STUDIES ON WATER SOLUBLE VITAMIN REQUIREMENTS IN *Cichlasoma urophthalmus* (GUNTHER 1862).

by

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THIS THESIS IS SPECIALLY DEDICATED

TO

MY SON AND DAUGHTER

CARLOS CRISTIAN AND CARLA CRISTINA

AND TO MY FRIEND, FELLOW AND HUSBAND

CARLOS

TO MY PARENTS, BROTHERS AND SISTERS

TO THE MEMORY OF DR. ALEJANDRO VILLALOBOS

FIGUEROA

Page.

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ABSTRACTS.

Recently, studies on the Mexican native cichlid Cichlasoma urophthalmus, have shown it to be a strong candidate for culture in the region, but work on its nutritional requirements is lacking.

In this study the qualitative requirements for the eleven water soluble vitamins were determined. Three experiments were carried out to determine the quantitative requirements of vitamin C, pyridoxine and calcium pantothenate for C.urophthalmus, based on growth response, food conversion ratio, histopathological changes and mortality.

It was demonstrated that C. urophthalmus requires at least 9 of the eleven essential water soluble vitamins: pyridoxine, pantothenic acid, vitamin C, riboflavin, biotin, niacin, thiamin, choline and inositol. Folic acid and cyanocobalamin did not affect performance of fish in short term experiments.

C.urophthalmus required 40mg of ascorbic acid/Kg diet for normal growth and 110mg/Kg diet to prevent deficiency signs. Fish fed vitamin C deficient diets developed anorexia, reduced growth, haemorrahges,

exophthalmus, lordosis, short operculae, loss of scales, erosion of skin and fins as external signs of deficiency. Histologically they suffered severe gill and bone changes, muscle atrophy, necrosis of the hepatocytes and pancreas. In addition various pathological abnormalities in the ganglion cells were reported for the first time in fish fed a vitamin C deficient diet. Fish tuberculosis was present in the fish fed vitamin C deficient diets, and it is concluded that adequate levels of vitamin C help prevent Mycobacterium infection.

C.urophthalmus fry were found to require 5mg of pyridoxine/Kg diet for normal growth and health, and when fed a pyridoxine deficient diet showed the commonly described deficiency signs of loss of appetite, retarded growth, lethargy, rapid breathing, nervous disorders and high mortality. There were no histological signs of deficiency probably due to the early cessation of growth and rapid mortality. An overdose of pyridoxine did not cause toxicity or reduced growth.

Diets for C.urophthalmus had to be supplemented with at least 80 mg of calcium pantothenate/Kg diet to achieve maximum growth and food conversion ratios and to avoid external and histological deficiency signs. C.urophthalmus showed anorexia, reduced growth, fin and

skin haemorrhages and high mortalities as external signs of deficiency of this vitamin and practical dietary levels of 120 to 160 mg/Kg are recommended. The histological changes noted were clubbed gills, necrosis of the pancreas and glycogen deposition. Hepatic ceroidosis due to calcium pantothenate deficiency was reported here for the first time.

The data are discussed in relation to those available for other fish species.

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Pages VI – XIV

LIST OF TABLES.

PAGINATION
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LIST OF FIGURES.

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LIST OF PLATES

GENERAL INTRODUCTION

The group of African fish known collectively as tilapia, is the most widely cultured from the family Cichlidae and there is a great deal of important literature on their biology and culture (Balarin, 1979; Pullin and Lowe-McConnell, 1982; Fishelson and Yaron, 1983).

On the other hand, the American cichlids have been poorly studied with regard to determining their potential for aquaculture. Neverthless, there has been some research on their zoogeography, taxonomy (Miller, 1976; Bussing, 1976) and behaviour (Thorson, 1976). At present, interest has been generated in Mexico to study the native fish species in order to establish their aquaculture potential. Mexico as a neotropical country is rich in fish species of which many can probably be cultured. Cichlasoma is an important genus of the American cichlids which comprises more than 90 species (Miller, 1976); many of these do not reach marketable size, but some of them are important from the ornamental point of view (Axelrod, 1967). However, other larger species are commercially exploited through artisanal fisheries. Cichlasoma urophthalmus is one of these, and is distributed in fresh and brackish water from the Atlantic coast in Mexico to Central America (Miller, 1976) and is one of the most important species of the native cichlids in Mexico.

There are important fisheries for this species in various Gulf of Mexico states (Resendez, 1981), including the Yucatan Peninsula. C. urophthalmus, commonly recognized as "mojarra castarrica" or "mojarra prieta", is very well accepted in local markets where it is often preferred to tilapias due to its firm flesh qualities and absence of muddy off flavours (Martinez and Ross, 1986).

There are only two studies on the biology of this species. Resendez, (1981) described the environmental parameters in which this species is distributed in Laguna de Terminos, Campeche, Mexico. Chavez, Mattheeus and Perez Vega, (1983) described its life cycle in Rio San Pedro, Tabasco, Mexico and explained that this species reproduces in freshwater from May to August and is an omnivorous feeder with a tendency also to be carnivorous.

It was selected on the basis of its characteristics to study its potential for aquaculture.

Initial studies on its basic biology in its natural habitat and laboratory, have demonstrated that C. urophthalmus has a high potential for aquaculture due to its wide salinity tolerance, high fecundity, good feed conversion ratio, general hardiness and easy handling.

It adapts well to culture conditions, is quite disease resistant and can be reared at high densities (Martinez and Ross, 1986; Martinez-Palacios PhD Thesis, 1987).

Water quality and appropriate diet are the most important factors for the success of a species being cultured. For this reason studies have been carried out on the oxygen (Martinez and Ross, 1986), temperature and salinity requirements (Martinez-Palacios PhD Thesis, 1987) of C. urophthalmus to cover the most important environmental factors that can limit its growth. Since dietary protein governs growth and is often the most expensive component in satisfactory fish diets, two experiments on the protein requirements at two different temperatures have also been carried out (Martinez-Palacios, PhD Thesis, 1987).

It has been observed that vitamin deficiency can be a major difficulty in the culture of many species, however, and thanks to the development of artificial diets (Hashimoto, 1972) many trials have been relised to meet the nutritional requirements of fish for water and fat soluble vitamins.

In spite of 40 years of research on fish vitamin requirements. little work has been done on the amounts needed by warmwater fish. The National Research Council (NRC, 1983), in a review of the nutrient requirements of

warmwater fish and shellfish reported the studies of vitamin requirements for 6 species of fish, catfish (Ictalurus punctatus), common carp (Cyprinus carpio), red sea bream (Chrysophrys major) and eel (Anguilla anguilla and A. japonica). Recently, isolated work has been carried out on other tropical fish, John and Mahajan (1979) worked with cyanocobalamin and folic acid in Labeo rohita: Agrawal and Mahajan, (1980a) worked with vitamin C in the major carp Cirrhina mrigrala; Kissil, Cowey, Adron and Richards, (1981) found the nutritional requirement of pyridoxine in Sparus aurata; Agrawal and Mahajan, (1980b, 1983) realized experiments with vitamin C and pyridoxine in Channa punctatus; Limsuwan and Lovell, (1981) studied the intestinal synthesis and absorption of cyanocobalamin in channel catfish; Mahajan and John, (1981) studied the effects of cyanocobalamin and folic acid deficiency in Channa punctatus; Lovell and Limsuwan (1982) determined the intestinal synthesis and dietary non essentiality of cyanocobalamin for O. niloticus; Lovell and Buston, (1984) worked with biotin supplementation in practical diets for channel catfish and Soliman, (1985) made a series of experiments related to vitamin C in O. niloticus and O. mossambicus. These works show the recent interest for determining the nutritional requirements of warmwater fish. Table 1 shows which species of coldwater and warmwater fish have been studied with regard to their qualitative and/or

TABLE 1 Studies on qualitative (Q_1) and quantitative (Q_2) requirements of coldwater and warmwater fish to date. (*) = the fish is able to synthetize the vitamin and the fish do not require supplementation. 1 Yone, 1975 IN Tacon, 1985. The data were obtained from tables 2.4, 2.5, 2.6, 2.6, 2.7, 2.8, 2.9, 2.10, 2.11, 3.6, 4.4 and 5.3 and the references are quoted in them.

Species	Thiamin B_1		Riboflavin B_2		Pyridoxine B_6		Cyanocobalamin B_{12}		Pantothenic acid		Niacin		Biotin		Folic acid		Choline		Inositol		Ascorbic acid	
	Q_1	Q_2	Q_1	Q_2	Q_1	Q_2	Q_1	Q_2	Q_1	Q_2	Q_1	Q_2	Q_1	Q_2	Q_1	Q_2	Q_1	Q_2	Q_1	Q_2	Q_1	Q_2
Salmo gairdneri	♦	♦	♦	♦	♦	♦			♦	♦	♦	♦	♦	♦	♦	♦*	♦	♦	♦		♦	♦
S. trutta			♦	♦	♦	♦			♦	♦	♦	♦										
S. salar			♦	♦	♦	♦			♦	♦	♦				♦				♦			
Salvelinus fontinalis			♦	♦	♦	♦			♦	♦	♦	♦	♦									
Oncorhynchus tsawytscha	♦		♦	♦	♦	♦	♦		♦	♦	♦		♦		♦		♦					
O. kisutch	♦		♦	♦	♦	♦			♦		♦		♦		♦		♦		♦		♦	♦
Salvelinus namaycush									♦	♦	♦				♦		♦	♦				
Scophthalmus maximus	♦	♦	♦		♦	♦																
Cyprinus carpio	♦	+NR	♦	♦	♦	♦	♦	♦*	♦	♦	♦		♦	♦	♦	+*	♦	♦	♦	♦	♦	+*
Ictalurus punctatus	♦	♦	♦	♦	♦	♦	♦	♦*	♦	♦	♦	♦	♦		♦		♦		♦	+*	♦	♦
Chrysophrys major[1]	♦		♦		♦		♦		♦		♦		♦		♦		♦		♦	♦	♦	
Anguilla anguilla	♦	♦	♦																			
A. japonica	♦		♦		♦		♦		♦		♦		♦		♦		♦		♦		♦	
Channa punctatus					♦		♦								♦						♦	
Labeo rohita							♦								♦							
Cirrhina migrala																					♦	♦
Sparus aurata					♦	♦	♦	♦*	♦													
Oreochromis niloticus							♦	♦*													♦	♦
O. mossambicus																					♦	♦
Seriola quinqueradiata					♦				♦													
Clarias batrachus	♦		♦		♦				♦		♦				♦						♦	
[1]Cichlasoma urophthalmus	♦		♦		♦	♦	♦		♦	♦	♦		♦		♦							
																	♦		♦		♦	♦

quantitative requirements for the eleven essential water soluble vitamins. It is obvious from this review that in spite of the numerous studies on vitamins since 1947 (McLaren, Keller, O'Donnel and Elvehjem, 1947a,b), few have been carried out on warmwater fish and most of them are on the qualitative rather than quantitative requirements. The species of warmwater fish which have been studied most for both the qualitative and quantitative requirements for the eleven vitamins are Cyprinus carpio and Ictalurus punctatus. For these two species Table 2 shows the nutritional requirements of each vitamin in mg/kg diet obtained by different researchers. The importance of these studies on each species is clear, due to the differences observed in the requirement values. To support that fact, the earliest nutritionist knew that each species of trout must be considered as more or less independent and that what may be true of one species does not neccesarily apply to others (Davis, 1927). Furthermore, it is well known that nutritional requirements vary widely not only between species, but also with fish size, stress, nutrient balance and rearing temperatures (Halver, 1976; NRC, 1983). Halver, (1976) observed that -"sparing effect of one vitamin or vitamin precursor on others has not been throughly investigated, and specific differences in requirements of fish on different carbohydrate or fat intake and different protein intake have not been

TABLE 2 Vitamin requirements (mg/kg diet) for the eleven water soluble vitamins on Cyprinus carpio and Ictalurus punctatus.

	Cyprinus carpio	Ictalurus punctatus	References
Thiamine	NR	1.0	9,16
Riboflavine	4-10	9	5,8,17,24
Pyridoxine	5.4	3	2,23
Niacin	28	14	6,15
Pantothenic acid	30-55	10-15	5,13,22,26
Biotin	0.1	R	10,27
Choline	2000-4000	R	3,12
Inositol	440	NRS	3,7
Cyanocobalamin	NRS	NRS	4,11,25
Ascorbic acid	NRS	25-200	14,18,19,20,21
Folic acid	NR	R	1,3

R= Required
NR= Not required
S= The organism is able to synthetize the vitamin

REFERENCE

1. Aoe, Masuda, Saito and Takeda, (1957)
2. Ogino, (1965)
3. Duerre, (1966)
4. Kashiwada and Teshima, (1966)
5. Ogino, (1967)
6. Aoe, Masuda and Takeda, (1967)
7. Aoe and Masuda, (1967)
8. Aoe, Masuda, Saito and Komo, (1967)
9. Aoe, Masuda, Mimura, Saito and Komo (1969)
10. Ogino, Watanabe, Kakino, Iwanaga and Mizuno (1970)
11. Kashiwada, Teshima and Kanagawa, (1970)
12. Ogino, Uki and Watanabe, (1970)
13. Halver, (1972)
14. Yamamoto, Sato, Ikeda, (1977)
15. Andrews and Murai, (1978)
16. Murai and Andrews, (1978 a)
17. Murai and Andrews, (1978 b)
18. Lim and Lovell, (1978)
19. Murai, Andrews and Bauernfund, (1978)
20. Yamamoto, Sato, Ikeda, (1978)
21. Sato, Yoshinaka and Yamamoto, (1978)
22. Andrews and Murai, (1979)
23. Murai and Andrews, (1979)
24. Takeuchi, Takeuchi and Ogino, (1980)
25. Limsuwan and Lovell, (1981)
26. Wilson, Dewsar and Poe, (1983)
27. Lovell & Buston, (1984)

adequately measured"- for example thiamine is related to the carbohydrate intake, then thiamine must be particularly important for herbivorous species (NRC, 1983). Likewise requirements for pyridoxine are related to the protein level (Hardy, Halver and Brannon, 1979) consequently pyridoxine is exhausted first in carnivorous fish fed low pyridoxine diets (Halver, 1976). Furthermore it is noticeable that the recent studies on warmwater fish have demonstrated that some of the tropical species are able to synthesize some of the vitamins, which is interesting as practical diets can be elaborated without these vitamins, thus reducing food costs. The differences in the apparent rate of intestinal synthesis of cyanocobalamin between tilapia and channel catfish indicates that the dietary requirement for various vitamins may be markedly different among fish (Lovell and Limsuwan, 1982). Consequently more work needs to be done to determine specific requirements of these water-soluble vitamins for different species of fish. With time, more differences are going to appear between the requirements of cold and warmwater fish, and undoubtedly one of the reasons for these differences is going to be the higher metabolic rate of the warmwater fish.

The objective of this thesis is to determine the qualitative requirements of the essential water soluble vitamins in the first instance and to initiate a series

of experiments to assess the quantitative requirements for these same vitamins based on growth response, gross signs of deficiency, feed conversion ratios, histopathological changes and mortality of Cichlasoma urophthalmus fry.

Chapter 1.

GENERAL MATERIALS AND METHODS

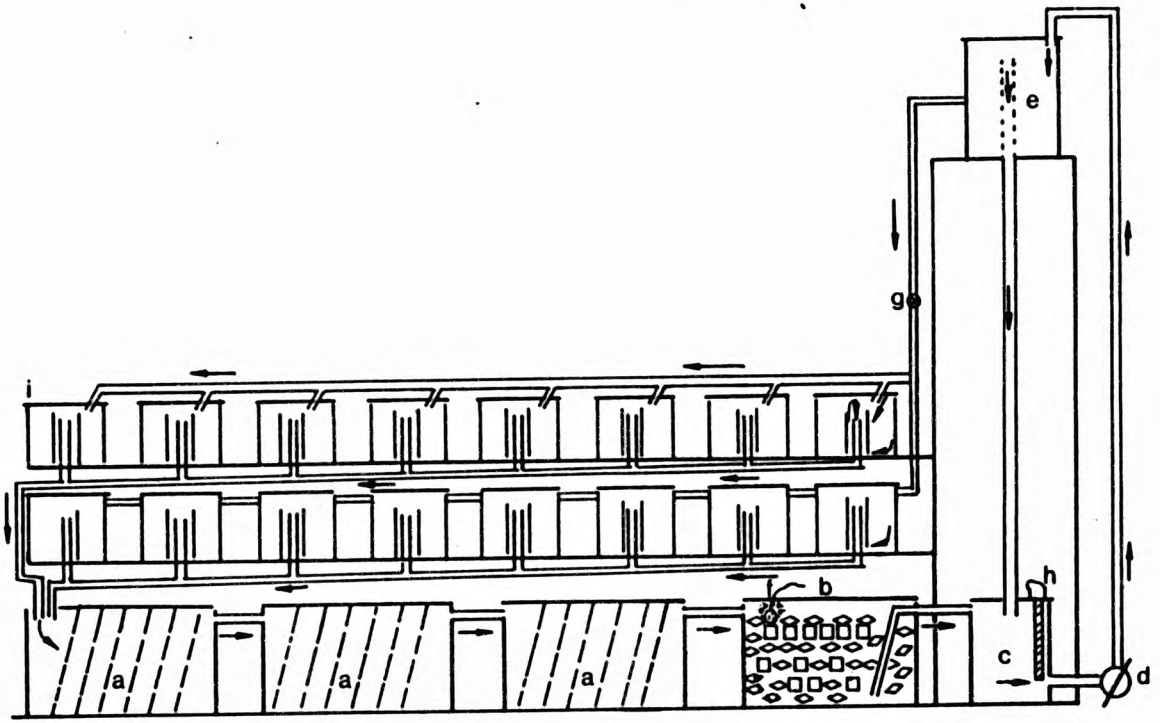
1.1. The experimental system.

All experiments were carried out in a water recirculation system consisting of 36 polypropylene 20 l circular tanks (Plate 1.1). The system was supplied with tap water and allowed to settle for a week in a concrete tank of 32 cubic metres capacity, after which the water was aereated for a further week in the recirculation system. The water entered each tank through a tangential pipe to give a circular flow and at a rate of 1 litre/minute. The tanks had central standpipes with collar for self cleaning. Effluent water from each tank passed through 3 sedimentation tanks of 150 l. capacity, each containing 4 sheets of fibre glass to aid sedimentation. Water then passed through a biological filter, which consisted of sacks of polystyrene crumbs used to increase the surface area for bacterial action. After the filter, the water collected in a sump from which it was pumped with a centrifugal pump (0.5 HP) to the header tank (Fig.1.1). The number of tanks utilized from these systems varied with each experiment.

Each system had a constant temperature of 28°C through the use of a 2 kilowatt water-heater made at Centro de Investigaciones y Estudios Avanzados Unidad Merida, Mexico (CINVESTAV). The system was installed in a closed laboratory with 25°C ± 2°C ambient temperature and a

Plate 1.1. Recirculated water system. 1. Header tank. 2. Experimental tanks. 3. Settlement tanks. 4. Biofilter.

Figure 1.a. Experimental recirculating system used in all the experiments. a) sedimentation tanks; b) biological filter; c) sump; d) pump; e) header tank; f) air-stone; g) valve; h) heater; i) experimental tanks. The arrows show the direction of the water flow.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controlled photoperiod of 12 hours light and 12 hours dark. Due to the hardness of the water (320 ± 10 ppm Ca) the rubber stopper device used in the tanks to control the water flow (Martinez, Flores and Olvera, 1986) became partially blocked during the experimental period with precipitated salts and required routine examination and cleaning to maintain a constant water flow.

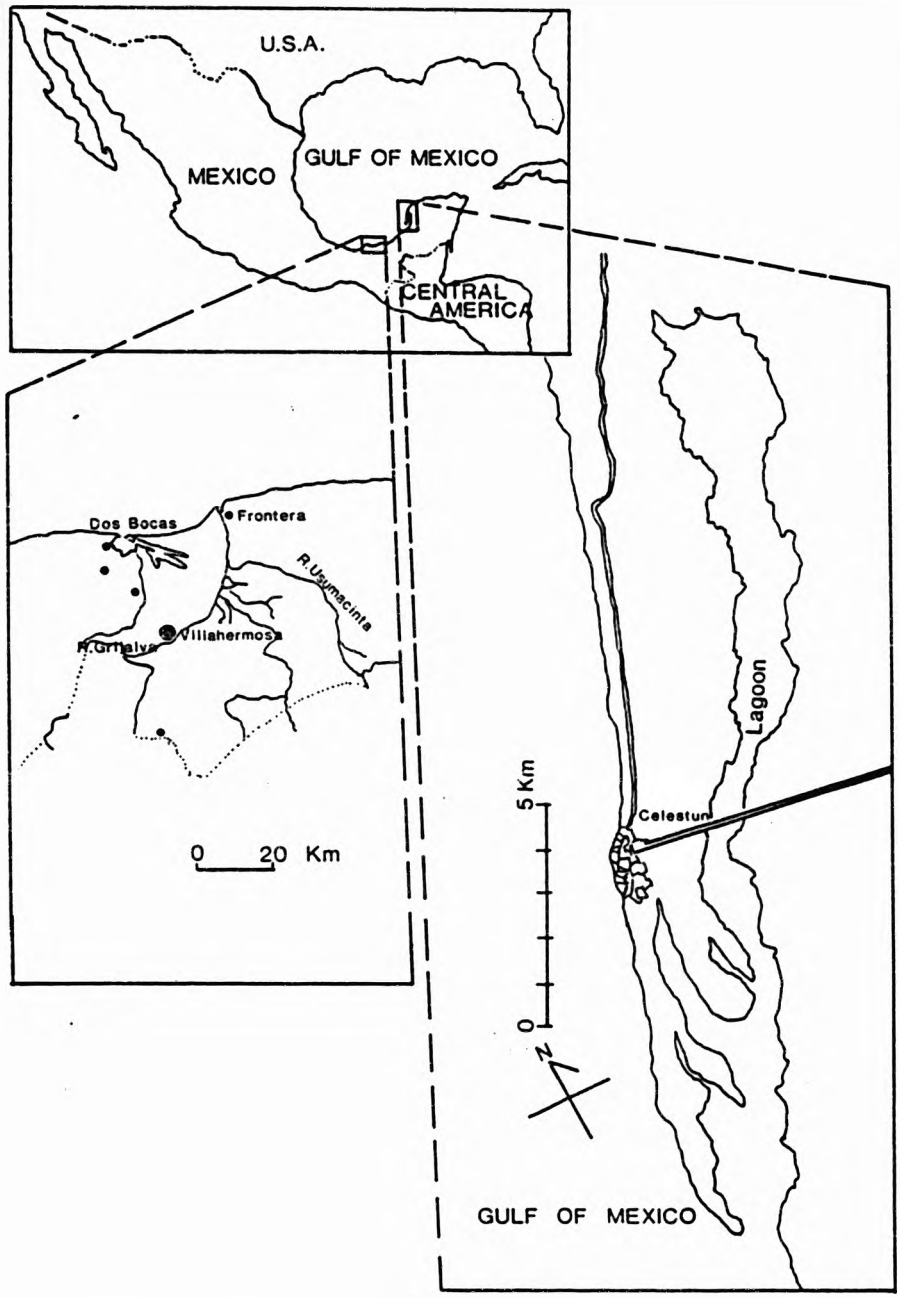
During the first experiment there was a bloom of Chironomid larvae in the tank sediment, which necessitated the rejection of the results from the last 15 days. To avoid any possible additional introduction of mosquito or other insect larvae into the system during subsequent experiments, two commercially available electric flying insect killers, with bright UV light as an attractant, were installed and the waste water channel in the centre of the room was cleaned frequently with a strong hypochlorite solution. The sediments in the settling sediment collecting tanks were examined weekly by microscope to ensure there were no insect larvae or other organisms that could interfere with the experiments. Tanks were cleaned weekly to avoid any build-up of food and faeces. Nitrite, ammonia and pH were measured fortnightly (Lind, 1979) to ensure there was no build up of these substances in the system. Details of measured parameters are noted in the appropriate experimental section.

1.2. The experimental fish.

Batches of between 2500 to 4000 fry in the size range of 105 to 200 mg. were used in the experiments. They were obtained from two sources, fish for the first two experiments were from a pair of broodstock captured from Dos Bocas, Tabasco, Mexico (fig.1.2.). After positive identification by Dr. Robert R. Miller, Curator Emeritus of Fishes, Michigan University, fish were grown and fry were obtained from the rearing tanks of the wet laboratory at CINVESTAV.

Fish fry for the other three experiments were obtained from the Celestun Lagoon, Yucatan (fig 1.2.). The fish were carefully captured from the same nest, and one batch of fish was sufficient for the Vitamin C, Pyridoxine and Calcium Pantothenate experiments.
Prior to acclimation in the experimental system, the fish were carefully examined for parasites and diseases; only Trichodina sp were found as external parasites in both batches of fish and Malachite green at 1ppm was applied for 1 hour, once daily for a week. Before establishing them in the experimental system, the fish were kept in quarantine for at least 15 days in fibre glass tanks of 1 cubic meter capacity, with aeration and one complete water change daily. During quarantine and subsequent acclimation to the experimental system the fry were fed

Figure 1.b. Geographical location of the Celestun Lagoon in Yucatan and the River Dos Bocas, Tabasco in which, adults and fry of C.urophthalmus were collected.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MEXICO
GULF OF MEXICO
CENTRAL AMERICA
Dos Bocas
Frontera
R.Usumacinta
R.Grijalva
Villahermosa
0 20 Km
Lagoon
Celestun
5 Km
0
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GULF OF MEXICO

with a balanced diet containing 50% protein based on fish meal, formulated and prepared at CINVESTAV. (Table 1.1).

1.3. Experimental diets.

1.3.1. Experimental diet formulation.

The complete vitamin test diet (basal diet) used for all the experiments is shown in Table 1.2. Vitamin and Mineral Mixture was supplied by Dr. Albert Tacon, Fish Nutritionist, FAO. All the vitamins was supplied by Roche except vitamin C (L-Ascorbic Acid Sodium salt) which was supplied by Sigma.

To ensure that there were no traces of endogenous vitamin, batches of 50 g of vitamin free casein were washed with hot 70% methanol according to the method of Aoe, Masuda, Saito and Komo, (1967) and quickly dried out using a Buchner funnel and a vacuum pump.

Fish oil and corn oil were submitted to the peroxide value test (AOAC, 1980) to ensure that there were no oxidised oils in the diets, only oils with less than 4 meq/Kg were used to prepare the diets. BHT was added to the fish oil and stored in the fridge while used.

Rations containing full vitamin mix were used as a control and experimental diets contained a specific vitamin deleted or different levels of one vitamin.

1.3.2. Diet preparation.

Table 1.1 Balanced diet containing 50% protein given to the fish before experimental treatment.

	%
Fish meal	75.8
Corn starch	14.7
Fish oil	0.5
Soybean oil	3.0
Mineral mix	1.5
Vitamin mix	3.0
Sodium alginate	1.5
	100

TABLE 1.2 Basal diet, mineral and vitamin mixture of the experimental diets.

Basal Diet (%)		Mineral Mixture (g/kg of food)		Vitamin Mixture (mg/kg or I U /kg)	
Casein vitamin free	48.5	$MgSO_4 \cdot 7H_2O$	10.4340	Thiamine mononitrate (B_1)	35.0
Dextrin	9.0	KCl	2.0000	Riboflavin (B_2)	25.0
Starch	18.0	NaCl	2.4000	Calcium pantothenate	90.0
Fish oil (Cod)	8.0	$FeSO_4 \cdot 7H_2O$	1.0000	Biotin	0.5
Corn oil	4.0	$ZnSO_4 \cdot 7H_2O$	0.2200	Folic acid	7.5
Carboxymethyl cellulose	2.0	$CuSO_4 \cdot 5H_2O$	0.0314	Cyanocobalamin (Vit. B_{12})	0.05
Vitamin premix	3.0	$MnSO_4 \cdot H_2O$	0.0769	Niacin	220.0
Mineral premix	4.5	$COSO_4 \cdot 7H_2O$	0.0236	Pyridoxine HCl (B_6)	30.0
$Ca\ HPO_4 \cdot 2H_2O$	3.0	$CaIO_3$	0.0118	Ascorbic acid (Vit C)[3]	1000.0
	100.0	$CrCl_3 \cdot 6H_2O$	0.0051	Choline chloride	1000.0
				Myo-Inositol	250.0
				Retinol acetate (Vit A) I U	6500.0
				Cholecalciferol (Vit D_3) I U	1000.0
				DL-Alpha Tocopherol acetate (Vit E)	300.0
				Menadione sodium bisulfite (50% vit k_3)	12.0
				BHT	25.00
				Ethoxyquin	100.00

Vitamin free casein, starch, dextrin, carboxymethyl cellulose flour and $CaHPO_4.2H_2O$ were carefully weighed individually on a Mettler Balance and mixed together by shaking in batches of 3-4 kg in a large plastic bag to avoid loss of materials during mixing. The ingredients were mixed continuously for half an hour. Mineral components were then carefully weighed on an analytical Mettler balance, ground up together in a plastic Moulinex blender and mixed by shaking in a plastic container with cap for 15 minutes before adding them to the main mixture.

While the vitamins were being quantified, all the ingredients previously weighed were mixed for another hour and later portioned out into capped plastic containers as required. Then the corresponding vitamin mixture was added and the diet mixed for about half an hour in a rotatory mixer (Plate 1.2) and later placed in a fridge while other diets were completed. The required amount of corn oil was added together with a small amount of distilled water to obtain a crumble then the mixture was pelleted by extrusion in a Hobart food mixer modified for small amounts of experimental diets (300-1000 g) and to produce pellets 2 mm thick (Plate 1.3). The pellets were then dried at 35°C in a forced air convection dryer and later broken into smaller crumbles and screened into 0.84mm and 1.68mm according to the size of the fish. At

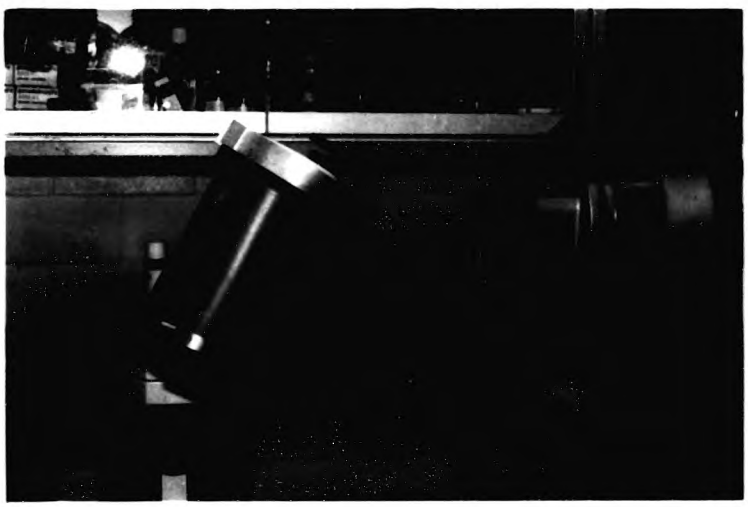
this stage fish oil was added to be used not only as a source of animal fat but also as an attractant. Most of the food was stored in the freezer (-15 to -20°C) until required, while a small portion (about 3 g) of each diet was placed in the fridge (4°C) and weighed daily as required.

Once the diets had been prepared samples were taken to determine the proximate analysis of all the diets using the Macro-Kjeldhal Tecator/Kjeltec System 1003 distilling unit (AOAC, 1980) for crude protein. The fat content was determined by extracting dried samples for 4 hours using a soxhlet apparatus and petroleum ether (40:60 °C boiling range) and measuring, by weight difference, the amount of ether soluble material extracted. Ash content was determined by heating a preweighed sample within a silica crucible in a muffle furnace at 450'C for 12 hours. Moisture was determined by drying a weighed sample in a drying oven at 105°C for 24 hours (AOAC 1980).

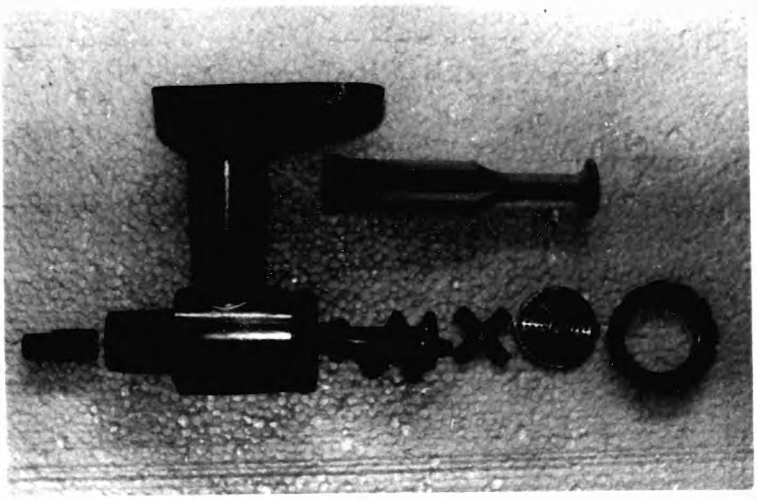
1.4. Fish Care.

