Development of a multitissue microfluidic array for assessing changes in gene expression associated with channel catfish appetite, growth, metabolism, and intestinal health

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Abstract

Large-scale, gene expression profiling methods allow for high throughput analysis of physiological pathways at a fraction of the cost of individual gene expression analysis. Systems, such as the Fluidigm quantitative PCR array described here, can provide powerful assessments of the effects of diet, environment, and management on physiological pathways affecting production parameters. A targeted microfluidic PCR array was designed and validated, for channel catfish (*Ictalurus punctatus*) representing key pathways involved in appetite, growth, metabolism, and intestinal inflammation for their potential to provide insight into the effects of diet and dietary supplements on these important physiological processes regulating feed efficiency and growth. With few exceptions, PCR primers were designed from *Ictaluridae* gene sequences published in GenBank. PCR amplicons from primers designed outside of *Ictaluridae* were sequenced to verify gene identity. All target gene primers were initially validated via conventional real-time qPCR (RT-qPCR). Combined hypothalamus/pituitary, hepatic, stomach, and intestinal tissue were used to validate a 48.48 microfluidic PCR array to analyze multitissue gene expression. Use of the Fluidigm array resulted in reliable cycle threshold levels (Ct), efficiencies (E), and quality threshold scores (QS) for all but eight genes examined. Of the potential reference genes included in the panel, alpha-tubulin (TUBA) had a high QS, E, and acceptable Ct. The high throughput application of this technology, relative to conventional RT-qPCR, for assessing dietary effects on these pathways is demonstrated. Development of this targeted multi-tissue microfluidic array paves the way for the rapid evaluation of regulatory pathways in response to alternative feeding strategies, dietary formulations, and supplementation, as well as environmental and management effects for improving channel catfish culture and validates a cost-effective, dynamic, gene expression platform for use with other cultured fishes.

Keywords:

Channel catfish; gene expression; array; microfluidic

1. Introduction

Large-scale gene expression analysis is imperative for many biological studies. With a variety of ways to assess gene expression available, including RNA-seq, conventional real-time-qPCR (RT-qPCR), and microfluidic dynamic arrays, it is essential to perform a cost analysis of various methods prior to gene expression analysis. RNA-seq, a relatively expensive option, allows for the profiling of the entire transcriptome, the entire set of RNA included in a population of cells, not just specified target genes, and produces a large quantity of data, most of which would be unnecessary in analyzing the behavior of certain physiological pathways. Real-time quantitative PCR amplifies and measures targeted DNA segments, but is often limited to 1 – 4 genes of interest on a PCR plate, due to limited numbers of fluorophores which can be detected per well. When RT-qPCR is performed on a 7900HT Fast Real-Time PCR System (Life Technologies, Carlsbad, CA, USA) using a 384-well plate, it costs about \$0.40 per reaction, not including labor (Dr. Mark Band, Roy J. Carver Biotechnology Center, University of Illinois, personal communication), with each reaction run in three replicate wells to account for technical errors.

Using the Fluidigm qPCR system (Fluidigm Corporation, South San Francisco, CA, USA), the expression of multiple genes (up to 96) can be measured in multiple samples (up to 96 on a single chip), allowing for 9,216 reactions on a single chip array (Spurgeon et al., 2008), representing a combination of the flexibility of qPCR, with the high throughput gene specific targeting obtained from microarrays. The nanofluidic chip, or integrated fluidics circuits (IFCs), employed in this technique allow for an increase in sample throughput that is several orders of magnitude greater than conventional RT-qPCR techniques. These chips are made up of fluidic networks that automatically combine samples and assay master mixes. According to the Fluidigm Corporation (2015), there is huge savings in operational efficiency as well. For example, a gene expression study containing 2,000 samples and 48 genes can be completed using 42 Fluidigm 48 x 48 arrays requiring only 4,032 liquid transfer steps on chips, but would require 1,000 96-well plates and 192,000 steps using conventional RT-qPCR. They equate this to 4.5 days using

the Fluidigm system and 100 days by conventional RT-qPCR. Precision and accuracy also increase with less transfer steps. Furthermore, each reaction can be run for about \$0.13 per reaction, not including labor, and replicates can be omitted due to the accuracy of microfluidic distribution of samples and reagents, in lieu of manual pipetting (Dr. Mark Band, personal communication). This type of analysis could be particularly useful for large scale targeted gene expression in cultured fish.

Channel catfish, *Ictalurid punctatus*, are an important aquaculture species in the United States and China. In the U.S., channel catfish food fish production is estimated to be over 143,000 MT (USDA, 2016) and represents the nation's largest finfish aquaculture industry. Production of channel catfish in China may be even higher, estimated around 150,000 MT (China Fishery Bureau, 2009). Even given its economic importance, research tools, such as a published genome or well annotated EST libraries, do not yet exist for this species. As such, the development of targeted assays for measuring gene expression requires the identification of species specific gene sequence for primer development followed by primer validation and optimization. Validation of multiple primer sets for use with the Fluidigm quantitative PCR array could provide powerful assessments of the effects of alternative dietary formulations, as well as many other situations, on physiological pathways affecting production parameters in a catfish.

