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Nutritional impacts on gene expression in the surface mucosa of blue catfish (*Ictalurus furcatus*)

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ABSTRACT

Short-term feed deprivation is a common occurrence in both wild and farmed fish species, due to reproductive processes, seasonal variations in temperature, or in response to a disease outbreak. Fasting can have dramatic physiological and biological consequences for fish, including impacts on mucosal immunity which can, in turn, change host susceptibility to pathogens. Culture and selection of blue catfish (*Ictalurus furcatus*) has gained importance as the production of a channel catfish × blue catfish (*Ictalurus punctatus* × *I. furcatus*) hybrid has increased in the Southeast US. Following a recent examination of fasting-induced impacts on mucosal immunity in channel catfish, here we utilized Illumina-based RNA-seq expression profiling to compare changes in blue catfish gill and skin after a brief (7 day) period of fasting. Transcriptome sequencing and *de novo* assembly of over 194 million 100 base-pair transcript reads was followed by differential expression analysis. Fasting altered a total of 530 genes in the surface mucosa, including genes regulating the immune response, energy metabolism, mucus production, cellular cytoskeletal structure, cell proliferation, and antioxidant responses. In particular, fasting perturbed arginine synthesis and metabolism pathways in a manner likely altering macrophage activation states and immune readiness. Our findings highlight key mediators of the critical interaction between nutrition and immunity at points of pathogen adherence and entry.

1. Introduction

Blue catfish, *Ictalurus furcatus*, are valued in the United States as a trophy fishery for their capacity to reach large sizes, sometimes exceeding 45 kg. Indeed, at maximum size, they are one of the largest freshwater fishes in North America (Graham et al., 1999). Early on, researchers studied the food habits of the species (Brown and Dendy, 1961), and recognized their potential for crossing with the farmed channel catfish (*Ictalurus punctatus*) to produce a faster growing hybrid fish (Giudice, 1966). While blue catfish attain a larger maximum size than channel catfish, their early growth has been found to considerably lag that of channel catfish (Bosworth et al., 1998; Giudice, 1966). However, blue catfish have been recognized for their superior disease resistance to several pathogens (Bosworth et al., 2003; Wolters and Johnson, 1994). Many of the differing phenotypic attributes of blue catfish, relative to channel catfish, are likely connected to its evolved habitat usage and feeding habits. Blue catfish have an affinity for swift water and deep channels, feeding there at irregular intervals on prey such as gizzard shad or threadfin shad (Graham et al., 1999). Increased usage of a hybrid catfish (*I. punctatus* female × *I. furcatus* male) by the U.S. aquaculture industry has stimulated renewed efforts to study, further domesticate, and carry out genetic selection on blue catfish parental strains used for crosses.

Alterations in feeding regimen are common practice in the modern aquaculture industry (Larsson and Lewander, 1973; Navarro and Gutiérrez, 1995). Short-term food deprivation strategies may be used as part of a seasonal feeding pattern (Pottinger et al., 2003), in response to overproduction, or as a response to disease outbreaks (Shoemaker et al., 2003; Wise et al., 2008). We recently examined the immune consequences of short-term (7 d) feed deprivation in channel catfish skin and gill mucosa (Liu et al., 2013). There, we observed the striking regulation of important immune effectors including iNOS and lysozyme C in a manner associated with susceptibility to the Gram-negative bacterial pathogen *Flavobacterium columnare*, the causative agent of columnaris disease (Peatman et al., 2013). Indeed, fasted catfish are significantly more susceptible to *F. columnare* infection (Shoemaker et al., 2003). Given the differing feed strategies of blue catfish and their differential infection rates when exposed to *F. columnare* (Arias et al., 2012), we wished to examine here the comparative impact of short-term fasting on *I. furcatus* immune health. While previous comparisons of channel catfish and blue catfish immune responses have utilized microarray platforms for transcriptomic profiling (Li et al., 2013a,b; Peatman et al., 2007, 2008), here we

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assembled a high-quality *de novo* blue catfish transcriptome from RNA-seq short reads prior to expression analysis. Shared and unique signatures of the fasting response in channel catfish and blue catfish may highlight important mechanisms regulating teleost mucosal immunity as well as providing genetic markers for selection of superior parental lines for hybrid catfish production.

2. Methods

2.1. Experimental animals and tissue collection

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. Juvenile blue catfish (61.2 ± 6.7 g) were stocked into four 600 L tanks with 30 fish per tank with forced air aeration and flow-through well water at 24.8 ± 0.02 °C, pH 7.7, and dissolved oxygen of 7.4 ± 0.3 mg/L.

