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Director of Ph.D. School: Prof. Dr. Sci. Tamás Bánszki

Theses of the dissertation

Subject leader
Dr. László Pócsi
assistant professor
Ph.D.

Effects of different vitamin C forms on European catfish (*Silurus glanis* L.) and on a sturgeon hybrid (*Acipenser ruthenus* L. x *Acipenser baeri* Brandt)

By:
Gyöngyösi Gyuláné dr. univ. Papp Zsuzsanna
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1. INTRODUCTION, PRELIMINARIES

Since it was demonstrated in the beginning of XX. century, that scurvy, which is one of the most feared disease of the human, caused by lack of vitamin C, more and more researches have studied physiological role and requirements of this vitamin also for different animals. Ever since had been proved, that the well-known compound - its trivial name is L-ascorbic acid - has several important physiological effects. Among others vitamin C is an essential cofactor of hydroxylation reactions go on in human and animal organs, it has a key role in the protection against dangerous reactions of free radicals and stress effects, as well as it is antioxidant and immunstimulant. While all plants can produce vitamin C, the humans and some animals are not able to synthesise of this essential vitamin due to lack of gulonolacton oxidase enzyme.

The fact, that vitamin C is essential not only for human but also for guinea pigs, was discovered already in the beginning of XX. century. As fish are live in natural waters, or reared in fish ponds can easily obtain the required amount of vitamin C, that is why it took researches long time to discover the need of this vitamin for fish.

However it was observed, that rainbow trout fed with formaline dried meat manifested lordosis and scoliosis, but as the development of their symptoms took a long time, some months or a year, it was not considered as scurvy. As intensive fish breeding spreaded in the world, symptoms similar to human scurvy occurred several times in different fish species. It was described as deficiency disease due to lack of vitamin C, for several fish species, like rainbow trout, channel catfish, salmons and sea bass, in the beginning of the seventies. Effect of vitamin C was studied for larvae of some fish species from the middle of nineties.

Some years later sturgeons were found with capability of vitamin C synthesis. It was described to the end of nineties, that while teleostei fish species are not able to synthesise vitamin C, some primitive fish like sturgeon or sharks have a sufficient amount of gulonolacton oxidase enzyme to produce L-ascorbic acid.

An unknown stable vitamin C form, ascorbate-2-sulfate was isolated at the end of sixties from *artemia cysts*. For this time the compound was detected in tissues of some fish species. After this many researchers adapted an attractive hypothesis, that fish can store vitamin C in sulfate form. However hypothesis seems to disapprove, because ascorbate-2-sulfate was not detectable in fish with more precise methods. As L-ascorbic acid is a very unstable compound, the research on bioactivity of its sulfate-ester helped to produce other stable ascorbate forms (phosphate, palmitate, and glucoside). Phosphate esters of L-ascorbic acid were proved to be most applicable in fish breeding, because they are stable and fish can hydrolyse ascorbate-2-phosphates with acidic phosphate enzymes already in the digestive tract.

Precise determination of vitamin C is difficult because it is an unstable and reactive compound. L-ascorbic acid and dehidro-L-ascorbic acid - the most frequent natural forms - are well detectable with spectrophotometry and by HPLC (high pressure liquid chromatograph). The industrial produced stable vitamin C forms are detectable with both methods, but there isn’t a generally accepted analytical method for the assay of the most important ascorbate forms from the same extraction of a sample.

Study of vitamin C requirement of fish is a complicated work. Approximate values of minimal ascorbate needs are determinable with examination of growth and mortality symptoms of scurvy in fish. Complex research are necessary to determine the optimal vitamin C levels of fish food, with assay of ascorbate concentration in different fish tissues, hystological studies, and observation of changes in stress situations. Although it is an important question, only few information are available about overfeeding of vitamin C.

However vitamin C is essential for some intensive reared fish species which is important for Hungarian aquaculture, research was initiated only in the beginning of nineties, probably because intensive fish breading has a small volume in Hungary. While quality requirements of fish nutrition are increasing on the way to join EU, more attention would be needed for contents and examination for vitamins as well as other micronutrients. Support of this study was provided mainly by Research Programme: "Cooperation in Science and Technology with Central and Eastern European Countries" of Commission of the European Communities CIPACT93140 (PL928019).

