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Supplemental effect of dietary nucleotides on hematological profile, hepatic biomarkers, antioxidant capacity, and digestive functions in Sterlet sturgeon, *Acipenser ruthenus*

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This study investigated the effects of dietary nucleotides (NTs) on hematological indices, hepatic biomarkers, antioxidant capacity, digestive functions, and intestinal histomorphology of Sterlet sturgeon (*Acipenser ruthenus*). Over 10 weeks, five diets with varying levels of NTs (0 g/kg, 1.5 g/kg, 2.5 g/kg, 3.5 g/kg, and 5.0 g/kg) were fed to triplicate groups of the fish (initial weight: 95.33 ± 1.23 g) in a flow-through system. The results indicated that final weight and relative growth rate reached the highest values in fish fed with the 5.0 g/kg NTs supplemented diet ($p < 0.05$). The fish fed NTs-supplemented diets also had lower feed conversion ratios than those fed the basal diet ($p < 0.05$). While total leukocytes were increased by increasing the dietary NTs supplementation to the highest value in the fish fed with 3.5 g/kg NTs, no significant differences were obtained in RBC, Hb, MCHC, HCT, and eosinophil values among the experimental groups ($p > 0.05$). The highest WBC count was seen in the fish fed with 3.5 g/kg NTs compared to the control group ($p < 0.05$). The serum hepatic enzyme levels generally decreased with higher NTs supplementation, although alanine transaminase significantly increased at the 5.0 g/kg level ($p < 0.05$). The antioxidant capacity was improved in the fish fed with NTs at 0.25 and 0.35 g/kg ($p < 0.05$), while the serum malondialdehyde level was decreased up to 3.5 g/kg NTs but it was increased at 5.0 g/kg ($p < 0.05$). The protease and amylase activities peaked in the fish receiving 3.5 g/kg NTs ($p < 0.05$), with the highest lipase activity obtained in 2.5 g/kg NTs ($p < 0.05$). The intestinal histology revealed that the fish fed with NTs at 3.5 g/kg exhibited the greatest villus height and width, along with more goblet cells ($p < 0.05$). Based on the second-order polynomial regression analysis, the optimum dietary levels of NTs for positive effects on physiometabolic responses and intestine functions of the Sterlet sturgeon lies in the range of 2.2–3.6 g/kg.

Keywords Digestive enzymes, Hematology, Intestinal histomorphology, Metabolic enzymes, Sterlet sturgeon

Aquaculture is one of the prime food-producing segments **following** the land-based agriculture[1]. In this context, sturgeon farming has received considerable attention from both environmental and economic points of view, because these fish are on the IUCN red list due to the destruction of their natural environments and overfishing for meat and caviar harvests[2,3]. Sterlet sturgeon, *Acipenser ruthenus*, is a potamodromous species with great potential for freshwater aquaculture worldwide, exhibiting shorter time to reach the sexual maturation, higher resistance to captive conditions, and lower weight to reach the marketing weight[4]. Fish nutrition is the main

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definitive factor for successful and sustainable aquaculture. Hence, improving aquafeed with functional feed additives leads to enhanced feed utilization efficiency, growth performance, immune and antioxidant defense responses, and reduced mortality rate during the rearing period[5–8].

Exogenous nucleotides (NTs) have been recently considered as feed additives in animal production[9,10]. These organic molecules are responsible for various biological processes in the body based on their key structure in DNA, RNA, and ATP. They are also involved in the synthesis of intracellular compounds used for signal transduction such as cAMP and cGMP[10,11]. Animals gain their required NTs in three ways including direct *de-novo* synthesis using amino acids, salvage pathway from dead cells, and through the diet[12].

NTs play a crucial role in maintaining the optimal function and high turnover rate of the gastrointestinal tract[10]. Dietary exogenous NTs can positively influence the gastrointestinal tract by enhancing the protein and DNA content in the intestinal mucosa, improving the maturation status of the small intestinal epithelium, increasing intestinal villus height, and boosting the activity of digestive enzymes[10,13]. Research has shown that the activity of digestive enzymes increases with higher dietary levels of NTs in the guts of rainbow trout, *Oncorhynchus mykiss*[13], zebrafish, *Danio rerio*[14], and olive flounder, *Paralichthys olivaceus*[15]. Additionally, the inclusion of NTs in the diet can enhance intestinal structure, resulting in a greater surface area for nutrient absorption, as observed in Atlantic salmon, *Salmo salar*[16] and turbot, *Scophthalmus maximus*[17].

The antioxidant system could be also influenced with dietary NTs by boosting the production of antioxidant enzymes, maintaining redox balance, and regenerating antioxidant molecules like glutathione. Numerous studies have shown that dietary NTs can enhance the antioxidant capacity of various teleost fish like rainbow trout[13], turbot[17], red seabream, *Pagrus major*[18], hybrid tilapia, *Oreochromis niloticus* × *O. aureus*[19], and Nile tilapia, *O. niloticus*[20,21]. However, there is limited information regarding the dietary effect of NTs on the antioxidant defense system in sturgeon aquaculture.

Our previous study illustrated the positive effect of dietary NTs supplementation on performance, body composition, and serum biochemistry of the Sterlet sturgeon[22]. Therefore, the present study aimed to answer the remaining questions about the response of Sterlet sturgeon to dietary NTs supplementation by considering its effects on hematological parameters, liver enzyme activity, antioxidant capacity, and digestive function of this species. This approach will provide a more holistic understanding of the NTs requirement and utilization in sturgeon feeding to produce more efficient and sustainable diets under farming conditions.

Materials and methods

Diet preparation

To prepare the experimental diets, a commercial pellet feed free from exogenous NTs was purchased from Biomar (Nersac, France) and used to prepare the experimental diets. The diet was finely ground in a hammer mill, sieved to remove large particles, and finally supplemented with different levels of NTs (i.e. 1.5, 2.5, 3.5, and 5.0 g/kg) purchased from Vannagen (Chemoforma, Augst, Switzerland) containing purified adenosine-5-monophosphate (AMP), disodium guanidine-5-monophosphate (GMP), disodium thymidine-5-monophosphate (TMP), disodium uridine-5-monophosphate (UMP), cytidine-5-monophosphate (CMP) at ratios of 1:1:1:1:1 and purified RNA. The powdered diets were then blended with the supplemented NTs for 30 min to ensure mixture homogeneity and were moistened with water to form a stiff dough. The dough was passed through a meat grinder (ME6051131-1600 W, Moulinex, France) with an appropriate diameter (3 mm) and air-dried at room temperature using an electronic fan until the moisture level was reduced to less than 10%. The resulting strands were then broken up and sieved into convenient pellet sizes before being stored in a freezer at -20 °C. The basal diet with no NTs supplementation was prepared in the same procedure and served as the basal diet. The proximate composition and ingredients of the experimental diets are shown in Table 1.

