Effects of dietary vitamin C and soybean lecithin in the nutrition of brown bullhead (Amelurus nebulosus L.) fingerlings

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Abstract

The effects of different forms of vitamin C and soybean lecithin on growth performance, feed utilization and body composition of brown bullhead (Amelurus nebulosus, Lesueur 1819) were evaluated during a 9-week growth trial. A special interest was to investigate a possible combine effect of the various nutritional components. The diets used contained three forms of vitamin C (crystallized ascorbic acid, encapsulated L-ascorbic acid and Ca-L-threonate) (100 mg/kg) with and without the combination of soybean lecithin. Besides control diet (K), one more diet was supplemented with soybean lecithin (L) only. One-hundred-ninety-two brown bullhead of about 45 g initial body weight was randomly divided in 24 tanks (115 L each). Testing conditions included 8 fish per tank, with triplicate tanks for treatment. All diets with supplemented components had higher final weight. Specific growth rate, feed conversion rate and condition factor were significantly higher with encapsulated vitamin C diets (CC, CC), followed by the results of enriched ascorbic acid diets. Vitamin C and lecithin supplementation showed positive influence on significantly higher number of erythrocytes, haematocrit, triglycerides and total protein. Vitamin C content of muscle and liver tissue was not uniform and was significantly higher in AA, CC, CC and AA feeding groups. The fatty acids profile of muscle and liver tissue showed that phospholipids from soybean lecithin and vitamin C diets enhanced the quality of usable part of the fish body. Combine supplementation of vitamin C and soy lecithin indicated positive production effects, but did not cause a statistically significant difference.

Introduction

In modern intensive aquaculture, feed formulations may account for more than 50% of the total production costs. Increasing feed efficiency, especially by improving the metabolic assimilation of dietary nutrients, is of high priority in contemporary animal production. Any reduction in feed costs is bound to have a direct positive effect on profitability of aquaculture production (Sakai, 1999; Francis et al., 2005; Ibrahim et al., 2010). Ascorbic acid (AA) (Vitamin C) is an essential micronutrient for fish. Invertebrates, insects, most fishes, some birds, guinea pigs, bats and primates are not able to synthesize ascorbic acid (Henrique et al., 1998). The inability to synthesize vitamin C is due to the lack of the enzyme L-gulono-lactone oxidase (GLO, EC1.3.8.8) which catalyzes the conversion of L-gulonolactone into ascorbic acid in the liver and kidney (Roy and Guma, 1958). Thus, these animals must depend upon a dietary supplement of this vitamin. There have been several benefits attributed to ascorbic acid supplementation in fish such as growth, survival, reduction of skeletal deformities, immunomodulatory and stress response (Dabrowski, 1992). As ascorbic acid is very labile and is prone to be destroyed during diet preparation and storage (Hilton et al., 1977; Halver, 1989), it has been difficult to establish the minimum requirement for each species. However, significantly higher concentrations of vitamin C than necessary for normal growth can increase the resistance of some fish species from Ictaluridae family to some pathogen bacteria and enhance their immune system (Durve and Lovell, 1982; Li and Lovell, 1985; Duncan and Lovell, 1994). In conditions of intensive farming, the biological instability of ascorbic acid may cause a significant problem. The availability of more stable forms of ascorbic acid has allowed for more accurate assessments of the vitamin C requirements in fish (Teshima et al., 1993; Shiau and Hsu, 1995, Henrique et al., 1998). Various coated forms of ascorbic acid, such as ethyl cellulose, cyclocelxtrins or lipids have been used to increase the retention of the vitamin in fish feeds. Protective coating slows its degradation under high heat and moisture conditions (Uddin et al., 2001). Nevertheless, approximately 50% of the supplemental ascorbic acid is destroyed during the manufacture of extruded catfish feeds (Lovell and Lim, 1978) and excess ascorbic acid is added to commercial formulations to ensure that an adequate concentration of the vitamin is retained during the processing. Elevated concentrations of vitamin C in fish feeds have different effects on some fish species depending on fish size, the differences in diet formulation, the species and culture system (NRC, 1993). Vitamin C metabolite L-threonic acid or its calcium salt, calcium threonate increases cells uptake of vitamin C. Comparing calcium threonate with ascorbic acid more ascorbate activity is provided within the human cells (Fay and Verlangieri, 1991). Calcium threonate in rats with a genetic bone disorder prevents scurvy four to five times more effectively than the ascorbic acid and has a dominant role in determining the activity of vitamin C (Verlangieri et al., 1991). Lecithin plays important roles in lipid and carbohydrate metabolism in the liver of the fish; also, it is an essential component of biomembrane systems in all eukaryotic cells. Soy lecithin bio-surfactant property makes it suitable for use as an agent that enhances the emulsification of dietary lipids in the fish intestine (Hertrampf and Piedad-Pascual, 2000). Lecithin is known to be a consistent source of highly bioavailable phospholipids to crustaceans and fish. The positive role of phospholipids in food was proved for several marine and freshwater fish species, particularly for their early stages (Kanazawa et al., 1981, 1983; Rumsey and Smith, 1990; Geurden et al., 1995, 1997; Tocher, 2008). Phospholipids can reduce the oxidation of vitamins A, C and E. Thus, lecithin also enhances the utilization of these vitamins in aquacultural species. Levels of phospholipids in soybean oils range from 1.48 to 3.08 percent, which is considerably higher than the 0.5 percent usually find in other veg-
etabolic oils (Daniel, 2004). Therefore, soybean lecithin is considered the most valuable and efficient plant source of natural phospholipids with the best profile of polyunsaturated fatty acids (Paltaug and Hermetter, 1990). A catfish (Ictalurus spp.) is the dominant aquaculture species in the US, far exceeding farm-raised trout, salmon, tilapia, craw fish and shrimp in both, volume and value (Kinnucan, 1992). Brown bullhead (Ictalurus nebulosus) is a member of Ictaluridae family. It is omnivorous, benthic fish species, intensively cultured in several European countries (Buttner, 1992; Mordenti et al., 1994). Estimation of brown bullhead production in Italy for 2010 was 250 tons (EFSA, 2011). Another Ictalurid, the channel catfish (Ictalurus punctatus), besides in the US, it is also being produced in Latin America and in China (Tucker, 2000), and it has been very extensively studied (Peres et al., 2003; Twibell and Wilson, 2004; Welker et al., 2007; Durland et al., 2010; Li et al., 2010).