In order to examine the role of nutritional status on host mucosal immune response, fish were subjected to two treatments with two replicate tanks per treatment. Fish in treatment group 1 were fed to satiation three times daily with a standard catfish ration (35% protein, 2.5% fat; Delta Western, Indianola, Mississippi). In treatment 2, fish were withheld feed for 7 d. All fish were sacrificed on day 7, euthanized with tricaine methanesulfonate (MS 222) at 300 mg/L (buffered with sodium bicarbonate) before tissues were collected.

At the termination of the study equivalent portions of the gill and skin were isolated from matching locations on each fish and stored in RNALater (Ambion, Life Technologies, Grand Island, New York) at −80 °C until RNA extraction. Three pools (five fish each) of tissue were generated from each condition. Equal amounts of tissue were collected from each fish within a pool. Samples were immediately placed in RNAlater 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.

2.2. RNA extraction, library construction and sequencing

Extractions were performed according to the manufacturer's directions using an RNeasy Plus Universal Mini Kit (Qiagen, Valencia, California). 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 skin and gill were pooled together to generate 3 pooled replicates for RNA-seq library construction.

RNA-seq library preparation and sequencing were 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 1–12. Finally, amplified library yields were 30 μl of 19.8–21.4 μg/μl with an average length of ~270 bp, indicating a concentration of 110–140 nM. After KAPA quantitation and dilution, the libraries were clustered 12 per lane and sequenced on an Illumina HiSeq 2000 instrument with 100 bp PE reads.

2.3. De novo assembly of sequencing reads

The *de novo* assembly was performed on blue catfish cleaned reads using Trinity (Grabherr et al., 2011). 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 subsequent assembly. Briefly, the raw reads were assembled into the unique sequences of transcripts in Inchworm via greedy K-mer extension (k-mer 25). After mapping of reads to Inchworm contig bundles, Chrysalis incorporated reads into deBruijn graphs. Butterfly then processed the individual graphs in parallel, reporting full-length transcripts and paralogous genes. In order to reduce redundancy, the assembly result was passed to CD-Hit version 4.5.4 (Li and Godzik, 2006) and CAP3 (Huang and Madan, 1999) for multiple alignments and consensus building. 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.

2.4. Gene annotation and ontology

The assembled 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 2.6.6, which is an automated tool for the assignment of gene ontology terms (Gotz et al., 2008). 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.

2.5. Identification of differentially expressed contigs

The high quality reads from each sample were mapped onto the Trinity 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 fed and fasted group with three replications in each group in blue catfish with corrected p-value < 0.05 (Baggerly et al., 2003). After scaling normalization of the RPKM values, fold changes were calculated (Robinson and Oshlack, 2010). Analysis was performed using the RNA-seq module and the expression analysis module in CLC Genomics Workbench. 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.

2.6. 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 Benjammini–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 < 0.05.

2.7. 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. Primers were designed based on contig sequences (Supplementary Table 1). 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). 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), 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. Sequencing of short expressed reads from blue catfish gill and skin

Illumina-based RNA-sequencing (RNA-seq) was carried out on gill and skin samples from blue catfish. Reads from different samples were distinguished through the use of multiple identifier (MID) tags. A total of 194 million 100 bp high quality reads were generated on an Illumina HiSeq 2000 instrument in a single lane with blue catfish. Greater than 22 million reads were generated for each of the six libraries. After removing ambiguous nucleotides, low-quality sequences (quality scores < 20) and short reads (length < 30 bp), the 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 SRP020252.

3.2. De novo assembly of blue catfish gill and skin transcriptome

Trinity generated approximately 264,152 contigs with average length of 1045.2 bp and N50 size of 2309 bp in its initial assembly, with 30,487 contigs longer than 1000 bp. After removing redundancy using CD-Hit and CAP-3, about 96.83% of contigs were kept, resulting in a final assembly of 255,766 contigs with average length of 1003.5 (Table 1). Over 80.95% of reads were mapped in pairs (both paired ends assembled in the same contig).

3.3. Gene identification and annotation

BLAST-based gene identification was performed to annotate the blue catfish gill/skin transcriptome and inform downstream differential expression analysis. After gene annotation, 79,344 Trinity contigs had a significant BLAST hit against 17,890 unique zebrafish genes (Table 2). 15,805 unigenes were identified based on hits to the zebrafish database with the more stringent criteria of a BLAST score $\geqslant 100$ and E-value $\leqslant 1e{-}20$. The same BLAST criteria were used in comparison of the Trinity reference contigs with the UniProt and nr databases. The largest number of matches was to the NR database with 82,247 contigs with putative gene matches to nr and 21,204 quality unigene matches (Table 2).