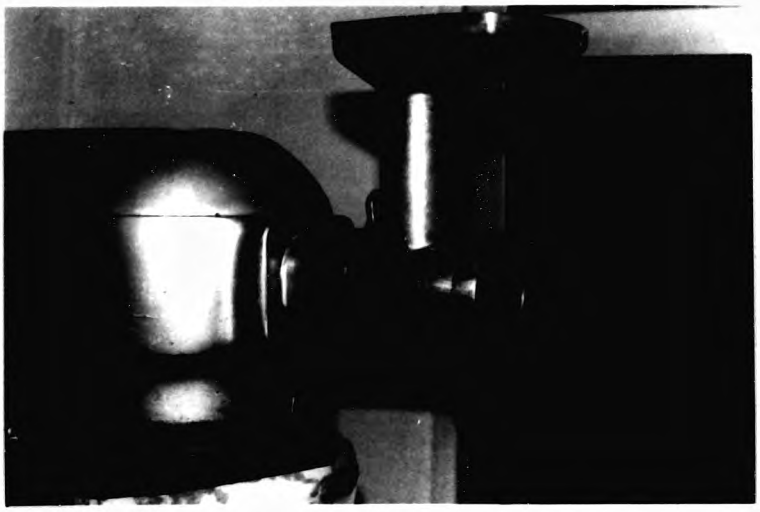
Each diet was tested in triplicate for different periods of time, as required for the particular experiment. The number and initial weight of fish also varied in each experiment according to the stock and supply. Fish were fed three times a day for six days and

Plate 1.2. Rotatory mixer for 300 to 600g of experimental diets.



Plates 1.3. (A,B). Modified Hobart mill to extrude and pelletize 300 to 1000g of experimental food.





twice on Sundays. Care was taken when giving the food, to provide only a small amount at a time to be sure that the fish ate it all. When food was seen floating, no more food was added. Fish were fed ad libitum during all the experiment. Each group of fish was routinely examined for physical and behavioural abnormalities. Any mortality and abnormality was recorded when observed. Weights of the dead fish were registered and the feeding rate was changed for the following period. All fish at the end of the experiments were examined using a Slit-lamp Microscope (Oculus-Karl Zeiss) to detect possible gross changes in the eyes.

1.6. Histological studies.

During the first and second experiments two fish from each experimental diet together with any moribund animals were killed with a benzocaine overdose. They were then immediately fixed in 10% buffered formalin and subsequently paraffin wax embedded for sectioning and histological observations Sections at 5 microns were stained using the Haematoxylin-Eosin technique. Mortalities at the end of the experiment refer only to fish dying and those moribund fish removed, not the pair taken for sampling.

With respect to the other three experiments, only moribund fish and 5 others from each diet were preserved at the end of the experiment for histological observations. Special stains were used in some of the studies and are expressed in the respective materials and methods sections.

1.6.Growth and Nutritional parameters measured.

Fish from all the experiments were weighed and counted every 15 days, and feeding rates were changed to agree with the new total weight. Food intake was recorded daily. With growth and food intake results the following parameters were obtained.

1.-SPECIFIC GROWTH RATE (SGR %/ day).

$$SGR = \frac{\text{Log e Final weight - Log e Initial weight}}{\text{Time (days)}} \times 100$$

2.-INDIVIDUAL WEIGHT GAIN (IWG mg/day)

$$IWG = \frac{\text{total weight gain each 15 days}}{\text{n at the end of the 15 days}} \times \frac{1000}{\text{Total time (days)}}$$

where n=number of fishes

3.-INDIVIDUAL FOOD INTAKE (IFI mg/Day)

$$IFI = \frac{\text{Total food intake during each 15 days}}{\text{n at the end of each 15 days}} \times 1000$$

time (days)

4.-FOOD CONVERSION RATIO (FCR)

$$FRC = \frac{\text{Individual food intake}}{\text{Individual weight gain}}$$

5.-WEIGHT GAIN (Wg %)

$$Wg = \frac{\text{Final weight - Initial weight}}{\text{Initial weight.}} \times 100$$

1.7. Statistical Analysis

Analysis of variance and Duncan's multiple range and F tests were employed in evaluating the experimental results (Parker, R.E., 1979 and Duncan, 1955).

CHAPTER 2.

QUALITATIVE REQUIREMENTS OF *Cichlasoma uropthalmus* (Gunther) FOR THE ELEVEN WATER SOLUBLE VITAMINS.

2.1. INTRODUCTION.

McLaren, Keller, O'Donnell and Elvehjem were the first pioneer fish nutritionists to report tentative qualitative and then quantitative requirements of nine water soluble vitamins (McLaren et al., 1947a, b). Afterwards numerous efforts were made to find a suitable synthetic diet, on which normal growth could be mantained for extended periods (Wolf, 1951). Finally it is important to say that the first step in the right direction for studying the nutritional requirements of fish was the establishment of a synthetic basal diet for salmonids. (Halver, 1957). Applications of this diet to salmon, trout and other species were rapid and produced dramatic results, finding specific deficiency syndromes when one of the required vitamins was deleted from the vitamin mixture (Halver, 1972).

Cichlasoma urophthalmus is possibly one of the most important Mexican cichlids with aquaculture potential. To determine the latter it is necessary to culture intensively its initial juvenil stages. However, a fundamental knowledge of its nutritional requirement is still lacking, and it is well known that, with the exception of the water supply, no single factor is more important in determining the success or failure of a hatchery than the daily diet of the fish. Deficiency signs for the essential water soluble vitamins have been

demonstrated in various species of fish. An initial experiment with the use of a synthetic diet and the deletion of the eleven essential vitamins in different diets has been utilized by many workers in different fish species to find, in the first instance, the qualitative requirement for those vitamins (McLaren et al., 1947; Wolf, 1951; Halver, 1957; Halver and Coates, 1957; Kitamura, Suwa, Ohara and Nakaguwa, 1967; Dupree, 1966; Arai, Nose and Hashimoto, 1972; Buttep, Sitasit and Boonyaratpalin, 1985).

The objective of this study is to demonstrate the qualitative requirements for the eleven water soluble vitamins of C. urophthalmus fry, based on growth parameters, changes in behaviour, gross signs of deficiency, histopathological changes and mortality. The results of this experiment will demonstrate which are the most critical vitamins in terms of the parameters used, and they will be used to design a series of experiments to meet the quantitative requirement of each vitamin in this species.

2.2. FIRST SHORT TERM INITIAL EXPERIMENT.

The objective of this experiment was to determine the qualitative requirement of C. urophthalmus for six

water soluble vitamins based on growth response, behavioural changes, gross signs, histopathology and mortality. Parallel to this main objective, this experiment was designed also to determine the number of fish per experimental tank, the appropriate feeding rate, and the acceptance of the artificial diet by observing fish behaviour plus feeding and growth rate. The aim of the above was to make recommendations for future experiments.

2.2.1. MATERIALS AND METHODS.

2.2.1.1. Experimental design and diets. Groups of 30 native Cichlidae (Cichlasoma urophthalmus) obtained at CINVESTAV Merida (Section 1.2.) with initial weights of between 0.127 to 0.134 g. were placed in 21 tanks of the recirculation system as mentioned in section 1.1. The fish were acclimated to the system and to the basal diet (6% of the body weight per day) for 1 week. Diets were designed as explained in chapter 1 (Section 1.3). A basal diet was used as a control and six others were prepared in the same way deleting thiamine, riboflavine, calcium pantothenate, niacin, pyridoxine and biotin respectively. Diet storage and parameters measured on the fish as well as statistical and histological treatments have been previously described (Chapter 1).

After the adaptation period, the fish from 3 tanks

received the vitamin complete diet as a control and the other 18 tanks received the respective vitamin deleted diet. Each diet was tested in triplicate for a period of 45 days.

2.3. SECOND EXPERIMENT.

The objective of this experiment was to determine the qualitative requirements of C.urophthalmus for nine water soluble vitamins.

2.3.1. Experimental design and diets.

Groups of 20 native C.urophthalmus fry obtained at CINVESTAV (Section 1.2) with initial weights of between 0.105 to 0.111g were placed in 30 tanks of the recirculation system (section 1 1). Fish were acclimated to the system and to the basal diet (5% of the body weight per day) for one week . After that period fish were fed with 10 different diets. A basal diet was used as control (diet 1) and diets with the deletion of one of the following nine different water soluble vitamins were also prepared; thiamine, riboflavine, niacin, biotin, folic acid, choline, inositol, cyanocobalamine and ascorbic acid . The preparation of diets was as described in chapter 1 (section 1.3). Fish were fed with these diets for 30 days ad libitum throughout the 90 day experiment. Storage of the diets, nutritional parameters

measured, histological treatments and statistical analysis are described in section 1.3; 1.5; 1.6 and 1.7 respectively.

2.4. RESULTS OF THE FIRST EXPERIMENT.

2.4.1. Behaviour, signs of deficiency and mortality.

The fish on the control diet (diet 1) ate voraciously and looked healthy during all the experiment. However in the last fifteen days they grew too big for the experimental tanks and they started to fight due to the space constraints causing mortalities which reached 26.5% at the end of the 45 day trial. This indicated that 30 fish/tank was not a good density and necessitated a reduction for further experiments to between 15 and 20 fish per tank. The fish from all treatments accepted the diet and ate it voraciously until the appearance of deficiency signs.

Fish on the thiamine and riboflavine deficiency diets (diets 2 and 3) showed normal appearance and ate voraciously for the first 15 days, but afterwards they lost their appetite and grew slowly with no mortalities at the end of the experiment. The fish with thiamine deficiency did not show any external signs in comparison

with the riboflavine deficient fish which had a slight congestion over the head, mouth plus pectoral and dorsal fins during the last 15 days.

Fish on the Pantothenic acid deficient diet (diet 4) showed an appetite reduction on day 18 of the experiment. They showed normal behaviour during 22 days of the trial, but the fish later acquired a dark colouration and lay on the tank bottom swimming suddenly to the surface but falling again when they stopped moving their fins. On day 24 of the experiment, all fish from the three replicas had total anorexia, dark colouration, abnormal swimming, lethargy, with very thin bodies and fast ventilation; some of them also showed congestion in the snout and dorsal and caudal fins. By day 36 all the fish were dead.

Fish from the niacin deficient diet (diet 5) showed an irregular appetite, alternating decreased and normal feeding, but never had total anorexia. They never showed any abnormal behaviour or appearance.

The Pyridoxine deficient diet (diet 6) was the most critical deficiency, the fish showed the first signs on day 8, with dark colouration and total anorexia. Later the fish rested on the bottom of the tank with rapid, gasping breathing, interspersed with sudden fast and

erratic swimming at any time, but mainly when the tank was disturbed, sometimes swimming vertically with head upwards followed by sudden fits and convulsions. The mortality started to appear 10 days after the first food intake, and 100% mortality occurred 19 days later. The fish on the biotin deficient diet showed a reduction in appetite by day 20, but they never showed total anorexia. A slight congestion over the whole body was seen in most of the fish giving them a pink and translucent coloration. Some of the fish did not move one of the pectoral fins

2.4.2. Growth parameters.

The mean growth response and performance data of C. urophthalmus given for the seven experimental diets are shown in Figure 2.1 and Table 2.1. There were no significant differences at $P<0.01$ in the initial weights of the fish. At the end of the experiment values of final weight, individual weight gain, individual food intake, weight gain (%) and specific growth rate of the fish fed the control diet were significantly different from the rest of the diets at $P<0.01$, except in the case of the weight gain and specific growth rate values for fish fed diets 5 and 7 (niacin and biotin deficiency) in which the figures were similar to the control fish. With regard to the food conversion ratios, fish fed diets 1, 5 and 7 (control, niacin and biotin deficiency) had the lowest

Figure 2.1. Growth Response of the "mojarra" Cichlasoma urophthalmus fed diets containing six different vitamin deficiencies. D1= Control; D2= thiamine deficiency; D3= riboflavine deficiency; D4= calcium pantothenate deficiency; D5= niacin deficiency; D6= pyridoxine deficiency; D7= biotin deficiency.

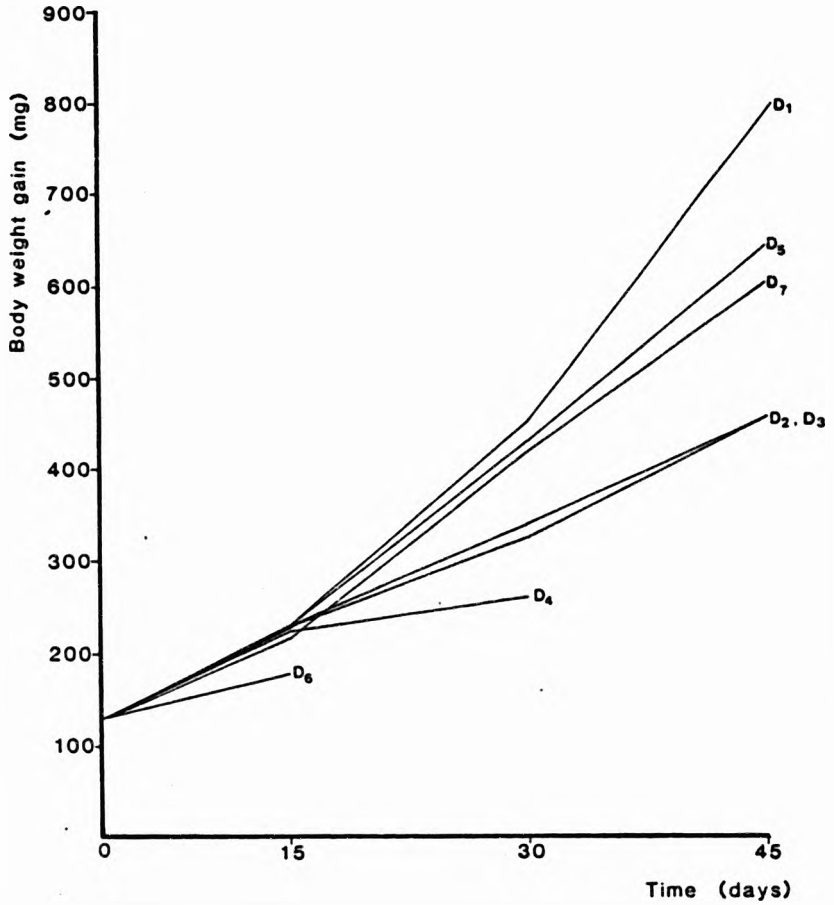

900
800
700
600
500
400
300
200
100
Body weight gain (mg)
0
15
30
45
Time (days)
D1
D5
D7
D2, D3
D4
D6

TABLE 2.1 Effects of thiamine, riboflavine, niacin and biotin deficiency on growth food conversion and survival of Cichlasoma urophthalmus during a 45 day experimental period.

Diet	Initial[1] Weight (mg)	Final[1] Weight (mg)	Individual[1] Weight gain (mg/day)	Individual[1] Food Intake (mg/day)	Food[1] Conversion Ratio	Weight[1] gain %	Specific[1] growth rate	Survival %
1. Control	127[a]	787.2[a]	14.544[a]	15.413[a]	1.059[b]	520.67[a]	4.056[a]	73.5
2. Thiamine deficiency	128[a]	456.9[c]	7.308[c]	9.441[c]	1.290[a]	256.41[b]	2.822[bc]	94.4
3. Riboflavine deficiency	132[a]	454.1[c]	7.182[c]	8.403[c]	1.169[ab]	244.89[b]	2.751[c]	93.3
4. Calcium pantothenate deficiency	131[a]	---	---	---	---	---	---	0
5. Niacin deficiency	129[a]	647.1[b]	11.467[b]	12.773[b]	1.131[b]	401.55[ab]	3.567[ab]	96.6
6. Pyridoxine deficiency	128[a]	---	---	---	---	---	---	0
7. Biotin deficiency	127[a]	505.8[b]	10.648[b]	11.651[b]	1.095[b]	377.03[b]	3.471[ab]	74.4

1. Values within the same column which bear different letter are significantly different at $P<0.01$

values and were significantly different at $P<0.01$ from diets 2 and 3.

2.3.3. Histopathological changes.

During the experiment no histopathological signs were found in the fish fed diets deficient in thiamine, riboflavine, niacin, pyridoxine and biotin. Histopathological results of the fish fed diet deficient in calcium pantothenate were similar to those found in the qualitative requirement experiment for this vitamin and they are referred to in chapter 5 (Section 5.3).

2.5. RESULTS OF THE SECOND EXPERIMENT.

2.5.1. Water quality.

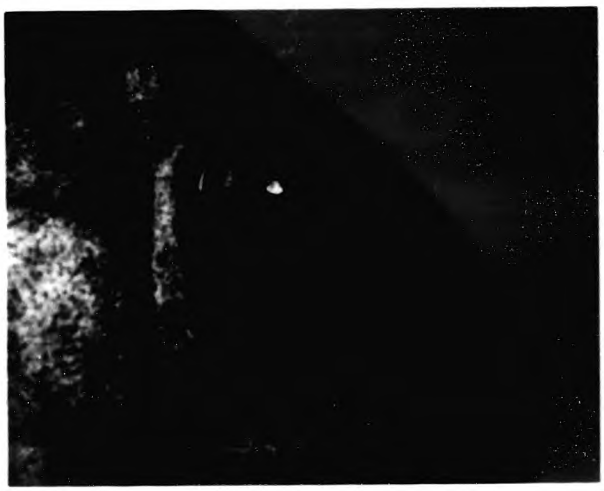
Average water quality readings for the experimental period were:
Temperature 27.76°C (Range 26.46 to 29.06).
Nitrites 0.008 ppm (Range .001 to .015ppm).
Ammonia .068ppm (Range .014 to .122 ppm).
pH 8.59 (Range 8.53 to 8.64).

2.5.2. Results on behaviour, deficiency signs and mortality.

Fish on the control and cyanocobalamin deficient diets were healthy, had normal behaviour and ate voraciously during the whole trial. During the experiment one or two larger fish in each tank attacked and killed the smallest, resulting in a final survival of 70% and 80% respectively. Fish on the thiamine, niacin, folic acid, choline and inositol deficient diets never showed abnormal behaviour or external signs of the deficiency, except that they had an irregular appetite, but never developed total anorexia.

Some of the Riboflavine deficient fish showed, on day 49 of the experiment, congestion in pectoral fins and mouth. Afterwards the fish acquired a dark colouration and some of them showed a marked reduction in appetite and became very thin. Later, some of these fish were found moribund on the bottom of the tank and were subsequently killed with an overdose of benzocaine. Post-mortem examination showed that they had a dark colouration, pale gills and liver and some of them had small haemorrhages in the inferior part of both eyes (Plate 2.1). With the use of a slit-lamp microscope no other deficiency signs were found in the eyes of these fish. However as these fish started to show signs in the last fifteen days of the experiment, 2 fish from each tank (6 in total) were killed with benzocaine and preserved for histological analysis as for the other diets at the end of the experiment, but the fish on this dietary regimen were fed for one more week,

Plate 2.1. Riboflavine deficient fish showing a small haemorrhage in the eye.



and during this time, fish started to die in large numbers showing the same signs as described above.

Fish on the biotin deficient diet had similar signs to the fish from experiment 1 fed the same deficient diet. The first sign appeared on day 42 and was congestion and/or small haemorrhages in the pectoral and caudal fins. In some fish the caudal fin was held as a "closed fan" giving to it a triangular shape, sometimes the fish failed to move one or both pectoral fins and showed a reduction in appetite but never total anorexia. By day 56 most of the fish acquired these signs and started to die, giving a 53.33% survival at the end of the experiment.

The vitamin C deficient fish showed the first signs of deficiency on day 52. These signs were marked haemorrhage in the head and around the eyes, slight exophthalmia, congestion in ventral, anal and dorsal fins and operculae as well as very pale gills. Three days later many other fish had exophthalmus, dark colour and some of them showed a swollen abdomen. Pale gills, liver and spleen were also seen. At the end of the experiment fish with Vitamin C deficiency reached 56.66% mortality.

2.5.3. Growth parameters.

The mean growth response and performance data of the C.urophthalmus given the ten experimental diets are shown in Figure 2.2 and table 2.2. There were no significant differences ($P<0.01$) in the initial weights of the fish from all treatments. Final weight and individual weight gain values for fish fed the control diet, plus those of cyanocobalamin and folic acid deficiency were significantly different ($p<0.01$) from fish fed the other deficient diets. Fish fed diet 1 (Control) and diet 9 (Cyanocobalamin deficiency) had the highest final weight, individual weight gain (%) and specific growth rate, followed by diet 6 (folic acid deficiency) and being significantly different from the rest of the diets. In respect to food conversion efficiency, fish fed diet 3 (riboflavine deficiency) had the highest value, followed by diets 5, 10, 2 and 8 being significantly different from the other treatments.

2.5.4. Histopathological changes.

Fish from all diets showed some fatty degeneration in the hepatopancreas and slight oedema in the gill epithelial cells. Different from those pathologies, fish fed diets 1, 2, 3, 4, 6, 7, 8, and 9 (Control, thiamine, niacine, Folic acid, Choline, Inositol and

Figure 2.2. Growth response of Cichlasoma urophthalmus fry fed diets containing nine different deficiencies. Numbers at the end of each line expressed the control diet and the deficient diets from table 2.2.

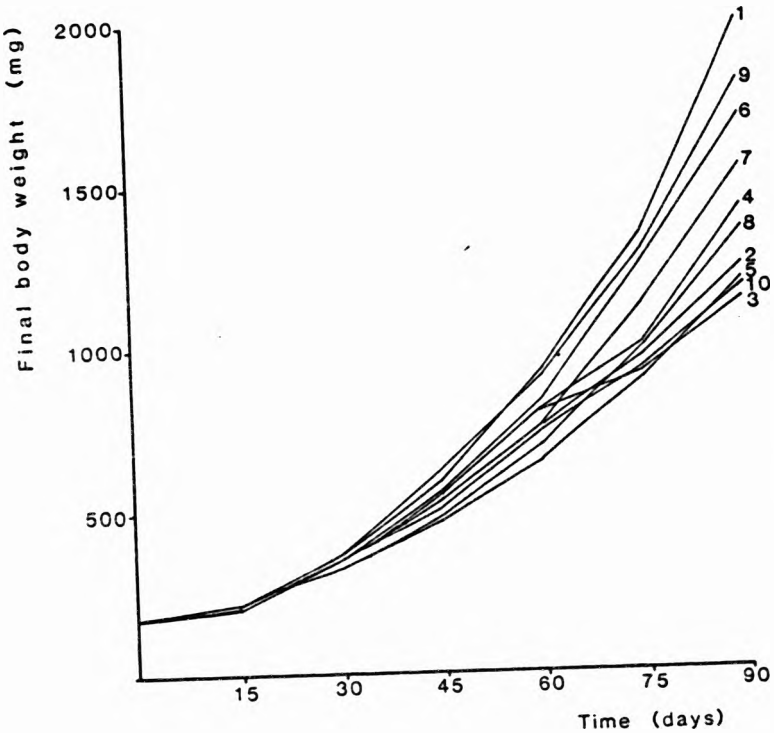

Final body weight (mg)
2000
1500
1000
500
15
30
45
60
75
90
Time (days)
1
9
6
7
4
8
2
5
10
3

TABLE 2.2 Effects of nine water soluble vitamin deficiencies on growth food conversion and survival of Cichlesoma urophthalmus during a 90 day experimental period.

Diet	Initial[1] Weight (mg)	Final[1] Weight (mg)	Individual[1] Weight gain (mg/day)	Individual[1] Food intake (mg/day)	Food[1] Conversion Ratio	Weight[1] gain %	Specific[1] growth rate	Survival %
1. Control	107^{a}	2000^{a}	20.705^{a}	29.938^{a}	1.450^{b}	1776.23^{a}	3.25^{a}	70
2. Thiamine (deficiency)	108^{a}	1240^{c}	13.012^{cd}	22.801^{bc}	1.754^{ab}	1051.56^{de}	2.71^{cd}	76.66
3. Riboflavine (deficiency)	107^{a}	1140^{c}	11.243^{c}	24.912^{abc}	2.233^{a}	966.86^{e}	2.62^{d}	73.33
4. Niacin (deficiency)	107^{a}	1420^{bc}	15.419^{bcd}	24.590^{abc}	1.595^{b}	1236.66^{cde}	2.87^{bcd}	86.66
5. Biotin (deficiency)	108^{a}	1200^{c}	11.294^{c}	20.981^{c}	1.962^{ab}	1019.57^{de}	2.68^{d}	53.33
6. Folic acid (deficiency)	105^{a}	1710^{ab}	17.7607^{ab}	24.478^{abc}	1.379^{b}	1484.86^{abc}	3.09^{ab}	81.66
7. Choline chloride (deficiency)	105^{a}	1550^{bc}	16.1352^{abc}	24.879^{bc}	1.542^{b}	1357.19^{bcd}	2.97^{abc}	78.33
8. Inositol (deficiency)	109^{a}	1360^{bc}	13.9422^{bcd}	23.281^{bc}	1.669^{ab}	1153.97^{cde}	2.80^{bcd}	85
9. Cyanocobalamin (deficiency)	108^{a}	1890^{a}	20.092^{a}	28.372^{ab}	1.417^{b}	1653.22^{ab}	3.18^{a}	80
10. Ascorbic acid (deficiency)	110^{a}	1180^{c}	11.515^{c}	20.309^{c}	1.765^{ab}	978.36^{e}	2.64^{d}	56.66

1. Values within column which bear different superscript were significant different at $P < 0.01$.

Cyanocobalamin deficiencies respectively) did not show any histopathological signs of deficiency at the end of this experiment.

Fish with biotin deficiency showed only haemorrhages in the pectoral fins as a result of this nutritional lack.

Fish with vitamin C deficiency had similar histopathological changes to those with the same deficiency (diets 1, 2 and 3) in the qualitative requirement experiment for this vitamin (Chapter 3) and they are fully described in section 3.3.5 of this chapter.

2.6. DISCUSSION.

For the first time C.urophthalmus fry were fed with a purified diet to meet their nutritional requirements. The basal diet was very well accepted by the fish which ate it voraciously until the appearance of deficiency signs. It was observed during the experiments that the composition of the basal diet proved to be good enough to support a good growth rate and food conversion ratio to be compared with the deficient diets.

It was demonstrated that under the conditions of these

experiments, C.urophthalmus fry had a requirement for 9 of the eleven water soluble vitamins and they were: pyridoxine, pantothenic acid, riboflavine, vitamin C, biotin, thiamin, inositol, niacin, and choline, while no differences in terms of growth and pathological response were observed in fish fed diets 9 and 6 (cyanocobalamin and folic acid).

In the case of the first experiment the most critical vitamins were pyridoxine and calcium pantothenate due to the early appearence of deficiency signs (days 8 and 18 respectively) and 100% mortality was reached on day 19 and 36 respectively. These findings are in agreement with the results of different researchers in many species of warm and coldwater fish studied, however to avoid repetitions these observations are examined and broadly discussed in the particular chapters of this study relating to the quantitative requirements of pyridoxine and calcium pantothenate (Chapters 4 and 5).

In the second experiment the most critical vitamins were riboflavine, vitamin C biotin and thiamine. Fish fed on the vitamin C deficient diet, were shown to have reduced appetite and growth together with a series of external signs similar to those observed in the experiment examining the quantitative requirement for vitamin C in this species, and as is the case of the

pyridoxine and calcium pantothenate, the results are fully described and discussed in chapter 3.

With respect to fish fed the thiamine deficient diet in both experiments, the only signs of deficiency showed were irregular appetite, reduced growth rate and poor food conversion ratio, being significantly different from the control diet. Thiamine deficiency has been studied in 9 species of fish (table 2.3), using different criteria, size of the fish and experimental periods. Gross signs of deficiency also varied and usually led to reduced appetite, total anorexia and reduced growth, but most of the other 11 different gross signs observed were apparently specific.

C.urophthalmus showed reduced appetite in less time than the other species, but it could be due to the smaller initial size of the fish in comparison with the initial size of the other species. Little work has been done looking for the quantitative requirement of this vitamin; McLaren et al., (1947); Cowey, Adron, Knox and Ball (1975); Kreutzman and Lehmitz (1976); Murai and Andrews, (1978), reported that Salmo gairdneri, Scophthalmus maximus, Anguilla anguilla and Ictalurus punctatus required between 1 to 10; 0.1; 0.6 to 2.6; and 1.0mg of thiamine/Kg diet respectively.

Halver and Tiews (1979) Halver 1982) reported that

TABLE 2.3 Thiamine (B_1) required, time for development of clinical signs, gross external signs observed in different species of fish and the different criteria, temperature of culture and initial size of the fish used during the experimental period.

Species	Temperature °C	Initial weight of the fish (g)	Criteria used	Exp. time (days)	Time of signs appearance (days)	mg of thiamine acid/kg diet	Gross signs of deficiency	Reference
Salmo gairdneri	—	12	3,7,8	196	—	1,10	2	1
Oncorhynchus tshawytscha	11-21	1.49	1,2,3,4,8	112	—	R	1,9,10,11	2
Oncorhynchus kisutch	12-20	7	1,2	112	84		1,7,25,31,32	3
Ictalurus punctatus	—	2	1,2,8	168	63	R	1,2,5,11	4
Cyprinus carpio	—	7.4		91	—	nR	—	5
		2.5		112	—	nR	—	5
		19.5		56	—	nR	—	5
Cyprinus carpio	—	5.3	1,2,9,10	77	35	R	1,13,16	6
Anguilla japonica	27 ± 1	0.75	1,2,5	112	56	R	1,2,7	7
	25 ± 1	1.0					1,2,8	
Anguilla japonica	25 ± 1	1 - 4	1,2,8	116	49	R	1,2,7,14	8
Poikilo-therm fishes						10-15		9
						10		9a
Scophthalmus maximus	10-14.9	18	1,6	112	84	0.6-2.6	1	10
Anguilla anguilla	19 ± 1	80	3,7	90	—	0.1	1,7	11
Ictalurus punctatus	27 ± 0.5	9	1,2,3,8	140	42	1.0	1,2,3,4,15	12
		6.3						
Cichlasoma urophthalmus	28°	.108	1,2,4,8	45	15	R	1,2,3	13
		.128		90	20	R		

Criteria Used

1. Growth
2. Gross signs
3. Haematological changes
4. Histological changes
5. Carcass composition
6. Tissue enzyme activities
7. Moisture and fat content in the liver
8. Mortality
9. Effect of antithiamine
10. Vitamin content in the tissues

Gross signs of deficiency

1. Anorexia (reduced appetite)
2. Reduced growth
3. Poor FCR
4. Dark colouration
5. Lethargy
6. Pin head condition, pinched abdomen
7. Haemorrhages in body and fins
8. Ataxia and flexion or winding of the trunk
9. Muscle atrophy
10. Convulsions prior to death
11. Loss of equilibrium
12. Loss of control of dorsal and pectoral fins
13. Colour change = transparent condition
14. Dark faeces
15. Mortality
16. Congestion of the fins and skin

Reference

1. McLaren et al., (1947)
2. Halver, (1957)
3. Halver & Coates,(1958)
4. Duerre, (1968)
5. Ase, Masuda, Saito, Kono, (1967)
6. Ase,et al., (1969)
7. Hashimoto, Arai and Nose, (1970
8. Arai, Nose and Hashimoto,(1972)
9. Halver, (1972)
9a. Halver, (1982)
10. Cowey, Adron, Knox and Sall (1975)
11. Kreutzmann and Lehnitz, (1976)
12. Murai and Andrews, (1978)
13. This study

coldwater fish require between 10-15 mg of thiamine/Kg diet. Apparently coldwater fish have a higher requirement for this vitamin than warmwater fish, but due to the different fish weights and criteria used and general paucity of data, this can not be conclusive.

C.urophthalmus fed a riboflavine deficient diet showed reduced appetite, low feed efficiency and reduced growth, and were the group with the lowest final weight, weight gain (%) and specific growth rate together with the worst food conversion ratio. The fish showed congestion in pectoral fins and mouth, small haemorrhages in the eyes, dark colouration and high mortalities. Riboflavin requirements have been studied in 13 species of fish (Table 2.4), the gross signs varied both intra and interspecifically, and as expressed in the thiamine case, this was probably due to the different fish size, experimental time and criteria used to determine the requirement. The most common signs were reduced appetite, poor growth, congestion and haemorrhages of the skin and fins, photophobia and increased mortalities. Ocular opacity (cataracts) resulting from lenticular or corneal abnormalities was associated with riboflavine deficiency in many species of fish (Halver, 1957; Phillips and Brockway, 1957; Coates and Halver, 1958; Dupree, 1966; Halver, 1972a; Poston, Riss, Rumsey and Ketola, 1977; Takeuchi, Takeuchi, and Ogino, (1980); Hughes, Riis,

TABLE 2.4 Riboflavin required, time for development of clinical signs, gross external signs observed in different species of fish and the different criteria, temperature of culture and initial size of the fish used during the experimental period.

