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Effect of dietary ascorbic acid on the susceptibility of steelhead trout *(Salmo gairdneri)* to nitrite toxicity

by

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Abstract: Four diets, each containing different levels of ascorbic acid were fed to duplicate groups of steelhead trout *(Salmo gairdneri)* in two four-week periods. Tolerance to nitrites increased when the concentration of ascorbic acid was high. Flow-through bioassays in fingerlings showed that when the temperature increased the percent of methemoglobin in their blood also increased.

The tolerance to nitrite toxicity was less in large fish than in those smaller fed the same concentration of ascorbic acid. Possibly ascorbic acid acts in the reduction of methemoglobin to hemoglobin, and also it has a protective effect against stress in the fish. A "safe" level of 200 mg/kg of ascorbic acid in practical diets was reached.

Today many countries are employing water reuse systems for the culture of warm and cold water species in both fresh water and marine environments. To control the environment, a nitrification process is generally used to maintain a safe concentration of ammonia, the first metabolite produced by the fish. In the nitrification process, nitrite is the intermediate product, which under certain conditions may increase significantly, producing a serious hazard to fish growth and survival (Spotte, 1970). Crawford and Allen (1977) studied the possible role of nitrite toxicity on pond fish mortality, in juvenile Pacific Salmon (*Oncorhynchus* sp.) reared in sea water ponds fertilized with treated domestic waste waters from the city of Arcata, Northern California.

Nitrite oxidizes the respiratory blood pigment nemoglobin (Hb) to ferrihemoglobin (Ferri-Hb) in the erythrocyte, inducing methemoglobinemia. Unlike oxyhemoglobin, methemoglobin (met-Hb) is a true oxidation product of Hb, the iron in Met-Hb is therefore incapable of transporting oxygen (Bodansky, 1951). Because it is unable to act as an oxygen carrier, Met-Hb in sufficiently high concentrations causes hypoxia and cyanosis (Miale, 1967).

Environmental nitrite in concentrations as low as 0.5 mg per liter becomes toxic to Chinook salmon *(Oncorhynchus tshawaytscha)* and rainbow trout *(Salmo*

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gairdneri) in fresh water (Cameron, 1971; Smith and Williams, 1974; Russo *et al.*, 1974; Westin, 1973; Smith and Russo, 1975). This toxic response can be characterized by changes in blood nitrite and methemoglobin.

Crawford and Allen (1977) reported 70% mortality in Chinook salmon fingerlings *(Oncorhynchus tshawaytscha)* in a 48 hour static bioassay at a concentration of 27 mg/l NO^-_2-N. Perrone (1978) has found that high levels of nitrite in the water produce stress and high ferrihemoglobinemia in coho salmon. Changes in plasma glucose, lactate, liver and muscle glycogen have been consistently reported in salmonids exhibiting ferrihemoglobinemia (Black *et al.*, 1961). In addition, many scientists have demonstrated that catecholamines and 17 hydroxycorticosteroids of various salmonids vary in direct proportion to physical or chemical stimuli (Hane *et al.*, 1966; Donaldson and McBride, 1967; Fagerlund, 1967; Nakano and Tomlinson, 1967; Wedemeyer, 1969). Some environmental factors that protect against nitrite toxicity are: the addition of chloride ions (Perrone and Meade, 1977); and the addition of calcium chloride and pH control (Wedemeyer and Yasutake, 1978). Injection of methylene blue (Bortz, 1976); and high dietary ascorbate have also been shown to exert a protective effect (Cameron, 1971). Ascorbic acid is an essential vitamin for fish fed on practical diets, however, the requirements are in relation to several factors such as: type of diet; processing; moisture content; storage time; environmental temperature; humidity, and possibly, size, age and genetic makeup of the fish (Halver *et al.*, 1969).

The objective of this study was to investigate the protective effect of dietary ascorbic acid on steelhead trout subjected to different levels of nitrite in the water.

MATERIAL AND METHODS

Diet and feeding methods for steelhead (Large fish): the dietary ingredients were selected on the basis of nutritional value and availability of the product and four test diets were prepared as follows:

Diet 1 was not supplemented with ascorbic acid and served as a control; diet 2 was supplemented with 200 mg/kg ascorbic acid of control diet; diet 3 was supplemented with 1000 mg/kg of control diet, and diet 4 was supplemented with 2000 mg/kg of control diet. The composition and calorie content for these diets were identical except for the vitamin C content. The diet composition and the level of vitamin C in each diet are listed in Tables 1 and 2.