3.4. Identification and analysis of differentially expressed genes

Differential expression analysis in comparison to fed control samples was made for the fasted blue catfish (Table 3). A total of 530 genes (unique annotated contigs with significant BLAST identities) were differentially expressed greater than 1.5-fold, with 186 up- regulated genes and 344 down-regulated genes (Supplementary Table 2). Short read coverage within differentially expressed contigs is critical for accurate quantification of expression levels. We obtained good coverage of differentially expressed contigs, with an average of 1039.74 reads/contig.

3.5. Enrichment and pathway analysis

A total of 966 GO terms including 255 (26.40%) cellular component terms, 297 (30.75%) molecular functions terms and 414 (42.86%) biological process terms were assigned to 530 unique gene matches. The percentages of annotated catfish sequences assigned to GO terms are shown in Supplementary Fig. 1. The differentially expressed unique genes were then used as inputs to perform enrichment analysis using Ontologizer 2.0. A total of 53 terms with p-value (FDR-corrected) < 0.05 were considered significantly overrepresented. Ten higher level GO terms were retained as informative for further pathway analysis (Supplementary Table 3). The GO terms included cellular amino acid metabolic process, carboxylic acid metabolic process, organic acid metabolic process and immune response.

Based on enrichment analysis and manual annotation and literature searches, representative key genes were arranged into 6 key categories, including immune response, metabolism, mucus-related, cytoskeletal, cell cycling/proliferation and antioxidant/oxidative stress (Table 4). Imputed putative functional roles of these genes are covered in Section 4.

Table 1
Summary of *de novo* assembly results of Illumina sequence data from blue catfish gill and skin using Trinity.

Contigs	264,152
Large contigs ($\geqslant$1000 bp)	30,487
N50 (bp)	2309
Average contig length	1045.2
Contigs (After CD-HIT-EST + CAP3)	255,766
Percentage contigs kept after redundancy removal	96.83%
Average length (bp) (After CD-HIT-EST + CAP3)	1003.5
Reads mapped in pairs (%)	80.95%
Reads mapped to final reference (%)	86.45%

Table 2
Summary of gene identification and annotation of assembled blue 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.

	Blue catfish		
	Zebrafish	UniProt	NR
Contigs with putative gene matches	79,344	63,163	82,247
Annotated contigs ⩾ 500 bp	68,529	56,501	70,650
Annotated contigs ⩾ 1000 bp	57,043	48,229	58,253
Unigene matches	17,890	19,367	27,871
Hypothetical gene matches	1060	0	4068
Quality unigene matches	15,805	16,651	21,204

Table 3
Statistics of differently expressed genes between fasted and fed blue catfish. Values indicate contigs/genes passing cutoff values of fold change ⩾ 1.5 ($p < 0.05$).

Fed (control)	Fasted blue catfish
Up-regulated	186
Down-regulated	344
Total	530
Reads per contig	1039.7

3.6. 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. Samples from fasted and fed blue catfish (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. 1, QPCR results were significantly correlated with the RNA-seq results at each timepoint (average correlation coefficient 0.87, p-value < 0.001; Fig. 1). All examined genes had the same direction of differential expression by both methods indicating the reliability and accuracy of the Trinity reference assembly and RNA-seq-based transcriptome expression analysis.

4. Discussion

Short-term feed deprivation (or fasting) events punctuate the lives of many aquaculture species. Fish are commonly removed from feed for short periods prior to and following transfer, harvest, or other stressor events. Fish also may be fed irregularly during colder weather or due to market oversupply. Feed deprivation is also a common response to disease outbreaks, although success appears to differ among species and pathogens. Fasting has been reported to increase survival of Atlantic salmon (*Salmo salar*) to *Vibrio salmonicida* and of channel catfish to *Edwardsiella ictaluri* (Damsgård et al., 1998; Wise and Johnson, 1998). Conversely, feed deprivation has been observed to increase mortality in *F. columnare*-infected channel catfish (Klesius et al., 1999; Shoemaker et al., 2003) as well as increase *Aeromonas salmonicida* mucus adhesion in chinook salmon, *Oncorhynchus tshawytscha* (Pirhonen et al., 2003).

While changes in feeding regimen may simply alter the physical interactions of fish and their association with feed-adherent bacteria (Wise and Johnson, 1998), several studies indicate that feed deprivation likely impacts a combination of factors including fish physiology, the aquatic environment, and, even, the commensal microbiome. A few studies have demonstrated that the physiological changes brought about by fasting may also alter immune readiness. Krogdahl and Bakke-McKellep observed rapid 20–50% decreases in intestinal tissue mass and enzyme activities after two days of fasting in Atlantic salmon (Krogdahl and Bakke-McKellep, 2005). Feed deprivation can also depress the immune response in Atlantic salmon liver during acute bacterial infections (Martin et al., 2010). Baumgarner et al., utilizing proteomic analyses, observed changes in important mediators of the innate immune response in intestinal epithelia after 4 weeks of feed deprivation in rainbow trout (Baumgarner et al., 2013).