The aim of this research was to study the effects of different forms of vitamin C and soybean lecithin on the growth and tissues composition of brown bullhead. Particular interest was to investigate the possibility of combine effects of these two nutrients.

Materials and methods

Fish and feeding trial

Brown bullhead fingerlings were obtained from a commercial fish farm. Prior to the experiment, the fish were acclimated to the experimental conditions for 7 weeks during which they were fed a diet (K0) without additional supplements (vit. C or soy lecithin). In the trial, this feed was used as control diet. One-hundred-ninety-two brown bullheads, with an initial average weight of 45 g, were then measured and randomly distributed in twenty-four separate 115 L rectangular tanks (8 fish per tank) inserted into a partially re-circulating system (10% of daily water renewal) for 9 weeks. Eight experimental diets in triplicate groups were randomly assigned within the 24-tank system. Low-pressure electrical blowers provided aeration via air stones while blowers provided aeration of air circulation of the 24-tank system, low-pressure electrical blowers provided aeration via air stones while dissolved oxygen (DO) levels were maintained at the average of 9.5 mg L⁻¹. Daily amounts of dissolved oxygen were measured by Winkler titration method (Winkler, 1888), pH value (7.53-8.10) with glass electrode (pH-meter ISKRA MA 9507) and total ammonia (0.08-0.7 mg N L⁻¹) was measured using colorimetric method by Nessler (US EPA, 1992). Tap water was filtered out with filter system (size: h=1000 mm, ø=300 mm) consisting of magnetic pipe, aquarium sponge, ceramic cylinders, synthetic fibres and granular active carbon. Each tank was supplied with primary biological filter. The rearing tanks were supplied by filtered water at a flow rate of 2.5 L min⁻¹. Water temperature was measured daily and maintained at 21±0.5°C. During the experimental period the fish were subjected to a 13:11-h (light:dark) photoperiod under fluorescent lightning.

Experimental diets

Eight isonitrogenous (38% crude protein) and isolipidic (7% crude lipid) diets based on fish meal and adequate vitamin and mineral supplement were used in addition to the ascorbic acid and soybean lecithin (Tables 1 and 2). The protein percentage utilized in the diet was reported to be the optimum for the growth of Ictalurus punctatus fingerlings (Robinson and Li, 2002). Triplicate groups of fish were hand-fed three times a day (at 07:30 AM, 02:00 PM and 08:30 PM) in total quantity of 2.5% of live body mass. The control group of fish (K) was fed with the basal diet while the other seven groups received basal diet with the addition of, either different forms of vitamin C (100 mg kg⁻¹ of dry diet) (CC, encapsulated vitamin C diet; AA, enriched ascorbic acid diet; Ca, Ca-L-threonate diet), soybean lecithin (2.5%) (L) or with the combination of these two components (CC₄L, AA₄L, Ca₄L) (Table 3). Additionally, we also used the adaptation feed (K0) group of fish to compare blood and tissue analyses results. The diets were processed by blending the dry ingredients into a homogenous mixture. The mixture was passed through laboratory pellet mill at 3.2 mm diameter at the Pliva Research Centre, Kalinovica, Croatia.

Sampling

At the beginning of the growth trial, 24 fish were sampled for blood via heart puncture and other 8 fish from an initial fish pool were dissected for liver and muscle and stored at -20°C for composition analysis. At the end of the trial prior to sampling, fish was deprived of food for 24 h. After the fish had been euthanized (1-2 min in 100 mg L⁻¹ MS-222) (Fluka, Sigma-Aldrich, St. Louis, MO, USA) (Topi Popovi et al., 2012) the same protocol of slaughter was followed for each tank. Six fishes from each rearing tank were weighed for individual whole body composition, collected for blood sampling via heart puncture by heparinized syringes and dissected for liver/viscera weighing. Muscles were also dissected from both sides of the fillets without skin. Liver and muscle tissue were stored for composition and fatty acid analysis at -20°C. Plasma was separated by centrifugation and stored at -70°C until the analysis of total protein, triglyceride and glucose were done. The analyses of the vitamin C content in the diets stored at room temperature were done on three occasions: at the beginning of the trial, 9 and 18 weeks after.