Ethics statement

All experimental protocols were approved [Islamic Azad University, Science and Research Branch, Tehran, Iran]. All methods were carried out in accordance with relevant guidelines and regulations. The experiment was conducted in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Fish rearing and feeding conditions

Healthy Sterlet sturgeon were obtained from the International Sturgeon Research Institute, Guilan, Iran, and adapted to the experimental conditions in circular cement tanks (diameter: 3 m, height: 1.2 m) in a flow-through system (9.3 l/min flow rate) with continuous aeration for 2 weeks, while fed to apparent satiation with the basal diet (without NTs supplementation). At the end of the acclimation period, 12 fish (average initial weight ± SE: 95.33 ± 1.23 g) were randomly stocked in fifteen flow-through 500-L tanks equipped with continuous aeration. Each experimental diet was randomly assigned to three replicates of tanks. The experimental animals were fed by hand to apparent satiation five times daily (04.00, 08.00, 12.00, 16.00, and 20.00) for 10 weeks. During the experimental period, the water temperature was 17.0 ± 0.5 °C, dissolved oxygen 8.4 ± 0.2 mg/L, pH 7.8 ± 0.2 and ammonia 0.14 ± 0.01 mg/L. A photoperiod of 12 h light (08:00 to 20:00 h) and 12 h dark was maintained during the experiment.

Sample collection and growth assessment

After 10 weeks of the feeding trial, all fish were starved for 24 h and anesthetized with clove oil (extracted from clove flower buds; *Syzygium aromaticum*, family Myrtaceae) at 0.070 mg/L. Then, the total number and weight of fish in each tank were determined to calculate the biomass and other biometric attributes. Subsequently, nine fish from each treatment (three fish per tank) were randomly selected to collect blood samples from the caudal veins using a 5-ml sterile syringe containing heparin as the coagulant. An aliquot (500 µl) of each blood sample was separated in heparinized tubes for complete blood count (CBC) assays, while the remaining blood was left

Ingredients (g/kg in dry weight)	Dietary levels of nucleotides (g/kg)				
	0 (Control)	1.5	2.5	3.5	5.0
Commercial sturgeon pellet[a]	985.0	985.0	985.0	985.0	985.0
NTs (Vannagen™)[b]	0.0	1.5	2.5	3.5	5.0
Vitamin C[c]	10.0	10.0	10.0	10.0	10.0
Sand	5.0	3.5	2.5	1.5	0.0
Total (g)	1000.0	1000.0	1000.0	1000.0	1000.0
Proximate composition (g kg^{-1})					
Fiber	30.0	30.1	29.9	30.2	29.8
Protein	469.7	469.9	470	470.1	470.3
Fat	160.1	161.0	159.9	160.9	160.3
Ash	69.0	69.1	68.9	69.5	69.0
Phosphorus	11.4	11.3	11.0	11.5	11.7
Calcium	14.1	14.0	13.9	14.2	13.8
Sodium	4.2	4.1	4.0	4.3	4.0
NFE[d]	200.3	201	200.1	200.4	200.1
Gross energy (MJ/kg)[e]	21.8	21.4	21.6	21.2	21.5

Table 1. The proximate composition and ingredients of experimental diets. [a]BioMar 3 mm (Nersac, France). [b]Vannagen™ (Chemoforma, Augst, Switzerland). [c]L-ascorbic acid-2-phosphate (thermostable source of vitamin C), Kemin Co., Belgium. [d]Nitrogen free extract, NFE = 1000 - (protein g/kg + lipid g/kg + Ash g/kg + fiber g/kg). [e]Gross energy (MJ/kg) = (0.0242 × g/kg protein + 0.0366 × g/kg lipid + 0.029 × g/kg fiber + 0.017× g/kg NFE).

to clot at 4 °C for a period of 12 h into non-heparinized tubes to isolate serum samples. Serum was separated by centrifugation at 3500 ×g for 15 min at 4 °C using a 3-30k Sigma centrifuge (Laborzentrifugen GmbH, Osterode am Harz, Germany) and stored at -80 °C for serum biochemical analysis. The blood smears were also prepared from a blood drop of the sampled fish for evaluation of differential leucocyte counts.

The sampled fish were then euthanized with an overdose of clove oil (1 mg/L) to remove the mid-intestine by dissection on ice. The sampled intestines were separately washed with phosphate-buffered saline (PBS, pH 7.5) and fixed in 4% polyoxymethylene for histological evaluation. The whole digestive tract of another three fish per tank was removed after euthanizing with the same procedure described above and washed with cold distilled water. The washed intestines were separately homogenized into chilled buffer solution (KCl 50 mM, Tris-HCl 50 mM, and CaCl 20 mM, pH: 7.5) at a ratio of 9:1 (v/w) using a homogenizer (PT 10–35 GT, Polytron®, Kinematica, Lucerne, Switzerland). The homogenates were centrifuged at 10,000 ×g for 15 min at 4 °C. Finally, the resultant supernatants were collected into 1.5-ml microtubes and stored at -20 °C to assay digestive enzyme activity.

Growth performance and feed utilization of Sterlet sturgeon were evaluated by calculating relative growth rate (RGR), feed conversion ratio (FCR), and feed intake (FI) based on the following equations:

$$\mathrm{RGR}\ (\%/\mathrm{day}) = 100 \times \frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{initial weight (g)} \times days}$$

$$\mathrm{FCR} = \frac{\text{feed consumed (g)}}{\text{fish weight gain (g)}}$$

$$\mathrm{FI\ (g/fish)} = \frac{\text{feed consumed (g)}}{\text{fish number}}$$

CBC measurements

The erythrocyte (RBC; red blood cells), leukocyte (WBC; white blood cells), and differential leukocyte (lymphocytes, neutrophils, monocytes, and eosinophils) counts were calculated according to the Blaxhall and Daisley[23] method. The hemoglobin (Hb) concentration was measured according to the cyanomethaemoglobin method[24]. The hematocrit (HCT) value was estimated using hematocrit capillaries and centrifuging at 10,000 ×g for 10 min at ambient temperature[25]. The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were also calculated using the following equation[26]:

$$\mathrm{MCV\ (Fl)} = \frac{\mathrm{HCT\ (\%)} \times 10}{\mathrm{RBC\ (million/mm3)}}$$

$$\mathrm{MCH\ (pg)} = \frac{\mathrm{Hb\ (g/dL)} \times 10}{\mathrm{RBC\ (million/mm3)}}$$

$$\mathrm{MCHC\ (g/dL)} = \frac{\mathrm{Hb\ (g/dL)} \times 100}{\mathrm{HCT\ (\%)}}$$

Serum biochemical measurements

Hepatic enzyme activity

The level of liver enzymes activity in the serum samples was determined using a biochemical auto-analyzer (RA1000, Technicon Instruments, NY, USA). The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in the serum were measured based on the method of Reitman and Frankel[27] using an enzymatic colorimetric test in 546 nm. One unit of AST and ALT was defined as the amount of enzyme required at 37 °C (pH 8.0) to generate 1.0 µmol/min glutamate and pyruvate, respectively. Determination of alkaline phosphatase (ALP) in the serum was performed based on the spectrophotometric method described by Bowers and McComb[28]. One unit of ALP was defined as the amount of enzyme that hydrolyzes 1 µmol of 4-nitrophenyl phosphate in 1 min at 37 °C under assay conditions.