2. OBJECTS

Original objects were drawn up in 1994 according to the international and national knowledge, but that was developed work with attention to followed literature. This subject is filling the gap in Hungary. For this reason we wanted to have answers during our experiments for the most frequent questions of researchers, so the main aims of this study are:

1. To develop an HPLC (high-pressure liquid chromatograph) method to determine the natural and the most important industrially produced vitamin C forms in small quantities using the same extract of different tissues and food.

2. Effects of natural and industrially produced vitamin C forms on European catfish (*Silurus glanis* L.). Determination of vitamin C requirements for fish with 5-200 g average weight, with attention for:

- Growth, mortality, symptoms of scurvy and collagen contents of fish fed with different levels and forms of vitamin C;

- Incorporation and saturation of natural and industrial produced vitamin C in different tissues;
- Excretion of vitamin C;
- Effects of vitamin C in different stress situations;
- Effects of vitamin C for larvae.

3. Effects of vitamin C on sturgeon hybrid (*Acipenser ruthenus* L. x *Acipenser baeri* Brandt) with attention for:

- Growth, mortality, symptoms of scurvy and collagen contents of fish fed with different levels and forms of vitamin C;
- Examination of ability of vitamin C synthesis;
- Excretion of vitamin C;
- Effects of vitamin C in different stress situations.

3. MATERIALS AND METHODS OF RESEARCH

A fish species European catfish (*Silurus glanis* L.) and a sturgeon hybrid (*Acipenser ruthenus* L. x *Acipenser baeri* Brandt) were studied in our experiment. Fish were chosen according to different properties:

- They must be well rearable in intensive system;
- They must have importance in Hungarian aquaculture;
- Two fish – a species and a hybrid - were chosen, European catfish (which is not able to synthesise) and a sturgeon hybrid (which is able to produce vitamin C).

Experiments on nutrition as well as on environmental and infection caused stresses were performed in the recycling system of Research Institute. Basic feeding treatments with different levels and forms of vitamin C were carried out

before individual experiments. Fish with 5-150 g average body weight were reared in 100 l volume tanks. 400 l volume EWOS tanks were used in some stress experiments. Flow rate was 7 $lmin^{-1}$, temperature was 22-23˚C and dissolved oxygen was 80-90% in the tanks. The period, density and size of fish were different during the individual experiments.

An approximately vitamin C free basic diet was applied as control in all experiments, in suitable size for fish. Supplementation of vitamin C was sprayed to the diet and all of that stored at -18˚C until feeding. According to our objects different observations and experiments were connected to the basic feeding treatments (Table 1). The period, density and size of fish were different during the individual experiments.

Table 1: **System of observations connected to basic feeding treatments**

Basic treatment	**Fish species**	**Experimental food ($mgkg^{-1}$)**	**Observations, connected experiments**
1.	European catfish	AA 0, 10, 100, 1000, 10000	Growth, mortality, scurvy, histology; Vitamin c status and saturation; Digestibility of ascorbate-2-phosphates and –sulfate; Stresses: confinement, formaline, and high nitrite.
2.	Sturgeon hybrid	AA 0 and 1000	Digestibility of ascorbate-2-phosphates and -sulfate
3.	European catfish	AA 0 and 1000; AMP 100, 200, 400; APP 150, 300, 600	Growth, mortality, scurvy; Vitamin C status and saturation; Collagen concentration in the cartilage; ^{14}C uptake; excretion of vitamin C forms.
4.	Sturgeon hybrid	AA 0 and 1000; AMP 100, 200, 400; APP 150, 300, 600	Growth, mortality, scurvy; Collagen concentration in the cartilage; Vitamin C status; Excretion of vitamin C forms.
5.	European catfish	AS 0, 100, 1000, 10000; APP 45 , 450	^{14}C uptake; Oxalate in tissues; Stresses: hypoxia.
6.	Sturgeon hybrid	AA 0, 10, 100, 1000, APP 450	Activity of gulonolactone oxidase; Stresses: hypoxia, high nitrite, and starvation.

