# ADVANCES IN FISH AND CRUSTACEAN NUTRITION AND AQUAFEED BIOTECHNOLOGY

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# COURSE MANUAL

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# ROLE OF VITAMIN C ON THE DISEASE RESISTANCE MECHANISM OF FISH

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## 1.0 Introduction

Diseases exert heavy economic losses in fish culture due to mortality, morbidity, poor product quality and costs associated with chemotherapy. Aquaculturists are, therefore, interested in developing cost-effective management strategies that can either prevent the outbreak or reduce the severity of epizootics. One management strategy currently under exploration is nutritional modification. The influence that dietary factors may have on disease outbreaks in cultured fishes has been recognized since many years. Earlier, investigators concentrated on the effects of various diets on the incidence and severity of common infectious diseases. Later, they attempted to determine the mechanisms for some of the observed nutritional effects.

1.1 Profound changes in the immune response are some of the earliest manifestation of malnutrition. Studies on higher vertebrates have shown that these changes arise through effects upon the thymolymphatic system, which can be seen automatically in the form of thymic involution, splenic atrophy and thinnning of intestinal lymphoid tissue. Some of these changes occur directly as a consequence of general weight loss and aminoacid deficiency, others arise indirectly in response to stress-mediated alterations in steroid catabolism (Mc Farlane and Path, 1977). The visible changes in thymolymphatic morphology are reflected immunologically by decreased phagocytic index, impaired hypersensitivity responses, subnormal lymphocyte transformations, altered immunoglobulin synthesis and other phenomena, which render the animal susceptible to diseases.

1. 2 In homeotherms specific dietary deficiency is known to modulate the immune system. Such specific nutritional factors include vitamins, proteins, lipids and minerals. Most of the research which has been conducted on nutrition and immunity has focused on mammals and birds; however, over the past few decades many investigators have conducted similar studies employing fish. Our knowledge of the fishes' immune system and nonspecific disease resistance factors has increased, as also the methodology for examining mechanisms of diet-induced effects on infectious disease. In early fish nutrition studies, requirements were determined based strictly on growth, feed conversion and lack of deficiency syndrome. Of late attention has been focused on the complex interactions of nutrients, physiological effects, disease susceptibility and overall health.

1. 3 Inadequate nutrition is one of the several factors, which results in stress and render the fish more susceptible to disease. In contrast to extensive type of farming systems where fish obtain all or part of their dietary nutrient needs from naturally available food organisms, fish in intensive conditions rely more on the provision of a nutritionally complete diet. Here, diet can have significant effects on disease resistance mechanisms and hence on infectious disease incidence and severity. A number of nutrients, mainly vitamins, minerals and fatty acids have been implicated in this regard.

Of the various nutrients in the diet, vitamins are a group of complex substances, which are essential for a wide variety of metabolic processes. Fish require vitamins in their diets for growth, health and general body function. Vitamin deficiency has been found to be associated with several pathological signs in fish. Among the various vitamins, vitamin C has attracted the attention of fish health researchers, probably owing to its role in disease resistance and immune response. It is also a vitamin that has long been implicated in disease resistance in homeotherms. The antiscorbutic vitamin has a distinctive and important role in fish nutrition and as such has been extensively studied.

**1. 4** Ascorbic acid deficiencies have been commonly found in intensively cultured fish due to its instability during processing and storage of feeds and its rapid depletion *in vivo* during stress. Besides the essentiality for growth, vitamin C has been demonstrated to play an important role in functioning of the immune system when supplied at levels higher than standard doses in several fish groups.

## 2. 0 Ascorbic acid

**2. 1** Ascorbic acid is optically active and the L- form (L-ascorbic acid) is the physiologically active form. L- ascorbic acid is a strong reducing agent. This is unstable in solution and is easily oxidised to form dehydroascorbic acid, which is less active than reduced form. Because ascorbic acid is extremely sensitive to degradation during processing and strorage in fish diets, its derivatives with sulphate or phosphate at the C-2 position in the lactone ring have been used to increase resistance to oxidation.

