

Research Article

Dietary effects of ascorbic acid and cobalt chloride on some biological parameters and gene expression in *Acipenser baerii*

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Abstract

This research investigated the effects of dietary cobalt chloride (CoCl₂) and ascorbic acid (AA) on growth, physiological, and molecular parameters in Siberian sturgeon (*Acipenser baerii*). Two hundred seventy juveniles were divided into eight groups fed diets containing 2 or 4 mg/kg CoCl₂ and 200 or 800 mg/kg AA for 12 weeks. Results showed significant differences in final weight, length, weight gain, and growth rate among treatments ($p < 0.05$), indicating differences when compared to the control group. However, no significant difference was observed in specific growth rate, condition factor, Average daily growth, or protein efficiency ratio compared to the control ($p > 0.05$). The lowest and highest AA/cobalamin levels were found in the control and the 4 mg/kg CoCl₂ with 200 mg/kg AA, and the 2 mg/kg CoCl₂ groups, respectively ($p < 0.05$). Maximum serum iron level was observed in fish fed 800 mg/kg CoCl₂, and the lowest glucose was in the control ($p < 0.05$). The cortisol level was significantly higher in 2 mg/kg CoCl₂ with 200 mg/kg AA group compared to the control, which had the lowest levels ($p < 0.05$). The highest *hsp70* and *p450* gene upregulation, as well as the highest *GH* and *igf-I* expression, occurred in the 4 mg/kg CoCl₂ with 200 mg/kg AA group, with the lowest expression in the control ($p < 0.05$). Overall, 4 mg/kg CoCl₂ and 200 mg/kg AA supplementation had more positive effects on growth performance, hematological and biochemical indices, and the modulation of genes associated with growth, stress response, and immunity in Siberian sturgeon.

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Introduction

Micronutrients, encompassing vitamins and minerals, play a pivotal role in various physiological and biological processes in aquatic animals. These essential nutrients contribute significantly to health maintenance and growth enhancement (Dawood and Koshio, 2018; Khanjani and Sharifinia, 2020). Vitamins, particularly AA, are vital micronutrients known for their immune-stimulating properties. They promote macrophage activity, cell proliferation, and the production of cytokines and antibodies (Rahman *et al.*, 2019; Rahmati, 2020). Unlike mammals that synthesize AA, the majority of fish species, including those unable to effectively convert L-gluconolactone, lack this ability due to a deficiency in the essential enzymes required for AA production. Consequently, dietary AA supplementation is necessary for normal growth and physiological processes (NRC, 2011; Khan *et al.*, 2015; Trichet *et al.*, 2015). The AA requirement of fish is influenced by various factors, including species-specific physiology, environmental conditions, age, diet, and genetics. This variability necessitates species-specific studies for optimal dosing. Research has identified diverse optimal AA levels across different species. For instance, studies indicated 150-250 mg/kg for rainbow trout (*Oncorhynchus mykiss*) (Adel and Khara, 2016), 400 mg/kg for red pacu (*Piaractus brachipomus*) (Hosseini *et al.*, 2015), 45.3 mg/kg for grouper (*Epinephelus malabaricus*) (Lin and Shiau, 2005), 120 mg/kg for barred knifejaw (*Oplegnathus fasciatus*) (Wang *et al.*, 2003), 160 mg/kg for puffer (*Takifugu rubripes*) (Eo and Lee,

2008), 60 mg/kg for tilapia (*Oreochromis karongae*) (Nsonga *et al.*, 2009), 100 mg/kg for vundu (*Heterobranchus longifilis*) (Ibiyo *et al.*, 2007), and 200 mg/kg for beluga sturgeon (*Huso huso*) (Falahatkar *et al.*, 2006). These variations highlight the diverse nutritional requirements among species and the importance of species-specific studies.

Ascorbic acid is also critical for growth performance, reproduction, and immunity in fish (Gouda *et al.*, 2020). The confident effects of AA supplementation on growth and immune function in finfish have been well documented. Consequently, ensuring adequate AA levels in fish diets is crucial for achieving optimal health and productivity in aquaculture (Moreau and Dabrowski, 2000; Rahman *et al.*, 2018).

Fish hematology (Cytologic and Serologic indices) offers valuable insights into both physiological function and overall health, making it a valuable factor in aquaculture management (Percin and Konyalioglu, 2008; Percin *et al.*, 2010). Biochemical studies, by measuring cortisol (as a stress indicator), glucose (as a carbohydrate metabolism indicator), albumin and total protein (as liver function and nutritional status indicators) in the blood, provide valuable information about internal organ function, metabolic status, and stress response in Siberian sturgeon. These parameters help us evaluate the effects of dietary supplements on the health and performance of this species (Roche and Bogé, 1996).

Cobalt, an essential trace mineral, has been recognized for its crucial role in various metabolic processes, particularly serving as a coenzyme in the conversion of

ribonucleotides to deoxyribonucleotides (Hall and Hall, 2020). It is important to note that the optimal levels of CoCl_2 supplementation reported in the literature can vary depending on factors such as the species of fish, age, size, sex, feed composition, feeding practices, and rearing conditions, reflecting differences in metabolic and functional demands for micronutrients (Biesalski and Jana, 2018). Deficiencies in essential micronutrients and vitamins can induce severe anemia in various fish species (Brunt and Austin, 2005; Cheng *et al.*, 2006). Dietary inclusion of CoCl_2 and cobalt sulfate in fish diets has been shown to promote cobalamin (Vitamin B_{12}) synthesis by gut bacteria (Blust, 2012; Lall and Kaushik, 2021). The gut microbiome of fish utilizes cobalt for the production of vitamin B_{12} , a critical cofactor involved in metabolic pathways linked to lipid and carbohydrate energy metabolism (Hossain *et al.*, 2022). Furthermore, CoCl_2 is essential for preventing pernicious anemia, and cobalt salts function as catalysts in the synthesis of various pigments in fish (Tonye and Sikoki, 2014). Vitamin B_{12} deficiency induces several deleterious outcomes in fish, including decreased hemoglobin levels, reduced appetite, growth impairment, and anemia (Stoica *et al.*, 2004; Boachie *et al.*, 2020). Moreover, cobalt supplementation is known to positively influence growth parameters, carcass composition, muscle manganese and iron content, and notably, the modulation of genes involved in growth regulation, as demonstrated in Golden mahseer (Younus *et al.*, 2020). Iron is an essential element for fish growth and metabolism, and its deficiency can lead to

various physiological problems. Furthermore, cobalt is one of the most important elements that has the greatest interaction with dietary iron, such that during iron deficiency in the body, cobalt absorption increases, and the reverse is also true (González-Montaña *et al.*, 2020).

