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Effects of different levels of dietary manganese on growth, blood parameters, immunity indices, blood biochemical and tissue accumulation of elements in young beluga, *Huso huso*

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ABSTRACT

Manganese (Mn), functioning as a microelement, serves as a crucial cofactor in numerous enzyme systems. This element is indispensable for regulating the nervous system, bone growth, and reproduction, while also playing a pivotal role in carbohydrate metabolism. This research endeavours to establish the optimal dietary Mn range for young beluga, *Huso huso*. A cohort of 180 young beluga, with an average initial weight of 266 ± 3.05 g, underwent a two-week acclimatization period to adapt to their environment. The fish were systematically allocated into 6 treatments, each with three replications, encompassing concentrations of 5 (Mn_1), 10 (Mn_2), 15 (Mn_3), 20 (Mn_4), and 25 (Mn_5) mg of Mn sulphate monohydrate ($MnSO_4$, H_2O) per kg of diet. A control treatment (Mn_0), devoid of $MnSO_4$ supplementation, was also included. The feeding regimen involved 3 time-daily feedings, constituting 1.8% of the biomass, sustained over two months. Biometric growth indices at the end of each month revealed no statistically significant differences across diverse treatments ($p > 0.05$), but the maximum values of these indices were observed in the control and Mn_3 treatments, respectively. However, the number of red blood cells (RBCs) and white blood cells (WBC), as well as haemoglobin (Hb) levels, exhibited significant differences ($p < 0.05$). Additionally, significant variations were observed in the plasma triglyceride and cholesterol levels ($p < 0.05$). Mn_2 treatment demonstrated the highest significant activity of lysozyme and total immunoglobulin (Total Ig) levels ($p < 0.05$). Conversely, immunoglobulin M (IgM) levels and complements activity (C_3 and C_4) remained non-significant across Mn_0, Mn_1, and Mn_2 treatments ($p > 0.05$). Despite the noteworthy level of tissue Mn aggregation ($p < 0.05$), no significant differences were noted in the tissue cumulation of Ca, Fe, and P elements within muscle, blood, cartilage, and liver ($p > 0.05$). Based on the obtained results, it is concluded that the optimal supplementation level of $MnSO_4$ required for young *H. huso* falls within the range of 10-15 mg kg^{-1} of diet.

Keywords: Beluga, *Huso huso*, Diet, $MnSO_4$, Immunity, Biochemical, Element accumulation.
Article type: Research Article.

INTRODUCTION

Great sturgeon, *Huso huso* is a very suitable species for aquaculture in inland waters, due to its fast growth, adaptability and the possibility of reproduction in rearing conditions (Falahatkar *et al*. 2009; Madadi & Khara 2016). Notwithstanding the pivotal role minerals play in diverse physiological processes, encompassing growth, organ functionality, and skeletal structure development, there is a notable scarcity of research addressing the role and efficacy of mineral nutrition in aquatic animals (Antony Jesu Prabhu *et al*. 2016; Mir Rasekhian *et al*. 2022). On the other hand, one of the important problems of commercial aquaculture is the improvement of formulated diets to increase the growth and the health of aquatic animals (Chebanov & Billard 2001). Manganese (Mn) stands out among the scarce minerals due to its pivotal cofactor function in numerous enzyme systems. This element is

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indispensable for regulating the nervous system, bone growth, reproduction, and plays a crucial role in carbohydrate metabolism, contributing to the overall health of fish (Asaikkutti *et al*. 2016). Contrary to some studies reporting no significant impact of Mn supplementation on growth indices in *Salmo salar* (Maage *et al*. 2000) and *Carassius auratus* (Pan *et al*. 2008), research conducted by Sotoudeh *et al*. (2019) in *Oncorhynchus mykiss* demonstrated a significant effect of dietary Manganese supplements on safety indicators. Furthermore, the addition of Manganese supplements significantly enhanced Mn concentrations in the whole body and vertebral column of *Labeo rohita* (Musharraf & Khan 2021). Studies across different species revealed optimal dietary Mn levels, such as 13-15 mg kg^{-1} in *O. mykiss* (Ogino & Yang, 1980; Satoh *et al*. 1991, 1992), 7.5-10.5 mg kg^{-1} in *S. salar* (Maage *et al*. 2000), 1.16-4.16 mg kg^{-1} in *Larimichthys crocea* (Zhang *et al*. 2016), approximately 12.7 mg kg^{-1} in young hybrid grouper (*Epinephelus lanceolatus* × *E. fuscoguttatus*; Liu *et al*. 2018), 1.8-7.8 mg kg^{-1} in *Heteropneustes fossilis* (Zafar & Khan 2019), and 4.9-10.9 mg kg^{-1} in *L. rohita* (Musharraf & Khan 2021). These findings collectively underscore the species-specific nuances in dietary Mn requirements for optimal growth and physiological well-being. Despite the existing body of research on the significance and optimal dosage of Mn in various farmed fish species, regrettably, no such study has been undertaken for farmed sturgeon. Consequently, this research was designed and executed to address this gap in knowledge. The objective of this study is twofold: firstly, to uncover crucial insights into the role of Mn in farmed sturgeon, and secondly, to ascertain the suitable quantity of Mn supplementation required in the diet of young belugas. Through this investigation, we aim to contribute valuable information to the understanding of sturgeon nutrition, particularly in the context of Mn requirements.