**2. 2** In animals, ascorbic acid is synthesised from glucose *via,* the glucuronic acid pathway of metabolism (Chatterjee *et al.*, 1975). In this biosynthetic pathway L- gulonolactone oxidase is the terminal enzyme. It converts L-gulono-1, 4-lactone to 2-keto-L-gulonolactone, which is spontaneously converted to ascorbic acid.

**2. 3** The involvement of vitamin C in disease resistance and immune response has been established particularly in species lacking the enzyme gulconolactone oxidase, which permits vitamin C to be synthesized from glucose. Ability to synthesise ascorbic acid is absent in insects, other invertebrates and many species of marine and fresh water fish, guinea pigs, bats, primates, human beings and some highly evolved birds. Lack of L-gulonolactone oxidase is a common genetic defect. Recently the gene for L-gulonolactone oxidase has been cloned and sequenced (Nishikimi et al., 1994). In the case of fish, the ability to synthesise ascorbic acid is lacking in most species (Dabrowski, 1990). Requirement of vitamin C and the occurrence of its deficiency syndromes *viz.*, structural deformities, retarded growth, hemorrhages and delayed wound healing have been reported extensively in two groups of fishes, the salmonids and ictalurids. Although it is well known that young rainbow trout are susceptible to scurvy, Primbs and Sinnhuber(1971) demonstrated that ascorbic acid is not essential in the diet of larger trout. It is, therefore, expected that the requirement for ascorbic acid may change as the fish becomes larger.

**2.4** Vitamin C requirement of common carp has also been investigated. Kitamura (1969) recorded deficiency syndrome similar to those reported in trout and salmon. But, Ikeda and Sato (1964) using radioactive tracer techniques reported the ability of this carp to biosynthesise vitamin C by the same mechanism as has been found in rat, although the rate of synthesis in the carp may have been too low to meet the requirement for rapid growth. Yamamoto *et al.* (1978) studied the distribution of L-gulonolactone oxidase in 12 species of

fish and detected enzyme activity in the hepatic tissues of *Cyprinus carpio, Carassius carassius cuvieri, Tribolodon hakonensis* and *Parsilurus asotus,* all of which belong to cypriniformes. But the enzyme activity was not detected in any of the other fishes tested such as rainbow trout, amago (*Oncorhynchus masou macrostomus),* ayu (*Plecoglossus altivelis)* eel (*Anguilla japonica),* red sea bream (*Pargus major),* yellow tail (*Seriola quinqueradiata)* and *Tilapia nilotica.* These findings suggest that some fishes are able to synthesise ascorbic acid and be independent of a dietary source of the vitamin under normal conditions.

2. 5 Chatterjee (1973) has reported L-gulonolactone oxidase activity is not present in the kidney or liver of Indian major carps such as *Labeo rohita, Catla catla, Cirrhinus mrigala and also in Labeo calbasu.* Agrawal and Mahajan (1980) and Mahajan and Agrawal (1980) reported dietary need for vitamin C and deficiency syndromes in *Cirrhinus mrigala.*

2. 6 Soliman *et al.,*(1985) assessed L-gulonolactone oxidase activity in kidney and liver tissue of 14 teleosts. Nine tilapias, two cyprinids and three salmonids were investigated. Enzyme activity was detected only in liver and kidney tissues of common carp and kidney tissue of *Oreochromis spilurus* and *Oreochromis aureus.* There was no detectable level of L-gulonolactone oxidase activity in grass carp, a cyprinid species. Of the four species of Tilapia tested only 2 species showed enzyme activity. This shows that the essentiality of dietary ascorbic acid should be determined separately, species by species and that even species of the same genus may differ.

## 3. 0 Biochemical functions of Vitamin C :

### 3. 1 Collagen synthesis

3.1.1 Ascorbic acid functions as a cofactor in collagen synthesis. The stability of collagen depends upon its triple helix structure, which in turn depends on the presence of the unique aminoacid, hydroxy proline. Ascoroic acid stimulates collagen synthesis by stimulating hydroxylation of proline which is catalysed by prolyl hydroxilase. The function of ascorbic acid in the stimulation of collagen synthesis is to keep the non-heme iron of prolyl 4-hydroxylase in the active state. During the catalytic reaction, a highly reactive iron-oxygen complex, a ferryl ion is produced, which subsequently hydroxylates an appropriate proline residue.