Growth in fish is regulated by the *gh/igf-I* axis. Growth hormone (GH) stimulates growth by affecting protein, carbohydrate, and fat metabolism (Rahimi *et al.*, 2012). However, insulin-like growth factor-1 (*igf-I*) is a more stable indicator of growth and is influenced by nutrition (Duan *et al.*, 2010). Therefore, *igf-I* gene expression can be used as an indicator of growth rate in fish (Larsen *et al.*, 2001). Heat shock protein (*hsp70*) genes play a role in response to various stresses, including heat shock and infection, and maintain cellular homeostasis (Basu *et al.*, 2002). cytochrome *p450* genes are involved in the metabolism of toxic substances and hormones (Arukwe and Goksoyr, 2003), and their expression can be altered by environmental and nutritional conditions (Van der Oost *et al.*, 2003). Therefore, changes in *p450* gene expression can be used as an indicator to assess the impact of pollutants and stressful conditions on fish health (Stegeman, 1994).

The Siberian sturgeon (*A. baerii*) is an introduced species of sturgeon to Iran and has gained attention from researchers due to its ability to live in freshwater, tolerance to environmental fluctuations, adaptation to low temperatures, acceptance of a wide range of food items, and high growth potential (Falahatkar, 2018). These characteristics make the Siberian sturgeon a suitable model for investigating the

effects of dietary supplements on growth and metabolism. Among various sturgeon species, the Siberian sturgeon is recognized as one of the most important and globally cultured species, making it a suitable option for aquaculture purposes (Eslamloo *et al.*, 2012). Additionally, it is used as a biological model in physiological and nutritional studies (Fontagné *et al.*, 2006). This species is widely cultivated due to its adaptability to temperature variations, rapid growth rate, and the fact that it reaches sexual maturity relatively early (Eslamloo *et al.*, 2012; Falahatkar and Poursaeid, 2014).

Former studies have analyzed the individual implications pertaining to CoCl₂ and AA, impacts on growth, stress responses, and hemato-biochemical indices in fish (Falahatkar *et al.*, 2006; Hernandez *et al.*, 2012; Rahimi *et al.*, 2012; Tonye and Sikoki, 2014; Guo *et al.*, 2015; Abbas and Javed, 2016; Pourgholam *et al.*, 2016; Akdemir *et al.*, 2017; Djissou *et al.*, 2019; Ibrahim *et al.*, 2020; Li *et al.*, 2021; Aboseif *et al.*, 2022; Li *et al.*, 2022).

Given the worldwide growth of sturgeon aquaculture, the rapid growth potential of Siberian sturgeon, and the known importance of AA and CoCl₂ in aquatic organisms nutrition, this research carried out aimed to determine the combined effects of dietary CoCl₂ and AA micronutrients on some growth parameters, hematological and biochemical indices, and the expression of *hsp70*, *p450*, *gh*, and *igf-1* genes in *A. baerii* for order suitable doses of these supplements to sturgeon fish diet.

Materials and methods

Ethical considerations

This research was performed in compliance with the Care or Use of Laboratory Animals Act. The experimental protocol received approval from the pertinent Local Ethics Committee for Animal Use in Iran (Ahmadi Noorbakhsh *et al.*, 2021).

Rearing system

The current research was performed at the Research Center of Fisheries Sciences and Marine Techniques Lahijan Branch, Islamic Azad University (Langroud, Guilan, Iran), in the summer of 2021. In this study, 270 fish with a mean initial weight and initial total length of 11.5±0.3 g and 12.9±0.1 cm, respectively, were divided into 27 fiberglass tanks (each 350 L) with a stocking rate of 10 fish per tank. Each tank was supplied with an aeration system, and water inflow was set at 1 L/min. The physicochemical parameters of the water, including temperature, pH, and oxygen, were measured 22.5±0.5°C, 7.2±0.1, and 6.3±0.3 mg/L, respectively (WTW Company, Weilheim, Germany).

Experimental design

In this study, experimental subjects were fed diets supplemented with 2 and 4 mg/kg of CoCl₂, and 200 and 800 mg/kg of AA (Falahatkar *et al.*, 2006) (Table 1). A commercial diet (Faradaneh Co., Shahre Kord, Iran) was used, with the following nutritional composition: 54% crude protein, 14% fat, 3% fiber, 11% ash, 11% moisture, and 2% phosphorus, with a diameter of 1.2 mm. The experiment consisted of eight experimental treatments and a control, with each having three replicates. The AA (powder) was obtained from Science Laboratory, Aras Bazar, Qazvin, Iran, and

CoCl₂ (CAS No. 7791-13-1) was from (Merck, Darmstadt, Germany). Ascorbic acid and CoCl₂ were dissolved in 10 mL water and then sprayed onto the pellets. All experimental diets including the control group, were coated using 1% gelatin. The control group was fed the basal diet without any added supplements. The fish were cultured with experimental diets for 12 weeks, during which the ration each day was 3% of their individual body weight, one quarter of the total meal at each time (6 a.m., 12 p.m., 6 p.m. and 12 a.m.) Biometric

measurements were used to adjust the feed ration every two weeks to support sturgeon growth. The actual concentrations of AA and CoCl₂ in the experimental diets were determined by iodometric titration and atomic absorption spectrophotometry (AA-6800; Shimadzu, Kyoto, Japan), respectively. In the sample of Faradaneh diet, AA concentration was 690 mg/kg and CoCl₂ was 0.05 mg/kg by adding different experimental concentrations of these supplements.

Table 1: Description of the diet treatments used for Siberian sturgeon, *Acipenser baerii*.

Treatments	Details
Control	The standard feeding group consisted of a basal diet without any supplementation of CoCl ₂ or AA, representing the standard feeding practice without the addition of the experimental additives.
2 CoCl ₂	Cobalt chloride (2 mg/kg) not supplemented with AA
4 CoCl ₂	Cobalt chloride (4 mg/kg) without AA
200 AA	Ascorbic acid (200 mg/kg) not supplemented with CoCl ₂
800 AA	Ascorbic acid (800 mg/kg) without CoCl ₂
200 AA+2 CoCl ₂	Ascorbic acid (200 mg/kg) + Cobalt chloride (2 mg/kg)
800 AA+4 CoCl ₂	Ascorbic acid (800 mg/kg) + Cobalt chloride (4 mg/kg)
800 AA+2 CoCl ₂	Ascorbic acid (800 mg/kg) + Cobalt chloride (2 mg/kg)
200 AA+4 CoCl ₂	Ascorbic acid (200 mg/kg) + Cobalt chloride (4 mg/kg)

In sample of Faradaneh food have AA is 690 mg/kg and CoCl₂ is 0.05 mg/kg.

