TOPIC AREA

SUSTAINABLE FEED TECHNOLOGY AND NUTRIENT INPUT SYSTEMS

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PELLET FEED IMPROVEMENTS THROUGH VITAMIN C SUPPLEMENTATION FOR SNAKEHEAD CULTURE

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Sustainable Feed Technology and Nutrient Input Systems/Experiment/16SFT01UC

Collaborating Institutions and Lead Investigators

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Objective

To improve cost-effective feeds for snakehead aquaculture in Vietnam and Cambodia, specifically by: (i) determining optimal vitamin C requirement in practical diets in laboratory and pond trials (Vietnam); and (ii) to evaluate cost-effectiveness of pellet diets with optimal vitamin C for hapa growout (Cambodia).

Significance

Aquaculture of freshwater carnivorous and omnivorous fish species in Cambodia and Vietnam has been highly dependent on inland fisheries of low value fish for sourcing key dietary nutrient inputs. Adequate pelleted diets, with minimal content of fish meal (FM), are needed to overcome the use of small-size fish (SSF) harvested from the Mekong for aquaculture. From 2007-2015 researchers at Can Tho University (CTU) in Vietnam, working with US collaborators at the University of Rhode Island (URI) developed a formulated feed that reduces the use of SSF and FM content without decreasing growth performance and marketability. Results of the research were disseminated directly to feed manufacturers and more than ten aquaculture fish feed manufacturers in the Mekong Delta now make pelleted diets containing a mixture of FM and soybean meal (SBM). In 2015, more than 90% of snakehead farmers (who produce 99% of the total production of snakehead) in 13 provinces the southern region of Vietnam, including the Mekong Delta, were using these diets instead of SSF, thereby reducing fishing pressure on the small-scale fish in the Lower Mekong Delta. In An Giang, Dong Thap and Tra Vinh provinces, mainly snakehead culture provinces, about 2500 farmers now use pelleted feed. However, in the latest phase of this project, in our commercial-scale farm trial in An Giang province, about 20% of fish that were fed this diet (vs. an SSF diet) developed vertebral column abnormalities. Anecdotal reports from farmers in the region also indicate that this "hunchback" condition is a problem for them. Pictures and X-rays of the fish suggest that this (technically, lordosis and scoliosis) is a classic case of vitamin C (ascorbic acid or AA) deficiency in the diet. Several benefits have been attributed to AA supplementation in fish such as growth, survival, reduction of skeletal deformities, immunoactivity and stress response. Dietary AA can enhance resistance to bacterial infection in fish, but AA requirements may depend on species and their

physiological conditions (Darias et al., 2011). Fish health is an important issue for snakehead culture; bacterial disease is a serious problem (Duc et al., 2012; Duc et al., 2013). Farmers also indicate that the hunchback problem is greatest when fish are fed to satiation; reduced rations appear to lead to less incidence of the condition. We therefore hypothesize that the condition is due to ascorbic acid (AA) deficiency in the diets of fully fed, fast-growing fish, but that AA levels are sufficient for the slower growth of poorly fed fish. Although we have not found any literature on the specific dietary requirements for AA of our research species, *Channa striata* or *Channa micropeltes* (or any other member of the family Channidae for that matter), the requirement for most fish species are considered to be 50 mg/kg (NRC, 1993). One study of the related *Channa punctata* indicated that 2 g/kg of AA provided greater resistance to toxicity of the pesticide endosulfan than did 1 g/kg, i.e., 2000 mg/kg vs. 1000 mg/kg (Sarma et al., 2009). The amount of AA that we used in our previous research in laboratory tanks and farm trials (80-150 mg/kg) (Hien et al., 2015; Hien et al., submitted *a*) appeared to us sufficient at the time. We saw a few abnormal fish in the laboratory experiment or pond trials at CTU. In the farm trials, abnormal fish were only seen at the farm in An Giang province, where the fish were grown for 6 months to a size of about 400 g, not at the farm in Dong Thap province, where fish were only grown for 4 months to about 200 g and where farmers added additional vitamin premix to the diet. In any case, it appears that additional research on AA requirements for snakehead is necessary to solve this issue of abnormal fish. It may be that the stress on a fast-growing fish like snakehead in a densely stocked pond demands higher levels of AA than we anticipated.

