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Primitive Actinopterigian fishes can synthesize ascorbic acid

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Abstract. Amphibians and reptiles evolved with the capacity to synthesize ascorbic acid. Some higher vertebrates, like bats, guinea pigs, primates, and humans have lost the microsomal enzyme gulonolactone oxidase, and in cases of ascorbic acid deficiency suffer from symptoms of scurvy. The question of whether the capacity to synthesize ascorbate is also present in lower vertebrates could throw light on the evolution of this pathway. In order to find out whether ascorbic acid synthesis took place in two primitive Actinopterigian fish, the paddlefish (*Polyodon spathula*) and the white sturgeon (*Acipenser transmontanus*) were fed with a scorbutogenic diet or diet(s) supplemented with a graded level of ascorbic acid. We found no growth depression nor external symptoms of scurvy, which would be pronounced in modern bony fishes (*Teleostei*) under similar conditions. The tissue level of ascorbate in both these primitive species indicated that vitamin C in intestine and liver is not depleted when fed a scorbutogenic diet. Gulonolactone oxidase activity was found in the kidneys of the Actinopterigian fishes. Thus, I question the accepted evolutionary pathway for ascorbic acid biosynthesis in lower vertebrates and suggest that the modern bony fishes, *Teleostei*, lost their ability to express the gulonolactone oxidase genes after they had separated during the Silurian from their common ancestor with the coelacanths (*Latimeria*) and Dipnoi.
Key words. Vitamin C; sturgeon; gulonolactone oxidase; teleost; *Chondrostei*; *Acipenser*.

In its final step, ascorbic acid (vitamin C) synthesis is catalyzed by the enzyme gulonolactone oxidase[1–3]. This enzyme is missing in humans, other primates[4], guinea pigs and bats[1,2,5], and consequently, these animals and humans require a dietary intake of vitamin C to prevent scurvy. Teleost fish, which represent the most numerous vertebrate taxonomic group on earth, also seem to be unable to synthesize ascorbic acid[3], and dietary deficiency results in overt signs of scurvy, biochemical disorders, and mortality[6–8]. Some earlier reports[1,9] suggested that the biosynthesis of vitamin C first developed in the kidneys of amphibians; however, a report on the synthesis of ascorbic acid in Australian lungfish[10], a representative of fishes which diverged during the Silurian period from the common ancestor of modern bony fishes, indicated that a re-examination of the primitive fishes, *Chondrichthyes*, *Holostei* and *Chondrostei* should be undertaken.

Materials and methods

Two representatives of North American *Chondrostei*, the paddlefish (*Polyodon spathula*) and the white sturgeon (*Acipenser transmontanus*) were investigated in a series of dietary experiments; gulonolactone oxidase was assayed to confirm the results on the subcellular level with an additional species, the lake sturgeon (*A. fulvescens*).

The feeding trial was conducted with juvenile paddlefish of 0.45 ± 0.21 g, distributed in conical fiberglass tanks (60 l capacity) and supplied with dechlorinated city water (0.5 l min^{-1}) at 21 ± 0.5 °C. Two diets were tested in triplicate for 70 days with 21 fish per tank. The diets contained fish meal as a major component[11]. The first diet was devoid of ascorbic acid, and diet 2 was supplemented with ascorbic acid at 120 ppm (F. Hoffmann La Roche, Nutley, NJ, USA). Diets were pelleted in a laboratory mill and freeze-dried to avoid ascorbate degradation. The ascorbate analysis[12] of diet 2 revealed 62 ppm. Fish were fed ad libitum and periodically weighed. After 70 days, 4–5 fish per treatment were sacrificed and total ascorbic acid and dehydroascorbate[12] were analyzed in the liver and posterior intestine (rectum).