Species	Temperature °C	Initial weight of the fish (g)	Criteria used	Exp. time (days)	Time of signs appearance (days)	mg of Riboflavin/kg diet	Gross signs of deficiency	Reference
Salmo gairdneri	—	3.5	1,4,8	196	—	5-15	3	1
Salvelinus fontinalis					—	.44-.68 *		2
Salmo trutta						.44-.68 *		2
Oncorhynchus tshawytscha	11-21	1.49	1,2,8,11	112	—	R	1,4,10,14,16 17,18,19	3
Oncorhynchus kisutch	12-20	0.5	1,2	112	—	— ?	—	4
Ictalurus punctatus	—	10	1,2,11	168	126	R	1,2,4,5	5
Cyprinus carpio	22-25	1.5 12.8	1,5,8	56 42	15	4-6.2	1,2,3,5,12	6
Cyprinus carpio	23 ± 1	2.8	1,5	42		7-10	1,2,3,13,14	7
Salmo gairdneri						20-30		8
S. trutta						20-30		8
Oncorhynchus kisutch						20-25		8
O. tshawytscha						20-25		8
Anguilla japonica	25 ± 1	4.5	1,2,11	112	49-70	R	1,2,3,6,14	9
Ictalurus punctatus	—	9	1,2,6,7,8	140	56	9	2,11,12	10
Coldwater fish		6.3		84		10-20 10		11 12
Salmo gairdneri	15-18	1.5	1,2,3,4,5	56	—	6	1,2,4,5	13
Cyprinus carpio	20-23	2.4		42	—	7	1,2,3,5	13
Salmo gairdneri	8.3	11.2	6	140	98	3	1,2,6,12,20	14
Salmo gairdneri	15 ± 0.5	7.4	1,2,3,4	140	84	4	1	15
Salmo gairdneri	15 ± 0.5	—	1,5,7,10,11	98	—	—	1,5,12	16
Salmo gairdneri	15	1.7 - 11	1,2,6,7,9	56-112	—	R	1,2,5,8,7 8,9,10	17
Salmo gairdneri	8.3	4.3	1,3,7,12	112	—	—		18
	8.3	3.2		140	—	—		18
	15	0.5		112	—	—		18
Salmo gairdneri	15		1,2,5,10	112	28	3.6-6.6	1,2,5,6,7 8,9,10	19
	15			70				
	10			70				
Clarias batrachus	—	4-5	1,2,11	168	35	R	1,3,4,8	20
Cichlasoma urophthalmus	28	0.109 0.129	1,2,6,11	45 90	15 49	R R	1,2,3,8,12,15	21

Criteria used

1. Growth
2. Gross signs
3. Carcass composition
4. Lipid content in the liver
5. Maximum storage in the tissue
6. Histological changes
7. Feed conversion ratio
8. Haematological changes
9. Biochemical changes in the liver (Erythrocyte glutathione reductase activity)
10. Hepatic D-amino-acid oxidase (DAAO) activity
11. Mortality
12. Effect of excess dietary riboflavin

Gross signs of deficiency

1. Reduced appetite
2. Poor growth
3. Congestion and haemorrhages
4. Cataracts
5. High mortalities
6. Lethargy
7. Apparent muscular weakness
8. Rapid operculae movements
9. Severe fin erosion
10. Light skin colouration
11. Short body duration
12. Poor feed conversion ratio
13. Nervousness
14. Photophobia
15. Haemorrhagic eyes
16. Dim vision
17. Incoordination
18. Abnormal pigmentation of fins
19. Striated constriction of the abdominal wall
20. Hyperplasia fusion of epithelial lamellae

Reference

1. McLaren et al., (1947)
2. Phillips and Brockway, (1957)
3. Halver, (1957)
4. Coates and Halver, (1958)
5. Dupree, (1966)
6. Ando et al., (1967)
7. Ogino, (1967)
8. Halver, (1972)
9. Arai et al., (1972)
10. Murai and Andrews, (1978)
11. Halver, (1979)
12. Halver, (1982)
13. Tanenchi et al., (1980)
14. Hughes, Mills, Bickham and Ramsey (1981)
15. Woodward, (1982)
16. Woodward, (1983)
17. Woodward, (1984)
18. Hughes (1984)
19. Woodward, (1985)
20. Buttner, Sitholt, Groenveldsolin (1985)
21. This study

Nickum and Rumsey, 1981; Hughes, 1985). However Woodward (1984) has consistently failed to observe large numbers of cataracts or other eye lesions among riboflavin deficient rainbow trout. His experiments showed that in 6 different experiments with Salmo gairdneri of varying sources and weights, cataracts occurred at a low frequency in all experiments and were no different from those of either satiety fed or pair fed control. In this respect some of the C.urophthalmus at the end of the experiment showed small haemorrhages in the eye, but no other abnormalities, either gross or histological were observed. As can be seen in Table 2.4, cataracts (gross sign number 4) as signs of riboflavin deficiency were not a consistent feature, even in the same species.

The requirements in the different species varied from 3 to 30 mg/Kg dry diet, but this varied even in the same species, for example the level for Salmo gairdneri varied in the different experiments from 3 to 30mg/Kg diet (Table 2.4). These differences indicate that it is necessary to ensure that the methodology and criteria for determining the nutritional requirements are uniform.

C.urophthalmus fed niacin deficient diets showed irregular appetite and reduced growth as the only signs of deficiency . Niacin requirements have been studied in 10 species of fish (Table 2.5), gross signs of deficiency varied widely between species and most commonly included

TABLE 2.5 Niacin required, time for development of clinical signs, gross external signs observed in different species of fish and the different criteria, temperature of culture and initial size of the fish used during the experimental period.

Species	Temperature °C	Initial weight of the fish (g)	Criteria used	Exp. time (days)	Time of signs appearance (days)	mg of niacin per kg diet	Gross sign of deficiency	Reference
Salmo gairdneri	—	12	3,8,9	196	—	1-5	2,21	1
Salvelinus fontinalis	8-11.5	—	1,4	56	—	3-4,1 *	2	2
Salmo trutta						3-4,1		
Salmo gairdneri						3-4,1		
Salmo gairdneri	—	4-5	1,2,3	175	—	6	—	3
Oncorhynchus tshawytscha	11-21	1.49	1,2,3,5,9	112	91	R	1,9,10,11,12	4
Oncorhynchus kisutch	12-20	—	1,2	112	—	R	1,3,13,17,18	5
Ictalurus punctatus	—	5	1,2,9	210	168	R	1,2,14,15,16,19	6
Cyprinus carpio	23 ± 1	1.2	1,2,9	56	—	28	2,5,20	7
Anguilla japonica	25 ± 1	1.2	1,2,9	126	56	R	1,2,10,22,5,23	8
Ictalurus punctatus	—	9 6.3	1,2,3,5	140 84	42	14	2,4,5,7,19,20	9
Coldwater fish						120-200 150		10 10a
Salmo gairdneri	9	0.78	1,2,5,7	112	—	18	1	11
Clarias batrachus	—	4-5	1,2,9	168	84	R	1,2,5,6,10,13,19	12
Cichlasoma urophthalmus	28	107-129	1,2,5,7,9	98	—	R	1,2	13

Criteria used

1. Growth
2. Gross signs
3. Haematological changes
4. Pantoate storage in the tissues
5. Histological changes
6. Intestinal synthesis
7. Behaviour
8. Moisture and fat content in the liver
9. Mortality

Gross signs of deficiency

1. Reduced appetite
2. Reduced growth
3. Fin head condition
4. Anaemia
5. Haemorrhages
6. Exophthalmia
7. Deformed jaws
8. Slow slime disease
9. Lesions in colon & stomach
10. Jerky or difficult motion
11. Anaemia
12. Muscle spasms
13. Loss of equilibrium
14. Stress susceptibility
15. Rigid bodies
16. Opercles spread
17. Loss of control of dorsal and pectoral fins.
18. Colour transparent condition
19. Lethargy
20. Mortality
21. Swollen gills
22. Dark colour
23. Ataxia and incoordination

Reference

1. Petersen et al., (1947)
2. Phillips & Brockway (1947)
3. Wolf, (1951)
4. Halver, (1957)
5. Halver & Coates, (1958)
6. Duerre, (1966)
7. Aoe, Masuda and Tanada, (1967)
8. Arai, Nose & Hashimoto, (1972)
9. Andrews & Murai, (1978)
10. Halver, (1979)
10a. Halver, (1982)
11. Poston & Wolfe (1985)
12. Buttner et al, (1986)
13. This study

* mg/kg body weight/day

reduced appetite and growth. C.urophthalmus showed only these signs, while another 21 signs were observed in other species, it could be that with longer experimental time more deficiency signs appear. In respect to the quantitative requirement this has been established in 5 species, Salvelinus fontinalis. Salmo trutta and Salmo gairdneri (Phillips and Brockway, 1957); Cyprinus carpio (Aoe, Masuda and Takada, 1967), Ictalurus punctatus (Andrews and Murai, 1978) and Salmo gairdneri (Poston and Wolfe, 1985) (Table 2.5).

C.urophthalmus fed the biotin deficient diet showed reduced appetite in both experiments, but they never showed total anorexia. Afterwards the fish were observed with congestion in all the body, lighter colour, haemorrhages in pectoral and caudal fins with caudal fins contracted to a triangular form. At the end of the experiment the fish started to die. Biotin requirement has been studied in 9 species of fish (Table 2.6) and the same pattern of gross deficiency signs as for previously mentioned vitamins was observed, in which sixteen different gross signs were reported and only reduced appetite and reduced growth were present in most of the deficient fish studied. Lighter skin colour was observed in Salmo gairdneri (Castledine, Cho, Slinger, Hicks and Bayley., 1978) Ictalurus punctatus (Lovell and Buston, 1984), and C.urophthalmus. Caudal fin contraction forming

TABLE 2.6 Biotin required, time for development of clinical signs, gross external signs observed in different species of fish and the different criteria, temperature of culture and initial size of the fish used during the experimental period.

Species	Temperature °C	Initial weight of the fish (g)	Criteria used	Exp. Time (days)	Time of signs appearance (days)	mg of biotin per kg dry diet	Gross signs of deficiency	Reference
Salmo gairdneri	—	12	10,11,12	196	—	0.09-0.25	4,13	1
Salmo gairdneri	—	4-5	1,2,11	175	—	NR	—	2
Salvelinus namaycush						.0068 -.027 *	4,16	
Salvelinus fontinalis						.0068-.027 *	4,16	3
Salmo trutta						.043-.088 *	4,16	3
Oncorhynchus tshawytscha	11-21	—	1,2,8,11 12	112	—	R	1,11,12	4
Oncorhynchus kisutch	12-20	0.56	1,10	112	74	R	1,9,10	5
Ictalurus punctatus	—	5	1,2,15	210	—	NR	—	6
Carassius auratus	24-25	2	1,10			R	4	7
Cyprinus carpio	20	3.6	1,8,10,11	90	60	0.1	1,4,8	8
Anguilla japonica	25 ± 1	2.3	1,2,12	78	56	R	4,14	9
Salvelinus fontinalis	8.3	1.2	4,5,6,7	140	21	R	1,4,5	10
Salvelinus namaycush	9	2.2	1,3,4	140	—	0.1;0.5-1.0	4,5	11
Salmo gairdneri	14.5-15.5	—	1,2,5,6,7,8	196 168	28 32	0.26	2,6,7	12
Ictalurus punctatus	30	2.9	1,2,8,11 6,7	154	98	R	4	13
Coldwater fish						1-5.2 1		14 14a
Salmo gairdneri	15 ± 2	25	1,2,8	54	—	R	4,5	15
Ictalurus punctatus	28 ± 1	2	1	119	77	R	1,2,3	16
Cichlasoma urophthalmus	28	107 129	1,2,8,12	45 90	20 42	R	1,2,4,10 15,17	17

Criteria used

1. Growth
2. Gross signs
3. Feed utilization
4. Level of swimming stamina
5. Carcass composition
6. Liver fatty acid composition
7. Enzime activities
8. Histopathology
9. Different dietary lipid and/or carbohydrate
10. Biotin content in the tissues
11. Haematology
12. Mortality

* mg/kg body weight/day

Gross signs of deficiency

1. Reduced appetite
2. Lighter skin colour
3. Highly sensitive to abrupt movements
4. Reduced growth
5. Low FCR
6. Pale gills, protruded beyond the operculae
7. Enlarged livers
8. Reduced activity
9. Emaciated bodies
10. Caudal fins contracted to form a triangular point
11. Spastic convulsions
12. Lesions in colon
13. Anaemia
14. Abnormal swimming
15. Slight congestion and small haemorrhages in the fins
16. Blue slime disease
17. Mortality

References

1. McLaren et al., (1947)
2. Wolf,(1951)
3. Phillips and Brockway,(1957)
4. Halver, (1957)
5. Coates & Halver,(1958)
6. Owsley, (1966)
7. Tomiyama and Ohno, (1967)
8. Ogino, Watanabe, Kakimo, Iwanaga and Mitsuno, (1970)
9. Arai,Nose & Hashimoto, (1972)
10. Poston & McCartney (1974)
11. Poston, (1976)
12. Castledine, Cho, Slinger Hilton and Bayley, (1976)
13. Robinson & Lovell, (1978)
14. Halver, (1979)
14a. Halver, (1982)
15. Walton, Casey and Adron (1984)
16. Lovell & Buston, (1984)
17. This study

a triangular point was observed in Oncorhynchus kisutch (Coates and Halver, 1958) and in C.urophthalmus. Little work has been undertaken to look for the quantitative requirement of biotin for the different species. McLaren et al., (1947) determined for Salmo gairdneri a requirement of between .05 and .25 mg/Kg diet; Ogino, Watanabe, Kakino, Iwanaga and Mizuno, (1970) determined for Cyprinus carpio 1 mg/Kg diet; Poston (1976); reported for Salvelinus namaycush a requirement of 0.1 mg/kg diet for optimum growth and feed conversion, but from 0.5 to 1.0 mg/Kg diet was needed for optimum swimming stamina. Castledine et al., (1978) reported for Salmo gairdneri a requirement of 0.26 mg/Kg diet; Halver, (1982) reported 1 mg/kg for coldwater fish.

Fish fed on choline, inositol and folic acid deficient diets did not show external signs of deficiency and at the end they exhibited only slightly reduced growth. Lack of folic acid made no significant difference at the time of this experiment, while choline and inositol were in the border line of significance and probably with more time of feeding this fish could separate clearly from the control. The reason for these results could be due to the effects of casein, in spite of the fact that it was washed with hot methanol to eliminate the possible traces of vitamins, it still may have contained enough content to maintain the fish in

good conditions during the experiment. The studies of these three vitamins are shown in Tables 2.7; 2.8; 2.9.

Folic acid requirement has been studied in 3 warmwater and 5 coldwater fish (Table 2.7). Cyprinus carpio is the only species to show neither a definite requirement nor deficiency syndrome even when kept on the folic acid deficient diet for a long period (Aoe, Masuda, Saito, Takada, 1957) and later, Kashiwada, Kanazawa and Teshima (1971) demonstrated that folic acid is synthetized by intestinal bacteria of carp. All the other species studied demonstrated a need for folic acid to maintain normal growth and health, and were shown to have different gross deficiency signs that vary widely between species, the most common being reduced appetite and growth together with dark colouration. It would be neccessary to feed C. urophthalmus over a longer period of time with a folic acid deficient diet to determine it's essentiality.

Choline has been studied in 9 species of cold and warmwater fish (Table 2.8), few deficiency signs have been observed in those fish in comparison with fish fed diets deficient in the vitamins from tables 2.3 to 2.7, the only common sign being reduced growth. The quantitative requirement has been established for Salmo gairdneri as between 50 and 100 mg/Kg diet (McLaren et

TABLE 2.7 Folic acid requirement, time for development of clinical signs, gross external signs observed in different species of fish and different criteria, temperature of culture and initial size of the fish used during the experimental period.

Species	Temperature °C	Initial Weight of the fish (g)	Criteria used	Exp.Time (days)	Time of signs appearance	mg of folic acid/kg diet	Gross signs of deficiency	Reference
Salmo gairdneri	—	12	3,7,9	196		1-5	2	1
Salmo gairdneri	—	4-5	1,2,3	175		R	1,8,15	2
Cyprinus carpio	—	2.5	1,2,3,4	112	—	NR	—	3
Oncorhynchus tshawytscha	11-21	1.49	1,2,3,8,9	112	70	R	1,4,6,8,10	4
Oncorhynchus kisutch	12-20			112		R	1,7,12,13,14	5
Ictalurus punctatus	—	5	1,2,9	210		R	1,2	6
Oncorhynchus kisutch	10	0.70	1,2,3	98		R	1,2,4,5,8,9,11	7
Cyprinus carpio	20-25	100	5	10	—	IS	—	8
Anguille japonica	25 ± 1	± 2.3	1,2,9	116	63	R	1,4	9
Coldwater fish						5-10 S		10
Labeo rohita	18-28	2.35 ± 0.82	1,2,3,6	105	—	R	1,2,3,4,7,8	11
Channa punctatus	—	32.5 ± 0.98	1,2,3,9	90		R	1,2,6,10	12
Clarias betrachus	—	4-5	1,2,9	168	42	R	1,2	13
Cichlasoma urophthalmus	28	109	1,2,8,9	90	—	NR	1,2,6,10	14

Criteria Used

1. Growth
2. Gross signs
3. Haematological changes
4. Maximum storage in the tissue
5. Intestinal synthesis
6. Haemopoiesis
7. Moisture and fat content in liver
8. Histological changes
9. Mortality

Gross signs of deficiency

1. Reduced appetite
2. Reduced growth
3. Low FCR
4. Dark colouration
5. Mortality
6. Lethargy
7. Pin head condition
8. Anaemia
9. Exophthalmus
10. Fragility of caudal fin
11. Distended abdomen with ascites fluid
12. Loss of equilibrium
13. Loss of control of dorsal and pectoral fins
14. Colour transparent condition
15. Blue slime disease

Reference

1. McLaren et al., (1947)
2. Wolf, (1951)
3. Ako, Masuda, Saito and Takase, (1957)
4. Halver, (1957)
5. Halver & Coates (1958)
6. Dupree, (1966)
7. Smith & Halver, (1969)
8. Kashiwada Kanekawa, Tashima, (1971)
9. Arai et al., (1972)
10. Halver (1979), Halver (1982)
11. John and Ranajan, (1979)
12. Ranajan & John, (1981)
13. Guttman et al., (1985)
14. This study

TABLE 2.8 Choline required, time for development of clinical signs, gross external signs observed in different species fish and the different criteria, temperature of culture and initial size of the fish used during the experimental period.

Specie	Temperature °C	Initial Weight of the fish (g)	Criteria used	Exp. time (days)	Time of signs appearance (days)	mg of choline acid/kg diet	Gross signs of deficiency	Reference
Salmo gairdneri	—	12	3,5,7	196		50-100	2	1
Oncorhynchus tshawytscha	11-21	1.49	1,2,3,4,7	112		R	1,3,4	2
Oncorhynchus kisuth	12-20			112		R	2,5	3
Ictalurus punctatus	—	9	1,2,7	252		R	1	4
Cyprinus carpio	20-23	3.5	1,2,4,7	60		2000-4000	—	5
Oncorhynchus tschawytscha	—					600-800		6
Oncorhynchus kisutch						600-800		6
Cyprinus carpio						1500-2000		6
Anguilla japonica	25 ± 1	2.3	1,2,7	116	14	R	1,2	7
Chrysophrys major						R		8
Coldwater fish						600-800		9
Ictalurus punctatus						NR		10
Coldwater fish						3000		11
Cichlasoma urophthalmus	28	.106	1,2,4,7	90	—	R	2	12

CRITERIA USED
1. Growth
2. Gross signs
3. Haematological changes
4. Histological changes
5. Intestinal syndrome
6. Moisture and fat content in the liver
7. Mortality

GROSS SIGNS OF DEFICIENCY
1. Anorexia
2. Reduced growth
3. Low FCR
4. Haemorrhages
5. Emaciated fish
6. Mortality

REFERENCE
1. McLaren et al., (1947)
2. Halver, (1957)
3. Halver & Coates, (1959)
4. Dupree, (1966)
5. Ogino, Uki, Watanabe, (1979 b)
6. Halver, (1972)
7. Arai et al., (1972)
8. Yone, (1975), In Lacon, (1985)
9. Halver, (1979)
10. Burtle (1981) In NAS (1983)
11. Halver, (1982)
12. This study

al., 1947); Salvelinus namaycush as 1000mg/Kg diet (Ketola, 1976) and Cyprinus carpio with a requirement of 2000 to 4000 mg/Kg diet (Ogino, Uki and Watanabe, 1970) Halver, (1982) reported a requirement of 3000 mg/Kg diet for coldwater fish.

Inositol deficiency has been studied in 7 species of fish. C.uroophthalmus fed a diet deficient in inositol showed as mentioned before, both reduced appetite and growth and these are common signs in other fish, but C.uroophthalmus did not show other signs such as haemorrhages, lesions in the skin, decomposition of mucosa, or sloughing off of scales, fins and epidermis observed by McLaren et al., (1947); Halver and Coates (1958) and Aoe and Masuda (1967) (Table 2.9). Requirements for inositol have been determined in Salmo gairdneri (McLaren et al., 1947); Cyprinus carpio and Chrysophrys major (Yone, Furuchi and Shitanda, 1971), as being between 250 to 900 mg/Kg diet.

Cyanocobalamin deficiency has been studied in 9 species of fish (Table 2.10), but it has been demonstrated that Cyprinus carpio (Kashiwada and Teshima, 1966; Kashiwada, Teshima and Kanazawa, 1970), Ictalurus punctatus (Dupree, 1966; Limsuwan and Lovell, 1981) and Oreochromis niloticus (= Tilapia nilotica) (Lovell and Limsuwan, 1982) do not require this vitamin for normal

TABLE 2.9 Inositol required, time for development of clinical signs, gross external signs observed in different species of fish and the different criteria, temperature of culture and initial size of the fish used during the experimental period.

Species	Temperature °C	Initial weight of the fish (g)	Criteria used	Exp. time (days)	Time of signs appearance (days)	mg of inositol acid/kg diet	Gross signs of deficiency	Reference
Salmo gairdneri	—	12	3,7,8	196		250-500	2,8	1
Salmo gairdneri	—	4-5	1,2,3	175		R	3	2
Oncorhynchus kisutch	12-20			112		R	1,2,9	3
Ictalurus punctatus	—	5	1,2,5	210		60	—	4
Cyprinus carpio	23 ± 1	6.3 6.8 33.3 8.8-9	1,2,4,8	112	28	440	1,2,5,6,7	5
Chrysophrys major	25	51-53	1,4,5	110		550-900	1,2	6
Anguilla japonica	25 ± 1	1.23	1,2,8	116	63	R	1,2	7
Calanotor fish						200-400 400		8 8
Cichlasoma urophthalmus	28	.189	1,2,8,9	90	—	R	1,2	9

Criteria used

1. Growth
2. Gross signs
3. Haematological changes
4. Maximum storage in the tissues
5. Tissue enzime activities
6. Moisture and fat content in the liver
7. Carcass composition
8. Mortality
9. Histological changes

Gross signs of deficiency

1. Reduced appetite
2. Reduced growth
3. Anaemia
4. Haemorrhages
5. Skin lesion
6. Skin, scale, epidermis sloughed off
7. Dark colouration
8. Fin degeneration
9. Mortality

Reference

1. McLaren et al., (1947)
2. Wolf, (1951)
3. Halver & Coates, (1958)
4. Dupree, (1966)
5. Ako and Mosuda (1967)
6. Yone, Furuchi and Shitenda,(1971)
7. Arai et al., (1972)
8. Halver, (1979)
   Halver, (1982)
9. This study

TABLE 2.10 Cyanocobalamin required, time for development of clinical signs, gross external signs observed in different species of fish and the different criteria, temperature of culture and initial size of the fish used the experimental period.

Specie	Temperature °C	Initial Weight of the fish (g)	Criteria used	Exp. time (days)	Time of signs appearance (days)	mg of B_{12} acid/kg diet	Gross signs of deficiency	Reference
Salmo gairdneri	—	4–5	1,2,3	175	—	NR		1
Oncorhynchus kisutch	11–21	1.48	1,2,3,5,9	112	—	A	1	2
Cyprinus carpio	23 ± 1		1,2,6	—	—	IS NR	—	3
Ictalurus punctatus	—	9	1,2,9	252	147	NR	1,2	4
Cyprinus carpio	20–25	70 120	1,2,6	—	—	NR IS	—	5
Anguilla japonica	25 ± 1	2.24	1,2,9	116	56	A	1,2	6
Labeo rohita	18–28	2.35 ± 0.82	1,2,3,7	105	—	A	1,2,3,4,6,7	7
Coldwater fish						0.15–0.02 0.02		8 8
Channa punctatus	—	32.5 ± 0.98	1,2,3,9	90	45	A	1,2,5,8	9
Ictalurus punctatus	28 ± 1	7.1 400 400	4 6,4 8	168 6 1	—	IS NR	— — —	10 10 10
Tilapia nilotica	28	7.1	1,3,4,6	112	—	IS NR	—	11
Cichlasoma urophthalmus	28	108	1,2,5,9	90	—	NR ?	—	12

Criteria used

1. Growth
2. Gross signs
3. Haematological changes
4. Maximum storage in the tissues
5. Histological changes
6. Intestinal synthesis
7. Haemopoiesis
8. Absorption of the vitamin through the gills
9. Mortality

Gross signs of deficiency

1. Anaemia
2. Reduced growth
3. Low FCR
4. Dark colouration
5. Lethargy
6. Pin head condition
7. Anaemia
8. Fragility of caudal fins

Reference

1. Wolf, (1951)
2. Halver, (1957)
3. Kashiwada and Tashima, (1966)
4. Dupree, (1966)
5. Kashiwada, Tashima and Kanazawa, (1970)
6. Arai et al., (1972)
7. Jann and Ranajan, (1979)
8. Halver, (1979)
   Halver, (1982)
9. Ranajan & John, (1981)
10. Limsuwan and Lovell, (1981)
11. Lovell & Limsuwan, (1982)
12. This study

growth, erythrocyte formation, good food conversion ratio and lack of deficiency signs as this vitamin is synthesized in the intestine of these three species. Significant amounts of cyanocobalamin were found to be synthesized in the intestine of channel catfish (Limsuwan and Lovell, 1981), and a year later Lovell and Limsuwan (1982) reported that the apparent rate of intestinal synthesis of this vitamin in O.niloticus fed the same cobalt supplemented diet was eight times higher than the value found in channel catfish fed the same diet under similar conditions and they explained that these differences of apparent synthesis rate could be due to the coprophagic characteristic and distinctive digestive tract in the former which has coprophagic habits and a much longer digestive tract than the channel catfish. C.urophthalmus fed a cyanocobalamin deficient diet did not show differences with the control diet and never showed abnormal behaviour or external and histopathological deficiency signs. From these results and from the data observed in Table 2.11 it is interesting to observe that C.uropthalmus was fed with the deficient diet over a shorter period than tilapia and channel catfish, but the initial size of the former was much smaller having as a consequence an increase in weight gain (%) much higher than the other two species. It is generally understood that smaller fish have higher requirements than bigger fish (Hilton, Cho and Slinger,

TABLE 2.11 Experimental condition, weight gain and food habits of three species of fish fed diets deficient in vitamin B_{12}

Species	Exp. time (weeks)	Initial Weight (g)	Weight gain (%) fish fed the basal diet + cobalt	Food habits	Digestive tract	Requirement	Intestinal synthesis	Reference
Ictalurus punctatus	20	7.1	336	omnivorous	short	NR	yes	Limsuwan and Lovell (1981)
Tilapia nilotica	16	7.1	800	herbivorous	long	NR	yes	Lovell and Limsuwan (1982)
Cichlasoma urophthalmus	12	0.108	1653.22	carnivorous with occational omnivorous	short	NR	?	This study

1978) and due to that, their stored vitamins are more rapidly utilized and exhausted as they have a more rapid metabolic rate. The fact that C.urophthalmus fry were fed a deficient diet for a shorter time but had a higher growth rate could counter out each other and allow a comparison between this data and that obtained for channel catfish and tilapia (Table 2.10). In respect to food habits there is a clear difference between these three species, tilapia being herbivorous, channel catfish omnivorous and C.urophthalmus carnivorous with occasional omnivorous habits (Martinez, PhD Thesis, 1987). C.urophthalmus did not show a requirement for cyanocobalamin in this experiment, however the basal diet was not tested to determine the possible content of this vitamin, and further experiments of longer duration would be necessary to determine its essentiality and the possibility of intestinal synthesis.

With respect to the histopathological findings, those fish fed the basal diet showed some fatty liver change in the hepatopancreas as well as slight oedema in the basal epithelium of the gill being frequently observed in the fish from all treatments in both experiments. This result probably indicates an imbalance or a deficiency in some of the nutritional components of the basal diet. Unfortunately the histological analysis of these two experiments were realized afterwards and

these problems were observed when the quantitative experiment for vitamin C, pyridoxine and calcium pantothenate were running, and the same pathological findings were also observed in all treatments of these studies and they are discussed in the respective chapters. Apart from the latter no histopathological changes were observed in the control diet and in the case of the following deficient diets; pyridoxine, thiamine, riboflavine, niacin, biotin, folic acid, choline inositol and cyanocobalamin.

Histopathological changes were observed in the fish fed vitamin C and calcium pantothenate deficient feeds and they are referred to in their respective chapters (Chapter 3 and 5 respectively).

CHAPTER 3.

DETAILED VITAMIN C REQUIREMENTS AND ITS DEFICIENCY SYNDROME.

3.1. INTRODUCTION.

Nutritional diseases caused by the deficiency of some nutrients in the diet were recognized in man and in domestic animals hundred of years ago (Halver, 1972a). Fish in their natural habitats seldom show signs of nutritional diseases because natural aquatic food ussually provides a balanced nutritious diet especially with respect to certain essential factors such as vitamins and minerals (Lovell 1975; NRC, 1983). In contrast to extensive and semi-intensive culture systems, where fish obtain all or part of their nutrient requirements from naturally available food organisms, fish maintained under intensive culture systems rely totally on the provision of a nutritionally complete diet throughout their culture cycle. For many farmed fish species the development of complete artificial rations (for use within intensive culture conditions) has proceeded despite the lack of information available on basic nutrient requirements (Tacon, 1985).

Under intensive culture systems, and in the absence of natural food organisms, dietary vitamin deficiencies may arise through feed processing and storage, leaching of water soluble vitamins (Andrews

and Murai, 1975; Hilton, Cho and Slinger 1977a,b), presence of dietary anti-vitamin factors, dietary vitamin toxicity and dietary antibiotic addition (Tacon, 1985). Vitamin C is highly unstable in practical diets for fish and for this reason numerous studies have been carried out to identify the loss of vitamin C from the diets and the factors affecting its stability. Other researchers have run trials to identify the most stable form of vitamin C and the biological activity of these forms (Andrews and Murai, 1975; Hilton, Cho and Slinger, 1979a,b; Hilton, Cho and Slinger, 1978; Lim and Lovell, 1978; Murai, Andrews and Bauerfeind, 1978; Slinger, Razzaque and Cho, 1979; Soliman, 1985).

The ability of most vertebrates to biosynthesize Vitamin C is well documented and few animals, including primates, guinea pigs and some birds and bats, are known to require a dietary supply of this vitamin (Chaterjee, 1973; Scott 1975). In fish, feeding studies have shown that many freshwater and marine species are also dependant on exogenous sources of ascorbic acid (vitamin C). The first work on the essential nature of dietary ascorbic acid for fish was carried out by McLaren et al., (1947) and Kitamura, Ohara, Suwa and Nakagawa, (1965). Following these initial studies, other aspects of Vitamin C have been studied in 10

different species of fish with importance in aquaculture. Experiments to analyse the ability of 21 species of fish to synthesize Ascorbic Acid were carried out by Primbs and Sinnhuber (1971); Wilson, (1973); Yamamoto, Sato, Ikeda, (1977, 1978); Sato, Yoshinaka, Yamamoto, (1978); Hilton, Cho, Brown and Slinger, (1979b); Soliman, Jauncey and Roberts, (1985). Their results determined an inability to synthesize ascorbic acid in all the species except the carp Cyprinus carpio, and tilapia Oreochromis niloticus and O.aureus.

Knowing the essential nature of ascorbic acid, studies were then carried out on the nutritional requirement for vitamin C for various species of fish, based on growth parameters, behaviour, gross pathology and histopathology (Halver, Ashley, Smith, 1969; Arai, Nose and Hashimoto, 1972; Lovell, 1973; Andrews and Murai, 1975; Ashley, Halver and Smith, 1975; Hilton et al., 1978; Lim and Lovell, 1978; Lovell and Lim, 1978; Mahajan and Agrawal, 1979; Halver and Tiews,, 1979; Agrawal and Mahajan 1980a, 1980b; Miyazaki, Plumb, Li and Lovell, 1985; Soliman, 1985).

Ascorbic acid has been shown to be required for collagen biosynthesis and plays a key role in wound healing, in this aspect several studies were carried out in fish showing that vitamin C is a vital co-factor

in the hydroxylation of proline to hydroxyproline and thus in the synthesis of collagen (Halver,1972; Wilson and Poe, 1973; Ashley et al., 1975; Lovell and Lim, 1978; Sato, Yoshinaka and Ikeda, 1978, 1982; Yoshinaka, Sato and Ikeda, 1978; Mahajan and Agrawal, 1979; Jauncey, Soliman and Roberts, 1985).

