Regulation of saturation and depletion of ascorbic acid in rainbow trout

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Rainbow trout (Oncorhynchus mykiss) *juveniles were fed fish meal-based diets supplemented with 0, 30, 60, 120, 240, and 480 mg ascorbic acid kg^{-1} dry diet or 15, 60, and 240 mg ascorbate monophosphate (expressed as ascorbate equivalents) kg^{-1}. Significant differences in both growth and instantaneous mortality rate between two compared strains (London, OH USA and Mount Shasta, CA USA) were found in fish fed the vitamin C-free diet. Deficiency symptoms included anorexia and lethargy, but no acute signs of lordosis or scoliosis were observed before heavy mortality after 9 weeks. Diets with supplemental ascorbate monophosphate supported significantly better growth and resulted in lower mortality than diets supplemented with equivalent amounts of ascorbic acid. Ascorbate concentrations in tissues (liver, kidney, and intestine) during the saturation period were higher in the groups fed 60 and 240 mg ascorbate monophosphate/kg than in groups supplemented with corresponding or higher levels of ascorbic acid. Significant differences in mortalities were experienced in fish with a different ascorbate status during the depletion phase. Kaplan-Meier analysis indicates that initial ascorbate level during the depletion phase can influence survival of fish. Analyses of ascorbate concentration during depletion revealed that the half-life ($t_{1/2}$) amounted to 42.8 to 50.2 days for the liver and 47.8 to 79.6 days for the kidney. These data imply that a conservation of tissue ascorbate is not operative in juvenile rainbow trout. We propose a two-compartmental model as describing ascorbate depletion pattern in juvenile rainbow trout.* (J. Nutr. Biochem. 5:204–212, 1994.)

Keywords: Vitamin C; ascorbic acid; ascorbate monophosphate; depletion kinetics; half-life; juvenile trout

Introduction

Previous studies have described the retention and depletion profiles of ascorbic acid in rainbow trout;[1,2] however, the half-life of the depletion phase ($t_{1/2}$) was not determined and experiments were performed with different sizes of fish. Hilton et al.[1] suggested that the ascorbic acid requirement is higher in young, small fish; this suggests the differences in the depletion and retention rate in tissues of fish during ontogeny. Tucker and Halver[3] used radiolabeled ascorbic acid and found that in rainbow trout weighing 250 g, the half-life of ascorbate in the liver and the kidney amounted to 28 and 17 days at 15° C, respectively.

Separate reports recently described the effect of the dietary ascorbate level on the depletion kinetics of ascorbic acid in guinea pigs of different ages[4] and attempted to explain the regulation of ascorbic acid metabolism based on the modulation of nutrient transporters at the intestinal level.[5] In a classic study, Penney and Zilva[6] concluded that the saturation or initial concentration of guinea pig tissues with ascorbate does not prolong the depletion time and, consequently, the appearance of scurvy. The down regulation of ascorbate uptake was, on the other hand, evident in adult guinea pigs fed a high-ascorbate diet, whereas in hypovitaminosis, the uptake of ascorbate was not changed.[5]

The purpose of this work was to determine retention and depletion profiles of ascorbic acid in juvenile rainbow trout and to analyze any inherent differences in the kinetics that may be due to dietary treatment or genetic variation. The retention and depletion profiles of ascorbic acid should be studied in tissues rather than in blood plasma,[7] because only specific tissues might reflect accurately the ascorbic acid concentration in respect to the appearance of scurvy. We also measured the survival of fish fed a scorbutic diet when

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Table 1 Formulation of basal diet fed to rainbow trout

Ingredient*	Concentration (g/kg dry diet)
Fish meal (menhaden 60% protein)	450
Corn gluten meal (60% protein)	120
Blood meal (spray dried)	100
Whey, dried	100
Dextrin	79
Cod liver oil	130
Lecithin (refined soybean)	1
Mineral mixture†	20
Vitamin mixture‡	5
Choline chloride (50%)	2

*Dietary ingredients were commercially obtained. Dextrin, cellulose, cod liver oil, and lecithin were from ICN Biomedical, Cleveland, OH USA. Cellulose provided bulk in vitamin and mineral mixtures.

†The mineral supplement provided the following (g/kg of dry diet) : $CaHPO_4 \times 2H_2O$ 3.75, $Ca\ CO_3$ 4.25, KH_2PO_4 3.5, Na_2CO_3 2, $MnSO_4 \times H_2O$ 0.088, $FeCl_3 \times 6H_2O$ 0.125, $MgSO_4$1.5, KIO_3 0.0025, $CuSO_4 \times 5H_2O$ 0.0075, $ZnCl_2$ 0.0375, $CoCl_2 \times$ 6H2O 0.0005, Na_3SeO_3 0.0005, $Na_2MoO_4 \times 2\ H_2O$ 0.002.

‡The vitamin supplement provided the following amounts per kg of dry diet: α-tocopheryl acetate 75 mg, biotin 2.5 mg, inositol 200 mg, niacin 5 mg, thiamin HCL 15 mg, Ca-pantothenate 50 mg, folic acid 5 mg, menadione 5 mg, pyridoxine HCL 7.5 mg, vitamin B12 0.02 mg, vitamin D_3 430 $\times$ 10^3 IU, vitamin A 15 mg, riboflavin (B_2) 12.5 mg. The vitamins were from F. Hoffmann-La Roche, Nutley, NJ USA.

The basal diet sums to 999 g. The remainder is accounted for vitamin C or dextrin.

it originated from a wide range of dietary ascorbate. By examining the depletion of tissue ascorbate and the accompanying mortality, we addressed the problem of the development of vitamin C dependency in fish. Using an animal model, we addressed a basic question relevant for the higher vertebrates, including humans.