As many catfish pathogens can gain initial entry through surface mucosal tissues (Beck et al., 2012; Li et al., 2012a; Menanteau-Ledouble et al., 2011), we wished here to examine the impact of fasting on immune components in these tissues. Similarly, Vieira et al. examined impacts of a 1 week feed restriction regimen on skin healing and scale regeneration in sea bream utilizing an oligo microarray (Vieira et al., 2011). Intriguingly, a recent report indicates that feed deprivation may additionally cause significant changes in microbial density and community composition in the cutaneous mucus of Atlantic salmon (Landeira-Dabarca et al., 2013).

We previously utilized RNA-seq expression analysis to examine impacts of 7 d feed deprivation in channel catfish. There, over 1500 genes were significantly dysregulated by fasting in channel catfish skin and gill (Liu et al., 2013). In contrast, here a relatively small number of genes (530) were changed following the same fasting treatment in blue catfish. Of the 530 differentially expressed genes in blue catfish, more than half (51.3%) were also differentially

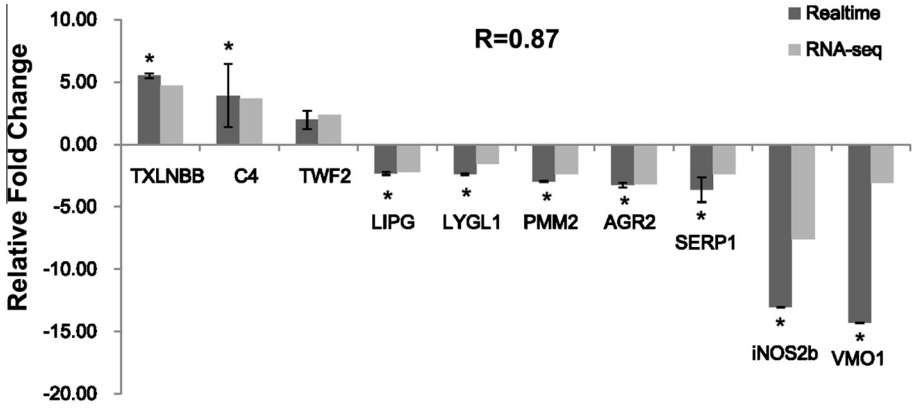

Fig. 1. Comparison of relative fold changes between RNA-seq and QPCR results in fed and fasted blue catfish. Gene abbreviations are: Taxilin beta b, TXLNBB; Complement c4, C4; Twinfilin-2, TWF2; Endothelial lipase precursor, LIPG; Lysozyme g-like 1, LYGL1; Phosphomannomutase 2, PMM2; Anterior gradient protein 2 homolog precursor, AGR2; Stress-associated endoplasmic reticulum protein 2, SERP1; Nitric oxide synthase 2b, inducible, iNOS2b; Vitelline membrane outer layer protein 1 homolog, VMO1.

Table 4
Differentially expressed genes in the gill and skin between fasted and fed blue catfish in different functional classifications. Positive values indicate higher expression in fasted blue catfish, while negative values indicate higher expression in fed blue catfish. Shared (*) indicates genes also differentially expressed in channel catfish (Liu et al., 2013).