To understand the role of vitamin C in particular species, biochemical studies have been carried out to identify and measure storage, distribution, uptake, half-life, turnover rates and tissue storage, of the major forms of vitamin C (Ikeda, Sato and Kimura, 1963; Hilton Cho and Slinger, 1977a; Hilton, Cho and Slinger, 1977; Hilton, Brown and Slinger, 1979a,b; Tsujimura, Fukuda, Kasai and Kitamura, 1981; Tucker and Halver 1984a, 1984b; Soliman, 1985). Other biochemical studies in fish have examined the interaction of vitamin C with other vitamins such as vitamin A, Myo-Inositol and Alpha-tocopherol, the result of these studies showing that there is no interaction between vitamin A and vitamin C, while Myo-Inositol is converted to L-ascorbic acid. The administration of vitamin E stimulates the biosynthesis of vitamin C in carp (Ikeda and Sato, 1966; Ikeda, Sato and Taguchi, 1966; Hilton, Cho and Slinger, 1978b).

Vitamin C has also been studied in fish to determine the impact of this vitamin on blood

parameters. Hilton et al., (1978a) demonstrated that scorbutic trout developed a gradual but progressive anaemia, Wilson and Poe, (1973) observed that there was a marked reduction in the serum alkaline phosphatase activity in the scorbutic catfish and in the same species Lim and Lovell, (1978) found significantly lower haematocrits in the deprived fish. Agrawal and Mahajan, (1980a) in a detailed study on the haematological changes due to vitamin C in Channa punctatus reported normochromic normocytic anaemia which later developed into a normochromic macrocytic anaemia after 150 days of deficiency. Soliman, (1985) found that scorbutic O. niloticus and O. mossambicus had a decrease in haemoglobin, haematocrit and mean corpuscular hemoglobin values.

Vitamin C is also involved in detoxification reactions and several studies suggest that there is an increased tolerance by fish to environmental pollutants when ascorbate stores are high in carp and rainbow trout (Yamamoto, Ishii, Sato and Shizunori, 1977; Yamamoto, Hayama and Ikeda, 1981). However Lanno, Slinger and Hilton, (1985) found that dietary ascorbic acid supplementation up to 10,000 mg/Kg did not significantly alleviate dietary copper toxicosis in juvenile rainbow trout.

Beneficial effects of elevated levels of vitamin C on resistance to infection have been reported for humans and guinea pigs and recently this aspect has been studied also in fish (Schrauzer, 1979; Durve and Lovell, 1982; Bell, Higgs and Traxler., 1984; Li and Lovell, 1985).

The role of vitamin C in fish reproduction has been observed by Sandness, Ulgenes, Brackan and Utne, (1984) and Soliman, (1985) where ascorbic acid supplementation of broodstock feed improved both hatchability and fry condition in rainbow trout and two species of tilapia.

Mahajan and Agrawal, (1980b) studied the role of vitamin C in calcium uptake by fish and demonstrated that an optimum vitamin C supply in the diet of intensively cultivated fish would enable the fish to utilize properly the available dissolved calcium in the water, thus resulting in healthy fish stocks, good growth and total productivity.

Finally Thomas, (1984) studied the influence of some environmental variables on the ascorbic acid status of mullet, Mugil cephalus L., tissues. Their results suggested an involvement of ascorbic acid in osmo- or ion-regulatory functions of teleost gills,

salinity and thermal adaptation mechanisms in neural tissue, and the response of renal tissue to adverse environmental stimuli.

Cichlasoma urophthalmus is an American native cichlid with high potential for aquaculture due to its wide salinity tolerance, high fecundity, good feed conversion ratios, general hardiness and easy handling as well as ready acceptance in the local markets (Martinez and Ross, 1986). Various investigations on different aspects of their biology have been carried out to asses it's potential for aquaculture. As this species must be cultured intensively, at least during the early phases of growth, and considering the significance of vitamin C in aquaculture, this study was carried out to investigate the nutritional requirement for this vitamin in Cichlasoma urophthalmus based on growth rate, food conversion ratio, gross signs of deficiency and histopathological changes produced.

3.2. MATERIALS AND METHODS.

3.2.1..Experimental design and diets.

Groups of 20 native fish of Cichlasoma

urophthalmus obtained from Celestun Lagoon, Yucatan, Mexico (Section 1.2.), with initial weights of between 0.160 to 0.170 g., were placed in 30 tanks of the recirculation system (section 1.1). Ten different diets were prepared as previously described in section 1.3. Each diet was provided with one of ten different levels of ascorbic acid; 0; 40, 80, 160, 320, 640, 1280, 2560, 5120 and 10240 mg per kilogram of diet. Fish were acclimated to the system and to the basal diet for one week. After that period fish started to receive the experimental diets. Fish were fed with these diets Ad libitum during the 90 day of the experiment. Special care was taken in preparation and storage of the diets (section 1.3.2.) as well as in the feeding of the animals (section 1.4).

Procedures during the experiment, nutritional parameters measured and statistical analysis were described previously (Section 1.4; 1.6 and 1.7 respectively).

3.2.2.. Chemical analyses.

3.2.2.1. Peroxide value.

Fish oil and corn oil were analysed to determine the peroxide value to ensure there was no rancidity in the diets as described in section 1.3.1.

3.2.2.2. Ascorbic acid content in the diets.

After preparing the diets, a sample of each ingredient was analysed to determine the possible traces of ascorbic acid contained in each element through the McGown, Rusnak, Lewis and Tillotson, (1982) method. The same analysis was performed out on the ten different diets, one day after their preparation as well as at the end of the experiment, in order to determine in each, the real values of the ascorbic acid at the begining of the experiment and the possible loss of activity during the trial.

3.2.2.3. Methods of proximate analysis.

Once the diets were prepared and using the methods described in section 1.3.2. one sample was taken to determine the proximate analysis of all diets because all of them had the same composition except for one vitamin value.

3.2.3. Histological studies.

Fish were killed, sectioned and processed as previously described (section 1.6.), some sections were also stained with the Periodic acid-Schiff reaction to identify possible granules of glycogen in the liver; Van Gieson's stain to determine possible changes in the collagen; Ziehl-Nielsen technique to determine acid-

fast bacilli; Cresyl fast violet for Nissl substance; Giemsa stain for protozoans and the Gless Marshland impregnation method for axons. (Drury and Wallington, 1980).

3.2.4. X-Ray techniques.

Fish with visible changes in the skeleton as well as 4 fish from diet 1, 14 fish from diet 2 and 11 fish from diet 3 were killed with an overdose of benzocaine, immediately frozen with liquid nitrogen, preserved in this way for 15 days and then X-rayed using a Watson Mobilix ward type x-ray machine. Exposures used routinely were 53 Kv, 50 mA, 2s on Kodak Industrex-C film, developed and fixed in DX-80 and FX-40 (Kodak).

3.3. RESULTS.

3.3.1.Water quality.

Water quality during the experiment was:

Temperature 28°C (Range 26.5 to 29.5).

Ammonia 0.068ppm (range 0.034-0.102).

Un-ionized ammonia 0.005ppm (range 0.002 and 0.008 ppm).

Nitrites 0.023 ppm (range 0.012-0.032).

pH 8.1.

3.3.2. Proximate analysis.

The results of the proximate analysis of the diets are given in table 3.1.

3.3.3. Ascorbic acid content in the diets.

The quantity of ascorbic acid detected in the components of the basal diet is expressed in Table 3.2. The ascorbic acid added to the diets and the ascorbic acid values after processing and at the end of the experiment are show in Table 3.3.

3.3.4. Nutritional parameter results.

The mean growth response and performance data of Cichlasoma urophthalmus given the ten experimental diets is shown in Fig 3.1 and Table 3.4. There were no significant differences ($P<0.01$) in the initial weight; however final weight, individual weight gain, weight gain (%) and specific growth rate differed significantly ($P < .01$) between diet 1 (with total deletion of vitamin C) and the other diets. The opposite was true of food conversion ratio, diet 1 had the highest value and was significantly different to the other diets ($P < 0.01$). Finally in respect of individual food intake there was no significant difference (P 0.01) between treatments.

Table 3.1 Proximate analysis of the experimental diets (wet weight basis) used in the experiment of vitamin C requirement.

Crude Protein	44.20
Lipids	10.19
Ash	5.41
Moisture	6.21

Table 3.2 Results of the vitamin C values obtained in the materials used to prepare the basal diet.

Material	g Vit. C/kg
Corn Oil	.01
Cod Oil	.01
Casein (vitamin free)	.0075
Carboxymethyl cellulose	0
Starch	0
Dextrin	0

Table 3.3 Ascorbic acid remaining in the diets after processing and at the end of the experiment.

Diet	Ascorbic Acid added to the diets (mg/kg of food)	Ascorbic Acid in the diets after processing (mg/kg of food)	retention %	Ascorbic Acid in the diets at the end of the experiment (mg/kg of food)	retention %
1	0	0.0	-	0.0	-
2	40	40.0	100	10.0	25
3	80	78.0	97.5	75.0	96.15
4	160	110.0	68.75	75.0	68.18
5	320	207.5	64.84	110.0	53.01
6	640	537.5	83.98	467.5	86.97
7	1280	1247.5	97.46	1100.0	88.17
8	2560	2487.5	97.17	2462.5	98.99
9	5120	4637.5	90.57	4537.5	97.84
10	10240	9980.0	97.46	6887.5	69.01

Figure 3.1. Mean growth response of Cichlasoma urophthalmus fed with ten different levels of vitamin C, during 90 days of experimental period. (1-10= experimental diets as shown in Table 3.3.).

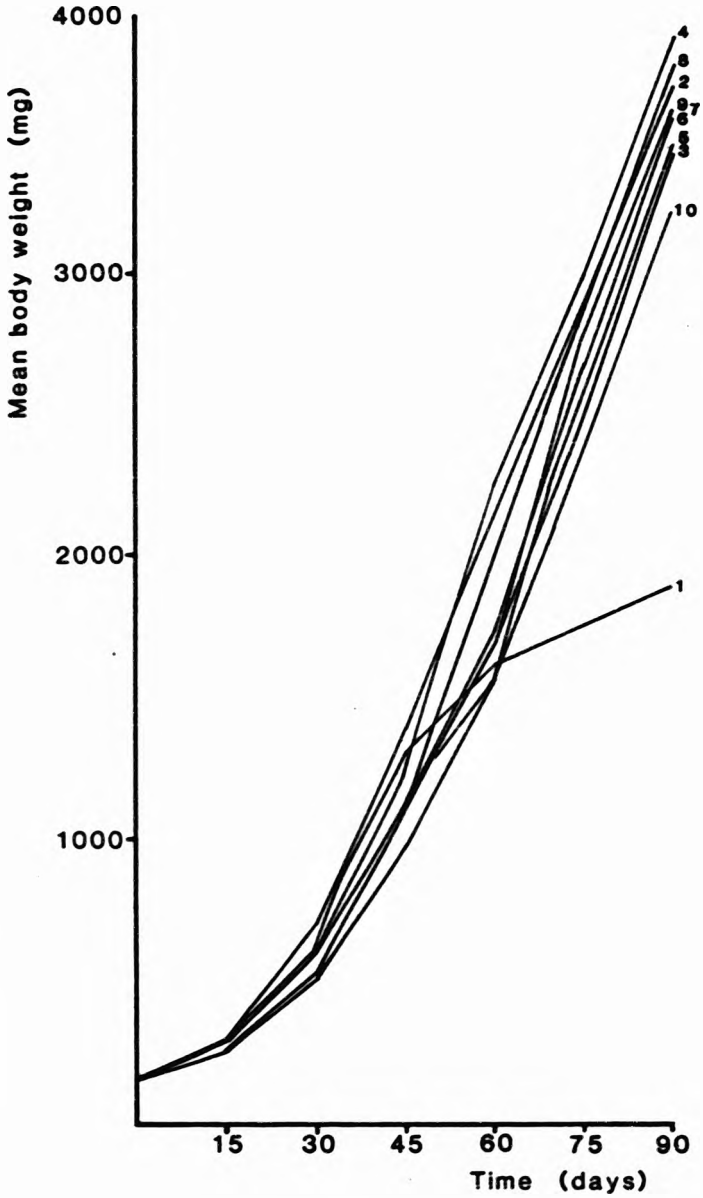

4000
3000
2000
1000
Mean body weight (mg)
15
30
45
60
75
90
Time (days)
4
8
2
9
7
6
5
3
10
1

Table 3.4 Effects of ten different levels of Ascorbic Acid on growth, food conversion and survival of Cichlasoma urophthalmus during 90 day experimental period.

Diet	Mg of Ascorbic acid/kg of diet	Initial Weight (mg)	Final[1] Weight (mg)	Individual[1] Weight gain (mg/day)	Individual food intake (mg/day)	Food[1] Conversion Ratio	Weight[1] gain %	Specific growth Rate	survival %
1	0	170	19[b]	19.67[c]	53.69[a]	2.7[a]	1017[b]	2.67[b]	21.6
2	40	167	3690[a]	33.68[b]	60.24[a]	1.9[b]	2109[a]	3.43[a]	28.33
3	79	162	3440[a]	35.35[b]	56.68[a]	1.6[b]	2029[a]	3.39[a]	76.6
4	110	168	3960[a]	39.69[b]	67.77[a]	1.7[b]	2188[a]	3.48[a]	61.6
5	270.5	168	3580[a]	37.54[b]	57.38[a]	1.5[b]	2032[a]	3.40[a]	75
6	537.5	167	3600[a]	37.75[b]	56.21[a]	1.5[b]	2044[a]	3.40[a]	85
7	1247.5	166	3570[a]	48.33[a]	54.65[a]	1.1[b]	2037[a]	3.40[a]	85
8	2487.5	164	3790[a]	37.96[b]	59.67[a]	1.6[b]	2202[a]	3.48[a]	56.6
9	4637.5	164	3590[a]	37.31[b]	63.26[a]	1.7[b]	2089[a]	3.4[a]	60
10	9990.0	167	3250[a]	34.05[b]	55.28[a]	1.6[b]	1842[a]	3.3[a]	60

1 Values within the same column which bear different letter are significantly different at P 0.01 and P 0.05.

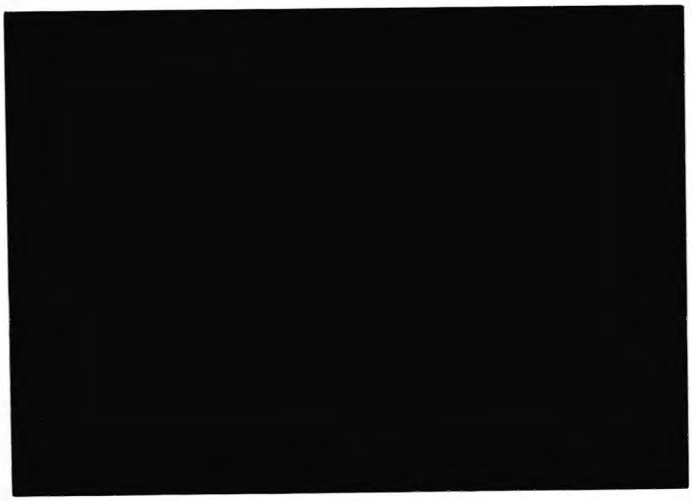
3.3.5. Behaviour, signs of deficiency and mortality.

Fish from diet 1 (total deletion of vitamin C) showed normal behaviour and appearance for 30 days, thereafter they started to reduce their appetite slightly and some had dark colouration and started to die without any other apparent signs. On the 45th day of the experiment the first obvious gross signs of deficiency were recorded with some fish showing dark colouration, others with obvious short operculae, one fish with haemorrhages in the head and eyes (Plate 3.1) and many fish with erosion of the skin and fins.

On the 49th day another fish was seen with eye haemorrhages and exophthalmia, (Plate 3.2), dark colour and swollen abdomen (Plate 3.3). From this day until the last day of the experiment all the fish from this diet showed a variety of the signs mentioned above and also became very thin and showed other signs such as iritis (Plate 3.4) (Personal communication Dr. A. Baqueiro: Ophthalmologist), a deformation of the lower jaw and the operculae became shorter and shorter until total exposure of the gills occurred (Plate 3.5). The abdomen was pinched and the flesh very flaccid (Plate 3.6). At the end of the experiment mortality was 78.33%.

Plate 3.1. Fish on vitamin C deficient diet showing pronounced haemorrhages in the head and eyes.

Plate 3.2. Marked haemorrhages and exophthalmia in the eyes of C.urophthalmus with zero vitamin C in the diet.



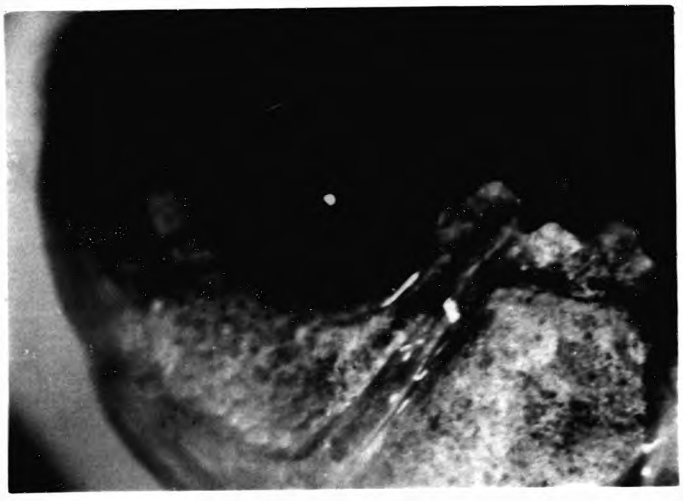


Plate 3.3. Fish on vitamin C deficient diet, showing exophthalmia, swollen abdomen and haemorrhages.

Plate 3.4. C.urophthalmus with iritis, haemorrhaghes, exophthalmia, short operculae, dark colour and erosion of the fins due to vitamin C deficiency.

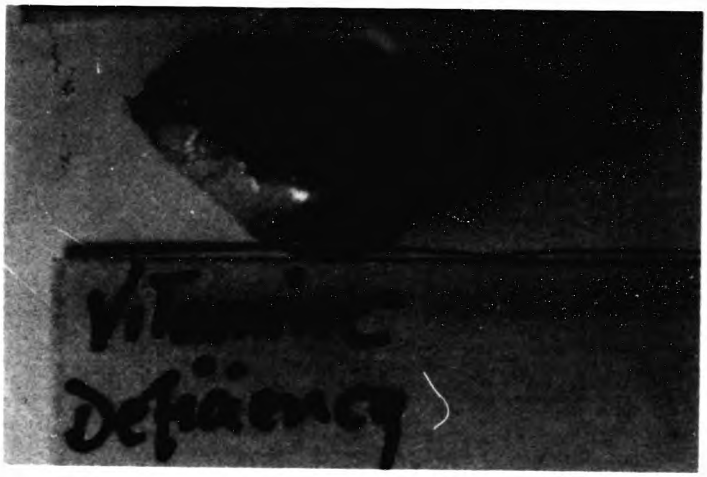
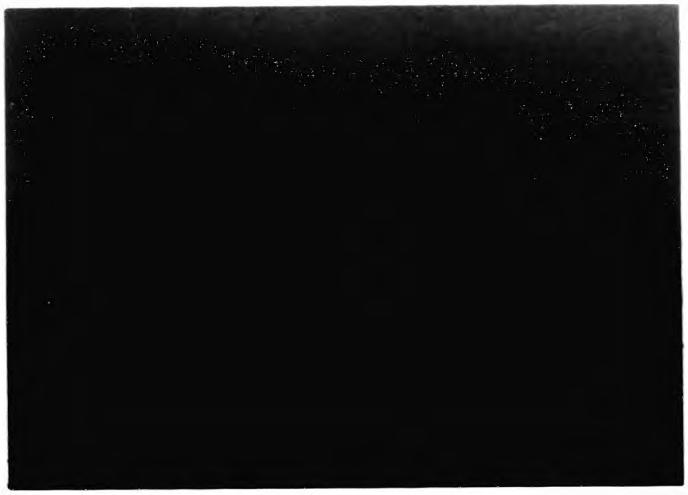
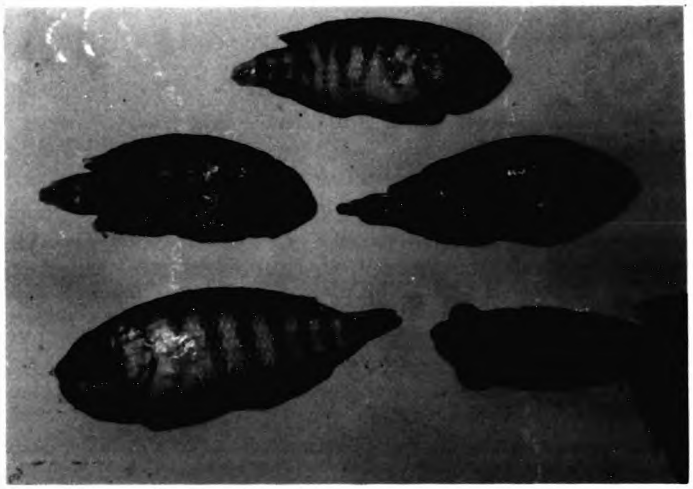

Deficiency

Plate 3.5. Extremely short operculae with total exposure of the gills, erosion of the skin and fins and haemorrhages in the eyes in C.urophthalmus after 90 days of total deletion of vitamin C in the diet.

Plate 3.6.Fish with dark colour, erosion of the fins, eyes haemorrahges, exophthalmia, iritis, short operculae, pinched abdomen and flacid flesh.





Fish from diets 2 and 3 had normal behaviour and appearance for 30 to 40 days, then on the 45th day of the trial, one fish from each diet was observed with deformation of the vertebra at the level of the tail (Plate 3.7), some of the fish had erosion of the skin and fins and a slightly dark colour. Most fish were apparently normal. No other different signs were registered during the rest of the experimental period, except that more fish acquired erosion of the fins and skin with loss of scales (Plate 3.8) and the mortality was higher in diet 2 than diet 3 (Table 3.4).

Fish from diets 4 to 10 did not have any gross signs of deficiency, but fish on diet 10 were very slow to eat and were frequently observed to be hungry, swimming to the surface to take food, then immediately rejecting it, such behaviour continuing until eventually small quantities were eaten, then another portion was administered. At other times the fish left the food floating or on the bottom for some time, eating it later. The mortality observed with these diets was due to the aggressive fish behaviour of some of the biggest fish in each tank (Table 3.4).

3.3.5.Histological changes.

The histological results described for the fish fed with total deletion of vitamin C in this section,

Plate 3.7. Fish fed with diets 2 and 3 with vertebral deformity. Normal fish at the bottom of the plate on diet 10.

Plate 3.8. Erosion of the skin, loss of scales and eroded fins in C.urophthalmus fed diets 2 and 3.

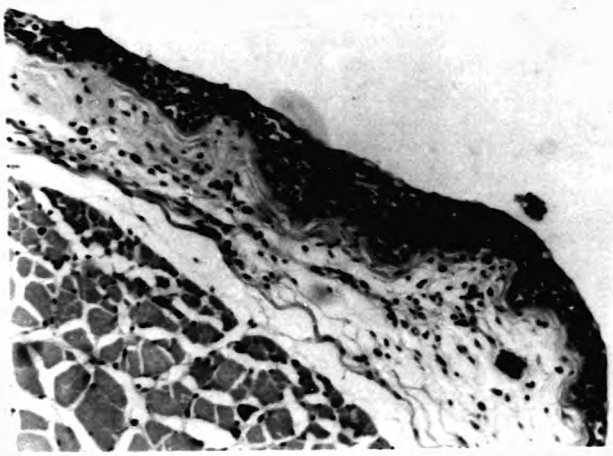
apply to those fish from diet 1 in this experiment as well as those fish from diet 10 in the second experiment (Chapter 2).

Histological sections of the skin from those fish fed with total deletion of vitamin C and diets 2 and 3, revealed an extensive mixed inflammatory response with many eosinophilic granular cells present in the epidermis. The epidermis was also affected by hydropic or vacuolar degeneration and in some areas degenerative changes in the basal cells had caused a complete disarragement of this cell layer, the dermis was observed frequently with inflammatory response, mainly with the presence of large number of eosinophilic granular cells (Plates 3.9, 3.10). The sections of the skin from diet 4 to 10 were normal in appearance.

Muscle sections from diet 1 and 2 of this experiment showed a range of pathological changes varying from a slight inflammatory response indicated by small numbers of lymphocytes in between the fibres to changes in the position and number of nuclei at the periphery or embedded within individual fibres (Plate 3.11), vacuolation and necrosis (Plate 3.12) granular degeneration (Plate 3.13) and fibre loss (Plate 3.14). Muscle sections from the fish with total deletion of vitamin C in experiment 2 (Chapter 2, diet 10) showed

Plate 3.9. Epidermis with extensive inflammatory cell infiltration, hydropic degeneration and atrophy of the basal cells. (H & E 312.5X).

Plate 3.10. Epidermis with severe inflammatory response, hydropic degeneration and atrophy of the basal cells. Obvious eosinophilic granule cells and acidophilic cells as well as lymphocytes are seen in epidermis (H & E 1250X).



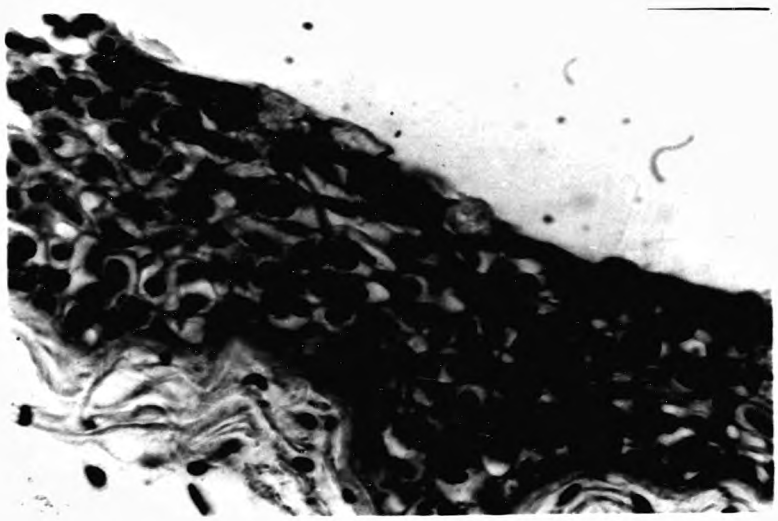
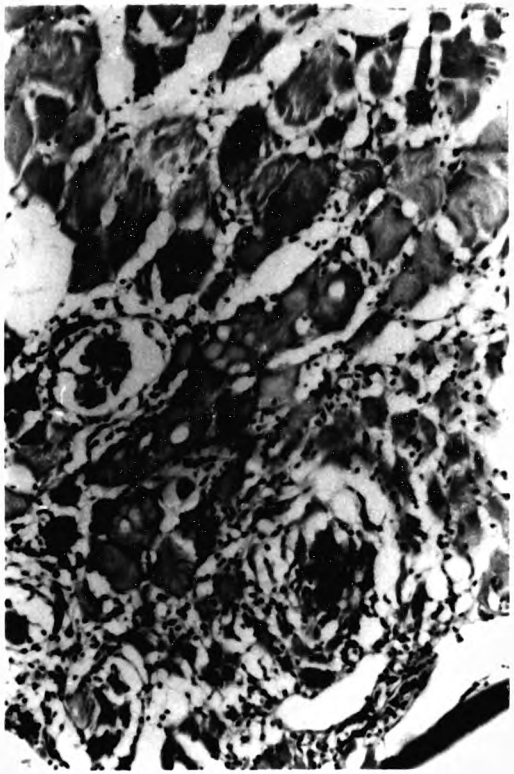


Plate 3.11. Nuclear changes in the muscle fibres of the fish fed diet 1. Note the changes in number and position of the nuclei (H & E 500X).

Plate 3.12. Muscle necrosis of the fish fed diet 1, vacuolar degeneration, cellular infiltration and disintegration of the muscle fibres are present (H & E 500X).



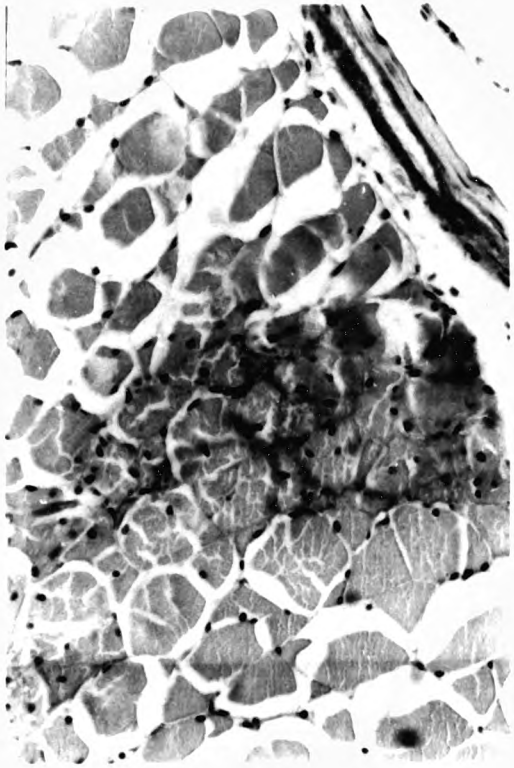
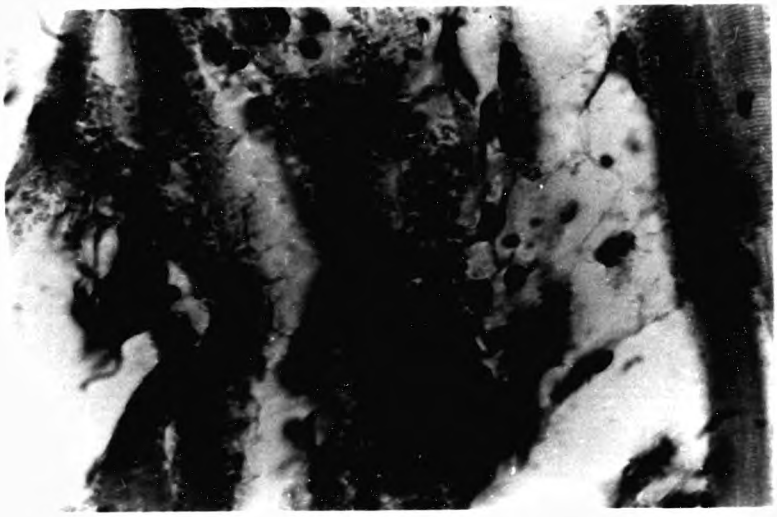
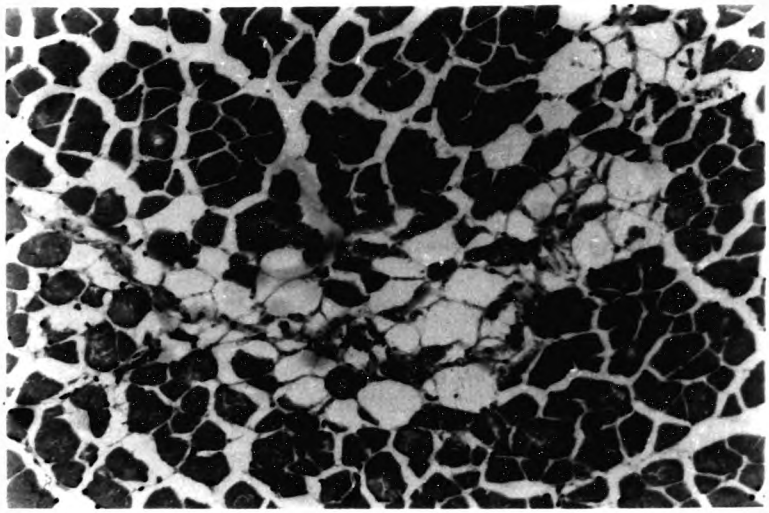


Plate 3.13. Muscle granular degeneration of the fish fed diet 1 and 2 (H & E 1250X).

Plate 3.14. Muscle of the fish fed diets 1 and 2 in which is obvious fibre loss (H & E 312.5X).





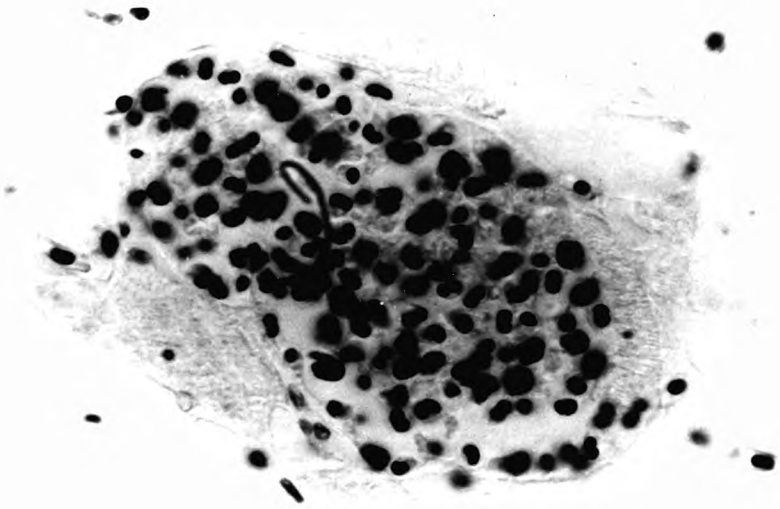
similar but less extensive pathological changes.

