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Ascorbate-2-sulfate as a dietary vitamin C source for atlantic salmon (*Salmo salar*): 1. Growth, bioactivity, haematology and humoral immune response

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Abstract

The present experiment shows that ascorbate-2-sulfate (AS) is not equivalent to ascorbic acid (AA) as a dietary vitamin C source for Atlantic salmon (*Salmo salar*). Within reasonable feed supplemental levels AS does not provide the tissues with adequate supplies of vitamin C to secure optimal physiological functions as demonstrated by biochemical and haematological analyses.

AS could not be detected in the liver of fish fed either AA or AS, nor in vitamin C – deprived salmon, suggesting that AS is not the natural storage form of vitamin C in this species.

There were no significant differences in antibody production against a soluble artificial antigen (NIP_{11}-LPH) in fish fed 500 and 5000 mg AA/Kg dry diet or equivalent amounts of AS during a period of six weeks at a water temperature of 7.2°C.

Introduction

According to Halver *et al.* (1975) ascorbate-2- sulfate (AS) is equivalent to ascorbic acid (AA) as a vitamin C source for young rainbow trout (*O. mykiss*). Tucker and Halver (1984, 1986) concluded that AS is stable in feed, readily absorbed and is the major storage form of vitamin C in fish where it is converted to AA as needed to maintain circulating AA levels. However, other studies have indicated that AS may be less efficient than AA as a dietary vitamin C source for rainbow trout (Tsujimura *et al.* 1978), channel catfish (*Ictalurus punctatus*) (Murai *et al.* 1978), ayu (*Plecoglossus altivelis*) and tilapia (*Oreochromis niloticus*) (Tsujimura *et al.* 1981; Soliman *et al.* 1986). In man, guinea pigs and rhesus monkeys AS has been shown to possess little or no vitamin C activity (Kuenzig *et al.* 1974; Machlin *et al.* 1976; Tsujimura *et al.* 1982).

The biochemical functions of AA suggest that it is important for the maintenance of fish health, including resistance to infectious diseases. Studies with terrestrial animals have demonstrated associations between the dietary level of AA and several defense mechanisms against pathogens, environmental factors and unspecific stress reactions. These include, drug metabolizing enzymes, protection against heavy metals, the metabolism of essential elements, steroid synthesis, inhibition of lipid peroxidation and specific and nonspecific immune responses (Hornig *et al.* 1984).

The impact of dietary factors on immunocompetence in fish is a relatively new field of research and has recently been reviewed by Landolt (1989). Studies with AA have attracted most interest so far where resistance in terms of the ability of the host to withstand pathogen challenge has been measured as well as in studies on specific and nonspecific im-

mune phenomena. In a series of experiments with channel catfish it was demonstrated that a higher than normal dietary level of AA increased the resistance to bacterial infection (Durve and Lovell 1982). Furthermore, a megadose of AA (100 times the normal growth requirement) significantly enhanced antibody production and complement activity (Li and Lovell 1985). Blazer (1982) reported decreased total serum iron-binding capacity and phagocytic index of peritoneal macrophages in rainbow trout fed 120 mg of AA/kg dry diet compared to fish maintained on a diet containing ten times this dietary level of AA. Navarre and Halver (1989) found improved survival against bacterial challenge and improved humoral antibody production in rainbow trout fed five or ten times the growth requirement levels of AA. In a study with parasite infection in rainbow trout Wahli *et al.* (1986) reported an AA immune-mediated decrease in mortality in fish infected with the holotrichous ciliate *Ichthyophthirius multifilis*.

On the other hand, Bell *et al.* (1984) failed to demonstrate any positive effects of elevated dietary levels of Na L-ascorbate-2-sulfate (AS) on survival time of sockeye salmon (*Oncorhynchus nerka*) inoculated with *Renibacterium salmoninarum* which causes bacterial kidney disease (BKD) in salmonids. Survival time was in fact found to be inversely related to the dietary AS level when the diets contained low levels of Zn and Mn. Fish from the same dietary treatment were also inoculated with formalin-killed *Aeromonas salmonicida*, the bacterial pathogen that causes furunculosis. No significant effect of diet on the production of antibodies against this bacterial vaccine was observed. In these studies Bell *et al.* (1984) used AS as a vitamin C source supplied in the diet equivalent to 100 mg AA/kg and ten and hundred times this level.