Juvenile white sturgeon (size 4.0 ± 0.24 g) were divided between 15 tanks which held 15 fish each, and given the experimental diets for 113 days. Fish were fed twice daily with diets of fish meal[11] and the feeding rate was adjusted to 2% b. wt after periodical weighing. Circular tanks (50 l each) with a continuous flow of water (16–18 °C) were used. An ascorbate monophosphate Mg salt derivative (Showa Denko America, NY, USA) was used as the supplement because of its heat resistance and excellent bioavailability to the fish[11,13]. Diets (# 2–5) were supplemented with 13.0, 47.6, 95.3, 190.5, and 376 ppm ascorbic acid equivalents. To avoid the effect of stress after weighing, after 120 days, 3–5 fish per group were sacrificed and tissues individually dissected to analyze total ascorbate and dehydroascorbate, as described earlier[12]. To avoid large variations among

individuals within a group and to obtain an 8-fold b. wt increase in anticipation of scurvy signs, the largest fish from all treatments were chosen for tissue analysis. The average body weights (g) in group (diet #) 1–5 were: 29.1 ± 7.1 (n = 4), 36.7 ± 6.2 (n = 3), 39.0 ± 7.8 (n = 5), 36.2 ± 3.1 (n = 3), and 38.8 ± 0.6 (n = 3), respectively. This approach strengthens the value of tissue ascorbate analysis because the b. wt gain was close to 8–10-fold, which is considered to be sufficient for signs of ascorbate deficiency to be manifested in teleost fish[7,14].

Tissues were homogenized fresh (white sturgeon) or after storage at −82 °C (paddlefish and lake sturgeon, *Acipenser fulvescens*) in phosphate buffer, and assay conditions were as described earlier[3] for both colorimetric ascorbate determination and direct assay. The insert in figure 3 shows the absorbance spectra of kidney preparation from white sturgeon in the direct spectrophotometric assay recorded every 10 min. Final assay conditions included 20 mM L-gulonolactone, 5 mM glutathione in 0.1 M phosphate buffer (pH 7.4) and an increase in absorbance at 265 nm was measured (A_{256} = 18.13 per l mM/cm^2). The assay based on colorimetric determination of ascorbate formed[3] produced very similar results.

Results and discussion

Neither growth depression nor increased mortality (below 5%) was found when the paddlefish (fig. 1) and the white sturgeon (fig. 2) were fed a scorbutogenic diet for 70 and 120 days, respectively. The final weights of white sturgeon were 16.0 ± 1.4, 14.3 ± 3.1, 21.6 ± 6.0, 19.2 ± 4.1 and 20.9 ± 5.7 g for diets # 1–5, respectively.

There was no indication of declining ascorbate concentration in the tissues of fish fed a diet devoid of ascorbic acid. This finding contrasts with experiments on teleost fish, where the deficiency in dietary ascorbate resulted in a depletion in tissue ascorbate because the catabolic rate of body ascorbate, expressed as the half-life of body ascorbic acid, was 4–10 days[6,11]. Differences were only significant among the groups when they were fed diets devoid of ascorbate as supplemented with the high level (fig. 2). This is in contrast to the teleost fish, rainbow trout, where an increase in dietary ascorbate resulted in a linear increase in the tissues[7,8,13]. However, the situation in white sturgeons is similar to the response of ascorbate-synthesizing mammals, such as mice[15], where only high doses of dietary ascorbate increased the tissue storage level of vitamin C. These results suggest that the exogenous, dietary ascorbate interacts with the elimination and/or biosynthesis of this compound in white sturgeon, and a control feedback mechansim might be present. Furthermore, the activities of ascorbate synthesizing enzyme in the mouse liver[16] were not influenced by an exogenous ascorbate intake as high as 1–5% of the diet. Similarly, gulonolactone oxidase activity in the kidney of white sturgeon from groups 1 and 5 (data not shown) did not differ significantly.

Under conditions used in the present studies, the enzyme reaction monitored by the change of absorbance at 265 nm remained linear for at least 2 h at 25 °C (fig. 3, insert). The synthesis rates shown for *Chondrostei* correspond on the rate of synthesis in kidney of an amphibian and bird, and in mammalian liver (fig. 3).