Methods and materials

Materials

Rainbow trout *(Oncorhynchus mykiss)* strains from London Hatchery (Ohio USA) and from Mount Shasta Hatchery (California USA) were the subject of these studies. Eggs were obtained from a single pair of 3-year-old parents from each hatchery and were incubated in The Ohio State University aquaculture laboratory. Initial weight of fish taken to the experiment was 267 ± 62.7 mg and 320.2 ± 24.9 mg for London and Shasta strains, respectively. Fish were divided into 28 experimental groups of 100 fish each and stocked in 20-L tanks with a water flow of 0.5 to 1.0 L/min. Tanks were supplied with city water dechlorinated with carbon filters. After 6 weeks of the trial, to avoid overstocking, only 50 fish were left in each tank.

The composition of the diet was based on the formula given by Cho and Cowey[8] *(Table 1)*. Nine diets, supplemented with 0, 30, 60, 120, 240, and 480 mg of ascorbic acid (AA) or 15, 60, and 240 mg ascorbate monophosphate-magnesium salt (AMP) kg^{-1} dry diet were prepared. AA was supplied by F. Hoffmann-La Roche, Nutley, NJ, and AMP by Showa Denko, Tokyo. AMP amounts are expressed as ascorbate molar equivalents. After processing, (freeze-dried) feeds were stored at 0° C until use. Six groups of London

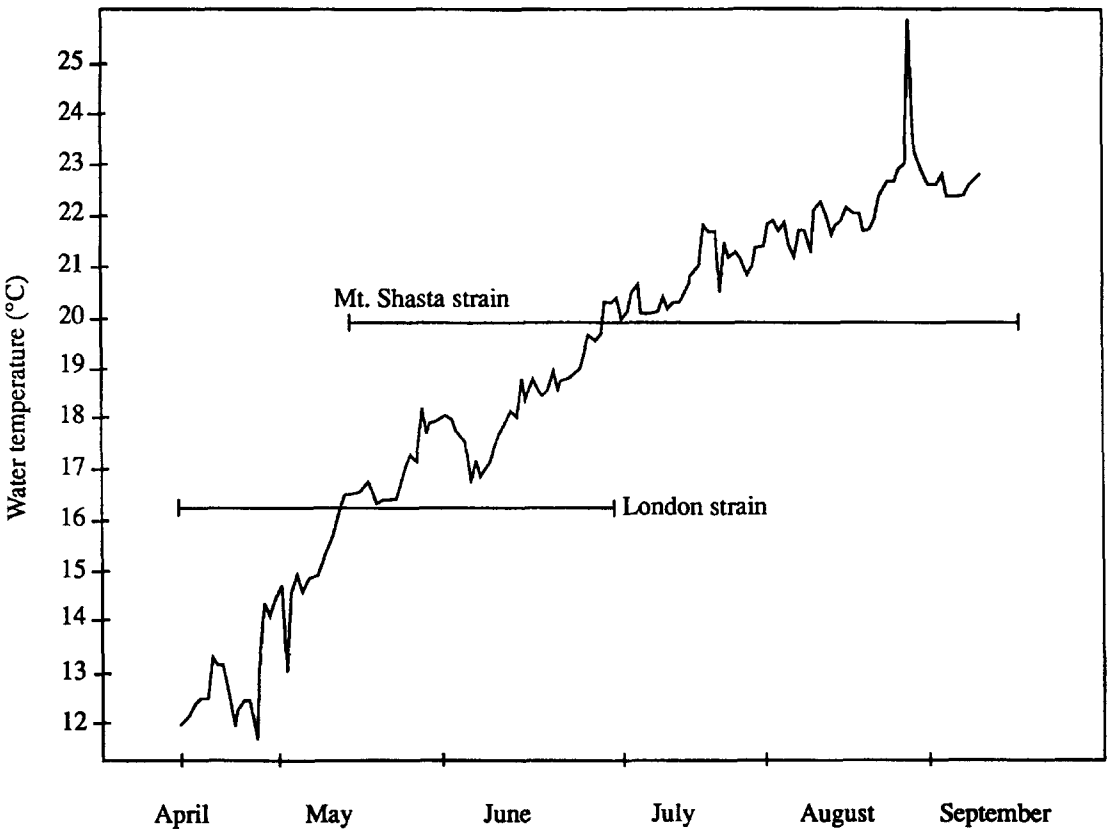

Figure 1 Mean water temperatures and duration of feeding (saturation-depletion) experiments with rainbow trout juveniles.

