Aquaculture 464 (2016) 213–221

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Short communication

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

Julie C. Schroeter [a], Brian C. Peterson [c], Brian C. Small [b,*]

[a] Center for Fisheries, Aquaculture, and Aquatic Sciences, Department of Animal Science, Food, and Nutrition, Southern Illinois University, Carbondale, IL 62901, USA
[b] Aquaculture Research Institute, Department of Fish and Wildlife Sciences, University of Idaho, Hagerman, ID 83332, USA
[c] Warmwater Aquaculture Research Unit, United States Department of Agriculture/Agricultural Research Service, Stoneville, MS, 38776, USA

ARTICLE INFO

Article history:
Received 28 January 2016
Received in revised form 23 June 2016
Accepted 24 June 2016
Available online 26 June 2016

Keywords:
Channel catfish
Gene expression
Array
Microfluidic

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 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.

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 9216 reactions on a single chip array (Spurgeon et al., 2008), representing a combination of the flexibility of qPCR, with the high

* Corresponding author.
E-mail address: bcsmall@uidaho.edu (B.C. Small).

http://dx.doi.org/10.1016/j.aquaculture.2016.06.036
0044-8486/

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 2000 samples and 48 genes can be completed using 42 Fluidigm 48 × 48 arrays requiring only 4032 liquid transfer steps on chips, but would require 1000 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 catfish.

Because of the importance of feeds and feeding in aquaculture, following genes were chosen to evaluate changes in gene expression associated with appetite regulation. 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) 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 (Cerda-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 (Cerda-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 often correlated to appetite and at least partially regulated by many 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., 2008). 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 (GHRL) (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, known 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