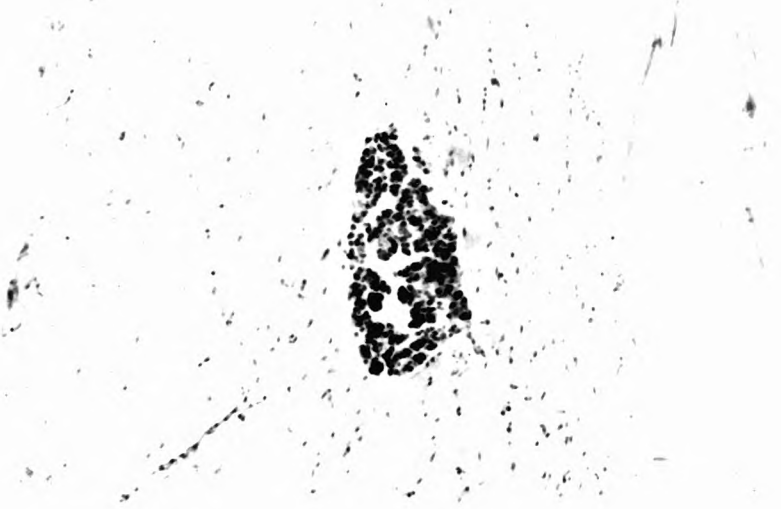
The muscle of the fish from the 10 diets of the present experiment, contained cysts of Myxobolus sp. (Plate 3.15) (Christina Sommerville, personal identification). Muscle bundles in the area around the Cysts in diets 1 and 2 occasionally appeared normal in structure (Plate 3.16) but in most cases an associated inflammatory response was seen (Plate 3.17). The changes described previously were also observed associated with Myxobolus cysts, however these pathological reactions were thought to be associated with vitamin C deletion as the same changes were found in those fish free from cysts from experiment 2 (Chapter 2). Muscle sections from diet 3 to 10 were normal in appearance except in the area immediately adjacent to cysts in which a slight inflammatory response was occasionally present.

The Myxosporidian cysts appeared in great numbers in diets 1 and 2, mainly in the muscular tissue, but some small cysts were also recorded in areas such as the heart muscle, thyroid gland, the choroid gland of the eyes and in various nerve ganglions. However in diets 3 to 10 the cysts were recorded only in the muscle and in much lower numbers than in diets 1 and 2 usually being isolated and in most cases no more than 3 cysts per histological section.

Plate 3.15. Cyst of Myxobolus sp. in the muscle of C.urophthalmus (Giemsa 1250X).

Plate 3.16. Cyst of Myxobolus sp. embedded in the muscle of C.urophthalmus. No associated inflammatory reaction is observed (PAS 312.5X).





Gills from diets 1, 2 and 3 showed a variety of changes including thickening of the primary lamellae (Plate 3.18), intracellular oedema with obvious epithelial separation (plate 3.19) hyperplasia (Plate 3.20) and telangiectasis (Plate 3.21). The bony supporting structures of the gills were atrophic, twisted and deformed (Plate 3.22) or were thickened with enlarged bizarre osteocytes (Plates 3.23). Gills from diet 4 to 10 sometimes showed slight intracellular oedema and slight hyperplasia in some areas but never the damage reported before for diets 1 to 3.

Fish from diets with total deletion of vitamin C (from both experiments) and diet 2 were found to be infected with presumptive Mycobacterium spp. (positive identification of the acid fast bacteria through the Ziehl-Neelsen technique). Miliary tuberculosis was found in many of these fish with anterior and posterior kidney, spleen and choroid gland being most commonly affected. The kidney and spleen were enlarged with numerous tubercles of different sizes and different stages of caseation (necrotic areas containing tubercle bacilli) (Plates 3.24, 3.25, 3.26). In some cases the tuberculosis was wide spread through all the viscera, and peritonitis was present (Plate 3.26). Nodules of caseous material and epithelioid cells were also observed in the pancreas, stomach intestine, heart and

Plate 3.17. Cyst of Myxobolus sp. embedded in the muscle fibres of C.urophthalmus. There is a lymphocytic infiltration. (H & E 312.5X).

Plate 3.18. Thickening of the primary lamellae and intracellular oedema of the secondary lamellae on the gills of the fish fed diets 1, 2 and 3 (H & E 312.5X).

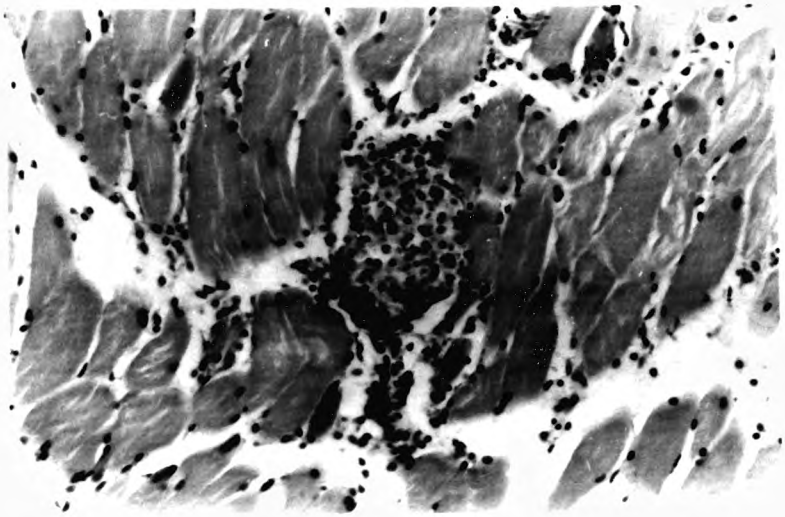


Plate 3.19. Obvious epithelial separation of the secondary lamellae of the gills of the fish fed diets 1, 2 and 3 (H & E 312.5X).

Plate 3.20. Gills from the fish fed diet 1, 2 and 3 showing hyperplasia (H & E 125X).

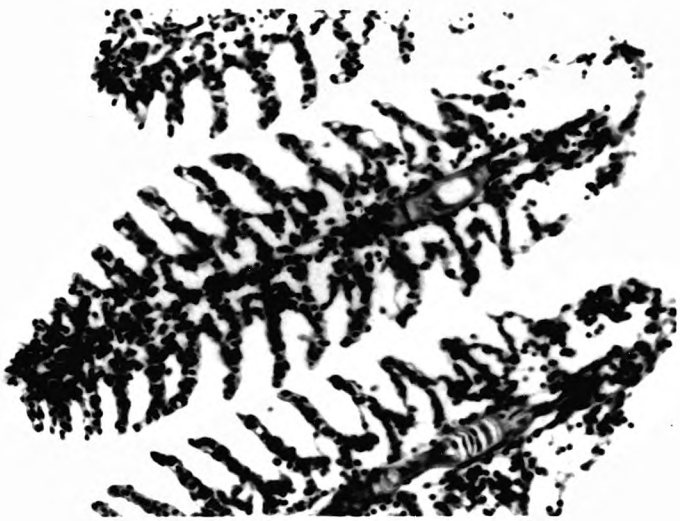


Plate 3.21. Gills from the fish fed diets 1, 2 and 3 showing epithelial separation, hyperplasia and telangiactasis (H & E 125X).

Plate 3.22. Distortion of the gills from fish fed diets 1, 2 and 3 (PAS 312.5X).

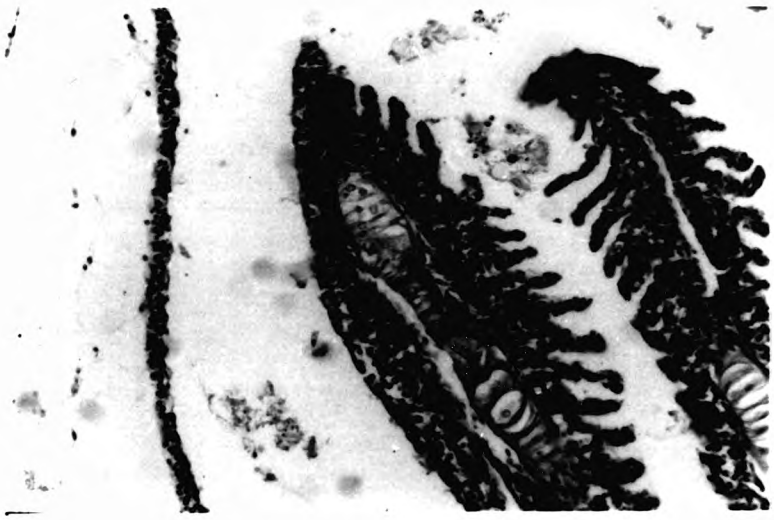
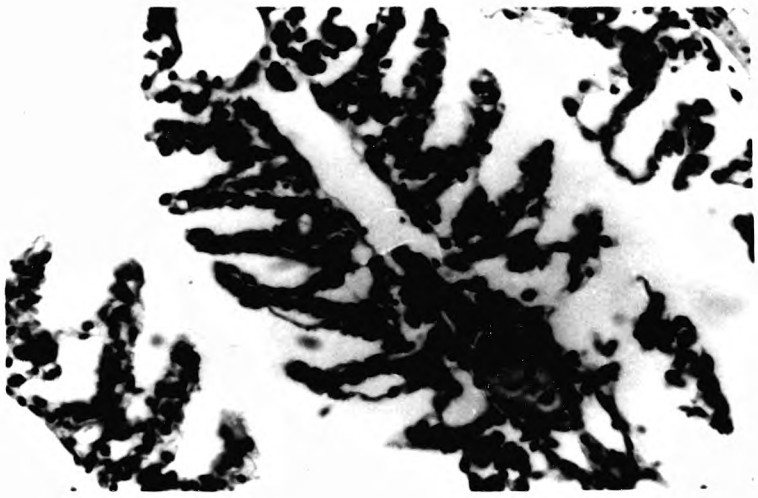


Plate 3.23. The central bony support is thickened with enlarged bizarre osteocytes (ZN 312.5X).

Plate 3.24. Anterior kidney swollen due to the Mycobacterium sp. infection. Nodules full of caseous material and epithelioid cells surrounding them, fills almost all the kidney. Some haematopoietic tissue is observed in the outer area of the kidney (H & E 78.75X).



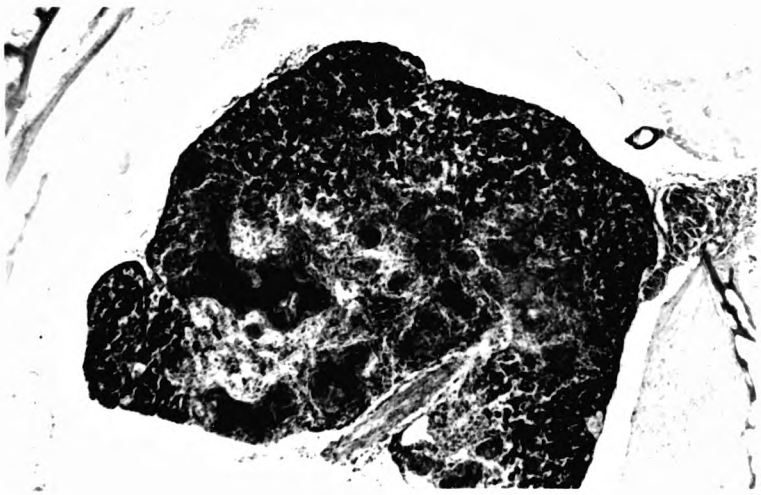
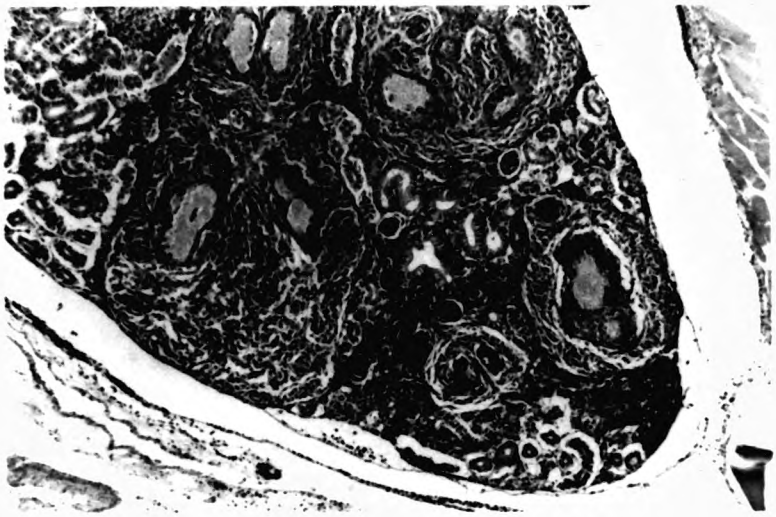
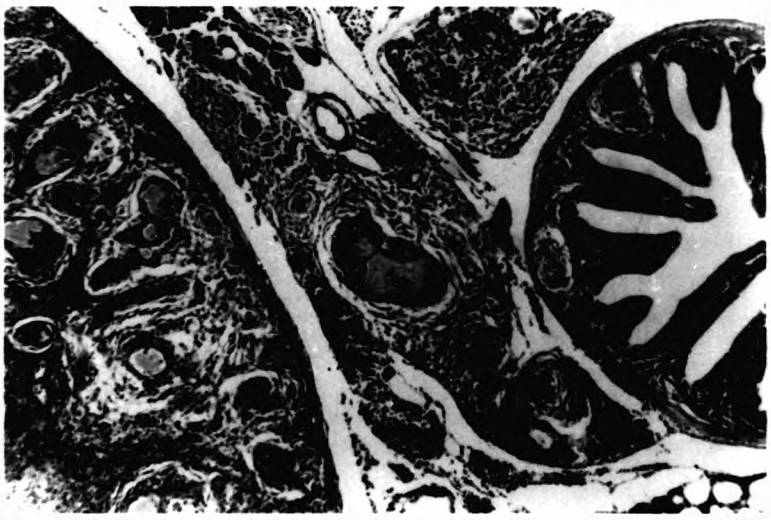


Plate 3.25. Posterior kidney affected by Mycobacterium sp. Some glomeruli and tubules are still unaffected (H & E 125X).

Plate 3.26. Peritonitis and swollen spleen from the fish affected with tuberculosis (H & E 125X).





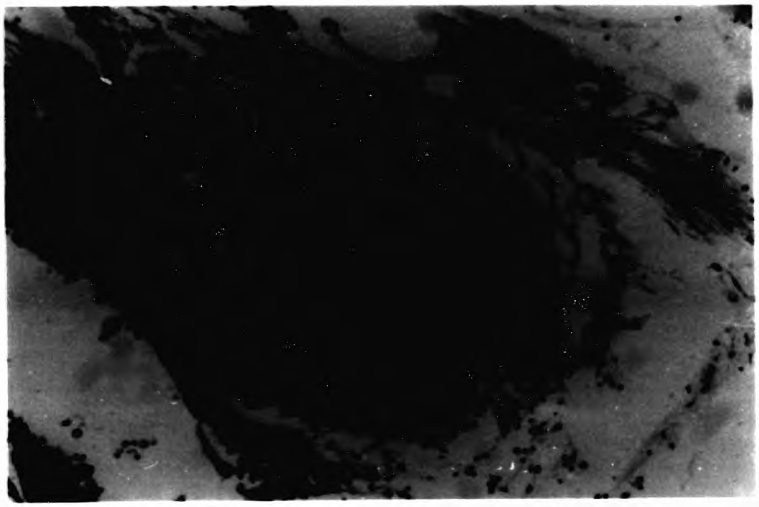
twice in the muscle. Fish from diet 3 to 10 were not infected with tuberculosis.

The ganglions were abnormal from the fish fed diets with total deletion of vitamin C and diet 2. The pathology observed in these ranged from slight inflammation represented by the presence of eosinophilic granule cells and lymphocyte infiltration (Plate 3.27) as well as a considerable amount of melanin deposition (Plate 3.28) to central chromatolysis in which there was swelling of the nerve cells, with eccentrically situated nuclei and dissolution of the Nissl granules.The cytoplasm was swollen and pale with vacuolation and in some cells the nuclei had disappeared (Plates 3.29, 3.30, 3.31). In one case neurone cells from the brain were also observed swollen and vacuolated (Plates 3.32, 3.33). Ganglions from the fish fed diets 3 to 10 were normal.

Fish fed diets 1 and 2 had affected livers. Focal necrosis of the hepatocytes was sometimes seen in which there was a lymphocytic infiltration and a mass of nuclei showing karyorrhexis and karyolysis (Plate 3.34). Most of the hepatocytes around this focal point were normal. In other cases focal necrosis was present together with a pronounced swelling of the hepatocytes and there were extensive areas of focal fatty

Plate 3.27. Ganglion cells with lymphocytic infiltration (H & E 312.5X).

Plate 3.28. Ganglion cells with considerable amount of melanin deposition (H & E 312.5X).



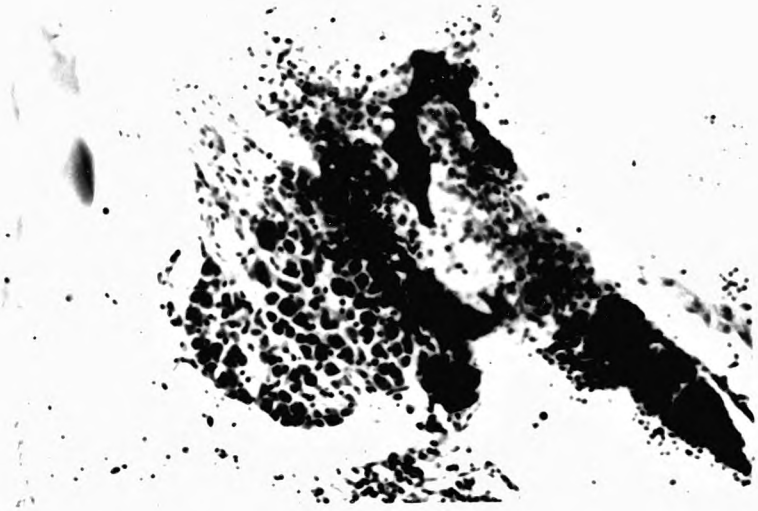
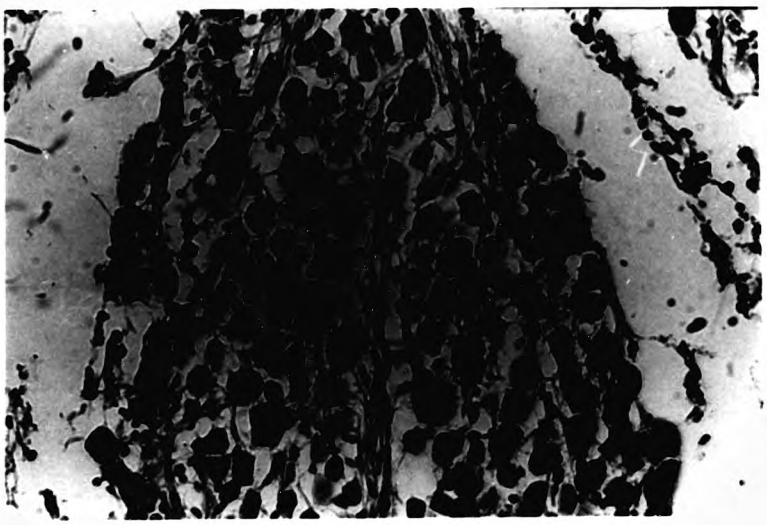


Plate 3.29. Liquefactive necrosis of the ganglion cells in which the lymphocytic infiltration is obvious, the cytoplasm is swollen and pale with vacuolation. The nuclei is swollen and marginal and in many cells the nuclei have disappeared (Cresyl fast violet 500X).

Plate 3.30. A magnification of the plate 3.29 in which the details described above are clear (Cresyl fast violet 1250X).



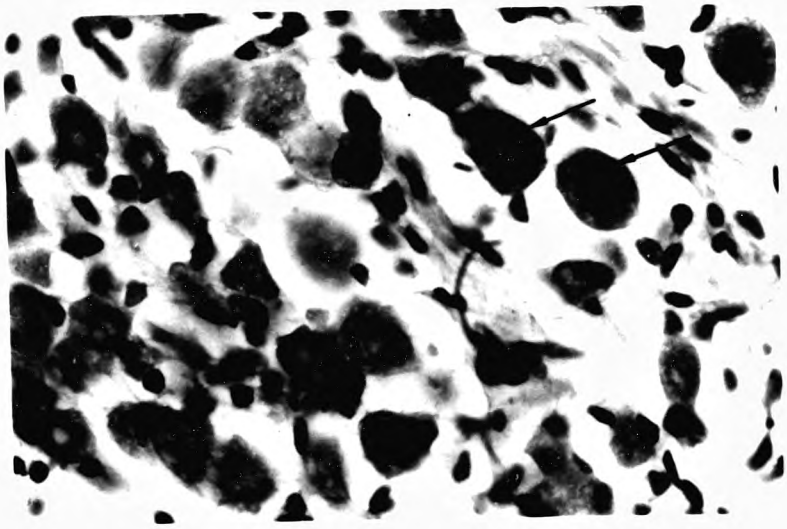
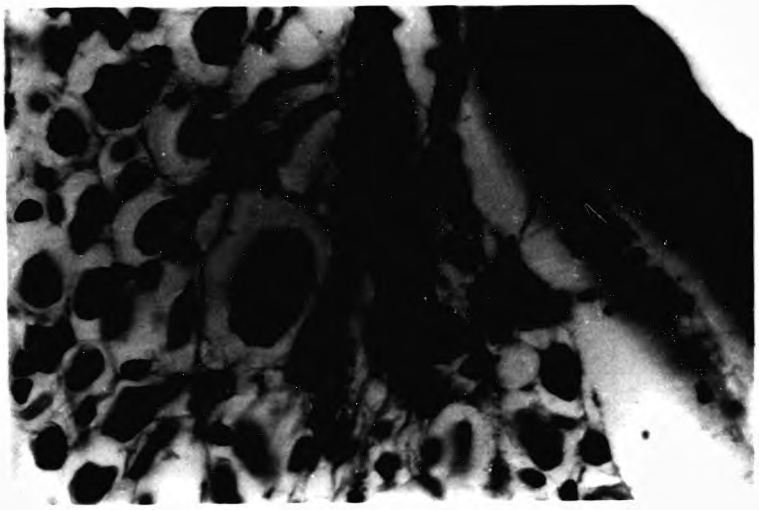


Plate 3.31. Nerve cells of the ganglion with vacuolation, dissolution of the Nissl's substance, some cells without nuclei and at the right side some eosinophilic granules (Cresyl fast violet 1250X).

Plate 3.32. Ischaemic neuronal injury manifested by shrinkage of the cytoplasm which looses its details and becomes vacuolated (H & E 500X).



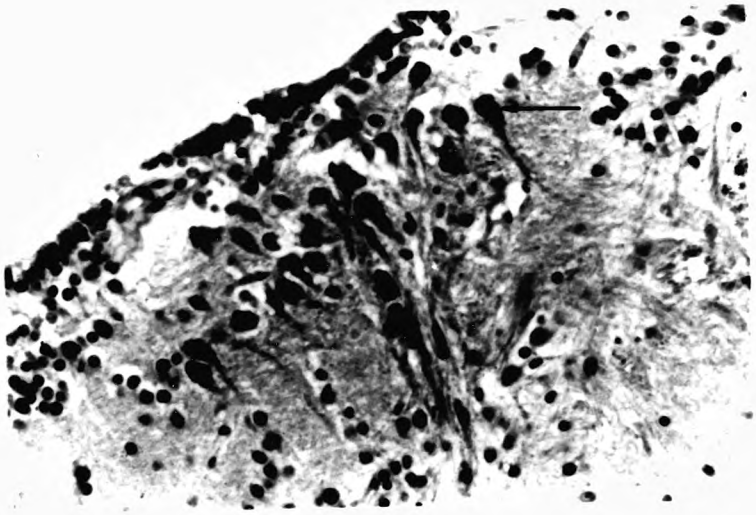
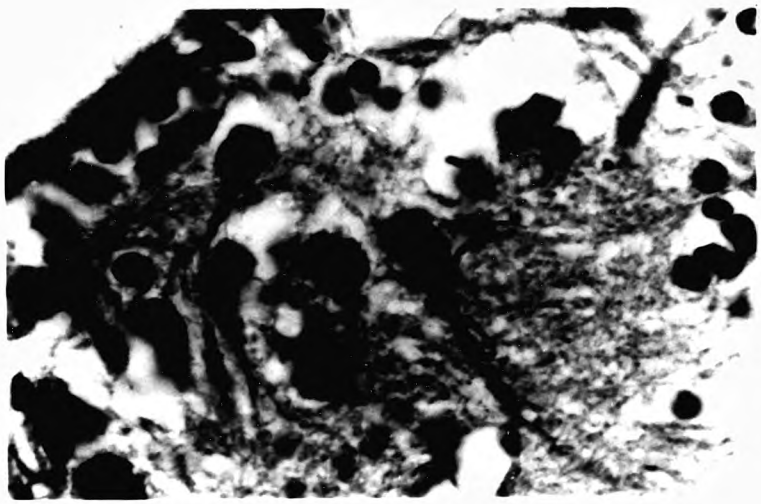
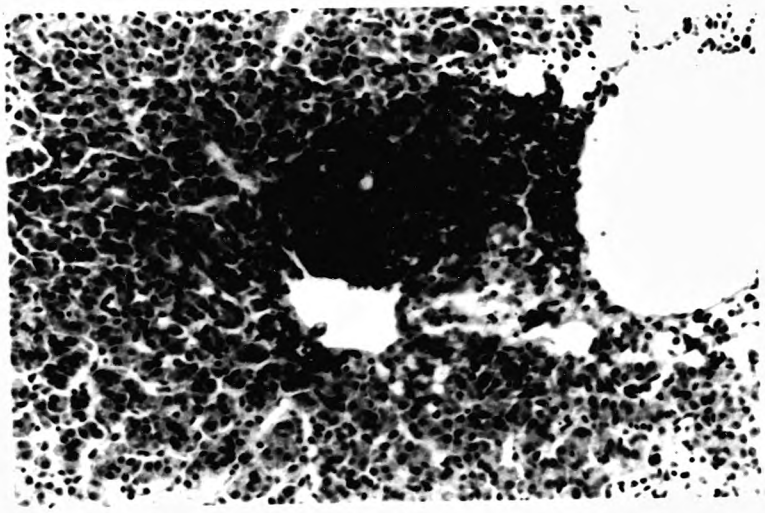


Plate 3.33. Magnification of Plate 3.32 in which the vacuoles in the cytoplasm of the injured neurones are evident (H & E 1250X).

Plate 3.34. Necrosis of the liver. The necrotic area is a mass of undifferentiated cells with many pycnotic nuclei (H & E 312.5X).





degeneration (Plate 3.35). Staining by the PAS reaction revealed a lack of glycogen in particular in the more central areas of the liver which were oedematous.

In the hepatopancreas of the fish fed diets 3 to 10, extensive areas with fatty degeneration were frequently observed. The pancreas of the fish fed diets 1 and 2 showed major changes with shrinkage of the acinar cells, pycnosis of the nuclei and a great reduction in zymogen granules (Plate 3.36).

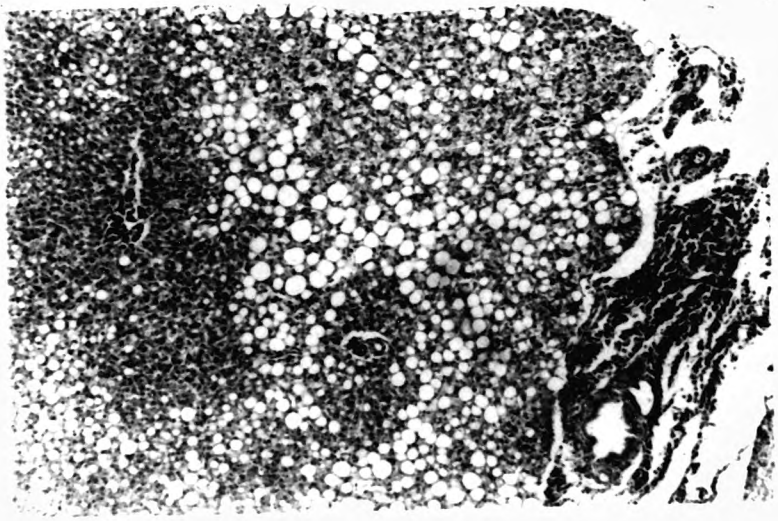
In the fish fed diet 1, 2 and 3 the structures described under the name of Rodlet cells were found (Morrison and Odense, 1978). Use of the Gless-Marsland stain for axons, counterstained with zafranin (.5% sol) clearly reveals that these cells appears in large numbers in the vein endothelium (Plate 3.37). Sometimes isolated rodlet cells were observed in the lumen of the vessels, probably due to artefacts. Isolated numbers of rodlet cells were observed in the vein endothelium in the fish fed diets 4 to 10.

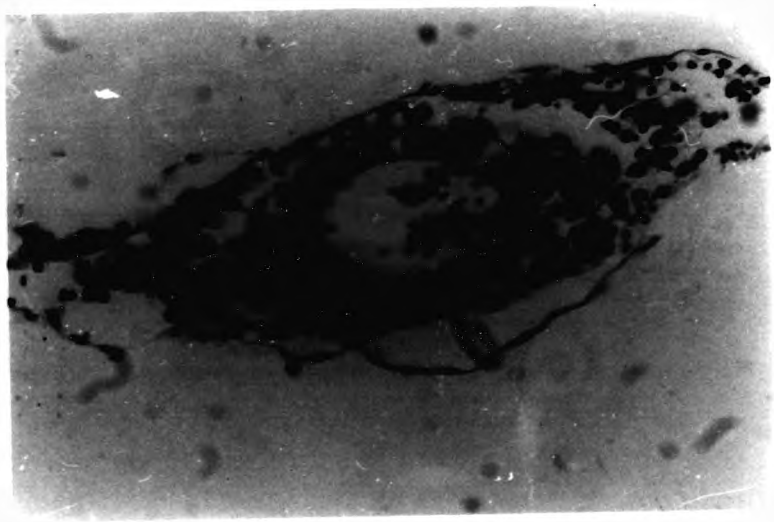
3.3.6. X-Ray results.

The results of X rays taken of the fish show only 2 cases with severe spinal deformity. One fish (fish at the top of Plate 3.7) from diet 2 showed lordosis, the

Plate 3.35. Extensive areas of fatty degeneration is observed in the hepatopancreas of the fish fed diets 1 to 10 (H & E 125X).

Plate 3.36. necrosis of the pancreas in which the acinar cells are smaller in size with pycnotic nuclei, reduction of the zymogen granules and lymphocytic infiltration (H & E 500X).





curvature in the caudal area being almost 90'. Plate 3.38 shows a fish from diet 3 with some vertebras compressed in the tail giving to the fish a different shape at this level. Plate 3.39 shows a fish fed diet 1, with very short operculae, the eye cavity is enlarged and the tip of the mouth is more rounded than the normal fish (Plate 3.40).

Plate 3.37. Large number of rodlet cells are observed the venous endothelium of the fish fed diets 1, 2 and 3 (H & E 1250X).

Plate 3.38. Lateral X rays of a fish fed diet 2. Lordosis of the spinal column is clear at the tail level, the curvature is almost 90'.

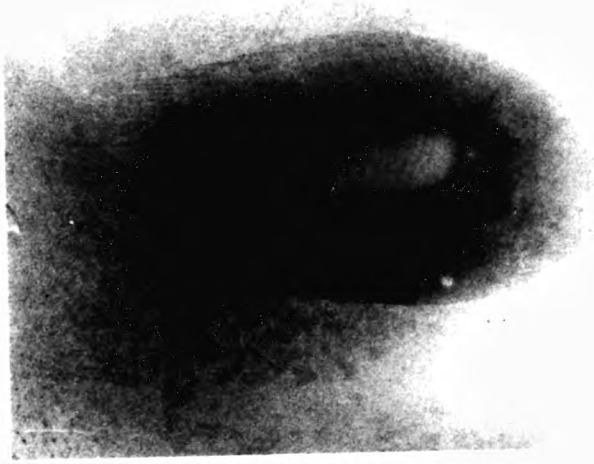


Plate 3.39. A fish fed diet 3 with some compressed vertebrae in the tail giving to the fish a different shape at this level.

Plate 3.40. Fish fed diet 1, with severe abnormalities in the head bones. Short operculae and rounded tip of the mouth are obvious.

Plate 3.41. Lateral X-Rays of a normal fish.