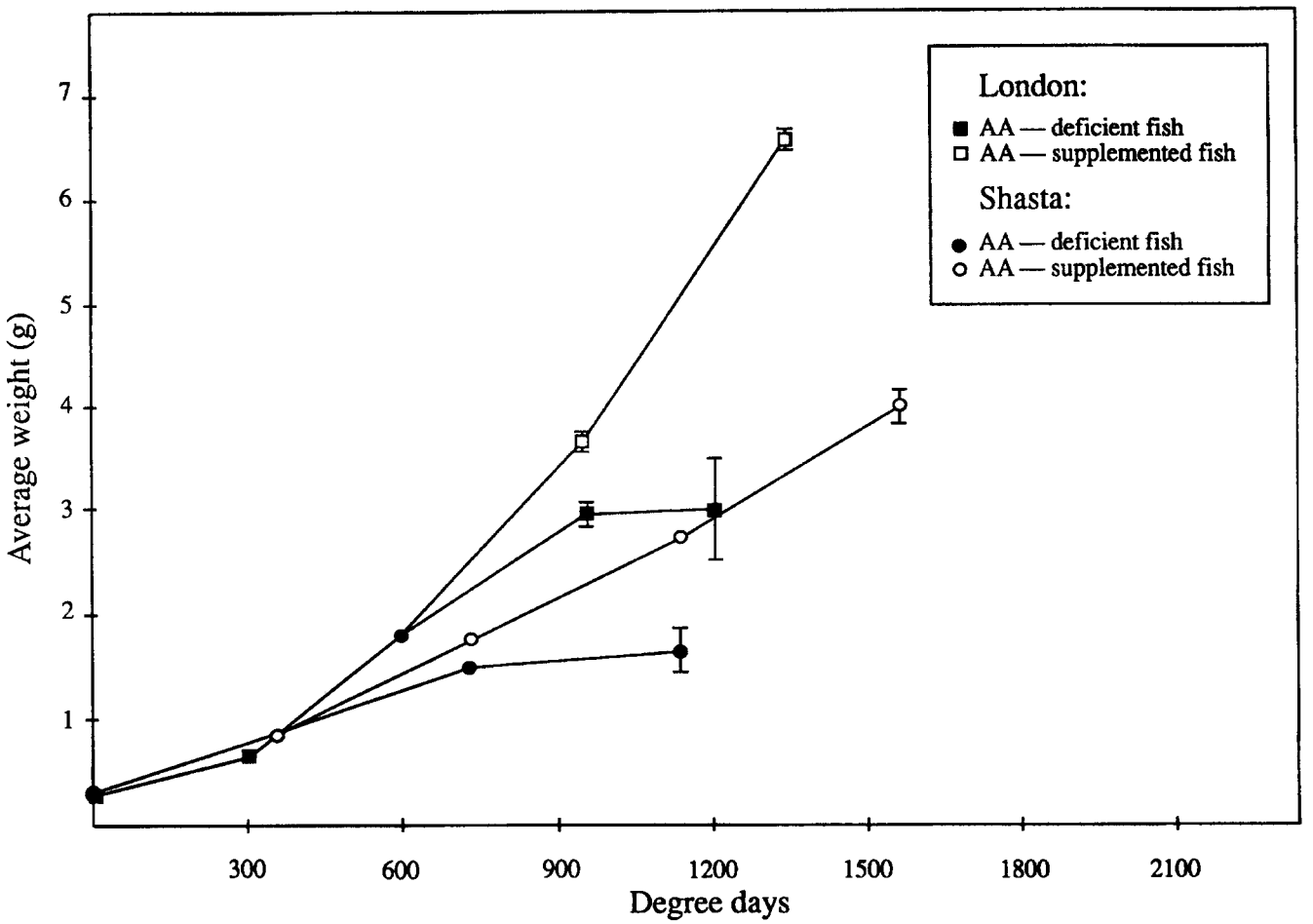

Figure 2 Average body weights of rainbow trout fed a scorbutic diet and 120 AA diet. The duration of the experiment is expressed as the sum of daily average water temperatures (degree-days). In this and other figures, when standard deviations are smaller than the symbols, they are not shown.

strain were formed: three fed ascorbate-free diet and three with diet supplemented with 120 mg AA kg^{-1}. In the experiment with the Mount Shasta strain, all nine diets were used, each fed to three groups, with the exception of an ascorbate-free diet, which was fed to four groups of fish. Fish were fed 7 days a week, three times a day. The daily feeding level was 2 to 6.5% of body weight, depending on body weight and water temperature changes *(Figure 1)*.

The saturation phase of the experiment was conducted for 18 and 12 weeks for the Mt. Shasta and the London strains, respectively. Afterwards, fish were the subject of the depletion phase of experiment for 7 and 6 weeks, respectively. Average body weights were determined every 3 weeks throughout the experiment by weighing the entire group and counting fish. Mortalities were recorded daily. For ascorbate concentration analysis, three fish per tank were sampled every 3 weeks during the saturation phase and each week during the depletion phase after 24 hours of food deprivation. They were anesthetized with MS 222 (50 to 100 mg/L) water solution and weighed. Afterwards, the whole fish were quickly frozen in liquid nitrogen and stored at −82° C for further analysis. Perichloric acid and EDTA were purchased from GFS Chemicals, Columbus, OH, USA. Potassium phosphate, monobasic salt was obtained from J.S. Baker Inc., Philipsburg, NJ, USA and MS 222 from Argent Chemical Labs, Redmond, WA, USA. All other chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA unless stated differently in the text.

Ascorbate analysis

Dissected tissue or whole fish were homogenized in 50 g/L trichloroacetic acid (TCA) in 250 mmol/L $HClO_4$ containing 0.8 g/L EDTA using an Omni 5000 homogenizer, and centrifuged at 29,000 *g* for 30 min in a J2-21 Beckmann centrifuge at 4° C. Supernatants were tested for total ascorbic acid using the dinitrophenylhydrazine (DNPH) method modified by Dabrowski and Hinterleitner.[9] Diets were homogenized in H_2O, centrifuged at 29,000 *g* for 30 minutes at 4° C and tested for AMP by the high performance liquid chromatography (HPLC) method described by Showa Denko.[10] KH_2PO_4NaCl as a mobile phase was used at a flow rate of 0.7 mL/min. AMP was detected by the UV detector at 257 nm. The actual monophosphate concentration in the diets determined using the HPLC method was found to be 17.35, 71.08, and 354.07 mg/kg.

Mortality and survival analysis

Instantaneous mortality rates were calculated using the formula: $Z = \ln N_t - \ln N_0)/t$ (where N_t equals number of fish at the end of the experiment period, N_0 equals number of fish at the beginning of

the experiment period, and t equals duration of the period in days.[11] Estimates of survivorship functions ($\hat{S}(t)$) were constructed according to product-limit method developed by Kaplan and Meier:[12]

$$\hat{S}(t) = \pi^{1}(n-r)/(n-r+1)$$
$$t_r \leq t$$

where n is the total number of fish with known survival time, censored or not. All n survival times are indexed in order of increasing magnitude such that $t_1 \leq t_2 \leq \ldots \leq t_n$ and r runs through those indices for which $t_r \leq t$ and t_r is uncensored.[13]

Statistical analysis and pharmacokinetics

For the purpose of statistical analysis, each tank was assigned one degree of freedom. The differences among groups were compared using analysis of variance (ANOVA) at a level of statistical significance $P < 0.05$. Influence of diet on survival was tested by Kruskal-Wallis K-sample test for censored data, followed by Gehan's generalized Wilcoxon test to compare Kaplan-Meier survivorship functions ($\hat{S}(t)$) of various groups.