The aim of the present investigation was to evaluate the efficiency of AS as a vitamin C source for Atlantic salmon (*Salmo salar*) described by means of growth parameters and biochemical and haematological analyses. A further aim was to study the effects of elevated dietary levels of AA and AS on the humoral immune response in this species.

Materials and methods

Experiment 1

Young Atlantic salmon from the Aquaculture Station Matre were fed a practical dry pelleted salmonid diet (Albrektsen *et al.* 1988) without supplementation of AA for four months before set up of Experiment 1. Body weight was recorded at the start (19.9 ± 5.5 g, n = 477) and at the end of the feeding trial which lasted for twelve weeks. Sixty-seven fish were stocked in 1.5 × 1.5 m aquaria supplied with flowing fresh water at a mean temperature of 7.2 ± 3.1°C (recorded daily).

Two aquaria of fish were assigned to each of five experimental diets. These comprised the basal diet without any vitamin C supplementation, and diets to which was added 500 or 5000 mg of crystalline ascorbic acid (AA)/kg or dipotassium ascorbate-2-sulfate (AS) in equimolar amounts. These groups are referred to as 0, 500 AA, 5000 AA, 500 AS and 5000 AS, respectively. The feeds were kept frozen at −20°C until the day used. The fish were fed to satiety by means of automatic feeders.

Analyses of AA and bound ascorbic acid (BA) in liver were carried out initially, after 6 weeks and after 12 weeks. AA and BA were analysed by a method modified according to Tucker (1983) and Schuep (1984). The analytical principle involved high performance liquid chromatography (HPLC) separation of an ethyl acetate extracted dehydroascorbic acid-2,4-dinitrophenylhydrazine (DNPH) complex with spectrophotometric detection at 515 nm as described by Schuep *et al.* (1984). Hydrolysis conditions were carried out according to Tucker (1983), but recent studies at our institute (unpublished) have shown that the compound identified as AS using this analytical procedure may constitute another form of ascorbic acid. Therefore, we use the expression "bound ascorbic acid" (BA) for the high temperature hydrolysis DNPH complex (Waagbø *et al.* 1989).

AS was determined by HPLC as described by Schuep *et al.* (1989) in liver samples from all feeding groups at the end of the experiment.

Proximate analyses (dry matter, protein (N × 6.25), lipid and ash) were determined in liver and

whole fish (exclusive the liver) by standard methods (Lie *et al.* 1988).

Determinations of haemoglobin (Hb), haematocrit (Hct), red blood cell count (Rbc), leucocrit (Lct), middle cell volume (MCV), middle cell Hb (MCH) and middle cell Hb concentration (MCHC) were carried out at the end of the feeding trial according to Sandnes *et al.* (1988). Five fish from each tank were anaesthetized with benzocaine and analyses were performed on heparinized blood which was collected by severing the caudal fin.

Hydroxyproline (OH-proline) in the vertebral column was analysed as described by Albrektsen *et al.* (1988). The samples were prepared by cooking the fish in a microwave oven before removing the flesh. The values are given as % of vertebral protein.

Antibody production against an artificial antigen, NIP_{11}-LPH (4-hydroxy-3-iodo-5-nitrophenyl acetic acid as hapten bound to *Limulus polyphemus* hemocyanin as carrier protein; molar ratio 11 per 100,000 MW of LPH), was determined as a measure of the humoral immune response as described by Killie (1987). Seven fish in each tank were immunized six weeks before the end of the experiment by intraperitoneal injection of 100 μl of NIP-LPH (1.0 mg/ml) in Freund's complete adjuvant (50/50). Blood was collected by cutting the caudal fin after placing the fish in iced water for a few minutes. Blood from three nonimmunized fish per tank was used as a control. Individual sera were obtained by centrifuging the blood samples after clotting at 3,000 × g for 10 min.

Antibodies were assessed by measuring the binding to antigen-coated (NIP-BSA) 96-well polystyrene microtiter plates, performing standard ELISA techniques (Espelid *et al.* 1987). Preliminary, pooled samples of control sera and test sera from all groups were titrated in the range 1:50 to 1:6,400. The final determination of antibodies in individual sera was found to be representative at a dilution of 1:100 in this experiment.