Serum antioxidant capacity

Superoxide dismutase (SOD, U/mg protein) activity was evaluated by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm due to O_2 generated by the xanthine/xanthine oxidase system[29]. One unit of SOD activity was defined as the amount of protein causing 50% inhibition of NBT reduction rate. Catalase (CAT, U/mg protein) activity was determined by measuring the enzymatic decomposition of H_2O_2 at 240 nm as described by Aebi[30]. One unit of CAT activity was defined as the enzyme concentration required to transform 1 mmole/min H_2O_2. Glutathione peroxidase (GPx, U/mg HGB) activity was determined according to Kraus and Ganther[31] by measuring the oxidation rate of NADPH at 412 nm in the presence of exogenous reduced glutathione, cumene hydroperoxide, and glutathione reductase. One unit of GPx activity was defined as the amount of enzyme necessary to oxidize 1 µmole NADPH/min.

The total antioxidant capacity (T-AOC, µmol/mg protein) was determined using the ferric reducing antioxidant power (FRAP) method[32]. The reduction of ferric ions to ferrous form by antioxidants present in the liver sample caused a colored complex ferrous–tripyridyl-S-triazine formation whose concentration was spectrophotometrically measured at 593 nm. The antioxidant capacity of the sample was determined by comparing its absorbance to that of a standard curve generated using known concentrations of Trolox. One unit of T-AOC was defined as the level of antioxidants present in a sample that can neutralize a specific amount of free radicals or oxidants. Malondialdehyde (MDA, nmol/mg protein) level was determined as an index of lipid peroxidation by measuring the pink color absorbance generated by the reaction of thiobarbituric acid reactive substances with MDA at 530 nm based on the method of Yagi[33].

Digestive enzyme activity assay

The protease activity was determined by the casein-hydrolysis method of Furné, et al.[34], where one unit of enzyme activity represents the level of enzyme required to catalyze the formation of 1 µg of tyrosine per minute under assay conditions. Lipase activity was analyzed by the method of Winkler and Stuckmann[35], where one unit of enzyme activity was defined as the amount of free fatty acid released from triacylglycerol per minute under assay conditions. Amylase activity was determined by the starch-hydrolysis method of Bernfeld[36], where one unit of enzyme activity represents the amount of enzyme that produces one µmol of maltose per minute under assay conditions. The protein content was determined according to the method developed by Lowry, et al.[37] using bovine serum albumin as the standard. All digestive enzyme activities were expressed as U/mg protein.

Histological measurements

The intestinal histological assay was performed according to the method described by Gava, et al.[38] method. The formalin-fixed intestinal samples were processed for histological examination including dehydration in graded ethanol concentrations and equilibration in xylene. The fixed intestinal samples were then paraffin-embedded and cut by an HM 310 rotary microtome (Microm, Germany). The sections (~4 µm) were stained with haematoxylin and eosin (HE). Afterward, morphological structures of stained tissues were analyzed under a light microscope by multiple magnifications equipped with an onboard camera (Zeiss, Cyber-Shot, Japan). The scale bar identification and magnification proof were performed using Measure software version 1.1.0.

Statistical analysis

All data were subjected to statistical verification by R software (R studio v. 4.3.2; RStudio Inc., Massachusetts, USA). Normality and homogeneity of variances were checked with Kolmogorov-Smirnov and Levene's tests, respectively. One-way analysis of variance was used to determine whether significant differences occurred between the treatments. When overall differences were found, the significances between means were evaluated using Turkey's HSD test. All differences were considered significant at the probability of less than 0.05 and the results were presented as means ± SE (standard error). Orthogonal polynomial contrasts were also used to assay the linear and quadratic effect of NTs supplementation on experimental variables in Sterlet sturgeon.

Results

Growth and feed efficacy

At the end of the 10-week feeding trial, the highest FW and RGR were obtained in fish fed with the 5.0 g/kg NTs-supplemented diet ($p < 0.05$), while they had no significant difference with the respective values in fish fed with the 3.5 g/kg NTs-supplemented diet (Table 2). No significant difference was also recorded in the level of FW and RGR among fish fed diets supplemented with 1.5, 2.5, and 3.5 g/kg NTs ($p > 0.05$). The FCR value in fish

Parameter	Dietary levels of NTs (g/kg)				
	0 (Control)	1.5	2.5	3.5	5.0
IW	95.00 ± 1.52^{a}	95.33 ± 1.45^{a}	94.67 ± 0.67^{a}	96.00 ± 1.16^{a}	95.67 ± 1.33^{a}
FW	179.33 ± 3.52^{a}	196.33 ± 20.49^{b}	190.67 ± 8.95^{ab}	200.33 ± 16.7^{bc}	215.33 ± 4.33^{c}
RGR	1.27 ± 0.08^{a}	1.51 ± 0.26^{ab}	1.45 ± 0.15^{ab}	1.55 ± 0.28^{bc}	1.78 ± 0.10^{c}
FCR	1.48 ± 0.003^{a}	1.38 ± 0.04^{b}	1.39 ± 0.03^{b}	1.40 ± 0.02^{b}	1.20 ± 0.10^{c}
FI	120.48 ± 5.07^{a}	142.28 ± 17.14^{b}	137.26 ± 6.30^{b}	142.93 ± 13.10^{b}	178.08 ± 3.61^{c}

Table 2. Effect of dietary nucleotides (NTs) supplementation on growth performance of Sterlet sturgeon, *Acipenser ruthenus*, after 10 weeks*. *Values are presented as means ± SE of three replicates per treatment ($n = 3$) with three fish per replicate. The values with different letters within the same row are significantly different ($p < 0.05$). IW (g), initial weight; FW (g), final weight; RGR (%/day), relative growth rate; FCR, feed conversion ratio; FI (g/fish), feed intake.

Parameter	Dietary levels of NTs (g/kg)				
	0 (Control)	1.5	2.5	3.5	5.0
WBC	4.90 ± 0.26^{a}	4.93 ± 0.48^{a}	6.43 ± 0.17^{ab}	9.13 ± 0.35^{b}	6.33 ± 0.69^{ab}
RBC	0.70 ± 0.69	0.71 ± 0.45	0.66 ± 0.63	0.63 ± 0.41	0.64 ± 0.19
Hb	5.93 ± 0.15	5.93 ± 0.20	5.43 ± 0.03	5.23 ± 0.07	5.30 ± 0.12
HCT	28.00 ± 0.58	28.33 ± 0.97	24.67 ± 0.13	24.33 ± 0.33	25.33 ± 0.20
MCV	398.67 ± 3.28^{b}	401 ± 4.65^{b}	372 ± 6.66^{a}	387.33 ± 3.18^{ab}	396.67 ± 6.98^{b}
MCH	84.53 ± 0.50^{b}	84 ± 0.96^{b}	80.63 ± 1.22^{a}	83.33 ± 0.64^{ab}	83.03 ± 0.76^{ab}
MCHC	21.17 ± 0.09	20.93 ± 0.12	21.81 ± 0.18	21.47 ± 0.27	21.06 ± 0.19
Neut	13.40 ± 0.88^{a}	12.36 ± 0.33^{a}	14.70 ± 1.20^{ab}	17.60 ± 0.88^{b}	15.30 ± 1.10^{ab}
Lym	83.00 ± 1.15^{b}	83.27 ± 0.33^{b}	79.33 ± 2.19^{ab}	75.40 ± 0.88^{a}	80.34 ± 2.03^{ab}
Mono	3.32 ± 0.88^{a}	3.65 ± 0.31^{a}	5.00 ± 0.58^{b}	5.76 ± 0.67^{b}	4.00 ± 0.51^{ab}
Eos	1.00 ± 0.08	1.00 ± 0.09	1.50 ± 0.21	1.30 ± 0.30	1.00 ± 0.18