The literature on the transfer of ascorbic acid through the aquatic food chain indicates[6,11] that the tissue is variable in *Teleostei* fish and reflects fluctuations in dietary intake. The ascorbic acid concentration in microalgae eaten by zooplankton (rotifer, copepods and cladocerans) is very high (0.11–1.62%)[17] and thus provides a rich source to fish feeding on zooplankton[18]. Ecological transfer of ascorbate in the phytoplankton–zooplankton–fish food chain has been only fragmentarily examined and amounted to 5–20% (ref. 6). Alterations of ascorbate transfer may cause some changes in ecosystems, particularly in situations with environmental stressors or an ontogenic shift to detritivorous feeding[19]. My finding of ascorbate synthesis in

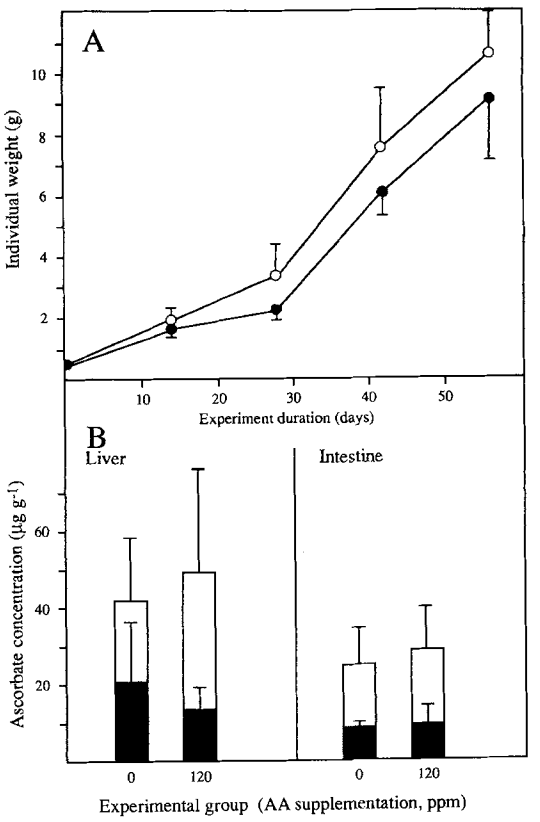

Figure 1. *A* Growth of paddlefish fed a scorbutogenic diet (–●–) or diet supplemented with ascorbic acid (120 ppm) (–○–). No significant difference in the final body weight or mortality was noticed. The weight gain was 19- and 23-fold (for fish fed non-supplemented and AA-supplemented diets, respectively) after 56 days of feeding.

B Concentration of the dehydroascorbate (filled columns) and the total ascorbate (blank columns) in liver and intestine of fish fed diets with and without ascorbic acid supplementation. Vertical bar represents SD of 3–5 animals.