Acknowledgements: AA = L-ascorbic acid; AMP = ascorbate-2-monosulfate; APP = ascorbate-2-polysulfate

Two experiments were carried out with larvae. Effects of enrichment with L-ascorbic acid or ascorbate-2-phosphate of first live food (tubifex) was studied. Results of spontaneous infection with *Ichthyophthirius multifiliis* were followed in control as well as group fed with ascorbate-2-phosphate supplemented diet.

Vitamin C concentrations of different tissues and food were determined with our HPLC method and with a known spectrophotometric assay. Collagen concentrations of cartilage were detected with gravimetric method. Oxalate content was analysed with KIT of Sigma Co.

4. THE MOST IMPORTANT ESTABLISHMENTS OF DISSERTATION

4.1. Determination of natural and industrially produced vitamin C forms with HPLC

A method was developed to assay four forms of vitamin C: L-ascorbic acid, dehydro-L-ascorbic acid, ascorbate-2-mono- and polyphosphate as well as ascorbate-2-monosulfate.

Sample preparation

A new combination of different possibilities founded in literature was developed for sample preparation. The whole L-ascorbic acid content was oxidased with specific enzyme reaction (ascorbate oxidase) to do a background correction. L-ascorbic acid was stabilised with ice cold perchloric acid and determined directly. Dehydro-L-ascorbic acid was reduced with 1,4-dithioerythritol to L-ascorbic acid. Ascorbate-2-phosphate was hydrolysed and originated L-ascorbic acid was measured. Ascorbate-2-sulfate (AMS) was determined in sulfate form using coinjection of AMS standard (Figure 1).

HPLC conditions

Mobil phase was an aqueous buffer of 0.04 $moll^{-1}$sodium acetate with 0.05 $mmoll^{-1}$EDTA and 0.5 $mmoll^{-1}$ tetrabutilammonium dihydrogen phosphate, pH 3,76 and 1000 ml of it was mixed with 24 ml methanol.

A Waters HPLC system was used with Rheodyne injector, Waters 510 pump, Waters 490E multiwavelenght UV detector. The elution was carried out on Nova Pak C_{18} column (5µm, 3.9 x 3.0 cm). Flow rate of mobile phase was 0.6 $mlmin^{-1}$.