3.1.2 The hydroxyproline to proline ratio is a useful indicator of vitamin C deficiency in fish at an early stage of development. The mechanical strength of connective tissue is dependent upon the activity of lysyl oxidase which is also regulated by ascorbate. Because of its role in collagen synthesis, ascorbic acid is essential for the maintenance of an effective epithelial barrier and for normal wound healing in fish.

### 3.2 Protection of microsomal membranes against lipid peroxidation and oxidative damage of proteins/protection of collagen against oxidative damage.

3.2.1 One of the most famous functions of vitamin C is its ability to quench oxygen radicals arising from cellular respiration. Lipid peroxidation and protein degradation are mediated by cytochrome P 450 and specifically prevented by ascorbic acid. Ascorbate also prevents $O_2^-$ initiated free-iron independent protein oxidation.

3.2.2 Ascorbic acid is also known to protect the collagen of the extracellular matrix from oxidative degradation. The extracellular matrix contains numerous macrophages that undergo oxidative burst during phagocytosis and release reactive metabolites such as superoxide anion, hydrogen peroxide, redox proteins and metalloproteinases. These are antimicrobial substances, however they may compromise host responses by causing oxidative damage of collagen. The oxidised collagen subsequently undergoes proteolysis by metalloproteases. As in the case of microsomal proteins, ascorbic acid apparently prevents collagen oxidation. Once collagen oxidaion is prevented, the subsequent proteolytic degradation of collagen is also prevented. This imparts a specific important role to ascorbic acid for the protection of collagen in the extracellular matrix of mammalian tissues. Therefore, a potential and apparently specific role of ascorbic acid is to protect tissues against oxidative damage both at the intracellular and extracellular levels. Scurvy symptoms in acute ascorbic acid deficiency is apparently a premortal syndrome of severe oxidative damage, leading to oxidation of tissues.

3.2.3 Environmental pollutants result in increased production of reactive oxygen, leading to increased oxidative degradation of proteins. Mixed function oxidases are involved in the metabolism of xenobiotics, toxicants, steroids and drugs. Induction of mixed function oxidase activity is directly related to ascorbate concentrations in the media (Zannoni *et al.*, 1982). An increased tolerance of fish to environmental pollutants has been observed when tissue stores of ascorbate are high (Halver, 1985). Effect of pesticides and heavy metal poisoning is diminished when ascorbate intake is high.

3.2.4 Vitamin C also functions as a general water-soluble redox reagent, a regulator of steroid synthesis, a modulator of the hexose monophosphate shunt and an activator of hepatic microsomal hydroxylases. Ascorbic acid plays a role in the transformation and utilisation of folic acid and in the absorption of iron. Dietary ascorbic acid facilitates absorption of iron in the gut, and spleen levels are affected by ascorbate tissue levels. Owing to the involvement of ascorbic acid in the synthesis of steroid hormones, the vitamin also has an effect on reproduction. The influence of vitamin C on the strucure and functioning of thyroid gland and iodine metabolism has been demonstrated in birds, mammals and fish.

## 4.0 Role of vitamin C on disease resistance and immune response

4.1 The acquisition of reliable techniques for the *in vitro* and *in vivo* assessment of cellular and humoral immune functions in mammals has been associated with a re-emergence of interest in ascorbate as a possible mediator of increased immunity. This has led to identification of the cellular immune functions which can be increased *in vitro* and *in vivo* by ascorbate as well as the concentrations of the vitamin which cause stimulation of these functions.