Table 3. Effect of dietary nucleotides (NTs) supplementation on hematological parameters of Sterlet sturgeon, *Acipenser ruthenus*, after 10 weeks*. *Values are presented as means ± SE of three replicates per treatment ($n = 3$, number of tanks) with three fish per replicate. The values with the different letters within the same row are significantly different ($p < 0.05$). WBC ($\times 10^3$/μl), white blood cell; RBC ($\times 10^6$/μl), red blood cell; Hb (g/dL), hemoglobin; HCT (%), hematocrit; MCV (Fl), mean corpuscular volume; MCH (pg), mean corpuscular hemoglobin; MCHC (g/dL), mean corpuscular hemoglobin. Neut (%), neutrophil; Lym (%), Lymphocyte; Mono (%), Monocyte; Eos (%), Eosinophil (%).

fed diets supplemented with different concentrations of dietary NTs was significantly lower compared to those fed the basal diet ($p < 0.05$), with the lowest value of 1.20 ± 0.1 in the fish fed diet supplemented with the highest NTs level ($p > 0.05$). The highest and lowest FI were also observed in fish fed with the 5 g/kg NTs and basal diets, respectively ($p < 0.05$).

Hematological parameters

Table 3 shows the effect of dietary NTs on the hematological profile of Sterlet sturgeon after 10 weeks. The values of RBC, Hb, and MCHC as well as HCT and eosinophil percentages showed no significant differences among the experimental groups ($p > 0.05$). The highest count of WBC was obtained in the fish fed with 3.5 g/kg NTs-supplemented diet compared to those fed the basal and 1.5 g/kg NTs-supplemented diets ($p < 0.05$). A significantly lower value of MCV was obtained in fish fed the diet supplemented with 2.5 g/kg NTs than in other experimental groups, except for those fed the diet supplemented with 3.5 g/kg NTs. The lowest MCH content was also observed in the fish fed diet supplemented with 2.5 g/kg NTs, which was significantly lower than those fed with the basal and 1.5 g/kg NTs-supplemented diets ($p < 0.05$). The highest and lowest percentages of neutrophils and lymphocytes were observed in the blood of fish fed with the 3.5 g/kg NTs-supplemented diet ($p < 0.05$). Also, the monocyte percentage reached the highest value in the fish fed supplemented diet with the 3.5 g/kg NTs, although no significant difference was obtained between those fed diets supplemented with 2.5 and 5.0 g/kg NTs ($p > 0.05$).

Hepatic enzyme activity

The ALP, AST, and ALT concentrations in the serum of Sterlet sturgeon fed with different levels of NTs are presented in Table 4. At the end of the 10-week feeding trial, the serum ALP and AST values showed a decreasing trend in NTs-added groups compared to those in the control group ($p < 0.05$). A significantly decreasing trend was also observed in the serum ALT of the fish fed diets supplemented with dietary NTs ($p > 0.05$), while no

Parameter	Dietary levels of NTs (g/kg)				
	Control (0)	1.5	2.5	3.5	5.0
ALP	374.00 ± 22.34^{c}	225.00 ± 5.131^{ab}	213.67 ± 6.64^{a}	262.00 ± 15.28^{b}	285.00 ± 6.43^{b}
AST	578.00 ± 11.15^{c}	398.33 ± 16.29^{b}	222.00 ± 25.94^{a}	167.00 ± 27.62^{a}	455.67 ± 1.45^{b}
ALT	22.67 ± 1.20^{d}	17.33 ± 1.20^{c}	12.00 ± 0.58^{b}	8.67 ± 0.88^{a}	20.67 ± 0.88^{d}

Table 4. The effects of dietary nucleotides (NTs) supplementation on serum hepatic enzymes of Sterlet sturgeon (*Acipenser ruthenus*) after 10 weeks*. *Values are presented as means ± SE of three replicates per treatment ($n = 3$, number of tanks) with three fish per replicate. The values with the different letters within the same row are significantly different ($p < 0.05$). ALP (U/l), alkaline phosphatase; AST (U/l), aspartate aminotransferase; ALT (U/l), Alanine aminotransferase.

Parameter	Dietary levels of NTs (g/kg)				
	0 (Control)	1.5	2.5	3.5	5
SOD	1.43 ± 0.14^{a}	1.45 ± 0.18^{a}	1.90 ± 0.21^{b}	2.55 ± 0.27^{c}	1.81 ± 0.13^{b}
CAT	3.57 ± 0.08^{a}	3.75 ± 0.13^{a}	4.12 ± 0.26^{b}	4.34 ± 0.24^{b}	3.45 ± 0.15^{a}
GPx	25.14 ± 2.99^{b}	37.42 ± 1.52^{c}	47.09 ± 4.23^{d}	26.97 ± 1.08^{b}	22.25 ± 1.85^{a}
T-AOC	44.87 ± 2.15^{a}	44.50 ± 3.30^{a}	61.88 ± 2.84^{b}	57.40 ± 6.08^{b}	42.43 ± 3.35^{a}
MDA	1.91 ± 0.13^{ab}	1.95 ± 0.11^{b}	1.62 ± 0.08^{a}	1.60 ± 0.6^{a}	2.27 ± 0.1^{c}

Table 5. Effect of dietary nucleotides (NTs) supplementation on serum antioxidant capacity of Sterlet sturgeon, *Acipenser ruthenus*, after 10 weeks*. *Values are presented as means ± SE of three replicates per treatment ($n = 3$, number of tanks) with three fish per replicate. The values with the different letters within the same row are significantly different ($p < 0.05$). SOD (U/mg protein), superoxide dismutase; CAT (U/mg protein), catalase; GPx (U/mg protein), glutathione peroxidase; T-AOC (μmol/mg protein), total antioxidant capacity; MDA malondialdehyde (μmol/mg protein).

Parameter	Dietary levels of NTs (g/kg)				
	Control	1.5	2.5	3.5	5
Protease	10.17 ± 1.27^{a}	13.5 ± 0.02^{ab}	14.46 ± 1.02^{b}	18.31 ± 2.25^{c}	14.72 ± 1.21^{b}
Lipase	2.75 ± 0.28^{a}	3.78 ± 0.12^{ab}	4.15 ± 0.71^{c}	3.92 ± 0.82^{b}	3.65 ± 0.35^{ab}
Amylase	22.76 ± 3.11^{a}	29.85 ± 0.765^{ab}	30.51 ± 2.26^{ab}	46.04 ± 7.22^{c}	31.67 ± 2.13^{b}

Table 6. Effect of dietary nucleotides (NTs) supplementation on digestive enzyme activity (U/mg protein) of Sterlet sturgeon, *Acipenser ruthenus*, after 10 weeks*. *Values are presented as means ± SE of three replicates per treatment ($n = 3$) with three fish per replicate. The values with the different letters within the same row are significantly different ($p < 0.05$).

significant difference was observed in ALT of fish fed the basal diet and those fed with 5.0 g/kg NTs-supplemented diets ($p > 0.05$).

