NUTRITIONAL CONDITIONING DURING LARVAL DEVELOPMENT TO IMPROVE PRODUCTION AND FEED EFFICIENCY AND ESTABLISHMENT OF BENEFICIAL GUT FLORA IN TILAPIA CULTURE

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Sustainable Feed Technology and Nutrient Input Systems/Experiment/16SFT02NC

Collaborating Institutions and Lead Investigators

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Objectives

1. Evaluate the effectiveness of nutritional conditioning on tilapia feed efficiency and production.
2. Identify key factors (gene networks) associated with improved nutrient utilization in response to larval nutritional conditioning in tilapia.
3. Characterize changes in gut microbial communities in response to nutritional conditioning and identify those that may be associated with improved nutrient absorption in fish.

Significance

Global production of farmed Nile tilapia (*Oreochromis niloticus*) has increased exponentially since 1985, with over 2.4 million metric tons consumed in 2010 (FAO, 2013). In Bangladesh, Nile tilapia comprises a significant source of per capita caloric and protein intake, with production increasing 30-fold from 1999-2007 (Hussain, 2009). Total production is second only to carps (Apu, 2014). Feed ingredients typically include fishmeal, other animal meal or byproducts and plant material (soybean products) as primary sources of protein for fish growth. High quality protein is critical for animal growth and health and requirements vary depending on age and size of the fish. Commercial feeds for pond growout contain ~30% protein while juvenile tilapia may require up to 40% protein for proper growth (El-Sayed, 2006). Work in mammalian models demonstrates that nutrient contributions early in pre- or postnatal development influence growth and immune function later in life (Lucas, 1998). This process, known as conditioning (also programming or imprinting), when established at critical periods in the animal's development, lead to life-long changes in the function of key elements of an organism's physiology. By altering early nutritional components, nutritional conditioning can result in more efficient uptake and utilization of nutrients from the diet thus increasing growth and health parameters in the organism later in life. The process of nutritional conditioning is also observed in poultry. Nutritional conditioning of energy and minerals can influence uptake and utilization in chickens (Angel and Ashwell, 2010; Ferket, 2013). Broiler chickens had increased retention of phosphorous from their diets following feeding of a phosphorous-deficient diet for the first 90 hours post-hatch (Angel and Ashwell, 2010).

A better understanding of how finfish acquire and utilize nutrient inputs is requisite for future improvements in aquaculture production efficiency because feed constitutes anywhere from 50% to 80% of total variable production costs. Nothing is known about the effectiveness of applying nutritional conditioning to tilapia culture, this despite strong evidence that the phenomenon is likely to occur across all vertebrates, including fish. A few studies have looked at energy uptake and utilization following conditioning in rainbow trout (*Oncorhynchus mykiss*) and the European sea bass (*Dicentrarchus labrax*), both carnivorous fishes. High dietary glucose diets fed to rainbow trout juveniles for a short period showed there was long-term modifications to carbohydrate digestion (Geurden et al., 2007). European sea bass juveniles fed a HUFA-deficient diet initially were able to metabolize lipids more efficiently than

those fed a high HUFA diet (Vagner et al., 2007). Here we will evaluate if nutritional conditioning can be applied to tilapia, and assess if reductions in the amount of protein in the diet of larval tilapia can subsequently lead to improved feed or protein efficiency during later growout of fishes.

Currently, the underlying mechanisms explaining how larval nutritional conditioning strategies can potentially achieve equivalent production yields with less protein in the feed is poorly understood. Some evidence suggests that during periods of fasting, nutrient uptake efficiency in the intestine is intrinsically enhanced, leading to a more-efficient uptake of nutrients at the next feeding period (Ali et al., 2003; Picha et al., 2006). Thus, decreasing the amount of select nutrients early in the life of the fish may increase the uptake and utilization of those nutrients during the growout phase of fish culture. Using a transcriptomic approach, we will evaluate the suite of genes in the intestine that are differentially expressed in fish that are fed a protein deficient diet early in larval development versus those who received a diet containing traditional levels of protein. This analysis will further our understanding of how protein uptake efficiency may be achieved for greater optimization of feeding protocols in the future.