We have concentrated our research, development and outreach efforts in Vietnam due to the ban on snakehead culture in Cambodia. Cambodia banned snakehead culture in 2005 because the use of SSF by snakehead farmers caused a user conflict with the human population who used SSF as a source of protein. A major rationale for our research has been to demonstrate to the Cambodian government that snakehead can be raised profitably on pellet feeds, that snakehead culture methods based on SSF can be discarded, and that the ban can be lifted. With the lifting of the ban on snakehead aquaculture in April 2016, the research will focus on snakehead culture technology (breeding, hatchery-rearing, and pond culture) being transferred from Vietnam to Cambodia scientists, specifically to the Fisheries and Aquaculture Research and Development Center (FARDeC) facility at Prey Veng. The next stage of the technology transfer should cover the dietary aspects of snakehead culture (fish nutrition and feed formulation). We propose that Dr. Hien of CTU will lead the work to provide training on fish nutrition, feed formulation and feeding strategies to IFReDI staff at CTU, including a trip to snakehead feed manufacturers in Vietnam and training in equipment use and conducting fish nutrition studies. This will be followed by several trips by CTU personnel to Cambodia for testing improved snakehead feed and installation of software for feed formulation at FARDeC.

Quantified Anticipated Benefits

The results of this study will further define optimal pelleted diets for snakehead in Vietnam and eliminate the hunchback abnormality problem and also improve fish health seen on some farms there. Perhaps more importantly, it will provide further demonstration to the Cambodian government that snakehead culture based on pelleted feed should be allowed in Cambodia. Finally, it will train IFReDI personnel to be able to conduct their own future studies in fish nutrition and feeding for snakehead and other species of interest.

These experiments will also allow the U.S. Participant in this investigation, Dr. Bengtson, to continue his studies of plant protein replacements for fish meal from temperate to tropical species.

The experiments will result in undergraduate and graduate thesis research at CTU, as well as training of IFReDI personnel, providing further capacity building in trained graduates who will work in the aquaculture industry in the Lower Mekong Basin.

On a broader scale, use of plant products to replace fish products in aquaculture diets is a major area of research worldwide. This project will add to the knowledge base for the formulation of diets that will make aquaculture more sustainable globally, as have our previous publications.

Finally, further adoption of soybean-based diets by the snakehead industry will provide increased markets for operators of feed-mills and potentially for the U.S. soybean industry.

Research Design and Activity Plan

Location

- Formulation of diets will be done through collaboration between CTU and URI based on information about chemical composition of ingredients. Manufacture of the diets will be done at CTU, which has a small fish-feed mill (for floating feed, 200kg/hour), as will analysis of diet composition: protein, lipid, mineral, fiber, and energy.
- The laboratory feeding trial will be conducted in a wet lab at CTU.
- The post-feeding trial bacterial challenge experiment will also be done in a wet lab at CTU.
- A pond trial will be conducted in experimental ponds with hapas at CTU by CTU researchers.
- Training of IFReDI researchers will take place at both CTU and FARDeC.
- Installation of software for feed formulation will take place at IFReDI.
- Based on the CTU pond trial results, a confirmatory pond trial will take place at FARDeC.

Methods

This study will comprise interrelated parts:

(i) Effects of variation in dietary vitamin C (AA) in snakehead.

Null hypothesis: AA level does not lead to significant differences in performance of snakehead, as measured by survival, growth, feed conversion ratio (FCR), protein efficiency ratio (PER), certain blood parameters (see below).

Based on the several benefits attributed to AA in other studies of fish nutrition, and the basal diet from our previous work with SBM replacement of FM in diets for snakehead fish, we propose to test the null hypothesis that AA level does not lead to differences in performance of snakehead. Although our previous work with diets based on soy protein concentrate (SPC) supplemented with mannan oligosaccharides (Hien et al., submitted *b*) was very promising, availability of SPC in Southeast Asia is problematical, so we choose to work with the more available SBM. We will conduct a feeding trial in the CTU laboratory, followed by a bacterial challenge, as outlined below. The lowest supplemented AA value is in the mid-range of what we have been using, the highest supplemented levels are those used by Sarma et al., (2009), and we have included two intermediate values in between. The feed will be analyzed to verify AA according to Nelis et al., (1997).