Antioxidant capacity

Table 5 shows the effect of dietary NTs supplementation on the fish antioxidant capacity. The highest serum SOD was seen in the fish fed the diet supplemented with 3.5 g/kg NTs ($p < 0.05$). The highest serum CAT was also obtained in the fish fed the diet supplemented with the 3.5 g/kg NTs, although it had no significant difference with that in fish fed diet supplemented with 2.5 g/kg NTs ($p > 0.05$). The serum GPx reached its highest concentration in the fish fed the diet supplemented with 2.5 g/kg NTs ($p < 0.05$). The highest serum T-AOC was also obtained in fish fed with 2.5 g/kg NTs-supplemented diet, which had no significant difference **compared to** that in fish fed with 3.5 g/kg NTs-supplemented diet ($p > 0.05$). On the other hand, a significantly decreasing trend was observed in the serum MDA in the fish fed diet supplemented with NTs up to 3.5 g/kg ($p < 0.05$), while those received the highest level of dietary NTs showed the highest serum concentration of MDA.

Digestive enzyme activity

After 10 weeks of feeding with different levels of dietary NTs supplementation, the highest activities of protease and amylase were obtained in fish fed with the 3.5 g/kg NTs-supplemented diet ($p < 0.05$), while no significant difference was recorded in the activities of these digestive enzymes between fish fed the other NTs-added supplemented diets ($p > 0.05$; Table 6). The highest activity of lipase was observed in fish fed the 2.5 g/kg NTs supplemented diet ($p < 0.05$). However, the lipase activity of fish fed the basal diet had no significant difference from that in the fish fed with NTs-supplemented diets at 1.5 and 5.0 g/kg ($p > 0.05$).

Intestine histomorphology

The histo-morphological characteristics of the intestine (mid-gut) samples are shown in Fig. 1 (a-e). The histological examination of the intestine in all groups showed that Sterlet sturgeon fed with the 3.5 g/kg NTs supplemented diet had significantly the highest and widest villi ($p < 0.05$), while the control group, 1.5, and 5.0 g/kg NTs had no significant differences (Fig. 1-f).

Table 7 illustrates the histopathological features of the mid-gut samples of Sterlet sturgeon fed with different levels of dietary NTs for 10 weeks. Fish fed the diet supplemented with dietary 2.5 and 3.5 g/kg NTs had a higher density of goblet cells (GC) than those in other experimental groups. The necrosis (N) of enterocytes and microvillus was not observed in fish fed with 3.5 g/kg NTs-supplemented diet, while the severe N was recorded in fish fed the 5.0 g/kg NTs-supplemented diet. The vacuolation (V) of enterocytes had low levels in the fish fed with NTs-supplemented diets at 2.5 and 3.5 g/kg compared to those fed with 1.5 and 2.5 g/kg NTs. No hemorrhage (He) was observed in the fish fed with 1.5, 2.5, and 3.5 g/kg NTs-supplemented diets, while the highest abundance of He was seen in the fish fed with 5.0 g/kg NTs-supplemented diet followed by those fed the basal diet.

Principal component analysis and Pearson's correlation of variables

The PCA was performed to appraise correlations among the variables in Sterlet sturgeon fed with different levels of NTs (Fig. 2). The first two PCA-biplots were 68.7% (52.1% and 16.6%). The PCA ordination yielded three distinct clusters group 1 consisted of some variables in the basal diet, group 2 consisted of the variables in diets supplemented with 1.5 and 5.0 g kg NTs, and group 3 consisted of variables in the diet supplemented with the 2.5 and 3.5 g/kg NTs. This axis seemed to be associated with the NTs interaction because group 3 was on the right and up, group 2 was on the left and up, and group 1 was on the right and up.

Moreover, the antioxidant and some hematological and digestive enzymes parameters including WBC, SOD, CAT, MDA, T-AOC, neutrophil, eosinophil, monocyte, amylase, lipase, and protease values were at the upper right of the PCA. However, the hepatic enzymes (ALP, AST, and ALT) and hematological indices (MCH, MCV, Hb, HCT, and RBC) were at the upper left side of the PCA. As shown in Fig. 3, the antioxidant responses (except for MDA) have positive correlations with some hematological parameters (WBC, neutrophil, eosinophil, monocyte, and MCHC) and digestive enzymes, while they had negative correlations with the liver enzymes (ALP, AST, and ALT).

Optimum rate of dietary NTs

The optimum dietary levels of NTs in the Sterlet sturgeon for the best performance are summarized in Table 8. Based on the second-order polynomial regression analysis, the best NTs dietary supplementation levels required for the Sterlet sturgeon to reach the lowest ALP, AST, and ALT values were 2.87, 2.90, and 2.79 g/kg, respectively. The best results for CAT, GPx, T-AOC, and MDA values were also obtained at dietary NTs levels of 2.60, 2.27, 2.61, and 2.22 g/kg, respectively. To achieve the highest activity of protease, lipase, and amylase, dietary NTs levels were finally predicted to be 3.62, 3.09, and 3.5 g/kg. Accordingly, the optimum dietary level of NTs for Sterlet sturgeon lies in the range of 2.2–3.6 g/kg.

Discussion

According to the available literature, the beneficial effects of dietary NTs have been proven on the growth performance of different aquaculture fish species[22,39–42]. Asaduzzaman, et al.[41] reported that dietary NTs (1–8 g/kg) upregulated the expression of major growth-related genes in Nile tilapia. Tie, et al.[43] conducted a 60-day feeding trial to investigate the effects of dietary NTs levels on growth performance, proximate composition, and some physicochemical responses of grass carp (*Ctenopharyngodon idella*) and reported that dietary NTs supplementation increased the growth of grass carp with increased protein and lipid in muscle.

Hematological indices are commonly used as health biomarkers in fish because they can be influenced by many biotic and abiotic factors such as age, gender, water quality, seasonal patterns, stress, and nutrition status[44]. However, the standard values and reference intervals of these parameters are still undefined for some fish species, especially sturgeons. A few studies have been conducted on the effects of dietary NTs on the hematological parameters of fish. In the current study, dietary NTs could significantly affect some of the CBC values in Sterlet sturgeon, although RBC, HCT, Hb, MCHC, and eosinophil were not affected by dietary NTs. Dietary supplementation of NTs could not significantly influence RBS counts in rainbow trout[45], Beluga[46], and catla, *Catla catla*[47]. Barros, et al.[48] did not observe a significant difference in hematological indices of tilapia fed diets supplemented with NTs at 0.5–4 g/kg after 60 days. Karimzadeh, et al.[49] stated RBC and Hb were not influenced by both NTs sources, but the HCT percentage was increased by the addition of both NTs sources at 1.5% and 0.5% in kutum (*Rutilus kutum*). Welker, et al.[50] reported no alteration in the HCT level of channel catfish in response to dietary NTs at 1, 3, 9, and 27 g/kg for 60 days. In this study, the higher levels of RBC, HCT, and Hb in the control fish and the decreasing trend in the NTs-treated groups, although not significantly different can be attributed to the positive role of NTs in the body. Increasing these indices is not always advantageous, for instance, the increased HCT level can be typically a reaction to the stress response[51] and an increase in RBC counts can be generally due to conditions such as low oxygen levels, kidney disease, and poor heart and lung functions, in which the body increases erythrocytes to compensate for the lack of oxygen under stressful conditions[52,53].