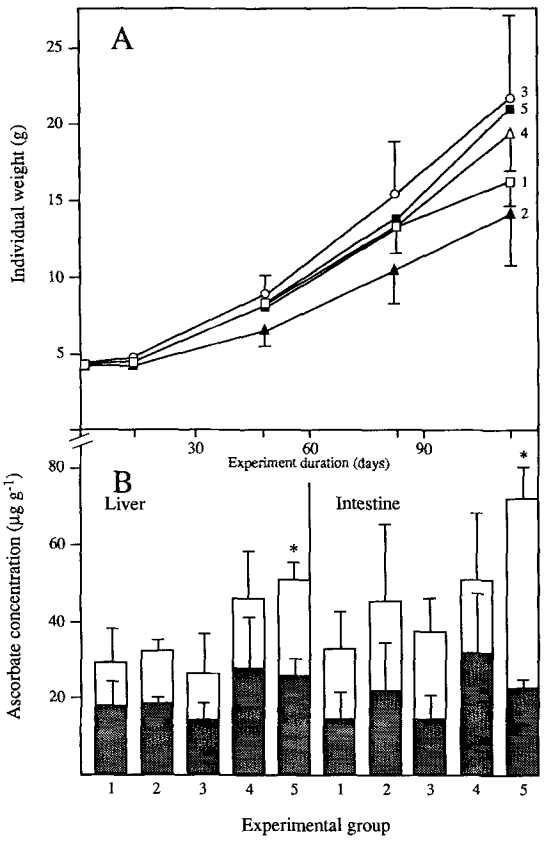

Figure 2. *A* Growth of white sturgeon fed a scorbutogenic diet (# 1) and diets supplemented with graded level of ascorbic monophosphate magnesium salt (# 2–5). There were no significant differences between the final weights of fish ($p < 0.05$). *B* Tissue concentration of the dehydroascorbate (filled columns) and the total ascorbate (blank columns). The only significant differences in total ascorbate concentrations were found in both liver and intestine between groups 1 and 5 ($p < 0.01$).

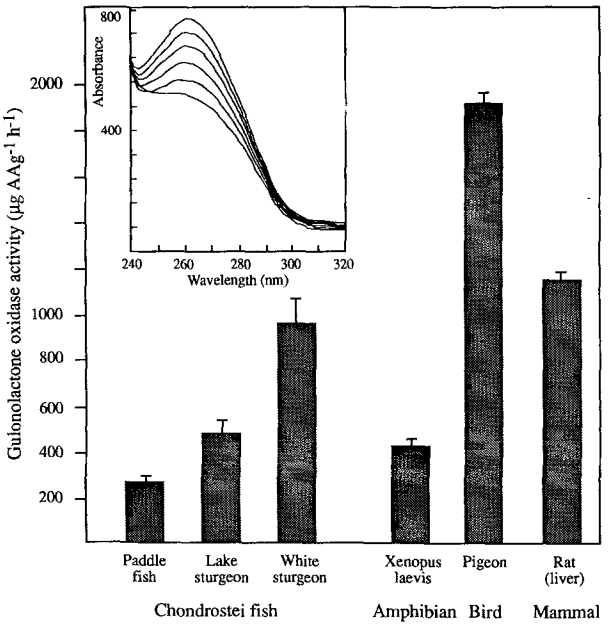

Figure 3. Comparison of gulonolactone oxidase activity in *Chondrostei* fishes with those in higher vertebrates (from previously published results[3]). Insert figure presents the results of direct spectrophotometric assay with a maximum absorbance at 265 nm characteristic for the ascorbic acid. Activity data are shown as a mean, where a vertical bar represents SD of 3–5 animals.

primitive Actinopterigian fishes suggest that this fish has an advantage in comparison to a scurvy-prone teleost fish, which depends on a food-chain transfer of ascorbic acid.

Although my results shed new light on the distribution and evolution of the ascorbate synthesis in vertebrates, they also suggest that the questions of when, why, and how the gulonolactone oxidase activity was lost should be reconsidered. The taxonomic position of the *Teleostei* is under discussion[20], but they are generally considered to have evolved in the Triassic–Cretaceous periods when the rate of morphological evolution, correlating with the rate of speciation, reached a maximum[21]. During this period divergence apparently occurred, and a phylogenetic comparison of nucleotide sequences provides evidence that *Chondrostei* (sturgeon) and *Holostei* (living representatives, *Amia* sp. and *Lepisosteus* sp.) are monophyletic groups[22]. The debate over which group of the primitive Actinopterigians (*Chondrostei*, *Holostei* or *Polypterus* sp.) is most closely related to the modern Actinopterigians (*Teleostei*) might be reignited with this new method, the gulonolactone oxidase activity assay. The phylogenetic relationship of *Latimeria* to bony fishes is uncertain[21] and might be addressed similarly, using information from comparative biochemistry, in concert with other information from phylogenetic characters.

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