Nutritional conditioning may also affect the microbial colonization of the gut. The establishment of beneficial microflora can affect nutrient availability and gut health (Marques et al., 2010). The emerging field of metagenomics has substantial implications for sustainable aquaculture, as diet, feeding strategy, and other environmental factors strongly influence the diversity and constitutive abundance of intestinal microbiota in both humans and fish (Al-Harbi and Uddin, 2004, 2005; De Filippo et al., 2010; Heikkinen et al., 2006). In aquacultured finfish, new research has shown that probiotic maintenance of beneficial gut flora can promote growth, greater nutrient availability, and better stock health (Nayak, 2010; Welker and Lim, 2011). In our previous AquaFish Innovation Lab studies, we found that tilapia fed on alternate days in fertilized ponds produced similar growth and survival, but improved feed efficiency by 100%, compared with fish fed daily. Fish also had a higher diversity of microbes in their intestines that may benefit nutrient processing and uptake. Here we will further build on this work to determine whether tilapia intestinal microbial composition and diversity varies with nutritional conditioning and identify key microbes that may be associated with increased protein uptake and utilization. Together, the identification of beneficial microbes that improve protein uptake and nutrient utilization may benefit current research into the application of probiotic supplements in feed for further enhancement of feed efficiency in fish.

This investigation will target a method to improve production efficiency of tilapia, namely through reducing the amount and cost of feed needed to produce a kg of fish. Since >50% of the costs associated with feed is protein, practical approaches that improve its utilization has tremendous application to global tilapia production (El-Sayed, 2006). Previously, our research showed that Nile tilapia and milkfish (seacages and ponds; *Chanos chanos*) can be grown to market size in monoculture with significant cost savings through implementation of alternate-day feeding versus daily feeding (50% feed reduction; Bolivar et al., 2006; Borski et al., 2011; De Jesus-Ayson and Borski, 2012). Our work over the past year also indicates similar responses with tilapia grown in ponds in Bangladesh. We will determine the proper length of time for nutritional conditioning of Nile tilapia larvae that will lead to enhanced feed efficiency with minimal impact on growth and survival of the fish. If successful, this refined strategy will be tested in tilapia pond culture in Bangladesh using the alternate day feeding strategy, an approach that could provide substantial costs savings for tilapia farmers in Bangladesh while also mitigating negative environmental impacts associated with excessive nutrient loading.

Quantified Anticipated Benefits

1. A new method of nutritional conditioning in rearing post yolk-sac fry will be developed that improves nutrient uptake and utilization and production efficiency of tilapia in culture.
2. The technology has potential to increase household income of farmers in Bangladesh, thereby contributing to greater food security. The work could also be applied to improving returns for farmers in the US who predominantly culture tilapia in recirculating aquaculture systems.

3. Improvement of environmental water quality and fish stock health through enhanced nutrient utilization in tilapia pond culture could be realized through use of nutritional conditioning and reduced nutrient inputs.
4. We anticipate that a 20% or greater savings in feed costs, translating to ~10-15 % increase in on-farm profitability might occur with nutritional conditioning.
5. Key genes responsive to nutritional conditioning and associated with improved protein utilization will be identified for future optimization of fry feeding protocols or for selective breeding of tilapia.
6. Beneficial gut microflora associated with improved nutrient absorption may be identified that could be further developed as probiotics.
7. One graduate student and one postdoctoral fellow will receive training on sustainable farming practices and genomic technologies enabling aquaculture research.
8. Development of nutritional imprinting protocols that require lower protein diets for tilapia growout, will enhance tilapia production efficiency not only within the host country, but also may significantly benefit both the global and domestic (US) tilapia industry.

Research Design and Activity Plan

Location

Initial investigations will be performed the Grinnell's Fish Laboratory, North Carolina State University and pond investigations will be performed at the Fisheries Field Laboratory, Bangladesh Agricultural University providing the opportunity to extend advanced genomic capabilities to collaborating institutions within the host country (BAU).

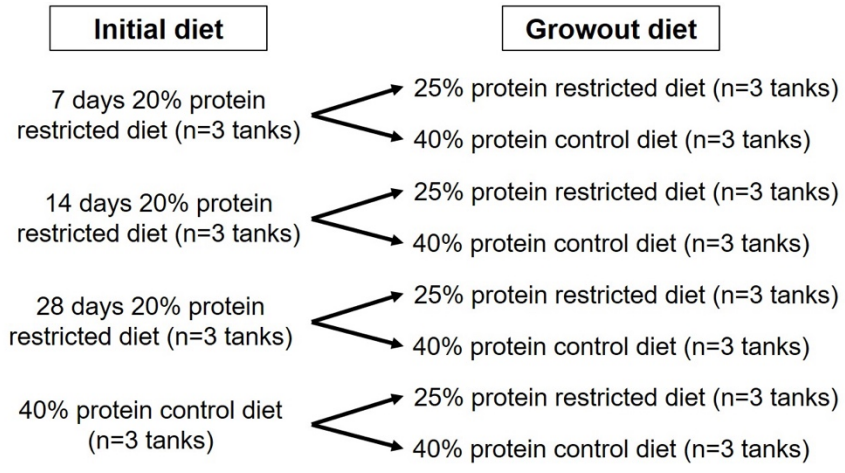
Methods

Experiment 1. Evaluate the effectiveness of nutritional conditioning on tilapia feed efficiency and production.