Gene name	Contig_ID	Fold change	Shared
Immune response			
CC chemokine SCYA113	comp32889_c0_seq1	−2.74	*
C-C motif chemokine 19-like precursor	comp43298_c0_seq1	−2.18	*
Chemokine (C–C motif) ligand 20-like precursor	comp68669_c0_seq3	−3.06	*
Chemokine CCL-C5a precursor	comp80779_c0_seq1	−2.20	*
Complement C4–1	comp73101_c1_seq3	3.58	
Complement C4-B	comp73101_c1_seq4	3.65	*
Immunoglobulin light chain	comp69623_c0_seq2	−2.14	*
Interferon stimulated gene, 15 kda	comp74997_c0_seq3	−2.34	
Interferon-induced GTP-binding protein Mx-a	comp105321_c0_seq1	−2.11	
Interferon-induced very large GTPase 1-like	comp75989_c0_seq1	2.48	*
Interleukin 2 receptor, gamma a precursor	comp39229_c0_seq2	1.70	*
Liver-expressed antimicrobial peptide 2 precursor	comp57828_c0_seq2	2.01	
Lysozyme g-like 1	comp44998_c0_seq1	−1.54	*
Matrix metalloproteinase-9 precursor	comp74970_c0_seq1	−1.74	
Microfibril-associated glycoprotein 4-like	comp42529_c0_seq1	−4.62	*
Myxovirus (influenza virus) resistance G	comp75986_c0_seq14	4.14	*
Nitric oxide synthase 2b, inducible	comp67082_c0_seq1	−7.58	*
Ribonuclease T2 precursor	comp73039_c0_seq1	3.61	
SAM domain and HD domain-containing protein 1	comp76039_c0_seq14	1.81	*
Serine protease 27	comp79901_c0_seq1	−5.95	*
Solute carrier family 3, member 2a (CD98)	comp77285_c0_seq1	1.58	
Vitelline membrane outer layer protein 1 homolog	comp70718_c0_seq1	−3.05	*
Metabolism			
Acyl-coa synthetase long-chain family member 1	comp67922_c0_seq5	2.25	
Aminopeptidase N-like isoform 1	comp75259_c2_seq2	−2.29	
Angiopoietin-related protein 4 precursor	comp44269_c0_seq1	3.71	*
Apolipoprotein L, 1	comp75496_c3_seq36	1.83	*
Argininosuccinate synthase	comp42610_c0_seq1	−3.57	*
Butyrate response factor 1	comp34043_c0_seq1	1.57	
cAMP-responsive element modulator-like isoform 5	comp65814_c0_seq2	−3.26	
Carnitine O-palmitoyltransferase 1, liver isoform	comp65812_c0_seq1	2.17	*
Carnitine palmitoyltransferase I-like	comp67927_c1_seq1	3.93	
Diacylglycerol O-acyltransferase 2	comp62095_c1_seq1	−10.48	*
Early growth response protein 1	comp75198_c0_seq1	2.66	
Epidermal growth factor-like protein 6	comp49974_c0_seq2	4.46	
Estradiol 17-beta-dehydrogenase 12-A	comp101922_c0_seq1	−2.02	*
Fatty acid binding protein 11b	comp61501_c1_seq1	−1.81	
Fatty acid binding protein 6	comp78067_c0_seq1	−4.80	
Fatty acid-binding protein 2, intestinal	comp40663_c0_seq1	−3.29	*
F-box only protein 32 (atrogin 1)	comp61421_c0_seq2	6.80	*
Folylpolyglutamate synthase	comp71730_c0_seq1	−1.61	
Growth hormone receptor b precursor	comp75735_c1_seq22	1.75	*
High affinity cationic amino acid transporter 1	comp70704_c1_seq1	−1.61	*
Malonyl-coa decarboxylase, mitochondrial	comp73662_c6_seq15	2.99	*
Mannose-1-phosphate guanyltransferase alpha-B	comp65505_c0_seq1	−2.92	*
Mannose-1-phosphate guanyltransferase beta	comp73271_c2_seq2	−2.37	*
Metalloreductase STEAP4	comp74585_c0_seq2	2.20	
MTHFD2	comp62326_c0_seq2	−2.30	*
Muscle RING finger 1 (MURF1)	comp81139_c0_seq1	2.04	*
N-acetylglutamate synthase (NAGS)	comp42507_c0_seq1	−2.05	*
Ornithine aminotransferase (OAT)	comp80784_c0_seq1	−1.63	*
Ornithine carbamoyltransferase (OTC)	comp75609_c0_seq3	−2.05	
Ornithine carbamoyltransferase (OTC)	comp75609_c0_seq5	−1.84	
Ornithine decarboxylase antizyme 2	comp35906_c0_seq1	1.90	*
Probable alpha-ketoglutarate-dependent hypophosphite	comp63151_c0_seq1	−13.06	*
Probable glutathione peroxidase 8	comp69704_c3_seq1	−2.28	
Prolyl 4-hydroxylase subunit alpha-2 precursor	comp42358_c0_seq2	−1.98	
Putative ferric-chelate reductase 1 precursor	comp75103_c0_seq1	−2.39	*
Pyruvate dehydrogenase [lipoamide] kinase isozyme 4	comp75401_c0_seq1	3.04	*
Pyruvate dehydrogenase kinase isozyme 2-like	comp44023_c0_seq2	2.89	*
Serine incorporator 1-like	comp72008_c0_seq2	5.25	
Solute carrier family 25	comp69443_c0_seq4	2.74	*
Solute carrier family 38, member 3	comp74794_c8_seq2	−2.50	
Stearoyl-CoA desaturase 5	comp21911_c0_seq1	−2.24	*
Ubiquitin carboxyl-terminal hydrolase 28 (USP28)	comp70158_c0_seq3	3.42	*
UDP-glucose pyrophosphorylase 2	comp72828_c1_seq5	−2.08	
Mucus-related			
Anterior gradient protein 2 homolog precursor (AGR2)	comp34194_c0_seq1	−3.20	*
Cellular retinoic acid-binding protein 2-like	comp62244_c0_seq1	−1.97	*
Galactose-3-O-sulfotransferase 3-like	comp62910_c0_seq1	−2.02	
IgGFc-binding protein-like	comp35503_c0_seq1	−1.82	*