Experiment 2

When Experiment 1 was terminated the 0 group fish were stocked in one aquarium and fed the AA deficient diet for another seven weeks. The fish were then distributed into two tanks and further fed for two weeks the diets supplemented with 500 AA and 500 AS, respectively. It was not possible to keep a control group feeding on the AA deficient diet during this period due to high mortality.

During these last two weeks ten fish were sampled initially and five fish in each tank after one week and after two weeks of feeding. Blood Hb and liver AA and BA content were determined as described above.

Simultaneously, the duplicates fed 500 AA and 500 AS in Experiment 1 were stocked in one tank each and fed the same diets for a further seven weeks, after which both were fed the AA deficient diet for another two weeks. Samples were collected and analyses performed as described previously.

Statistical methods

Statistical evaluation of experimental data was carried out by means of the Mann-Whitney U test using a Luxor ABC 806 computer.

Results

Experiment 1

The final fish weight after the feeding period of twelve weeks was significantly less in fish given the AA/AS deficient diet ($p < 0.05$) compared with the remaining dietary treatments (Table 1). The fish fed the 5000 AA diet showed the highest final weight which was significantly higher than in the 500 AS and 5000 AS groups. The hepatosomatic index was highest in fish fed the vitamin C deficient diet, but there were no other diet-related significant differences (Table 1). Mortality was negligible in all groups.

Proximate analyses of liver and whole fish (without liver) revealed no significant differences between groups. Mean values (g/100 g) were 21.4, 14.0, 61.2, 5.9 and 29.1, 16.6, 52.3, 7.0 for dry matter, total lipid, protein and ash in liver and whole fish, respectively.

Table 1. Growth and hepatosomatic index of Atlantic salmon fed increasing levels of ascorbic acid (AA) or ascorbate-2-sulfate (AS) in the diet for 12 weeks

Dietary AA/AS [mg/kg d.w.]	Initial weight [g ± SD (n)]	Final weight [g ± SD (n)]	Hepatosomatic index [% ± SD (n)]
0 AA/AS	19.9 ± 5.5 (477)	32.9 ± 9.2 (124)	1.19 ± 0.32 (10)
500 AA		36.4 ± 9.9 (125)	1.05 ± 0.21 (10)
5000 AA		38.8 ± 9.5 (130)	1.14 ± 0.16 (10)
500 AS		35.3 ± 9.0 (109)	1.04 ± 0.17 (10)
5000 AS		35.9 ± 11.7 (134)	0.99 ± 0.13 (10)

Table 2. The concentration of ascorbic acid (AA) and bound ascorbic acid (BA) in the liver and vertebral OH-proline of Atlantic salmon fed different levels of ascorbic acid (AA) or ascorbate-2-sulfate (AS) after six and twelve weeks

Dietary AA [mg/Kg d.w.]	Weeks after start of feeding	Liver AA[1] [μg/g w.w.]	Liver BA [μg/g w.w.]	Ratio BA/AA	Vertebral OH-proline [% of protein]
0	6	12 ± 4	1 ± 2	0.04 ± 0.10	–
500 AA	6	138 ± 47	56 ± 32	0.37 ± 0.22	–
5000 AA	6	252 ± 59	194 ± 84	0.80 ± 0.37	–
500 AS	6	33 ± 25	6 ± 8	0.21 ± 0.25	–
5000 AS	6	31 ± 7	16 ± 7	0.53 ± 0.21	–
0	12	6 ± 2	0 ± 0	0.00 ± 0.00	0.90 ± 0.10
500 AA	12	166 ± 35	75 ± 35	0.46 ± 0.20	1.07 ± 0.07
5000 AA	12	309 ± 63	130 ± 58	0.45 ± 0.23	1.12 ± 0.07
500 AS	12	27 ± 16	10 ± 5	0.41 ± 0.15	1.11 ± 0.06
5000 AS	12	85 ± 30	42 ± 19	0.49 ± 0.24	1.07 ± 0.06

Data are shown as mean ± SD (n = 6 at six weeks and 16 at twelve weeks); [1] Initial values (n = 10): AA: 6 ± 2 μg/g; BA: 1 ± 1 mg/g w.w.

