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Pellet Feed Improvements through Vitamin C Supplementation for Snakehead, *Channa striata* (Bloch 1793), Culture

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Abstract

Vitamin C, or ascorbic acid (AA), is important in growth and physiological functions of fish. Six soybean meal-based (SBM) diets containing 0, 125, 250, 500, 1000 and 2000 mg.kg^{-1} of AA were fed to snakehead, *Channa striata* (Bloch 1793) (initial weight 6.63 $\pm$ 0.16 g) for 8 weeks in the laboratory. Survival of snakehead in the 250 and 500 mg.kg^{-1} treatments was significantly higher ($P < 0.05$) than the control, but not in the other treatments. Final weight and specific growth rate (SGR) of fish in all the AA-supplemented diet treatments were significantly higher ($P < 0.05$) than the control. Feed intake (FI), feed conversion ratio (FCR), and protein efficiency ratio (PER) also differed significantly, but not in a clearly dose-dependent manner. The requirement of AA for snakehead was calculated to be 277 mg.kg^{-1}. No vertebral anomalies were observed. Erythrocyte count was significantly ($P < 0.05$) higher at 1000 and 2000 mg.kg^{-1} than at 0 and 125 mg.kg^{-1}. Fish in 250 mg.kg^{-1} had significantly ($P < 0.05$) higher leukocyte count than those in 0, 1000, and 2000 mg.kg^{-1}. A bacterial challenge test with *Aeromonas hydrophila* revealed that 500 and 1000 mg.kg^{-1} had the lowest cumulative mortality. In an on-farm trial, SBM-based and commercial diets augmented with 0, 500, 750, or 1000 mg.kg^{-1} AA were fed to *C. striata* for 23 weeks. All AA-supplemented SBM-based diets provided significantly ($P < 0.05$) higher final weights and overall yield than did all the AA-supplemented commercial diets. Maximum survival (85.3 %), final fish weight (573.5 g), yield (293.3 kg.hapa^{-1}) and profit (0.38 USD.kg fish^{-1}), as well as minimum FCR (1.16), production cost (1.12 USD.kg fish^{-1}) and feed cost (0.98 USD.kg fish^{-1}) were obtained with the 500 mg.kg^{-1} SBM diet.

Keywords: *Channa striata*, ascorbic acid, growth, immune response

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Introduction

Aquaculture of freshwater carnivorous and omnivorous fish species in Cambodia and Vietnam has been highly dependent on inland fisheries of small-size fish (SSF) for sourcing key dietary nutrient inputs (Hien et al. 2015a). Adequate pelleted diets, with minimal content of fish meal (FM), were developed to overcome the use of SSF harvested from the Mekong for aquaculture (Hien et al. 2015a; 2016a). In 2015, more than 90 % of snakehead farmers, who produce 99 % of the total production of snakehead, in 13 provinces in the southern region of Vietnam, including the Mekong Delta, were using these pelleted diets instead of SSF, thereby reducing fishing pressure on the small-scale fish in the Lower Mekong Delta (Hien et al. 2016a).

However, in a commercial-scale farm trial in An Giang province, about 20 % of fish fed this pelleted diet (vs. an SSF diet) developed vertebral column abnormalities (Hien et al. 2016b). 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 condition (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 (Ai et al. 2006). 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 with bacterial disease identified as a serious problem (Duc at al. 2012; 2013). Farmers also indicate that the hunchback problem is frequently seen when fish are fed to satiation; reduced rations appear to lead to low incidence of the condition. Thus it was hypothesized that AA level is not the limiting nutritional factor when fish are not fed an optimal ration, but it may become so when fish are fed a full ration and are growing at maximum rates.

Although there was no literature on the specific dietary requirements for AA of *Channa striata* (Bloch 1793) or other member of the family Channidae, the requirement for most fish species is considered to be 50 $mg.kg^{-1}$ (NRC 2011). One study on *Channa punctata* (Bloch 1793) indicated that 2000 $mg.kg^{-1}$ of AA provided greater resistance to toxicity of the pesticide endosulfan than did 1000 $mg.kg^{-1}$ (Sarma et al. 2009). Hien et al. (2015b; 2016b) used 80–150 $mg.kg^{-1}$ of AA in laboratory tanks and farm trials, which were sufficient given the NRC standard cited above, but saw a few abnormal fish in the laboratory experiment and pond trials.

In the commercial farm trials conducted by Hien et al. (2016b), abnormal fish were only seen at the farm in An Giang province, where the fish were grown for 6 months to about 400 g. Whereas no fish abnormality was seen at the farm in Dong Thap province, where fish were only grown for 4 months to about 200 g and fed additional vitamin premix to the diet (Hien et al. 2016b).

Thus, additional research on AA requirements for snakehead was necessary to solve the issue of abnormal fish. It could be possible that the stress on a fast-growing snakehead in a densely stocked pond demands higher levels of AA than were previously anticipated.