Because of the importance of feeds and feeding in aquaculture, appetite regulation is one such pathway of interest. As such, the following genes were chosen to evaluate changes in gene expression associated with appetite. Cocaine and amphetamine regulated transcript (CART) is an anorectic hypothalamic peptide which has been shown to inhibit feed intake in mammals (Stanley et al., 2001) and fish (Volkoff and Peter, 2000). Cholecystokinin A and B (CCKa and CCKb, respectively) are represent two CCK genes identified in channel catfish and expressed in the gastrointestinal tract and brain, which have many physiological roles but primarily function as a satiety signals (Volkoff et al., 2005). Ghrelin (GHRL) is a peptide that is highly expressed in the stomach, and moderately expressed in the brain of

fish, acting as both a growth hormone secretagogue and stimulator of feed intake (Kaiya et al., 2003a; Kaiya et al., 2003b; Unniappan et al., 2002; Unniappan et al., 2004). Two ghrelin receptor genes have been identified in channel catfish (GHSR1 and GHSR2) and are predominantly expressed in the pituitary (Small et al., 2009). The melanocortin 4 receptor is a protein that is likely involved in the circadian rhythm of feeding in fish and down-regulates feeding behavior (Jangprai et al., 2011). Neuropeptide Y (NPY) is a member of the NPY family of peptides, and is involved in the regulation of feed intake in fish, where it upregulates feeding behavior (Narnaware et al., 2000). Peptide YY (PYY), another member of the neuropeptide Y family, is primarily expressed in the gut endocrine cells (Cerdeira-Reverter et al., 2000), but has also been detected in the central nervous system in teleost species (Soderberg et al., 1994), where it acts as an anorexigenic factor (Gonzalez and Unniappan, 2010). Pro-opiomelanocortin (POMC), which is expressed in the arcuate nucleus (Cerdeira-Reverter et al., 2003), is a precursor molecule for several molecules, which are heavily involved in the regulation of energy metabolism and food intake in fish (Volkoff et al., 2005). Vasoactive intestinal polypeptide receptor (VIPr) binds to vasoactive intestinal polypeptide (VIP), where upon binding it acts to induce smooth muscle relaxation and inhibit gastric acid secretion, among other actions (Chow 1997). Each of these genes plays an important role in appetite regulation and as such, energetics, in fish.

Growth is closely related to appetite, and is often partially regulated by some of the same genes. In addition to the genes listed above, growth regulation can be evaluated by changes in expression of the following genes. Insulin-like growth factor 1 (IGF1) is primarily expressed in the liver, and its release is stimulated by growth hormone (GH) (Anderson et al., 2004). Insulin-like growth factor 1 is a mitogen and involved in cell differentiation and proliferation. Insulin-like growth factor 2 (IGF2) is also highly expressed in the liver, as well as muscle of many fish, playing roles in embryonic development and adult growth (Radaelli et al., 2003). Growth hormone (GH) stimulates growth and cell regeneration in a variety of species, in part by stimulating the liver to produce IGF1, which enacts many of GH's growth-

promoting effects (Anderson et al., 2004). Growth hormone works through specific cell membrane receptors (GHR1 and GHR2) which trigger a phosphorylation cascade, resulting in its physiological effects (Saera-Vila et al., 2005). These receptors are located primarily in the liver, adipose tissue, and skeletal muscle (Saera-Vila et al., 2005). Intuitively, growth-hormone-releasing-hormone (GHRH), a hypothalamic hormone, is partially responsible for stimulating release of growth hormone from the pituitary (Volkoff et al., 2005). In addition to regulation of GH release by GHRH, GH release is also regulated by hypothalamic pituitary adenylate cyclase-activating peptide (PACAP) (Volkoff et al., 2005; Gahete et al., 2009), and the gut peptide ghrelin (GHL) (Unniappan and Peter, 2005). The release of GH is inhibited by the hypothalamic hormone, somatostatin (SST) (Peng and Peter, 1997), of which there are two forms in channel catfish, SST-14 and SST-22 (Dixon and Andrews, 1985), and whose receptors, SSRs, are widely expressed in the brain (Lin et al., 2000).

Metabolism is closely correlated with appetite and growth, and like many physiological processes is regulated by a number of genes. Glucose-6-phosphate dehydrogenase (G6PD) is a cytosolic enzyme involved in the pentose-phosphate pathway leading to production of NADPH as an energy source (Criss and McKerns, 1968). Glucose-6-phosphatase (G6Pase) is a hepatic enzyme that plays a role in blood glucose homeostasis by causing hydrolysis of glucose-6-phosphate to glucose and phosphate, allowing the stored glucose in the liver to be released in to the blood (Enes et al., 2009). Carnitine palmitoyltransferase 1A (CPT1A) is an enzyme which plays a role in fatty acid oxidation by bringing fatty acids into the mitochondria for β -oxidation (Tang et al., 2013). Fatty acid synthase (FAS) is an enzyme involved in lipogenesis, know to catalyze the conversion of acetyl-CoA and malonyl-CoA to the saturated fatty acid palmitate (Tang et al., 2013). Hexokinase (HK) is an enzyme that catalyzes the conversion of glucose to glucose-6-phosphate (Wilson, 2003). Trypsin (TR) is a serine protease that is involved in protein catabolism by hydrolyzing peptide bonds (Villalba-Villalba et al., 2013). Pepsinogens, such as pepsinogen A (PEPA) and pepsinogen C (PEPC), are inactive precursors to pepsin that are released by the

chief cells of the stomach, where hydrochloric acid (HCl) triggers the activation of pepsinogen by conversion to pepsin (Feng et al., 2008). Alpha-amylase (α -amylase) is an enzyme responsible for hydrolysis of starch and glycogen to primarily yield maltose (Froystad et al., 2006). Glucagon receptor (GCGr) is a protein that is activated by glucagon binding, and is the mechanism by which glucagon's effects to raise the concentration of glucose in the bloodstream are mediated (Irwin, 2014; Moon, 1998). Glucocorticoid receptors, GR1 and GR2 are the receptors through which the effects of corticosteroids are mediated (O'Connora et al., 2013). Insulin receptors, such as IRa and IRb, are transmembrane glycoproteins that bind insulin outside the cell, and initiate biological activities inside the cell (Caruso and Sheridan, 2011).