Analytic methods

Proximate analysis of the diets, liver and muscle homogenates were done by standard methods (AOAC, 1990). Crude protein (N x 6.25) was determined by the Kjeldahl method. Crude lipid was determined by the dichloro ether extraction method by Soxtec System HT. Dry matter was oven-dried for 24 h at 105°C. Crude ash was incinerated at 550°C in a muffle furnace for 24 h. Fatty acids were determined using the gas chromatography with flame ionization detector (FID) and a glass column (100-300 cm x 0.2-0.4 cm) filled with polyester stationary phase type, the GP 5%-20% DB5 (diethylene glycol succinate). Red blood cell count was done manually by microscope in the counting chamber. Hematocrit was determined by a microhematocrit direct method. Capillaries for microhematocrit (Laborgeraete Hirschmann, Eberstadt, Germany) filled with blood and centrifuged for 5 minutes at 10,000 rev/min in a centrifuge for

| Table 1. Experimental diet design. |
|-----------------------------------|
| **Diet**                     | **Additives to basal feed** |
| K                              | None (control)               |
| L                              | Soy lecithin                 |
| AA                             | Ascorbic acid                |
| AA₄L                           | Ascorbic acid + soy lecithin |
| CC                             | Protected (encapsulated) vitamin C |
| CC₄L                           | Protected (encapsulated) vitamin C + soy lecithin |
| Ca                             | Ca-L-threonate               |
| Ca₄L                           | Ca-L-threonate + soy lecithin |


Data collection

As growth measurements were done once a week, the feeding rate was adjusted accordingly. At the end of the experiment the following production parameters were determined: weight gain (WG) was calculated as (final mean weight - initial mean weight); specific growth rate (SGRw) (% day\(^{-1}\)) as 100 \(\times\) ln (final mean weight) - ln (initial mean weight) / days; feed conversion ratio (FCR) as dry feed intake / wet weight gain; the condition factor (CF) was calculated as 100 \(\times\) (live weight, g) / (body length, cm\(^2\)); the viscero-somatic index (WSI) as 100 \(\times\) (viscera weight) / (body weight); the hepatosomatic index (HSI) as 100 \(\times\) (liver weight) / (body weight); standard length increments (SLI) = final standard length (cm) – initial standard length (cm); specific length increments (SGSRI) (% day\(^{-1}\)) as 100 \(\times\) ln (final mean standard length) - ln (initial mean standard length) / days.

Statistical analyses

All experimental diets were assigned according to a completely randomized design. Data were analyzed by one-way analyses of variance ANOVA in order to determine the effect of treatments, Fisher’s LSD test followed

Table 2. Ingredients and proximate composition of the experimental diets administered to brown bullhead fingerlings.

| Ingredients, g kg\(^{-1}\) as fed basis | K | L | AA | AAL | CC | CCL | Ca | CCL |
|----------------------------------------|---|---|----|-----|----|-----|----|-----|
| Fish meal                              | 300 | 300 | 300 | 300 | 300 | 300 | 300 | 300 |
| Soybean meal                           | 215 | 215 | 215 | 215 | 215 | 215 | 215 | 215 |
| Wheat meal                             | 255 | 255 | 255 | 255 | 255 | 255 | 255 | 255 |
| Corn meal                              | 115 | 115 | 115 | 115 | 115 | 115 | 115 | 115 |
| Inactive brewing yeast                 | 50  | 50  | 50  | 50  | 50  | 50  | 50  | 50  |
| Soybean oil                            | 30  | 30  | 30  | 30  | 30  | 30  | 30  | 30  |
| Vitamin mineral mix                    | 20  | 20  | 20  | 20  | 20  | 20  | 20  | 20  |
| Binder                                 | 15  | 15  | 15  | 15  | 15  | 15  | 15  | 15  |
| Soybean lecithin                       | 25  | 25  | 25  | 25  | 25  | 25  | 25  | 25  |
| Crystallized AA                        | -   | -   | 0.1 | 0.1 | -   | -   | -   | -   |
| Ethyl cellulose encapsulated AA        | -   | -   | -   | -   | 0.1 | 0.1 | -   | -   |
| Ca-L-threonate                         | -   | -   | -   | -   | -   | -   | 0.1 | 0.1 |

Proximate composition, %

| Dry matter                             | 90.15 | 90.79 | 90.1 | 90.1 | 90.15 | 90.34 | 90.02 | 90.16 |
| Crude protein                          | 38.62 | 38.43 | 38.71 | 38.68 | 38.70 | 38.59 | 38.77 | 38.8 |
| Crude lipid                            | 6.88  | 7.12  | 6.56  | 6.24  | 7.10  | 6.90  | 6.94  | 7.00  |
| Crude fibre                            | 2.74  | 2.90  | 2.92  | 2.86  | 2.80  | 2.76  | 2.72  | 2.86  |
| Ash                                    | 8.82  | 9.05  | 8.88  | 9.00  | 9.11  | 8.70  | 8.90  | 9.00  |
| NFE                                    | 33.09 | 33.29 | 33.03 | 33.32 | 33.24 | 33.39 | 32.69 | 32.50 |

K, control diet; L, soy lecithin diet; AA, ascorbic acid diet; AAL, ascorbic acid + soy lecithin diet; CC, encapsulated vitamin C diet; CCL, encapsulated vitamin C + soy lecithin diet; Ca, Ca-L-threonate diet; C, Ca-L-threonate + soy lecithin diet; NFE, nitrogen free extract (NFE = %Dry matter (%Protein+% Ash+% Lipid+% Fibre); vitamin- mineral mix provided the following: retinol, 12,000 U; cholecalciferol, 1500 U; tocopherol, 50 U; thiamine, 11 mg; riboflavin, 13 mg; niacin, 19 mg; pantothenic acid, 35 mg; pyridoxine, 11 mg; folic acid, 2 mg; cyanocobalamin, 0.1 mg; choline, 500 mg; inositol, 80 mg; Mn, 35 mg; Zn, 30 mg; Fe, 20 mg; Ca, 1 mg; I, 0.5 mg; Co, 0.06 mg

Table 3. Haematological parameters of brown bullhead fingerlings fed experimental diets for 9 weeks.