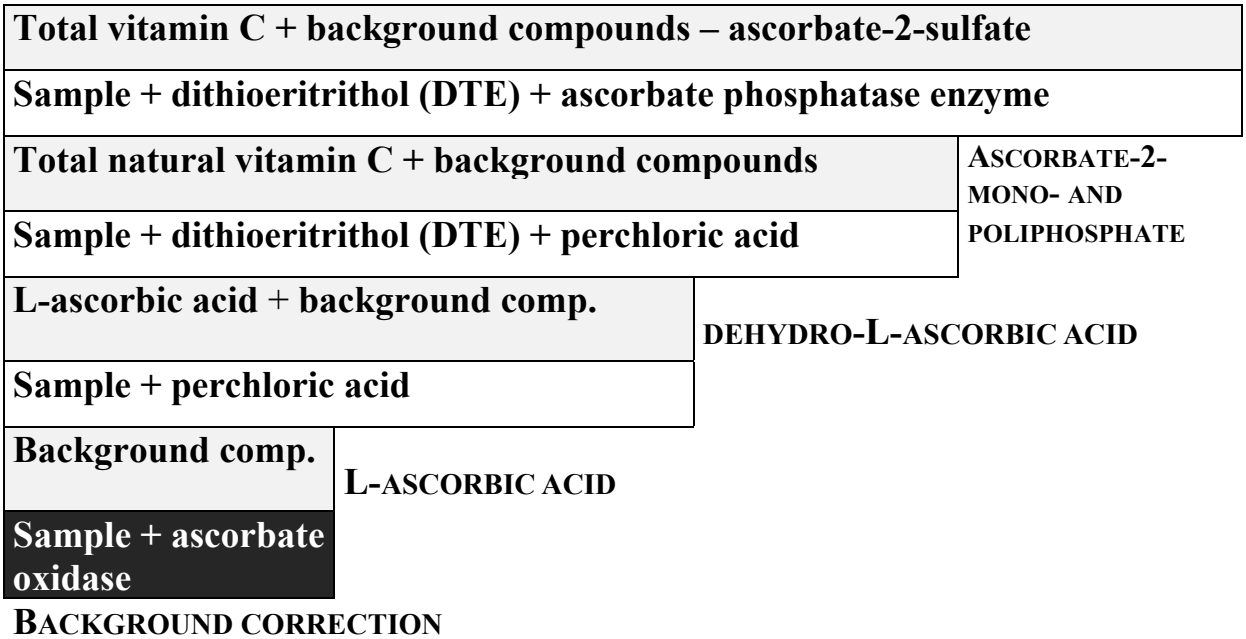

Figure 1: **Vitamin C determination with HPLC: Theoretical summary of sample preparation**

The method was used with some changes for determination of different enzyme activities (ascorbate phosphatase and sulfatase as well as gulonolacton oxidase).

2.2. Vitamin C requirements of European catfish (*Silurus glanis* L.)

Growth of fish fed with approximately vitamin C free diet was significantly ($p<0.05$) lower than in groups fed with any levels of L-ascorbic acid (AA) (Figure 1). Slightly higher mortality (10-15%) was detected in fish fed with AA free diet, while it was 5-10% in other groups fed with L-ascorbic acid supplemented diets.

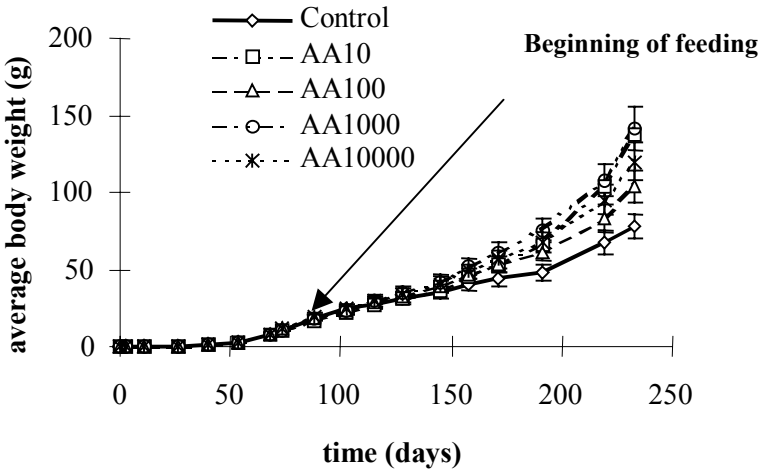

Figure 2: **Growth of European catfish *(Silurus glanis* L.*)* fed with graded levels of vitamin C**

According to the hystological analysis, numerous degenerative phenomena were found on slides of ribs cartilage of European catfish fed with approximately ascorbate free diet.

Several deformities, like osteoporosis or large quantities of intercellular fluid were found on slides of rib cartilage from European catfish fed with approximately ascorbate free diet. Cells were hyalinized and nucleus were almost absent. Slides obtained from cartilage of fish fed with 10 $mgkg^{-1}$ AA showed some slight deformities. Many nucleus were absent from new cells. Cartilage cells of fish fed with 100 $mgkg^{-1}$ AA were well organised.

The most striking pathological changes were in cartilage tissues of fish fed with 1,000 or 10,000 $mgkg^{-1}$ L-ascorbic acid. An unusual density of nucleus, contraction of cartilage tissues and a large quantity of intercellular fluid were found on the slides. Symptoms of hypervitaminosis might cause by oxalate originated from L-ascorbic acid.

Changes in ascorbate concentration were followed through four months of first basic feeding treatment. Total vitamin C concentration in liver and kidney of AA10 group significantly ($p<0,05$) lower than it was found in fish fed with at least 100 $mgkg^{-1}$ L-ascorbic acid supplementation.