In addition to genes involved in appetite, growth and metabolism, a suite of genes were also chosen to evaluate the inflammatory response, as studying their expression in response to alternative dietary formulations, particularly those high in plant feedstuffs, will increase our knowledge of how dietary changes can induce intestinal enteritis. Interleukin-1 beta (IL1b) is an endocrine-signaling molecule produced in multiple cell types, which is involved in the activation of T cells, B cells, of cytotoxic activity in macrophages and natural killer (NK) cells, and also causes secretion of antibodies (Kono et al., 2002). Interleukin-8 (IL-8) is a chemokine that is produced by many cell types, including macrophages, that plays a role in inflammation by recruiting and mediating neutrophils, and inducing healing of wounds through promoting angiogenesis (Li and Yao, 2013). Suppressor of cytokine signaling 1 (SOCS1) and suppressor of cytokine signaling 3 (SOCS3) are proteins involved in inflammation, which play a role in the negative feedback loop by reducing cytokine signaling. Transforming growth factor beta (TGFb) is a protein that has primarily suppressive immunoregulatory actions, including down-regulation of MHC expression, cytokine production, B- and T-cell proliferation, and NK and macrophage activation (Harms et al., 2000).

The objective of this study was to develop and validate targeted PCR primers and a high throughput, multitissue microfluidic array for assessing changes in gene expression associated with channel catfish appetite, growth, metabolism, and intestinal health for use in evaluating regulatory pathways associated with the nutritional physiology of channel catfish, while also providing a cost-effective, dynamic, gene expression platform for use with other cultured fishes.

2. Methods

2.1 Tissue Collection and Storage

The following experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Southern Illinois University Carbondale (Protocol number: 14-052). For initial PCR primer validation, one fish (~100 g) was rapidly caught and euthanized using a sodium bicarbonate-buffered 250 mg L⁻¹ solution of tricaine methanesulfonate (MS-222, Western Chemical, Inc., Ferndale, WA, USA) until cessation of opercular movement (AVMA, 2013). Immediately following euthanasia, hypothalamus, pituitary, liver, and proximal intestine were excised. Samples were immediately placed into RNase-free microcentrifuge tubes containing 1 mL TRI REAGENT®RT (Molecular Research Center, Inc., Cincinnati, OH, USA), flash frozen in liquid nitrogen, and stored at -80°C until later RNA isolation for analysis of target gene expression.

2.2 RNA Isolation and cDNA Conversion

Total RNA was isolated according to the manufacturer's protocol for TRI REAGENT®RT (Molecular Research Center, Inc., Cincinnati, OH, USA), converted to cDNA, and analyzed for target gene expression in each of the tissue samples. Total RNA concentration and purity were assessed by measuring the absorbance at 260nm and 280nm on a Nanodrop™ 2000 spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA), and samples were spot-checked for quality using an Experion™ Automated Electrophoresis System (Bio-Rad Laboratories, Inc., Berkeley, CA, USA). One

microgram of total RNA was used to develop single-stranded cDNA, using a High-Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Life Technologies, Carlsbad, CA, USA).

2.3 Primer Design and Sequence Verification

A complete list of oligonucleotide primers for real-time qRT-PCR are presented in Table 1 with full names and source. Potential reference genes (18S, EFA, GAPDH, TUBA) were selected according to Small et al. (2008). Primers were designed from sequences published in GenBank (<http://www.ncbi.nlm.nih.gov/>) using the Beacon Designer 7 program (PREMIER Biosoft, Palo Alto, CA, USA). Optimal primer pairs were selected based on primer length, T_m, target amplicon length, and incidence of secondary structures; and were evaluated based on PCR-product quality and lengths after electrophoresis on a 1.4% agarose gel.

Since MCR4, TGF- β 2, and FAS products were designed from sequences outside of the genus *Ictaluridae*, the QIAquick Gel Extraction Kit and protocol (Qiagen, Venlo, Netherlands) were used to extract and purify MCR4, TGF- β 2, and FAS products, and these products were then cloned and transformed using the TOPO® Cloning Reaction Kit (Life Technologies, Carlsbad, CA, USA) and the One Shot® chemical transformation protocol (Life Technologies, Carlsbad, CA, USA), following standard manufacturer's protocol. Plasmid DNA was isolated using the GeneJET Plasmid Miniprep Kit (Life Technologies, Carlsbad, CA, USA). A sequencing reaction was performed on amplicons for MCR4, TGF- β 2, and FAS using plasmid-specific primers and the BigDye® Terminator Kit (Life Technologies, Carlsbad, CA, USA), following standard manufacturer's protocol. Sequencing reactions consisted of 1 μ l BigDye v. 3.1, 5 μ l 5X Sequencing Buffer, 1 μ l primer (3.2 μ M), 1 μ l plasmid DNA, and 7 μ l H₂O. Sequencing reaction products were then cleaned using Sephadex columns (General Electric Healthcare, Little Chalfont, Buckinghamshire, UK), and sequenced using an ABI 3130 XL genetic analyzer (Applied Biosystems, Foster City, CA, USA). Sequences were then determined using SEQUENCHER 4.6 software (Gene Codes Corporation, Ann Arbor, MI, USA). Constructed (query) sequences (Table 2) were then aligned against

those in a select target database using the Basic Local Alignment Search Tool (BLASTn) program provided by the National Center for Biotechnology Information, to verify the percentage of similarity with the sequences of origin.

2.4 Conventional RT-qPCR Primer Validation

Targeted gene primers (Table 1) were initially validated on individual tissues from the one fish (described above) via conventional RT-PCR using the SsoFast™ EvaGreen® Supermix protocol (BioRad®, Hercules, CA, USA). Each 10 µl reaction consisted of 5µl SsoFast EvaGreen supermix, 0.5 µl (10 µM) of each primer, 3 µl of RNase/DNase-free H₂O, and 1 µl of cDNA template. The reactions were loaded into a 96-well plate, and qRT-PCR was performed on an CFX96 Real-Time System (BioRad®), using a thermocycling protocol consisting of an enzyme activation step at 95°C for 30 s, followed by 40 cycles of denaturation (98°C for 5 s) and annealing/extension (65°C for 5 s), and finally, melt curve analysis was conducted by cycling from 65°C to 95°C by 0.5°C increments for 5 s per step. The melt curve was used to verify that there was no amplification of non-target genes. PCR amplification efficiency was later determined on pooled samples analyzed with the Fluidigm system as described below.