Growth parameters

To assess growth parameters and feed conversion efficiency, the weight and total length of each group of 270 fish were measured biweekly throughout the experimental period. Prior to measurement, fish were anesthetized using clove powder at a concentration of 150 mg/L (Mohseni,

2015). Fish weight was measured using a DK-300 digital scale (ZhiHeng, Shanghai, China) with an accuracy of 1 g, and total length was measured using a graduated ruler with an accuracy of 1 mm (Falahatkar and Poursaeid, 2014):

Condition factor (CF = $100 \times \text{body weight (g)} / \text{total length (cm)}^3$)

Growth rate (%) (GR = $100 \times (\text{final weight} - \text{initial weight} / \text{days})$)

Weight gain (g) (WG = $\text{final weight (g)} - \text{initial weight (g)}$)

Body weight increase (%) (BWI = $100 \times [\text{final body weight (g)} - \text{initial body weight (g)}] / \text{initial body weight (g)}$)

Specific growth rate (%/day) (SGR = $100 \times [\ln \text{final weight (g)} - \ln \text{initial weight (g)}] / \text{rearing period (day)}$)

Food conversion ratio (FCR = $\text{feed intake (g)} / \text{weight gain (g)}$)

Protein efficiency rate (PER = $\text{wet weight gain (g)} / \text{protein intake (g)}$)

Average daily growth (%) (ADG = $100 \times \text{final weight (g)} - \text{initial weight (g)} / \text{initial weight (g)} \times \text{rearing period (day)}$)

Survival rate (%) (SR = $100 \times (\text{number of fish at final} / \text{number of fish at start})$) was assessed at the end of the experiment.

Biochemical indices

Fish were fasted for 24 hours prior to blood collection. Blood was collected from the caudal region using 2 mL syringes. After collecting 2 mL of blood (from 3 fish per treatment), 0.5 mL of blood was transferred to numbered 1 mL heparinized vials for complete blood count (CBC) analysis, and the remaining 1.5 mL was transferred to numbered 2 mL vials for serum preparation for blood biochemical analysis. Samples were transported to the laboratory in a cooler containing dry ice. Siberian sturgeon blood samples were centrifuged at room temperature at 3000 rpm for 10 minutes (Hestaran Teb, Tehran, Iran), and the serum was separated (Kazemi *et al.*, 2010).

The measurement of AA in blood samples (n=81 including 3 samples per each 3 replicates of 9 treatments) was conducted following a modified version of the Roe and Kuether (1942) method. This approach involved oxidizing AA by copper sulfate, forming a red-colored hydrazone in a robust acid solution containing 2,4-dinitrophenylhydrazine. This color's optical density (OD) was then read at a wavelength of 520 nm using a Biochrom spectrometer (Cambridge, England). Iron, protein, and albumin levels were assessed spectrophotometrically. Iron was determined at a wavelength of 600 nm using a colorimetric method (Higgin, 1981). Protein quantification was performed using the Biuret colorimetric method at 540 nm (Koller and Kaplan, 1984). Albumin concentrations were determined using the BCG assay with a Pars Azmun kit (Karaj, Iran), employing a photometric method at 546 nm. The difference between total protein and

albumin values yielded the globulin levels. Lactate dehydrogenase (LDH) levels were measured using a quantitative photometric method (Pars Azmun, Karaj, Iran) at 340 nm (Hoseini *et al.*, 2011). Cortisol levels were determined using an ELISA kit (Monobind Inc., Lake Forest, California, USA) possessing inter-assay and intra-assay coefficients of variation of 3.22% and 5.96%. Glucose concentrations were determined using commercially available kits from Pars Azmun (Karaj, Iran) and a Unico UV/Vis 2100 spectrophotometer (Chicago, USA) at 546 nm (Bartonkova *et al.*, 2017).

Gene expressions

At the end of the experiment, 27 samples, including three fish from each nine treatments were randomly selected. Liver and brain tissues were collected under sterile conditions for gene expression analysis of *igf-I*, *p450*, *hsp70* and *gh*, respectively. Prior to tissue collection, fish were anesthetized by immersion in a 0.5 g/L clove powder solution and small sections of liver and brain tissues were excised and promptly transferred to sterile microtubes. The microtubes were then immediately cryopreserved in liquid nitrogen and subsequently stored at -80°C until RNA extraction (Safari *et al.*, 2016).

Total RNA was extracted from approximately 100 mg of homogenized tissue using the Biozol kit (Biozol-Bioflux-Bioer) according to the manufacturer's protocol. For RNA extraction, samples were pulverized to a fine powder using a pre-chilled mortar and pestle with liquid nitrogen. *beta-actin* gene was used as the reference gene for normalization of gene

expression data. Small sections of each tissue were promptly transferred to microtubes, immediately cryopreserved in liquid nitrogen and subsequently maintained at -80°C for subsequent processing. To assess the RNA both qualitative and quantitative methods were employed following extraction from the tissues. Qualitative assessment involved agarose gel electrophoresis (1%). Quantitative evaluation was conducted using a BioPhotometer, measuring the 260/280 absorbance ratio. cDNA synthesis was carried out using a commercial kit from Gent Bio (Gent Bio, Daejeon, Korea). For cDNA synthesis, 1 μL of oligo primer was admixture with 15 μL of the prepared RNA in new tubes, and the volume was brought to 10 μL with nuclease-free water. The mixture was deposited on a heat block at 65°C for 1 min, followed by immediate transfer to ice. Reverse transcription was performed by adding 10 μL of the master mix to each sample. The admixture incubated at 50°C for 60 minutes and 70°C for 10 minutes. The resulting cDNA solution (120 μL) kept stored at -20°C . Standard PCR was carried out with 2 μL of

the diluted cDNA sample (1:10 dilution), 1 μL of both forward and reverse primer, 3 μL of sterile, nuclease-free water, and 5 μL of PCR premix at 59°C . PCR was conducted in specialized tubes with 4 technical replicates for each treatment. Each tube contained a 20 μL reaction mixture consisting of 10 μL Cybergreen amplicon buffer, 1 μL of each Oligonucleotide primer (both forward and reverse) for the target and reference genes, 8 μL of water, 0.2 μL of tag enzyme, and 8 μL of the diluted cDNA. To optimize real-time PCR conditions, varying volumes (1.10, 1.20, and 1.50 μL) of the mixed cDNA samples from different treatments were prepared and amplified in quadruplicate with both target and reference primers at 59°C . Additionally, standard curves were generated for each primer to assess the efficiency of the assay, as described by Awad *et al.* (2011) and Safari *et al.* (2016). Gene expression levels were quantified using the $-\Delta\Delta\text{CT}$ method ($\Delta\Delta\text{CT} = \Delta\text{CT}$ (target gene) $- \Delta\text{CT}$ (reference sample)) (Livak and Schmittgen, 2001) (Table 2).

Table 2: Oligonucleotide primers for quantitative PCR analysis of gene expression in Siberian sturgeon, *Acipenser baerii*.