1. SBM diet + 0 mg AA/kg feed
2. SBM diet + 125 mg AA/kg feed
3. SBM diet + 250 mg AA/kg feed
4. SBM diet + 500 mg AA/kg feed
5. SBM diet + 1000 mg AA/kg feed
6. SBM diet + 2000 mg AA/kg feed

The laboratory experiment will be conducted in a manner similar to those we conducted previously (Hien et al., 2015). Experimental units are 500-L tanks. In this trial we will use seven replicate tanks per treatment for greater statistical power, especially for the subsequent bacterial challenge. The stocking density will be 80 fish/tank. At the beginning of the experiment, fish (initial weight about 4-5 g) will be weighed. Fish will be fed to satiation twice a day (0800 and 1600 hrs) and the amount of feed consumed by the fish in each tank will be recorded daily by removing and weighing (dry weight) excess feed to

ascertain intake. Amounts of feed provided per replicate will be recorded so that feed conversion ratio (FCR) and protein efficiency ratio (PER) can be calculated at the end of the experiment. The water will be maintained at 28±2^{o}C. Water quality (temperature, pH, DO) will be measured by meters twice daily (0700 and 1500 hrs) and ammonium, nitrate and nitrite will be measured weekly by test kit. Any dead fish will be recorded and removed daily. The experiment will last eight weeks, at the end of which fish will be measured, weighed and then used in the bacterial challenge experiment. Blood samples of a subset of experimental fish will be taken at the end of experiment and examined for white blood cell count, red blood cell count, lysozyme activity and glucose level, using methods that we have used in our research during the last two years (Hien et al., submitted b). Any skeletal disorders will be documented via photographs and X-rays. Data from a tank are pooled (i.e., no pseudoreplication) and only one number representing average growth per fish (specific growth rate, SGR) is used per replicate. Based on the cost of feed ingredients used for each diet, feed cost per kg of fish produced will be calculated for each treatment. Data analysis is by one-way analysis of variance (ANOVA), following arc-sine square-root transformation of the proportionate data to insure normality. Tukey's HSD test is used to determine specific differences among means if the ANOVA indicates that significant differences are present.

(ii) Bacterial challenge experiment

Null hypothesis: AA levels in the feeding trial above do not lead to significantly different survival in a post-trial bacterial challenge.

The bacterial challenge experiment will be conducted at the end of the feeding trial. The six treatments in the feeding trial will be subdivided, such that fish from five tanks per treatment will be IP injected with 0.1 mL of bacterial strain *Aeromonas hydrophila* CD1012 based on the lethal dose (LD_{50}) of 1.16×10^5 CFU/mL (Duc et al.., 2013) and fish from two tanks per treatment will be IP injected with 0.1 mL of physiological saline (0.85%) as control (Ward et al., 2016) (tanks will be randomly selected prior to the feeding trial to avoid bias). This bacterial challenge experiment will last 2 weeks, as in our previous work (Hien et al., submitted *b*). During the 14 days post-inoculation, fish will continue to be fed their respective diets, and activity and cumulative mortality will be noted daily. For moribund fish, clinical signs will be observed by gross inspection, and lesions will be sampled directly for bacteria. Re-isolation and re-identification of bacteria will be carried out according to methods of Barrow and Feltham (1993) and PCR method will be used to speciate the re-identified bacterial strains. The cumulative mortality will be recorded daily.

1. SBM diet + 0 mg AA/kg feed (saline 0.85% injection), control (2 reps)
2. SBM diet + 0 mg AA/kg feed (*Aeromonas hydrophila* injection) (5 reps)
3. SBM diet + 125 mg AA/kg feed (saline 0.85% injection), control (2 reps)
4. SBM diet + 125 mg AA/kg feed (*Aeromonas hydrophila* injection) (5 reps)
5. SBM diet + 250 mg AA/kg feed (saline 0.85% injection), control (2 reps)
6. SBM diet + 250 mg AA/kg feed (*Aeromonas hydrophila* injection) (5 reps)
7. SBM diet + 500 mg AA/kg feed (saline 0.85% injection), control (2 reps)
8. SBM diet + 500 mg AA/kg feed (*Aeromonas hydrophila* injection) (5 reps)
9. SBM diet + 1000 mg AA/kg feed (saline 0.85% injection), control (2 reps)
10. SBM diet + 1000 mg AA/kg feed (*Aeromonas hydrophila* injection) (5 reps)
11. SBM diet + 2000 mg AA/kg feed (saline 0.85% injection), control (2 reps)
12. SBM diet + 2000 mg AA/kg feed (*Aeromonas hydrophila* injection) (5 reps)

Data will be analyzed by the Kaplan-Meier log-rank survival test, with pairwise Holm-Sidak multiple comparison procedure where appropriate (Ward et al., 2016).