2.5 Tissue collection and Fluidigm validation

Nine channel catfish of approximately 100 g each were euthanized using a sodium bicarbonate-buffered, 250 mg L⁻¹ overdose solution of MS-222 for tissue collection. Fish remained in the solution for 10-min after the cessation of opercular movement (American Veterinary Medical Association; AVMA 2007). Immediately following euthanasia, hypothalamus with intact pituitary, liver, stomach, and intestine were quickly removed from each of the individual channel catfish ($n = 9$ channel catfish x 4 tissue groups = 36 SAMPLES). Hypothalamus and pituitary were processed together as a combined tissue sample. Samples were placed in RNase-free microcentrifuge tubes containing 1 mL TRI Reagent

RT[®] (Molecular Research Center, Cincinnati, OH, USA), flash frozen in liquid nitrogen, and stored at -80°C. RNA isolation and cDNA conversion were performed as previously described.

A pre-amplification step was conducted prior to gene expression according to the manufacturer's protocol (Fluidigm Corporation, South San Francisco, CA, USA). In a PCR plate, 3.75 µL of Pre-Mix was aliquoted for each sample, and 1.25 µL of 100 ng µL⁻¹ cDNA were added to each well containing premix, resulting in a pre-amplification reaction volume of 5 µL per well. The reactions were mixed briefly by vortexing, centrifuged, and placed in a thermal cycler. The thermocycling protocol consisted of the following steps: a hold step at 95°C for 2 min, a denaturation step of 10 cycles at 95°C for 15 s, an annealing/extension step of 10 cycles at 60°C for 4 min, and a final holding temperature of 4°C.

Exonuclease I was diluted to 4 U/µL according to the manufacturer's protocol (Fluidigm Corporation, South San Francisco, CA, USA), and 2 µL of diluted Exonuclease I was added to each pre-amplification reaction. Reactions were then vortexed, centrifuged, and placed in a thermal cycler with the following program: Digestion step at 37°C for 30 min, and inactivation step at 80°C for 15 min, and a final hold temperature of 4°C. The final PreAmp and Exo I products were then diluted in 18 µL of TE Buffer to create a 5-fold dilution, and stored at -20°C until later assayed.

Sample Pre-Mix was prepared by the combination of 180.0 µL 2X SsoFast EvaGreen Supermix (BioRad[®]) with Low ROX and 18.0 µL of 20X DNA Binding Dye Sample Loading Reagent. 3.3 µL of Pre-Mix was combined with 2.7 µL of PreAmp and Exo I-treated sample, vortexed for 20 s, centrifuged for 30 s, and stored at 4°C until loading into the chip. An assay mix was prepared by the combination of 3.0 µL 2X Assay Loading Reagent, 2.7 µL 1X DNA Suspension Buffer and 0.30 µL 100 µM each mixed forward and reverse primers. The assay mix was then vortexed for 20 s and centrifuged for 30 s.

Control line fluid was injected into each accumulator on the chip, and the chip was primed on the IFC Controller MX via the Prime (113x) script. Following priming, the chip was removed from the controller, 5 μ L of each assay and 5 μ L of each sample were pipetted into their respective inlets on the chip, the chip was returned to the IFC Controller, and the Load Mix (113x) script was run to load the samples and assays into the chip. After the Load Mix script was completed, the loaded chip was then run on the chip reader using the 96 x 96 chip thermal cycling parameters. Ct values, quality threshold scores (QS) and efficiencies (E) were calculated from a pooled sample of all four tissue types from all nine fish using the Fluidigm Real-Time PCR Analysis Software. PCR efficiency was determined by serial dilution (1:1, 1:9, 1:17, 1:81, 1:243) of the pooled sample. Quality threshold scores were calculated in the Fluidigm Real-Time PCR analysis software as a means of assessing the amplification curve of each gene by comparing each amplification curve to an ideal exponential curve. Quality threshold scores range from 0 to 1, where 0 is a flat line and 1 is a perfect sigmoidal curve.

2.6 Demonstration of high throughput targeted gene expression

For the purpose of demonstrating the high throughput nature of the Fluidigm system, relative to conventional RT-qPCR, the array developed and validated in this study was applied to samples collected from a larger study in which channel catfish were fed four diets formulated to meet channel catfish requirements, but having different combinations of dietary protein ingredients (Schroeter, unpublished). For demonstration purposes, these diets have been labeled 1-4. All data were transformed by square root to reduce the influence of very large and very small data points. Replicate diet samples were then clustered using the UPGMA method, conducted on a S17 Bray-Curtis similarity resemblance matrix. Gene variables were then further transformed by standardization, (shifting the population mean to “0” and the standard deviation to “1”) to ensure that all variables were “shaded” at roughly the same level of expression. After standardizing, genes were clustered using UPGMA method on a S17 Bray-Curtis

similarity matrix. Following this, a shade plot/heat map was generated, all conducted on the statistical software package Primer v7 (Primer-E Ltd, Plymouth, UK).

3. Results

When using the Basic Local Alignment Search Tool (BLASTn) on the NCBI Website, sequenced amplicons generated from primers designed from non-*Ictaluridae* sequences came back with high sequence similarity to their intended target gene sequences: MCr4, TGF-B2, and FAS (Table 2). Fluidigm validation of the genes in Table 1 resulted in the majority of the genes having sufficient Ct levels (5 – 23), efficiencies, and quality threshold to be considered reliable (Table 3). Thresholds for efficiency were set at $R^2 \geq 0.9$ and for QS at 0.65 using a baseline correction and auto detectors for the Ct threshold method. Genes which failed to meet these criteria were 18S, POMC, VIP, SSR, PEPC, CCKb, TGF-B2, and MCR4 (Table 3).