The diets were prepared by pelleting the food into a soft moist product that required frozen storage until fed.

One hundred and sixty randomly selected steelhead trout approximately 16 months old were obtained from the silo culture system of Animal Science Laboratories, University of Rhode Island. The fish were weighed, measured and stocked at 20 fish per lot in 150 liter flow-through culture units. All treatments were duplicated.

Throughout this research large fish were those between 37 and 42 g and average length between 12 and 18 cm.

Each diet was fed to the duplicate lots of fish twice daily. Daily feeding was calculated using the following equation (Croker, 1972):

$$RE = \frac{300 \times C\ (T - 32)}{RTU \times L}$$

RE = Percent of body weight or pound per 100 pound of fish
300 = Constant
C = Feed conversion factor
RTU = Degree days (above 32°F) required per inch of growth
T = Temperature in F
L = Length in inches

TABLE 1

Composition of the basic test diet

(diet N° 1)

Component	%	g/kg
Fish Meal	35	350
Soybean oil meal	5	50
Dried skim milk	10	100
Wheat millings	5	50
Vitamin mix	1	10
Cod liver oil	2	20
Corn oil	10	100
Ethoxyquin *	0.025	3
Gelatin **	2	20
Water	30	300

* Added to the oil (antioxidant)
** Dissolved in cold water and then heated to 80 C prior to blending.

The steelhead trout were weighed and measured at the beginning and at the end of the feeding experiments (4 weeks). A Yellow Spring Instrument Model 51 B meter was used to measure the dissolved O_2 and the temperature of the water, pH was determined with a model Corning pH meter.

Nitrite experiments — Large fish: Flow-through biossays were carried out with steelhead trout of different sizes, fed with the test diets during four weeks.

The following considerations given to the bioassays were a minimum of ten steelhead trout in four 130 liter rectangular plastic tanks and subjected to 5,10 and 15 mg/l of NO^-_2 level test solutions. Mechanical failures reduced this original ten fish per concentration in the last bioassay to a minimum of six. Nitrite was allowed to build up to the final concentration in the test tanks over a 9–12 hour period. Fish were acclimated to the tanks for 24 hours prior to introduction of the toxicant; they were not fed during the bioassays or during acclimation.

The ground water was aereated in an elevated holding tank of up to 95% of oxygen saturation. Supplemental aeration was occasionally used in the bioassay tanks. Water was supplied by gravity feed at a rate of 1 liter per minute. Ground water quality during each test remained constant.

Solutions having different concentration of sodium nitrite (reagent grade) were metered directly into the water of test fish with a microgear pump and in the last six experiments by a monostal cassette pump.

TABLE 2

Vitamin Mix in all the diets and levels of ascorbic acid*

Component	mg/kg
Thiamine hydrochloride	15
Riboflavin	25
Pyridoxine hydrochloride	20
DL-Calcium Pantothenate	50
Niacin	200
Folacin	10
Cyanocobalamin	0.02 (active)
Choline chloride	800
d-Biotin	1.6
Vitamin A	2 000 (I.U.)
Vitamin E	50
Vitamin K (Menadione sodium bisulfite)	30
Levels of ascorbic acid	**mg/kg**
Diet number one	0
Diet number two	200
Diet number three	1 000
Diet number four	2 000

* The formula of Vitamin mix is that reported by Halver 1972.

Dead fish were removed, counted, weighed and recorded over periods of 24, 48, 72 and 96 hours. Flows and water quality were checked at six-hour intervals to ensure consistency in the test solution. Water was analyzed for nitrite, pH, dissolved oxygen and temperature during each bioassay.

Ten fish of similar age and weight which had been fed with a test diet containing 200 mg of ascorbic acid per kilogram served as controls. They were left in the 150 liter flow-through culture units. These controls were maintained under the same basic environmental conditions as the fish in the experiment, with the exception that no nitrite was added to the water. The highest nitrite observed in the water of the controls was 0.008 mg/l NO^{-}_{2} and Met-Hb values were determined.

A Yellow Spring Instrument Model 51 B oxygen meter was used to determine dissolved oxygen and temperature in the control and test tanks, a small sample of water was taken to measure the pH with a Corning Model 7 pH meter, and nitrite concentration was determined according to procedures described by the American Public Health Association (1971).

Blood samples were taken from fish surviving the bioassay to determine Met-Hb as percent of the total hemoglobin using the spectrophotometric method described by Dubowski (1960).

