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Two examples of nutritional pathology related to vitamin E and C deficiencies

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Abstract — *Symptoms recalling a nutritional pathology and more precisely a process of lipid peroxydation were observed during the last years in seabass* ***Dicentrarchus labrax*** *and other fishes cultured in tropical marine condition,* ***Ocyurus chrysurus*** *and* ***Lutjanus analis****. They included dark coloration, skin ulceration, lethargy, anorexy, emaciation. Histological examinations showed hepatic fatty degenerative lesions, pancreatitis, muscular degeneration and retinal atrophy. Additional vitamins E and C in the food suppressed tha pathological symptoms. Several experiments were carried out using sea bass which received a lightly oxidized food. The previously evoked clinical or histological signs were not achieved in spite of an increase of the level of hepatic, muscular or blood malondialdehyde and of the conjugated dienes of the perivisceral fat. In the same time, hepatic and muscular tocopherol decreased. These experiments also showed the feasibility and the interest of different analyses in the characterization of such a pathology : hemolysis test and activity of enzymes such as erythrocytic catalase or superoxyde dismutase and plasma glutathione peroxydase.*

Another nutritional disease is worth describing again, the Granulomatous Hypertyrosinaemia. It was essentially investigated in turbot ***Scophtalmus maximus*** *but other fish species such as sea bream* ***Chrysophris aurata*** *are often affected. The disease is both characterized by an increase of the plasma tyrosinaemia and the coming out of visceral and muscular granulomatous nodules. Microscopic needle shaped crystals of tyrosine may be found in lesions and in subcorneal deposits. The experimental ascorbic acid deficiency induces the pathology and on the contrary a sufficient providing of vitamin C rapidly leads to normal tyrosinaemia and reduced eye lesions. As in mammals, ascorbic acid seems to plan an anti-oxydant role in protecting the p-hydroxyphenyl-pyruvate dioxygenase, the enzyme acting in the main way of the tyrosine metabolism. The tyrosine crystals could directly induce an inflammatory specific response with development of granulomatous nodules.*

In practise, the knowledge of the risks and the distribution of a good quality food must prevent such diseases.

INTRODUCTION

It may be surprising to imagine that the present good knowledge of fish nutrition may let subsist any possible nutritional pathology. However, vitamin E and C deficiencies have been recently mentioned in 2 diseases described on marine fish raised in the West Indies : Summer Mortality of seabass *Dicentrarchus labrax* (Gallet de Saint Aurin, 1987) and Blindness-Melanism Syndrome (Raymond, 1968).

This review presents several experiments made in LPAA to check these aetiological hypothesis. Attempts to reproduce clinical signs described on these diseases are made by feeding fish vitamin C and E deficient diet, further containing oxidized lipids.

Granulomatous Hypertyrosinaemia is then briefly described as directly related to vitamin C deficiency.

A number of the collected data are here presented in order to discuss possible nutritional aetiologies and mechanisms of the diseases described in the West Indies.

I. PATHOLOGY OF CULTURED FISHES IN THE WEST INDIES

1.1. Observations

« Summer mortality of seabass »

The disease occurs on net-pen reared fish less than one year old when water temperature is over 29-30°C, from July to September. An unusual behaviour is mentioned : whirling swimming or, at rest, recognizable position : oblique, head down, sometimes belly up. The fact that fish are repetitively hitting the net can be related to blindness. Anorexia is noticeable.

Histopathological examinations were carried out on different organs :

Liver : lipid vacuolisation is particularly significant during summer season, simultaneously with degeneration phenomena (nucleolus swelling, chromatin margination). There is some ceroid deposits.

Kidney : vacuolisation of tubular epithelium, increasing throughout the hot season.

Stomach : degeneration of gastric glands.

Guts : desquamation of intestinal mucosa with atrophy of villosities and epithelial necrosis.

Pancreas : atrophy, cytoplasmic densification, pycnosis.

Spleen : congestion and hemosiderin deposits in macrophages and melanomacrophage centres.

Muscle : cloudy swelling, vacuolization, necrosis.
Eyes : retinal atrophy (2 cases).

The lower haematocrit, haemoglobin and erythrocyte number characterize an anaemic condition, while the decreasing levels of plasma total protein and cholesterol indicate a metabolic disorder. Cytolysis (high Na^+ and K^+ with hydrolysis of intracellular adenosine triphosphate increases with clinical signs. Abnormaly high amounts of malondialdehyde (MDA) are detected in liver of fish presenting clinical symptoms.