Three heat maps were generated using samples collected from a larger study in which channel catfish were fed four diets formulated to meet channel catfish requirements, but having different combinations of dietary protein ingredients (Schroeter, unpublished). Tissue specific gene expression is presented to demonstrate the potential volume of gene expression data generated using a single 48 x 48 array in feed trial. Clustering demonstrates similarities in changes in gene expression among certain pathway related genes and across dietary treatments, demonstrating the power of targeted, higher throughput gene expression.

4. Discussion

The regulation of appetite, growth, metabolism, and intestinal health in channel catfish is controlled by the expression of many genes and gene products in a variety of overlapping physiological pathways. Expression of these genes, which is dependent on many factors, can generally be described

as the result of changes in the nature and number of interactions between organ systems or axes with the environment and diet, which as a whole influence the level of DNA transcription. In this experiment, a gene-targeted channel catfish specific microfluidic PCR array was validated for assessing the changes in gene expression associated with appetite, growth, metabolism, and intestinal health. While specifically validated for evaluating physiological pathways in channel catfish research, this array can be dynamic in that it allows for easy target gene modification through the use of different gene specific primers. In order for the expression of genes to be assessed relative to changes in study parameters, they must first be assessed relative to an internal reference. Four genes, 18S ribosomal RNA (18S), alpha tubulin (TUBA), elongation factor 1 α (EFA), and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were chosen as potential reference genes based upon previously published results from our laboratory (Small et al., 2008). Of these four potential reference genes, alpha tubulin (TUBA) had the highest quality threshold score (0.942 ± 0.0004 ; Table 3), and came up after about 11 cycles. Elongation-factor 1 α came up at around 16 cycles, with a lower quality threshold score (0.80 ± 0.006 ; Table 3); and GAPDH came up at around cycle 10.49, and also had a high quality threshold score (0.938 ± 0.006 ; Table 3). 18S ribosomal RNA (18S) came up very early, at 4 cycles or less. Of these potential reference genes, TUBA would be the best reference gene for the tissues tested, assuming no effect of treatment on TUBA expression is validated.

The Ct (cycle threshold) is the number of PCR cycles required for the fluorescence level to come above the threshold level and be detected above background fluorescence (Life Technologies Corporation 2012). This value is inversely related to the amount of target gene in the sample. The pre-amplification step, which increases the number of copies of a target gene in a sample to a detectable level, is recommended for all cDNA samples, due to the small volumes sampled by the Fluidigm IFCs. While this step corrects for genes of low incidence, it can over-amplify and cause genes to be so concentrated that they express in the very early cycles of PCR, making it impossible to calculate an efficiency score. In

addition to 18S, POMC was also over-amplified, with undiluted samples yielding a low quality threshold score and coming up very early in the cycling period, just after the first cycle. However, a 1:3 dilution yielded good efficiencies. Therefore, if using a pre-amplification step when measuring hypothalamic POMC, it would be appropriate to then dilute samples three-fold prior to pre-amplification.

A few genes in this study failed to break the cycle threshold, meaning the amount of target gene expression in the sample was nonexistent or too low to be detected. It should be noted that general cut-off for reliable quantification in this assay system is 23 cycles. Based on individual sample cycle threshold scores (Table 3), SSR and VIP came up very late in the cycle period, after cycles 28 and 27, respectively. For this reason, it appears SSR and VIP expression was too low to quantify. In addition to SSR and VIP, CCKb also came up relatively late in the cycling period, at an average of about 24 cycles. Therefore, CCKb wasn't expressed at a high enough level to achieve a Ct which allowed for calculation of an efficiency score. Peterson et al. (2012) also found that Ct levels were low for CCKb when measured in the intestine, brain, and hypothalamus of channel catfish. Transforming-growth factor-beta 2 (TGFB2) didn't come up at all, suggesting the transcript may be too low for detection or not expressed at all in the tissues examined.

A dilution series (1:1, 1:9, 1:17, 1:81, 1:243) was used in the present study to calculate PCR efficiency (Life Technologies Corporation 2012), which is the R^2 for the $\Delta Ct/\Delta$ concentration regression relationship. The dilution series was made up of pooled cDNA samples from all the tissues evaluated, so that the efficiency of the genes could be measured across all tissues at one time, and so space was maximized on the plate. In the case of PEPC, threshold cycle (Ct) levels in individual undiluted samples were acceptable, coming up around 12 cycles, with an excellent quality threshold score (1.0 ± 0.00). However, in the averaged pooled sample Ct scores (Table 3), PEPC didn't come up until the end of cycle 23, and PCR efficiency could not be calculated. Serial dilution of individual samples may be necessary for genes over-diluted in pooled samples, such as PEPC, when using this methodology.

The melt curve analysis tracks the change in fluorescence when double-stranded DNA with incorporated dye molecules melts into single-stranded DNA due to a steady increase in temperature (Life Technologies Corporation 2012). This analysis is used to ensure reaction specificity, as a single peak in the melt curve indicates that only one product was amplified (Life Technologies Corporation 2012). Melanocortin Receptor 4 (MCR4)'s melt curve showed an indistinct melt curve with possible amplification of multiple products, as indicated by two low-level peaks within the curve. Likely for this reason, Ct levels of MCR4 did not increase proportionally with serial dilution. In initial conventional real-time PCR validation of primers using the CFX96 Real-Time System (BioRad®), the MCR4 melt curve yielded only one amplification peak, so it is unclear why problems occurred using the microfluidic array.