Null hypothesis 1: No significant differences in growth, feed efficiency or survival rates are observed by use of larval nutritional conditioning strategies for tilapia culture.

Null hypothesis 2: No significant differences in growth or economic parameters in pond-based culture are observed in nutritional conditioning of larvae.

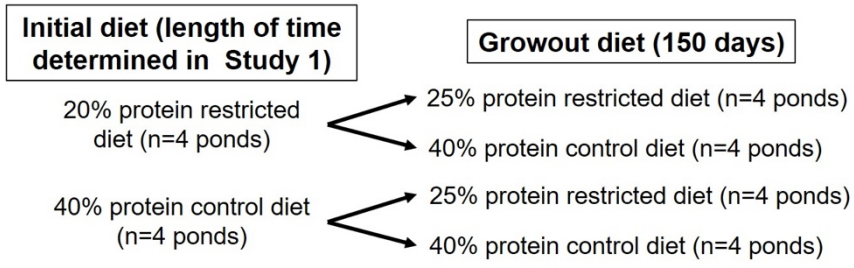
The aim of this investigation is to identify if nutritional conditioning of protein in post yolk-sac fry can lead to more efficient tilapia production. Restricted and normal protein diets will be produced at the NCSU feed mill with a nutritional profile as recommended by Mjoun et al., (2010). We will reduce the total dietary crude protein (while maintaining a constant amino acid profile, total energy) by 50% of the requirement from 40% to 20% during the developmental period immediately following yolk-sac absorption when first feeding occurs. Post yolk-sac Nile tilapia will be produced at the NCSU hatchery facility. Fry will be randomly assigned to one of 4 treatment group and restricted of protein for either 0, 7, 14, or 28 days and then subsequently fed a normal (40% crude protein; Mjoun et al., 2010; El-Sayed, 2006) or submaximal protein diet (25% crude protein) lower than the recommended level. This experiment will determine the longest restriction period for nutritional conditioning that will limit deleterious effect on fish survival and growth. Also by feeding fish a submaximal feed



of 25% crude protein it might be possible to achieve growth similar to those fish fed the recommended level (40%), but at substantially lower cost and with optimal utilization of the protein available (see Figure below). The control diet will have a nutritional profile as recommended by Mjoun et al., (2010). Each of the treatments will be grown in triplicate tanks (n=3; N=24).

The trial in tanks will be conducted for 120 days. Growth parameters (length and weight) will be monitored throughout the production period and for analyses of growth and adjustment of feeding rates. Feed rates will be fixed for all groups and will follow that used for tilapia production (15% bw/day for 0-15 g fish, 8% bw/day for 15-60 g fish, and 5% thereafter). Samples of tilapia anterior intestine tissue, fecal material from the intestine, and muscle tissue will be collected at 28 and 120 days from the time of first feeding for further analysis at NCSU (see Studies 2 and 3). Water quality parameters (*e.g*. ammonia, nitrites, nitrates, dissolved oxygen, and salinity) will be measured weekly. Temperature will be recorded daily. Specific growth rates (SGR) and feed conversion ratios (FCR) will be collected as production parameters. Growth and production (SGR, FCR) parameters for all treatments will be tested for significant differences using Analysis of Variance (JMP, SAS industries). The economic benefit of nutritional conditioning to production of tilapia will be determined from the amount and cost of feed needed to produce a kg of fish.

Should the nutritional conditioning tank trials in Experiment 1 work, we will conduct an additional experiment to evaluate the effectiveness of this technology in pond-based culture in Bangladesh for use by hatcheries and farmers for improving production efficiency of tilapia culture. Protein-restricted fry and growout feed will be produced by at a local feed manufacturer (CP or alternate) using the nutritional recommendations similar to Mjoun et al., (2010) and formulated by Dr. Ferket. Nile tilapia post yolk-sac fry will be obtained from a local hatchery and placed in hapas within a pond at BAU and fed a normal or protein restricted diet for a period determined from Experiment 1.



After this, control and nutritionally conditioned fry fish will be raised in hapas up to 2-3 g on a 25% or 40% crude protein diet. Fish will then be distributed to ponds and continued on the 25% or 40% crude protein diet for 150 days (N=16 ponds, 4 replicates/group; 100 m^2, 1.5 m depth ponds; 4 fish/m^2). All fish will be fed the respective diets on alternate days at a rate of 10 – 3% body weight/day. Our previous work shows that alternate day feeding produces of similar growth and yield as daily feeding (unpublished, current AquaFish report). All ponds will be fertilized weekly at 28 kg N and 5.6 kg P/ha. Differences in growth, yield, FCR among groups will be determined. A marginal benefit-cost analyses will also evaluate if there is a significant cost savings in rearing protocols that incorporate nutritional conditioning in tilapia.

