# QUANTIFICATION OF VITAMIN C (ASCORBIC ACID) REQUIREMENTS OF *HETEROBRANCHUS LONGIFILIS* FINGERLINGS.

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**Abstract**

A feeding experiment was conducted to quantify the minimum dietary vitamin C requirement of *Heterobranchus longifilis* fingerlings and the minimal dietary inclusion levels that will meet these requirements after degradation from feed processing and storage. Fish were fed a basal diet with 42.5% crude protein for a conditioning period of 2 weeks. Following conditioning, fingerlings with initial mean weight, 2.3 ± 0.3 g were stocked as groups of 20 fingerlings into 30 litre tanks in a mini-flow through experimental system and fed the basal diet and experimental diets supplemented with 0, 50, 100, 150, 200, or 250 mg of L-ascorbic acid $kg^{-1}$ diet obtained from TUYIL PHARM. INDUSTRIES, Ilorin, included into the basal diet by replacing part of the silica component and fed to triplicate groups for 20 weeks. Fish fed the control (0 mg vitamin C $kg^{-1}$) diet exhibited deficiency signs including lordosis, caudal fin deformity, skin erosion and significantly suppressed weight gain and higher condition factor. Protein efficiency ratio and specific growth rate were significantly improved with increasing levels of vitamin C up to 200mg $kg^{-1}$ diet. Tissue (liver, kidney, gills and muscle) ascorbate concentration generally reflected dietary inclusion levels with the significant lowest level occurring in the control groups. Vitamin C analysis after feed processing revealed 18-21% loss. The least mean squares error regression analysis of weight gain data on inclusion level of vitamin C revealed that the minimum dietary requirement of *H. longifilis* is 82.2 ± 0.2 mg vitamin C $kg^{-1}$ diet which corresponds to 100 mg of vitamin C $kg^{-1}$ diet based on data from this study.

**Key words:** Vitamin C, requirements, deficiency signs, *Heterobranchus longifilis*.

## Introduction

Vitamin C (Ascorbic acid) belongs to the water-soluble group of vitamin. Its importance in fish diets and characteristics had been described by various authors (Brander and Pugh 1977; Lim and Lovell, 1978; De Silva and Anderson, 1995; Dabrowski, 2001). Ibiyo *et al.*, (2006) also has a detailed account of it. Lack of the enzyme gulonolactone oxidase responsible for the synthesis of vitamin C in many fish's liver and kidney (Dabrowski, 1990; Fracalossi *et al.*, 2001) demands dietary inclusion to meet the nutritional requirement for optimum performance of the developing fish (Dabrowski, 1990; Ai *et al.*, 2004).

*Heterobranchus* species is now widely cultured in Nigeria. Its protein requirements has been established as 40% and 42.5% (Fagbenro *et al.*, 1992 and Eyo, 1995) respectively. The preliminary work to test whether *H. longifilis* needs vitamin C in its nutrition revealed that vitamin C is a necessity in this fish species (Ibiyo *et al.*, 2005; 2006). Unlike Sturgeons *acipenser* that possess gulonolactone dehydrogenase and can synthesize ascorbic acid from glucose; therefore they have little dependence on dietary sources for vitamin C (Dabrowski, 2001b). Nevertheless, there is a dearth of information on the actual level of vitamin C required by *Heterobranchus longifilis* for optimum growth. Although, 50 mg vitamin C $kg^{-1}$ diet was able to eliminate the deficiency symptoms observed in the group that was devoid of vitamin C it might not be the optimum level required by *H. longifilis* (Ibiyo *et al.*, 2005; 2006). This work was designed to study the vitamin C requirements of *H. longifilis* fingerlings and the minimal dietary inclusion levels that will meet these requirements after degradation from feed processing and storage.

## Materials and Methods

A completely randomised design with three replicates was used for the experiment. Fingerlings of *H. longifilis* were obtained and conditioned for 2 weeks prior to the start of the experiment. Feed preparation and storage was as described by Ibiyo *et al.*, (2005; 2006). Sample was taken for proximate analysis. During acclimatization they were fed a basal diet with 42.5% crude protein which finally served as the control diet (Table 1). Feed supply was stopped 2 days to the commencement of the feeding experiment. After acclimatization the fingerlings were weighed into 30-litre circular plastic tanks in a mini-flow through experimental system previously described by Madu (1989). The average initial weight was $2.3 \pm 0.3$ grams. The replicates with 20 fingerlings each were randomly allocated to the treatments. Some of the fingerlings were sacrificed and taken for analysis of initial vitamin C content and proximate analysis.