As shown in Table 2, the liver AA concentration (μg/g w.w.) increased in fish fed all AA- or AS-supplemented diets, except for the 500 AS group during the last six weeks of the experiment. Salmon fed AA as a vitamin C source showed significantly ($p < 0.001$) higher liver AA concentrations than the AS dietary groups. With a tenfold increase in the dietary AA level (from 500 to 5000 mg/kg) the liver AA concentration was nearly doubled after both six and twelve weeks. In the AS – fed groups this degree of differentiation was first seen after twelve weeks.

The liver BA concentration exhibited similar pattern as that of AA as regards dietary vitamin C supplementation (Table 2). The ratio between the concentration of BA and AA in the liver showed wide variations after six weeks, but ranged within 0.41 (500 AS) to 0.49 (5000 AS) in fish fed any form or level of vitamin C supplementation after twelve weeks. No AS was detected in any samples from fish fed either AA or AS.

The percentage of OH-proline in the vertebral protein was significantly reduced ($p < 0.005$) in the AA/AS deficient group at the end of the experiment compared to the remaining groups; the latter were not significantly different (Table 2).

Table 3 comprises the haematological data after twelve weeks of feeding which was six weeks after antigen injection. The Hb, Hct and Rbc values represented anaemic values in the vitamin C – deprived fish, while immunization caused a general reduction in these parameters. The MCV and MCH were lower in antigen treated fish than in the control fish. The MCHC was affected to a lesser extent. Lct values differed significantly between samples, but the differences were not related to dietary treatment.

Table 3. Haematological values in immunized and nonimmunized (control) Atlantic salmon fed increasing levels of ascorbic acid (AA) or ascorbate-2-sulfate (AS) for 12 weeks

Dietary [mg/kg]	Treatment	Hb [g/100 ml]	Hct [%]	Rbc [$\times 10^{12}$/l]	MCV [$\times 10^{-15}$l]	MCH [$\times 10^{-6}$g]	MCHC [g/100 ml]	Lct [%]
0	immunized	3.0 (1.0)	19 (7)	0.50 (0.15)	383 (85)	62 (19)	17.4 (8.0)	0.21 (0.07)
	control	3.6 (1.4)	26 (10)	0.60 (0.20)	423 (53)	61 (10)	14.3 (1.4)	0.22 (0.20)
500 AA	immunized	6.1 (0.7)	34 (4)	0.75 (0.09)	456 (24)	81 (6)	17.9 (0.8)	0.39 (0.14)
	control	6.4 (0.8)	37 (3)	0.78 (0.06)	472 (35)	82 (9)	17.2 (1.1)	0.48 (0.21)
5000 AA	immunized	5.7 (0.8)	36 (4)	0.74 (0.08)	487 (59)	77 (5)	16.1 (2.3)	0.36 (0.31)
	control	6.6 (0.6)	40 (4)	0.79 (0.07)	502 (34)	84 (6)	16.8 (2.0)	0.27 (0.17)
500 AS	immunized	5.0 (1.1)	29 (5)	0.64 (0.11)	448 (50)	78 (15)	17.5 (2.6)	0.28 (0.07)
	control	6.1 (1.0)	34 (5)	0.74 (0.11)	471 (44)	83 (9)	17.7 (2.2)	0.33 (0.10)
5000 AS	immunized	6.1 (0.7)	35 (4)	0.77 (0.08)	451 (28)	80 (7)	17.7 (1.3)	0.34 (0.13)
	control	6.1 (0.9)	36 (5)	0.72 (0.09)	495 (24)	85 (8)	17.2 (1.5)	0.28 (0.12)

Data are shown as mean (SD), n = 10.

The median and range of antibody responses in immunized and control fish are shown in Table 4. The antibody responses were significantly elevated ($p < 0.05$) in the immunized fish compared with the control in all groups. Fish fed the vitamin C – deficient diet showed the lowest values, but there were no statistical differences in antibody responses in stimulated fish between any of the dietary groups.

Experiment 2

After the 0 group fish had been fed vitamin C – deficient diet for another seven weeks after the termination of Experiment 1, they were severely anemic (Table 5) and mortality increased (results not shown). In Experiment 2, the initial Hb level was 2.0 g/100 ml and after feeding the 500 AS diet for two weeks Hb increased, but clearly less than in fish fed the 500 AA diet. Dietary AS did not increase the liver tissue concentrations of AA and BA as efficiently as did dietary AA. The relationship between AA and BA in the liver of salmon fed the 500 AA diet after one and two weeks indicated a kind of conservation mechanism of AA during this period when the dietary supply of AA was sufficient after a period of depletion.