Fish were anesthetized individually with MS–222 (tricaine methanesulfonate) in a 500 mg/l concentration. Total lengths and weights were recorded. After severing the caudal peduncle, the blood from each fish was mixed with 10 ml of M/60 phosphate buffer; 0.5 cc of triton X–100 was added to induce hemolysis, and the solution was mixed well; it was centrifuged to eliminate turbidity, and the supernatant was used for subsequent analysis. A Spectronic 70 was used for Met-Hb determination at an absorption spectra of 360 nm.

The surviving fish from each bioassay were submitted to subjective observations in relation to the general condition of each individual, signs of stress and the color of the gills and body.

Using fish of the same age and size, as the test populations, and fed with commercial feed, similar bioassays were carried out to compare their percentage of methemoglobin to fish fed with the test diets.

A second experiment was carried out with steelhead trout fingerlings.

Four diets were prepared similar to the diets used in the first experiment (see Tables 1 and 2).

Prior to the feeding experiment the 150-liter culture units were treated for 15 days with stock solution of formalin malachite green (Roberto and Shepherd, 1975).

Two hundred and forty randomly selected steelhead trout approximately 5 months old were obtained from the circular flow-through culture system of the Animal Science Laboratories, University of Rhode Island. The fish were weighed, measured and stocked in duplicated 30-fish lots in the culture units. The fish were acclimated for one week prior to testing .

Nitrite experiments — Small fish: Throughout this research, small fish ranged between 3.16 and 3.53 g, with lengths between 3.5 and 5 cm.

Following methods used in the first bioassay experiment, ten fish were placed in each of four 150 liter rectangular plastic tanks and subjected to 10, 15, 20 and 30 mg/l of NO^{-}_{2} level test solutions.

The ground water was aereated and water quality was measured using the same procedures and equipment described in the first experiment. Ten fish of similar age and weight fed the 200 mg ascorbic acid/kg diet served as controls.

Analyses of Me-Hb were made following the spectrophotometric method described by Dubowski (1960), total lengths and weights were recorded.

Statistical analysis of time series (tsp) by ordinary least squares was used for the analysis of the relation between methemoglobin percentage in the blood and concentration of nitrite in the water, concentration of ascorbic acid in the feed, and temperature of the water.

Two other studies were undertaken. The first was with large fish involving nitrite toxicity at the level of 10 mg/l NO^{-}_{2}, using 60 fish initially and following the same methods described before, to verify the results obtained during the first studies. The second study was with small fish, following the methods described in the corresponding phase of the first experiment.

These small fish were tested for 96 hours without addition of nitrite, using only the normal concentration in the ground water. This study was carried out to discover the possible effect of starvation on the production of Met-Hb.

RESULTS

Large fish: In the control steelhead trout the following averages were observed: 24.81 ± 4.23 Ferri-Hb, 15.32 cm length, and 43.05 gm weight.

The level of NO^{-}_{2} in the system averaged 0.01 mg/l and the level of chloride in the water was 15.6 ± 1.5 mg/l (Perrone and Meade, 1977).

The results of the 96-hour bioassays with a concentration of 5,10 and 15 mg/l of nitrite in the environment of steelhead trout fed at different levels of ascorbic acid are presented in Table 3. All the fish were not affected at a level of 5 mg/l

TABLE 3

The effect of Ferri-Hb in steelhead trout fed with different levels of ascorbic acid/96 h

Level of ascorbic acid/test diet	Nitrite (mg/l)	Exposure time (h)	Mortality (%)	Met-Hb mean + SD (%)	Temperature Range (°C)	Mean length (cm)	Mean weight (gm)
0 mg/kg of ascorbic acid	5	96	–	22.1 ±0.965	15–16	16.97	50.06
200 mg/kg of ascorbic acid	5	96	–	22.76 ± 2.04	15–16	17.15	51.11
1 000 mg/kg of ascorbic acid	5	96	–	22.6 ± 1.69	15–16	16.5	47.24
2 000 mg/kg of ascorbic acid	5	96	–	22.61 ± 1.81	15–16	17.36	52.6
o mg/kg of ascorbic acid	10	96	–	39.54 ± 17.42	13–14	16.48	48.15
200 mg/kg of ascorbic acid	10	96	–	23.56 ± 2.72	13–14	16.88	49.8
1 000 mg/kg of ascorbic acid	10	96	–	25.77 ± 5.35	13–14	16.81	39.95
2 000 mg/kg of ascorbic acid	10	96	–	20.39 ± 1.39	13–14	16.45	45.67
o mg/kg of ascorbic acid	15	96	10	40.12 ± 17.10	14–15	16.9	50.5
200 mg/kg of ascorbic acid	15	96	–	41.56 ± 24.82	14–15	17.23	53.39
1 000 mg/kg of ascorbic acid	15	96	20	35.84 ± 21.02	14–15	17.08	53.36
2 000 mg/kg of ascorbic acid	15	96	20	33.09 ± 11.76	14–15	17.57	57.30