Six iso-nitrogenous diets containing graded levels of vitamin C (L-ascorbic acid) 0, 50, 100, 150, 200 or 250 mg $Kg^{-1}$ dry diet are being evaluated. The L-ascorbic acid was obtained from TUYIL PHARM. INDUSTRIES, Ilorin. The increasing vitamin C content was achieved by substituting the silica in the basal diet (Table 2) with vitamin C. Representative samples of the diets were analysed for vitamin C content to determine percentage retention of vitamin C immediately after feed processing and after one week of feed storage. Each of the six dietary treatments was fed for 20 weeks to the randomly assigned replicate tanks. A fixed feeding regime of 5% body weight per day divided into two equal feeds and given between the hours of 800 900 and 17.00 18:00 was adopted except on sampling days in which, time of feeding was altered. The feed supply at 5% body weight continued in the first 8 weeks of the experimental period after which it was reduced to 3% body weight per day due to poor feed acceptability of the control group. Prevention of microbial growth that could possibly alter the experiment was as described by Ibiyo *et al.*, (2005). Feed preparation was carried out fortnightly to prevent long storage.

Sampling was carried out fortnightly with the fish bulk weighed per tank and feeding rate subsequently adjusted. During each weighing, time of draining and washing of tanks mortality and the condition of fish were observed and recorded. Photographs of fish with observed abnormalities were taken with a digital camera at each time of occurrence. At the end of the experiment, six (6) fish per replicate of diet 2 to 6 and three (3) in diet 1 (control treatment) were sacrificed for blood samples to determine some blood parameters and kidney, liver, gills and whole body samples were pooled from fish of each replicate to determine vitamin C content and proximate analysis of the final fish. Micrographs of the gills were also taken with ZEISS-Stemi 2000-C Photomicrograph. X-ray of the fish from with and without vitamin C were taken at the General Hospital, New Bussa.

The water quality parameters were monitored by the staffs of Limnology Division of National Institute for Freshwater Fisheries Research and average value for temperature, dissolved oxygen, hydrogen ion concentration (pH) and conductivity were 29.5°C, 5.8 mg $l^{-1}$, 7.2 units and 220 µmhos $cm^{-3}$ respectively.

Proximate composition analysis was carried out using the Association of Analytical Chemists methods (AOAC, 2000). Crude fat was determined using petroleum ether (40 -60 Bp) extraction method with Soxhlet extractor. Vitamin C concentration was determined by titrimetric and high-performance liquid chromatography methods. The Pack cell volume (PCV) or haematocrit was determined by the microhaematocrit method and haemoglobin was determined using the cynomethaemoglobin method and the coulter haemoglobinometer (Coulter, U.K.).

**Table 1: Composition of the basal diet**

| Ingredients | Inclusion levels |
|---|---|
| Clupeid meal (65%) | 30.00 |
| Soybean meal (45%) | 35.00 |
| Groundnut cake (40%) | 13.82 |
| Maize bran (12.5%) | 13.35 |
| Oil | 2.00 |
| Starch | 2.00 |

| | |
|---|---|
| Bone meal | 2.00 |
| Vitamin C-free Premix* | 1.00 |
| Methionine | 0.50 |
| Salt | 0.25 |
| Silica | 0.08 |
| Total | 100 |
| **Proximate composition** | |
| Crude protein % | 42.26 |
| Crude fat % | 11.55 |
| Crude Fibre % | 1.20 |
| Ash % | 8.89 |
| NFE % | 27.91 |
| Moisture content % | 8.19 |
| Metabolizable energy (Kcal/100g) ** | 375.00 |

** Metabolizable energy (Kcal/100g) is based on standard physiological values of 4.5, 3.3 and 8 Kcal/g for protein, carbohydrate and fat respectively (Brett and Grooves, 1979; Panaflorida , 2002).