Gene	Accession sequence Number Abbreviation	Primers	Junction Temperature ($^{\circ}\text{C}$)	Primer Efficiency (%)
<i>igf-1</i>	AB512770.1	F: GACACGCTTTGTGTGTGGAG R: ACTCGTTCACGATGCCCTGTGGTG	59	95
<i>gh</i>	AB517597.1	F: TGTGGCTCTCATGAGGGAT R: CTGCATTTTCATCACTTTCAGG	59	95
<i>p450</i>	JX013935.2	F: GTCATCTGTGCCATGTGCTT R: TCTTGTCGAAGGAGCGGTAG	59	95
<i>hsp70</i>	KF000408.1	F: CGCTGGCCTTAATGTTCTCC R: GCGCTTGAACCTCTGCAATGA	59	95
<i>beta-actin</i>	AY878120.1	F: TTGCCATCCAGGCTGTGCT R: TCTCGGCTGTGGTGAA	59	95

Statistical analysis

The data distribution was assessed for normality using the Kolmogorov-Smirnov test, while Levene's test was used to evaluate the homogeneity of variances. To assess the mean differences, a two-factor ANOVA, including CoCl₂ and AA as factors, was conducted to evaluate the observed differences among the various treatments. Mean comparisons were conducted using Duncan's multiple-range test. Statistical analyses were performed using SPSS software (version 26, IBM, USA) with statistical significance set at $p < 0.05$ (95% confidence interval).

Results

Growth performance

The highest final weight, final length, and WG were recorded for the diet containing 4 mg CoCl₂ per kg commercial diet ($p < 0.05$). The analysis revealed no significant differences in BWI, FCR, SGR, GR, CF, ADG, and PER between the treatment groups and the control group ($p > 0.05$). There was a significant interaction between CoCl₂ and AA, but only for final weight and length ($p < 0.05$; Table 3).

Table 3: The growth performance of *Acipenser baerii* fed diets containing CoCl₂ and AA after 12 weeks (mean $\pm$ SE) (n= 81).

Treatments	Parameters												
	Initial weight, (g)	Final weight, (g)	Initial length, (cm)	Final length, (cm)	Weight gain, (g)	Body weight increase (%)	Condition factor	Growth rate, (%)	Food conversion ratio	Specific growth rate, (%/day)	Average daily growth (%)	Protein efficiency ratio	Survival rate (%)
Control	11.5 ± 0.0	97.0 ± 3.5 ^d	12.9 ± 0.3	30.4 ± 0.7 ^{bcd}	85.4 ± 3.4 ^d	742.3 ± 28.8	0.3 ± 0.0	101.7 ± 4.1 ^d	2.9 ± 0.0	2.5 ± 0.0	8.8 ± 0.3	0.7 ± 0.0	100
2 CoCl ₂	11.5 ± 0.1	108.3 ± 1.5 ^{ab}	12.9 ± 0.3	31.6 ± 0.0 ^{ab}	96.8 ± 1.6 ^{ab}	841.8 ± 20.6	0.3 ± 0.0	115.2 ± 1.9 ^{ab}	2.8 ± 0.0	2.7 ± 0.0	10.0 ± 0.2	0.7 ± 0.0	100
4 CoCl ₂	11.6 ± 0.3	110.9 ± 1.2 ^a	12.9 ± 0.1	32.4 ± 0.1 ^a	99.3 ± 1.0 ^a	857.4 ± 12.9	0.3 ± 0.0	118.3 ± 1.2 ^a	2.8 ± 0.0	2.7 ± 0.0	10.2 ± 0.2	0.7 ± 0.0	100
200 AA	11.5 ± 0.2	107.3 ± 4.8 ^{ab}	12.9 ± 0.2	30.1 ± 1.0 ^{cd}	95.7 ± 4.8 ^{ab}	830.0 ± 45.4	0.4 ± 0.0	114.0 ± 5.7 ^{ab}	2.8 ± 0.0	2.7 ± 0.1	9.9 ± 0.5	0.7 ± 0.0	100
800 AA	11.5 ± 0.6	106.2 ± 0.2 ^{abc}	12.9 ± 0.1	31.3 ± 0.1 ^{abc}	94.7 ± 0.6 ^{abc}	821.1 ± 48.1	0.3 ± 0.0	112.8 ± 0.7 ^{abc}	2.8 ± 0.0	2.6 ± 0.1	9.9 ± 0.6	0.7 ± 0.0	100

Table 3 (continued):

Treatments	Parameters												
	Initial weight, (g)	Final weight, (g)	Initial length, (cm)	Final length, (cm)	Weight gain, (g)	Body weight increase (%)	Condition factor	Growth rate, (%)	Food conversion ratio	Specific growth rate, (%/day)	Average daily growth (%)	Protein efficiency ratio	Survival rate (%)
2 CoCl ₂ + 200 AA	11.4 ± 0.4	100.7 ± 1.1 ^{abcd}	12.9 ± 0.2	30.5 ± 0.3 ^{bcd}	89.2 ± 1.3 ^{bcd}	783.4 ± 34.2	0.4 ± 0.0	106.2 ± 1.6 ^{bcd}	2.8 ± 0.0	2.6 ± 0.0	9.3 ± 0.4	0.7 ± 0.0	100
4 CoCl ₂ + 800 AA	11.5 ± 0.4	103.7 ± 1.8 ^{bcd}	12.9 ± 0.1	31.1 ± 0.1 ^{abcd}	92.2 ± 2.0 ^{abcd}	802.3 ± 41.4	0.3 ± 0.0	109.7 ± 2.4 ^{abcd}	2.8 ± 0.0	2.6 ± 0.1	9.6 ± 0.5	0.7 ± 0.0	100
2 CoCl ₂ + 800 AA	11.4 ± 0.2	98.8 ± 2.3 ^{cd}	13.0 ± 0.0	29.7 ± 0.3 ^d	87.4 ± 2.5 ^{cd}	765.4 ± 33.3	0.4 ± 0.0	104.0 ± 2.4 ^{cd}	2.9 ± 0.0	2.6 ± 0.0	9.1 ± 0.4	0.7 ± 0.0	100
4 CoCl ₂ + 200 AA	11.5 ± 0.5	108.4 ± 2.3 ^{ab}	13.0 ± 0.0	32.2 ± 0.3 ^a	96.9 ± 2.3 ^{ab}	844.1 ± 42.8	0.3 ± 0.0	115.4 ± 2.7 ^{ab}	2.8 ± 0.0	2.7 ± 0.1	10.0 ± 0.5	0.7 ± 0.0	100
Two- Way ANOVA													
	Final weight		Final length		Weight gain	Body weight increase	Condition factor	Growth rate	Food conversion ratio	Specific growth rate	Average daily growth	Protein efficiency ratio	
CoCl ₂	0.038*		0.001*		0.600	0.379	0.057	0.045*	0.225	0.362	0.379	0.346	
Ascorbic acid	0.642		0.039		0.112	0.940	0.098	0.659	0.060	0.935	0.940	0.929	
Ascorbic acid + CoCl ₂	0.002*		0.018*		0.946	0.141	0.234	0.002*	0.551	0.123	0.141	0.108	

Non-identical symbols within a row indicate a significant differences ($p < 0.05$) between treatments for that specific parameter.