4.2 Recent work has shown that ascorbate mediated immunostimulation is related to the antioxidant activity of the vitamin. Ascorbic acid can effect disease resistance in fish in numerous ways. It acts as a biological agent for hydrogen transport and hence involved in a variety of enzyme reactions. It acts synergistically with vitamin E and selenium to maintain activity of glutathione peroxidase and superoxide dismutase. Thus it helps in preventing oxidative damage to neutrophils and other phagocytes during respiratory burst activity, thereby helps to enhance motility and phagocytosis by these cells. It is also a necessary factor in the regulation of the synthesis of corticosteroids. During stress conditions production of cortisol is regulated to check its immunosuppressive action. In homeotherms vitamin C has also shown to enhance complement activity, iron metabolism, antibody

responses and a variety of other immune functions. It is also believed to be important in phagocytic cells, although reports are often conflicting. Phagocytic cells generate reactive metabolites such as superoxide anion, hydrogen peroxide and hypochlorous acid in response to membrane stimulation. These are antimicrobial substances, however, they may compromise host responses by causing oxidative damage.

4.3 Specific effects on a variety of non-specific resistance mechanisms and the specific immune response have also been reported in fish. Unfortunately, there are conspicuous conflicts that may possibly be explained by differences in total diet composition, actual tissue levels of ascorbic acid achieved under various experimental designs, difference in antigens used and species differences.

4.4 Lovell and Lim (1978) recognised that pond held channel catfish (*Ictalurus punctatus*), stocked at medium densities and fed ascorbic acid deficient feed, were more susceptible to *Aeromonas hydrophila* infections than those fed supplemented feeds. Durve and Lovell (1982) observed increased resistance to *Edwardsiella tarda* infections in channel catfish with dietary supplementation of ascorbic acid.

4.5 Nonvaccinated channel cat fish fingerlings, fed purified diets containing 0 to 3000 mg ascorbic acid / kg feed for 13 weeks, also exhibited a dose dependent mortality pattern to *Edwardsiella ictaluri* (Li and Lovell, 1985). They also studied the complement activity by testing the serum ability to induce haemolysis of sensitised SRBC. Sera from fish fed 0 mg/kg had significantly lower levels of haemolytic activity than those fed supplemented feeds. Ascorbic acid deficiency also significantly reduced phagocytosis of *E. ictaluri* by channel catfish neutrophils. High dose of ascorbic acid also enhanced humoral antibody production to *E. ictaluri*. Hence megadoses feeding of vitaminn C has actually shown to increase resistance in channel catfish. Quite contrary to the above observations, Li *et al.*(1993) observed that elevated dietary vitamin C concentrations did not improve resistance of channel catfish to *E. ictaluri* infection.

4.6 Navarre and Halver (1989) observed that in challenges of rainbow trout with *Vibrio anguillarum*, resistance to infection was directly related to dietary vitamin C level, ranging from 0 to 20 times the requirement. Bacterial gill disease, moving stress, weighing stress and overcrowding resulted in significantly higher mortalities in fish fed the unsupplemented feeds than in the fish receiving ascorbic acid. High doses of ascorbic acid enhanced humoral antibody production to *V.anguillarum*.

4.7 Verlhac *et al.*(1993) demonstrated that dietary supplementation of vitamin C enhances the non-specific immune parameters such as serum complement activity and phagocytosis of rainbow trout. The C1q component of complement has collagen-like helical regions which are rich in hydroxyproline residues. Supplementaion of vitamin C would have helped in collagen synthesis thereby increasing the complement activity in the supplemented group. Megadoses of ascorbic acid also affected the mortality rate of rainbow trout experimentally exposed to the protozoan parasite, *Ichthyophthirius multifiliis*. Ascorbic acid is also reported to affect resistance to challenge with viral hemorrhagic septicemia agent in rainbow trout (Meier *et al.*, 1991). Dunier *et al.*(1995) reported, dietary vitamin C supplementation at higher levels can prevent, to some extent, immunosuppression in rainbow trout on exposure to an organochlorine insecticide, lindane. Wagboe *et al.* (1993) noticed increased lysozyme activity in head kidney, serum complement and iron in ascorbic acid supplemented Atlantic salmon.