| Blood parameter | K | L | AA | AAL | CC | CCL | Ca | CCL | K0 |
|-----------------|---|---|----|-----|----|-----|----|-----|----|
| RBC             | 1.03 ±0.08\(^a\) | 1.13 ±0.09\(^b\) | 2.16 ±0.09\(^a\) | 2.26 ±0.03\(^a\) | 2.21 ±0.13\(^a\) | 2.36 ±0.05\(^a\) | 1.18 ±0.17\(^c\) | 1.22 ±0.08\(^b\) | 1.03 |
| HTC             | 18.28 ±0.75\(^c\) | 18.77 ±0.58\(^c\) | 30.32 ±0.82\(^c\) | 30.95 ±0.38\(^c\) | 30.51 ±0.82\(^c\) | 32.00 ±0.93\(^c\) | 26.47 ±0.91\(^c\) | 25.68 ±2.25\(^b\) | 17.22 |
| MCV             | 179.17 ±14.22\(^c\) | 167.80 ±15.53\(^c\) | 140.61 ±7.97\(^d\) | 136.96 ±2.89\(^d\) | 138.71 ±8.24\(^d\) | 135.73 ±4.44\(^d\) | 228.44 ±30.59\(^d\) | 212.26 ±26.82\(^a\) | 169.82 |
| TRIGL           | 9.49 ±2.75\(^c\) | 8.56 ±0.63\(^c\) | 6.89 ±1.1\(^c\) | 7.97 ±1.29\(^c\) | 8.68 ±1.19\(^c\) | 9.8 ±1.49\(^c\) | 7.55 ±2.26\(^c\) | 9.04 ±2.34\(^c\) | 2.27 |
| TPROM           | 40.75 ±5.93\(^d\) | 48.37 ±4.43\(^c\) | 45.97 ±8.11\(^d\) | 51.23 ±4.77\(^d\) | 59.87 ±11.16\(^d\) | 56.03 ±2.65\(^d\) | 47.42 ±3.01\(^d\) | 46.72 ±4.42\(^d\) | 19.52 |
| GLU             | 11.12 ±0.85\(^c\) | 9.05 ±1.02\(^c\) | 7.93 ±1.03\(^c\) | 5.64 ±1.31\(^c\) | 5.37 ±0.75\(^c\) | 5.51 ±0.68\(^c\) | 9.63 ±1.75\(^c\) | 8.51 ±0.99\(^c\) | 2.34 |

K, control diet; L, soy lecithin diet; AA, ascorbic acid diet; AAL, ascorbic acid + soy lecithin diet; CC, encapsulated vitamin C diet; CCL, encapsulated vitamin C + soy lecithin diet; Ca, Ca-L-threonate diet; CaL, Ca-L-threonate + soy lecithin diet; K0, adaptation period diet. RBC, red blood cells number (10\(^6\) mL\(^{-1}\)); HTC, haematocrit; MCV, mean cell volume of red blood cells; TRIGL, triglycerides (mmol L\(^{-1}\)); TPROM, total protein serum (g L\(^{-1}\)); GLU, glucose (mmol L\(^{-1}\)). Values are means ±SD of three replicate. \(^*\)Values within the same row with different superscripts are significantly different (P<0.05).
in order to detect significant differences between the groups. The results were considered significant at P<0.05. The software used was Statistica 7.0 (StatSoft Inc., 2004).

Results

Growth performance, HSI and VSI of brown bullhead fingerlings fed the experimental diets for 9 weeks are presented in Figure 1 and Table 4. Significant differences of SGRw, FCR and CF among the diets are shown in Figure 1. A diet where encapsulated vitamin C was added (CC) had significantly higher FBW and WG than all the other groups. The results of SGRw showed a strong significant difference among diets CC (1.04) and CC (0.99) compared to K (0.65) and diet CC compared to Ca (0.78). SGRw of control diet (K) showed significantly lower values than other diets. FCR was highest (poorest) in diet K (3.46) with a significant difference compared to CC (2.21), CC (2.30), AA (2.41) and AA (2.48). Diet with added crystallized ascorbic acid and soybean lecithin (AAL) had significantly highest CF (2.07) followed by diets AA (2.48) and CC (1.99). As expected, there was a significant difference of SGRsl between the control diet (K) (0.12) and the diets CC (0.24), CC (0.21), Ca (0.20), AA (0.20) and L (0.19).

Also, significant higher values of SGRsl were determined for diets Ca (0.17) and AA (0.18). The values of the HSI and VSI did not vary among experimental treatments. During the experimental feeding period, mortality wasn’t recorded in any of the feeding groups. Haematological parameters of brown bullhead fed on experimental diet for 9 weeks are given in Table 3. The number of red blood cells (RBC) showed significant differences between AA (2.16), AA (2.26), CC (2.21), CC (2.36) and Ca (1.18), Ca (1.22), K (1.03), L (1.13) diets. Also, Hematocrit showed similar results between the same diets with the exception of significant difference between Ca (26.47), Ca (25.68) and K (18.28), L (18.77). MCV was significantly higher in diets supplemented with calcium threonate Ca (228.44) and Ca (212.2). The values of serum triglycerides are affected differently regarding the addition of vitamin C or soybean lecithin in diets. Combination of lecithin and vitamin C enriched diets (AA, CC) had significantly higher triglyceride values. Significantly lower value was in AA diet (6.89). Plasma total proteins were significantly higher in diets CC (59.87) and CC (56.03). Significantly lower serum glucose concentration was measured in CC (5.37), CC (5.51) and AA (5.64) diets. Except for the MCV, the results from adaptation group K0 were generally lower. Vitamin C content in the diets, liver and muscle tissue of brown bullhead fed on experimental diets for 9 weeks are given in Table 5. Level of ascorbic acid in the analyzed feed samples stored at +4°C were significantly different. Highest concentration of the vitamin C after 9 weeks of storage was in CC (73.3) and CC (72.6) diets. Ca and Ca diets had an average of 90% loss of vitamin C. After 18 weeks, CC diet showed no change in concentration, while the AA and AA showed a strong