An asterisk (*) denotes the presence of significant interaction effects between cobalt chloride and ascorbic acid at $p < 0.05$.

Treatment Groups: Control: Basic diet without any added cobalt chloride or ascorbic acid. Treatment 1: 2 mg cobalt chloride per kg of diet. Treatment 2: 4 mg cobalt chloride per kg of diet. Treatment 3: 200 mg ascorbic acid per kg of diet. Treatment 4: 800 mg ascorbic acid per kg of diet. Treatment 5: 2 mg cobalt chloride + 200 mg ascorbic acid per kg of diet. Treatment 6: 4 mg cobalt chloride + 800 mg ascorbic acid per kg of diet. Treatment 7: 2 mg cobalt chloride + 800 mg ascorbic acid per kg of diet. Treatment 8: 4 mg cobalt chloride + 200 mg ascorbic acid per kg of diet.

Biochemical indices

Levels of vitamins C and B12 were lower in the control group than those of the treatment groups ($p<0.05$). The highest AA level was detected in *A. baerii*, fed 4 mg/kg CoCl₂ and 200 mg/kg AA ($p<0.05$), while the maximum vitamin B₁₂ level was observed in those fed 2 mg CoCl₂ per kg diet ($p<0.05$). Regarding minerals levels, the treatment fed 800 mg AA per kg diet

exhibited maximum iron concentrations ($p<0.05$). Glucose level was lower in the control group than those of treatments groups ($p<0.05$). Ultimately, the treatment had a significant impact on cortisol levels, with the peak level observed in fish fed 2 mg CoCl₂+200 mg AA per kg diet, whereas the control group exhibited the minimum level ($p<0.05$; Table 4).

Table 4: Biochemical indices of Siberian sturgeon, *Acipenser baerii*, following 12 weeks of dietary CoCl₂ and AA supplementation (mean $\pm$ SE) (n= 81).

Treatments	Parameters							
	Ascorbic acid (mg/mL)	Vitamin B ₁₂ (mg/mL)	Iron (mg/dL)	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Glucose (mg/dL)	Cortisol (ng/mL)
Control	6.3 $\pm$ 0.1 ^f	81.7 $\pm$ 1.6 ^h	65.4 $\pm$ 1.8 ^e	1.3 $\pm$ 0.0 ^e	0.6 $\pm$ 0.0 ^e	0.7 $\pm$ 0.0 ^b	32.7 $\pm$ 0.6 ^c	91.6 $\pm$ 3.4 ^e
2 CoCl ₂	6.5 $\pm$ 0.0 ^e	155.0 $\pm$ 2.6 ^a	61.6 $\pm$ 2.4 ^e	1.6 $\pm$ 0.1 ^a	0.7 $\pm$ 0.0 ^b	0.9 $\pm$ 0.1 ^a	38.7 $\pm$ 0.8 ^b	131.0 $\pm$ 0.4 ^b
4 CoCl ₂	6.6 $\pm$ 0.0 ^d	135.0 $\pm$ 2.7 ^{ab}	67.8 $\pm$ 0.6 ^{cd}	1.5 $\pm$ 0.0 ^{ab}	0.7 $\pm$ 0.0 ^b	0.8 $\pm$ 0.0 ^{ab}	40.7 $\pm$ 0.6 ^b	126.0 $\pm$ 2.3 ^b
200 AA	7.2 $\pm$ 0.0 ^c	103.0 $\pm$ 2.0 ^g	73.2 $\pm$ 0.5 ^{ab}	1.4 $\pm$ 0.0 ^b	0.6 $\pm$ 0.0 ^c	0.8 $\pm$ 0.0 ^{ab}	42.7 $\pm$ 0.2 ^b	98.0 $\pm$ 1.1 ^d
800 AA	7.3 $\pm$ 0.0 ^b	112.7 $\pm$ 1.2 ^f	74.0 $\pm$ 0.9 ^a	1.3 $\pm$ 0.0 ^c	0.6 $\pm$ 0.0 ^c	0.7 $\pm$ 0.0 ^b	42.0 $\pm$ 0.2 ^b	93.3 $\pm$ 0.6 ^{de}
2 CoCl ₂ + 200 AA	7.2 $\pm$ 0.0 ^c	142.7 $\pm$ 0.8 ^{bc}	64.9 $\pm$ 1.1 ^e	1.6 $\pm$ 0.0 ^a	0.8 $\pm$ 0.0 ^a	0.8 $\pm$ 0.0 ^a	54.7 $\pm$ 0.6 ^a	147.0 $\pm$ 0.8 ^c
4 CoCl ₂ + 800 AA	7.4 $\pm$ 0.0 ^b	130.3 $\pm$ 0.6 ^c	69.7 $\pm$ 0.5 ^{bc}	1.5 $\pm$ 0.0 ^a	0.9 $\pm$ 0.0 ^a	0.6 $\pm$ 0.0 ^c	55.3 $\pm$ 0.4 ^a	116.3 $\pm$ 0.6 ^c

Table 4 (continued):

Treatments	Parameters							
	Ascorbic acid (mg/mL)	Vitamin B ₁₂ (mg/mL)	Iron (mg/dL)	Total protein (g/dL)	Albumin (g/dL)	Globulin (g/dL)	Glucose (mg/dL)	Cortisol (ng/mL)
2 CoCl ₂ + 800 AA	7.4 ± 0.1 ^b	145.7 ± 2.1 ^b	66.6 ± 1.0 ^{cd}	1.5 ± 0.0 ^a	0.9 ± 0.0 ^a	0.6 ± 0.0 ^c	53.0 ± 0.4 ^a	97.7 ± 1.0 ^d
4 CoCl ₂ + 200 AA	7.9 ± 0.1 ^a	139 ± 1.6 ^{cd}	69.2 ± 0.7 ^c	1.6 ± 0.0 ^a	0.8 ± 0.0 ^a	0.6 ± 0.0 ^c	53.7 ± 0.8 ^a	93.0 ± 2.4 ^{de}

Two-Way ANOVA								
	Ascorbic acid	Vitamin B ₁₂	Iron	Total protein	Albumin	Globulin	Glucose	Cortisol
CoCl ₂	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
Ascorbic acid	0.000*	0.003*	0.000*	0.000*	0.000*	0.000*	0.000*	0.000*
Ascorbic acid + CoCl ₂	0.000*	0.060*	0.060*	0.000*	0.000*	0.000*	0.196*	0.000*