The depletion study (Table 5) revealed a somewhat similar mechanism, with an increase in the BA fraction after two weeks although the total vitamin C content in the liver decreased. Blood Hb was not negatively affected in the course of two weeks without a dietary supply of vitamin C.

Discussion

The analytical data presented in this study show that AS is not equivalent to AA as a dietary vitamin C source for Atlantic salmon. The liver retention of vitamin C in forms of AA and BA was clearly reduced when feeding AS in the diet. The concentration of total ascorbate (AA + BA) in the liver at the end of Experiment 1 showed that AS supported 22% (500 mg/kg) and 30% (5000 mg/kg) of the levels found for the AA equivalent diets. No AS could be detected in any group. The haematological data confirm a reduced efficiency of AS by showing a slower recovery towards normal values after depletion when feeding 500 mg AS/kg.

Kuenzig *et al.* (1974) showed that AS is devoid of antiscorbutic activity in the guinea pig and that total hepatic ascorbate levels were similar to those observed in scorbutic animals. Moreover, Machlin *et al.* (1976) found no antiscorbutic effect of AS in the rhesus monkey. AS has been suggested to be a urinary metabolite of AA in man (Baker *et al.* 1971) whereas Tillotson and McGown (1981) were unable to detect AS in monkey urine. Tucker and Halver (1984, 1986) concluded that AS is readily absorbed from feeds and that AS is the major storage form of vitamin C in fish and can be converted to AA as

needed to maintain circulating AA levels. These statements are mainly based on feeding experiments and metabolic studies with rainbow trout using ^{14}C[AA] and ^{35}S[AS], and analyses of the ascorbate forms in the tissues using complex – forming reactions with DNPH and anion exchange HPLC (Halver *et al.* 1975; Tucker 1983). Further, a study describing the purification of an enzyme catalyzing the hydrolysis of AS from rainbow trout liver has been published (Benitez and Halver 1982). Our experience suggests that the accuracy and precision of these methods limit interpretation of the analytical data and that the DNPH assay does not measure AS specifically (Waagbø *et al.* 1989). In the present study, a modification of the DNPH method was used to determine AA (incubated at 37°C) and an AA fraction which complexes DNPH at an elevated incubation temperature (100°C). We have named this latter fraction "bound ascorbic acid" (BA) and it is not detected by a direct AS – specific HPLC assay (Schuep *et al.* 1989) applied on the same material. The analytical data from Experiment 2 in the present study suggest that BA constitutes a source of metabolizeable AA. Thus, our findings fit into a model with two or more body pools (or compartments) of ascorbate in fish, but do not support AS to be a storage form.

A radioautograph of rainbow trout intubated with ^{35}S[AS] showed fixation of radioactive material throughout the body identical with those areas observed in fish intubated with ^{3}H-4[AA] and ^{14}C-1-[AA] (Halver *et al.* 1975). Fixation of radioactive material was seen mainly in supporting tissues and major metabolic organs such as the liver and the kidney. Hornig (1975) reported a similar incorporation of ^{35}S[AS] in the guinea pig, an animal in which AS has no antiscorbutic activity (Kuenzig *et al.* 1974). A question can be raised as to whether AS *per se* is incorporated, or only the sulfate moiety of the molecule, as a similar autoradiographic appearance was seen in the guinea pig after injection of inorganic $^{35}SO_4^{2-}$ (Hornig 1975).

The natural-ingredient feed used in the studies reported here has been shown to produce vitamin C deficiency in rainbow trout (Albrektsen *et al.* 1988). Also, Hilton *et al.* (1978) have demonstrated vitamin C deficiency in this species using a practical diet. In the unsupplemented feed used in the present study no traces of AA or AS could be detected upon analysis (results not shown). If AS is a natural and stable storage form of vitamin C in fish, one should expect to detect it in fish meal and subsequently fish meal-based diets should supply some degree of vitamin C activity in farmed fish. This is not the case according to the present experiment and experience from several other studies with salmonids at our institute.