Table 4. Growth performance and morphometric indices of brown bullhead fingerlings fed experimental diets for 9 weeks.

|       | K       | L       | AA      | AAL     | CC       | CC      | Ca      | CaL     |
|-------|---------|---------|---------|---------|----------|---------|---------|---------|
| IBW   | 45.06±0.83 | 45.46±0.67 | 45.54±0.3 | 45.53±0.45 | 45.53±0.7 | 45.83±0.15 | 45.74±0.58 | 46.16±0.55 |
| FBW   | 67.41±4.29 | 76.16±2.32 | 80.55±1.95 | 81.69±2.29 | 84.22±2.64 | 87.42±2.55 | 74.27±2.46 | 78.38±2.06 |
| WG    | 22.35±0.53 | 30.7±0.49 | 35.01±2.11 | 36.16±0.74 | 38.89±1.23 | 41.59±2.24 | 28.53±1.41 | 32.22±2.01 |
| SLL   | 1.05±0.08  | 1.71±0.11 | 1.6±0.08  | 1.59±0.12  | 1.57±0.03  | 2.11±0.13  | 1.45±0.11  | 1.8±0.07  |
| SGRsl | 0.12±0.05  | 0.19±0.07  | 0.16±0.08  | 0.20±0.09  | 0.21±0.07  | 0.24±0.08  | 0.17±0.07  | 0.20±0.06  |
| HSI   | 3.65±0.59  | 3.44±0.4  | 3.51±0.47  | 3.29±0.32  | 3.47±0.44  | 3.27±0.77  | 3.78±0.56  | 3.68±0.45  |
| VSI   | 9.55±0.54  | 10.09±2.5  | 10.65±1.2  | 10.42±1.3  | 10.55±1.9  | 10.08±2.0  | 9.92±1.4  | 9.91±1.9  |

K, control diet; L, soy lecithin diet; AA, ascorbic acid diet; AAL, ascorbic acid + soy lecithin diet; CC, encapsulated vitamin C diet; CC, encapsulated vitamin C + soy lecithin diet; CC, Ca-L-threonate diet; Ca, Ca-L-threonate + soy lecithin diet. IBW, initial body weight (g fish⁻¹); FBW, final body weight (g fish⁻¹); WG, weight gain (g); SLI, standard length increments (cm); SGRsl, specific growth rate; FCR, feed conversion ratio; CF, condition factor. Values are means ±SD of three replicates. a-d Values within the same figure with different superscripts are significantly different (P<0.05).
decrease of 84%. Ca and CA diets showed no vitamin C concentration after 18 weeks of storage. The variations in liver and muscle vitamin C concentration correlated with those of diets concentration. As expected, diets resulting in a significantly higher amount of ascorbic acid in liver were CC (145.8) and CC (145.6) and also in the muscle tissue CC (87.7) and CCL (88.4), followed by AA and AA diets. Basic chemical parameters and fatty acid composition of muscle and liver tissues of brown bullhead fingerlings fed on experimental diets for 9 weeks are given in Tables 6, 7 and 8. The chemical parameters in liver and muscle tissue showed significant differences depending on the diet. The highest concentrations of muscle protein were in AA (18.06), CC (18.00), CC (17.91) and AA (17.86) diets and significantly different from all the other diets. Also, AA (2.97), CC (2.89) and AA (2.83) diets, with highest concentrations of muscle crude fat, were significantly different from diets L (2.65), CA (2.66), CCL (2.66) and CA (2.69). Significantly lower amount of moisture in muscle tissue contained the diets CC (77.60) and CA (77.80). Highest concentration of liver crude fat was found in K (2.92) and AA (2.88) diets that showed a significant difference to L diet (2.68). Average concentrations of ash in muscle showed no significant difference. Compared to all the other diets, K showed lower concentrations of crude fat in liver and muscle tissue and lower muscle crude protein. The results of the fatty acid composition of liver and muscle tissues showed a significant difference between the diets. Palmitic acid (16:0) represented the largest percentage of saturated fatty acid, but showed some variation among the diets. The lowest values found in muscle tissue were observed in diets Ca (12.05) and K (12.54) while the highest percentage share in total lipids were determined by the diets CC (13.81) and L (13.56). This ratio was almost identical to the relation established in the liver tissue, but in a higher share of percentage. Oleic acid (18:1) was dominant in unsaturated fatty acids profile of muscle and liver tissue. The differences between the groups were those of liver tissue, ranging from the lowest value of the group K (17.36), to the highest percentage share of CC group (26.55). This difference was statistically significant, and was also presented in relation to the diets CA, AA, and CA. Also, we obtained significantly higher results on DHA 22:6 ω3 in the liver (AA, CA, CC, CCL) and muscle tissue (CC, CCL) in brown bullhead fish fed on vitamin C diets regardless of the lecithin supplementation.