The results of above mentioned experiment indicated, that European catfish (*Silurus glanis* L.) was unable to synthesise L-ascorbic acid, thus this species are depend on a dietary source of vitamin C. It can be concluded that a minimum dietary requirement for normal growth and mortality, as well as against macroscopic symptoms of scurvy in European catfish approximates 10 $mgkg^{-1}$ L-ascorbic acid equivalents.

According to histological studies and results on saturation of liver, brain and kidney with ascorbate, the optimum need of vitamin C is 100-200 $mgkg^{-1}$ L-ascorbic acid for 50-200 g average weight European catfish. Feeding through a longer period than a month is not suggested for this species because of microscopically detected deformations in cartilage tissues.

Ascorbate-2-monosulfate wasn’t found in tissues of European catfish. From this observation it can be concluded that fish aren’t able to store vitamin C in this form. AMS wasn’t hydrolysed by incubation with tissues of digestive tract, thus the results of "in vitro" studies suggest that European catfish hasn’t sufficient ascorbate sulfatase enzyme activity.

“*In vitro*” hydrolysis of ascorbate-2-mono- and poliphosphate was studied in homogenised tissues of digestive tract (intestine, kidney, and liver). AMP was hydrolysed quickly by natural acidic phosphatase enzymes. Growth of control group was significantly lower than other fish fed with any forms of vitamin C

supplemented diet in the 3rd basic feeding treatment after four month. Mortality of control group was 40 %, while less than 10 % fish was died in the other groups during this period. According to this results industrial produced ascorbate-2-mono- and poliphosphate is a good vitamin C source for European catfish.

Kidney, liver and brain were saturated with ascorbate in 60 days. There wasn't substantial difference in tissue ascorbate concentration of groups fed with different levels and forms of vitamin C. According to this results it may conclude that 100 $mgkg^{-1}$ AMP or 150 $mgkg^{-1}$ APP is an optimal dietary level for European catfish.

1-^{14}C labelled L-ascorbic acid were completely discharged in two weeks, so this species does not store vitamin C fed continuously with that. Tissues of European catfish fed with ascorbate free diet were depleted in 50-60 days.

Enrichment of first live food (tubifex) of larvae is suitable with any form of vitamin C. Chopped tubifex absorbed any vitamin C form dissolved in water. Vitamin C concentration of natural tubifex was 5-10 μgg^{-1}. Ascorbate concentration of tubifex enriched with L-ascorbic acid or with ascorbate-2-polphosphate was 60-80 μgg^{-1}. Larvae fed with Vitamin C enriched tubifex had lower mortality in possible infection with *Ichthyophthirius multifiliis*.

Changes in ascorbate concentrations of studied organs in three environmental stress model with chemical character (formaline, high nitrite, and hypoxia) were shown that vitamin C requirement of fish increased temporary (Table 2).

According to this result fish might require a higher, sometimes 10,000 $mgkg^{-1}$ ascorbate supplementation in the diet. Increasing of dietary ascorbate level is recommended in case of water pollution or if there is a need to apply a bath with chemicals.

Table 2: **Changes of total vitamin C concentration in tissues of European catfish (*Silurus glanis* L.) in different stress situations**

Stress	*Brain*	*Liver*	*Kidney*
Confinement	↓	↓	**nd**
Nitrite	↑	↑	**nd**
Formaline	↑	↓	**nd**
Hypoxia	↓	↑	↑↓

Acknowledgement: ↓ = decreased in all groups; ↑ = increased in all groups; ↑↓ = changed; nd = it wasn't determined

According to this result fish might require a higher, sometimes 10,000 μgkg^{-1} ascorbate supplementation in the diet. Increasing of dietary ascorbate level is recommended in case of water pollution or if there is a need to apply a bath with chemicals.