* Provides per kg diet: Vitamin A, 50000 IU, Vitamin $D_3$ 25000 IU, Vitamin E 160mg, Vitamin K 8mg; Vitamin $B_1$ 12mg; Vitamin $B_2$ 22mg; Vitamin $B_5$ 20mg; Vitamin $B_{12}$ 220 mg; Biotin 4 mg; Zinc 320 mg; Iodine 6 mg; Calcium pantothenate 46 mg; Cupper 34 mg; Cobalt 1.2 mg; Selenium 0.48 mg; Antioxidant 480 mg; Choline chloride 0.1 mg.

**Table 2: Composition of the experimental diets**

| Ingredients | % inclusion levels | | | | | |
|---|---|---|---|---|---|---|
| | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 | Diet 6 |
| Vitamin C carrier | 0.00 | 0.015 | 0.03 | 0.045 | 0.06 | 0.07 |
| Silica | 0.08 | 0.065 | 0.05 | 0.035 | 0.02 | 0.005 |
| Clupeid meal (65%) | 30.00 | 30.00 | 30.00 | 30.00 | 30.00 | 30.00 |
| Soybean meal (45%) | 35.00 | 35.00 | 35.00 | 35.00 | 35.00 | 35.00 |
| Groundnut cake (40%) | 13.82 | 13.82 | 13.82 | 13.82 | 13.82 | 13.82 |
| Maize bran (12.5%) | 13.35 | 13.35 | 13.35 | 13.35 | 13.35 | 13.35 |
| Vegetable Oil | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| Starch | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| Bone meal | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 | 1.50 |
| Vitamin C free Premix* | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| Methionine | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Chromic oxide | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Salt | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| Total | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 | 100.00 |

* As in Table 1

## Calculations and Statistical Analysis

The following variables were calculated:

Weight Gain (WG) = $W_t$ $W_0$

Specific growth rate (SGR) = (ln $W_t$ ln $W_0$) X 100/t

Feed efficiency ratio (FER) = Wet WG in g/dry feed fed in g (Hardy and Barrows, 2002)

Condition factor = $\frac{Wt \times 100}{L^3}$

Survival = $N_t \times 100/N_0$

Where WG is weight gain; $W_t$ and $W_0$ were final and initial fish weights, respectively; L is final length of each fish; $N_t$ and $N_0$ were final and initial numbers of fish in each replicate, respectively; t is the experimental period in days.

The data obtained from the trial were subjected to one way analysis of variance. When ANOVA identified significant difference among groups, multiple comparison tests among means were performed using Duncan's new multiple range test with SPSS 10.0 for windows in the computer. For each comparison, statistically significant differences were determined by setting the aggregate type I error at 5% ($P<0.05$). Weight gain and specific growth rate data were also subjected to least square regression analysis to estimate the minimum dietary vitamin C requirement of *Heterobranchus longifilis* fingerlings.

## Results and Discussion

The results of the vitamin C retention analysis after feed processing are presented in Table 3. The loss of vitamin C during feed processing ranged from 18 21%. The higher the level of inclusion the greater the quantity lost. The growth performances of fish in the first seven weeks in this trial were similar to that observed in the preliminary studies of Ibiyo *et al.*, (2005; 2006) to establish necessity of vitamin C in the nutrition of *Heterobranchus longifilis* fingerlings. The weight gain of *H. longifilis* fed the control diet was significantly ($P<0.05$) reduced compared with fish fed the diets with graded levels of vitamin C (Table 4). Feed efficiency of fish fed the various diets also responded similarly to weight gain, with fish fed the basal diet (control) exhibiting significantly reduced performance with respect to these parameters. Those fish fed the basal diet also had a significantly ($P<0.05$) lower percentage survival compared with fish fed diets with supplemental levels of vitamin C. The significant differences also revealed that the supplemented group would have performed better if feed supply was not minimised after the 8th week due to unacceptability of feed exhibited by the control group. These results agree well with previous studies on some other fish (Eya and Mgbenka 1990; Al-moudi *et al.*, 1992; Gouillou-Coustans *et al*, 1998; Shiau and Hsu, 1999; Sealey and Gatlin 1999; Wang *et al.*, 2003; Ai *et al.*, 2004). The reduction in growth performance of fish fed the control diet in the present study seems to indicate that AA has a specific effect on growth as first suggested by Ram (1966).