Leukocytes are important immune cells with diverse functions that help the host to combat foreign germs[54]. Dietary NTs can affect the maturation and proliferation of leukocytes[55]. In this regard, neutrophils are the first line of defense among leukocytes, when fish encounter pathogen attacks to eliminate foreign agents[56]. In the current study, the highest count of WBC and neutrophil percentage were obtained in the fish fed with 3.5 g/kg NTs-supplemented diet for 10 weeks. Monocytes are the other immune cells with phagocytic and chemotactic

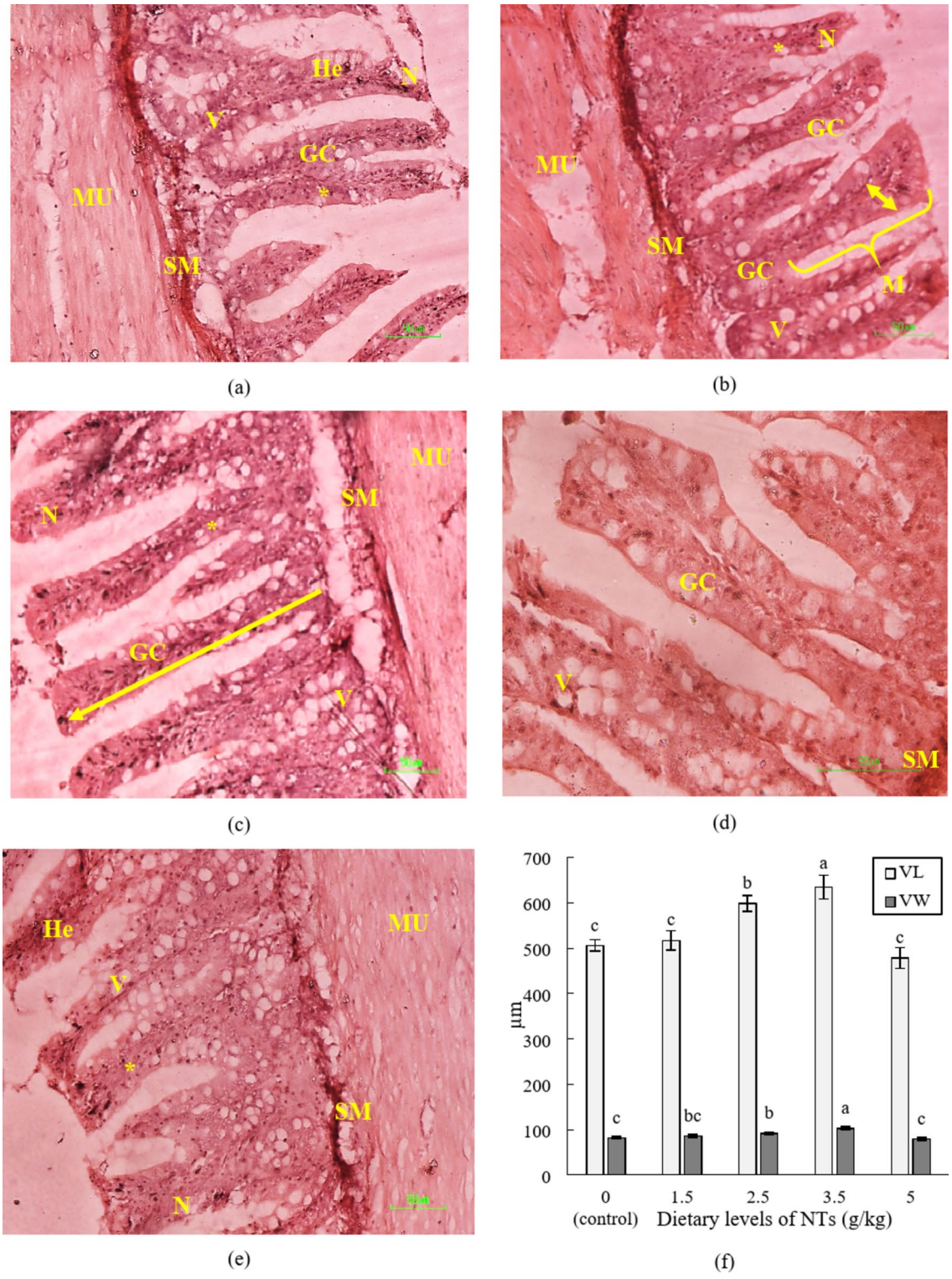

Fig. 1. Histomorphological features of the mid intestine of Sterlet sturgeon (*Acipenser ruthenus*) fed diets supplemented with different levels of nucleotides (NTs) for 10 weeks. Cross section from midgut samples; control (**a**), 1.5 (**b**), 2.5 (**c**), 3.5 (**d**), 5.0 (**e**) g/kg NTs, and comparison of morphological parameters (**f**). Values are presented as means ± SE of three replicates per treatment ($n = 3$, number of tanks) with three fish per replicate. The values with the different letters on each column are significantly different ($p < 0.05$). GC, Goblet cell; N, necrosis; He, hemorrhage; V, vacuolated enterocytes; MU, muscular layer; SM, submucosal layer; M, mucous layer; VL, villus length; VW, villus width; double arrow, expansion of lamina propria; *, contraction of the lamina propria; big arrow, the length of intestinal folds (All images are captured after H&E staining under 2.4 optical zoon provided by camera at 40× magnification, scale bar = 50 µm).

Parameters	Dietary levels of NTs (g/kg)				
	Control (0)	1.5	2.5	3.5	5
Goblet cells (GC)	+	++	++	++	++
Necrosis (N)	+	+	+	–	++
Vacuolation (V)	+	++	+	+	++
Hemorrhage (He)	+	–	–	–	++

Table 7. Relative abundance of goblet cells (GC), necrosis (N), vacuolation (V), and hemorrhage (He) in the midgut samples of Sterlet sturgeon (*Acipenser ruthenus*) fed diets supplemented with different levels of nucleotides (NTs) for 10 weeks*. * (–), no observed, (+) low, (++) medium, (+++) relatively high and (++++) high density.