4.8 The results of most of the studies suggest that vitamin C requirement for maximising immune functions and infectious disease resistance among fin fish is extremely high relative to requirements defined according to other indices. At the same time well designed studies in many cases have failed to reveal an influence of dietary vitamin C megadoses on immunological indices or resistance to experimental infections. In view of the high level of redundancy within the immune system, small effects of vitamin C megadoses, such as have been reported with regard to antibody responses and complement haemolytic activity are probably inconsequential. Nevertheless, high concentrations of dietary vitamin C clearly can confer resistance to acutely and chronically lethal doses of pathogenic bacteria. Possible mechanisms include both antioxidant and pro-oxidant actions (Hemila, 1992). Enhancement of vitamin C dependent antioxidant potential can confer protection against mortality from acute experimental infection in animals (Oda *et al.*, 1989). On the other hand, vitamin C, at high concentrations can inactivate bacteria and viruses directly by way of a pro-oxidant interaction with metal ions (Hemila, 1992). These effects are best regarded as accruing from the use of vitamin C as a drug rahter than as a nutrient (Woodward, 1994).

4.9 Most of the studies on vitamin C are done in salmonoids and channel cat fish. There is a scarcity of information in the role of vitamin C on the disease resistance and immune responses in Indian species of cultivable fishes. The work which have been already carried out mainly concentrates on the vitamin C requirement and deficiency syndromes in species like green snake head (Mahajan and Agrawal, 1979), Mrigal (Mahajan and Agrawal, 1980) and in rohu (Hasan *et al* 1993).

4.10 The studies by Sobhana(1997), in two species of Indian major carps, mrigal and rohu have clearly shown that supplementation of vitamin C at levels of 1000 mg/kg diet or above can play important role in increasing the disease resistance of *naïve* fish and in enhancing the protection in immunised fish. However, such an enhanced protection was not related to increased antibody levels. The role of vitamin C in enhancing the magnitude of the protective inflammatory response in Indian major carps was obvious in the findings. The enhanced inflammatory response could be a result of enhanced complement activity, phagocytosis and other factors. Complement is a non-specific serum factor which is an important chemical mediator that can influence various events in the inflammatory response such as histamine release from mast cells, vasodialation, vascular permeability and chemotaxis of phagocytes to the area of injury.The products of complement activation can act as opsonins thereby increasing phagocytosis of complement coated particles and can also cause target cell lysis by the formation of membrane attack complex. In addition, vitamin C is involved in a variety of other enzyme reactions and it is suggested that, vitamin C supplementation would have enhanced the release of several other cytokines and other chemical mediators thereby enhancing inflammatory response. Vitamin C supplementation also improved the formation of fibrous granulation tissue during the healing process of the inflammatory response, for which collagen is an important component.

4.11 The use of wound healing effects as a model for the collagen moderation effect of vitamin C deficiency was pioneered in fish by Halver *et al.*(1969), who created sutured surgical lesions in the dorsum or flank of salmonids, and showed that healing did not occur in the absence of this vitamin. And most of the studies on the effect of vitamin C on wound healing in fish have been performed on cold water species. Halver (1972), studied the rate of collagen synthesis and wound repair in rainbow trout and coho salmon in relation to supplementation of ascorbic acid and found that '0' level dietary ascorbate failed to repair the sutured surgical lesions made on the dorsum and flank, even after 3 week post trauma

period. No evidence of granulation tissue or collagen formation was observed histologically and the wounds could be easily teased apart. The vitamin C supplemented groups exhibited rapid wound and tissue repair.

4.12 Among the warm water teleosts, the channel cat fish, *Ictalurus punctatus* and the Nile tilapia (*Oreochromis niloticus)* have been investigated for wound healing in relation to dietary ascorbic acid intake (Lim and Lovell, 1978; Jauncey *et al.*, 1985). Lim and Lovell (1978) observed that in channel cat fish, all wounded fish, regardless of dietary ascorbic acid intake, showed superficially healed wounds after 10 days. Healing of the epidermal and dermal regions and skin was also judged to be complete. The rate of healing of somatic muscle was reported as proportional to the dietary ascorbic acid level. Fish fed a diet devoid of vitamin C had replacement of muscle by dense immature collagen fibers.
The wound healing response in relation to ascorbate supplementation of the diet indicates the importance of the ascorbate status of fish as a predisposing factor to disease outbreak in intensive aquaculture, where injuries from handling and rearing techniques are common.

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