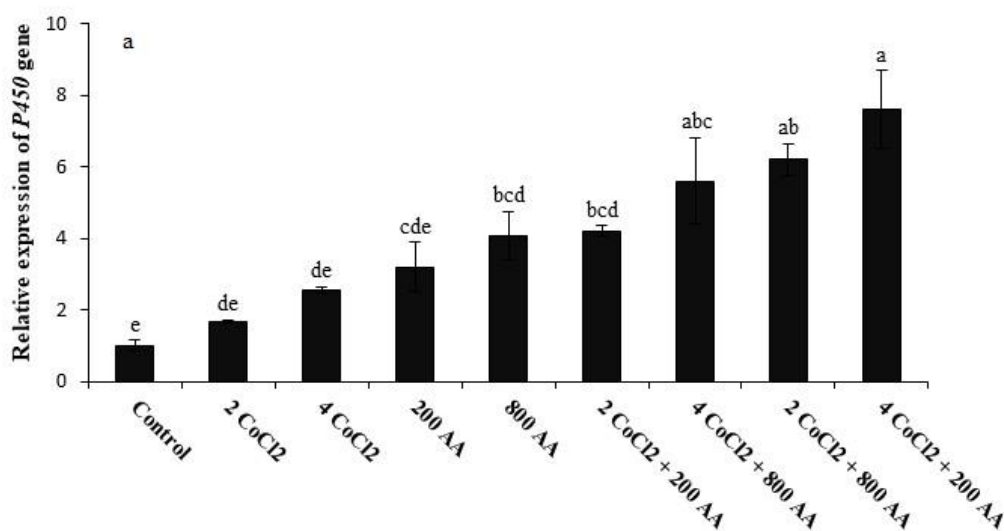
Non-synonymous letters in the column indicate a significant difference ($p < 0.05$).

The asterisk (*) indicates the presence of significance and interaction effects at the level of $p < 0.05$.

Gene expression

The highest upregulation of *hsp70* and *p450* mRNA levels was observed in the group fed 4 mg/kg CoCl₂ with 200 mg/kg AA, which were significantly higher than those of the control group ($p < 0.05$).

Additionally, peak expression levels of *GH* and *igf-1* were seen in the fish receiving 4 mg/kg CoCl₂ and 200 mg/kg AA in their diet, while the lowest expression was found in the control group ($p < 0.05$; Fig. 1).



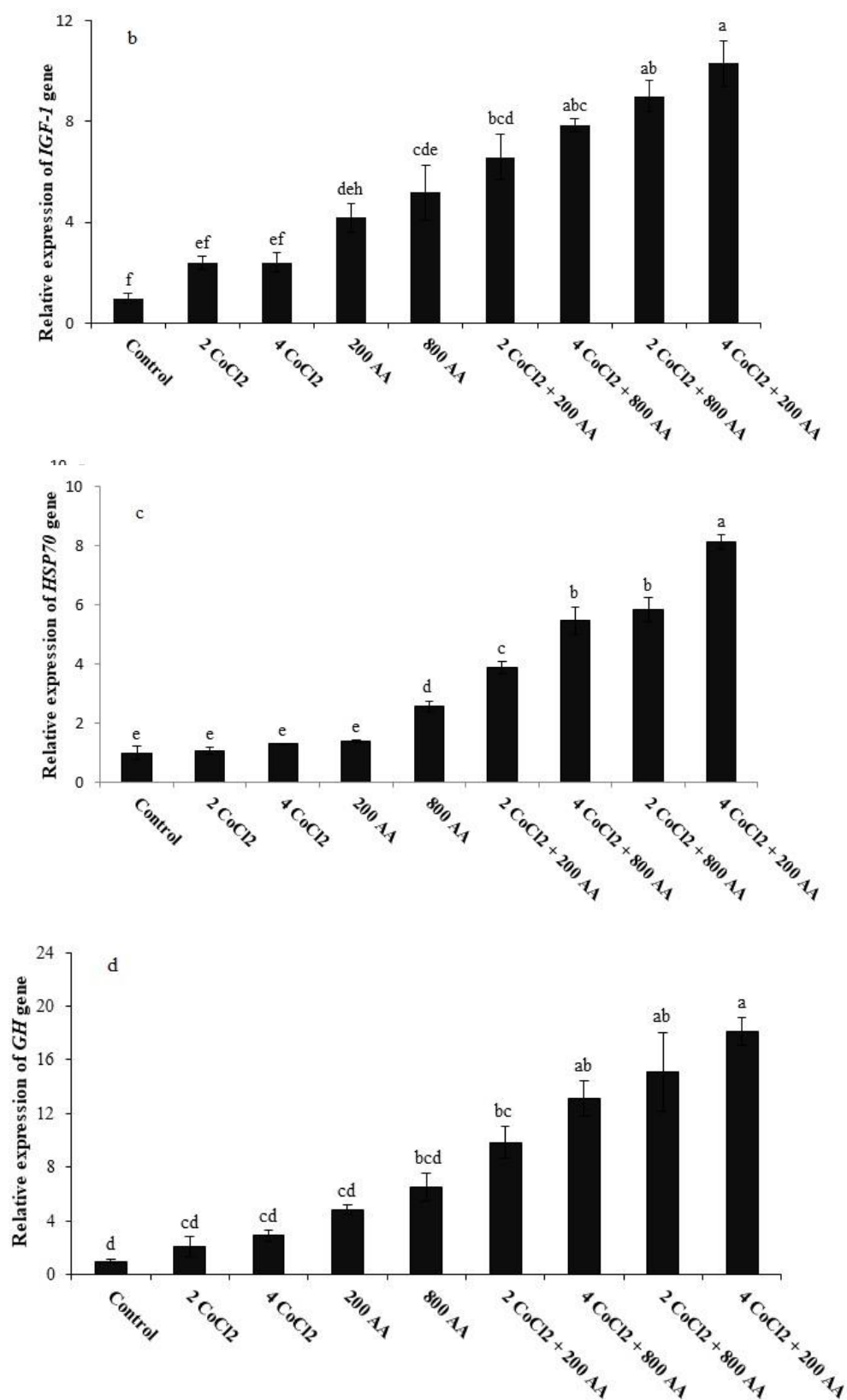


Figure 1: The effect of treatment uses of AA and CoCl₂ on relative expression of *p450* (a), *igf-1* (b), *hsp70* (c) and *GH* (d) after 12 weeks of feeding. Non-synonymous letters in the column indicate a significant difference ($p < 0.05$) ($n = 27$).