Cambodia banned snakehead culture in 2005, because the feed used by snakehead farmers incorporated SSF causing a user conflict with the human population which also relies on SSF as a source of protein. However, the ban was lifted in 2016 after research by Hien et al. (2015a,b; 2016a,b; 2017) demonstrated that snakehead can be raised profitably on pellet feeds without the use of SSF. Thus the objective of the present study was to improve the cost-effectiveness of feeds for snakehead aquaculture in Vietnam and Cambodia, specifically by determining the optimal vitamin C requirement in practical diets in laboratory and pond trials.

Materials and Methods

Laboratory feeding trial

Six treatment diets were formulated, based on the lowest AA supplementation levels by Hien et al. (2015a, 2016a) and highest levels by Sarma et al. (2009), with three intermediate values and a control. The treatments were: 1) SBM diet + 0 mg AA.kg^{-1} feed; 2) SBM diet + 125 mg AA.kg^{-1} feed; 3) SBM diet + 250 mg AA.kg^{-1} feed; 4) SBM diet + 500 mg AA.kg^{-1} feed; 5) SBM diet + 1000 mg AA.kg^{-1} feed; and 6) SBM diet + 2000 mg AA.kg^{-1} feed (Table 1). The feed was analyzed to verify AA according to methods of Nelis at al. (1997).

The laboratory experiment was conducted in a similar manner to that of Hien et al. (2015b). Experimental units were 500-L tanks, with five replicate tanks per treatment. The stocking density was 80 fish.tank^{-1}. Average fish initial weight was 6.63 $\pm$ 0.16 g. Fish were fed to satiation twice daily (0800 and 1600 h) and the amount of feed consumed by the fish in each tank was recorded daily by removing and weighing (dry weight) excess feed to ascertain feed intake.

Amounts of feed provided per replicate were recorded so that feed conversion ratio (FCR) and protein efficiency ratio (PER) could be calculated at the end of the experiment. Water temperature, dissolved oxygen and pH were monitored daily with a YSI 556 meter (Yellow Springs Instruments, Yellow Springs, Ohio, USA), whereas total ammonia nitrogen (TAN) and nitrite (NO_2^-) were monitored weekly by the indophenol and diazonium methods, respectively (APHA 2012). Dead fish were recorded and removed daily.

Table 1. Ingredients used in, and proximate composition of, diets used in the snakehead *Channa striata* laboratory feeding trial with varying levels of L-ascorbate-2-monophosphate.

Ingredients	Composition (%)
Kien Giang fishmeal	35.8
Defatted soybean meal	33.4
Cassava	8.26
Rice bran	15.0
Premix mineral and vitamins	2.0
Oil	3.08
Carboxymethyl cellulose	0.40
Lysine	0.40
Methionine	0.28
Fish solution	1.50
Phytase	0.02
Total	100
Crude protein	45.0
Crude lipid	8.91
Ash	12.5
Fiber	2.15
NFE	31.5
Energy ($kJ.g^{-1}$)	19.69

Premix mineral and vitamin ($unit.kg^{-1}$): Vitamin A 2,000,000 IU; Vitamin D 400,000 IU; Vitamin E 6 g; Vitamin B_1 800 mg; Vitamin B_2 800 mg; Vitamin B_{12} 2 mg; Calcium D Pantothenate 2 g; Folic acid 160 mg; Choline Chloride 100 g; Iron (Fe^{2+}) 1 g; Zinc (Zn^{2+}) 3 g; Manganese (Mn^{2+}) 2 g; Copper (Cu^{2+}) 100 mg; Iodine (I^-) 20 mg; Cobalt (Co^{2+}) 10 mg. Fishmeal was from Kien Giang. Cassava and rice bran were local products. CMC; methionine and lysine were products of Evonik.

The experiment was conducted for 8 weeks, at the end of which fish were weighed to the nearest 0.01 g on an electronic balance and then used in a bacterial challenge experiment (described below). After 8 weeks, three fish from each tank were randomly collected and blood was withdrawn for analysis of erythrocytes (RBC), leukocytes (WBC), and lysozymes. The remainder of the fish were then transferred to the bacterial challenge experiment. RBCs were counted by the usual method using the Neubauer chamber and Natt-Hedrick solution (Natt and Hedrick 1952). WBCs were stained by Wright and Giemsa solution and counted (Hang et al. 2013). Lysozyme was analysed by the method of Ellis (1990). Any skeletal disorders were documented by photographs and X-rays. Data from each tank were pooled and only one number representing average growth per fish (specific growth rate, SGR) was used per replicate.

Data analysis was by one-way ANOVA, following arc-sine square-root transformation of the proportionate data to insure normality. Duncan's multiple range test was used to determine specific differences among means if the ANOVA indicated that significant differences were present. Estimation of the vitamin C requirement for fish growth in this trial was by the broken-line regression method (Robbins et al. 1979).