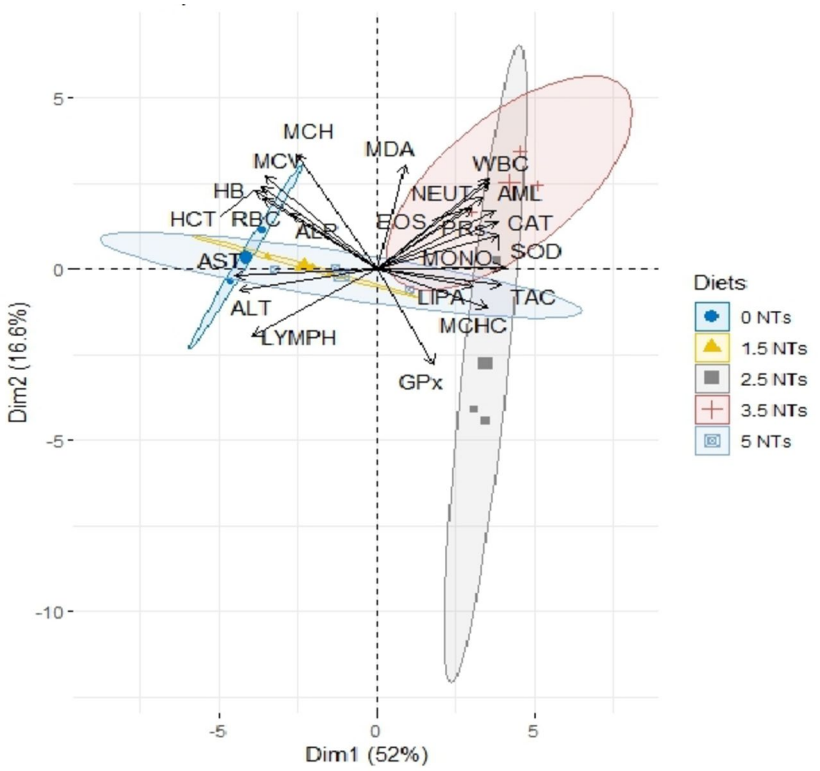

Fig. 2. The principal component analysis (PCA) between the serum biochemical parameters of Sterlet sturgeon (*Acipenser ruthenus*) in response to dietary nucleotides (NTs) after 10 weeks. Blue points, control group; Yellow point, 1.5 g/kg NTs group; Black point, 2.5 NTs group; Redpoint, 3.5 NTs group; Gray point, 5.0 g/kg NTs group.

functions that produce lysozyme, an antimicrobial enzyme that plays a key role in nonspecific defense[39,57]. In this study, the monocyte percentage reached the highest value in fish fed with 3.5 g/kg NTs-supplemented diet, although it had no significant difference compared to those fed with NTs-supplemented diets at 2.5 and 5.0 g/kg. Similarly, Jha, et al.[47], Tahmasebi-Kohyani, et al.[45], and Reda, et al.[20] showed a significant increase in WBC counts in response to dietary NTs in catla, rainbow trout, and Nile tilapia, respectively. In the present study, the percentage of Lymph in the blood of fish fed 3.5 g/kg NTs-supplemented diet was lower than those fed the basal diet after 10 weeks. The duration of administration of dietary NTs showed a different effect on the fish immune components and disease resistance[20]. Leonardi, et al.[58] recorded an enhancement in the lymph percentage of rainbow trout after 60 days of NTs feeding, while this effect was decreased after feeding for 120 days. The inclusion of NTs in aquafeed can improve fish immune system such as phagocytosis activity[55,59,60], natural killer cells, and macrophage activation[61]. Altogether, the increased values of WBC, neutrophil, and monocyte in Sterlet sturgeon by increasing dietary NTs may confirm the idea that NTs supplementation can improve the innate immune system.

The liver has a wide range of functions in fish including detoxification, protein synthesis, and nutritional digestion[62]. The ALP is an important enzyme for breaking down proteins and is found in the liver but it is also made in bones, intestines, pancreas, and kidneys. The AST and ALT are other abundant hepatic enzymes that catalyze the transfer of amino groups to form the hepatic metabolites pyruvate and oxaloacetate, respectively. In

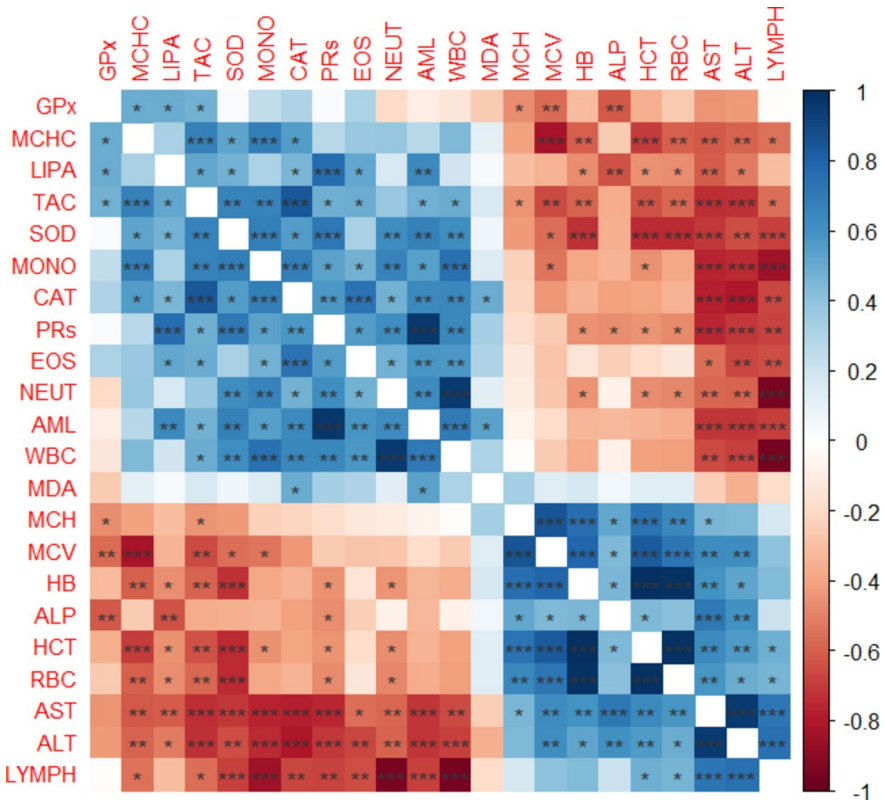

Fig. 3. Pearson's correlation between all studied parameters in Sterlet sturgeon (*Acipenser ruthenus*) in response to dietary nucleotides (NTs). The color intensity of boxes is proportional to the correlation values. Significant correlations are marked with no star: ($p \geq 0.05$), * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

Parameter	Equation	R^2	Optimum level (g/kg)
ALP	$Y = 17.406 \times^2 - 99.821x + 362.22$	0.854	2.87
ALT	$Y = 1.6012 \times^2 - 8.9487x + 23.987$	0.804	2.79
AST	$Y = 45.728 \times^2 - 266.18x + 610.33$	0.873	2.90
CAT	$Y = -0.1 \times^2 + 0.5192x + 3.4639$	0.683	2.60
GPx	$Y = -2.63 \times^2 + 11.931x + 26.012$	0.672	2.27
T-AOC	$Y = -2.1175 \times^2 + 11.056x + 41.954$	0.562	2.61
MDA	$Y = 0.0672 \times^2 - 0.2983x + 2.002$	0.627	2.22
Protease	$Y = -0.4982 \times^2 + 3.6074x + 9.7748$	0.8105	3.62
Lipase	$Y = -0.1238 \times^2 + 0.865x + 2.8159$	0.8407	3.09
Amylase	$Y = -1.3216 \times^2 + 9.2607x + 21.107$	0.5823	3.5

Table 8. Optimum dietary levels of nucleotides (NTs) for Sterlet sturgeon (*Acipenser ruthenus*) based on the significant quadratic relationships ($p < 0.05$) and polynomial regressions analysis*. *ALP(U/l), alkaline phosphatase; AST(U/l), aspartate aminotransferase; ALT(U/l) Alanine aminotransferase; T-AOC (µmol/mg protein), total antioxidant capacity; CAT(U/mg protein), catalase; MDA malondialdehyde (µmol/mg protein); GPx (U/mg protein), glutathione peroxidase; Protease (U/mg protein), Lipase (U/mg protein), Amylase (U/mg protein).