While other methods of large-scale gene expression analysis are available and widely accepted in the scientific world, the use of microfluidic dynamic arrays provide a flexible, efficient and cost-effective option for assessing the expression of several targeted genes in a variety of tissues, as demonstrated by the heat maps of gene expression presented in Figure 1. Stated earlier, the average cost per reaction of a traditional 384-well qPCR plate is \$0.40, with having to run reactions in triplicate to account for technical errors. Having to account for these potential inaccuracies reduces the number of wells available for different reactions, and increases the amount of labor required, which ultimately drives the overall cost up. Another option for large-scale gene expression analysis, RNA-seq, provides an extensive amount of gene expression information, the majority of which may be unnecessary for the targeted analysis of specific pathways, and requires complex analysis. This, coupled with its high cost, makes it an inefficient option for assessing the effects of alternative dietary formulations on the molecular regulation of targeted genes known to be involved in appetite, growth, metabolism, and intestinal inflammation.

The Fluidigm qPCR system can be operated while utilizing either a 12x12, 48x48, or 96x96 microfluidic array, resulting in a total of 144, 2,304, or 9,216 reactions per plate. The running of

replicate wells is unnecessary with the Fluidigm qPCR System because of the microfluidic distribution of samples and reagents, which eliminates the potential for human pipetting error. The Fluidigm qPCR system also employs software that allows for the timely calculation of quality threshold scores, efficiencies, cycle threshold scores, amplification curves, and melt curves, all of which are imperative to the development of an assay that is of inherent value to the gene expression studies.

In an era of functional feeds and pre- and probiotics, it is imperative to understand the mechanisms driving physiological and performance differences associated with feeds and feedstuffs. The development and validation of relatively large scale assays to target several genes, tissues, and pathways, such as the multi-tissue microfluidic array validated in this study, should have many uses in catfish research including assessing changes in gene expression associated with channel catfish appetite, growth, metabolism, and intestinal health. For those genes that were not successfully validated here, new primer sets can be developed and validated or primers for different genes selected to replace them. Additionally, 54 more genes could be added on a 96 x 96 array for a more comprehensive understanding of regulatory pathways in response to changes in catfish nutrition, as well as management, genetics, and environment. While many nutrition studies analyze the effects of changing dietary ingredients on production parameters, which provides a basic understanding of the effects of such ingredients on the overall performance of the animal, elucidating the specific mechanisms of gene regulation involved in various physiological pathways provides a more thorough explanation of the effects of dietary changes. The platform validated in this manuscript, with species specific primers, provides a “plug-and-play” assay for this type of research in channel catfish and demonstrates how microfluidic arrays can be cost effective in providing increased throughput over classical techniques used to measure gene expression.

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Table 1. Oligoprimers for measuring mRNA expression in channel catfish tissues using a Fluidigm qPCR microfluidic dynamic array.

Gene	Abbreviation	Primers	Sequence	Accession No.	Source Organism
18 S ribosomal RNA	18S	Sense	GGATTGACAGATTGATAGC	AF021880.1	<i>I. punctatus</i>
		Antisense	TCGTTCGTTATCGGAATT		
Alpha-amylase	AMYA	Sense	GAAGCTCTTGACAGGAA	CK 421713.1	<i>I. punctatus</i>
		Antisense	CGGATTCTCGTCAGTGTT		
Cholecystokinin a	CCKa	Sense	GGAGTGCTGAGGAGTATGA	JQ028716.1	<i>I. punctatus</i>
		Antisense	CGTGGTTGGTTGATTGATTG		
Cholecystokinin b	CCKb	Sense	CATGAGGAGTAATCATATTC	JQ028715.1	<i>I. punctatus</i>
		Antisense	GATAAAAGATCGGGACTA		
Carnitine palmitoyl-transferase 1a	CPT1A	Sense	CTGGATGATGAGGAATAC	JT418607.1	<i>I. punctatus</i>
		Antisense	CACAATGACTTCAACTTC		

Elongation factor					
alpha	EFA	Sense	TCTACCTATCCTCCTCTT	DQ353797.1	<i>I. punctatus</i>
		Antisense	CTTCTTCTCAAACTCTT		
Fatty acid synthase					
	FAS	Sense	CTGCCTCCTCATTCTACTAC	JN579124.1	<i>Tachysurus</i>
		Antisense	TGATGTTGCCGTGGTATT		<i>fulvidraco</i>
Glyceraldehyde-3-phosphate					
dehydrogenase	GAPDH	Sense	GGACATGCTATCACAGTT	NM_001201199.1	<i>I. punctatus</i>
		Antisense	GATTCCACAACATACTCAG		
Glucagon receptor	GCGr	Sense	ATCAACTTCTTCATCTTCATA	JT 409353.1	<i>I. punctatus</i>
		Antisense	CTTGTAGTCGGAGTATCT		
Growth hormone	GH	Sense	CTGTGTCTCGGCAACTTA	AF267989.1	<i>I. punctatus</i>
		Antisense	GCCAGGAGCGTATGTAA		

Growth hormone					
releasing hormone	GHRH	Sense	GAGTGACGAACAGGAAGT	FJ882999.1	<i>I. punctatus</i>
		Antisense	AAGGTTCTTGGTCAAATATC		
Ghrelin	GHRL	Sense	AGGATGTTACTGGATGTC	AB196450.1	<i>I. punctatus</i>
		Antisense	CGGTTATGAATCAGGAATC		
Ghrelin receptor 1	GHSR1	Sense	TCTGGCGAGTGTATGTAG	NM_001200321.1	<i>I. punctatus</i>
		Antisense	AGTATCTGTATCCTTCAAGTCA		
Ghrelin receptor 2	GHSR2	Sense	TCGTCTCCTTAGTTCTGT	NM_001200322.1	<i>I. punctatus</i>
		Antisense	GCCATAATCCTGTACTTCTT		
Growth hormone					
receptor 1	GHR1	Sense	GTTACATTATGCCATCCAA	NM_001200214.1	<i>I. punctatus</i>
		Antisense	GACCATCACATCACAGTA		
Growth hormone				Small and Waldbieser,	
receptor 2	GHR2	Sense	ATGTCGTTCCAGTGAGTT	unpublished	<i>I. punctatus</i>