« Blindness-melanism Syndrome »

The disease was firstly described as a « Loss of Scales Syndrome » by Raymond (1987) on indigenous fishes of the West Indies, especially *Ocyurus chrysurus* and *Lutjanus analis.* Fish show a loss of appetite, melanism, and an important decrease of weight. Some of them can hardly catch the pellets which also suggest blindness. Ulcerative skin lesion are often seen on the head, latero-dorsal body part, and fins. Ocular lesions such as keratitis and aphaky are sometimes detected.

In the liver, the excessive accumulation of fat in cytoplasm is often accompanied by nuclear atrophy and sometimes pycnosis. More seldom, intrahepatocytic ceroid deposits can be observed. The kidney presents a hyalin droplet degeneration of tubular epithelial cells, particularly on *Ocyurus chrysurus.* In the guts, desquamation of enterocytes begins on the top of villosities and possibly extends to almost the whole intestinal epithelium with, in that case, a flattening of the mucosa. Sub-epithelial structures remain untouched. A muscle degeneration appears on white as well as on red muscle and under different forms : hyalinisation, vacuolization, centronucleation. The retinal atrophy seems to be specific to the disease, which justifies its name : Blindness-Melanism Syndrome. The lesion is gradually developing, first on retinal center and then towards *Ora serrata* : the most internal tissue layers are first touched (ganglionic cells, inner plexiform and nuclear layers) and then external to be disorganized and fragmented. No more nucleated structure can be seen at the last stage.

A high level of tyrosinaemia is observed on the most affected fish. Further, low levels of plasma glucose, proteins, and cholesterol illustrate a nutritional disorder.

No evidence of any septicaemia is made on sea bass, neither on indigenous fishes. Parasitism is somewhat inconstant. Clinical, histopathological and biochemical data tend to prove nutritional (particularly vitamin C and E deficiencies) and environmental (light and temperature) aetiologies. This hypothesis is enhanced by the fact that distribution of food enriched with vitamins did reduce the problems. Accordingly, it seemed quite appropriate to verify this hypothesis with experiments reproducing field conditions : partially oxidized food, containing pretty low levels of vitamin E and C, hot temperature and high light.

1.2. Attempts to experimental reproduction of the disease (Table 1).

Experiment A :

Five hundred 80 g seabass raised in gradually increased temperature (until 31°C) are vaccinated against *Vibrio anguillarum* and kept in a heated

open circulating water system. Three batches are made, each of them divided into 4 identical tanks (4 repetitions). The basal diet contains 12 % of voluntarily oxidized oil *in vitro*. Its lipid fraction is then characterized with the following indices median values : peroxyde value (mEq./Kg of lipids) : 15.5; n moles MDA/g : 55. This diet is given to the B group. C and CE groups are fed the B diet supplemented with vitamin C (minimum measured 1 700 mg/Kg treated instead of 96 in B) and CE group with vitamin E (m.m. 550 mg/Kg instead of 36 in B and C).

Tab. 1. — *Experiments A, B, C. Experimental conditions and results*

EXPERIMENTS	A (batch B)	B (Batch Ox)	C (Batches Ox)
FOOD CHARACTERISTICS *			
MDA (nmoles TMP**/g)	55		500
POV (mEq/Kg Lipid)	15.5		350
VITAMIN E (mg/Kg)			
- supplementation	40	40	0
- measured in food	36-56		5
VITAMIN C (mg/Kg)			
- supplementation	710	710	50
- measured in food	96-404		
BHT (mg/Kg)			
- supplementation	100	100	0
CHOLINE (g/Kg)			
- supplementation	3.1	3.1	0
FISH : Seabass			
Initial mean weight (g)	102	3	86
Final mean weight (g)	170	30-50	162
WATER TEMPERATURE (°C)	28-31	18	20
EXPERIMENT DURATION (weeks)	13	45	11
RESULTS			
Hepatic E vitamin (µg/g)	56.2 ± 15.6	46.5 ± 21.5	3.4 ± 0.9
Muscular E vitamin (µg/g)	3.63 ± 0.93	2.5 ± 21.5	0.78 ± 0.13
Muscular TMP (n.mole/g)		3.71 ± 1.44	8.12 ± 5.23

* Characteristics of basal diets, with oxidized oil added and vitamin E and C deficiency
** TMP : tetramethoxypropane.

After 13 weeks, fishes supplemented with vitamin C (batches C and CE) have got double liver concentrations of vitamin C (70 mg/Kg). In batch CE, liver and muscular values of vitamin E are 3 times higher (respectively in liver and muscle : 156 and 13 mg/Kg) than in B and C.