Fish fed the basal diet began to exhibit overt signs of vitamin C deficiency apart from reduced weight gain which includes skin and opercula erosion (Plate 1), lordosis (Plate 2) and loss of equilibrium after 8 weeks. Tail deformities (Plate 3) were evident at the 14th week and it progresses till the end of the experiment. These deficiency symptoms have also been observed in several other fish species fed diets deficient in vitamin C including cyprinids (Dabrowski, *et al.*, 1988), ictalurids (Miyasaki *et al.*, 1985), cichlids (Soliman *et al.*, 1994; Shiau and Hsu, 1995), scophtalmids (Coustans *et al.*, 1990), African catfish (Eya and Mgbenka 1990) Korean rockfish (Lee *et al.*, 1998) and red drum (Aguirre and Gatlin, 1999) that were fed vitamin C deficient diets. The time of occurrence of deficiency symptoms in the control group in this trial is an indication that *H. longifilis* fingerlings were able to depend on stored ascorbate for nine weeks (i.e. 2 weeks of accliamatization plus 7 weeks into the experimental period) for normal physiological functions.

This time is however longer than that observed in juvenile Olive flounder, *Paralichthys olivaceus* (Wang *et al.*, 2002) which exhibited symptoms after 4 weeks into the trial period but similar to those observed in those fish mentioned above. The earlier occurrence of symptoms in Olive flounder was attributed to size of the fish used, the recirculating experimental system used which is more stressful and may require a higher vitamin C than those reared in a flow through system used in some other experiment as in this present study and the type of diet (Soliman *et al.*, 1994; Wang *et al.*, 2002). There were significant ($P<0.05$) difference in tissue ascorbate concentration and haematology of fish fed the graded levels of vitamin C. The vitamin C concentration in the liver, kidney, gills and muscle of fish fed graded levels of ascorbic acid were positively correlated with dietary levels of vitamin C (Wang *et al.*, 2002).

The haematocrit and haemoglobin improved significantly ($P<0.05$) with increasing graded levels of dietary vitamin C. The 50, 100 and 150 mg AA $kg^{-1}$ diet were significantly ($P<0.05$) better than the 200, 250 and 0 mg AA $kg^{-1}$ diet supplementation with respect to either of these parameters (Table 4). Soliman *et al.*, (1994) also reported a depression of these parameters in the fish fed diet devoid of vitamin C.

Due to the significant effect of dietary vitamin C on weight gain of *H. longifilis* fingerlings, this response was used to quantify the minimum dietary requirement. Least mean square error regression analysis of the weight gain data against graded levels of dietary vitamin C resulted in a requirement estimate of 82.2 $\pm$ 0.2 mg AA $kg^{-1}$ diet which corresponds with the 100 mg AA $kg^{-1}$ supplementation including that present endogenously before commencement of the trial.

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Table 3: Vitamin C retention

| Diets | Vitamin C content immediately after feed processing | | Vitamin C content after 1 week of storage | |
|---|---|---|---|---|
| | mg / Kg of diet | % | mg / Kg diet | % |
| 1 | 0.01 | 0 | 0.0 | 0 |
| 2 | 39.5 | 79.0 | 37.1 | 74.3 |
| 3 | 82.2 | 82.2 | 77.3 | 77.3 |
| 4 | 122 | 81.3 | 114.7 | 76.5 |
| 5 | 162 | 81.5 | 152.3 | 76.1 |
| 6 | 206 | 82.4 | 193.6 | 77.5 |

Table 4: Effects of vitamin C on growth parameters, liver ascorbate concentration and haematology of *H. longifil is* fingerlings fed the diets with graded levels of vitamin C (0 20 weeks).