		Antisense	CCAGGTTCACTGAGGTTA		
Glucocorticoid receptor 1	GR1	Sense	ATGAGATCAGGATGTCGTA	Small and Quiniou, unpublished	<i>I. punctatus</i>
		Antisense	GTAGAATCGTTGCCAGTT		
Glucocorticoid receptor 2	GR2	Sense	ACCTCAATGTTCTGTCA	Small and Quiniou, unpublished	<i>I. punctatus</i>
		Antisense	TAGCCTCTCCTCTCACTT		
Glucose-6- phosphatase	G6Pase	Sense	TGTGGAGTTATCTTAGGTATCATT	FD 209754.1	<i>Catfish</i>
		Antisense	CTTCAGGCTGGTGTGTA		
Glucose-6-phosphate dehydrogenase	G6PD	Sense	CCTGAATAACCACCATAGA	CK 411979.1	<i>I. punctatus</i>
		Antisense	CACCGATGTCCATATCTT		
Hexokinase 1	HK1	Sense	ATGTCGTCAATCTCCTAA	FD 343899.1	<i>I. punctatus</i>

		Antisense	CATAAGCACAAGTCATCA		
Insulin-like growth					
factor 1	IGF1	Sense	AAACACCAAAGAAACCTA	NM_001200295.1	<i>I. punctatus</i>
		Antisense	TTACATCCGATAGTTCCT		
Insulin-like growth					
factor 2	IGF2	Sense	ACACAACAATGATGACTTCTTC	NM_001200946.1	<i>I. punctatus</i>
		Antisense	TGCCGAGACTTGAGATGA		
Interleukin 1- beta	IL1b	Sense	TGCTAATGTTGTAATCGCTCTG	NM_001200220.1	<i>I. punctatus</i>
		Antisense	AAGTTCCTGGTCGGTGAA		
Interleukin 8	IL8	Sense	CAATCTAATACCGCTTACACTT	AY145142.1	<i>I. punctatus</i>
		Antisense	CCTTCATTCCTTGCTTCAG		
Insulin receptor b	IRb	Sense	CGTGTTATGTTTCATCAGA	FD369653.1	<i>I. punctatus</i>
		Antisense	AACCTGTTCTTCAACTAC		

Insulin receptor a	IRa	Sense	TGTAATCCTCGGACACCAT	FD267929.1	<i>I. punctatus</i>
		Antisense	TGGCTTATCTCAACGCTAA		
Melanocortin					
receptor 4	MCR4	Sense	CACCTCATCCTCATGATCTCCT	BC 163227.1	<i>D. rerio</i>
		Antisense	TGACCGAGTTGCACATAATGA		
Neuropeptide Y	NPY	Sense	CATTACATCAACCTCATAACG	NM_001200087.1	<i>I. punctatus</i>
		Antisense	GTATCTCTGCCTCTCCAA		
Pituitary adenylate					
cyclase-activating					
polypeptide	PACAP	Sense	CAGACGGATTGTTAGATAGA	NM_001200093.1	<i>I. punctatus</i>
		Antisense	CCTCTTCTCCTCACCTA		
Pepsinogen A	PEPA	Sense	GGATGTCTTCTCAGTCTA	NM_001201015.1	<i>I. punctatus</i>
		Antisense	TAGTAGGAGGTGTCAATC		
Pepsinogen C	PEPC	Sense	CTCAGCAGGACAGTTACG	CK 425295.1	<i>I. punctatus</i>

		Antisense	ATGGTGAAGGTCAGAGTTG		
Proopiomelanocortin	POMC	Sense	GGAGGACCACTGAAGAACTC	AY174051.1	<i>I. punctatus</i>
		Antisense	TACTGCTGGCTACTAGAACA		
Peptide YY	PYY	Sense	TTATGCTCATTGCTGTCT	JT409147.1	<i>I. punctatus</i>
		Antisense	AATACTACACGGCTCTCA		
Suppressor of					
cytokine signaling 1	SOCS1	Sense	AATCTATGCTAGAGGAGAG	FD 032636.1	<i>I. punctatus</i>
		Antisense	CATTCTTGAGCTTGTGAT		
Suppressor cytokine					
signaling 3	SOCS3	Sense	GTACACCACTATATGAACTCA	NM_001201254.1	<i>I. punctatus</i>
		Antisense	CCCTCCTGTGAAGATGTA		
Somatostatin					
receptor	SSr	Sense	TTGTATGGCTGATATGTT	AF127777.1	<i>I. punctatus</i>
		Antisense	AGAGTTGTGTAGAAGATG		

Somatostatin-14	SST14	Sense	CCGTCTGATGCCAAACTG	NM_001200330.1	<i>I. punctatus</i>
		Antisense	GAGCGTGACCTGGTGAG		
Somatostatin-22	SST22	Sense	AGAGAGACTCACTCTTCCT	J00945.1	<i>I. punctatus</i>
		Antisense	GGCTTCTTCCACCTGAAC		
Transforming growth					
factor-beta2	TGFβ2	Sense	AGCGAGGAGGAGTATTAC	NM 194385.1	<i>D. rerio</i>
		Antisense	AAGATGACATTCTCTGAAGG		
Trypsin	TR	Sense	CTACAGTTGAGACATAGC	GU 588177.1	<i>Ictalurus furcatus</i>
		Antisense	AACAGAGCAGTTCATTAG		
Alpha-tubulin	TUBA	Sense	CACTGGTATGTAGGAGAG	GH642228	<i>I. punctatus</i>
		Antisense	CCTCAATGGAATCAACAC		
Vasoactive intestinal					
polypeptide receptor	VIPr	Sense	TGCGACAGAAGATCAACT	JT411823.1	<i>I. punctatus</i>

		Antisense	AAGAGCGGAATCAACAAC		
Vasoactive intestinal					
polypeptide	VIP	Sense	TATTCACAGACAACTACAGT	NM_001114553.2	<i>D. rerio</i>
		Antisense	CTTCTTGACTTCGCTTTC		

Table 2. Channel catfish amplicon sequence using oligo primers designed from species outside of the genus *Ictaluridae* and identity to source sequence.