Bacterial challenge experiment

The bacterial challenge experiment was conducted immediately after the end of the 8 weeks feeding trial. The six treatments in the feeding trial were subdivided, such that fish from five tanks per treatment were intraperitoneally (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^{-1} (Duc et al. 2013). Fish from five other randomly chosen tanks were also IP injected with 0.1 mL of physiological saline (0.85 %) as control, following Ward et al. (2016). The bacterial challenge experiment lasted 2 weeks, as in the previous work of Hien et al. (2016a).

During the 14-d post-inoculation period, fish continued to be fed their respective diets, and activity and cumulative mortality were noted daily. For moribund fish, clinical signs were observed by gross inspection, and lesions were sampled for bacteria. Re-isolation and re-identification of bacteria were carried out according to methods of Barrow and Feltham (1993) and PCR was used to speciate the re-identified bacterial strains. Cumulative mortality was recorded daily. Results of the bacterial challenge experiment were analysed by one-way ANOVA followed by Duncan's multiple range test at significance level of $P \leq 0.05$.

Farm trial

Based on the results of the laboratory experiment and bacterial challenge, the effects of AA on snakehead in hapas in ponds to simulate farm conditions, was tested using the following treatments: 1) Commercial feed (formulation proprietary, not available); 2) Commercial feed + hand mixed AA at 500 mg.kg^{-1}; 3) Commercial feed + hand-mixed AA at 750 mg AA.kg^{-1}; 4) Commercial feed + hand mixed AA at 1000 mg AA.kg^{-1}; 5) SBM diet (same as Table 1) without AA; 6) SBM diet + 500 mg AA.kg^{-1}; 7) SBM diet + 750 mg AA.kg^{-1}; 8) SBM diet + 1000 mg AA.kg^{-1}.

The experiment was conducted in two large ponds at a commercial facility (only SBM diet without AA placed in one pond and the rest in the other pond) with four replicate hapas each. Stocking density was at 150 fish.m^2 and culture period was 23 weeks until market size was attained. Data on water quality parameters were collected daily (as described above) and data on fish survival and growth monthly. Based on the cost of feed ingredients used for each diet and other production costs, as well as sales price, feed cost.(kg of fish produced)$^{-1}$, production cost.(kg of fish produced)$^{-1}$ and profit.(kg of fish produced)$^{-1}$ were calculated. Any skeletal disorders were documented at the end of the experiment by photographs and X-rays. Data on fish survival and final weight, yield, FI, FCR and lysozyme were statistically analysed by two-way analysis of variance.

Results

Laboratory feeding trial

Water temperature in all the experimental tanks ranged from 27.1–30.1 ºC, dissolved oxygen from 5.3–5.9 $mg{\cdot}L^{-1}$, pH from 6.9–7.3, TAN from 2.1–3.2 and $NO^{2-} < 0.1$ $mg.L^{-1}$, which were within suitable range for the normal growth and development of snakehead. Survival was significantly higher ($P < 0.05$) in AA supplemented diets at 250 and 500 $mg.kg^{-1}$ than in the control (0 $mg.kg^{-1}$), but survival in all treatments with added AA did not differ significantly (Table 2). Final weight and SGR of fish in the 500 $mg.kg^{-1}$ treatments were significantly higher than the 125 $mg.kg^{-1}$ treatment, which in turn were significantly higher than the control, but they did not show a significant difference among the 250, 500, 1000 and 2000 $mg.kg^{-1}$ treatments (Table 2). Feed intake and FCR did not appear to vary in a dose-dependent manner, although in some cases significant differences were seen (Table 2). Values of PER in all the treatments to which AA had been added were significantly higher than that of the control, but did not differ among themselves (Table 2). Based only on the weight gain data, the vitamin C requirement for the *C. striata* in this experiment was estimated to be approximately 277 $mg.kg^{-1}$ (Fig. 1).