4.2. Effect of vitamin C for sturgeon hybrid (*Acipenser ruthenus* L. x *Acipenser baeri* Brandt)

Growth, mortality or collagen concentration of fish did not show difference independently of dietary vitamin C concentration or forms, during basic feeding treatments carried out at least four month. All groups were healthy without symptoms of scurvy. Gulonolacton oxidase enzyme activity was detected in the kidney of fish. Enzyme activity wasn't prevented by feeding with high dietary vitamin C level, at least one month. According to this result sturgeon hybrid can synthesise a suitable amount of L-ascorbic acid, so this species do not require dietary vitamin C.

However sturgeon hybrid doesn't store vitamin C in tissues, a temporary increase in ascorbate concentration was found in different organs of fish fed with AA.

Sturgeon hybrid was fed with diets supplemented with different levels of vitamin C (AA 0, 10, 100, 1000, APP 450). Effects of environmental stress (nitrite, hypoxia) were studied in 6th basic feeding treatment (Table 3). Significant increasing of total ascorbate concentration was found in the kidney of control group in both studied stress model. Slightly decreasing was observed in the kidney of AA 10, 100 and APP450 groups, while kidney ascorbate concentration dramatically decreased in fish fed with 1,000 μgkg^{-1} AA (Table 3).

Table 3: **Changes of total vitamin C concentration in tissues of sturgeon hybrid (*Acipenser ruthenus* L. x *Acipenser baeri* Brandt) in different stress situations**

Stress	*Brain*	*Hepatopancreas*	*Kidney*
Nitrite	↓	↑↓	↑↓
Hypoxia	*nc*	↑↓	↓

Acknowledgement: ↓ = decreased in all groups; ↑ = increased in all groups; ↑↓ = changed; nc = it wasn't changed

Sturgeon hybrids starved through 54 hours. Changes were observed in ascorbate concentration of kidney. Production of L-ascorbic acid was significantly decreased in fish fed with vitamin C.

However earlier results showed, that feeding with high level of vitamin C didn't occur inhibition in gulonolactone oxidase enzyme activity, this observation might conclude that there is a "*feed back*" reaction anywhere in L-ascorbic acid synthesis. Excess of prevention has a correlation (r^2=-0.801) with ascorbate levels of food (Figure 3).