Discussion

This research showed that supplementation with both AA and CoCl₂ positively affected the growth parameters of Siberian sturgeon. Notably, the diet that enriched with 4 mg CoCl₂ per kg demonstrated the most significant improvements in final weight and length, WG, and GR. This enhancement might be attributed to the synergistic actions of AA and CoCl₂ when it comes to promoting intestinal health and nutrient absorption. The diet containing 4 mg/kg CoCl₂ and 200 mg/kg AA significantly improved several biochemical indices, including the highest levels of ascorbic acid, total protein, albumin, and glucose, while also reducing cortisol levels in Siberian sturgeon (*A. baerii*). These findings are consistent with earlier research on Siberian sturgeon, including a study by Pourgholam *et al.* (2016), which demonstrated that dietary micronutrient with 800 mg/kg of AA for 12 weeks significantly improved growth parameters. It is important to consider that some research on two other species has shown different results. For example, Moreau and Dabrowski, (2000) and Gy. Papp *et al.* (1999) reported good growth performance in hybrid *A. ruthenus* × *A. baerii* even without AA supplementation. Furthermore, a study by Desimira *et al.* (2013) showed a negative correlation between SGR and AA levels in stellate sturgeon (*A. stellatus*), where the lowest SGR values were observed in both the control and the group receiving 800 mg/kg AA. These findings suggest that lower AA concentrations may be more beneficial for growth in certain juvenile sturgeon species. In contrast, dietary AA has consistently shown positive

effects on final body weight, SGR, and WG across different fish species, e.g., Nile tilapia (*O. niloticus*) (Ibrahim *et al.*, 2020). Aboseif *et al.* (2022) also demonstrated that 200 mg/kg AA of feed enhanced growth factors and intestinal mucosal epithelium structure in common carp (*Cyprinus carpio*), supporting our findings. The significant growth enhancement observed in Siberian sturgeon fed a diet containing AA due to its potential to elevate circulating GH levels, induce beneficial alterations in intestinal morphology, and enhance the gut's ability to absorb nutrients (Rahman *et al.*, 2018). The other reason is that stimulates the production of proteins could also contribute to the observed increase in WG and faster growth following supplementation (Chagas and Val, 2003).

Dietary supplementation with CoCl₂ and AA has shown promise in enhancing growth performance across various fish species. For instance, Anadu *et al.* (1990) demonstrated that *Tilapia zilli*, which received diets AA, exhibited superior growth in comparison to those receiving CoCl₂ or the control diet. Similarly, CoCl₂ supplementation has been linked to increased total length and weight in rainbow trout (*Oncorhynchus mykiss*), *C. carpio*, royal carp (*Astronotus ocellatus*), grouper (*E. malabaricus*), red-bellied tilapia (*Coptodon zillii*), Nile tilapia (*O. niloticus*), and Golden mahseer, *Tor putitora* (Yada *et al.*, 2002; Mukherjee and Kaviraj, 2009; Younus *et al.*, 2020). To ensure optimal growth and survival in economically important fish species like *O. spilurus* (Al-Amoudi *et al.* 1992), redlip croaker (*Larymichthys polyactis*) (Ai *et al.*, 2006), and parrotfish (*O. fasciatus*) (Wang

et al., 2003), adequate AA levels in fish feeds are essential. Ibrahim *et al.* (2020) further emphasized the positive influence of AA on *O. niloticus* growth, attributing it to the vitamin's role in elevating serum growth hormone levels, improving intestinal morphology, and enhancing nutrient absorption. In fish, *GH* and *igf-1* play crucial roles in regulating metabolic homeostasis and growth. Hepatic *igf-1* levels are often dependent on nutritional status. A considerable body of research has demonstrated the beneficial effects of AA and CoCl_2 on growth performance in a range of bony fish species (Dabrowski, 2000; Harsij *et al.*, 2020; Singh *et al.*, 2021; Delavari *et al.*, 2022; Ghafarifarsani *et al.*, 2022; Xu *et al.*, 2022). Growth enhancement was most significant with 4 mg CoCl_2 per kg; however, no significant differences were observed in SGR, ADG, PER, CF, BWI, FCR, and SR across the treatment groups. This lack of pronounced differences in growth parameters, despite varying AA supplementation levels in *A. baerii*, is potentially attributed to the presence of L-gluconolactone oxidase. This enzyme enables the de novo synthesis of L-ascorbic acid from D-glucose or D-galactose, potentially fulfilling the fish's ascorbic acid requirements for growth, immunity, and health even in the absence of dietary supplementation (Dabrowski, 2000; Moreau and Dabrowski, 2000). Consistent with our findings, many studies have demonstrated the beneficial effects of cobalt supplementation on growth, survival, protein synthesis, and glucose homeostasis in a range of fish species, including *O. mykiss* (Blust, 2012), and *C. carpio* (Wilson, 1991). Notably, Tonye and

Sikoki, (2014) observed significant growth enhancement in Nile tilapia fed a CoCl_2 -supplemented diet, even though FCR and carcass composition remained unchanged. These results suggest that CoCl_2 , even at a 0.1% inclusion level, possesses growth-promoting properties. In the current study, supplementing the diet with 4 mg/kg CoCl_2 alongside 200 mg/kg AA had the most pronounced positive impact on growth parameters in Siberian sturgeon. We observed a significant increase in WG with 4 mg/kg dietary CoCl_2 , with this group exhibiting the highest percentage of WG. Cobalt chloride is crucial for fish metabolism by enhancing muscular protein synthesis and nitrogen assimilation, contributing to overall growth and development. Additionally, the chlorine ions present in CoCl_2 activate digestive enzymes, potentially improving nutrient digestion and absorption.

Therefore, studying hematological and biochemical parameters is crucial for assessing fish growth and health status. Our findings indicate that serum biochemical indices, such as serum protein, albumin, and globulin concentrations, were significantly elevated in the groups supplemented with CoCl_2 and AA, compared to the control group. Total protein, which is synthesized by liver parenchymal cells, serves as an important clinical indicator of overall health and well-being, including nutritional status, immune function, and stress response in fish (Safari *et al.*, 2019). Elevated levels of total protein, albumin, and globulin may indicate an enhanced innate immune response (Wiegertjes *et al.*, 1996) and could be attributed to non-specific immune reactions

stimulated by AA and CoCl_2 supplementation. Furthermore, in contrast to the treatments, the control group had significantly lower iron levels. Siberian sturgeon groups that received diets with 200 mg and 800 mg of AA exhibited the highest iron levels, highlighting AA crucial role in iron metabolism. Ascorbic acid is necessary for releasing iron bound to ferritin in the liver, making it available for erythropoiesis (red blood cell formation). These findings underscore the complex interplay of various factors influencing blood indices and overall fish health, emphasizing the multifaceted effects of nutritional interventions on fish physiology. Ascorbic acid is also crucial for iron uptake in some fish species. Iron deficiency may be attributed to reduced dietary absorption and impaired iron metabolism due to a lack of AA. Without AA, ferric hemoglobin (Fe^{3+}) is converted to ferrous hemoglobin (Fe^{2+}), hindering plasma transport and cellular iron uptake. Ascorbic acid improves the absorption of iron from dietary sources, and a deficiency in AA can cause damage to iron mobilized from reticuloendothelial reserves (Elbaraasi *et al.*, 2004).