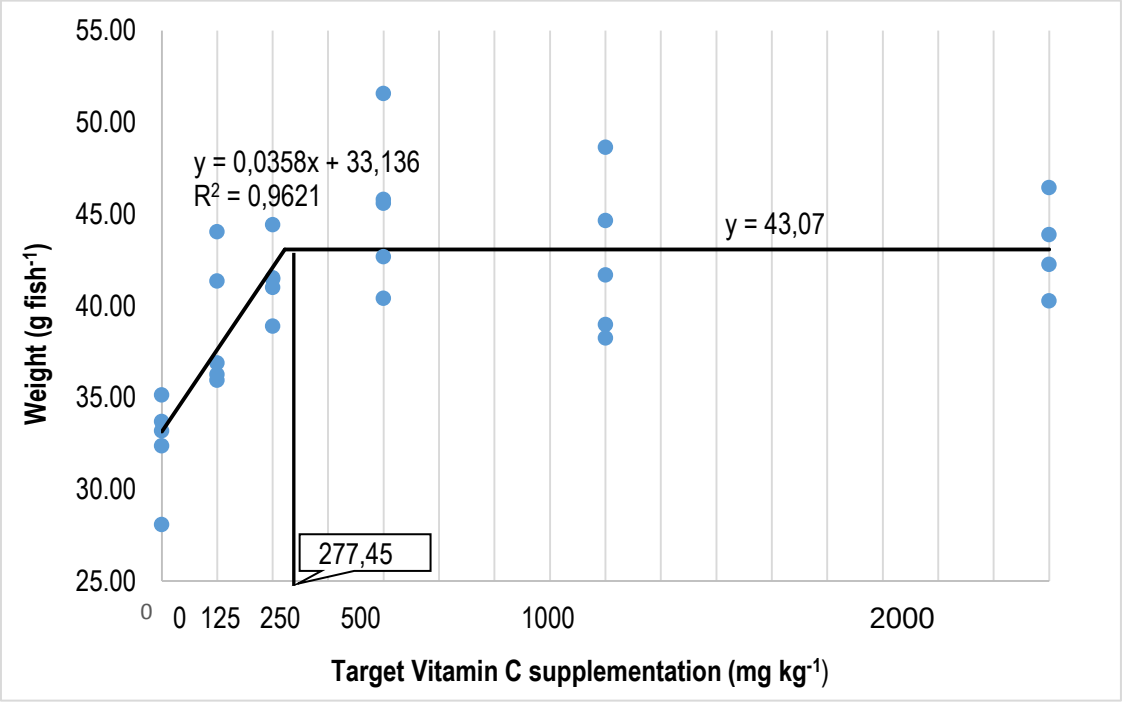

Fig. 1. Calculated requirement of dietary vitamin C on based on growth responses of snakehead fish, *Channa striata* in the 8-week laboratory feeding trial.

Table 2. Initial and final weights.fish^{-1}, specific growth rate (SGR), feed intake (FI), feed conversion ratio (FCR), and protein efficiency ratio (PER) of *Channa striata* fed diets with different levels of vitamin C (as L-ascorbate-2-monophosphate) for 8 weeks. Values (mean ± standard deviation in parentheses) in a column sharing a superscript letter are not significantly different ($P > 0.05$).

Target vitamin C supplementation (mg kg^{-1})	Initial weight (g)	Final weight (g)	SGR (%.d^{-1})	Survival (%)	FI (%.fish^{-1} day^{-1})	FCR	PER (%)
0	6.57^{a} (0.09)	39.06^{a} (2.63)	2.97^{a} (0.13)	76.5^{a} (6.3)	3.99^{ab} (0.27)	1.18^{c} (0.04)	1.52^{a} (0.14)
125	6.56^{a} (0.18)	45.45^{b} (3.66)	3.22^{b} (0.12)	84.8^{ab} (3.5)	4.27^{abc} (0.07)	1.14^{bc} (0.07)	1.79^{b} (0.12)
250	6.61^{a} (0.18)	48.07^{bc} (1.93)	3.31^{bc} (0.09)	88.8^{b} (7.7)	4.24^{abc} (0.21)	1.09^{abc} (0.04)	1.8^{b} (0.15)
500	6.60^{a} (0.10)	51.8^{c} (4.17)	3.43^{c} (0.14)	86.3^{b} (6.7)	4.29^{bc} (0.16)	1.06^{ab} (0.09)	1.92^{b} (0.11)
1,000	6.68^{a} (0.26)	49.1^{bc} (4.34)	3.32^{bc} (0.14)	83.5^{ab} (6.4)	3.97^{a} (0.24)	1.02^{a} (0.12)	1.9^{b} (0.16)
2,000	6.77^{a} (0.08)	49.97^{bc} 2.67	3.33^{bc} (0.08)	84.7^{ab} (6.7)	4.37^{c} (0.27)	1.12^{abc} (0.06)	1.8^{b} (0.13)

Fish in the 1000 and 2000 mg.kg^{-1} treatments had similar erythrocyte counts, and were significantly higher than those in the control and 125 mg.kg^{-1} treatments (Table 3). The highest leukocyte counts were seen in the 125 and 250 mg.kg^{-1} treatments, significantly higher than those in the 1000 and 2000 mg.kg^{-1} treatments, which in turn were significantly higher than those in the control (Table 3). At the end of the feeding trial, lysozyme levels in the control and 2000 mg.kg^{-1} treatments were similar, significantly less than those at 250, 500, and 1000 mg.kg^{-1} and the lysozyme levels in the 1000 mg.kg^{-1} treatment were significantly higher than those in any other treatment (Table 3). No abnormal backbones of snakehead were observed during 8 weeks culture in the laboratory trial.

Table 3. Effect of vitamin C (as L-ascorbate-2-monophosphate) supplemented feed on hematology and immune response of *Channa striata.* Pre-challenge lysozyme levels are after 8 weeks of the laboratory feeding trial, whereas post-challenge lysozyme levels are after 8 weeks of the laboratory feeding trial plus 4 days of the bacterial challenge. Values (mean ± standard deviation in parentheses) in a column followed by the same superscript are not significantly different.