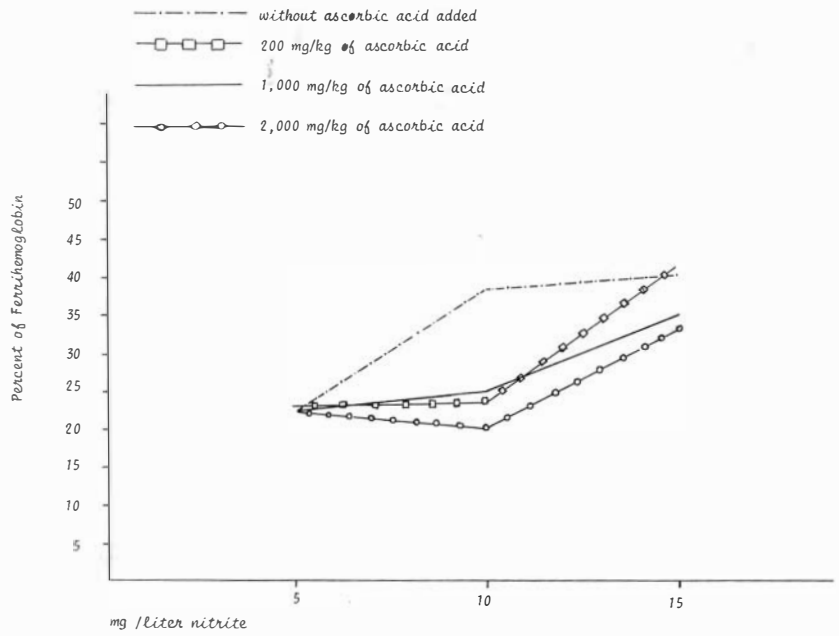

Fig. 1. Effect of different levels of nitrite on the production of Ferri-Hb to steelhead trout fed with different levels of ascorbic acid/96 h.

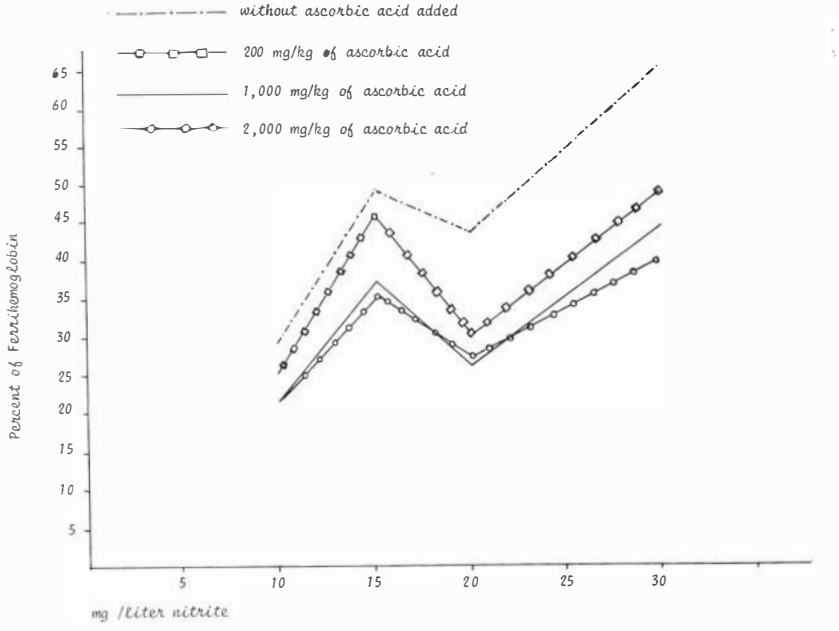

Fig. 2. Effect of different levels of nitrite on the production of Ferri-Hb to steelhead trout fingerlings fed with different levels of ascorbic acid/96 h.

NO^-_2 for 96 hours. When fish were exposed to 10 mg/l NO^-_2 over 96 hours a slight increase in Met-Hb occurred in those fed a diet without ascorbic acid. Low mortality and elevation in Met-Hb occurred in fish fed a non supplemented diet, and in fish fed high ascorbic acid diets for over 96 hours at 15 mg/l NO^-_2. The effect of different levels of nitrite on Met-Hb production in steelhead trout tested is shown in Figure 1. A comparison of these values showed that the toxicity of nitrite to steelhead trout increased with increasing nitrite concentration.