Of the AS feeding studies carried out in fish, only a few authors have presented analytical data on tissue levels of ascorbate forms. The experiments indicate antiscorbutic activity of AS in fish, but no authors have reported AS to be equivalent to AA with respect to organ retention of ascorbate (Murai *et al.* 1978; Tsujimura *et al.* 1978; Tsujimura *et al.* 1981; Soliman *et al.* 1986). However, the possible existence of two or more body pools of metabolizeable AA in fish complicates the interpretation of published data due to the methods of analysis applied.

In our opinion, the metabolism of vitamin C in fish should be critically re-evaluated. The first step should be to establish the exact mechanisms for absorption of AA and possible vitamin C-active derivatives hereof from the gut. In terrestrial animals, the absorption of AS is minimal and AS administered parenterally is rapidly excreted (Omaye *et al.* 1982). According to Rose (1981), AA is absorbed by an active Na^+ mediated transport mechanism in the ileum of the guinea pig and humans. No studies on the mechanisms of AA absorption from the gut seem to have been undertaken in fish. Murai *et al.* (1978) were unable to detect AS in the blood or in the liver in channel catfish fed AS in the diet. The absence of AS in the blood may indicate that AS was not absorbed *per se*. The absence of AS in the liver of AS- or AA-fed channel catfish (Murai *et al.* 1978) is in accordance with our findings in Atlantic salmon, suggesting that these species are not able to convert dietary AA or AS into body stores in the form of AS. Murai *et al.* (1978) suggest cold water fishes are able to utilize AS more effectively than the warm water catfish; Tucker and Halver (1984) subsequently concurred. Our data from studies with the cold water Atlantic salmon do not confirm that such species differences exist.

Table 4. Antibody responses in immunized and nonimmunized (control) Atlantic salmon fed increasing levels of ascorbic acid (AA) or ascorbate-2-sulfate (AS) for 12 weeks

Dietary AA/AS [mg/kg d.w.]	Treatment	n	Antibody responses[1] in ELISA (OD_{405} nm)	
			Median	Range
0	immunized	14	0.382	0.179 – 0.624
	control	6	0.231	0.141 – 0.285
500AA	immunized	14	0.459	0.278 – 0.999
	control	6	0.234	0.131 – 0.362
5000 AA	immunized	14	0.461	0.206 – 0.794
	control	6	0.221	0.056 – 0.486
500 AS	immunized	14	0.473	0.226 – 0.800
	control	6	0.250	0.134 – 0.340
5000 AS	immunized	14	0.438	0.163 – 0.626
	control	6	0.316	0.144 – 0.451

[1] Serum dilution 1:100.

Table 5. Blood haemoglobin and liver ascorbic acid (AA), bound ascorbic acid (BA), total AA and BA/AA ratio in: I. Atlantic salmon (50 g) fed ascorbic acid (AA) or ascorbate-2-sulfate (AS) diets (500 mg/kg) for 7 and 14 days after an AA deprived period of 9 months. II. Atlantic salmon (60 g) fed an AA deficient diet for 7 and 14 days after being prefed AA or AS diets (500 mg/kg) for nearly 6 months

Feed	Day[1]	Haemoglobin [g/100 ml]	AA [μg/g w.w.]	BA [μg/g w.w.]	Total AA [μg/g w.w.]	BA/AA ratio
I.						
0 AA/AS	0	2.0 ± 1.0	2 ± 2	3 ± 2	5 ± 2	1.4 ± 2.8
500 AA	7	3.4 ± 1.1	71 ± 17	16 ± 6	87 ± 23	0.2 ± 0.1
500 AA	14	3.8 ± 1.3	38 ± 20	134 ± 80	172 ± 100	3.0 ± 1.8
0 AA/AS	0	2.0 ± 1.0	2 ± 2	3 ± 2	5 ± 2	1.4 ± 2.8
500 AS	7	2.8 ± 0.8	5 ± 2	2 ± 3	8 ± 4	0.5 ± 0.5
500 AS	14	2.4 ± 0.4	6 ± 1	7 ± 6	13 ± 6	1.4 ± 1.3
II.						
500 AA	0	7.2 ± 0.6	152 ± 34	84 ± 32	236 ± 52	0.6 ± 0.2
0 AA/AS	7	7.7 ± 0.4	120 ± 13	40 ± 15	160 ± 28	0.3 ± 0.1
0 AA/AS	14	9.1 ± 1.0	39 ± 4	126 ± 31	165 ± 32	3.3 ± 0.9
500 AS	0	6.6 ± 0.8	19 ± 17	12 ± 5	31 ± 20	1.1 ± 0.9
0 AA/AS	7	6.8 ± 1.6	16 ± 4	7 ± 2	23 ± 5	0.4 ± 0.2
0 AA/AS	14	6.8 ± 1.7	10 ± 5	22 ± 11	33 ± 16	2.2 ± 0.3