### Table 5. The concentration of vitamin C in the diets and liver and muscle tissues of brown bullhead fingerlings fed experimental diets for 9 weeks.

| Diets, mg kg⁻¹ | Liver, μg g⁻¹ | Muscle, μg g⁻¹ |
|----------------|---------------|---------------|
|                | Beginning     | After 9 weeks | After 18 weeks |
| K (n=6)        | 0             | -             | -             |
| L (n=6)        | 0             | -             | -             |
| AA (n=6)       | 100           | 48.2          | 7.6           |
| AA (n=6)       | 100           | 47.4          | 8.2           |
| CC (n=6)       | 100           | 72.6          | 70.8          |
| CC (n=6)       | 100           | 73.3          | 73.4          |
| Ca (n=6)       | 100           | 9.1           | 0             |
| Ca (n=6)       | 100           | 11.0          | 0             |

### Table 6. Basic chemical parameters of muscle and liver tissues of brown bullhead fingerlings fed experimental diets for 9 weeks, on wet weight basis.

| Diet   | Lipid, % | Protein, % | Moisture, % | Ash, % | Liver Lipid, % |
|--------|----------|------------|-------------|--------|---------------|
| K0 (n=8) | 2.24±0.80° | 17.11±1.72° | 78.53±1.82° | 1.32±0.21 | 2.28±0.37° |
| L (n=12)  | 2.76±0.73° | 16.87±1.80° | 78.10±2.58° | 1.21±0.51 | 2.92±1.00° |
| AA (n=12) | 2.65±1.25° | 17.36±0.96° | 78.00±3.00° | 1.11±0.60 | 2.68±0.51° |
| Ca (n=12)  | 2.97±0.92° | 17.86±1.63° | 77.90±2.40° | 1.20±0.34 | 2.68±0.42° |
| AA (n=12) | 2.83±0.90° | 18.06±2.04° | 77.64±3.10° | 1.20±0.52 | 2.75±0.71° |
| CC (n=12)  | 2.89±0.71° | 17.91±1.10° | 77.84±1.81° | 1.16±0.40 | 2.80±0.46° |
| CC (n=12)  | 2.66±0.76° | 18.00±1.48° | 77.60±2.15° | 1.21±0.37 | 2.71±0.58° |
| Ca (n=12)  | 2.66±1.13° | 17.07±1.72° | 78.00±1.78° | 1.18±0.21 | 2.90±0.34° |
| Ca (n=12)  | 2.69±0.84° | 17.64±1.62° | 77.86±2.40° | 1.21±0.40 | 2.75±0.30° |

K0, adaptation period diet; K, control diet; L, soy lecithin diet; AA, ascorbic acid diet; AA, ascorbic acid + soy lecithin diet; CC, encapsulated vitamin C diet; CCL, encapsulated vitamin C + soy lecithin diet; CA, Ca-L-threonate diet; CA, Ca-L-threonate + soy lecithin diet. Values are means ±SD of three replicates. °Values within the same column with different superscripts are significantly different (P<0.05).
Discussion

The results of the production parameters are in agreement with the data from previous, similar studies on channel catfish (Andrews et al., 1989; Robinson, 1992; Li et al., 1993). Opposite results were found in rainbow trout (Kavadias et al., 1994; Takeuchi et al., 1996). Our blood results correspond to the results obtained by Duncan and Lovell (1994) on channel catfish (Ictalurus punctatus) and those by Papp et al. (1995) on European catfish (Silurus glanis). Diets with vitamin C supplementation, in an appropriate form (AA and CC), showed significantly higher RBC, higher HCT and lower MCV. The MCV is an index of the size of the RBCs and the results of MCV are derived from HCT, HGB, and RBC counts (Corbett, 2008). Results of high RBC count and hematocrit values are very important for the oxygen transport in the conditions of intensive culture. Triglycerides constitute the major class of neutral lipid and they are the primary class for lipid storage and energy provision (Tocher, 2003). The levels of triglycerides are considered to be major indices of the health status of teleosts, but they are not a reliable indicator of fish nutritional status (Wagner and Congleton, 2004). Kavadias et al. (2004) demonstrated that high concentrations of plasma triglycerides depend on the feeding rate, while low levels can be explained by fish low-energy diet. Juvenile shrimp (Litopenaeus vannamei) fed on the 3% soy lecithin diets showed higher triglyceride concentration in serum than those fed on the other experimental diets (Hu et al., 2011). Taking into account the positive effects of vitamin C on fish growth and with combine lecithin supplementation, higher results of serum triglycerides in diets supplemented with vitamin C and lecithin in our research are in agreement with the data from previous studies. Verhac and Gabaudan (1994) determined a stimulatory effect of vitamin C on the production of oxygen in salmonids; vitamin C not only acted as a radical-trapping antioxidant, but, when its concentration increased, it has also

Table 7. Composition of muscle tissue fatty acids of brown bullhead fingerlings fed experimental diets for 9 weeks, in percentage of total fatty acids.