At a concentration of 15 mg/l NO^-_2, the fish with a high level of methemoglobin could be divided into 2 equal subgroups; one with a light color and one with a dark color.

Initial reactions of the fish to nitrite toxicity at the level of 15 mg/l were generally the same for all the fish in agreement with the observations of Lam Son (1977), Crawford and Allen (1977), and Perrone (1978).

Table 4 contains the results of a nitrite study at a level of 10 mg/l NO^-_2. This study was undertaken for the purpose of comparing the response of the fish to 10 mg/l NO^-_2 in a second experiment. The results are similar to those obtained in the first experiment.

Small fish: In the control steelhead fingerlings, the following averages were observed: 22.29 ± 1.81 ferri-Hb; 10.13 cm length, and 11.05 gm in weight.

The results of 96-hour bioassays carried out with steelhead fingerling fed different levels of ascorbic acid and exposed to nitrite concentrations of 10, 15, 20 and 30 mg/l NO^-_2 are presented in Table 5. Fish were not affected by nitrite at a level of 10 mg/l NO^-_2, increases in percent of Met-Hb were observed at a level of 15 mg/l NO^-_2 with a temperature of 11 C, at a level of 20 mg/l NO^-_2 concentration and a temperature of 8 C the percent of Met-Hb decreases in the fish dietary ascorbic acid. When the concentration is at the level of 30 mg/l NO^-_2, low mortality and increases in the percent of Met-Hb are noted in fish with dietary ascorbic acid and greater mortality and increases in percent of Met-Hb are observed in fish not fed with ascorbic acid.

At a level of 15 mg/l NO^-_2 the fish with a high percent of Met-Hb, showed a light color, but a concentration of 30 mg/l NO^-_2 and a high percentage of Met-Hb separated them into two subgroups, one with a light color and the other darker, in equal proportions.

The effect on production of methemoglobin at the different levels of nitrite is shown in Figure 2. A comparison of the values shows the response to nitrite levels of fish not fed ascorbic acid and those fed different levels, and exposed to different temperatures for each experiment. At a level of 15 mg/l nitrite concentration, the temperature was 11 C; at 20 mg/l NO^-_2 the temperature was 8 C, and at 30 mg/l NO^-_2 the temperature was 10 C.

Steelhead fingerlings were considerably more tolerant to nitrite than the large fish. No mortality occurred when the fingerlings were exposed to approximately 20 mg/l NO^-_2 for over 96 hours.

Table 6 contains the results of a starvation study at the level of nitrite in the groundwater. This study was undertaken to find out if there was any effect of starvation on the production of Met-Hb. We found that no change had occurred in the fish in a 96-hour period.

The statistical analysis of time series (tsp) by ordinary least squares for large fish is shown in Table 7. The fraction of observed variation in y_1 that is explained by the regression is 0.2131. The t-test showed 0.01 level of probability that an increase of the level of nitrite in the water increases the percentage of methemo-

TABLE 4

The effect of Ferri-Hb in steelhead trout fed with different levels of ascorbic acid

Level of ascorbic acid/test diet	Nitrite (mg/l)	Exposure time (h)	Mortality (%)	Ferri-Hb mean + SD (%)	Temperature Range (°C)	Mean length (cm)	Mean weight (gm)
o mg/kg of ascorbic acid	10	96	–	38.13 ± 9.7	10–1	15.3	38.8
200 mg/kg of ascorbic acid	10	96	–	24.4 ± 3.19	10–1	15.38	36
1 000 mg/kg of ascorbic acid	10	96	–	26.04 ± 4.04	10–1	15.3	30.9
2 000 mg/kg of ascorbic acid	10	96	–	26.3 ± 4.97	10–1	15.9	40.8

globin in the fish. The t-test for the independent variables ascorbic acid and temperature were not significant, and the t-test showed 0.2 of probability for both. Temperature had a negative estimated coeficient because we started the experiment with the highest temperature and lowest level of nitrite.

The tsp by ordinary least squares for small fish is shown in Table 8. The fraction explained by the regression is 0.4149.

The t-test showed the 0.01 level of probability significant possitive regressions for all three independent variables: nitrite concentration, ascorbic acid, and temperature of the water.

Residuals were plotted against dependent and each explanatory variable to see if there is any relationship between them. However, in the case of nitrite we found a definite tendency for the residual to increase with the level of nitrite. This fact is indicative of non homogeneous variances.