Pharmacokinetic parameters and $t_{1/2}$ were calculated according to Blanchard et al.[14] We assumed the one compartment model as described by the formula $C(t) = C_o\exp(-kt)$, where C(t) equals tissue ascorbate concentration, C_o equals initial concentration, k equals decay coefficient, and t equals time in days; and two-compartment model as $C(t) = C_o(t)\exp(-kt) + C_o'(t)\exp(-k't)$, where C_o and C_o' equal initial model concentrations, k,k′ equals decay coefficients, and t equals time in days. The formula $t_{1/2} = \ln 2/-k$ was applied to calculate half-life time. A Marquard's algorithm[15] for nonlinear regression was employed to estimate

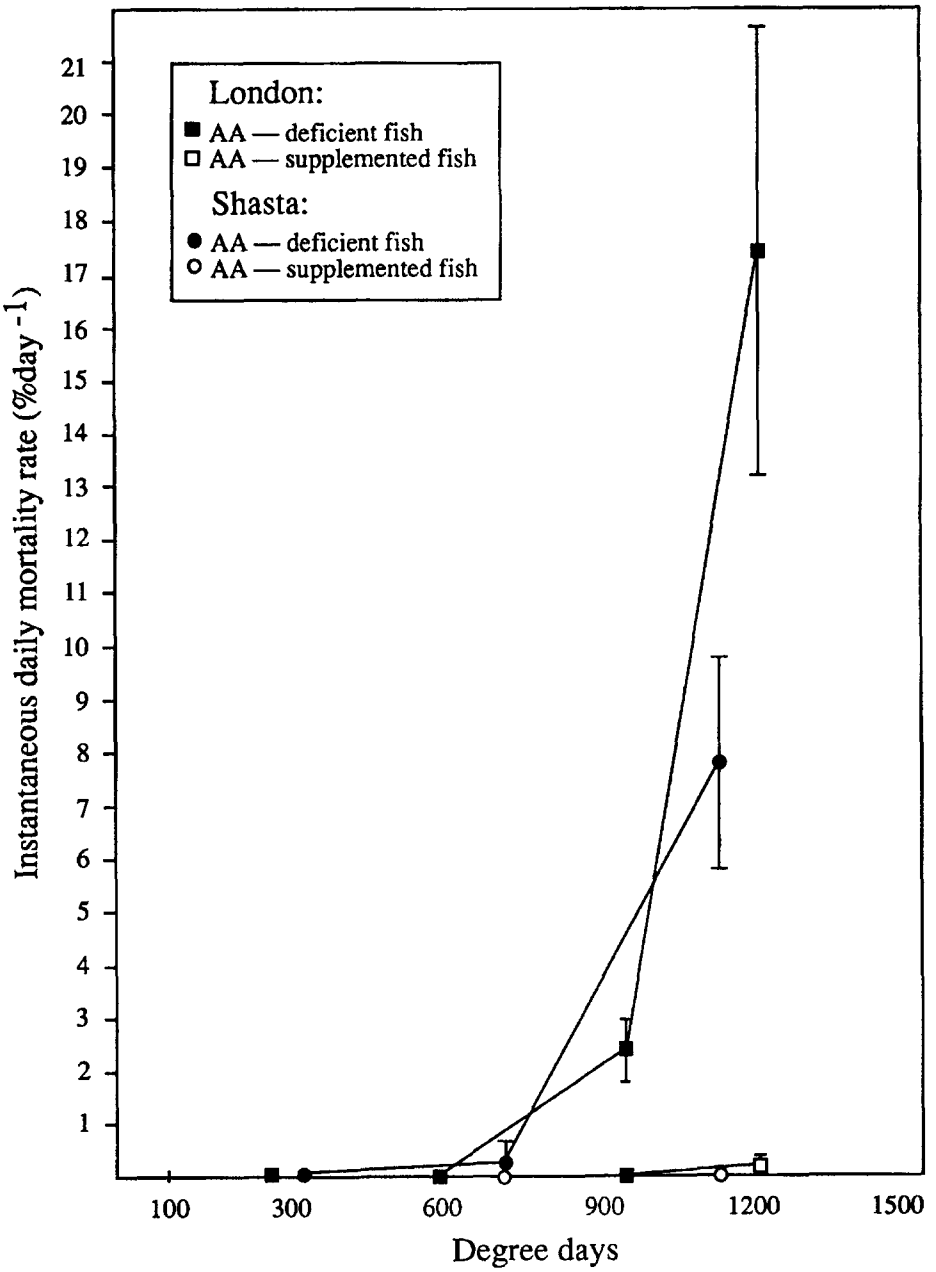

Figure 3 Mortality rates of rainbow trout juveniles of two strains fed a scorbutic or AA supplemented (120 mg/kg) diet.

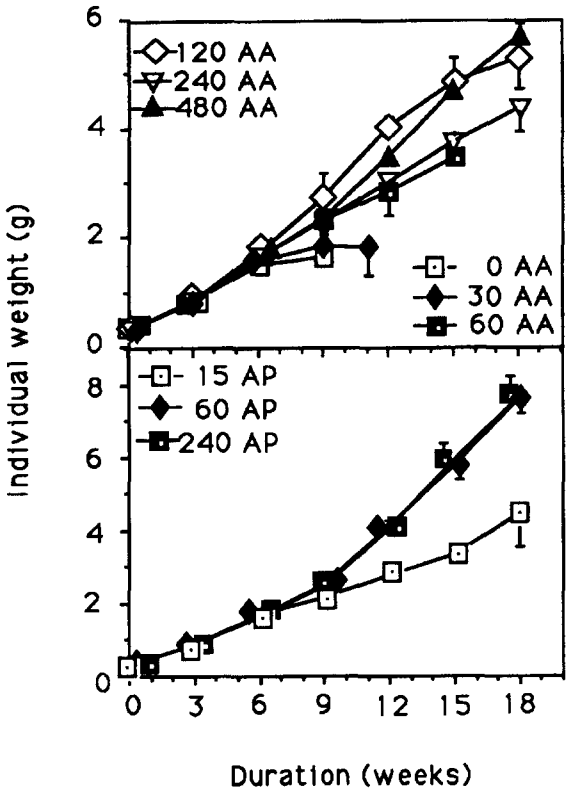

Figure 4 Average body weights of Mt. Shasta rainbow trout fed a diet devoid of (0 AA) or supplemented with AA or AP.