Table 4 (*continued*)

Gene name	Contig_ID	Fold change	Shared
Mucin-2-like	comp72744_c0_seq2	−3.37	*
Mucin-2-like	comp67982_c0_seq1	−2.08	*
Mucin-5AC-like	comp35601_c0_seq1	−2.02	*
Olfactomedin-like	comp74187_c0_seq4	−1.77	*
Rhesus blood group, C glycoprotein a	comp73593_c0_seq1	−2.09	
Cytoskeletal			
Collagen alpha-1(I) chain	comp56929_c1_seq1	−2.08	*
Collagen alpha-1(VII) chain	comp73350_c0_seq1	−3.29	
Collagen alpha-2(I) chain precursor	comp42781_c0_seq1	−2.24	*
Collagen, type I, alpha 1b precursor	comp56929_c1_seq4	−2.21	*
Cytoskeleton-associated protein 2	comp57955_c0_seq1	−2.39	*
Cytoskeleton-associated protein 4	comp32584_c0_seq1	−4.24	*
Fast skeletal myosin heavy chain isoform mmyh-11	comp79793_c0_seq1	−4.28	
Myosin heavy chain fast skeletal type 2	comp35276_c0_seq1	−3.01	*
Myosin heavy chain, fast skeletal muscle	comp77353_c0_seq1	−2.91	*
Myosin heavy chain, partial	comp36130_c0_seq1	−2.52	
Myosin-7	comp44875_c0_seq1	−3.61	
Taxilin beta b	comp73110_c1_seq3	4.72	*
Tubulin beta-2A chain	comp58109_c0_seq1	−2.25	*
Tubulin beta-6 chain isoform 2	comp45388_c2_seq1	−2.10	*
Cell cycling/proliferation			
Asparagine synthetase	comp70146_c0_seq2	−3.35	*
Borealin (cb373)	comp70245_c0_seq2	−2.20	*
CCR4-NOT transcription complex subunit 6-like	comp61866_c0_seq1	2.28	*
Cell division control protein 2 homolog	comp31769_c0_seq1	−2.01	*
C-Fos	comp69875_c0_seq4	9.91	
G2/mitotic-specific cyclin-B1	comp64201_c0_seq1	−1.69	*
Immediate early response 2	comp61772_c0_seq1	2.73	
Jun B proto-oncogene b	comp62366_c0_seq1	5.60	
Kruppel-like factor 4b	comp69691_c1_seq1	2.35	
Mitotic spindle assembly checkpoint protein MAD2A	comp102508_c0_seq1	−2.04	*
Nucleolar and spindle-associated protein 1	comp65272_c0_seq3	−1.89	*
Proto-oncogene c-Fos	comp69875_c0_seq8	6.63	
Proto-oncogene c-Fos-like	comp69875_c0_seq5	6.47	
SPARC precursor	comp56969_c1_seq1	−1.93	*
Transcription factor jun-B-like	comp62366_c0_seq2	8.60	
Tumor protein p53 inducible nuclear protein 2	comp72255_c4_seq6	2.29	
Antioxidant/oxidative stress			
Dual specificity protein phosphatase 1	comp57100_c0_seq1	2.80	
Glutathione peroxidase 1	comp60578_c1_seq1	1.95	
Glutathione synthetase	comp70551_c0_seq2	−2.41	
GTP-binding protein RAD	comp26157_c0_seq1	−4.83	
Peroxiredoxin-1	comp44782_c0_seq1	−1.56	*
Selenoprotein M precursor	comp33336_c0_seq1	−2.09	*
Selenoprotein Pa precursor	comp76216_c0_seq1	1.93	*

expressed in channel catfish (Table 4; Supplementary Table 4). The magnitude of fold change was greater in channel catfish than in blue catfish for 72% (198) of these genes. The broader and stronger transcriptomic response in channel catfish relative to blue catfish likely reflects differing abilities of the two species to withstand short periods of fasting. However, comparative analysis of pathways and key genes between the two species indicated a high level of conservation in the basic immune and metabolic mechanisms triggered by food deprivation in the surface mucosa. Below we highlight several of these important pathways and note shared and differing responses in blue catfish relative to channel catfish.

4.1. Nutri-immunological impacts

Recent studies have highlighted important mediators of the mucosal immune response in catfish (Peatman et al., 2013; Li et al., 2013a,b). These include both classical immune genes such as lysozymes as well as potentially fish-specific responses such as microfibril-associated glycoprotein 4 (Niu et al., 2011). As in channel catfish (Liu et al., 2013), short-term fasting of blue catfish perturbed many of these immune actors.