| Fatty acids | K (n=6) | L (n=6) | AA (n=6) | AAk (n=6) | CC (n=6) | CCk (n=6) | Ca (n=6) | CaL (n=6) |
|------------|--------|--------|---------|-----------|---------|---------|---------|---------|
| 12:0       | 0.1±0.01 | 0.11±0.08 | 0.14±0.08 | 0.10±0.08 | 0.12±0.08 | 0.12±0.06 | 0.12±0.06 | 0.11±0.08 |
| 14:0       | 2.5±0.10 | 2.63±0.09 | 2.92±0.12 | 2.64±0.09 | 2.58±0.13 | 2.63±0.09 | 2.78±0.17 | 2.60±0.10 |
| 16:0       | 12.5±0.08 | 13.56±0.16 | 13.55±0.18 | 13.17±0.21 | 13.41±0.18 | 13.81±0.19 | 12.05±0.19 | 12.74±0.21 |
| 16:1       | 7.47±0.42 | 8.26±0.10 | 8.46±0.14 | 8.82±0.15 | 8.32±0.15 | 8.55±0.21 | 7.18±0.16 | 8.76±0.09 |
| 18:0       | 3.63±0.08 | 3.42±0.31 | 3.84±0.18 | 3.70±0.21 | 3.52±0.14 | 3.74±0.18 | 3.78±0.31 | 3.50±0.20 |
| 18:1ω9     | 20.06±0.65 | 22.10±0.27 | 26.88±0.31 | 28.32±0.14 | 27.31±0.18 | 28.15±0.09 | 20.32±0.09 | 23.15±0.11 |
| 18:2ω6     | 9.97±0.20 | 11.63±0.31 | 11.87±0.17 | 12.30±0.23 | 13.71±0.14 | 14.91±0.32 | 10.07±0.20 | 12.09±0.20 |
| 18:3ω3     | 3.58±0.05 | 5.80±0.08 | 5.86±0.11 | 7.01±0.10 | 5.26±0.09 | 6.23±0.16 | 3.69±0.18 | 4.27±0.14 |
| 20:0       | 0.15±0.01 | 0.10±0.02 | 0.12±0.02 | 0.12±0.02 | 0.08±0.01 | 0.10±0.01 | 0.09±0.03 | 0.11±0.02 |
| 20:1ω9     | 1.04±0.33 | 1.33±0.13 | 1.34±0.28 | 1.42±0.21 | 1.38±0.28 | 1.62±0.09 | 1.00±0.13 | 1.16±0.30 |
| 20:2ω6     | 0.45±0.17 | 0.68±0.13 | 0.69±0.09 | 0.62±0.09 | 0.60±0.20 | 0.73±0.17 | 0.41±0.09 | 0.52±0.11 |
| 20:3ω6     | 1.07±0.21 | 1.30±0.21 | 1.44±0.30 | 1.72±0.14 | 1.53±0.22 | 1.78±0.24 | 1.02±0.18 | 1.29±0.18 |
| 20:4ω6     | 4.76±0.46 | 5.41±0.31 | 5.12±0.17 | 5.85±0.24 | 5.12±0.26 | 6.02±0.15 | 4.32±0.30 | 4.96±0.21 |
| 20:5ω3     | 5.75±0.12 | 6.78±0.29 | 6.78±0.2 | 7.22±0.18 | 6.40±0.11 | 7.61±0.17 | 5.54±0.13 | 6.27±0.09 |
| 22:4ω6     | 0.64±0.08 | 0.65±0.10 | 0.55±0.10 | 0.54±0.18 | 0.52±0.11 | 0.55±0.14 | 0.70±0.11 | 0.60±0.09 |
| 22:5ω3     | 3.22±0.25 | 3.83±0.16 | 2.22±0.23 | 2.13±0.11 | 2.16±0.17 | 2.16±0.25 | 3.07±0.09 | 3.39±0.11 |
| 22:6ω3     | 6.40±0.41 | 7.12±0.26 | 7.38±0.26 | 7.62±0.36 | 7.92±0.17 | 7.98±0.16 | 6.12±0.18 | 6.28±0.24 |
| SUMω3      | 18.95 | 22.08 | 22.24 | 23.98 | 18.42 | 20.21 | 21.74 | 23.98 |
| SUMω6      | 16.89 | 19.67 | 20.57 | 21.13 | 16.52 | 19.46 | 21.67 | 23.99 |
| Saturated fatty acids | 18.93 | 19.82 | 20.57 | 19.73 | 18.82 | 17.06 | 19.71 | 20.40 |
| Unsaturated fatty acids | 28.57 | 31.69 | 38.56 | 28.50 | 33.09 | 37.01 | 38.32 |
| Saturated fatty acids/ unsaturated fatty acids | 0.40 | 0.38 | 0.34 | 0.35 | 0.40 | 0.37 | 0.33 | 0.32 |