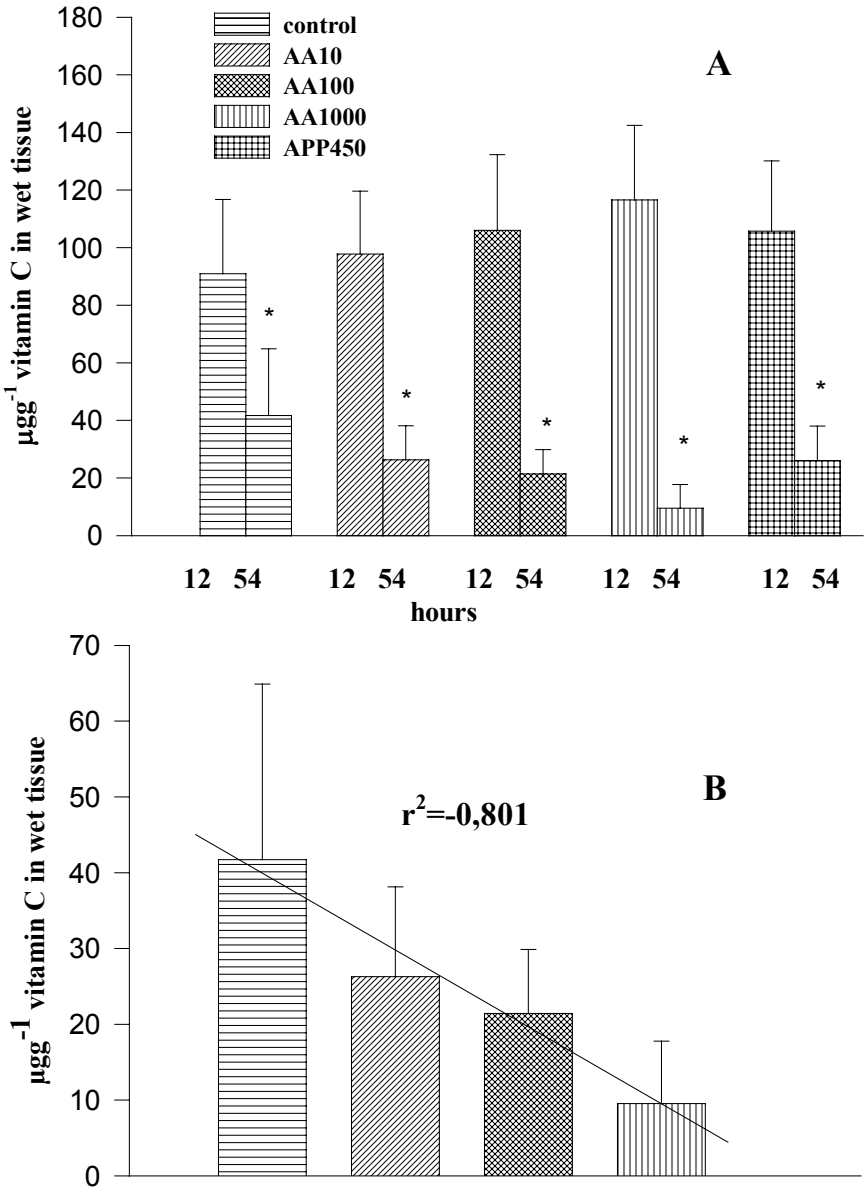

Figure 3: **A: Effects of starvation on changing of vitamin C concentration in the kidney of sturgeon hybrid (*Acipenser ruthenus* L. x *Acipenser baeri* Brandt)**
B: Correlation of vitamin C concentration in the food with decreasing of kidney ascorbate

* significant difference from control group

5. SUMMARY OF NEW SCIENTIFIC RESULTS

1. A new HPLC method was developed for determination of most important natural and industrially produced vitamin C. L-ascorbic acid, dehydro-L-ascorbic acid, ascorbate-2-phosphates and ascorbate-2-monosulfate might determine from the same extraction of sample in low concentrations. Determination limit is 0.5-5.0 $\mu g g^{-1}$ ascorbate in wet tissues.

2. A method was developed for enrichment of first live food (tubifex) of European catfish (*Silurus glanis* L.) Larvae fed with Vitamin C enriched tubifex had lower mortality in possible infection with *Ichthyophthirius multifiliis*.

3. Minimal (10-20 $mg kg^{-1}$ AA) and optimal (100-200 $mg kg^{-1}$ AA) requirements of European catfish (*Silurus glanis* L.) were determined. Sometimes can increase with 10-100 times more in some stress situation, like formaline bath is. However feeding with high levels of vitamin C must be avoided because fish might have tissue damages by oxalate.

4. L-ascorbic acid synthesis was demonstrated in kidney of sturgeon hybrid (*Acipenser ruthenus* L. x *Acipenser baeri* Brandt), so this fish do not require dietary vitamin C. Decreasing of produced vitamin C amount was proven in sturgeon hybrid fed with high dietary ascorbate levels.

5. Vitamin C synthesis in kidney of sturgeon hybrid (*Acipenser ruthenus* L. x *Acipenser baeri* Brandt), was increased in fish fed without dietary ascorbate during stress treatments. Vitamin C production was inhibited by "*feed back*" regulation in the kidney of fish fed with high dietary ascorbate levels, so

feeding of sturgeon might be injurious with diets which have higher AA levels than 10-20 $mgkg^{-1}$.

6. UTILIZATION OF THE RESULTS IN PRACTICE

The basic studies of dissertation have several practical aspects, so results can be applied directly in the practice. Let us point out some important observation.

According to results on studied fish, supplementation of food with vitamin C must do with high attention, because a fault might have effects only after some month. Breeder must to know if fish can produce vitamin C. More than 10-20 $mgkg^{-1}$ ascorbate concentration in the diet must be avoided in sturgeon nutrition, because this species can synthesise L-ascorbic acid. So, sometimes practically applied trout food isn't recommended for sturgeon.

Feeding of ascorbate depends on fish species with higher or lower levels of vitamin C, than the optimal dietary AA concentration might occur injury.

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