Target vitamin C supplementation (mg.kg^{-1})	Total erythrocytes count (×10^5 cells.mm^{-3})	Total leukocytes count (×10^{3}cells.mm^{-3})	Pre-challenge lysozyme (µg.mL^{-1})	Post-challenge lysozyme (µg.mL^{-1})
0	22.8^{a} (8.9)	108.1^{a} (51.3)	205.0^{a} (42.5)	241.1^{a} (29.3)
125	31.5^{a} (6.6)	355.8^{c} (80.5)	214.5^{ab} (30.7)	328.2^{b} (25.7)
250	32.4^{ab} (5.2)	385.1^{c} (53.7)	274.5^{bc} (31.8)	351.5^{b} (17.6)
500	35.1^{ab} (6.7)	313.2^{bc} (66.2)	313.0^{c} (46.6)	413.2^{c} (62.5)
1,000	44.7^{b} (6.8)	263.1^{b} (58.8)	372.5^{d} (24.3)	506.5^{d} (46.7)
2,000	45.1^{b} (16.5)	230.8^{b} (83.1)	212.0^{a} (41.1)	264.0^{a} (15.6)

Bacterial challenge experiment

After 14 d of the bacterial challenge, cumulative mortality was significantly higher in the control (56 %) and 2000 mg.kg^{-1} (54 %) treatments, low in the 125 mg.kg^{-1} (30 %) and 250 mg.kg^{-1} (34 %) and least in the 500 mg.kg^{-1} (20 %) and 1000 mg.kg^{-1} (14 %) treatments, although the last two listed treatments were not significantly different from each other (Fig. 2). Lysozyme levels measured 4 d into the bacterial challenge increased in all treatments compared to those measured prior to the start of the bacterial challenge (Table 3).

Lysozyme levels were significantly highest in the 1000 mg.kg^{-1} treatment, followed by those at 500 mg.kg^{-1}, which in turn were significantly higher than those at 250 and 150 mg.kg^{-1}, and were significantly higher than those at 2000 mg.kg^{-1} and the control (Table 3).

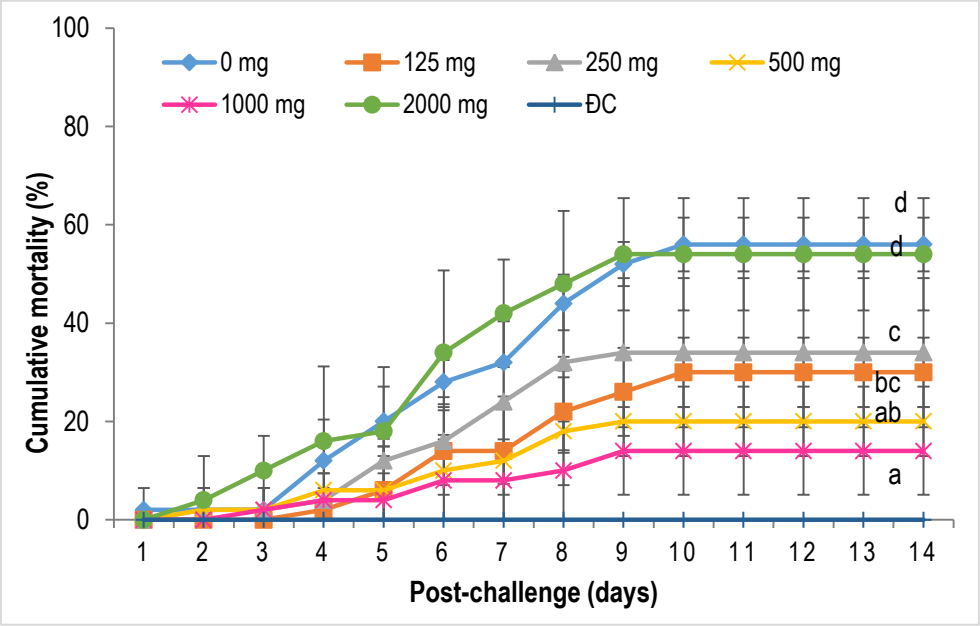

Fig. 2. Cumulative mortality over 14 d in snakehead fish, *Channa striata*, fed diets with different levels of vitamin C (as L-ascorbate-2-monophosphate) for 8 weeks, then inoculated intraperitoneally with *Aeromonas hydrophila* in the bacterial challenge experiment.

Farm feeding trial

During the 23-week experimental period, all water quality parameters (i.e. temperature, pH, DO, TAN, NO_2, and NH_3) were similar in the two experimental ponds (Table 5). Results of the two-way ANOVA indicated that diet (commercial vs. SBM) and AA level, as well as their interaction, were significant for all measured variables (final weight, yield, SGR, FI, survival, FCR, lysozyme, hunchback percentage, production cost, feed cost and profit), with two exceptions.

Level of AA was not significant for hunchback percentage and diet x AA level interaction was not significant for FCR. More specifically, final weight, yield, survival, FI and lysozyme levels of fish fed SBM with and without AA-supplemented diets were higher than those of the fish fed commercial feed with and without AA-supplemented diets, respectively, and FCR levels were lower (Table 6). The highest values of final weight, yield, and survival were observed in the fish fed with SBM diet + AA 500 mg.kg^{-1}, which were significantly different from the control (fish fed SBM diets + 0 mg.kg^{-1} AA); whereas among the treatment groups of commercial diets + AA, these indices were not significantly different (Table 6).