Cortisol, a principal stress hormone in teleost fish (Hsieh *et al.*, 2003), serves as a well-established indicator of the physiological response to stress and is crucial in regulating glucose metabolism, particularly in stress-induced hyperglycemia (Vijayan *et al.*, 1997). Stress initiates a cascade of physiological responses in teleosts, notably activating the hypothalamic-pituitary-interrenal (HPI) axis, leading to heightened catecholamine secretion and elevated serum cortisol

levels. Our results, consistent with those of Montero *et al.* (1999), demonstrate that higher concentrations of AA can effectively decrease cortisol levels, suggesting that AA may have anti-stress properties. The observed reduction in cortisol levels resulting from AA supplementation may be explained by its ability to inhibit steroidogenesis. Through its regulation of unsaturated fatty acids peroxidation, AA may limit the availability of cholesterol, an essential precursor for cortisol synthesis, ultimately reducing cortisol production (Ming *et al.*, 2012). Functionality is consistent with the results reported by Zhang *et al.* (2022), who found that dietary AA in *Pampus argenteus* enhanced immunity and mitigated stress responses. This investigation revealed that glucose concentrations were markedly lower for the control group compared to those receiving dietary treatments. Interestingly, among the various dietary treatments, the one featuring 2 mg/kg CoCl_2 and 200 mg/kg AA showed the peak cortisol level. This seemingly contradictory finding warrants further investigation to elucidate the complex interactions between AA, CoCl_2 , cortisol, and glucose metabolism in *A. baerii*. However, it is worth noting that the overall trend of reduced cortisol levels with AA supplementation supports its potential anti-stress properties. The results showed increased cortisol and glucose levels in the non-supplemented group when exposed to stress, followed by a reduction in these levels upon supplementing the diet with AA, which agrees with the findings of Mustafa *et al.* (2013) in Nile tilapia (*O. niloticus*). This further strengthens the evidence for the stress-mitigating and

adaptive benefits of ascorbic acid in fish diets.

CoCl₂ induces hypoxia response genes, including *hifa*, by mimicking hypoxia (Ji *et al.*, 2012). Our findings indicate that the levels of vitamin B₁₂ were elevated in the treatments that included CoCl₂ supplementation showing a significant increase relative those in the control group as well as the groups receiving other dietary regimens. In addition, among Siberian sturgeon fed various diets, those receiving a diet with 4 mg/kg of CoCl₂ and 200 mg/kg of AA displayed maximum expression of *hsp70* and *p450* genes. Peak gene expression levels of *GH* and *igf-1* were observed in the group fed the combined diet, while the control group showed the lowest expression these genes Growth hormone, a polypeptide hormone, is synthesized by the pituitary gland and is essential for a range of physiological processes, such as growth, carbohydrate, and protein metabolism, and energy balance. Growth hormone secretion stimulates *igf-1* production, which then carries out cell division processes. Thus, the production of *igf-1* depends on both the production and release of *gh*, ultimately regulated by the neuroendocrine *hpi* axis and nutritional factors, including food intake and nutrient absorption. Cytochrome *p450*, a type of hemoprotein, is highly present in the liver and involved in the first phase of xenobiotic substance metabolism (Parhar *et al.*, 2003). The findings of this study indicated substantial differences in the expression of genes among the different treatment groups and the control group. Dietary supplementation with both AA and CoCl₂ led to increased

levels of *gh*, *igf-1*, *hsp70*, and *p450* relative to the groups receiving only AA or CoCl₂ as well as the control group, with peak levels observed for the 4 CoCl₂+200 AA treatment. The heat shock response involves the upregulation of several *hsps*, including *hsp70*, *hsp90*, and *hsp60*, with *hsp70* playing a particularly crucial role in mitigating the effects of cellular stress (Zheng *et al.*, 2010). However, fish immunostimulants can reduce *hsp70* expression (Ahmadi *et al.*, 2014). Research by Ming *et al.* (2012) showed that dietary AA resulted in increased levels of *hsp70*, including its *mRNA*, in fish exposed to heat stress that were given ascorbic acid. The *p450* family comprises numerous enzymes that play a crucial role in detoxifying and metabolizing an extensive range of substances, both those produced naturally within the body and those foreign to it. (Miandare *et al.*, 2016). Downregulation of *p450*, *rpl6*, and *hsp70* gene expression was reported in treated *A. baerii* using barberry fruit extract, indicating increased tolerance against potential stressors in fish husbandry (Shekarabi *et al.*, 2022). Similar results were observed in the reduction of cytochrome C gene expression in *Ctenopharyngodon idella* using berberine (Yang *et al.*, 2019). The results of studies showed *igf-1* exhibits the greatest resistance in rainbow trout, while GH is the most sensitive component to downregulation of gene expression when exposed to cobalt and zinc. Ekinci *et al.* (2011) reported that *igf-1* exhibited the most resistance and GH the most sensitivity to decreased gene expression when exposed to cobalt and zinc in *O. mykiss*. In this investigation, the combined supplementation AA and CoCl₂

resulted peak expression levels of these genes. This suggests a potential synergistic effect of these nutrients on gene expression. Ascorbic acid and CoCl_2 may reduce *hsp70* gene expression by mitigating cellular stress, as demonstrated by Ming *et al.* (2010). According to Wan *et al.* (2014) demonstrated that dietary inclusion of AA (133.7-251.5 mg/kg) upregulated the mRNA expression of three *hsps* in *Megalobrama amblycephala* (juvenile blunt snout bream). In conclusion, the combined effects of CoCl_2 and AA promote growth and immune function in fish and have a positive impact on key physiological markers, suggesting improved resilience against stressors. These findings highlight the crucial role of dietary supplementation in aquaculture practices to optimize fish health and performance.

Cobalt chloride mimics hypoxic conditions by stabilizing the transcription factor *hif1 α* , a crucial regulator of numerous genes involved in growth and metabolism, notably those encoding *GH* and *igf-1*. Therefore, CoCl_2 -induced *hif1 α* activation likely contributes to the increased *GH* and *igf-1* gene expression. Furthermore, AA has immunomodulatory effects, and a robust immune system indirectly promotes growth. While CoCl_2 mimics hypoxia, AA is a cofactor in various enzymatic reactions. Their supplementation may improve overall nutritional status and metabolic homeostasis, creating a conducive environment for *GH* and *igf-1* gene expression and protein synthesis.

Conclusions

The data presented here show that supplementing the diet with AA and

CoCl_2 , especially when administered together at levels of 200 mg/kg AA and 4 mg/kg CoCl_2 , can enhance growth performance, hematological and biochemical indices, and the modulation of genes associated with growth, stress response, and immunity in Siberian sturgeon. Ascorbic acid exhibited a more substantial impact on these parameters compared to CoCl_2 . Thus, we propose supplementing the feed with this specific combination of AA and CoCl_2 to improve overall health, growth, and potential disease resistance in Siberian sturgeon. However, further studies must examine the effects over an extended period and possible interactions of AA and CoCl_2 with other dietary components in Siberian sturgeon and other commercially important fish species.

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Conflicts of Interest

The authors declare no competing interests.

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