However, general health status of fish is not affected : biometric and plasma parameters (glycaemia, total protein, cholesterol) are identical at the end of the experiment. No gross or histological lesions are observed. The levels of agglutinating antibody (anti-*Vibrio anguillarum*) remain the same in the 3 batches. There is no difference in values of hepatic lipids

nor in fatty acid composition, and MDA levels in liver. But, levels of MDA are superior in B fishes muscle and plasma B fishes ($P < 0.05$).

Experiment B (G. STEPHAN, 1988)

500 seabass (mean body weight : 3 g) are randomly divided into two 200 litres tanks. The temperature (18°C) is maintained constant throughout the experiment in an opened sea-water system. One group (T) is fed the basal diet with fresh oil added. The other one (Ox group) is fed the basal diet but with slightly oxidized oil. As in experiment A, no alteration of w3 total fatty acids was recorded. The batch T is maintained under alleviated light strength whenever the batch Ox is under constant artificial light.

Throughout this experiment, no mortality is noticeable. After 10 months, fish weight ranged from 30 to 50 g without any difference between the 2 batches. Fishes in batch Ox are darker than in batch T. No gross lesions are detected. Liver MDA values are increased at the end of the experiment, but similarly in the 2 batches (230 - 330 n moles TMP/mg fresh tissue). Conjugated dienes of perivisceral fat are significantly more abundant on batch Ox. On the other hand, gluthation peroxydase activity in plasma is decreased but there is no difference on erythrocytic enzymes (catalase, SOD). Liver concentrations of vitamin E remain similar in the 2 batches, but muscular values are significantly lowered on fish Ox.

Experiment C

This experiment is presently conducted in LPAA. Two batches (Ox) of seabass (90 and 190 g) are fed an oxidized diet, synthetized anti-oxydant -vitamin E - choline- deficient, poorly supplemented with vitamin C. Two control batches are fed corresponding regular food. Water temperature is around 20°C. After 11 weeks, a first sample does not show any difference in growth rate, biometrical data, haematological values hemolysis test, plasma transaminases and creatine-kinase, plasma glucose and total protein.

On the contrary, hepatic and muscular concentrations of vitamin E are dramatically lowered on fish not supplemented with vitamin E while muscular MDA level is increased.

1.3. Discussion

Most of the pathological signs described on fishes raised in the West Indies could not be reproduced in the laboratory. It is likely that experimental conditions were not drastic enough to test protection mechanisms.

In experiment A, fish size is rather important when fishes have to face high temperatures. They have probably got sufficient vitamin C and E stores. Body weight and body stores have been invoked by Cowey et *al.* (1981) and Lowell et *al.* (1984) as two important factors in the success of experimental reproduction of vitamin deficiencies. For Mocia et *al.* (1984), metabolic requirements of vitamin E and C on fish are highly dependent on their relative growth and the amount of body storage of vitamins. For them, differences on this particular point and also on

experiment duration (24 weeks instead of 16) can explain why they observed symptoms of vitamin E deficiency on rainbow trout while Cowey et *al.* (1981, 1983) could not. Nevertheless, at the end of our experiment A, hepatic and muscular levels of vitamin E on non supplement fish are down to 56 µg/g and 3.6 µg/g, respectively (from initial values 85 µg and 9.5 µg/g). These values are close to those detected on Atlantic salmon (45 and 1.4 µg) by Poston et *al.* (1976) and on carp (27 and 2.8 µg) by Watanabe et *al.* (1970), associated with clinical signs. Similarly, vitamin C levels in liver go down to 33 µg on non supplemented fishes, which approximate the 30 µg/g threshold value of deficiency defined by Saroglia and Scarano (1984) on sea bass.

Furthermore, concentrations of MDA (reflecting accumulation of degradation products of oxidized lipids) increase in plasma, and particularly in muscle of fishes not supplemented with vitamin E. If we had carried out the experiment on a longer period of time, it is possible that more important metabolic disorders would have occured, even inducing some clinical symptoms on the non supplemented fishes.

In experiment B, no clinical but biochemical disorders are detected on fishes fed an oxidized diet. On this batch, the level of hepatic vitamin E does not change a lot perhaps because of a still remaining sufficient amount of vitamin E and/or of other anti-oxydant products in food but the level of muscular vitamin E is significantly lower. An explanation could be a lesser transportation of vitamin E towards surrounding tissues. In fish indeed as in mammals, alphatocopherol is transported by low-density lipoproteins (Hung et *al.*, 1982). In other respects, *in vitro* assays showed the interaction of oxidized products upon the structure and the synthesis of lipoproteins. In the Blindness-Melanism Syndrome observed in the West Indies fishes, the retinal histopathology and the preventive effect of a vitamin E supplementation show some likeness to the human abetalipoproteinaemia (Raymond, 1988). Ox fishes are also characterized by a decrease of the glutathione peroxydase (GPX) activity. It could be related to a lesser bio-avaibility of selenium if in sea-bas as in catfish (Gatlin et *al.*, 1984) GPX exists in plasma only as a selenium dependent form. This decreased activity of the enzyme can lead to a lesser protection to food hydro-peroxides for the intestinal epithelial cells and explain the higher values of conjugated dienes in perivisceral fat.