Feed intake was highest in the 1000 mg.kg^{-1} AA-supplemented SBM diet group (FI = 6.4 % fish^{-1}.day^{-1}, significantly different from the SBM diet + AA 0 mg.kg^{-1}); and the lowest value of FCR, in the 750 mg.kg^{-1} AA-supplemented SBM diet group (FCR = 1.27), was significantly different from all commercial diets supplemented with AA ($P < 0.05$). Lysozyme levels of the AA-supplemented treatments were higher than those of the control group (no AA supplementation) in both SBM and commercial diets, and the highest lysozyme levels (287.3 and 286.8 µg.mL^{-1}) were found in fish fed diets containing 1000 mg.kg^{-1} AA-supplemented SBM or commercial feed, respectively.

Fish fed an SBM diet + 500 mg.kg^{-1}AA exhibited the lowest percent occurrence of abnormal backbones, production cost, feed cost, and the highest profit (Table 7). The highest percent occurrence of abnormal backbones was found in fish fed commercial diet + 500 mg.kg^{-1} AA. Fish fed the commercial diet + 750 and 1000 mg.kg^{-1} AA exhibited the highest production costs and the lowest profits. The highest percent occurrence of abnormal backbones, in the experimental group of fish fed commercial diet + 750 mg.kg^{-1} AA, was significantly higher than in the control (commercial + 0 mg.kg^{-1} AA).

Discussion

The result of the present study is the first in Southeast Asia to provide an estimate of AA requirements in practical diets for *C. striata* grown in ponds. Vitamin C is important in multiple processes in the growth, collagen formation, iron metabolism and hematology, reproduction, response to stressors, wound healing and immune response (NRC 2011). Thus, it is not surprising that the diets supplemented with AA promoted better growth of snakehead compared to the control. This result is consistent with the previous studies in hybrid tilapia, *Oreochromis niloticus* (Linnaeus 1758) × *Oreochromis aureus* (Steindachner 1864) (Shiau and Hsu 1995), tilapia, *Oreochromis spilurus* (Günther 1894) (Al-Amoudi et al. 1992), large yellow croaker, *Pseudosciaena* (= *Larimichthys*) *crocea* (Richardson 1846) (Ai et al. 2006), *O. niloticus* (Ibrahem et al. 2010) and rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (Adel and Khara 2016). The finding herein is in agreement with a previous study (Yousefi et al. 2013). Pal and Chakrabarty (2012) and Tewary and Patra (2008) pointed out that supplementation of vitamin C at 1200 and 1000 mg.kg^{-1} feed yielded better growth of African catfish, *Clarias batrachus* (Linnaeus 1758) and rohu, *Labeo rohita* (Hamilton 1822), respectively.

Results of experiments on vitamin C may differ due to differences in fish species, size, purity and sources of ascorbic acid and experimental conditions in different studies. The requirement of vitamin C for snakehead growth in this study was 277 mg.kg^{-1} feed, indicating that the level is higher than that used in previous diet studies with this species (Hien et al. 2015b, 2016b). Vitamin C requirement was 63.37 mg.kg^{-1} feed in *O. niloticus* × *O. aureus* (Shiau and Hsu 1999), 118 mg.kg^{-1} feed in parrot fish, *Oplegnathus fasciatus* (Temminck & Schlegel 1844) (Wang et al. 2003) and 43.5 mg.kg^{-1} feed in grouper, *Epinephelus malabaricus* (Bloch & Schneider 1801) (Lin and Shiau 2005).

Table 4. Water quality parameters in two experimental ponds (control and treatment, see text) used in the farm trial with *Channa striata* during the 23 weeks of culture. Values are mean ± standard deviation (in parentheses).

Experimental pond	Temperature (^oC)		pH		DO ($mg.L^{-1}$)		TAN ($mg.L^{-1}$)	NO_2 ($mg.L^{-1}$)	NH_3 ($mg.L^{-1}$)
	Morning	Afternoon	Morning	Afternoon	Morning	Afternoon			
Control	29.3 (0.90)	31.5 (0.63)	8.2 (0.1)	8.3 (0.1)	1.6 (0.4)	3.8 (0.2)	3.48 (0.35)	0.004 (0.002)	0.030 (0.010)
Treatment	29.2 (0.94)	31.3 (0.86)	8.1 (0.1)	8.2 (0.2)	1.8 (0.3)	3.7 (0.4)	0.55 (0.14)	0.061 (0.016)	0.005 (0.001)

Table 5. Initial and final weights.fish^{-1}, yield, survival, feed intake (FI), feed conversion ratio (FCR), and lysozyme levels of *Channa striata* fed soybean meal (SBM) diet and commercial feed (CF) with different levels of vitamin C (as L-ascorbate-2-monophosphate) for 23 weeks in the farm trial. Values (mean ± standard deviation in parentheses) in a column sharing a superscript letter are not significantly different ($P > 0.05$). Results of the two-way analysis of variance are given at the bottom.