| Parameters | Diet 1 | Diet 2 | Diet 3 | Diet 4 | Diet 5 | Diet 6 | SEM |
|---|---|---|---|---|---|---|---|
| **Growth indices:** | | | | | | | |
| Weight gain (g/fish) | $22.40^{d}$ | $35.2^{c}$ | $65.9^{a}$ | $66.2^{a}$ | $66.3^{a}$ | $55.7^{b}$ | 0.637 |
| Feed conversion ratio | $2.56^{a}$ | $1.92^{c}$ | $1.73^{d}$ | $1.76^{d}$ | $1.73^{d}$ | $2.02^{b}$ | 0.018 |
| Feed Efficiency ratio (%) | $38.95^{d}$ | $51.98^{b}$ | $57.47^{a}$ | $56.49^{a}$ | $57.53^{a}$ | $49.34^{c}$ | 0.511 |
| Protein efficiency ratio | $0.92^{e}$ | $1.22^{cb}$ | $1.35^{a}$ | $1.25^{b}$ | $1.14^{dc}$ | $1.12^{d}$ | 0.029 |
| Specific growth rate (%) | $1.66^{c}$ | $2.11^{b}$ | $2.37^{a}$ | $2.38^{a}$ | $2.43^{a}$ | $2.20^{b}$ | 0.042 |
| Survival (%) | $50.00^{a}$ | $83.33^{b}$ | $93.33^{b}$ | $95.00^{b}$ | $95.00^{b}$ | $96.66^{b}$ | 6.230 |
| Condition factor | $0.95^{a}$ | $0.74^{c}$ | $0.81^{ba}$ | $0.79^{bc}$ | $0.82^{ba}$ | $0.81^{ba}$ | 0.008 |
| **Tissue Ascorbic acid content** | | | | | | | |
| Liver (µg /g tissue) | $10.86^{d}$ | $45.83^{c}$ | $57.4^{b}$ | $67.93^{a}$ | $67.93^{a}$ | $67.90^{a}$ | 2.651 |
| Kidney (µg /g tissue) | $18.30^{a}$ | $42.10^{d}$ | $66.10^{c}$ | $80.63^{b}$ | $98.46^{a}$ | $98.53^{a}$ | 2.663 |
| Gills (µg /g tissue) | $26.00^{f}$ | $41.50^{e}$ | $47.53^{d}$ | $57.00^{c}$ | $69.33^{b}$ | $82.50^{a}$ | 0.063 |
| Muscle (µg /g tissue) | $5.23^{e}$ | $19.10^{d}$ | $27.5^{c}$ | $29.23^{b}$ | $37.86^{a}$ | $38.30^{a}$ | 0.301 |
| **Haematological indices:** | | | | | | | |
| Haematocrit (%) | $22.55^{b}$ | $27.14^{a}$ | $26.86^{a}$ | $26.45^{a}$ | $21.60^{b}$ | $22.42^{b}$ | 0.238 |
| Haemoglobin conc. (mg/dl) | $7.51^{b}$ | $9.045^{a}$ | $8.95^{a}$ | $8.815^{a}$ | $7.20^{b}$ | $7.47^{b}$ | 0.079 |

a-e Means in each row with different superscripts are significantly different (P<0.05).

conc. = Concentration

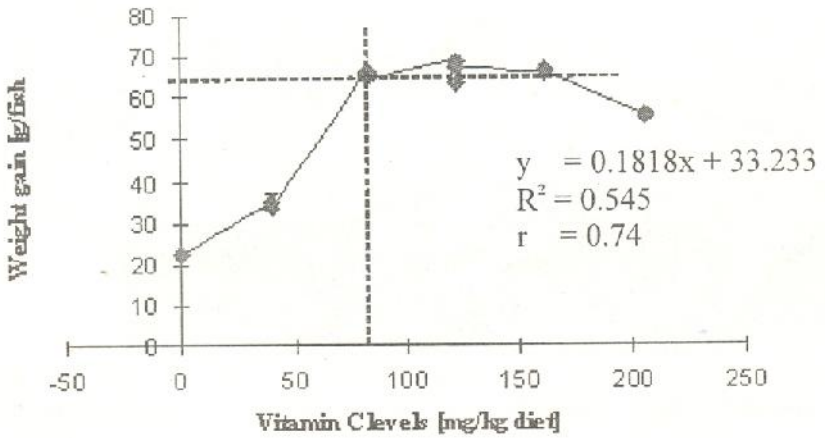


Fig. 1: Effect of dietary vitminE C on the weight gain of H. Longifilis fingerlings