In experiment C, the higher oxidation of lipids, the vitamin E deficiency and the lack of synthetic anti-oxidant in the food more rapidly induce the fall of both hepatic and muscular vitamin E. The increase in MDA level seems to be a first indication of the accumulation of decayed products of oxidized lipids.

II. GRANULOMATOUS HYPERTYROSINAEMIA (GH)

2.1. Observations

Tixerant et *al.* (1984) were the first to link a so called Granulomatous Syndrome observed on farmed turbots (*Scophthalmus maximus*) to a disorder in tysosine metabolism. Clinical signs of the disease are essentialy : 1) White yellowish or orange nodules, mostly on kidney but also on

the other viscera and muscle. 2) Subcutaneous white deposits, around the articulations or under the cornea, possibly hiding the pupil. It is also possible to remark cutaneous melanism, loss of weight, hepato-splenomegaly, adbominal dropsy and presence of urinary calculus. At microscopic examination, the white deposits show bushes of needle-shaped crystal, 30 - 40 µm long, located around the melanomacrophage centres or around the nodules. These crystals cannot be seen on histological sections (after paraffin embedding). However, two basic types of granulomatous nodules (GN) can be identified : 1) Solid homogenous GN 50 to 200 µm in diameter, constituted of elongated epithelioid cells concentrically arranged within a thin basal lamina. 2) Cystic or necrotic GN, larger than the previous type with a peripheral lamellar cell arrangement and a necrotic centre empty or containing cellular debris. An infra-red spectrometry allows to first identify L-tyrosine as the primary component of vesical calculus. Plasma and kidney analysis by ion exchange chromatography gives evidence of abnormally elevated levels of tyrosine. By its clinical aspects, the disease seems to be very similar to the nutrionally induced Granulomatous Condition in farmed turbot (Richards et *al.*, 1984) although the writers do not report any crystal in tissues. The Systemic Granuloma of sea-bream *Chrysophris aurata* (Paperna et *al.*, 1980) also appears to be related, since the presence of tyrosine is detectable in kidney and in urinary calculi. In other respects, hypertyrosinsemia was detected in *Charax puntazzo* (Messager, unpublished data) and, as above related, in the West Indies farmed *Ocyrurus chrysurus* and *Lutjanus analis*. More recently, examination made on sea bream from Turkey suggested this kind of pathological disorder (LPAA, unpublished data), and a presumptive evidence of the disease appeared in halibut *Hypoglossus hypoglossus* (Egidius, pers. com.).

2.2. Experimental approach

This work was made in order to identify, pathological processes and to bring solutions for its control. Considering routine catabolism of tyrosine in mammals, and hypertyrosinaemia process in humans, Messager, (1986) makes the hypothesis that the catabolism disorder of tyrosine on turbot could be related to vitamin C deficiency. This reductor agent is necessarily involved in the second pathway of tyrosine catabolism, to protect the parahydroxy-phenyl-dioxygenase, enzyme which catalyses the oxydation of p.HPP acid in homogentisic acid. To check this hypothesis, twenty four 130 g turbots exhibiting clinical signs of GH (subcorneal deposits, melanism, loss of weight) receive intraperitoneal injection : 0.2 ml of a solution of ascorbic acid for 14 of them (10 mg ascorbic acid per fish), or the same amount of saline for control fish. Tyrosinaemia is estimated by the fluorimetric method on blood samples before any injection and 7, 15, 30 days later. Seven days after ascorbic acid injection, 11 out of the 14 treated fish have got a normal tyrosinaemia (less than 60 µñ moles/l) which they keep until the 30th day. The injection of saline in control fishes does not affect tyrosinaemia which remains around 2000 - 3000 µ moles/l. A remarkable regression of corneal lesions is also observed on treated fishes. After 30 days, hepatic levels in vitamin C are the following : 2.3 µg/g on treated fishes.