The strongest down-regulation after fasting in blue catfish surface mucosa was seen in inducible nitric oxide synthase 2b (iNOS2b), previously established as the most likely teleost orthologue of mammalian iNOS (Lepiller et al., 2009). Macrophages utilize the NOS pathway for increased bacterial killing and disruption of pathogen virulence mechanisms (Bogdan, 2001). iNOS catalyzes the production of nitric oxide (NO) from L-arginine, and, therefore, is often an indicator of the readiness of mononuclear phagocytes to handle infection, or their activation state (Severin et al., 2010). Arginine, itself, is a semi-essential amino acid formed from glutamate or proline, and is best known for its role in the urea cycle (Baumgartner, 2011). Alterations in feed availability are expected to impact free amino acid concentration in surface mucosa (Saglio and Fauconneau, 1985), likely perturbing the urea cycle and arginine availability (Munder, 2009). Indeed, we observed evidence of dysregulation of arginine metabolism and uptake at several points in the cycle beyond iNOS2b (Table 4). Argininosuccinate synthase (ASS-1), which recycles intracellular citrulline to arginine, was down-regulated −3.57-fold by fasting. ASS1-deficient macrophages failed to produce NO, resulting in their inability to control mycobacterial infection (Qualls et al., 2012). The high-affinity cationic amino acid transporter 1 (CAT1), used by macrophages to import exogenous arginine (Cui et al., 2011; Hatzoglou et al., 2004), was suppressed −1.6-fold by fasting. N-acetylglutamate synthase (NAGS), an essential cofactor of carbamoyl phosphate synthetase

(CPS), utilizes glutamine to synthesize arginine (Munder, 2009). Here, NAGS was down-regulated −2.05-fold after short-term fasting. Other members of the urea cycle, ornithine carbamoyltransferase (OTC), ornithine aminotransferase (OAT), and an ornithine decarboxylase (ODC) inhibitor, ornithine decarboxylase antizyme 2, were also disturbed by fasting in a manner predicted to minimize arginine availability (Table 4). Similar trends were also observed in channel catfish following fasting (Liu et al., 2013). A shortage of exogenous arginine likely hinders the ability of mucosal macrophage populations to activate and proliferate, and could compromise the robustness of early mucosal immune responses to invading microbes (Bogdan, 2001; Baumgartner, 2011).

A population of immunocytes with disrupted arginine stores and/or metabolism may have widespread molecular and physiological consequences. A polarized type II immune response (Oliphant et al., 2011; Severin et al., 2010) would be expected to change the observed cytokine milieu (Table 4) and enhance gene expression of MHC class II. Indeed, the three genes with the "highest" fold-change induction (Supplementary Table 2), were all MHC class II genes. We did not include them in Table 4 as they had "0" captured reads in the fed state, making accurate fold change calculations difficult. However, in previous RNA-seq studies we have determined that genes involving "0" reads in one condition are significantly differentially expressed by QPCR (Peatman et al., 2013). One would also expect that a change in immune polarization would impact responses to catfish pathogens. Indeed, similar alterations in genes regulating arginine levels and associated byproducts were observed in fish with high susceptibility to columnaris. There also, columnaris-susceptible fish had lower levels of iNOS and urea cycle enzymes when compared with resistant fish both basally and at early timepoints following infection (Peatman et al., 2013). It is tempting to speculate, therefore, that lowered survival of fasted catfish exposed to *F. columnare* challenge (Shoemaker et al., 2003) may be, in large part, due to arginine-dependent macrophage activation patterns. Also worthy of mention here, and in need of functional follow-up studies to confirm, is the potential connection between altered arginine metabolism following fasting and *higher* survival in fasted, *E. ictaluri* infected fish (Wise and Johnson, 1998). Intriguingly, an inverse relationship between *E. ictaluri* and columnaris susceptibility may exist in the catfish host (LaFrentz et al., 2012). *E. ictaluri* relies on exogenous urea, the byproduct of arginine metabolism, to raise the pH within the macrophage phagolysosome to a level conducive to replication (Booth et al., 2009). Inhibition of the macrophage's arginase activity *in vitro*, blocked the ability of *E. ictaluri* to replicate intracellularly (Baumgartner, 2011). In the case of *E. ictaluri*, therefore, alterations in host arginine stores brought about by fasting may deprive the bacteria of an essential means of pathogenesis. Further functional studies are clearly needed to examine the complex interrelationships between feed deprivation, arginine metabolism, and bacterial disease susceptibility in catfish.