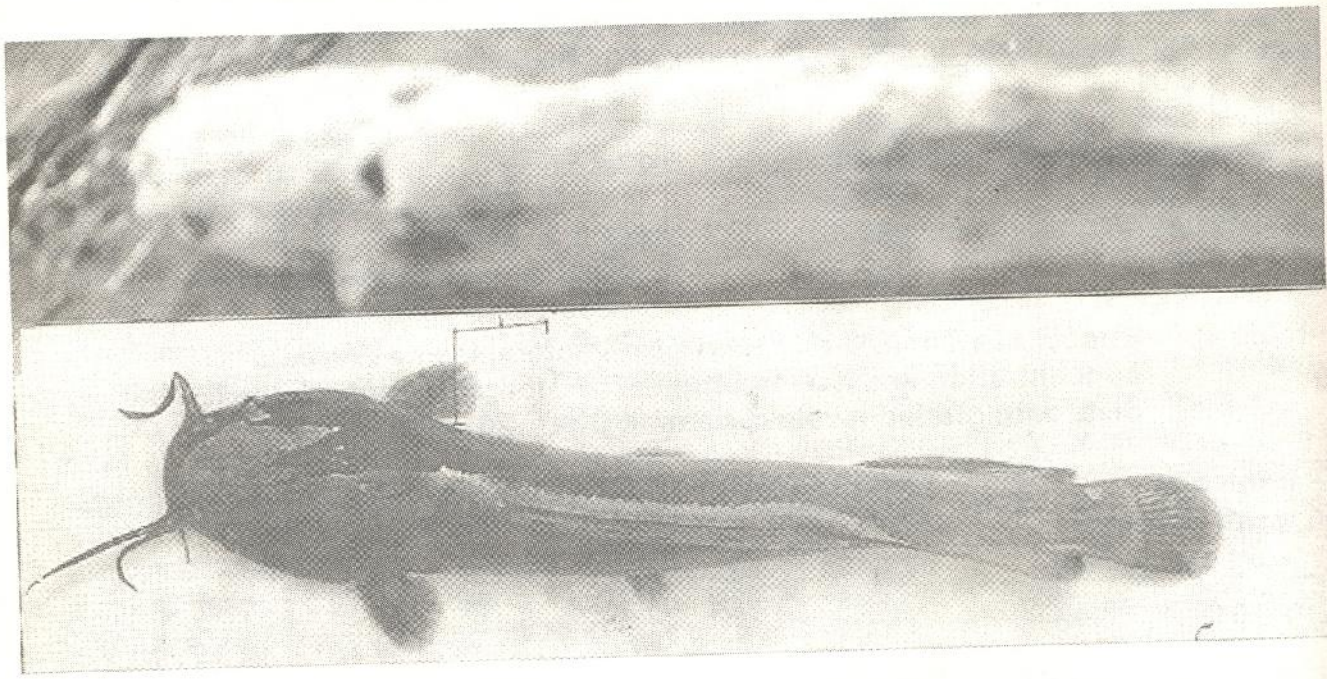

Plate 1 : Fish showing opercula and skin erosion

Plate 2: Fish fed vitamin C free diet showing symptoms of lordosis

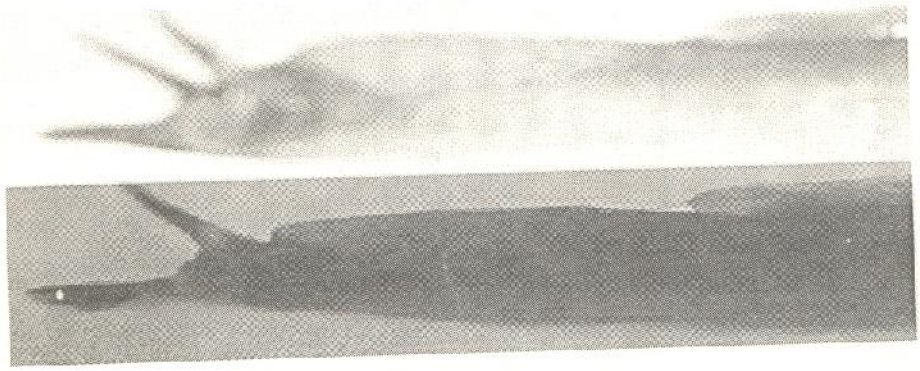

Plate 3: Fish showing tail erosion

Plate 4: Vertebra colon of Fish showing deformity between 8th and 11th vertebrae

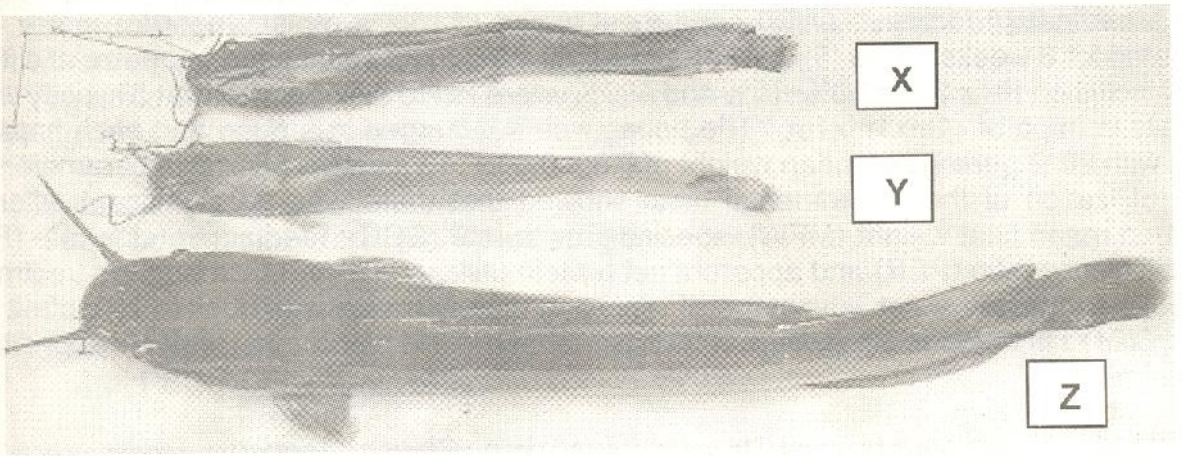