DISCUSSION

On the basis of the protective effect against nitrite toxicity, the level of ascorbic acid supplementation of 200 mg/kg appears to represent the appropriate requirement. Halver *et al.*, (1969) recommended that 200 mg/kg in the diet of trout and salmon raised in fresh water systems between 10–15 C would ensure reasonable tissue storage levels and furnish some excess for low level stress conditions. Sato *et al.*, (1978) observed that weight gains were improved by the addition of 200 mg/kg ascorbic acid to the diet. However, it is important to point out that the processing method of the feed is very important because certain conditions can cause the complete destruction of supplemental ascorbic acid (Hilton *et al.*, 1977).

The high levels of Met-Hb observed in control fish fed at 200 mg/kg ascorbic acid in our study differed from values reported by other researchers, Shterman (1970) reported Met-Hb levels of 2.7–39% for rainbow trout, and Cameron (1971) reported under "natural" conditions the percent of 2.9 Met-Hb for wild rainbow trout *(Salmo gairdneri);* however, he found 17% for hatchery reared rainbow trout. Perrone and Meade (1977) reported 20.9% Met-Hb for rainbow trout.

The results reported here describe a marked reduction in the toxicity of nitrite to small steelhead trout fed dietary ascorbic acid; in contrast, in larger trout under the same conditions, ascorbic acid only had a slight protective effect against nitrite toxicity. Smith and Williams (1974) reported that fingerling rainbow trout were less sensitive to nitrite toxicity than yearlings and Perrone and Meade (1977) reported that coho fry have a greater tolerance to nitrite than yearlings. In this study t-test value showed 0.01 probability that adding ascorbic acid to the diet consequently increased the tolerance of steelhead trout fingerlings to nitrite toxicity. The t-test showed 0.2 probability values of protective effect of ascorbic acid for large fish.

Cameron (1971) reported that ascorbic acid administered intravenously in rainbow trout reduced the amount of Met-Hb in the blood. In humans, ascorbic acid has been used successfully in the treatment of methemoglobinemia (Liany Frumusan, 1939).

The literature related to nitrite toxicity is conflictive, as indicated by studies carried out by Cameron (1971), Smith and Williams (1974), Russo *et al.*, (1974), Westin (1974) and Smith and Russo (1975). Their investigations have shown that nitrite becomes toxic at concentrations as low as 0.5 mg/l in Chinook salmon and

rainbow trout in fresh water. Nitrite tolerance is greater in water reuse systems, in brackish and in sea water systems used for rearing fish (Meade 1974, Crawford and Allen 1977). The information on nitrite toxicity in fresh water, brackish and sea water remains sparse and confusing, and it has to be related to the water chemistry of the system used.

Although the reduction in nitrite toxicity to steelhead trout in our bioassays is related with the increasing in ascorbic acid concentration in the basic diet, the possibiblity that another factor like the concentration of chloride and oxygen in the water, is also suggested. Lam Son (1977) reported that the increase of dissolved oxygen concentration increased the resistance of fingerling coho salmon to nitrite. Perrone and Meade (1977) indicated that nitrite toxicity is related to the concentration of chloride ion in the test system. Our bioassay water contained between 8.5–10 mg/l dissolved oxygen and 15.6 ± 1.5 mg/l chloride ion. Analyses are required to identify other protective factors that were beyond the scope of this study.

The results of the four 96-hour bioassays (Table 5) at temperatures of 10 C, 11 C, 8 C, and 10 C showed that nitrite became more toxic to steelhead trout fingerlings fed diets with and without added ascorbic acid when the temperature increased. The t-test statistic value for small fish showed 0.01 probability that as temperature increases, the percent of Met-Hb increased in the blood. From the same results it was shown that increases of ascorbic acid in the diet decreased the percentage of Met-Hb in the blood. The t-test statistic value for small fish showed 0.01 probability that as ascorbic acid increases in the diet, the percentage of Met-Hb in the blood of the fish decreases.

The data reported in Table 3, for large fish showed no significant results. The effects of temperature on nitrite toxicity to steelhead trout fingerlings was different from the observations made by Lam Son (1977). He found that nitrite becomes more toxic to coho salmon fingerlings at temperatures of 45 F and 60 F in comparison to 55 F.