(iii) Hapa trial based on SBM diet with optimal vitamin C (AA) level in snakehead

Null hypothesis: Source of feed (commercial vs. experimental SBM diet) and levels of added AA do not lead to significant differences in snakehead production to market size in experimental ponds.

Based on the optimal vitamin C (AA) requirement in snakehead from the results of the first experiment, we will test the effects of vitamin C (AA) in hapas to simulate farm conditions, as follows:

1. Commercial feed
2. Commercial feed + hand mixed optimal AA concentration requirement
3. Commercial feed + hand-mixed 1.5X optimal AA concentration requirement
4. Commercial feed + hand mixed 2X optimal AA concentration requirement
5. SBM diet without vitamin C (AA)
6. SBM diet + optimal AA concentration requirement
7. SBM diet + 1.5X optimal AA concentration requirement
8. SBM diet + 2X optimal AA concentration requirement

The experiment will be conducted in eight experimental ponds with three replicate hapas each. Stocking density will be 100 fish/m^2 and culture period will be 5-7 month until market size is attained. We will collect data on water quality parameters daily (as described above) and fish survival and growth monthly. We will also calculate feed cost of production per kg of fish (as described above). Any skeletal disorders will be documented at the end of the experiment by photographs and X-rays. Data on fish survival and growth, FCR and PER will be statistically analyzed by two-way analysis of variance.

Trainings and Deliverables

Publish one peer-reviewed journal article in English and one peer-reviewed journal article in Vietnamese, plus one fact sheet.

Training of IFReDI researchers by CTU researchers will take place at both CTU and FARDeC

CTU investigators will conduct a series of short-term training programs for IFReDI researchers in fish feeding, nutrition and processing

Short-term training will include: (1) fish nutrition and data analysis (theory and practical work); (2) snakehead feed formulation and manufacture (theory and practical work); (3) feeding strategy; and (4) improved snakehead processing technology.

CTU will help install feed formulation software at IFReDI

Following the training of IFReDI personnel at CTU, and based on the products available at the time, IFReDI and CTU personnel will decide on the optimal feed formulation software to be installed at IFReDI for future studies on aquaculture nutrition.

CTU will assist with a pond (hapa) feeding trial based on SBM diet at FARDeC

Based on the results of the pond trial at CTU, CTU researchers will work with IFReDI scientists to conduct a pond (hapa) feeding trial at FARDeC to confirm the CTU results using Cambodian snakehead. This trial will only use two ponds, one for the commercial feed without added AA and the other for the best-performing diet treatment from among the other seven treatments in the CTU pond trial with fish in three hapas in each pond. IFReDI personnel will assist with diet manufacture at CTU so that they learn how to conduct nutrition and feeding experiments from diet formulation and manufacture through conduct of the feeding trial and data analysis. Methods for this trial will follow those used at CTU. The null hypothesis will again be that there is no difference between the treatments. Statistical analysis will be by t-test. Financial data will be recorded from this trial (e.g., cost of fingerlings, cost of feed, hours of labor required, sale price of product, etc.), so that an economic analysis can be made of the profitability of snakehead aquaculture in Cambodia.

Long-term training of students at CTU

Two master's (MSc) students at CTU will conduct their thesis research as part of research items (i) – (iii) above.

Schedule

The duration of implementation of this proposed investigation will be 24 months, starting from 1 March 2016 to 28 February 2018.

Year 1 (March 2016 – February 2017):

- Dietary Vitamin C ascorbic acid (AA) feeding trial
- Bacterial challenge experiment
- Sample analysis and report writing
- CTU researchers will train IFReDI researchers in feeding and nutrition studies at CTU

Year 2 (March 2017 – February 2018):

- Hapa feeding trial at CTU
- Hapa feeding trial at FARDeC
- CTU will help install feed formulation software at IFReDI
- Sample analysis and report writing