Diet and target vitamin C supplementation (mg.kg^{-1})	Initial weight (g)	Final weight (g)	Yield (kg.hapa^{-1})	Survival (%)	FI (%.fish^{-1}.day^{-1})	FCR	Lysozyme (µg.mL^{-1})
SBM-0	9.00	461.7^{a}	190.6^{a}	69.1^{a}	5.40^{a}	1.40^{bc}	232.9^{a}
	-	(40.1)	(8.9)	(5.8)	(0.40)	(0.11)	(4.8)
SBM-500	9.00	573.5^{c}	293.3^{c}	85.3^{c}	5.69^{ab}	1.16^{a}	271.9^{b}
	-	(34.3)	(10.8)	(3.2)	(0.25)	(0.06)	(17.6)
SBM-750	9.00	556.2^{c}	268.3^{bc}	80.4^{bc}	6.00^{abc}	1.27^{ab}	283.7^{b}
	-	(19.2)	(8.6)	(2.2)	(0.05)	(0.01)	(4.9)
SBM-1000	9.00	565.4^{c}	264.6^{b}	78.3^{bc}	6.40^{c}	1.35^{bc}	287.3^{b}
	-	(44.2)	(7.5)	(4.7)	(0.55)	(0.12)	(9.5)
CF-0	9.00	398.9^{ab}	172.1^{a}	71.9^{ab}	6.07^{abc}	1.50^{c}	228.6^{a}
	-	(41.2)	(18.2)	(3.0)	(0.11)	(0.07)	(10.6)
CF-500	9.00	399.8^{ab}	185.9^{a}	77.7^{abc}	6.03^{abc}	1.47^{c}	224.1^{a}
	-	(28.1)	(9.1)	(4.2)	(0.19)	(0.08)	(16.8)
CF-750	9.00	398.0^{ab}	177.7^{a}	74.6^{ab}	6.08^{bc}	1.49^{c}	227.9^{a}
	-	(38.9)	(13.3)	(3.8)	(0.10)	(0.08)	(13.7)
CF-1000	9.00	376.0^{a}	173.3^{a}	76.8^{abc}	6.08^{bc}	1.48^{c}	286.8^{b}
	-	(22.6)	(12.0)	(1.8)	(0.05)	(0.02)	(22.9)
P values							
Feed		0.000	0.000	0.033	0.050	0.000	0.000
Vitamin C		0.018	0.000	0.000	0.013	0.017	0.000
Feed*vitamin C		0.006	0.000	0.049	0.020	0.051	0.001

Table 6. Hunchback (abnormal backbones), production costs (PC), feed cost, and profit (P) for fish raised on soybean meal (SBM) diet and commercial feed (CF) with different levels of vitamin C (as L-ascorbate-2-monophosphate) for 23 weeks in the farm trial. Values (mean ± standard deviation in parentheses) in a column sharing a superscript letter are not significantly different ($P > 0.05$).

Diet and target vitamin C supplementation ($mg.kg^{-1}$)	Hunchback fish (as % of total)	Production cost (USD.kg $fish^{-1}$)	Feed cost (USD.kg $fish^{-1}$)	Profit (USD.kg $fish^{-1}$)
SBM-0	6.28^{ab}	1.39^{bc}	1.18^{b}	0.12^{ab}
	(0.68)	(0.10)	(0.09)	(0.10)
SBM-500	5.37^{a}	1.12^{a}	0.98^{a}	0.38^{c}
	(0.85)	(0.06)	(0.05)	(0.06)
SBM-750	5.89^{a}	1.23^{ab}	1.07^{ab}	0.27^{bc}
	(0.61)	(0.02)	(0.01)	(0.02)
SBM-1000	6.75^{abc}	1.31^{bc}	1.14^{b}	0.20^{ab}
	(1.08)	(0.10)	(0.10)	(0.10)
CF-0	8.32^{bcd}	1.43^{c}	1.19^{b}	0.08^{a}
	(0.98)	(0.08)	(0.05)	(0.05)
CF-500	9.63^{de}	1.40^{bc}	1.17^{b}	0.11^{a}
	(1.49)	(0.07)	(0.06)	(0.07)
CF-750	11.1^{e}	1.43^{c}	1.19^{b}	0.07^{a}
	(0.81)	(0.06)	(0.06)	(0.05)
CF-1000	8.91^{cde}	1.44^{c}	1.18^{b}	0.07^{a}
	(1.23)	(0.04)	(0.02)	(0.04)
P values				
Feed	0.000	0.000	0.000	0.000
Vitamin C	0.118	0.003	0.017	0.003
Feed*vitamin C	0.009	0.025	0.049	0.015

