markers of the early inflammatory response. Also notable were several major histocompatibility complex (MHC) class I and class II genes which were differentially expressed between resistant and susceptible fish (Table 5). MHC genes can play critical roles in both innate and adaptive immune responses through antigen presentation and interactions with T cell subtypes and NK cells. Allelic and expression differences in genes in the MHC have been linked with differential susceptibility to diseases impacting a variety of vertebrate species (Morrison et al., 2006b; Dalgaard et al., 2009; Du et al., 2011). In summary, further work is needed to determine if/how differences in prevalence of interbranchial lymphocyte populations (Koppang et al., 2010) before and soon after infection contributed to the observed polarized immune signatures. These studies will be aided by growing resources of monoclonal antibodies directed at immune cell types in catfish (Edholm et al., 2010, 2011).

4.2. Mucin secretion and modification

Some of the most prominent differences in basal expression between resistant and susceptible catfish were found among mucin genes (Table 5; Fig. 1). Mucin levels (MUC5AC, MUC5AC-like, MUC2-like, MUC19-like) were dramatically higher in the gill of susceptible fish at 0 h when compared to resistant fish. These differences (between groups) were reduced upon experimental infection with *F. columnare*, and only modest induction of mucin expression was observed within either group at 1 h post-infection (Fig. 1). Mucins are large glycoproteins which can be divided into secreted (gel-forming and non-gel forming) and membrane-bound forms (Dharmani et al., 2009; Moran et al., 2011). In mammals, the secreted gel-forming mucins (MUC2, MUC5AC, MUC5B, MUC6, and MUC19) are the major constituent of mucus, forming a protective physiochemical matrix on mucosal surfaces. Additionally, mucins are extensively modified through addition of glycans (sugars) to various amino acid sites, offering protection from proteolytic enzymes. Increasingly, however, research into mammalian host-pathogen and host–commensal interactions is revealing that mucin production and glycosylation states can change dramatically during colonization and infection (Colomb et al., 2012; Mack et al., 1999, 2003; Mahdavi et al., 2002; Navabi et al., 2013). As particular host glycosylation states can be manipulated by microbes, factors linked with glycolipid metabolism and modifications such as fucosyltransferase 1 (FUT1) are now understood to be critical in pathogen binding dynamics and host susceptibility (Bao et al., 2012; Moran et al., 2011).

Our present understanding of the structure, abundance, and functions of fish mucins is very limited (Micallef et al., 2012; Provan et al., 2013) but a recent functional study in zebrafish revealed a role for retinoic acid (RA) in control of mucin expression and a mucosecretory phenotype in the gut (Oehlers et al., 2012). This study indicated that, as in mammals, RA may be a critical factor in regulating mucus differentiation and mucin gene expression in fish. Research in human bronchial epithelial cells has shown that secreted gel-forming mucin expression is RA-dependent and that control is mediated through the RA receptor (RARα) (Gray et al., 2001; Koo et al., 1999). Furthermore, RA-deprived bronchial epithelial cells are reported to become squamous, fail to produce mucin, and instead secrete large amounts of lysozyme (Yoon et al., 1999). RA is additionally known to possess broad tolerogenic/anti-inflammatory properties including suppressing expression of TNF-alpha and iNOS (Dheen et al., 2004). Intriguingly, in our results (Table 5), RARα is over 7-fold higher in susceptible fish at 0 h (Table 5), with modest, non-significant differences between groups following infection. Taken together, the immune and mucin profiles obtained by RNA-seq suggest a basal polarization in the gill mucosa with susceptible fish possessing a putative mucosecretory, tolerogenic phenotype which may predispose them to *F. columnare* infection. Furthermore, this polarization may be driven in part by RA-dependent mechanisms. Functional studies are currently underway to determine the impacts of Vitamin A and RA on mucin expression and goblet cell abundance in fish gill and to explore how differing dietary regimens or commensal microbial populations may affect these factors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.04.014.

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