Target	Sequence with primers (underlined)	Primer source		
		organism	Accession no.	Identity
MCR4	<u>TGACCGAGTTGCACATAATGAGAATCAGG</u>	<i>Danio rerio</i>	BC163227	83%
	TACATGTTGAAATGGGACATGAAGCAGAT			
	GCAGTACAGATTCTTTGGACAGGAGATCA			
	<u>TGAGGATGAGGTG</u>			
TGFB2	<u>AAGATGACATTCTCTGAAGGGTAGAAGG</u>	<i>D. rerio</i>	NM_194385	86%
	GCTGCTTGTCGATTTTGTGCACCTCCTTGG			
	<u>CGTAATACTCCTCCTCGCT</u>			
FAS	<u>CTGCCTCCTCATTCTACTACAAATTAAAGG</u>	<i>Pelteobagrus</i>	JN579124	94%
	CTGCTGACTGCTACATCCCATCTACCAA <u>AT</u>	<i>fulvidraco</i>		
	<u>ACCACGGCAACATCA</u>			

Table 3. Mean cycle threshold (Ct) values, PCR efficiency, and quality threshold scores (QS) following real-time PCR analysis channel catfish tissue using a Fluidigm qPCR microfluidic dynamic array.

Gene	Tissue ^a	CT	Efficiency	QS	Pass/Fail
18S	H/I/L/S	3.86±0.74	0	0.37±0.04	Fail
AMYA	I	15.38±0.58	0.9897	1.0±0.00	Pass
CART	H	16.02±0.26	0.9606	0.99±0.01	Pass
CCKa	H/I	13.60±0.36	0.9682	1.0±0.00	Pass
CCKb	H/I	>23	0	0.79±0.01	Fail
CPT1A	L	19.33±0.97	0.9781	0.95±0.02	Pass
EF1A	H/I/L/S/	16.27±1.00	0.9769	0.82±0.03	Pass
FAS	L	11.68±0.53	0.9992	0.99±0.00	Pass
G6P	L	10.26±1.64	0.9967	1.0±0.00	Pass
G6PD	L	17.57±0.34	0.9582	0.96±0.03	Pass
GAPDH	H/I/L/S	11.16±0.75	0.9928	0.94±0.02	Pass
GCR	L	18.47±1.80	0.9863	0.72±0.01	Pass
GH	H	5.65±.033	0.9978	1.0±0.00	Pass
GHSR1	H	16.91±0.07	0.9178	1.0±0.00	Pass
GHSR2	H	19.41±0.49	0.9891	0.91±0.05	Pass
GHR1	L	13.67±1.21	0.9689	1.0±0.00	Pass
GHR2	L	15.11±0.79	0.9862	0.99±0.01	Pass
GR1	L	14.63±1.15	0.9968	0.99±0.00	Pass
GR2	L	15.20±0.99	0.9907	0.98±0.01	Pass
GHRH	H	13.94±0.59	0.9745	1.0±0.00	Pass

GHRL	S	13.11±0.25	0.9764	0.95±0.03	Pass
HK	L	21.07±1.32	0.9857	0.99±0.00	Pass
IGF1	L	22.83±1.96	0.941	0.94±0.02	Pass
IGF2	L	14.29±1.56	0.9738	1.0±0.00	Pass
IL1B	I	15.98±0.96	0.9924	1.0±0.00	Pass
IL8	I	19.83±1.50	0.9523	0.99±0.01	Pass
IRa	L	13.53±0.71	0.9959	1.0±0.00	Pass
IRb	L	17.52±1.16	0.9307	0.90±0.02	Pass
MCR4	H	20.01±0.37	0	1.0±0.00	Fail
NPY	H	13.39±0.27	0.9731	0.99±0.01	Pass
PACAP	H	9.19±0.09	0.9958	0.96±0.04	Pass
PEPA	S	6.40±0.08	0.99	0.98±0.02	Pass
PEPC	S	>23	0.3484	1.0±0.00	Fail
POMC	H	1.35±1.35	0.998	0.04±0.04	Fail
PYY	H	14.65±0.18	0.9398	0.98±0.01	Pass
SOCS1	I	17.53±0.49	0.9691	0.96±0.01	Pass
SOCS3	I	14.93±0.47	0.9991	1.0±0.00	Pass
SS22	H	17.75±0.24	0.9936	1.0±0.00	Pass
SSR	H	>23	0	0.00±0.00	Fail
SS14	H	9.29±0.93	0.991	0.98±0.02	Pass
TGFB2	I	0.00±0.00	0	0.00±0.00	Fail
TR	I	10.59±0.95	0.9879	0.98±0.01	Pass
TUBA	H/I/L/S	12.24±0.35	0.9989	0.92±0.03	Pass
VIP	I	>23	0	0.08±0.08	Fail

VIPr	H	12.68±0.16	0.9843	1.0±0.00	Pass
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^aH = Pooled hypothalamus and pituitary; I = Intestine; L = Liver; S = Stomach

Figure Caption

Figure 1. Demonstration of the utility of the multi-tissue, microfluidic array validated for higher throughput, targeted, gene expression associated with channel catfish appetite, growth, metabolism, and intestinal health. Heat map results were generated using a single 48.48 array and are presented by tissue with key genes from the array for (A) Hypothalamus/Pituitary, (B) Stomach/Proximal Intestine, and (C) Liver. Data is part of a larger study in which channel catfish were fed four diets (Diet 1 – 4) formulated to meet channel catfish requirements, but having different combinations of dietary protein ingredients (Schroeter, unpublished).