3.4. DISCUSSION.

The percentage of retention of vitamin C in diet 2 after processing was 100% and at the end of the experiment, this diet had the lowest value of retention (25%). There is no satisfactory explanation of the results in ascorbic acid retention in this diet because all diets were subjected to the same treatment. After processing and after storage during 90 days, ascorbic acid remaining in diets 3 to 10 had different values of retention, ranging at the end of the experiment from 53.01 to 98.99%. The lowest values were obtained in diets 5,4 and 10 in which the ascorbic acid retained was 53.01, 68.18 and 69.01 respectively, while in diets 6, 7, 3, 9 and 8 the values varied from 86.97 to 98.99%. A possible explanation for the variance of these results could be that when the diets were taken out of the deep freeze in order to top up smaller containers held in a fridge, they were affected by the large temperature and humidity variation. Samples were moved to make sure that only food due to be fed over a period of a few days was exposed to the relatively higher fridge temperature of 4°C. A breakdown in the air conditioning system in the laboratory during some of the days in the experimental period resulted in a temperature difference from -15°C (freezer) to 30'C

(laboratory) and could easily have led to water accumulation in some of the flasks due to condensation. The accumulation of water could then have caused a loss of vitamin C (Hilton et al., 1977; Hilton et al., 1978; Tacon, 1985). The air conditioning breakdown occurred on a number of different occassions which might explain the variation in the vitamin C retention results. In this respect Hilton et al., (1977) found that freezer stored diets showed virtually no losses of ascorbic acid in the extruded diets. In this study diets 3, 8 and 9 showed similar results with those of Hilton et al., (1977), while values of diets 7, 6, 10, 4, 5 and 2 had lower and different values of retention, having no relation with the ascorbic acid added to the diets as was observed by Soliman, (1985) in which his results shows that the retention of dietary ascorbic acid was increased by increasing the dietary ascorbic acid level after processing and after storage.

In this work C.urophthalmus fed the deficient diet had severe growth reduction and poor food conversion ratios. Similar results have been obtained in different species of fish in which the authors studied the vitamin C requirements for growth and health, wound healing, collagen formation, resistance to diseases and blood parameters (Andrews and Murai, 1975; Lim and Lovell, 1978; Durve and Lovell, 1982; Li

and Lovell, 1985) for catfish (Kitamura et al., 1965; Ashley et al., 1975; Hilton et al., 1978; Sato et al., 1978; Halver et al., 1979; Sato, Kondo, Yosinaka and Ikeda, 1982) for Salmo gairdneri (Mahajan and Agrawal, 1980) for Cirrhina mrigala (Mahajan and Agrawal 1980) for Channa punctatus (Soliman 1985) for Oreochromis niloticus and O. mossambicus.

Fish fed with diets 2 and 3 in which several signs of deficiency were observed, grew as well as the fish fed diets with higher levels of vitamin C. This indicates that 40 mg of ascorbic acid/kg is enough for optimum growth in C. urophthalmus. Similar results were obtained by Andrews and Murai, (1975) for channel catfish; Halver et al., (1969) for coho salmon and rainbow trout; Hilton et al., (1978) for rainbow trout; Lim and Lovell (1978) and Li and Lovell, (1985) for channel catfish; Sato et al., (1978, 1982) for rainbow trout. Slightly different results were obtained by Mahajan and Agrawal (1980) in which growth rates increase appreciably by increasing the amount of ascorbic acid in the diet up to 600 mg but beyond this point no further increase was achieved in Cirrhina mrigala. Completely different behaviour was obtained by Soliman (1985) who found that megadose of vitamin C (3000, 4000 mg/kg of diet) produced growth retardation

in comparison to those fed the diet containing 1250 mg of ascorbic acid/kg. C. urophthalmus were fed with higher doses (9980 mg/kg of diet), and did not show any effects of toxicity or growth reduction. The only effect observed in the highest level (diet 10) was the rejection of the food by the fish, probably due to the fact that this high concentration of vitamin C perhaps gave to the food a strong acidic taste. The fish often left the food for some time in the water possibly until some of the vitamin C was leached into the water, then food was avidly eaten.

Abnormal behaviour and morphological changes in C.urophtalmus were noted after 30 days on the vitamin C deficient diet. Agrawal and Mahajan (1980) have critically reviewed the available literature about the appearance of deficiency syndromes, and they suggest that it depends not only on the stage of life at which deficient feeding begins, but also on the environmental temperature. They established this fact with only a small amount of data, but by adding and analysing the present results together with those obtained recently for other species (Table 3.5) a highly significant correlation ($r=0.8106$, $P<0.001$) was obtained between the temperature of culture and the appearance of deficiency signs. The linear equation for expressing the relation is $y= 32.6283-0.0928x$. The equation was

Table 3.5 Relationship between the appearance of deficiency signs in days, temperature and initial weight in different species of fish.

Species	Appearance of Deficiency signs (days)	Temperature °C	Initial Weight (g)	REFERENCE
Salmo gairdneri	238	10.5	48	Poston, (1967)
S. gairdneri	140	15	?	Hilton et al., (1978)
S. gairdneri	140	15	0.4	Ashley et al., (1975)
Oncorhynchus kisutch	140	15	0.3	Ashley et al., (1975)
S. gairdneri	140	15	-	Sato et al., (1978 b)
Ictalurus punctatus	70	29	2.3	Lim and Lovell, (1978)
I. punctatus	91	30.5	3-9.7	Li and Lovell, (1985)
I. punctatus	98	29	4	Durve and Lovell, (1982)
Channa punctatus	180	16 - 32	42.8 ± 4.26	Mahajan and Agrawal, (1980)
Anguilla japonica	63	25	1.19-1.23	Arai et al., (1972)
Cirrhina migrala	65	25 - 35	0.12	Mahajan and Agrawal, (1980)
Oreochromis mossambicus	63	29	1.5	Soliman, (1985)
O. mossambicus	35	28	2.92	Soliman, (1985)
O. niloticus	56	28	1.0	Soliman, (1985)
O. niloticus	30	25	2.48	Soliman, (1985)
Cichlasoma urophthalmus	30	28	0.17	This study

calculated using 16 data from 11 different papers and the regression line illustrates the equation (fig 3.2). However the validity of this relationship can only be tested in full when more data becomes available.

Temperature affects the rate of fish growth, and is probably the most important factor governing aquatic poikilotherms (Cincotta and Stauffer, 1984) and it seems that vitamin C requirement is also temperature related (Scott, 1975). Vitamin C requirement for the different species of fish found in the literature ranged from 25 to 4000 mg/kg, however the values change even in the same species, due to the age of fish used, methods of diet preparation and storage, genetic background, environmental conditions, stress and the other nutrients present in the diet (Halver et al., 1975, Hilton et al., 1978a; NRC, 1983). The criteria used to evaluate the vitamin C requirement also give different results. Analysing the data from Table 3.6 it can be seen that with the available results it is still difficult to determine if the requirement for ascorbic acid is greater at higher temperatures. More work must be done utilizing the same criteria and age of the fish to arrive at some conclusion.

The external signs of deficiency in C.urophthalmus were similar to those found in the literature for other tropical species. Relating again the temperature and in this case the gross external

Figure 3.2. This figure shows the highly significant correlation ($r=.8106$, $P < 0.001$) between the temperature of culture and the time for the appearance of deficiency signs.

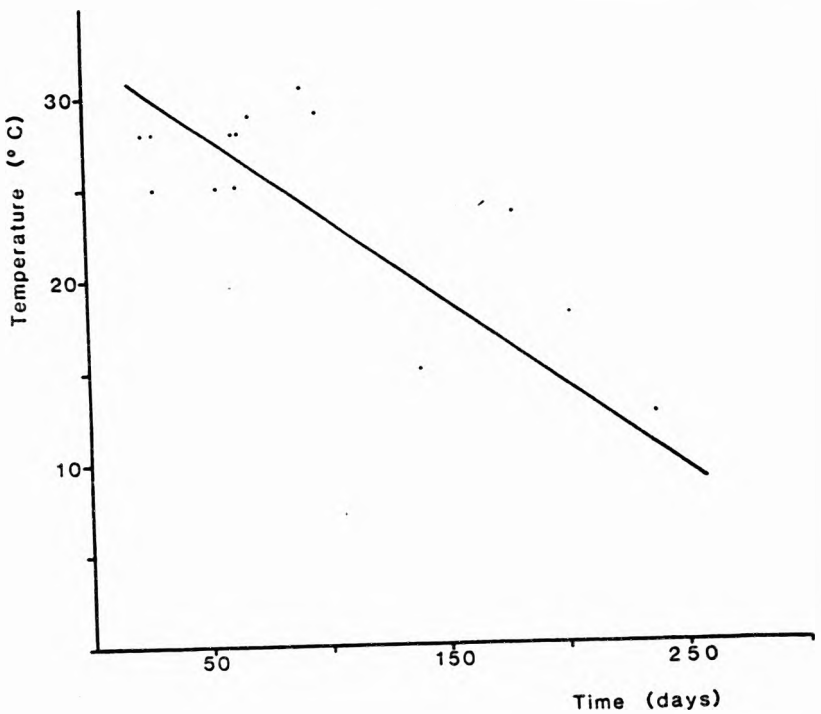

Temperature (° C)
30
20
10
50
150
250
Time (days)

Table 3.6 Vitamin C requirement of different species of fish, water temperature of culture and the various criteria used to determine the requirement.

Species of fish	Water Temperature C	Criteria	Vitamin C Requirement (mg/kg)	Reference
Salmo gairdneri	15	1; 3; 4	40	Hilton et al, 1978
	15	1	100	Halver, 1972
	15	5	500	Halver, 1972
	16-17	1; 6	50-100	Halver, Ashley, Smith, 1969
	15	1;2;3;5	100	Halver, Ashley, Smith, 1969
Oncorhynchus kisutch	15	1;3	50	Halver et al., 1969
		5	200	
	15	1;2; 3; 5	100	Ashley et al., 1975
Ictalurus punctatus	20	1;5	60	Lim and Lovell, 1977
	27	9	25	
		1	50	Murai et al., 1978
		3	200	
Ictalurus punctatus	27	1,10	50	Andrews and Murai, 1975
	29	1	30	
		8	150	Durve and Lovell, 1982
	29-32	1	30-300	
	x=30.5	8;11	3000	Li and Lovell, 1985
Cirrhina mrigala	25-35	1,9,12	650-750	Agrawal and Mahajan, 1980
	x=30			
Oreochromis niloticus	28	1;2;3;4;7;13	1250	Soliman , 1985
	28	5	4000	Jauncey et al., 1985
Oreochromis mossambicus	28	1,2,3,4,7,13	1250	Soliman, 1985
Cichlasoma urophthalmus	28	1,2,7,8,9,10	40	This study
		12	110	

1. Optimum growth
2. Gross signs
3. Tissue ascorbic acid saturation
4. Hemoglobin values
5. Wound healing
6. Collagen formation
7. Histopathology
8. Disease resistance
9. Spinal deformities
10. Food efficiency
11. Antibody production
12. Mortality
13. Biochemical changes

signs of scurvy in the different species of fish, it is evident that temperate species show less external gross signs than tropical fish (Table 3.7). The common gross signs are scoliosis, lordosis, haemorrhages, reduced growth, anorexia and high mortality. The difference in this aspect must be due to the high growth rate of the tropical species which require more vitamin C per unit time than temperate fish. The vitamin C stored in the tissue of growing fish is more rapidly absorbed, utilized and fixed in all of these areas in which vitamin C is known to be important such as tissue synthesis, collagen and cartilage formation, growth process, catabolic process, haematological involvement of anaemia prevention, calcium intake, tissue repair, resistance to disease, prevention of damage of pollutants and various biochemical relationships (Galloway, Garry and Hitchin., 1947; Evans and Hughes, 1963; Halver, 1972b; Ginter, Nemec and Bobek, 1972; Ashley et al., 1975, Yamamoto et al., 1977; Mahajan and Agrawal 1979; Agrawal and Mahajan, 1980; Hamilton and Bidloch, 1980; Sato et al., 1982a,b; Miyazaki et al., 1985; Jauncey et al., 1985). When the stored vitamin C is exhausted, the deficiency signs become evident and in fish with high growth rates, the signs appear earlier than in lower growth rate fish. As mentioned above, the requirements in warm water fish are more critical than in coldwater species, having as a

Table 3.7 Gross external signs of scurvy observed in different species of fish

Species	Gross external signs of scurvy													References
	1	2	3	4	5	6	7	8	9	10	11	12	13	
Salmo gairdneri	*	*	*	*	*	*	*						*	Kitamura et al 1965;Halver et al 1969; Halver 1972; Ashley et al, 1975; Hilton et al, 1977a, 1978a; Sato et al, 1978, 1982.
Oncorhynchus kisutch	*	*	*	*	*	*								Halver et al 1969;Halver 1972; Ashley et al 1975
Ictalurus punctatus	*	*	*	*	*	*		*	*			*	*	Lovell 1973; Andrews and Murai 1975; Lovell 1975;Lim and Lovell 1978; Durve and Lovell 1982; Miyazaki et al 1985.
Channa punctatus	*	*	*	*	*	*		*	*	*		*	*	Mahajan and Agrawal 1979
Cirrhina mrigala	*	*	*	*	*	*	*	*		*		*	*	Agrawal and Mahajan 1980b
Oreochromis niloticus	*	*	*	*	*	*	*		*		*	*	*	Soliman 1985
O. mossambicus	*	*	*	*	*	*	*		*		*	*	*	Soliman 1985
Cichlasoma urophthalmus	*	*	*	*		*	*	*	*	*	*		*	This study

1. Anorexia
2. Reduced growth
3. High mortality
4. Haemorrhages
5. Scoliosis
6. Lordosis
7. Exophthalmia
8. Dark colour or loss of pigmentation
9. Erosion of skin and fins
10. Loss of scales
11. Short operculae
12. Erratic and imbalanced swimming
13. Lethargy and lying prostrate

consequence more noticeable symptoms.

Histopathology of experimentally induced vitamin C deficiency fish has been described before for Salmo gairdneri. Oncorrhynchus kisutch. Ictalurus punctatus. Oreochromis niloticus and O. mossambicus (Ashley et al., 1975; Lim and Lovell, 1978; Soliman 1985; Miyazaki et al., 1985).

With respect to bone changes Ashley et al., 1975, found spinal curvature and severe vertebral dislocation as well as moderate to severe changes in skull and jaw cartilages and in developing bone and teeth in rainbow trout and coho salmon. Lovell, (1973) and Lim and Lovell, (1978) described for Ictalurus punctatus early vertebral deformities such as enlarged soft vertebrae and slightly curved spinal columns. Later there were weak and brittle vertebrae, greater curvature and broken backs. In a recent paper Miyazaki et al., (1985) made a full description of the histopathology of broken back syndrome in channel catfish fed a vitamin C deficient diet under laboratory conditions. Specimens with broken back syndrome had vertebral injury due to osteoporosis, abnormally increased ossification and dysplasia. Soliman, (1985) found in O.niloticus and O.mossambicus generalized bone changes associated with excessive production of chondrocytes and failure of

ossification of growing bone areas. Spinal vertebrae with centres of ossification from the growing vertebrae were enlarged and very cellular with no evidence of calcification in the points of spinal fracture. In the present experiment, only 1 fish from diets 2 and 3 had lordosis, unfortunately histological preparation could not be made from these fish as they had been frozen prior to their radiological examination but similar changes to those described before must be expected for this species. X rays also showed some bone changes in the head, but no histological changes were observed in the bones of other fish. Normal cartilage and ossification was observed except in the gill cartilage.

Gill changes observed for the deficient C.urophthalmus were similar to those described previously for vitamin C deficient rainbow trout, coho salmon, channel catfish and tilapia in which the gill support cartilage was severely distorted and the chondrocytes were deformed (Halver et al., 1969; Ashley et al., 1975; Lim and Lovell, 1978; Soliman 1985; Miyazaki et al., 1985).

Gill hyperplasia has not been reported for coho salmon and rainbow trout (Halver et al., 1969; Ashley et al., 1975). Lim and Lovell, (1978) described clubbed gills in channel catfish fed a vitamin C deficient diet

and also on fish fed a diet containing 30 mg/Kg of vitamin C, but they found it present to some degree in fish from all treatments, so they concluded that it was not a vitamin C deficiency sign. Soliman, (1985) reported significant hyperplasia of gill secondary lamellae of O.mossambicus fed vitamin C deficient diets, while this result was not observed in O.niloticus fed the same deficient diets. In the present study involved hyperplasia of the primary and secondary lamellae was observed in fish fed diets 1,2 and 3 while slight hyperplasia and oedema were observed in the primary and secondary lamellae of the fish in the rest of the treatments. The similar results in this aspect of deficiency observed by Lim and Lovell, (1978), Soliman, (1985) and this work suggest that vitamin C causes gill hyperplasia at least in some species of tropical fish.

Changes in the epithelia due to vitamin C deficiency have been briefly described by Ashley et al., (1975) in which one fish had severely vacuolated epithelial cells and pyknotic nuclei in the macula of the internal ear, and Miyazaki et al., (1985) in channel catfish observed that the dermis was extensively hyalinized and the epidermis was atrophic. In the present study it was clear that fish fed diets 4 to 10 had normal epidermis and dermis, while those with

lower levels showed a marked inflammatory response, vacuolar degeneration and degenerative changes. Krehl (1960) reviewing the role of Ascorbic acid in skin diseases has shown that this vitamin is concerned with the metabolism of tyrosine and Phenylalanine, which are precursors for the formation of the melanin in the skin. This factor could explain the loss of pigmentation in Channa punctatus fed the deficient diet during investigation, (Mahajan and Agrawal, 1979). The present study suggested also that vitamin C causes epithelial changes in C.urophthalmus.

Muscle atrophy has been observed in coho salmon, rainbow trout, channel catfish and tilapia in experiments with ascorbic acid deficiency concerned with wound healing. In all cases muscle atrophy apparently occurred in response to trauma , because it was only found surrounding the wounds (Halver et al., 1969; Halver 1972; Ashley et. al., 1975; Lim and Lovell 1978; Jauncey et al., 1985). Miyazaki et al., (1985) found in channel catfish that myomeres of the lateral musculature beside the deformed vertebrae was markedly atrophied but also in individual myomeres, myosepta and dermal dense connective tissue were atrophic and hyalinized. A range of muscle changes were observed in C.urophthalmus fed a diet deficient in vitamin C. These changes were observed in myosepta free of Myxobolus sp.

cysts but on occasion this damage were observed around them . In this respect Christensen and Lynch (1948) observed that scorbutic guinea pigs showed a reduction in the free glycine and glutamine content of muscle cells while aminoacids other than glycine and glutamine tended to be increased. This review suggests that vitamin C deficiency causes muscle pathology, but the effects of trauma in the wound healing experiments, or the lateral effects of the vertebral fractures and the damage caused by increased number of parasites makes this pathology more extensive.

Miyazaki et al., (1985) described for scorbutic channel catfish abnormal collagen fibres in visceral organs and the magnitude of the lesions varied among individual fish. In this experiment it is difficult to observe whether vitamin C caused abnormalities in many of the organs due to the effect of tuberculosis. However focal necrosis, swelling of the hepatocytes and extensive areas of fatty degeneration in the liver were clearly seen. In this respect Miyazaki et al., (1985) found that all hepatocytes in the liver lost glycogen and were atrophic, and intrahepatic blood vessels had a reduced number of collagen fibres and were infiltrated by macrophages. In the present study, fatty livers were observed in the fish fed the 10 experimental diets, there is not a clear explanation of these results but

it could be due to a number of different factors. Shimeno (1982) observed that the fat content of the body and the liver of yellowtail was greater in the groups fed a starch-enriched diet than in the group fed a starch free diet. Henderson and Sargent (1985) and Tacon, (1985) indicate that fatty livers have been oberved in deficiency of choline and essential fatty acids, or toxicity by oxidized fish oil. Oxidized fish oil was discounted as the cause of the fat deposition in the livers due to the precautions taken in the experimental design. To know the cause of this pathology must be the subject of future research.

Scorbutic C.urophthalmus showed necrosis in the pancreas, while in scorbutic channel catfish the number of pancreatic cells was decreased (Miyazaki et al., 1985) and in scorbutic tilapias a mild but noticeable leucocyte infiltration was observed in the peri-acinar lipid tissue (Soliman, 1985).

In this study various pathological changes were observed in the ganglion cells of scorbutic C. urophthalmus that ranged from slight inflammatory response to central chromatolysis. These changes seem not to be related with Mycobacterium sp. or Myxobolus sp. Infection. In this respect Sulkin and Sulkin, (1967) in an electron microscopic study to investigate

the changes in autonomic ganglion cells of guinea pigs during ascorbic acid deficiency and partial inanition, observed that the first changes noted in the ganglion cells of the scorbutic animals were a loss of free ribosomes and an increase in the number of agranular vesicles. In many instances the mitochondria became swollen, devoid of cristae and assumed round or irregular shapes. The Golgi complex showed vesicles and cisternae which were distended, enlarged and swollen, thus losing its classical structural appearance. Where the changes were severe, the whole cell had a vacuolated appearance. In some cells there was a displacement of the nucleus to the periphery of the cell and an irregular outer nuclear membrane due to an increase in the spaces between the two membranes. Another characteristic of the ganglion cells of the scorbutic animals was the appearance of lipofuscin pigment, a substance that is not observed in normal guinea pigs. They concluded with this data that marked alterations occur in autonomic ganglion cells of guinea pigs following a vitamin C-deficient diet. Their observations also indicated that the alterations observed during avitaminosis C are due to the lack of this vitamin and not to the effects of reduced food intake which accompanies a diet deficient in vitamin C. Sulkin, Sulkin and Rothrock, (1968) demonstrated that the alteration occurring in the ganglion cells during

vitamin C deficiency are reversible. Full recovery in guinea pigs takes approximately 28 days. Milby, Mefford, Chey and Adams, (1981) explained that a real understanding of the role(s) of ascorbate in CNS functioning remains obscure, however, there is a growing body of information from recent publications that it may be linked in some manner to neuronal transmission processes. Their experiments suggest that Ascorbic acid could exhibit a strong effect on modulation of various synaptic events and play a very important role in neuronal functioning, rather than just fulfilling the relatively passive roles of enzymatic cofactor and general reducing egent that have been assigned to it in the past. Hilton et. al., (1979b) reported the ascorbic acid level of the total brain tissue of the rainbow trout was consistently higher than the concentration in other tissues and Hilton et al., (1978a) have indicated that anorexia, lethargy and lying prostrate at the bottom of the tank in scorbutic rainbow trout is due to severe reduction of total ascorbate in the brain. Soliman, (1985) found high levels of Ascorbic acid in the brain of O. niloticus and O.mossambicus and he suggests a role in the functioning of the nervous system. The observations of the present study suggests that the neurological pathology observed in C. urophthalmus was due to Vitamin C deficiency.

Histological examination of the organs affected with fish tuberculosis revealed that the lesions were substantially similar to those observed for different species of fish (Wolke and Stroud, 1978; Giavenni Finazzi, Poli and Grimaldi, 1980; Leibovitz, 1980; Majeed, Gopinath and Jolly, 1981). In general lesions appeared as granulomatous foci, in varying stages of development and consisted of soft tubercles with a central area of caseous necrosis resulted in enlargement of the tubercle, although the zone of peripheral macrophages and lymphocytes became relatively thiner.

With regard to fish tuberculosis observed in this study it is important to analyse some aspects:

1) Fish tuberculosis was only found in all the fish fed diets 1, 2 and 3. Fish from diet 4 to 10 were free of the disease.

2) In two parallel experiments to this study, (chapter 5 and 6) fish from the same batch as those used in this experiment was used to determine the nutritional requirements for pyridoxine and calcium pantothenate. No case of fish tuberculosis was observed in the histological sections examined from all the treatments.

3) In two previous experiments that were carried out, fish from different sources were obtained to determine the qualitative requirements for the eleven water soluble vitamins, (Chapter 2) and fish tuberculosis was observed only in the diet 10 of the second experiment in which vitamin C was deleted, while the fish fed with the other ten deleted vitamins showed no signs of infection with Mycobacterium sp.

4)Mycobacteria were present in those fish used in these experiments, possibly as opportunistic pathogens (Sniezko, 1978) or perhaps as an infection through the ovarian pathway (Asburner, 1977).

5)Many studies with terrestrial animals have indicated an association between the dietary level of ascorbic acid and several mechanisms of the host defense system (Nungester and Ames, 1948; Ganguluy, Durieux and Waldman, 1976; Shilotri, 1977; Prinz, Bortz, Bregin and Hersh, 1977; Anderson, Ocsthuizen, Maretz, Theron and Van Rosburg, 1980; Fraser, Pavlovic, Kurahara, Murata, Peterson, Taylor and Feigen, 1980; Sakamoto, Kobayashi, Ishii, Katto and Shimazono, 1981). A concrete relationship between vitamin C deficiency and tuberculosis was observed by Mueller and Kies, (1962) in which guinea pigs with a vitamin C deficient diet, inoculated with Mycobacterium butyricum failed to

respond to tuberculin, and also restoration of vitamin C to these animals resulted in subsequent development of sensivity to tuberculin, indicating an inability to respond immunologically to Mycobacteria infection in the absence of sufficient vitamin C. Afterwards, Zweiman, Schoenwetter and Hildreth, (1966) based on the Mueller and Kies, (1962) work designed an experiment to determine the mechanism of depression of tuberculin skin reactivity in the scorbutic animals. They found that their results confirm the fact that experimental scurvy inhibits the manifestation of tuberculin skin reactivity in the actively sensitized guinea pigs. This was accompanied by an almost completely absent mononuclear inflammatory response.

Recently some work has been carried out on this aspect with fish. Lovell, (1973) and Lovell and Lim, (1978) reported that channel catfish fed diets deficient in ascorbic acid were more susceptible to bacterial infection than those fed diets supplemented with ascorbic acid. Durve and Lovell, (1982) found that up to 5 times the level of vitamin C than the minimum requirement for normal growth and bone development provided increased resistance to the pathogenic bacterium Edwarsiella tarda in channel catfish. They found that the nutritional requirement for vitamin C varies with metabolic function and that resistance to

bacterial infection increases with higher levels of dietary vitamin C.

In this study, diet 2 with 40 mg/Kg of vitamin C was enough for normal growth and diet 4 with 110 mg/Kg was enough to prevent the signs of scurvy and infection by Mycobacterium sp. Probably megadoses of vitamin C in C.urophthalmus provides increased resistance to bacterial infection as has been found by Durve and Lovell, (1982) and Li and Lovell, (1985). The mechanism involved in the resistance to fish mycobacterium by C. urophthalmus could be similar to those found by Li and Lovell, (1985) in which their study shows that dietary ascorbic acid is functional in antibody production, complement activity and engulfment of bacteria by phagocytosis. Megadoses of 3000 mg/Kg enhance the humoral immune response, antibody production and complement activity. With these observations it is possible to conclude that in this experiment a clear relation between fish mycobacterium and vitamin C deficiency exists.

No reference was found with respect to vitamin C and increased resistance to a major parasite such as the protozoan Myxobolus sp. However in this study a clear difference was observed between the large quantity of Myxobolus sp. cysts in fish fed diets 1 and

2 and the greatly reduced number of cysts observed in fish fed diets 3 to 10. It is possible therefore that vitamin C has not only a relation with bacterial resistance but also some involvement with the resistance to major parasites like this microsporidian. In this case fish with adequate levels of vitamin C do not completely eliminate the cysts but the fish responses perhaps prevent the dispersion of the parasites.

Rodlet cells have been reported in variable numbers from many epithelia in both freshwater and marine teleost fish (Morrison and Odense 1978). The Rodlet cell is an enigmatic cell described as either a parasitic or a glandular cell (Thelohan, 1892 and Plehn, 1906 In Modin, 1981; Viehberger and Bielek 1982; Bielek and Viehberger, 1983.). Whatever interpretation of these cells is given, in this study rodlet cells have been observed in large numbers in cases of vitamin C deficiency, this observation being in agreement with Dawe, Stanton and Schwartz, (1964) and Anderson, Roberts, Mackenzie and McVicar, (1976) in which they found rodlet cells in large numbers in conjuntion with pathological conditions.

Due to elevated levels of ascorbic acid has beneficial effects over the fish, because this vitamin

is involved not only in important metabolic functions but also in other factors such as immune response, reproduction, resistance to adverse environment, and also to prevent losses during processing and storage, it is recommended that practical diets should contain 100% more than the minimum required. However, in Mexico bulk amounts of food are delivered and must be stored for long periods under inadequate conditions such as high temperatures and high humidity, practical diets for C.urophthalmus should contain at least 2000 mg/Kg of diet (1818.18% more than the minimum required). It is also recommended that the price and disponibility in Mexico of other forms of ascorbic acid such as barium salt of L-ascorbic acid 2 sulphate and Glyceride coated L-ascorbic acid should be investigated since these have been shown to be more stable during processing, storage and leaching than L-ascorbic acid and the sodium L-ascorbic acid. (Soliman, 1985).

CHAPTER 4.

DETAILED PYRIDOXINE REQUIREMENTS AND ITS DEFICIENCY SYNDROME.

4.1. INTRODUCTION.

Pyridoxine and vitamin B6 are terms used to denote at least three chemically, metabolically and functionally related substances, Pyridoxine, Pyridoxal and Pyridoxamine, which in the form of their phosphates, are interconvertible *in vivo* (Dyke,1965; Snell and Haskell, 1971). Vitamin B6 is involved in a number of extremely important metabolic reactions of the alpha-aminoacids. Pyridoxal phosphate is the coenzyme of many aminotransferases (Chow, 1964; Dyke, 1965; Halver, 1982) and is important in other aspects of protein, lipid (especially fatty acids) and carbohydrates metabolism (Sauberlich, 1968; Halver, 1972, 1979).

Since Pyridoxine was established as an essential micronutrient more than 50 years ago (Gyorgy, 1934), various aspects of its physiological roles in man (Linkswiler, 1967) and in animals (Fuller, 1964) have been investigated.

The first qualitative requirement of this vitamin in fish was demonstrated in 1944 (Halver, 1972) and since then, studies have been carried out in 13 species of fish. Some of the studies recognize the qualitative requirement of this vitamin in various fish species, in

terms of growth, behaviour, gross signs of deficiency and mortality (Dupree, 1966; Kitamura et al., 1967; Sakaguchi, Takeda and Tange, 1969; Arai et al., 1972; Halver, 1972; Agrawal and Mahajan, 1983a). Other researchers have utilized various different criteria such as tissue enzyme activities, disease resistance, haematological and haematopoietic changes, vitamin B6 content in the tissues, food conversion ratio and histopathology. Also different levels of this vitamin in purified or semipurified diets were used to find the quantitative requirement for different species of fish (Ogino, 1965; Takeda and Yone, 1971; Halver, 1972; Smith, Brin and Halver, 1974; Adron, Knox and Cowey, 1978; Hardy, Halver and Brannon, 1979; Andrews and Murai, 1979; Kissil, Cowey, Adron and Richards, 1981; Agrawal and Mahajan, 1983b). In all these studies it was demonstrated that Pyrydoxine is essential for normal growth and health in various species of salmonids, in turbot (Scophthalmus maximus), gilthead bream (Sparus aurata), channel catfish (Ictalurus punctatus), common carp (Cyprinus carpio), snake-head (Channa punctatus), yellow tail (Seriola quinqueradiata), red sea bream (Chrysophrys major) and Atlantic salmon (Salmo salar)

In a previous experiment to identify the qualitative requirement of the water-soluble vitamins for Cichlasoma urophthalmus (Chapter 2), Pyridoxine was

the most critical vitamin in terms of the early appearance of deficiency signs and 100% mortality in 15 days. The objective of this study was to determine the quantitative requirement of Pyridoxine in terms of growth, food conversion ratio, behaviour, gross external signs of deficiency, mortality and histopathological changes.

4.2. MATERIALS AND METHODS.

4.2.1. Experimental design and diets.

4.2.1.1. First experiment. 300 fry of C. urophthalmus were captured at Celestun Lagoon Yucatan (Section 1.2.) with an initial weight of 0.093g and after a period of acclimatíon (Section 1.2.) were placed in 15 tanks of the recirculated water system (Section 1.1.). Five different diets were prepared as was previously described in section 1.3. Each diet contained one of 5 different levels of Pyridoxine (table 4.1). Fish were fed ad libitum during the experimental trial. Feeding methodology, care of the fish and nutritional parameters measured are explained in sections 1.4 and 1.6. Records of the behaviour, signs of deficiency and mortality were recorded daily.

4.2.1.2. Second experiment. 720 fish captured in Celestun lagoon (section 1.2) with an initial weight between 0.140 and 0.155g were placed in groups of 20 in 36 tanks of the recirculated water system (section 1.1). After the same quarantine and acclimation period described previously (section 1.2.), the fish were fed with eleven diets provided with different levels of Pyridoxine (Table 4.1). Methods of preparation and storage are described in Section 1.3.

The fish were fed ad libitum with these diets during 75 days of the experiment. Methods of feeding, fish care, parameters measured and records observed as well as statistical methods used are described in section 1.4, 1.6 and 1.7.

4.2.2. Pyridoxine content in the diets.

A sample of each diet was preserved at -20'C and afterwards, the pyridoxine content was evaluated microbiologically, utilizing Bacto-Pyridoxine "Y" Medium Code 0951 and Saccharomyces carlbergensis ATCC 9080 following the Difco Supplementary Literature methods (1964). The assay was carried out by the professional staff at the "Servicios de Control Analitico y evaluacion de Calidad" laboratories at CINVESTAV-Mexico.