K, control diet; L, soy lecithin diet; AA, ascorbic acid diet; AAk, ascorbic acid + soy lecithin diet; CC, encapsulated vitamin C diet; CCk, encapsulated vitamin C + soy lecithin diet; Ca, Ca-L-threonate diet; CaL, Ca-L-threonate + soy lecithin diet. Values are means ±SD of three replicates. *Values within the same row with different superscripts are significantly different (P<0.05).
undergone the self oxidation. Saroglia et al. (1990) concluded that the increase of temperature and storage time, positively correlated with the degradation of ascorbic acid in the pelleted feed. After 9 weeks of storage, the result of 70% of residual encapsulated vitamin C concentration in CC and CCL diets were similar to Skelbaek et al. (1990) results in a research on stability in fish feed and bioavailability to rainbow trout of two ascorbic acid forms. Our results of crystallized ascorbic acid concentration in AA and AAL diets were similar to the results obtained by Marchetti and Tossani (1990) and Volker and Fenster (1994) who concluded that the loss of crystalline ascorbic acid in pelleted feed during 12 weeks of storage was 44% and 34%, respectively. Ascorbic acid liver content is usually considered an indicator of the vitamin C status (Gabaudan et al., 1991; Sandnes and Waagbo, 1991; White et al., 1993) and has been described as a physiological indicator of stress (Wedemeyer and Yasutake 1977; Wedemeyer and McLeay, 1981; Thomas et al., 1982; Thomas, 1990). Also, because of the role that vitamin C apparently plays in the synthesis of corticosteroids (Sauberlich, 1984), feeding high levels of vitamin C has been proposed as beneficial in reducing the effects of physiological stress in fish (Jaffa, 1989; Hardie et al., 1991) and chickens (Brake et al., 1992; Purdie and Williams, 1992). We found that the highest level of vitamin C content in the liver and muscle tissues were in the diets supplemented with encapsulated and crystallized ascorbic acid, alone or in combination with the soybean lecithin. Similar results were obtained by Kanazawa et al. (1992) on Japanese amberjack (Seriola quinquemaculata), El Naggar and Lovell (1991) and by Li et al. (1998) in channel catfish (Ictalurus punctatus) and Grant et al. (1989) and Matusiewicz et al. (1994) on rainbow trout (Oncorhynchus mykiss). Due to the vitamin C deficiency, structural deformities such as scoliosis and lordosis have been observed in channel catfish (Wilson, 1973; Andrews and Murai, 1975; Lim and Lovell, 1978; Wilson et al., 1989). A value of less than 26 µg g⁻¹ of liver has been suggested to indicate the vitamin C deficiency in channel catfish (Lim and Lovell, 1978). Although, the concentration of the vitamin C in liver and muscle tissues of experimental diets K, L, Ca, Ca, Ca, suggests deficiency, neither abnormal fish deformations nor any of the characters common to the above deficiency disease were observed. Dabrowski (1986) reported that the deficiency of ascorbic acid is affected by fish size, feeding duration and previous nutritional status. Ai et al. (2006) reported that there was no ascorbic acid in the muscles of yellow croaker (initial body weight, 17.82 g) fed on ascorbic acid free diet, although vitamin C was still detected in its liver (16.6 µg g⁻¹ wet weight). This is similar to the results of Ai et al. (2004) who also detected no ascorbic acid in the muscle of sea bass, but, after feeding ascorbic acid free diet for 10 weeks, detected it in its liver tissue (16.5 µg g⁻¹ wet weight). Li et al. (1998) fed channel catfish (6.5 g initial weight) on basal diet which contained a residual amount (3.3 mg kg⁻¹) of ascorbate activity and after 10 weeks found 11.0 µg g⁻¹ wet weight of ascorbic acid concentration in liver tissue. In comparison to many other studies on the vitamin C requirements (Li et al., 1998; Gouillou-Coustans et al., 1998; Shiau and Hsu, 1999; Ai et al., 2004), in the present study, the fish size (45 g initial weight) was relatively larger. Depending on the diet and amount of supplementation, we found wide range of vitamin C content (11.8-
145.8 μg g⁻¹ in liver and (8.0-88.4 μg g⁻¹) in muscle tissues. These results, as well as those of other researchers, confirmed that large fishes retain vitamin C in their tissue longer than the smaller ones, which lose it more quickly. Consequently, it is important that the ascorbic acid functions be further investigated in terms of clinical deficiency signs when dealing with smaller fishes and experiments that last longer. Poston (1991a, 1991b) concluded that lecithin significantly increased the percentage of total fat in the body of rainbow trout and Atlantic salmon fry, while in the adult stage and with well-balanced food, the fat disappeared. Our results showed lower values of muscle and liver fat in fish fed with soy lecithin. Dietary phospholipids also may impart beneficial effects due to their emulsification properties and the role in lipid absorption and transport. Koven et al. (1993) demonstrated that the addition of lecithin to the diet of larval gilthead seabream (Sparus aurata) significantly increased the incorporation of free fatty acids into the neutral and polar fraction of body lipid. Moreover, in comparison to the fish fed on diets containing cuttlefish oil, the fish fed on dietary lecithin had both, increased consumption rate and incorporation efficiency, which indicates possible emulsification activity of the lecithin. Similar to our results, when Robinson (1992) (according to Hertrampf, 1992) reduced total body fat of muscle tissue by adding lecithin and choline in the feed mixture, the structure of unsaturated fatty acids significantly improved in favour of linoleic, linolenic and arachidonic acid. Takeuchi et al., (1992) supplemented phosphatidycholine (0.5, 1.0, 1.5, 2.0%) and phosphatidyl ethanolamine (1.5%) in diets for juvenile striped jack (Pseudocaranx dentex) and pointed out the supplementation of 1.5% phosphatidycholine, which promoted good growth up to 3.3 g in body weight. Also, in diets supplemented with phosphatidycholine and phosphatidylethanolamine, the structure of unsaturated fatty acids improved in favor of linoleic (18:2n-6) and α-linolenic (18:3n-3) acids, which is similar to our results on diets supplemented with lecithin. The level of fatty acids in the tissue of milkfish (Chanos chanos) was in general higher when fed on essential fatty acids and vitamin C-enriched live food diets than the fish fed on enrichment-free diets (Gapasin et al., 1998). Except for the calcium threonate (Ca, CaCl) diets, we obtained similar results in our research with the levels of palmitic (16:0), oleic (18:1n-9), eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids.

Conclusions

The data resulted from the present study indicate the dominant role of encapsulated vitamin C in comparison to other forms of vitamin C. Crystallized ascorbic acid also had a strong effect on individual weight gains, condition factor, SGR-w and FCR. According to the results on brown bullhead, calcium threonate did not justify the earlier findings on its potent activity on vitamin C. The haematological parameters showed positive influence of crystallized ascorbic acid and protected vitamin C, independently of soybean lecithin. Vitamin C content of muscle tissue was not homogeneous among different groups and was significantly higher in AA, CC, CC and AA feeding groups. Soybean lecithin added to fish feed supplemented with encapsulated form of vitamin C, indicated positive production effect.

This research showed that the use of soybean lecithin phospholipids together with vitamin C improved the fatty acid composition in muscle and liver tissues. In other words, the nutritional quality of eatable parts of fish body has been enhanced.

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Vitamin C-lecithin in diet of b. bullhead

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