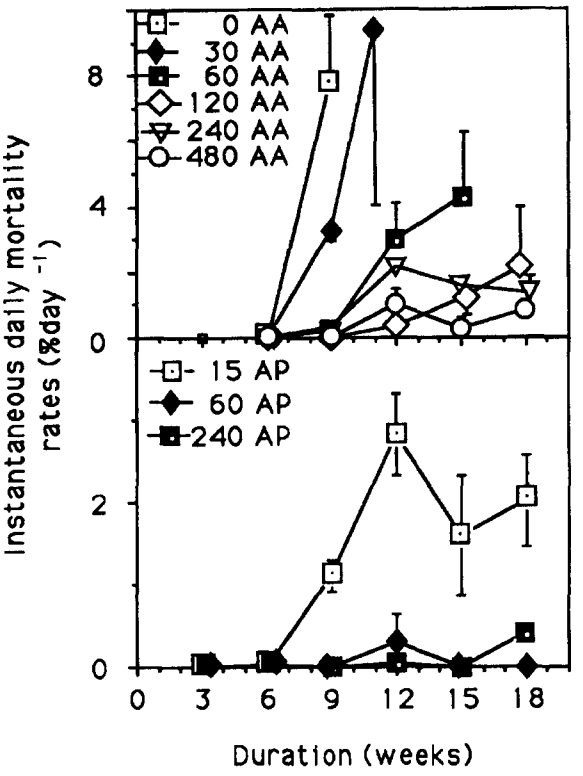

Figure 5 Mortality rates of the Mt. Shasta rainbow trout strain fed nine experimental diets.

parameters of the depletion models from the experimental data. F-ratio test and R^2 coefficient were used as the indicators of model fitness.

Results

Figure 1 illustrates the evolution of water temperature during the experiments for the London and the Mt. Shasta strains. To minimize the effect of water temperature for comparison of the performance of two strains, degree-days were introduced as the measure of thermal history. This expression allows us to compare poikilothermic fish across the physiological range of temperatures more precisely. Significant differences ($P < 0.05$) between strains in body weights of both supplemented groups (120 mg AA kg^{-1}) and groups fed a scorbutic diet were found *(Figure 2)*. There were no differences ($P < 0.05$) in body ascorbate concentration at the beginning of the trial. Mt. Shasta strain fish offered an ascorbate-free diet ceased growth earlier, but the mortality rate was higher in the London strain fish ($17.4 \pm 4.2\%$ day^{-1} for London versus $7.8 \pm 2.0\%$ day^{-1} for Mt. Shasta) *(Figure 3)*. The water temperature was closer to optimum for the growth of rainbow trout (17.2° C)[16] during the London strain experiment than during the Mt. Shasta strain experiment. Therefore, the growth rate differences seemed to be inherent to the strains. A higher growth rate resulted in a higher demand for ascorbate.

After 9 weeks, groups supplemented with 15 mg AMP and less than 60 mg AA kg^{-1} had significantly lower body weights (1.83 ± 0.23 g for 30 mg AA, and 2.31 ± 0.19 g and higher for 60 and above mg AA) *(Figure 4)* and higher mortality rates ($1.1 \pm 0.18\%$ day^{-1} for 15 mg AMP, $0.23 \pm 0.11\%$ day^{-1} for 60 mg AA, and 0 for 120, 480 mg AA and 60, 240 mg AMP) *(Figure 5)* than groups fed diets with 120 mg AA or 60 mg AMP kg^{-1} and over. Severe mortalities (over 8% day^{-1}) in groups fed 0 and 30 mg AA kg^{-1} eliminated them from the experiment after 9 and 11 weeks, respectively. Deficiency symptoms included anorexia and lethargy, but no acute signs of lordosis or scoliosis were observed before heavy mortalities.

Body and tissue ascorbic acid concentrations are presented in *Table 2 and Figure 6*, respectively.

The responsiveness of the daily mortality rate to the depletion phase of the experiment is illustrated in *Figure 7A*. In the groups that received the diets supplemented with 120, 240, or 480 mg AA kg^{-1} at the moment of entry into the depletion phase (18th week), the length of survival was not more than 25 days *(Figure 7B)*. On the contrary, none of the fish fed 60 or 240 mg AMP kg^{-1} died during this time. At the end of the depletion period (50 days) fish on the 240 mg AMP diet suffered 25% mortality.

Exposure of fish to a scorbutic diet produced a variable response in tissue ascorbate concentration *(Figure 8)*. Initial concentration at the start of desaturation determined the rate of ascorbate depletion.

Tissue ascorbic acid concentrations during the depletion phase were analyzed by nonlinear regressions. The half-lives ($t_{1/2}$) for the liver, the kidney, and the intestine are listed in *Table 3*. These analyses resulted in $t_{1/2}$ ranging from 42.8 to 50.2 days for the liver, 47.8 to 79.6 for the kidney, and

Table 2 Total ascorbate concentration (pmol/g wet tissue) in the whole body of Mt. Shasta strain fish

Duration of experiment	Diet number							
	0 AA	60 AA	120 AA	240 AA	480 AA	15 AMP	60 AMP	240 AMP
	Tissue AA concentration pmol/g wet mass*							
3 weeks	56 ± 6^{a}	74 ± 10^{ab}	106 ± 28^{ab}	90 ± 30^{ab}	88 ± 25^{ab}	68 ± 17^{a}	123 ± 18^{b}	309 ± 18^{c}
6 weeks	—	43 ± 9^{ace}	57 ± 4^{a}	45 ± 3^{c}	67 ± 3^{b}	41 ± 10^{e}	75 ± 7^{b}	308 ± 54^{d}
9 weeks	—	33 ± 4^{a}	52 ± 6^{b}	35 ± 4^{ac}	56 ± 10^{b}	37 ± 39^{ab}	61 ± 5^{bc}	183 ± 14^{d}

Initial concentration of ascorbate in the whole body was 87 ± 10 pmol/g wet mass (n = 3).
AA, ascorbic acid; AMP, ascorbate monophosphate.
*Value means ± SD.
n = 3 for all results.
Within rows, values that have no letters in common are significantly different, $P < 0.05$.