4.2. Energy metabolism

Short-term fasting also altered several key regulators of lipid and glucose metabolism in blue catfish (Table 4). The blue catfish response contained many of the same components previously observed following fasting in channel catfish (Liu et al., 2013). Among these were angiopoietin-related protein 4 (Angptl4), a known regulator of food intake in mammalian species (Kim et al., 2010) with roles as a central regulator of energy metabolism (Koliwad et al., 2012). New roles for this protein in the mucosa continue to be described with recent reports of a protective, anti-inflammatory role during lung injury (Wang et al., 2013) and a role in regulating host-commensal interactions (Korecka et al., 2013). Gene components of lipid accumulation, synthesis and metabolism pathways were almost universally down-regulated in the blue catfish surface mucosa. These included diacylglycerol acyltransferase (DGAT2), several fatty-acid binding proteins (2, 6, and 11) and Stearoyl-CoA desaturase-5 (Table 4).

Several genes known to have roles in proteolytic pathways induced by fasting/starvation were up-regulated in blue catfish. These include F-box only protein 32 (atrogin 1), muscle RING finger 1 (MURF1) and ubiquitin carboxyl-terminal hydrolase (USP28), induced 6.8, 2.0, and 3.4-fold, respectively. Fbox32 and MURF1 expression levels have served as indicators of proteolytic capacity in salmonids previously (Cleveland and Weber, 2013; Tacchi et al., 2012; Wang et al., 2011), and appear to respond sensitively to feeding status in Ictalurid catfish as well.

4.3. Mucus–related changes

We observed modest down-regulation of several mucins and mucus-related genes following 7 d fasting in blue catfish. A few previous studies have reported changes in mucin contents and dynamics following fasting or starvation (Caruso et al., 2011; Smirnov et al., 2004), but experimental conditions vary too greatly to compare with the present study. We speculate that reduced dietary uptake of key vitamins such as Vitamin A would lead to altered retinoic acid synthesis and a decreased rate of goblet cell proliferation and secretion (Oehlers et al., 2012; Tei et al., 2000). Consistent with such a theory, anterior gradient protein 2 (AGR2) was down-regulated 3.2-fold here. Knockout of AGR2 in zebrafish revealed that AGR2 was limited to intestinal goblet cells and required for terminal differentiation (Chen et al., 2012). Similarly, using mice lines, Li et al. (2012b) recently showed that Agr2 expression controlled goblet cell fate in lung epithelia. Previously we observed drastically different levels of mucin expression (including mucin-2-like and -5AC) between *F. columnare* resistant and susceptible channel catfish (Peatman et al., 2013). There, susceptible catfish had several hundred-fold higher levels of mucin expression accompanied by significantly higher numbers of goblet cells. Further study is needed to know whether these relatively small alternations in mucin dynamics would alter host-pathogen dynamics in blue catfish.

4.4. Cell cycling/proliferation

One final group of differentially regulated genes deserving discussion here was within the cell cycling/proliferation category (Table 4). While almost all major (>5-fold) gene expression differences following fasting in blue catfish were mirrored in channel catfish (Liu et al., 2013), the major exception were multiple c-Fos and Jun-B genes. These genes were strongly induced following fasting in blue catfish alone and were among the most highly up-regulated of all significant genes (Supplementary Table 2). The JUN and FOS families are composed of transcription factors linked to regulation of cell division and growth. Despite decades of study, a clear picture explaining their sometimes contradictory roles in cell proliferation, tumorigenesis and tumor suppression has yet to emerge (Milde-Langosch, 2005; Shaulian, 2010). A recent study revealing a link between nutrition and Jun-B and c-Fos, may shed some light on the expression changes seen here. There, mammalian cells subjected to amino acid limitation responded by activating the expression of four JUN/FOS members (Fu et al., 2011). These changes set off a signaling cascade regulating the amino acid response (Kilberg et al., 2005) to nutrient deprivation. The lack of similar JUN/FOS signals in channel catfish, despite widespread dysregulation of downstream cell cycling mediators (Liu et al., 2013), may potentially be explained by temporal differences in the fasting response. Future studies should extend the examination of expression profiles of key genes in both species to earlier and later timepoints.

5. Conclusion

RNA-seq-based expression profiling revealed a largely conserved response to feed deprivation in blue catfish when compared to channel catfish. Short-term nutrient restriction, while often having no apparent phenotypic consequences in farmed fish, rapidly impacts a variety of physiological processes in the surface mucosa. Perhaps the most important of these appears to be changes in arginine metabolism and iNOS2b levels which likely account, in part, for differential susceptibility to bacterial pathogens observed in catfish. Follow-up studies currently underway in our lab will examine in greater depth the functional roles of key genes regulating immuno-nutritional interactions at mucosal surfaces.

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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.dci.2013.12.014.

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