A second experiment is conducted by Messager et *al.* (1986). Its purpose is to experimentally reproduce natural disease in feeding fish on ascorbic acid deficient diet. Five hundred 200 days old turbots are divided into two size groups S and T (average body weight, 12 and 18 g). Fishes of each group are distributed into five 150 l tanks in an open sea-water system. Water temperature is 18°C. Fishes are fed on a food supplemented or not in vitamin C. At day 155, noticeable hypertyrosinaemia is detected on fishes fed on non supplemented food only : 67 % on group S and 27 % on group T. Some fishes also show subcorneal or renal deposits of tyrosine crystals and/or microscopic granuloma in kidneys : 67 % on group S and 6 % on group T. Compared to control fishes (1500 mg ascorbic acid/Kg dry food) the non-supplemented groups exhibit significantly lower growth. In other respects supplemented diets with 750 mg/Kg of ascorbic acid or ascorbyl palmitate give results similar to control. On the other hand, diet supplemented with ground fish and 600 mg/Kg ascorbic acid gives a poor growth and some cases of hypertyrosinaemia.

2.3. Discussion

Results confirm that a vitamin C deficiency in turbot can be the cause of hypertyrosinaemia and of tissual tyrosine deposits. According to the lysosomial theory of Goldsmith (1978), these crystals are then able to induce local inflammatory granulomatous response. Nodules, such as observed in GH were also produced in turbot intraperitoneally injected with talc or BCG suspensions (Balouet et *al.*, 1986). A nutritional aetiology (but not the role of ascorbic acid) was already suspected by Paperna et *al.* (1979) for the Systemic Granuloma of sea bream. As a matter of fact disease was experimentally carried out on sea bream (Paperna et *al.*, 1984) and on turbot (Richards et *al.*, 1984) fed on an altered food. It was suggested that prolonged storage could be accused.

It is well known that ascorbic acid is one of the most unstable dietary components. Hilton et *al.* (1977) showed the importance of its destruction during the preparation and the storage of a fish food. According to these authors, incorporated water is the main cause of the ascorbic acid degradation. Messager et *al.* (1986) show that almost the total amount of incorporated ascorbic acid quickly disappears in humid pellets incorporating or not ground fish.

However, several factors may be implicated in the process of the disease. In the above related experiment, the characteristic macroscopic nodules of the natural disease are not observed. On the other hand, smaller fish appear to be more sensitive. Fish age seems to be determining : Systemic Granuloma could not be reproduced in sea bream older than one year (Paperna et *al.*, 1984). Water temperature and particularly food consumption appear to be also important (Messager, 1986). Indeed, enzymatic inhibition level and further, the quantity of non catabolized tyrosine and tyrosine deposits, are dependent on the amount of ingested tyrosine or phenyl-alanine. As a matter of fact, throughout the course of the disease, while the nutritional status is impairing, and in spite of the setting up of the lesions, hypertyrosinaemia can regress and disappear. Sometimes weakest and thinnest turbots show remaining kidney lesions attesting a previous GH, but exhibit a subnormal blood tyrosine level.

CONCLUSION

The two diseases observed in the West Indies show a few similarities (hepatocyte and muscular degeneration, retinal atrophy), which can partly be attributed to vitamin E and C deficiencies. In the two cases, the pathology was limited on the field by supplementing the food with vitamin E and C. It does not necessarily mean that nutritional factors can utterly explain the described symptoms and lesions. The neurotrop virus reported by Gallet de Saint Aurin et *al.* (1987) may already take part in the « Summer Mortality of seabass ». There is no doubt that environmental aetiology plays a role in retinal atrophy (Raymond, 1988).

The carried out experiments did not reproduce the clinical signs of these diseases, but probably because the experimental methods did not gather the bad field conditions together. However, biochemical results show the begining of a pathological process susceptible to go forward to the symptoms described in the field pathology.

The role of ascorbic acid in the tyrosine catabolism explains why the vitamin C deficiency is directly related to the determinism of the GH. The observed hypertyrosinaemia in the West Indies cultured fish is certainly the mark of such a deficiency. Stress increases utilization of ascorbic acid (Wedemeyer, 1969) and the severe summer conditions and/or pathological agents can act in such a way.

A number of authors have discussed mechanisms of vitamin E and C interactions and their importance as chain breaking antioxidants in the in vivo autoxidation of polyunsaturated lipids of cellular membranes (Tappel, 1968; Lambelet et *al.*, 1985).

Alpha-tocopherol is pretty resistant in a food suitably protected from oxidative process. In the opposite, the unstability of vitamin C in the presence of various environmental factors (light, temperature, humidity, pH) explains still frequent deficiencies. They are attested by GH in some fish species but they are also undoubtedly related to many other pathological phenomena.

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