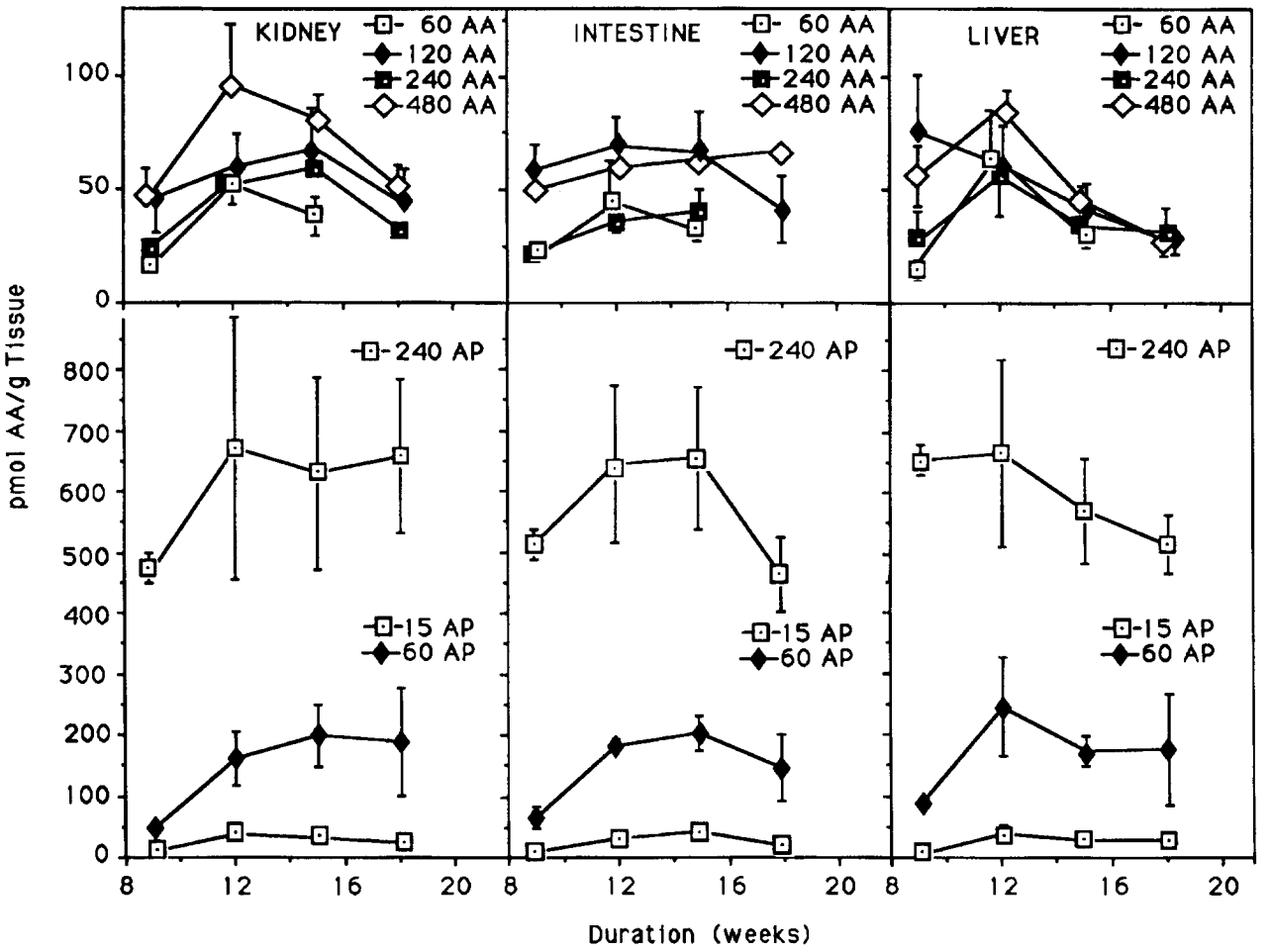

Figure 6 Effects of dietary level and form (AA or AP) of ascorbate on tissue concentrations of total ascorbic acid during the saturation experiment with the Mt. Shasta strain.

49.9 to 59.2 for the intestine. There were no significant differences in $t_{1/2}$ between strains of rainbow trout. In constructed depletion models, R^2 coefficients were higher for two-compartmental model (0.58 to 0.91) than for the one-compartmental model (0.52 to 0.85) for all tissues and all diets. Also, the F-ratio test indicated that the second exponential component contributed to the fitness of the model.