Table 4.1 Mg of Pyridoxine/kg dry diet obtained through the microbiological method.

	DIETS										
	1	2	3	4	5	6	7	8	9	10	11
Exp. 1	1.02	1.41	1.99	2.72	5.08	-	-	-	-	-	-
Exp. 2	0	1.85	3.17	5.61	10.7	24.42	32.76	66.52	94.20	426.24	642.62

4.2.3. Methods of proximate analysis.

As the same ingredients were used and mixed together in accordance with the procedures described in section 1.3., one sample was taken to determine the proximate analysis of all diets, following the methods described in the same section.

4.2.4. Histological studies.

All fish that were observed with deficiency signs during the experiment, and 5 fish from each diet at the end of the experiment, were killed with an overdose of benzocaine and processed as previously described (section 1.5) for histological studies.

4.3. RESULTS.

4.3.1. Water quality during the second experiment was : Temperature 28°C (Range 27.3 to 28.7).
Nitritos 0.024 ppm (range 0.006-0.059).
Ammonia 0.063 ppm (range 0.023-0.130).
Un-ionized ammonia 0.0102ppm (range 0.0037-0.021).
pH 8.45.

4.3.2. Pyridoxine content in the diets.

Table 4.1. shows the Pyridoxine values of the diets in each experiment obtained by the microbiological method.

4.3.3. Proximate analysis.

The results of the diet proximate analysis are given in Table 4.2.

4.3.4. Behaviour, signs of deficiency and mortality

Fish from the five diets of the first experiment ate voraciously during the 42 days of the experiment, except those fish which were showing signs of deficiency.

Fish on diet one (1.02 mg of Pyridoxine/Kg) showed the first deficiency signs on day 9. Fish had total anorexia consequently empty stomachs, dark colour, lethargy, some haemorrhages in the base of the anal fins and from this day, the fish started to die. On day 10 the first sign of nervous disorders were observed as rapid, erratic and circular swimming movements with periods of rest at the bottom of the tank coupled with rapid breathing, on day 34, 100% mortality was reached in this diet.

On day 14, some fish from diet 2 to 4 were observed with the same signs, having subsequently daily mortalities. At the end of the month, fish on diet 2 had a mortality of 83.34% and on day 42 the mortalities on

Table 4.2 Proximate analysis of the experimental diets (wet weight basis) used in the Pyridoxine (B_6) requirement experiment.

Moisture	6.50
Crude Protein	44.07
Lipids	10.48
ASH	5.32

diets 1 to 4 were 100, 95, 81.6 and 90% respectively.

Fish fed diet 5 with 5.08 mg of Pyridoxine/Kg at no point showed deficiency signs and at the end of the 42nd day the mortality was only 25%., this was due to one or two fish from each tank showing aggressive behaviour and attacking the rest.

Fish fed diet 1 (0.0mg of Pyridoxine/Kg diet) on the second experiment started to show on day 11 the same deficiency signs described previously and started to die on day 12, all fish from this diet being dead by day 19. Fish from diet 2 and 3 (1.85 and 3.17 mg of Pyridoxine/Kg diet respectively) showed the first signs of deficiency on day 15 (anorexia, lethargy and dark colouration) and started to die, but this time erratic swimming and convulsions were not observed. On day 60 there was a mortality of 66.7% and 52% on fish fed diets 2 and 3 respectively. Most of the surviving fish were moribund, subsequently the fish were killed, and preserved for histological observations.
Fish fed diets 4 to 11 never showed deficiency signs, behavioural changes or mortalities. In table 4.3 is given the percentage of survival on day 75.

Table 4.3 Effects of eleven different levels of Pyridoxine on growth, food conversion ratio and survival of Cichlasoma urophthalmus during a 75 day experimental period.

Diet	Mg of B_6/kg of diet	Initial[1] Weight (mg)	Final[1] Weight (mg)	Individual[1] Weight gain (mg/day)	Individual[1] Food Intake (mg/day)	Food Conversion Ratio	Weight gain (%)	Specific growth Rate	Survival %
1	0	148[a]	-	-	-	-	-	-	0
2	1.85	148[a]	-	-	-	-	-	-	33.3[2]
3	3.17	147[a]	-	-	-	-	-	-	48[2]
4	5.61	145[a]	2550[a]	34.68[a]	40.60[a]	1.19[a]	1658.0[a]	3.82[a]	95
5	10.7	145[a]	2850[a]	36.03[a]	44.43[a]	1.23[a]	1864.6[a]	3.97[a]	95
6	24.42	147[a]	2540[a]	31.86[a]	40.66[a]	1.28[a]	1625.0[a]	3.79[a]	97
7	32.76	145[a]	2610[a]	32.93[a]	42.62[a]	1.29[a]	1700.2[a]	3.85[a]	92
9	66.52	150[a]	2610[a]	32.79[a]	42.39[a]	1.29[a]	1641.4[a]	3.81[a]	92
9	94.20	146[a]	2420[a]	30.39[a]	39.35[a]	1.29[a]	1637.9[a]	3.75[a]	80
10	426.24	147[a]	2560[a]	32.20[a]	41.99[a]	1.30[a]	1639.4[a]	3.81[a]	100
11	642.62	141[a]	2620[a]	32.11[a]	42.38[a]	1.29[a]	1755.8[a]	3.90[a]	100

1. Values within the same column which bear a common superscript were not significantly different at P 0.01.
2. Survival at the 60 day experimental period, 0% survival at the 75 day of the experiment.

4.3.5. Nutritional parameters.

The initial fish weights from the two experiments were not significantly different ($p < 0.01$). As fish from diet 1 to 4 in the first experiment were dying continuously during the trial, it was not possible to obtain results for the nutritional parameters. Fish from diet 1, 2 and 3 (0, 1.85 and 3.17 mg of Pyridoxine/Kg) in the second experiment also had high mortalities during the trial but, fish from diet 4 to 11 had no significant difference in any of the nutritional parameters measured ($P<0.01$) by day 75 (Table 4.3).

4.3.6. Histological results.

There were no histopathological changes related to Pyridoxine deficiency in any of the diets. However as in the case of the fish from the experiments to determine Vitamin C (Chapter 3) and calcium panthotenate (Chapter 5) some gill pathology was observed in fish fed on the different diets, showing slight oedema and hyperplasia. Fatty liver degeneration was also observed in the hepatopancreas of the fish fed all the experimental diets. These changes were not considered to be due to the diets fed.

As these fish came from the wild they were already infected with Myxobolus sp. (Christina Sommerville, personal communication). The number of cysts observed

and the associated pathology of slight inflammatory response around the cysts and occasional muscle atrophy bore no relationship to the pyridoxine levels. Rodlet cells were observed in many slides, surrounding the endothelium of the vessels and once in the tubules of the kidney, again, the presence of these cells bore no relationship to the Pyridoxine levels.

4.4. DISCUSSION.

Fish from the two experiments fed diets containing 5 mg/Kg of pyridoxine or more, never showed behavioural changes, reduced growth, signs of deficiency and mortalities. This level corresponds well with the values obtained with respect to weight gain (%), specific growth rate and food conversion ratio in which no difference was observed between diet 4 with 5.61 mg/Kg diet and diets with higher levels. Consequently 5 mg/Kg of Pyridoxine is considered as the minimum dietary requirement for Cichlasoma urophthalmus.

This level is within the range of Pyridoxine requirements for other tropical fish or those grown at subtropical temperatures, e.g. turbot (Adron et al., 1978), table 4.4 shows these results.
Analysing the data in this table it seems that coldwater

fish have a higher requirement for Pyridoxine (10-20 mg/Kg diet) than tropical fish (1 to 6 mg/Kg diet), however, the information is still fragmentary to support this fact, in many cases it is known that Pyridoxine is required for normal growth and enzyme activity, however the requirement has not yet been fully established.

C.urophthalmus fed diets deficient in vitamin B6, showed many of the external characteristic signs of deficiency for this vitamin in the various species of fish studied to date. Examining the signs reported by other authors (Table 4.4) it is easy to see that some of them are common in all cases, for example loss of appetite, retarded growth, lethargy, rapid breathing, nervous disorders expressed as hyperirritability, violent, erratic and spiral swimming (=epileptic fits or convulsions) and high mortality.

Some signs vary with the species and these could due simply to interespecific variation in responses to the deficiency, but also due to the size of the fish as small fish have faster growth and in consequence show the characteristic external signs of deficiency in less time than in bigger fish and high mortalities occur before other external signs appear. In the present experiment, fish with an initial weight of less than 1 g. were used, and if this factor added to the fact that this species is a fast growing tropical fish, it is not

TABLE 4.4 Pyridoxine requirement, time for development of clinical signs, gross external signs observed in different species of fish and the different criteria, temperature of culture and initial size of the fish used during the experimental period.

Species	Temperature °C	Initial weight of fish (g)	Criteria used	Experimental Time (days)	Time of appearance (days)	B$_6$ Requirement mg/kg dry diet	Signs of deficiency	Reference
Cyprinus carpio	25	4-5.5	1,2,6,9	70	28-42	5.4	9,10,12,22 23	Ogino,(1968)
Ictalurus punctatus	7	12	1,2,3,4	168	63	R	9,6,8,10	Dupree,(1966)
Salmo gairdneri	7	4	1,2,3,4	-	-	R	1,6,10	Kitamura et al., (1967)
Seriola quinqueradiata	7	43.5	1,2,3,10	90	40	R	1,2,5,22 23,24	Sakaguchi et al., (1969)
Chrysophrys major	25	34.5	1,10,9,12 6	51	14-28	2-5 5-6	1,2,5,6,10 22	Takeda and Yone, (1971)
Salmo gairdneri	12-15		1,2,3,4	-	14-21	10-15	1,2,5,6,	Halver, (1972)
Salvelinus fontinalis						10-15	7,8,9,11 12	
Salmo trutta						10-15		
Salmo salar						10-15		
Oncorhynchus tshawytscha						15-20		
Oncorhynchus kisutch						15-20		
Salmo gairdneri	10	148	6,7,8	70	56	R	1,5,6,7,8	Smith et al., (1974)
Scophthalmus maximus	18° 0.5	18.66	1,2,6	84	56	1 2.5	1,10	Adron et al, (1978)
Oncorhynchus tshawytscha	-	Finger-lings	1,2,5	-	-	.38-.43 [a]	-	Halver, (1979)
Oncorhynchus tshawytscha	16	4	1,5,6	70	56	10 20-24	1,10	Hardy et al, (1979)
Ictalurus punctatus	27 ± 0.5	6.3 9 24.1	1,2,3	84 140	28	3	1,2,5,10 14,15	Andrews and Murai, (1979)
Sparus aurata	20-22	2.69 69.26	1,2,4 5,6,7	133 138	18 35	1.97 1.97	1,5,6, 10,13	Kissil et al., (1981)
Salmonids	—	—	—	—	—	10	—	Halver, (1982)
Channa punctatus	20-31.5	49.51	3,4	240	100	*	6,4,5,7, 20,21	Agrawal and Mahajan (1983 a)
Channa punctatus	20-31.5	49.51 ±21.4	8	240	180 240	*	4,2,5,1,9 16,17,18	Agrawal and Mahajan (1983 b)
Cichlasoma urophthalmus	25 29	.093 .147	1,2,3,4, ?	42 75	9 11	5	1,2,3,4, 6,7,10,25	This study

Criteria used

1. Growth
2. Mortality
3. Gross external signs of deficiency
4. Behaviour
5. FCR
6. Tissue enzyme activities
7. Histopathological changes
8. Haematological and haematopoietic changes
9. Vit. B_6 content in the tissues
10. Crude fat content in the tissues
11. Glucose resistance
12. Ratio of liver body weight

Signs of deficiency

1. Low growth
2. Anorexia
3. Dark colour
4. Lethargy
5. Hyperirritability, general nervous disorders
6. Cranial, skeletal and retinal oedema (ataxia,convulsions)
7. Rapid and gasping breathing
8. Flaring of opercula
9. Anaemia
10. High mortalities
11. Ataxia
12. Oedema of peritoneal cavity
13. Poor food conversion ratio
14. Tetany
15. Cranium blue coloration
16. Significant increase in haematological parameters
17. Integumentary oedema
18. Enlarged and mediated spleen and kidney
19. Opacity of lens
20. Muscular spasm
21. Parasitic metabolic part of the body
22. Exophthalmia
23. Skin disturbance
24. Scoliosis
25. Haemorrhage in the anal fins

surprising that signs appeared in less time (9-11 days) in comparison to other fish studied, (initial sizes ranged from 2.69 to 148 g.) in which the first signs were observed between 14 and 240 days. (Ogino, 1965; Dupree, 1966; Sakaguchi et al., 1969; Takeda and Yone, 1971; Halver, 1972; Smith et al., 1974; Adron et al., 1978; Hardy et al., 1979; Andrews and Murai, 1979; Kissil, et al., 1981; Agrawal and Mahajan, 1983a,b). To further support this observation, Kissil et al., (1981) stated "that the occurrence of abnormal behaviour at an earlier stage (18 days) in the fish of experiment 1 than in those of experiment 2 (35 days) is probably a function of fish size. The larger fish of experiment 2 are growing at a slower rate than are those of experiment 1; therefore vitamin deficiency signs would take longer to appear."

In C.urophthalmus fed Pyridoxine deficient diets, high mortalities were suffered and this situation was also observed in other species (Ogino 1965; Dupree 1966; Kitamura et al., 1967; Sakaguchi et al., 1969; Takeda and Yone 1971; Halver 1972; Smith et al., 1974; Adron et al., 1978; Halver 1979; Andrews and Murai 1979; Hardy et al., 1979; Kissil et al., 1981). Channa punctatus is an exception, Agrawal and Mahajan (1983b) found that this species was less sensitive to a prolonged dietary deficiency of Pyridoxine and 15% mortalities were recorded after 34 weeks, while in other species,

including C.urophthalmus 100% is reached between 2 to 8weeks.

Little work has been carried out describing histological changes in relation to Pyridoxine deficiency in fish. Smith et al., (1974), observed pathological abnormalities in Salmo gairdneri and Kissil et al., (1981) in Sparus aurata. Except for the slight gill pathology and the fatty liver degeneration observed in the fish fed all the experimental diets, no histopathological changes were observed in C. urophthalmus related with diets deficient in Pyridoxine. Similar results being obtained by Andrews and Murai, (1979) in which in spite of the severity of gross deficiency signs observed in Ictalurus punctatus the histological studies revealed no abnormalities. In this case they concluded that "-The pyridoxine content in the basal diet (1.2 mg/Kg) or intestinal synthesis was sufficient to prevent histological changes as has been reported in other animals (Griess and Scott, 1972), cessation of growth and death may have occurred prior to the onset of histological changes."-. In the case of C.urophthalmus absence of pathological changes could also be due to the possible synthesis of Pyridoxine in the intestine or to the early cessation of growth and mortality.

Andrews and Murai (1979) reported that catfish fed a diet with 2.2 mg of B6/kg, below the minimum requirement for this specie (3 mg/Kg), showed reduction of growth, as the only sign of deficiency in comparison with fish fed a diet unsupplemented with Pyridoxine (1.2 mg/Kg). In the latter severe nervous disorders, greenish-blue coloration and 100% mortality were recorded. Kissil et al., (1981) reported that the same behavioural and pathological changes were observed in Sparus aurata fed two levels below the minimum required, but these signs appeared later. In the present study, C. urophthalmus from the first experiment fed diets containing less than 5 mg/kg, (1.02; 1.41; 1.98; 2.72) showed the same behavioural and external signs of deficiency as those fish completly deprived of this vitamin (diet 2 Chapter 2 and diet 1 of the second experiment in this work), but the signs were observed later, whilst in diets 2 and 3 of the second experiment (1.85 and 3.17 mg/Kg) the fish only showed as signs of deficiency, anorexia, lethargy, dark coloration reduced growth and mortality. However this time erratic and spiral swimming was not observed. These results suggest that below the minimum level of Pyridoxine required for normal growth and health there is a level in which not all the characteristic gross external signs of Pyridoxine deficiency appear, and it is important to know this for future diagnosis.

Anaemia in fish has been associated with pyridoxine deficiency (Halver 1972, Smith et al., 1974 and Agrawal and Mahajan 1983b), However Andrews and Murai (1979) reported unexpected and significantly reduced haematocrit values, in fish from two experiments, fed diets with 20 and 30 mg of Pyridoxine/Kg diet. A third experiment was conducted by them to confirm this result, and as with the first two experiments, the fish fed 30 mg of B6/Kg had severe microcytic normochromic anaemia, however the severity of the anaemia was not enhanced by increasing the dietary pyridoxine level up to 300 mg/Kg of diet. In the present study, fish were fed at higher levels up to 300 mg/kg (426.24 and 642.62 mg/Kg of diet) during 75 days (a similar time to the experiments carried out by Andrews and Murai, (1974) in catfish, but haematological studies were not performed in this case, but fish fed with 5 mg/Kg and above this level, did not show any external signs of toxicity at the time of the experiment, and grew as well as the fish fed the minimum level of pyridoxine required. However, water soluble vitamins taken in high doses over long period of time may induce dependancy stages, and users may show signs of deficiency or withdrawal signs when the dose is lowered or the substance discontinued (Alhadeff, Gualtieri and Lipton, 1984). To prevent these possible effects over longer periods of time and for practical uses, high doses of Pyridoxine are not generally recommended.

However to prevent possible losses of this vitamin during processing and storage (Tacon, 1985) as well as to improve the possible role of this vitamin in disease resistance (Hardy et al., 1979) and to fulfill the multiplicity of biochemical functions of this vitamin a safety level of 50 to 100% (7.5 to 10 mg/Kg diet) to the minimum required should be present into practical diets (Halver, 1976) for C.urophthalmus .

CHAPTER 5.

DETAILED PANTOTHENIC ACID REQUIREMENT AND ITS DEFICIENCY SYNDROME

5.1. INTRODUCTION.

Pantothenic acid is a water soluble vitamin that functions as a part of the coenzyme A molecule in the metabolic release of energy from all three energy providing-nutrients, carbohydrate; fat and protein by way of the tricarboxilic acid (TCA) cycle. Pantothenic acid as a component of Coenzyme A, is also required for the synthesis of fat, fatty acid oxidation, pyruvate oxidation and other biological acetylations such as the conversion of choline to acetylcholine and the conversion of oxalacetic acid to citric acid. (Dyke, 1965; Brown, 1971; Halver, 1972, 1982; NRC, 1983).

Deficiency signs in animals are well defined but vary with the species, chickens suffer a specific dermatitis, retarded growth and nervous derangements and in rats, growth is retarded and a depigmentation of the fur occurs, whilst in man deficiency signs are virtually unknown, because definitive evidence has been obtained that pantothenic acid is synthesized by the intestinal flora (Krehl, 1960; Dyke, 1965; Gries and Scott 1972).

Pantothenic acid is also essential for normal physiology and metabolism of a growing fish (Halver, 1972). The most evident lesion in fish caused by pantothenic acid deficiency is in the gills. The first

work on gill disease was described by Davis, (1926, 1927) who concluded that it was a bacterial infection, however he did not present experimental evidence. Wolf, (1945) made a full description of dietary gill disease of trout based on 10 years of experience. He established that it is a non infectious syndrome, characterized by hyperplasia of the respiratory epithelium and that this failure was specifically due to the lack of pantothenic acid. This work was confirmed by Tunison, Phillips, Shaffer, Maxwell, Brockway and McCay, (1944) who found that the addition of pantothenic acid to the diet corrected the disease condition among fish showing gill damage due to a lack of this vitamin. After these original studies, further research was carried out looking for the qualitative and quantitative requirements of pantothenic acid and also to determine the pathological changes caused by this deficiency in different species of fish (McLaren et al ,1947; Rucker, Johnson and Kaydas, 1952; Halver, 1957; Phillips and Brockway, 1957; Coates and Halver, 1958; Phillips, Podoliak, Brockway and Vaughn, 1958; Dupree, 1966; Kitamura et al., 1967; Ogino, 1967; Halver, 1972; Ishi and Yamamoto, 1972; Murai and Andrews, 1975; Halver and Tiews, 1979; Murai and Andrews, 1979; Poston and Page, 1982; Wilson, Bowser and Poe, 1983; Karges and Woodward, 1984). Eller (1975) discussed the physiological problems involved in the gills of pantothenic acid deficient fish.

In the second chapter of this study the qualitative requirement of calcium pantothenate for Cichlasoma urophthalmus was demonstrated. The objective of this study was to determine the quantitative requirement of this vitamin in terms of growth, food conversion ratio, behaviour, gross external signs of deficiency, mortality and histopathological changes.

5.2. MATERIAL AND METHODS.

5.2.1. Experimental design and diets.

315 fry of Cichlasoma urophthalmus were captured at Celestun Lagoon Yucatan (Section 1.2.) with an initial weight of .160 to .175 g, and after a period of acclimation (section 1.2) they were placed in 21 tanks of the recirculation system (section 1.1.). Seven different diets were prepared as was previously described in section 1.3.. Each diet was provided with 7 different levels of calcium pantothenate (0, 10, 20, 40, 80, 160 and 320 mg/Kg dry diet). Fish received the experimental diets ad libitum during the trial. Feeding methodology, care of the fish and nutritional parameters measured are described in sections 1.4, 1.5, and 1.6.

5.2.2. Methods of proximate analysis.

One sample was taken to determine the proximate analysis of all diets, following the methods described in section 1.3.

5.2.3. Histological methods.

Histological methods have been described previously in section 1.7., but apart from these techniques, in this study some of the sections were also stained with the Periodic Acid Shiff reaction (PAS) for glycogen, long Ziehl-Nielsen (ZN) method for ceroids and Giemsa for protozoans (Drury and Wallington, 1980).

5.3. RESULTS.

5.3.1.Water quality.

During the experiment the following water quality parameters were recorded.

Temperature 28°C ±1.5.

Nitrites 0.026ppm (range 0.001-0.034).

Ammonia 0.050 ppm (range 0.013-0.107).

Un-ionozed ammonia 0.008ppm, (range 0.002 and 0.013ppm).

pH 8.20-8.40

5.3.2. Proximate analysis.

The results of the proximate analysis of the diets are given in table 5.1.

5.3.3. Behaviour, deficiency signs and mortality.

Fish on diet 1 ate voraciously during the first 15 days, but on day 8 they started to show rapid breathing, dark colouration, distended opercula and on day 11 a high mortality. All fish fed this diet died before day 30 of the experiment.

Fish on diet 2, ate voraciously for the first two weeks, then their appetite was greatly reduced and they started to show the first deficiency signs on day 22, these signs were rapid breathing, distended opercula, haemorrhages in fins and head, slight exophthalmus and sometimes dark colouration. From this day onwards the fish started to die, reaching 100% mortality on day 40.

Fish fed diet 3 ate well during the first month, after which there was a slight reduction in their appetite in comparison with fish fed diets 4, 5, 6 and 7. Thus after the first month there was a reduction in their weight gain (%). Fish fed diet 3 showed the first deficiency signs on day 48, one fish was observed moribund in the bottom of the tank with haemorrhages in one eye and on the fins, after day 48, the fish started

Table 5.1 Proximate analysis (wet weight basis) of the experimental diets used in the experiment of calcium pantothenate requirements.

Moisture	7.73
Crude Protein	45.41
Lipids	11.56
Ash	5.80

to die in large numbers with the same deficiency signs described before for diets 1 and 2, reaching 100% mortality on day 62. Fig 5.1. Shows that there is a direct relation between the time in which the first signs appeared and the time at which 100% mortality was reached for each Calcium Pantothenate level in the diet.

Fish fed diets 4, 5, 6 and 7 never showed abnormal behaviour or other gross deficiency signs at day 60 of the experiment. To be sure that fish fed diet 4 were not going to develop external signs of deficiency they were fed in the same way for a further 15 days, as were fish on diets 5,6 and 7. No mortality or deficiency signs were observed in any of these treatments, the survival at the end of the experiment was 77.78%, 74.55%, 88.88% and 86.66% respectively. (Table 5.2)

5.3.4. Nutritional parameter results.

Initial body weights of the fish on the seven diets was not significantly different at $P < 0.01$. Table 5.2 and Figure 5.1. show the growth response of the fish fed the 7 different levels of calcium pantothenate at the end of the 75 days experimental period. The highest value obtained in respect of final weight gain, individual weight gain, individual food intake, weight gain (%) and specific growth rate was reached in fish fed diet 6, however there was no significant difference

Figure 5.1. There is a direct relation between time of appeareance of deficiency signs (x) and time in which 100% mortality is reached (o) and calcium pantothenate in the diet.

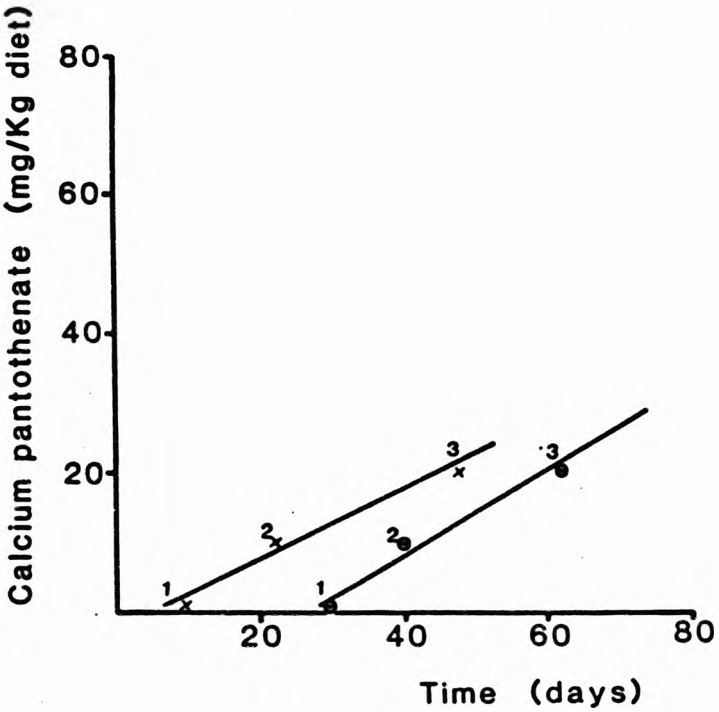
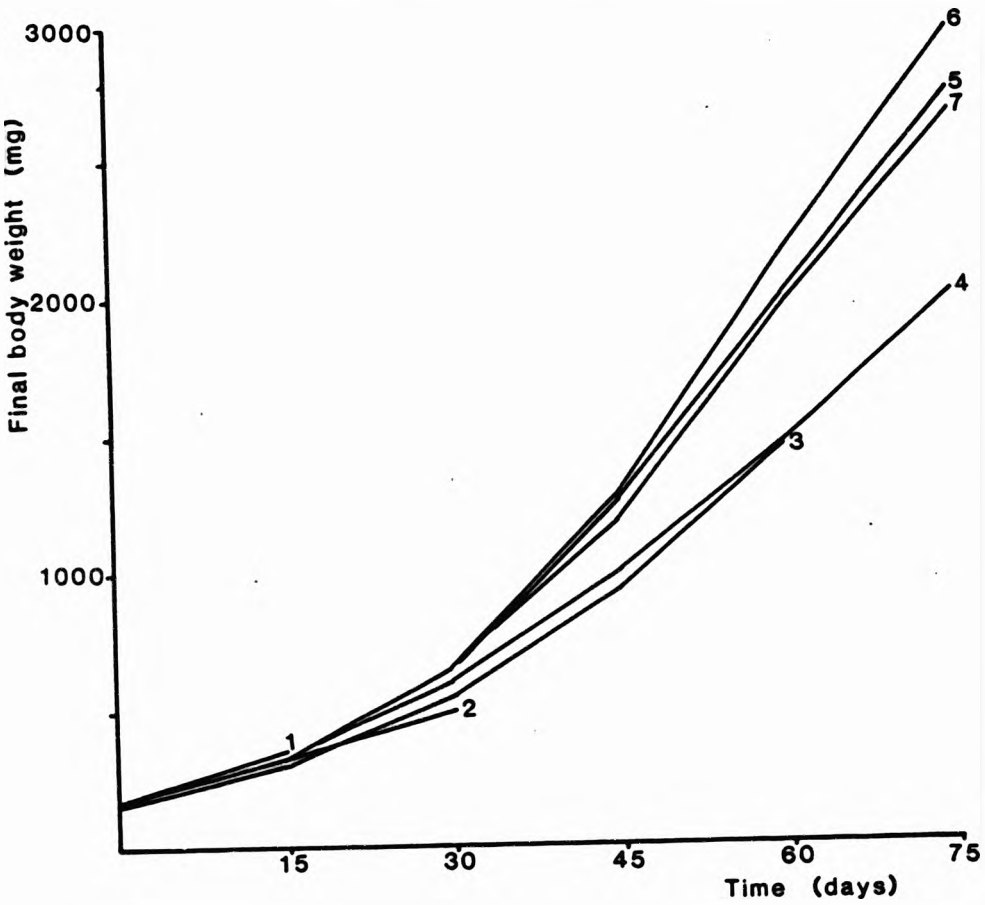

Calcium pantothenate (mg/Kg diet)
80
60
40
20
20
40
60
80
Time (days)
1
2
3
1
2
3

Table 5.2 Effects of seven different levels of Calcium pantothenate on growth, food conversion ratio and survival of Cichlasoma urophtalmus during a 75 day experimental period.

Diet	Calcium Pantothenate added to the diet (mg/kg)	Initial Weight (mg)	Final* Weight (mg)	Individual* Weight gain (mg/day)	Individual* ood Intake (mg/day)	Food* Conversion Ratio	Weight* gain %	Specific Growth Rate %	Survival (%)
1	0	171.5	-	-	-	-	-	-	0
2	10	165.0	-	-	-	-	-	-	0
3	20	164.0	-	-	-	-	-	-	0
4	40	166.33	2010.17	23.795	40.93	1.75	1108.09	3.31	77.78
5	80	165.33	2746.96	32.98	49.82	1.52	1556.82	3.73	75.56
6	160	173.33	2978.17	36.09	51.78	1.44	1617.84	3.79	88.89
7	320	165.67	2669.4	33.60	49.36	1.47	1514.55	3.71	86.66

* There was no significant difference at $P < 0.01$

Figure 5.2. Mean growth response of the fish fed 7 different levels of calcium pantothenate during a 75 day of experimental time.


3000
2000
1000
Final body weight (mg)
15
30
45
60
75
Time (days)
1
2
3
4
5
6
7

(P=0.01) between diets 4 to 7 in any of the nutritional parameters measured at this time.

5.3.5. Histopathological changes.

Marked interlamellar proliferative lesions were observed in gills of fish fed diets 1, 2 and 3 supplemented with 0, 10 and 20 mg/kg of calcium pantothenate respectively. The lesions ranged from oedema in the epithelial cells of the gills (Plate 5.1) to hyperplasia covering all the secondary lamellae thus giving the fish gills a clubbed appearance (Plate 5.2) and finally to the complete fusion of adjacent filaments (Plate 5.3).

Fish fed diets 4 to 7 had normal gills, however between some normal filaments, slight oedema in the base of the secondary lamellae was observed, but these lesions were never severe.

Livers from the fish fed the 7 different treatments showed fatty degeneration. These fatty deposits ranged, even in the same liver, from very slight to areas with all hepatocytes severely affected. Fish fed diets 1, 2, 3 and 4 had marked glycogen deposition (PAS positive) in the hepatic cells, while fish fed diets 5 to 7 showed slight or no glycogen deposition. Those fed diets 1 and

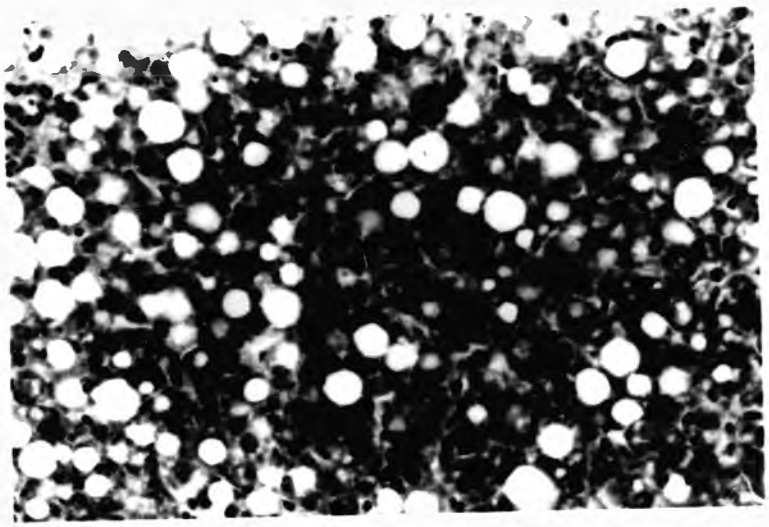
Plate 5.1. Gills of the fish fed diets 1, 2 and 3 showing marked oedema in the epithelial cells (H & E 312.5X).