Data are shown as mean ± SD; [1] Day 0 (n = 10); day 7 and 14 (n = 5).

Antibody production

The fish with signs of AA deficiency showed no statistical differences between any dietary group as regards circulating antibody levels. This is in contrast to humoral immune response studies in rainbow trout (Wahli *et al.* 1984; Navarre and Halver 1989) and in channel catfish (Lovell 1984). On the other hand, Bell *et al.* (1984) were not able to demonstrate any significant effect in sockeye salmon fed increasing levels of AS in the diet.

In the cited salmonid studies, the water tempera-

tures and the immunization periods were 8–9°C and 5 weeks (*Aeromonas salmonicida*) (Bell *et al.* 1984), 15°C and 7 weeks (*Ichthyophthirius multifiliis*) (Wahli *et al.* 1984) and 10–15°C and 6–8 weeks (*Vibrio anguillarum*) (Navarre and Halver 1989). It is possible that a relatively short immunization period (6 weeks) at a low water temperature (7.2°C) is not sufficient to elicit a high enough antibody production to reveal possible differences caused by dietary vitamin C. In the warm water channel catfish, Li and Lovell (1985) reported a significant increase in antibody production after three weeks against *Edwardsiella ictaluri* vaccinated twice and fed elevated dietary AA at 29–32°C. However, *in vitro* studies using leucocytes from catfish fed 50 and 5000 mg AA/kg diet showed no differences in mitogen (LPS and Con A) – stimulated proliferation. Stimulation of the proliferation with T-cell dependent antigen and PFC – responses showed no effects of the higher dietary AA supplementation (N. Miller, personal communication).

The artificial antigen used in the study reported here, NIP_{11}-LPH, has been shown to be successful as an antigen in immunological studies with Atlantic salmon (Killie 1987). However, this is a soluble antigen in contrast to particulate antigens (formalin-killed bacteria) which were used in the experiments mentioned above. Particulate antigens are known to give increased immune responses compared to soluble antigens and this may explain the low antibody responses in the immunized fish in the present study. However, Bell *et al.* (1984), using a particulate and thus a more potent antigen, found no differences in antigen responses with increasing levels of dietary AS.

The haematological values indicate physiological effects of immunization related to erythropoiesis and the metabolism of haemoglobin. This is most significant in fish receiving no form of dietary vitamin C. Stimulation of the humoral defence system implies mobilization of metabolic pathways susceptible to environmental and nutritional disorders. Haematological parameters are of value in assessing health and physiological status in fish, although not very specific (Sandnes *et al.* 1988). It is well documented that AA deficiency causes anemia in fish (Hilton *et al.* 1978; Halver *et al.* 1975; Waagbø *et al.* 1989), but the present study also reveals a reduction in blood haemoglobin and the number, size and haemoglobin content of circulating erythrocytes caused by immunization. These findings initiated further studies on the metabolism of trace elements related to immunological aspects of dietary AA and AS in fish as reported by Måge *et al.* (1990).

According to Landolt (1989), profound changes in the immune response are some of the earliest manifestations of malnutrition, and experimental evidence exists that dietary levels of some essential nutrients beyond the growth optimum level have positive effects. However, testing only for antibody-mediated protection may not give the full answer in nutrition/disease studies as fish seem to rely more heavily than higher vertebrates on non-specific and cell-mediated protection. Thus, a broader range of parameters to measure defence mechanisms should be applied in fish nutrition studies, preferably also including challenge tests. Further studies in this field may show that it will be of value to modify diets, otherwise considered nutritionally adequate, to increase the disease resistance.

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