Discussion

Genetic variation and dietary treatment are among factors that may influence ascorbate metabolism in both fish and higher vertebrates. Vitamin C deficiency with scurvy is caused by a hereditary deficiency in gulonolactone oxidase activity, the final enzyme in vitamin C synthesis.[17] In rainbow

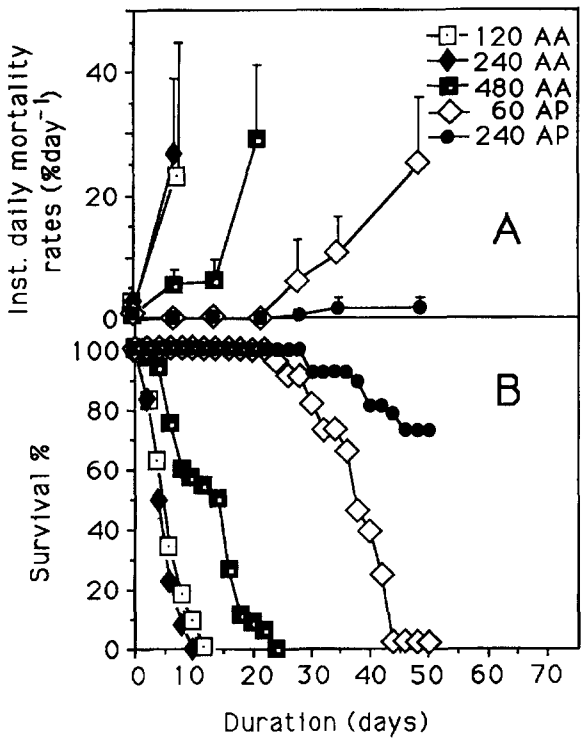

Figure 7 Mortality rates of rainbow trout during the depletion phase of the experiment. All groups were fed ascorbate-free diets; the treatments refer to a diet prior to this experiment. Survival was calculated according to the Kaplan-Meier model.

trout, the susceptibility to spinal deformity was found heritable by McKay and Gjerde,[18] leading them to speculate that the nutritional requirement may vary among strains of salmonid fish. To study how the genetic variation can affect ascorbate status, we compared two strains, each strain from a single pair of parents, in their responses to an ascorbate deficiency. Fish of the Mt. Shasta strain cease growth earlier and have lower mortality rates than the London strain when fed an ascorbate-free diet. This suggests that the growth rate is inherent to the strains and leads to earlier or delayed scurvy signs. We did not observe in either of the strains acute scurvy symptoms such as lordosis or scoliosis.[19] We did observe, however, anorexia, sometimes accompanied by exopthalmia and/or dark coloration. This can be explained by the fact that juvenile fish with a fast growth rate suffer from scurvy, but they die before full symptoms, including scoliosis and lordosis, can be developed.

While there is much research concerning the ascorbate requirement in rainbow trout bigger than those used in the present study, only Hilton et al.[1] suggested that the ascorbate requirement might be higher in young fish. Studies on guinea pigs showed that young guinea pigs have a higher ascorbate concentration in most tissues and different depletion patterns of ascorbate than adult guinea pigs.[4] Yet Blanchard et al.[7,14] did not find an essential difference in plasma and urine concentration between young and elderly humans during their vitamin C depletion study. This study was conducted on adults (in their twenties and sixties) and addressed more a question of aging than a stage of development. An animal vertebrate model seems to be excellent for following ascorbate metabolism throughout all the stages of life. Results of such a study, in which the ascorbate concentration in critical tissues—the liver, the kidney, the intestine—can be traced with an animal model, offers a unique opportunity to understand the ascorbate requirement during rapid growth. Karasov et al.[5] performed several experiments with intestinal ascorbate uptake in adult and young guinea pigs and concluded that the down regulation after switching from a high to a low ascorbate diet varies with age and therefore vitamin C dependency is unlikely. However, the intestinal regulation is only a minor step in determining the ascorbate status in tissues. In human subjects offered an ascorbate dose of 1 g day^{-1}, 75% of the ascorbate is excreted in the urine.[20] Therefore, in subsequent studies of ascorbate's metabolic role, tissue concentration, saturation, and depletion rate should be given the major priority. Berger et al.[21] demonstrated that when plasma was not yet saturated at 9.45 mmol/L ascorbate, the liver and kidney concentrations reached a plateau at 3.78 mmol/g tissue. Penney and Zilva[6] showed that the slopes of ascorbate disappearance were similar for all tissues, with the exception of the blood (slower rate). This was attributed to the "flushing" of ascorbate from tissues. It shows how an inaccurate picture might be obtained in the study where plasma concentration or intestinal uptake only are looked at. It stresses the importance of using animal models with the life stages differing by size, growth rate, and metabolic rate.

The instability of ascorbic acid in fish feeds leads to the necessity of its higher doses to assure the safety level in the diet or the use of a stable form like ascorbate phosphate. Our results with juvenile rainbow trout confirm earlier results with catfish[22] and yellowtail.[23] As in other fish species,[2,22-24] the ascorbyl phosphate supplement resulted in better growth and a lower mortality rate, as well as in higher tissue ascorbate concentration than in fish supplemented with equimolar amounts of ascorbic acid *(Figure 6)*. However, tissue ascorbate concentrations in rainbow trout in the present study were lower than reported previously by Johnston et al.[2] at the same range of the dietary ascorbate supplementation. This difference was most likely caused by the use of casein as the dietary protein source, resulting in higher growth rate of fish; thus, higher ascorbate supplementation is required to gain the same tissue concentration. Dabrowska et al.[25] indicated that the differences in growth rate of common carp fed an ascorbate-deficient diet resulted in a variable level of tissue ascorbate depletion. It seems, therefore, that the requirement for ascorbate is directly related to the metabolic rate in poikilotherms.

During the depletion phase of the experiment *(Figures 7 and 8)*, fish fed a diet supplemented with AMP, the equivalent of 240 mg AA, had a 100% chance of surviving for about 30 days while only 50% of those on a diet supplemented with a two-fold higher level of free ascorbic acid survived after 15 days. Survival during the depletion phase was dependent on the supplementation dose during the saturation phase. These results are contrary to the notion that the saturation of guinea pig tissues with ascorbate does not prolong the period of survival of the animal on a scorbutic diet.[6] We document the case with the rainbow trout model in which the increased mortality does bear a definite relationship to the concentration of ascorbate in tissues at the initiation of