Snakehead in the laboratory experiment fed the SBM diet + 500 mg kg^{-1} AA had the lowest FCR compared to the control, which was similar to results for African catfish, *Clarias gariepinus* (Burchell 1822) (Adewolu and Aro 2009), and significantly higher PER. Vitamin C is known to be positively related to protein metabolism (Yousefi et al. 2013). Vitamin C is a powerful antioxidant, protecting against oxidative damage to various tissues of fish including red blood cells (Sahoo and Mukherjee 2003). Red blood cells can act as oxidative status indicators (Pimpimol et al. 2012). In our study, fish fed the diets containing vitamin C had higher total erythrocyte and leukocyte counts than those fed the control diet. Total erythrocyte counts differed significantly between the control and treatments supplemented with AA at 1000 and 2000 $mg.kg^{-1}$. Similar results were observed by Andrade et al. (2007) for pirarucu, *Arapaima gigas* (Schinz 1822) receiving AA at 800 and 1200 $mg.kg^{-1}$ and by Pimpimol et al. (2012) for Mekong giant catfish, *Pangasianodon gigas* Chevey 1931, receiving AA at 750 $mg.kg^{-1}$. Moreover, total leukocyte count significantly increased in snakehead fed AA-supplemented diets, which was similar to results in rainbow trout (*O. mykiss*) (Rahimi et al. 2015), tra catfish, *Pangasianodon hypophthalmus* (Sauvage 1878) (Hang et al. 2015) and Japanese eel, *Anguilla japonica* Temminck & Schlegel 1846 (Shahkar et al. 2015).

Leukocytes reached their maximum level in AA-supplementation of 250 $mg.kg^{-1}$ and then declined as AA levels increased beyond that. Thus, AA supplementation may affect the blood composition of snakehead; however, further studies should be considered to evaluate the relationships between vitamin C and other haematological parameters. Lysozyme activity is an important index of innate immunity of fish and is ubiquitous in its distribution among living organisms (Saurabh and Sahoo 2008). Lysozyme is liberated by leukocytes and important in anti-microorganism activity (Pimpimol et al. 2012). In the present study, lysozyme activity increased in the serum of snakehead fish fed AA-supplemented diets compared to the control, both before and after challenge with *A. hydrophila*.

Lysozyme activity increased with level of AA from 250 to 1000 $mg.kg^{-1}$. This is consistent with the previous reports on *P. gigas* fed the dietary AA supplements at $\geq$ 250 $mg.kg^{-1}$ (Pimpimol et al. 2012) and on improved lysozyme activity with AA supplementation in cobia, *Rachycentron canadum* (Linnaeus 1766) (Zhou et al. 2012). The present study also revealed that lysozyme activity in snakehead fed diet supplemented with AA at 2000 $mg.kg^{-1}$ was not different from the control. This finding is in agreement with previous studies in channel catfish, *Ictalurus punctatus* (Rafinesque 1818) (Li et al. 1993), Atlantic salmon, *Salmo salar* Linnaeus 1758 (Thompson et al. 1993), and *R. canadum* (Zhou et al. 2012) showing lower lysozyme activity at higher AA levels. The bacterial challenge test indicated that cumulative mortality of snakehead decreased with increased AA levels, at least up to 1000 $mg.kg^{-1}$. Moreover, cumulative mortality was negatively correlated with the total erythrocyte and leukocyte counts and lysozyme activity. Similar results were found in *R. canadum* (Zhou et al. 2012) and they demonstrated the relationships among dietary AA supplementation, immunological parameters, and disease resistance. However, in the present study the highest AA-supplemented diet (2000 $mg.kg^{-1}$) did not appear to be effective for disease resistance, which has also been demonstrated previously by Li et al. (1993).

In the present study, results of the farm feeding trial were consistent with those of the laboratory feeding trial, demonstrating that dietary vitamin C can significantly improve the normal growth and other physiological functions in snakehead. Fish fed diets with AA supplemented at 500 mg.kg-1 in the farm trial (i.e., the next highest treatment level above the AA requirement calculated from growth results in the laboratory trial) yielded the lowest feed costs and highest profits. Additionally, the farm feeding trial showed that the SBM diet was a viable feed option for use in aquaculture of snakehead compared with the commercial feed. It appears that the diet X AA level interaction in the farm trial was largely due to AA level having much less effect on the commercial diet than on the SBM diet. We believe that this was caused by hand-mixing of the AA into the commercial diet, rather than fully incorporating it into the diet during production.

Conclusion

This study demonstrated that dietary AA supplementation is able to improve growth performance, immune responses, and survival of snakehead fish against *A. hydrophila* infection. The requirement of AA by snakehead was determined as 277 $mg.kg^{-1}$ feed in the laboratory study. The diet supplemented with vitamin C at 250 to 1000 $mg.kg^{-1}$ feed is an appropriate concentration range for improving growth performance and immunity of snakehead fish on fish farms. Soybean meal-based diet was a better diet used in culturing snakehead compared with the commercial diet. Further experiments should be considered to investigate correlations between vitamin C and other immune parameters, as well as disease resistance of snakehead against other pathogens.

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