the present study, the supplementation with NTs up to 3.5 g/kg led to a significant decrease in the serum AST and ALT levels. In agreement with our results, the decline in most of the above enzymes has also been reported in barramundi, *Lates calcarifer*[63], Caspian trout, *Salmo trutta caspius*[64], *Ancherythroculter nigrocauda*[65], Iridescent shark, *Pangasianodon hypophthalmus*[40], and European sea bass, *Dicentrarchus labrax*[66]. The serum ALP and AST of Sterlet sturgeon fed the basal diet in the present study reached the highest values compared to those fed with NTs-supplemented diets, while the highest ALT level was obtained in those fed the basal and 5.0 g NT/kg supplemented diets. Clifford and Story[67] stated that excessive dietary NTs in monogastric animals like fish might have toxic impacts leading to protein, lipid, and carbohydrate metabolism dysfunctions due to the deficient levels of urease activity, the enzyme involved in the nucleotide metabolism. Therefore, the increased level of ALT

in the 5.0 g /kg NT group could be justified based on this physiological pathway. Also, some studies showed a marked increase in ALT and AST levels in red seabream and Nile tilapia fed with higher levels of NTs[18,68]. Based on the present result, the decline in the serum hepatic enzymes could explain the potential beneficial role of the dietary NTs up to 2.90 g/kg to improve liver functions in Sterlet sturgeon based on the orthogonal polynomial contrasts.

The antioxidant defense system is a multicomponent mechanism with enzymatic and non-enzymatic elements to protect cells and tissues from oxidative stress such as internal reactive oxygen species (ROS) that are generated during the immune response and metabolic process[69,70]. The antioxidant defense system in fish is dependent on various biotic and abiotic factors such as feeding behavior and nutritional components[71]. In the present study, the highest serum T-AOC and CAT values were obtained in Sterlet sturgeon fed the diet supplemented with 2.5 g/kg NTs. The highest serum GPx was also obtained in the fish fed diet supplemented with 2.5 g/kg NTs, while the best serum SOD activity was recorded in the fish fed the diet supplemented with 3.5 g/kg NTs. A remarkable decreasing trend was observed in the serum MDA in Sterlet sturgeon fed with NTs up to 2.5 g/kg. In agreement with the present results, the improvement of antioxidant status (i.e. SOD, CAT, T-AOC, and GPx) has been reported in juvenile turbot[17,72], red sea bream[18], rainbow trout[13], Nile tilapia[21], and hybrid tilapia[19] when fish were fed with the diets containing NTs-supplementation. The decrease in serum MDA concentration was reported by Reda, et al.[20] in Nile tilapia and Xu, et al.[19] in juvenile hybrid tilapia fed with NTs-supplemented diets. Additionally, Tie, et al.[43] observed an upregulated *Nrf 2* gene expression, which is crucial in initiating the antioxidant enzyme gene expressions in grass carp after feeding with dietary NTs supplementation and suggested that increased nutrient availability with dietary NTs might lead to upregulation of the antioxidant-related gene expression. In addition, the serum MDA level was significantly decreased by elevating the dietary levels of NTs up to 2.5 g/kg in Sterlet sturgeon. It can probably indicate a decrease in lipid peroxidations. However, decreases in the serum SOD, T-AOC, GPx, and CAT and an increase in serum MDA level of fish fed the 5.0 g/kg NTs diet probably indicate an increase in free radicals and lipid peroxidation.

The fish intestine serves as the first protection line against aquatic pathogens and its structural integrity has a vital role in feed utilization[14,73]. It is widely accepted that digestive enzymes activity is a useful tool to reveal nutrient absorption, digestive capacity, and growth performance in fish[74,75]. In this context, improvement in aquafeed can modulate enzymatic activities and nutrient absorption capacity, leading to improved feed utilization and growth rate[76]. In the present study, the highest activity of protease and amylase was obtained in fish fed with 3.5 g/kg NTs-supplemented diet compared to those fed the basal diet, while the highest activity of lipase was observed in the fish fed with 2.5 g/kg NTs-supplemented diet. Previous studies also showed that the digestive enzymes activity were increased by dietary NTs in the gut of rainbow trout[13], zebrafish[77], and olive flounder[15]. The oral administration of NTs could improve intestinal structure by providing a higher area for nutrient absorption as observed in Atlantic salmon[16] and turbot, *S. maximus*[17].

The histological examination of the intestine in the present study showed that Sterlet sturgeon fed with 3.5 g/kg NTs had the most height and villus width of villi compared to those in the other experimental groups. Some research showed that dietary NTs might promote villi growth, resulting in higher nutrient absorption. In similar study, the mean fold height of the proximal, mid, and distal intestine as well as the total gut surface area of Atlantic salmon fed with 0.03% NTs-supplemented diet was significantly greater than those fed the control diet[16]. Investigation of the histopathological features of the mid-gut of Sterlet sturgeon showed fish fed the diet with 2.5 and 3.5 g/kg NTs had a higher density of goblet cells and low levels of vacuolation of enterocytes compared to other experimental groups. The necrosis of enterocytes and microvillus was not observed in the fish fed with 3.5 g/kg NTs-supplemented diet and no hemorrhage was observed in the fish fed with NTs-supplemented diets at 1.5, 2.5, and 3.5 g/kg. These outcomes illustrate that the dietary levels of NTs were safe for Sterlet sturgeon at certain concentrations. The highest abundance of He and N was seen in the fish fed with 5.0 g/kg NTs-supplemented diet, which may show the negative impacts of dietary NTs at the higher dose. The NT supplemented diet improves the intestine structure through increased intestinal fold height and enterocyte number in fish[72]. In previous studies, dietary NTs have been shown to improve performance in monogastric animals by promoting the renewal of small intestine epithelial cells and influencing the composition of the beneficial microbial community in the digestive tract[78]. In the intestine, exogenous NTs are important for rapidly dividing mucosal cells due to absent or limited *de-novo* nucleotide synthesis[79,80]. Dietary NTs may affect the maturation status of the small-intestinal epithelium since the enzymes are maturation markers of intestinal cells[81]. The improvement of the intestinal structure and increased villi surface area could increase digestive enzyme activities through dietary NTs supplementations[13], a similar mechanism might have led to enhanced digestive enzymes activity (i.e. protease, lipase, and amylase) in the present study.

In conclusion, the findings of the present study demonstrated that dietary nucleotides supplementation could improve the hematological indices, liver function, antioxidant capacity, digestive performance, and intestinal functions of Sterlet sturgeon. Therefore, the optimum dietary levels of NTs for positive effects on physiological functions of Sterlet sturgeon lay in the range of 2.2–3.6 g/kg NTs after a 10-week administration based on polynomial regression analysis.

Data availability

The data supporting the findings of this study are available upon request on reasonable request. Interested parties can obtain the data by contacting the corresponding author.

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Author contributions

Meigol Taklu was responsible for the methodology, investigation, and data curation. Houman Rajabi Islami and Seyed Pezhman Hosseini Shekarabi contributed to the conceptualization of the project, with Houman Rajabi Islami also serving as the project supervisor. Seyed Abdolmajid Mousavi and Ayoub Yousefi Jourdehi conducted the data curation and formal analysis. All authors reviewed and approved the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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