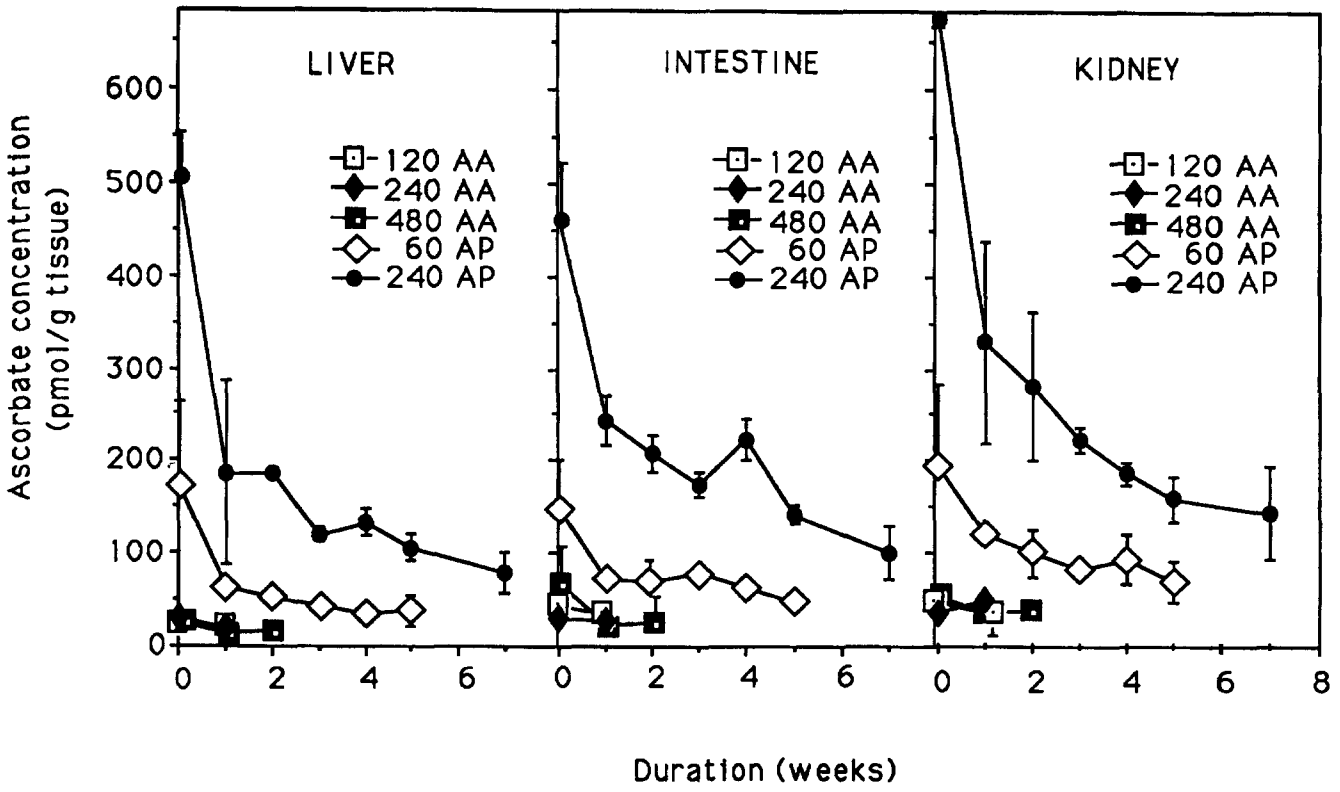

Figure 8 Effect of switching the experimental diet to a scorbutic diet on the tissue ascorbate concentration in juvenile rainbow trout. Group treatments refer to diets prior to the depletion phase.

Table 3 Half-life times of ascorbic acid in tissues estimated by one- and two-compartmental models

Tissue	Diet	Initial tissue AA concentration ($\mu g\ g^{-1}$)	Half-life times: one compartmental model	Half-life times: two compartmental model $t_{(1/2)1}$	Half-life times: two compartmental model $t_{(1/2)2}$
Mt. Shasta strain					
Liver	120 AMP	30.76	50.23	2.17	55.23
Liver	480 AMP	68.99	42.79	0.09	34.13
Intestine	120 AMP	25.66	59.24	0.21	59.65
Intestine	480 AMP	67.00	51.73	0.04	38.92
Kidney	120 AMP	33.82	79.62	2.77	55.04
Kidney	480 AMP	95.86	47.80	2.06	35.01
London strain					
Liver	120 AA	20.91	44.72	4.18	54.42
Intestine	120 AA	17.54	49.87	9.32	38.49
Kidney	120 AA	23.51	55.75	0.09	35.81

Values of $t_{1/2}$ expressed in days in two-compartmental models refer to the initial period of 1 to 9 days and the second depletion period of a much longer time.

avitaminosis *(Figures 7 and 8)*. In earlier studies, it was demonstrated that ascorbyl sulfate is not stored in the tissues of rainbow trout,[26,27] and its biosynthesis in mammals is negligible.[28] Therefore, the present results cannot be explained by the release of "stored" tissue ascorbate.[29]

In most studies,[14,30] the one-compartment model for ascorbate turnover is proposed based on the half-life of this compound. Both the R^2 and F ratio tests for one- and two-compartment regression models constructed for our results favor the two-compartment models as more accurate. However, the limited number of measurements during the first days of the depletion study produced a wide confidence range of the decay coefficient. Factors k and k′ approach a value close to 0, and because they appear in the denominator,

small differences among them produce big differences between $t_{1/2}$ for different tissues. Zloch and Ginter[4] proposed two-phase depletion models for juvenile guinea pigs. The half-life of ascorbate in young guinea pig livers and kidneys was estimated to be 5.9 and 3.4 days, respectively. Two-compartment models in the early life stages change to a one-compartment in the course of the development of the guinea pig. Our results with juvenile rainbow trout corroborate that finding. Adult rainbow trout growing at a slower rate may have a different pattern, corresponding to a one-compartment model. Blanchard's[7] study involved both young and elderly adult humans, and possibly the same two-compartment model for children might be a more accurate approximation. This difference in the first response to an ascorbate deficiency between juvenile and older individuals may be caused by changes in a regulatory mechanism in ascorbate metabolism. If this is a general pattern, the questions remain as to when and why such a change occurs.

Acknowledgments

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