Experiment 2. Identify key factors (gene networks) associated with enhanced nutrient utilization in response to larval nutritional conditioning in tilapia.
Null hypothesis 3: No significant differences in nutrient transporter mRNA abundance are observed in response to larval nutritional conditioning.

This investigation will identify those gene or gene networks that may be associated with elevated nutrient absorption in the Nile tilapia induced by nutritional conditioning (e.g. solute transporters, enzymes, immune factors, etc.) (Bröer, 2008). Standard RNA-Seq methods (Mortazavi et al., 2008) will be followed employing the Illumina HiSeq platform at the NCSU Genomic Sciences Laboratory (GSL). Samples (n = 8; N = 64) of tilapia anterior intestine (anterior). Expressed cDNA will be obtained from

these tissues using established procedures (see Picha et al., 2006) and mRNA expression will be determined by RNA Sequencing (RNAseq). For each treatment, a bar-coded amplicon library (n=8) will be constructed, 2 pools will be made, and run on 2 Illumina lanes for each pool (125 bp, single end reads). This design will provide a depth of ~ 75 million reads for the total library. The NCSU Bioinformatics Consulting and Service Core (BCSC) will be utilized for the High Performance Computing (HPC) environment with the CLC Workbench license. Gene expression using Real-Time quantitative PCR (qPCR) will be performed on the most highly predictive groups of differentially expressed genes (DEG) identified in the RNAseq experiment to validate the effects discovered in the transcriptome analysis. Artificial neural networks will be generated from the DEG between treatments using WEKA software (Hall et al., 2009). The residuals of each highly significant DEG will be input into a Modulated Modularity Clustering program (Stone and Ayroles, 2009) to group the highly significant differentially expressed genes and Gene Ontology (GO) term enrichment of each module will determine cluster function using DAVID Bioinformatics suite (Huang et al., 2009).

Experiment 3. Identification of key microbial factors promoting increased nutrient absorption by enterocytes in the tilapia gastrointestinal tract.

Null hypothesis 4: No significant differences in microbial abundance or diversity occur with nutritional conditioning in tilapia.

This investigation will assess how gut microbial flora is altered by larval nutritional conditioning strategies, and will build on previous studies. Samples of tilapia fecal material will be collected from the tank experiment (see Experiment 1) and analyzed at NCSU. Samples collected from fish will be pooled together according to treatment group (n=6). We propose the use of a pooled sample design to offset potential variability of microbiota within individuals, instead focusing on common patterns, which may be more reflective of changes with treatment group among the population as a whole. Environmental (genomic) DNA will be isolated from fecal samples using procedures outlined in the 16S Metagenomic Sequencing Library Preparation protocol for the Illumina MiSeq system. Using universal bacteria/archeal primers (Fadrosh et al., 2014), the V3 and V4 regions of 16S ribosomal RNA sequence will be amplified for all microbial constituents present within the pooled samples. For each treatment combination, a bar-coded amplicon library (n=48) will be constructed and run on 2 Illumina lanes (300 bp, paired end reads). The sequencing on an Illumina MiSeq platform will be performed at the NCSU Genome Sciences Laboratory (GSL). The QIIME metagenomic toolkit (Caporaso et al., 2010) will be used for operational taxonomic unit (OTU) picking and core diversity analysis. The bioinformatics package PICRUSt (Langille et al., 2013) will be employed to predict microbial functionality of the identified bacterial communities. These predictions will be used to determine if certain bacterial community profiles are associated with increased feed conversion and utilization by nutritionally conditioned tilapia.

Trainings and Deliverables

1. The findings from Studies 1-3 will be reported through the Technical Reports of the AquaFish Innovation Lab (FIR).
2. The results will be presented in the scientific proceedings of the World Aquaculture Society Annual meeting or a regional Aquaculture conference.
3. We anticipate that novel findings arising from nutritional conditioning, intestinal transcriptome and metagenomic studies (Experiments 2-3) will likely lead to high-impact publications within the peer-reviewed literature and will be useful in the development of probiotic supplements. These papers will be produced following completion of the project.
4. One graduate student and one postdoctoral fellow will receive training on sustainable aquaculture farming practices, molecular mechanisms of growth in fishes, and/or genomic technologies enabling aquaculture research.

Schedule

August 2016 to February 2017: Experiment 1 Larval Conditioning Trial at NCSU.
November 2016 to December 2017: Quantitative analysis of the gut transcriptome (Experiment 2), identification of microbial gut populations by metagenomic analysis (Experiment 3).
April 2017 to December 2017: Pond-based Experiment 1.
January to February 2018: Final Technical Report.