The maximum level of Met-Hb observed in our test was 90% in a moribund fish. In general, severe stress was noted when Met-Hb reached 60–70% of total Hb. However, stress did occur at levels as low as 40–60% in both large and small fish. Small fish were able to tolerate very high levels without showing signs of stress, although their gills and blood were brown. Behavioral variations and differences in physical conditions within the two groups of fish may be reflected in the variances in the respective tolerance of individuals to elevated Ferri-Hb levels.

Stressed fish have a high degree of activity; consequently the demand of oxygen is greater, but the oxygen carrying capacity of the blood decreases because the percent of Met-Hb is too high. Fish in better physical conditions with high stores of ascorbic acid may respond more favorably to the stress, thus decreasing their activity, with the oxygen demand being less. Ascorbic acid plays a dual role. First ascorbic acid decreases the stress in the fish, and second, a biological reducing agent, it acts in the reduction of Met-Hb to Hb.

Another alternative for the stressed fish is to derive a greater portion of their energy requirement from anaerobic glycolysis.

The behavior of the fish fed with and without added dietary ascorbic acid showed differences in large fish at a level of 15 mg/l NO^-_2, the skin of 50% of the fish changed from a normal light color to dark brown. Small fish at a level of 15 mg/l did not suffer any stress and did not exhibit changes in the color of the skin, even though Met-Hb was found to be between 40.4–65.68%. At 20 mg/l NO^-_2 only small fish fed a diet without ascorbic acid turned dark and

TABLE 5

The effect of Ferri-Hb in steelhead trout fingerlings fed with different levels of ascorbic acid/96 h

Level of ascorbic acid/test diet	Nitrite (mg/l)	Exposure time (h)	Mortality (%)	Met-Hb mean + SD (%)	Temperature pH Range (°C)		Mean length (cm)	Mean weight (gm)
o mg/kg of ascorbic acid	10	96	–	28.18 ± 7.67	9–10	6.4	10.13	10.99
200 mg/kg of ascorbic acid	10	96	–	25.2 ± 3.79	9–10	6.5	9.54	9.4
1 000 mg/kg of ascorbic acid	10	96	–	22.6 ± 4	9–10	6.5	9.92	9.58
2 000 mg/kg of ascorbic acid	10	96	–	22.1 ± 4.3	9–10	6.4	10.51	11.76
o mg/kg of ascorbic acid	15	96	–	46.12 ± 14.68	10–11	6.4	9.2	7.86
200 mg/kg of ascorbic acid	15	96	–	48.35 ± 19.29	10–11	6.4	9.12	9.99
1 000 mg/kg of ascorbic acid	15	96	–	37.06 ± 10.13	10–11	6.4+0.1	8.69	6.42
2 000 mg/kg of ascorbic acid	15	96	–	36.37 ± 8.30	10–11	6.4+0.1	9.07	7.91
o mg/kg of ascorbic acid	20	96	–	44.38 ± 9.74	8–9	6.4+0.4	11.11	14.93
200 mg/kg of ascorbic acid	20	96	–	30.36 ± 5.95	8–9	6.5+0.3	11.55	16.53
1 000 mg/kg of ascorbic acid	20	96	–	26.08 ± 4.42	8–9	6.4+0.4	11.61	18.16
2 000 mg/kg of ascorbic acid	20	96	–	27.99 ± 4.7	8–9	6.4+0.4	12.06	18.56

o mg/kg of ascorbic acid	30	96	30	61.25 ± 10.05	9–10	6.2+0.2	11.5	15.24
200 mg/kg of ascorbic acid	30	96	20	48.41 ± 19.41	9–10	6.3+0.5	11.06	13.92
1 000 mg/kg of ascorbic acid	30	96	10	44.72 ± 21.50	9–10	6.2+0.6	10.25	10.84
2 000 mg/kg of ascorbic acid	30	96	10	40.60 ± 17.60	9–10	6.2+0.5	11.47	16.5

TABLE 6

The effect of Ferri-Hb in fingerlings of steelhead trout fed with different levels of ascorbic acid, without any treatment with nitrites