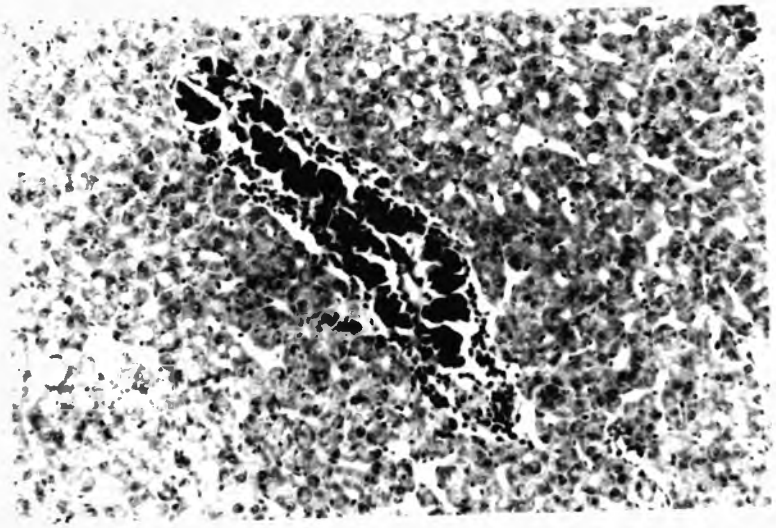
Plate 5.2. Gill of the fish fed diets, 1, 2 and 3 showing at the top of the plate some filaments with marked oedema and some hyperplasia in the base of the secondary lamellae. The filaments at the bottom of the plate shows marked hyperplasia that covers all the filaments and joins together the secondary lamellae and results in some fusion between filaments (H & E 78.75X).

Plate 5.3. Last stage of the gills from fish fed diets 1, 2 and 3 showing complete fusion of adjacent filaments (H & E 78.75X).

Plate 5.4. Hepatopancreas of the fish fed diets 1 and 2 showing granules and globules of ceroid of different sizes (arrows) and fatty degeneration (H & E 500X).

Plate 5.5. Necrosis of the pancreas of the fish fed diets 1, 2 and 3 in which there is obvious shrinkage of the cells, and no zymogen granules (H & E 312.5X).





2 also had some lipochrome pigments within the cytoplasm of many hepatocytes and within the spleen parenchyma. This material was present either as fine granules or distinct globules, stained golden with H & E, slightly pink with PAS and bright red with long ZN, indicating that it was ceroid. (Plate 5.4). In the liver, no infiltration of macrophages was observed, but in the spleen some macrophage-like cells were observed englobing some granules of this material.

Fish fed diets 1, 2 and 3 were observed with pycnotic nuclei in their pancreas together with a shrinkage of the acinar cells and very few, or sometimes no zymogen granules, indicating necrosis of the pancreas cells (Plate 5.5).

Fish fed diets 1 to 7 were seen to have cysts of Myxobolus sp., the cysts being observed mainly in the muscle, but they were also observed in the connective tissue of the dermis, in the sclera of the eye, in the heart and in the intestine. Related with the cysts, inflammation and other related pathologies were observed around the area in which they were situated (see chapter 3) having no relation with the levels of calcium pantothenate supplied to the diets. However larger numbers of cysts were observed in the fish fed diets 1,2 and 3 than in the fish fed diets 4, 5, 6 and 7.

Some rodlet cells were observed in the endothelium of veins from fish on all diets.

5.4.DISCUSSION.

Fish fed diets 4, 5, 6 and 7 which were supplemented with 40, 80, 160 and 320 mg/Kg of calcium pantothenate/Kg of diet, never showed gross external signs of deficiency, behavioural changes or high mortalities. They had the same growth response in respect to specific growth rate, weight gain (%) and food conversion ratio at the end of 75 days. With respect to the histopathological changes observed they all had normal gills. Livers from fish fed diets 5, 6 and 7 were free from glycogen deposition and hepatic ceroidosis. Taking these results into account it is clear that food for Cichlasoma urophthalmus fingerlings must be supplemented with at least 80 mg of calcium pantothenate/Kg diet to reach maximum growth, good food conversion ratios and avoid external and histological deficiency signs. Unfortunately, in the present study, facilities were not available to determine via a microbiological method, the exact amount of calcium pantothenate offered to the fish in the diet, but, assuming that the stability of calcium d-pantothenate is high in multivitamin premixes (Tacon, 1985) and the

losses during processing were in the range of 10% as were observed by Slinger et al. (1969), then the requirement is between 70 to 80 mg/Kg diet and this value is in the range of requirements observed for other species (Table 6.3). However it is recommended that the food should contain 160 mg pantothenic acid/Kg diet to avoid the risk of losses during pelleting or extrusion, and from the leaching of the vitamin from pellets (NRC, 1983).

Table 5.3 summarises the different studies on fish with respect to their pantothenic acid requirement. Observing this data, it is noticeable that the requirements observed to date are not higher than 80 mg/kg dry diet except in the case of the study by Murai and Andrews (1975) in which they observed a requirement of 250 mg/Kg, but this high level was perhaps due to their having used recently hatched channel catfish fry (Initial weight .028-.057g) in their research. However they did also point out that this apparent high requirement may be related to a differential rate of loss of pantothenate into the water from the small crumb feeds used. Four years later, they studied the requirement of this vitamin for the same species but this time they worked with fingerlings with an initial size of 10 and 22 g., and obtained a requirement of 10 mg of calcium pantothenate/Kg diet. (Murai and Andrews,

TABLE 5.3 Pantothenic acid required, time for development of clinical signs, gross external signs observed in different species of fish and the different criteria, temperature of culture and initial size of the fish used during the experimental period of time.

Species	Temperature °C	Initial weight of fish (g)	Criteria used	Experimental time (days)	Time of appearance of signs (days)	Pantothenic acid requirement (mg/kg diet)	Signs of deficiency	References
Salmo gairdneri	8	4.12-6.1	10	—	—	16-19	5,13	Tunison et al., (1944)
Salmo gairdneri	—	3.5	1,2,5	112	—	40	5	McLaren et al., (1947)
Oncorhynchus tschawytscha	—	approx. 2	3,4	70	—	R	4,5,19	Rucker et al., (1952)
Salvelinus fontinalis 1. namaycush 2. gairdneri 3. irutis	—	—	—	—	—	38.8-50	5,10,19	Phillips and Brockway, (1957)
Oncorhynchus tschawytscha	18	1.5	2	126	—	R	1,17,18 19	Halver, (1957)
O. kisutch	—	0.50	1,2,4	112	—	R	3,5,6,7	Coates & Halver, (1958)
Salmo salar	9-11	6.32	1,2,4	224	—	R	5,19	Phillips et al, (1958)
Ictalurus punctatus	—	10	1,2,4	184	43	R	1,5,6, 7,9,19	Dupree, (1966)
S. gairdneri	—	—	1,2,3	—	—	R	1,3,4,8	Kitamura et al, (1967)
Cyprinus carpio	23 ± 1	7	1,2,3	42	—	40-56	1,3,7, 11,14,15	Ogino, (1967)
Oncorhynchus tschawytscha	10-15	—	1,4,5,10	168	—	52,80	—	Halver, (1971)
Carassius auratus	21 ± 1	4.5	2,8	52	30	R	3,5,6,14 17	Ishii & Yamamoto, (1972)
Ictalurus punctatus	28 ±	.020 .057	1,2,4	42	14	250	3,5,7,19	Page & Andrews, (1975)
I. punctatus	27 ± 0.5	22	1,2,4	56	28	10	2,3,5,9 15,19	Page & Andrews, (1979)
Oncorhynchus tschawytscha						40-50		Halver, (1972)
O. kisutch						40-50		
S. gairdneri						40-60		
S. irutis						40-50		
Salvelinus fontinalis						40-50		
Cyprinus carpio						30-60		
Ictalurus punctatus						25-50		
Sparus aurata						*		
Scophthalmus maximus						*		
Anguilla japonica						*		
Seriola quinqueradiata						R		Halver, (1972)
Salvelinus namaycush	12.4	6.7	2,3,4,5, 6,7	70	35	R	2,3,4,7,12 15,16,19	Poston & Page (1982)
Ictalurus punctatus	26.7 ± 1.1	6.39-6.60	1,2,4,7,9	70	15	15	3,5	Wilson et al., (1983)
Clarias batrachus	—	4-5	1,2,4	168	21	R	1,2,3,5,7 9,11,12	Buttner et al., (1986)
Cichlasoma urophthalmus	28	.267	1,2,3,4,7 9	75	8	70-80	1,2,3,4,5 7,11,12,13 14,19	This study

* Denotes a requirement, the level of which has not been established.

Criteria used

1. Growth
2. External signs of deficiency
3. Behaviour
4. Mortality
5. Haematological values
6. Body composition
7. Histological changes
8. Liver changes
9. Feed efficiency
10. Maximum liver storage

External signs of deficiency

1. Loss of appetite
2. Total anorexia
3. Reduced growth
4. Thin and emaciated fish
5. Clubbed gills
6. Gills covered with exudate
7. Loss of activity (sluggishness)
8. Abnormal swimming
9. Crooked fins & skin
10. Blue slime disease
11. Haemorrhages of body surface
12. Distended operculae, rapid breathing
13. Prostration on the bottom of tank
14. Slight exophthalmus
15. Anaemia
16. Poor FCR
17. Anorexia and scarring
18. Cellular atrophy
19. High mortality

1979). Wolf, (1945) suggested that smaller fish have a higher calcium pantothenate requirement than bigger fish which is apparently the case when the results obtained from Murai and Andrews (1975, 1979) are observed, but from the available data to date (Table 5.3) it is clear that there is not enough information to confirm that suggestion. It is obvious from this review that there is a lack of information with respect to the requirements of calcium pantothenate for different sizes of fish. This fact is important from the fish health and economic point of view.

In table 5.4. The external deficiency signs of calcium pantothenate in the different species studied can be observed. It is clear that the number of external signs varied with the species but a few of them were common in most of the cases, such as anorexia, reduced growth, clubbed gills and high mortalities.

In respect to histological changes, most of the reports described gill lesions caused by pantothenic acid deficiency. Since the first study on this aspect from Tunison et al., (1944) and Wolf, (1945) it seems that most of the species fed a deficient pantothenic acid diet suffered proliferation of the gill epithelium which then grow together to form a clubbed shape and afterwards into a solid mass as reported by McLaren et

TABLE 5.4 Gross external signs of calcium pantothenate deficiency in different species of fish.

Species	Gross external signs of Calcium pantothenate deficiency																		Reference
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
Salmo gairdneri	*		*	*	*			*		*					*					1,2,4,9
Oncorhynchus tschawytscha	*			*	*												*	*	*	3,5
O. kisutch			*		*	*	*													6
Salvelinus fontinalis					*					*									*	4
S. namaycush		*	*	*	*		*			*		*			*	*			*	4,16
S. trutta					*					*									*	4
S. salar					*														*	7
Cyprinus carpio	*		*				*			*				*	*					10
Ictalurus punctatus	*	*	*		*	*	*		*				*		*				*	8,13,14,17
Cichlasoma urophthalmus	*	*	*	*	*		*				*	*		*					*	18

1. The numbers express the same external gross of deficiency indicated in table 3
2. The numbers indicate the same authors quoted in table 3.

al., (1947) Rucker et al., (1952); Wood and Yasutake, (1957); Coates and Halver, (1958); Dupree, (1966); Murai and Andrews, (1975, 1979); Poston and Page, (1982); Wilson et al., (1983); Karges and Woodward, (1984). Only Ishii and Yamamoto (1972) reported that, in their experiment with goldfish (Carassius auratus) the fish fed the deficient diet showed growth retardation, decrease of average body weight, haemorrhages in various parts of the body surface, slight exophthalmia and abnormal swimming but no histological abnormalities were observed in their gill filaments. C.urophthalmus was shown to have the same gill lesions reported previously in other fish species fed deficient diets. These lesions followed the same patterns for the characteristic nutritional gill disease described previously by Wood and Yasutake, (1957), Eller, (1975) and Karges and Woodward, (1984) in which a very early change of pronounced swelling of the epithelium on the lamellae ocurred, followed by hyperplasia that developed in a proximal direction from the distal extremity of the gill filaments and rapidly spread to cover the entire structure and fusing together to form a "rodlike" structure.

From gross observations gills were not observed covered with exudate, probably due to the small size of the fish, but histologically there was a clear

accumulation of cellular debris in the lesioned gills. Poston and Page, (1982) indicated that the exudate on gill filaments in trout deficient in pantothenic acid was possibly an accumulation of abnormal mucus and cellular debris, from a diminished metabolism and abnormal secretion of mucous cells, caused by reduced activity of cellular Coenzyme A.

In this study fatty liver degeneration was observed in the hepatic cells of the fish fed the 7 different diets. High levels of fat in fish livers are due to various factors such as choline and essential fatty acid deficiency or toxicity by oxidized fish oil (Henderson and Sargent, 1985; Tacon, 1985) or due to carbohydrate imbalance (Shimeno, 1982).

With respect to choline deficiency, in the second chapter of this study the essential nature of this vitamin for C. urophthalmus was observed, but no study has been carried out to determine this species requirement for this nutrient on this species. In the same way nobody has done research on the carbohydrate or essential fatty acid requirements and it is not possible to determine at the moment if these factors or a combination of them, caused the fatty livers in this experiment. Great care was taken to avoid oxidized oil when preparing the experimental diets, analysing the

oils before using them and chosing only those which less than 4 meq/Kg of peroxide. Furthermore, in the basal diet an antioxidant protection and vitamin E were used. The diets were carefully stored at between -20'C, and 4'C prior to use. Even though there was no test made to determine oil rancidity at the end of the experiment, no reason exists to think that oxidized oils were present in the diets.

With respect to fatty livers, Ishii and Yamamoto, (1972) demonstrated that no difference in the amount of fat droplets were recognized in liver cells of both control and pantothenic deficient fish. Poston and Page, (1982) observed that infiltration of fat was less than normal in livers of fish fed diets lacking supplemental pantothenic acid. In this study no difference was observed in the fat content of the fish fed the seven different diets.

In this study it was also noticed that the hepatopancreas of the fish fed diets 1, 2, 3 and 4 had glycogen deposition and fish fed diets 1 and 2 also had ceroid deposition in the spleen and liver cells. In this respect Isshi and Yamamoto, (1972) in a paper which deal with the use of both light and electron microscope to examine the liver cells of the goldfish (Carassius auratus) affected by pantothenic acid deficiency,

reported that liver cells contained many small granules slightly stained with eosin in the cytoplasm. These granules seemed to be large mitochondria and ultrastructurally the liver cells of the vitamin deficient fish showed conspicuous changes in cytological features, while in the fish fed the basal diet supplemented with calcium pantothenate, the normal cytoplasmic organelles were observed via electron microscope.

Poston and Page, (1982) found that livers from fish fed a pantothenic acid deficient diet were intensely PAS positive, indicating large deposits of glycogen or other polysaccharides, and they explained that high levels of liver fat and glycogen precluded satisfactory examination for abnormalities such as cytoplasmic vacuoles and hyaline bodies. Similar results and assumptions to those observed by Poston and Page, (1982) could also be applied for the deficient C. urophthalmus livers.

Poston and Page, (1982) observed also traces of glycogen in kidney tubules of trout fed the pantothenic acid deficient diets, this result was not observed in those of the deficient C. urophthalmus of this study.

Hepatic ceroidosis is the principal feature of lipoid liver degeneration and is associated with the feeding of rancid feed or a vitamin E deficient diet (Roberts, 1978; Smith, 1979; Roald, Amstrong and Landsverk, 1981; Holliman and Southgate, 1986). In this study only in the fish fed diets 1 and 2 extensive deposits of a red fat insoluble material were seen within many liver hepatocytes and between the spleen cells. As has been explained before there is no reason to suppose that the food was rancid or deficient in vitamin E. It is clear that this condition is associated with calcium pantothenate deficiency because fish fed adequate levels did not show these pathological signs. This is the first report in which ceroid in the livers of pantothenic acid deficient fish was observed. However the histological appearance of this ceroid deposition was similar to the typical ceroidosis reported as a result of oxidized oil or vitamin E deficiency in which there was a marked intracellular deposition of a pink-golden granular formation of different sizes within many of the hepatocytes and spleen phagocytes. Infiltration of free macrophages was observed only in the spleen. Analyzing again table 5.3 most of the histological changes reported for pantothenic deficient fish deals mainly with gill alterations and most of the studies have been carried out in cold water fish. Only Ishii and Yamamoto (1972) and Poston and page, (1982) reported

histological changes not just in the gills but also in various organs such as liver kidney and pancreas of the deficient fish. This could be a reason why this type of pathological response has not been reported prior to this study. Furthermore Ishii and Yamamoto, (1972) and Poston and Page, (1982) worked with bigger fingerlings (4.6g and 6.7 g respectively) than in this study (.167g), and the experimental time was 52 days and 70 days respectively, while this study lasted 75 days. Water temperatures of the other experimental systems were 21°C and 12.4°C respectively in comparison with this study in which the water temperature was 28°C, and consequently the growth rate of C. urophthalmus was higher than in Carassius auratus and Salvelinus namaycush used in the above mentioned previous studies.

It is also possible, as in the case of vitamin C deficient fish (Chapter 4) that more pathological signs appear in tropical fast growing fish than in temperate or coldwater fish, or that the time to get the same deficiency signs is shorter. In this respect Phillips et al., (1958) observed that water temperature and fish size had an effect in their studies and this was supported by the relatively slow rate of development of the deficiency signs of the three required vitamins. The deficiencies of pyridoxine, riboflavine and pantothenic acid occured at higher temperatures in about one half of the time required for them to show in colder water.

Changes in the pancreas were also observed by Poston and Page, (1982). They observed that acinar cells of fish fed supplemental biotin, and no pantothenic acid showed atrophy, vacuolated and granular cytoplasm and loss of cellular definition. In this study pancreas of the fish fed diets 1, 2 and 3 showed necrosis expressed as shrinkage of the acinar cells pycnotic nuclei and lack of zymogen granules. In these aspects Poston and Page, (1982) mention that if pantothenic acid is required for the synthesis of nucleic acid, as well as for high energy-transfer metabolism, a dietary deficiency of this vitamin would be expected to induce cellular lesions, such as abnormal deposits of glycogen, mitochondrial clumping, vacuolation, atrophy and eventual necrosis of the kidney tubules and degeneration of pancreatic acinar cells. Halver, (1982) explained that a dietary insufficiency of pantothenic acid impairs the normal metabolism within mitochondrial rich cells undergoing rapid mitosis and high energy expenditure, adding that structures such as the gill, kidney tubules and pancreatic acinar cells are normally affected by the deficiency of this vitamin due to the high levels of energy that are utilized for their normal processes such as osmorregulation, hydromineral homeostasis and enzyme synthesis.

Finally in this study it was observed that fish

from all diets were affected by Myxobolus sp. cysts, but a larger number of these parasites were observed in fish fed lower levels of calcium pantothenate. However it is not clear if this vitamin improves resistance to diseases or parasites in fish. In this respect in very early work Wood and and Yasutake, (1957) suggested that as it had been proved that pantothenic acid deficient mammals lose their resistance to pathogenic organisms, fish could will have the same response, although to date, no experimental work has been realized to confirm this suggestion.

GENERAL DISCUSSION.

Due to the recent interest in the aquaculture potential of the Mexican native cichlids, a series of studies on Cichlasoma urophthalmus has been initiated. This species was selected because it is appreciated in a wide region of the Gulf of Mexico, reaches good commercial size (600g) (Martinez and Ross, 1986) and initial studies have demonstrated that it has many of the characteristics that makes a species suitable for culture, such as wide salinity tolerance (0 to 35‰.), high fecundity (3000 to 7000 eggs) and good tolerance to low oxygen levels (two hours of virtual anoxia). It is also also known that it is a typical substrate spawner with biparental care and does not have sexual precocity problems as found in the tilapias. It can reproduce in different types of substrate on an all year round basis if appropriate environmental conditions are supplied. Is also known, through studies on its morphological characteristics and food habits in its natural habitat, that this species is a carnivorous fish (eating mainly invertebrates), without piscivorous habits but with some omnivorous tendencies. It has also been seen that in culture conditions, fry, juveniles and adults accept pelleted food and two experiments on its protein requirements demonstrated that in spite of its carnivorous habits, its protein requirements are similar

to those of the omnivorous or herbivorous tilapias such as Oreochromis niloticus, O.mossambicus, O.aureus and Tilapia zilli (Martinez-Palacios PhD Thesis, 1987).

The next step in the study of its culture potential, is to establish a small hatchery and to produce the fish in small scale intensive and semi-intensive systems such as cages, ponds, polyculture etc and to grow them to commercial size determining different aspects such as optimum stocking densities, testing different protein sources to reduce feed costs and in parallel with this, to make an study of its market potential.

To continue with the above, one of the most important factors for successfull experiments is to have an appropriate food for the early rearing stages. The protein requirement is the most expensive element in fish diets and has already been determined and some research has also been carried out on the digestibility of different carbohydrates (Martinez-Palacios PhD Thesis, 1987).

A lack of vitamins has also been seen to be a major problem in the diets of fish that are cultured intensively, and specifically water soluble vitamins due to the poor stability of many of them (Hilton et al.,

1977b; Tacon, 1985). To date there have been no studies on the requirements of any American cichlid, and vitamin requirements on African cichlids are rare, thus it is important to study these needs in C.urophthalmus. In this research a series of experiments was undertaken to determine the qualitative and quantitative requirements for the eleven water soluble vitamins for C.urophthalmus, based on growth response, gross signs of deficiency, food conversion ratio, histopathological changes and mortality.

C.urophthalmus accepted the basal diet offered very well and it supported good growth rates and good food conversion ratios compared with the vitamin deficient diets. However, histological observations indicated that the basal diet had some deficiency or an imbalance, due to the fact that the fish from all the experiments were observed to have fatty liver degeneration and slight oedema in the gill epithelium. Vitamin E deficiency was not the cause of these changes and oxidized fish oil was discarded as the cause of these signs due to the care taken in the avoidance of oxidized oils, the use of antioxidants in the diets and by ensuring adequate storage . As C.urophthalmus is mainly a carnivorous fish, it is possible that there is an imbalance in the dietary carbohydrates as there is a greater availability of more digestible carbohydrates

(dextrin) than the species requires. The excess may be stored in the liver in the form of fat (Shimeno, 1982). This problem will be the subject of future investigations.

In respect to the qualitative requirements, it has been demonstrated that under the conditions of these experiments, C.urophthalmus requires 9 of the 11 water soluble vitamins; Pyridoxine, pantothenic acid, riboflavine, vitamin C, biotin, thiamin and niacin in terms of growth and pathological response. Choline and inositol were on the border line of significance to be required for normal growth. It is likely that with a longer feeding trial a requirement for these vitamins would also be demonstrated.

The lack of folic acid made no significant difference when compared with the control diet during the time of this experiment (90 days). Cyprinus carpio has been shown to have an ability to synthesize folic acid, while the other species studied demonstrated a need for dietary folic acid to maintain normal growth and health, thus it is quite possible that C.urophthalmus also requires this vitamin. However, an experiment must be designed to determine its essentiality and its quantitative requirement.

Fish fed a diet with cyanocobalamin deficiency grew as well as the control diet and never showed deficiency signs. It has been demonstrated in recent studies that various species of warmwater fish such as Cyprinus carpio (omnivorous), Ictalurus punctatus (omnivorous) and Oreochromis niloticus (herbivorous), do not require this vitamin for normal growth, with a good food conversion ratio and lack of deficiency signs, as this vitamin is synthesised in the intestine of these 3 species (Kashiwada and Teshima, 1966; Kashiwada et al., 1970; Limsuwan and Lovell, 1981; Lovell and Limsuwan, 1982). It would be interesting, in a further long term experiment, to demonstrate if C.urophthalmus, being a mainly carnivorous fish, requires cyanocobalamin or if this species can also synthesise this vitamin in its intestine.

C.urophthalmus fed a diet with complete deletion of vitamin C, showed severe growth reduction and a poor food conversion ratio. These results are similar to those observed in other fish. However, fish fed diets 2 and 3 in which several deficiency signs were observed, grew as well as the fish fed diets with higher levels of vitamin C, indicating that C.urophthalmus requires 40 mg/Kg of ascorbic acid/Kg diet for optimum growth, and 110 mg/kg diet to prevent deficiency signs. Abnormal behaviour and morphological changes were noted after 30

days on the vitamin C deficient diets, and the external signs of deficiency were similar to those found in the literature for other tropical fish and included anorexia, reduced growth, haemorrhages, exophthalmos, lordosis, short operculae, loss of scales, erosion of skin and fins. Analysing the data obtained from different authors when cold and warmwater fish were fed with vitamin C deficient diets, it was observed that in temperate species, gross external signs appear later (Table 3.5) and show less external gross signs than tropical fish (Table 3.7). The difference in this aspect must be due to the high growth rate of the tropical species which requires more vitamin C/unit time than temperate fish.

Histologically, C.urophthalmus fed vitamin C deficient diets suffered severe pathological changes. Gill damage, such as severely distorted support cartilage and deformed chondrocytes, was similar to that observed in other fish such as salmondis, catfish and tilapias (Halver et al., 1969; Ashley et al., 1975; Lim and Lovell, 1978; Miyazaki et al, 1985 and Soliman, 1985). Gill hyperplasia was also observed and this pathological change was also noted in two tropical fish, Ictalurus punctatus (Lim and Lovell, 1978) and in O.mossambicus (Soliman, 1985), who came to the conclusion that vitamin C deficiency causes gill hyperplasia at least in some tropical fish species. In

the present study it was also observed that vitamin C deficiency caused epithelial changes in C.urophthalmus, these pathologies being marked inflammatory response, vacuolar degeneration and other degenerative changes.

Muscle atrophy related to vitamin C deficiency was observed in C.urophthalmus and after a review on this subject in other fish species studied it was concluded that vitamin C deficiency causes muscle pathology, but the effects of trauma, or the lateral effects of the vertebral fractures and the damage caused by parasites makes this pathology more extensive.

Focal necrosis of the hepatocytes and necrotic pancreas was observed in fish fed vitamin C deficient diets, similar changes being observed by Miyazaki et al., (1985) and Soliman, (1985) in channel catfish and tilapias.

For the first time various pathological changes were observed in the ganglion cells of a scorbutic fish, these abnormalities ranged from slight inflammatory response to central chromatolysis. Similar results were observed in scorbutic guinea pigs (Sulkin and Sulkin, 1967; Sulkin et al., 1968), however a real understanding of the role(s) of ascorbate in the central nervous

system remains obscure in spite of the growing body of information from recent publications related with vitamin C and the nervous system (Milby et al., 1981). A review on experimental scorbutic fish also revealed that vitamin C is highly related to the nervous system, due to high levels of ascorbate found in the brain of Salmo gairdneri, O.niloticus and O.mossambicus, and there is evidence that severe reduction of the total ascorbate in the brain produces anorexia and lethargy (Hilton et al., 1978a; Hilton et al., 1979b; Soliman, 1985). Thus, the observations of the present study indicate that the neuronal pathology observed in C.urophthalmus was due to vitamin C deficiency.

Fish tuberculosis due to Mycobacterium sp. infection was present in the fish used in this experiment and was probably also present in the fish from the other experiments (Chapters 2, 4 and 5) possibly as an opportunistic pathogen or perhaps as an infection through the ovarian pathway (Sniesko, 1978, Asburner, 1977). However, this disease was only evident in fish fed diets 1, 2 and 3 in the vitamin C experiment and in fish fed diet 10 of the second experiment described in chapter 2 in which vitamin C was deleted. With these results and after a review of the available literature related to ascorbic acid and several mechanisms of the host defense system with terrestrial

animals, and recent work carried out on immune response and vitamin C on fish, the conclusion was made that in the present study a clear relation between fish mycobacterium infection and vitamin C deficiency was observed. Diet 4 supplied with vitamin C at 110mg/Kg diet was found to contain the level to prevent fish Mycobacterium sp. infection.

In the muscle tissue of all fish used in the Vitamin C experiment the presence of Myxobolus sp. cysts was observed, and rodlet cells were found in the vessel endothelium, however, it was interesting to note that larger numbers of these parasites were observed in fish fed diets 1 and 2. In this respect, Durve and Lovell (1982) found that up to 5 times the minimum requirement level of vitamin C provides an increased resistance to the pathogenic bacterium Edwarsiella tarda in channel catfish. Thus, this indicates that adequate levels of vitamin C prevent the dispersion of these parasites in C.urophthalmus.

The most critical vitamin in terms of early appearance of deficiency signs and 100% mortality was pyridoxine, thus an experiment to determine its quantitative requirement was designed. From this, it was found that C.urophthalmus requires 5mg/Kg diet as the

minimum dietary requirement for normal growth, lack of deficiency signs and good food conversion ratio. This value is in the range of pyridoxine requirements for other tropical fish. The review of the available literature on the pyridoxine requirements for cold and warmwater fish demonstrated that coldwater fish have higher requirements (10-20mg/Kg diet) than warmwater fish (1-6mg/Kg diet). However, the requirements of many species are still unknown and more work must be done in this area to verify this assumption. C.urophthalmus showed the same common deficiency signs observed in other species such as loss of appetite, retarded growth, lethargy, rapid breathing, and nervous disorders expressed as violent, erratic and spiral swimming followed by high mortality. There were no histological signs of pyridoxine deficiency, probably due to the early cessation of growth and rapid mortality. It has been demostrated that anaemia is related to Vit B6 deficiency (Smith et al., 1974; Agrawal and Mahajan, 1983b), but also with an overdose (30-300mg/Kg diet) of the same vitamin (Andrews and Murai, 1979). In this study C.urophthalmus was fed with higher levels (426 and 642mg/Kg diet) but this species did not show any external signs of toxicity and grew as well as the fish fed the minimum requirement. To prevent possible losses of the vitamin during processing and storage, and to improve the possible role of this vitamin in disease

resistance as well as to fulfill its biochemical functions, it is recommended that for practical diets a safety factor of 50 to 100% (7.5 to 10mg/Kg diet) of the minimum requirement observed be incorporated.

Diets of C.urophthalmus fry must be supplemented with a minimum of 80 mg of calcium pantothenate/Kg diet to reach maximum growth and food conversion ratio and to avoid external and histological deficiency signs. However for practical purposes it is recommended that food should contain 120 to 160mg/Kg diet. C.urophthalmus fed pantothenic acid deficient diets was shown to have the common external deficiency signs observed in other species such as anorexia, reduced growth, haemorrhages of fins and skin and high mortalities. In respect to histological changes C.urophthalmus showed the characteristic clubbed gills for this deficiency. These changes were observed by many authors in different species of fish. Necrosis of the pancreas and glycogen deposition was also observed in the deficient C.urophthalmus, these pathologies are similar to those observed in Carassius auratus and Salvelinus namaycush (Ishii and Yamamoto, 1972; Poston and page, 1982). Hepatic ceroidosis was also observed in C.urophthalmus fed diets 1 and 2. This condition was not due to vitamin E deficiency or oxidized oil, and was restricted to diets 1 and 2 of this experiment. This is the first

report of hepatic ceroidosis due to pantothenic acid deficiency. Possibly this histological changes has not been reported before because most of the studies on pantothenic acid deficient fish deal with gill alterations and also because most of the studies have been carried out on coldwater fish, thus it is possible that as was the case of the vitamin C deficient fish in this study, more pathologies appear in tropical fast growing fish than in those of coldwater, or that the time to get the same deficiency signs is shorter.

Coche, (1978) in a report based on the papers of the Symposium on Finfish Nutrition and Feed Technology held in Hamburg in 1978, emphasises that "-recent research indicating the remarkable similarity, in quantitative requirements of most species of fish examined, for water soluble vitamin needs for normal growth but that carp appear to be an exception"-. Probably this was true at that time, because there was no available research data on different species of warmwater fish.

After a review of the research work carried out on both cold and warm water fish and their vitamin requirements together with a subsequent comparison with the data collected for C.urophthalmus it is evident that an analysis of the requirement for several species under

different environmental conditions should be made as many differences exist. For example it was noted that coldwater fish fed vitamin C deficient diets shows the gross external signs in a longer period of time than the warmwater fish (Table 3.5, Figure 3.2), and apparently this is dependent on the environmental temperature of culture. In respect to the number of gross external signs of deficiency observed, warmwater fish show a larger number than those of coldwater (Table 3.7).

In respect to histopathological changes, in this study C.urophthalmus a fast growing tropical fish showed other different pathologies due to vitamin C and calcium pantothenate deficiency than those reported before for other species with the same deficiency. It is possible that these could be due to the different metabolic rates that exist between cold and warmwater fish. The vitamins stored in the tissues of the fast growing fish cultured at higher temperatures are more rapidly absorbed, utilized and exhausted, and as a result deficiency signs become evident and signs appear earlier and are more critical than in coldwater fish.

Another important difference is that some tropical fish have the ability to synthesise some of the water soluble vitamins such as vitamin C, cyanocobalamin, Inositol and folic acid, this is an aspect that has not

been demonstrated in any cold water fish. This occurrence is important to determine, because practical diets can be prepared without this vitamin or with a low level as a safety factor, reducing the costs of the practical food.

During the review it was also evident that on many occasions it was difficult to compare the data between the results obtained even intraspecifically because the different authors used different temperatures of culture and different criteria to determine the requirement, and between species it was difficult to make a comparison because different sizes of fish and different criteria were used. To define the methodology for the determination of the quantitative requirements is not an easy matter, and only more research with different species will give in the future enough information to facilitate comparisons between groups of fish.

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