Plate 5: Fish showing disparity in growth and skin discolouration. X, Y are Fish fed vitamin C free diet (stunted and deformed) and Z is fish fed vitamin C supplemented diet (normal).

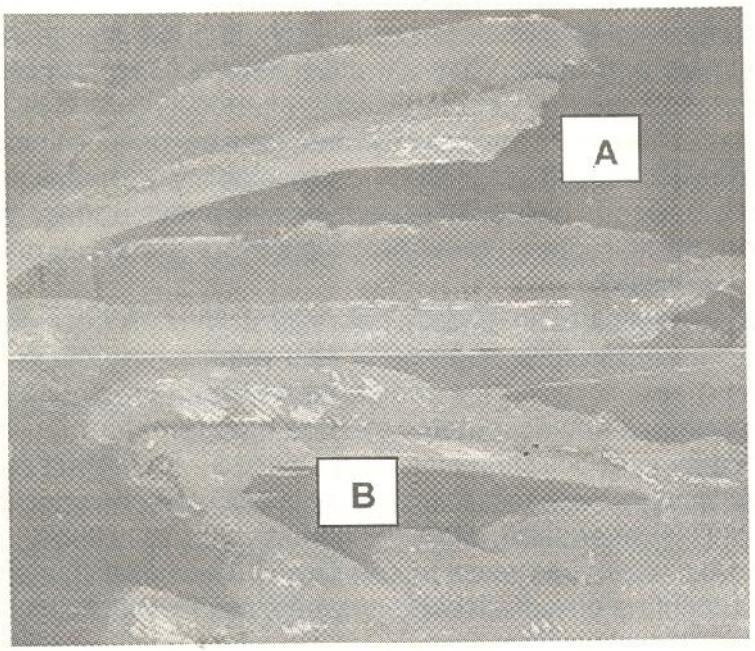


Plate 6: A and B are Micrographs of gills with normal and clubbed filaments from fish fed diet supplemented with 50 and 0 mg vitamin C respectively.