Level of ascorbic acid/test diet	Nitrite (mg/l)	Exposure time (h)	Mortality (%)	Ferri-Hb mean ± SD (%)	Temperature Range (°C)	pH	Mean length (cm)	Mean weight (gm)
o mg/kg of ascorbic acid	0.01	96	–	23.62 ± 3.13	9–1	6.7	9.95	9.77
200 mg/kg of ascorbic acid	0.01	96	–	23.37 ± 2.14	9–1	6.7	10	9.99
1 000 mg/kg of ascorbic acid	0.01	96	–	22.15 ± 0.55	9–1	6.8	9.44	8.37
2 000 mg/kg of ascorbic acid	0.01	96	–	20.96 ± 2.30	9–1	6.6	10.31	10.63

showed signs of stress at the end of 96 hours, with a 10% mortality. At 30 mg/l NO^-_2 fish with a high level of Met-Hb showed stress, and 50% of the skin changed from light to dark. In fish with a high percent of Met-Hb, the blood and gills were generally dark chocolate brown. Similar results have been reported by Konikoff (1975) in channel cat fish *(Ictalurus punctatus),* Lam Son (1977) with coho salmon (*Oncorhynchus* Kisutch), Smith and Williams (1974) with rainbow trout *(Salmo gairdneri)* and Chinook salmon *(Oncorhynchus tshawytscha).*

The mechanism of toxicity of nitrite to fish is not very clear. Through these bioassays with steelhead trout and from the observation made by Smith *et al.,* (1975) it is concluded that elevation of methemoglobin of fish exposed to nitrite should be one of the factors that produce stress and death of the fish. From the statistical analyses of time series by ordinary least squares for small fish, it was observed that the unexplained fraction by the regression represents 0.5851. Thus toxicity of nitrite in fish could be due to the combination of several factors like: 1) the increase of the methemoglobin in fish blood increases the affinity of the oxygen; 2) increases in methemoglobin in fish blood increase the degree of activity of the fish caused by stress, consequently fish required more oxygen; 3) nitrite itself is a toxicant. Smith and Williams (1974) reported that necrotic lesions and hemorrhage were consistently formed in the thymus gland of trout exposed to nitrite, and also pathological changes in the liver of trout exposed to 15 mg/l NO^-_2 N for 48 hours; 4) increases in environmental temperature increased nitrite toxicity in steelhead trout; 5) individuals in better physical condition can respond better to stress, reducing their activity, thus reducing oxygen requirement and respiration rates; 6) level of ascorbic acid and protein in the diet, a higher protein level increases the probability of elevating nitrite concentration in the environment due to bacterial action.

In summary, ascorbic acid is an essential vitamin for steelhead trout fed a practical diet; 200 mg/l is recomended as a minumum ascorbic acid level in the diet. However, the total requirement is dependent upon several factors related with the environment where the fish grow, such as quality of the water, temperature, dissolved oxygen. The concentration of ascorbic acid in the diet is also dependent upon feed storage conditions, such as: temperature, humidty, light, and moisture content of the diet.

TABLE 7

Ordinary least squares, dependent variable: methemoglobin

Right-hand Variable	Estimated Coefficient	Standard Error	T–Statistic
Intercept	32.441	8.582	3.78
Nitrite	1.171	0.230	5.08
Ascorbate	–0.002212	0.00144	–1.52
Temperature	–0.916	0.644	–1.42

R-Squared = 0.2131
Sum of squared residuals = 12729.7
Standard error of the regression = 11.1715
Sum of residuals = 0.326538E–02
Number of observations = 106
Mean of dependent variable = 28.1827
F-Statistic (3., 102.) = 9.20765

TABLE 8

Ordinary least squares, dependent variable: methemoglobin

Right-hand Variable	Estimated Coefficient	Standard Error	T–Statistic
Intercept	–44.83	12.88	–3.48
Nitrite	1.016	0.1152	8.82
Ascorbate	0.05902	0.001209	–4.88
Temperature	7.123	1.319	5.39

R-Squared = 0.4149
Sum of squared residuals = 21551.0
Standard error of the regression = 11.7915
Sum of residuals = 0.949097E–02
Number of observations = 159
Mean of dependent variable = 35.4576
F-Statistic (3., 155.) = 36.6413

RESUMEN

Grupos duplicados de truchas *(Salmo gairdneri),* fueron alimentados con 4 dietas conteniendo cada una de ellas diferentes niveles de ácido ascórbico. Cuando la concentración de ácido ascórbico fue alta, las truchas aumentaron su tolerancia a la toxicidad de nitritos disueltos en el agua.

Los experimentos con peces pequeños mostraron que cuando la temperatura aumenta, el porcentaje de metahemoglobina en la sangre del pez también aumenta.

Con igual concentración de ácido ascórbico en la dieta, los peces grandes son menos tolerantes que los pequeños a la toxicidad del nitrito disuelto en el agua. Posiblemente el ácido ascórbico actúa como agente reductor de la metahemoglobina a hemoglobina y agente protector de los peces en situaciones de "stress".

Un nivel de 200 mg/kg de ácido actúa como "seguro" en la confección de dietas prácticas para truchas.

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