MATERIALS AND METHODS

The investigation was conducted at the Shahid Dr. Beheshti Sturgeon Restoration and Genetic Conservation Centre, located in Rasht, Guilan Province, Iran. A total of 180 young beluga, with an average weight of 266 ± 3.05 g and an average length of 38.23 ± 0.49 cm, were utilized in this study. The research employed a completely random statistical design and was conducted under uniform rearing conditions. The Manganese (II) sulphate monohydrate ($MnSO_4$, H_2O) utilized in this study was procured from the Merck-Germany brand, identified by the code 1.05491.0250, and possessed a purity of 99%. For the preparation of experimental diets, GFS1 type concentrate diet with a 4 mm diameter from Faradane Company (Tehran, Iran) was employed. Initially, the diet underwent complete pulverization using an electric mill. Subsequently, the required amount of manganese sulphate ($MnSO_4$) was weighed, dissolved in double-distilled water, and added to the pulverized concentrate diet, ensuring thorough mixing. The resulting dough was then processed into edible pellets using a pellet machine equipped with 4 mm diameter of mesh size and subsequently dried. The prepared diet was stored in plastic bags at a temperature of 4 °C until the commencement of the experiment. The experiment comprised six treatments, each replicated three times. The selection of $MnSO_4$ concentrations for the treatments was guided by the study conducted by Ye *et al*. (2009). The control treatment (Mn_0) consisted solely of the base diet. Treatment 1 (Mn_1) included 5 mg, treatment 2 (Mn_2) 10 mg, treatment 3 (Mn_3) 15 mg, treatment 4 (Mn_4) 20 mg, and treatment 5 (Mn_5) 25 mg $MnSO_4$ per kg of diet. Each treatment and its respective repetitions were integral to the experimental design.

Growth and nutrition indicators

Following the anaesthesia of the fish using clove extract at a concentration of 200 mg L^{-1}, biometric measurements were conducted on days 0, 30, and 60 of the rearing periods, as outlined in the study by Hallajian *et al*. (2011). To ensure compliance with health standards and minimize physical injuries, feeding was suspended for a duration of 12 hours both before and after the biometric assessments. Growth indices were subsequently calculated using the relationships specified in equation 1, as detailed in the study of El Basuini *et al*. (2020). Equation 1: Standard relationships for calculating BWI, PBWI, SGR, CF, FCR and SR values (El Basuini *et al*. 2020).

$$\textbf{BWI (g)= Final weight (g)-Initial weight (g)}$$

$$\textbf{PBWI}=\left(\frac{\textbf{Final weight (g)} - \textbf{Initial weight (g)}}{\textbf{Initial weight (g)}}\right)\times 100$$

$$\textbf{DGR}=\left(\frac{\textbf{Final weight (g)} - \textbf{Initial weight (g)}}{\textbf{Duration (day)}}\right)\times 100$$

$$\textbf{SGR}=\left(\frac{\textbf{Ln final weight} - \textbf{Ln initial weight}}{\textbf{Duration (day)}}\right)\times 100$$

$$\textbf{CF}=\left(\frac{\textbf{Weight (g)}}{\textbf{Lenght (cm)}^3}\right)\times 100$$

$$\mathbf{FCR} = \mathbf{Feed\ intake\ (g)} / \mathbf{BWI\ (g)}$$

$$\mathbf{SR} = \left(\mathbf{Initial\ fish\ count - Casualty\ count} / \mathbf{Initial\ fish\ count}\right) \times \mathbf{100}$$

where, BWI is body weight increase, PBWI is percentage of body weight increase, DGR is daily growth rate, SGR is specific growth rate, CF means condition factor, FCR is food conversion ratio and SR is referred to survival rate.

Haematological parameters

At the end of the experiment, three fish were randomly chosen from each replicate for haematological investigations. Following anaesthesia using clove extract at a concentration of 200 ppm, blood was extracted from the caudal vein using a syringe. Subsequently, the obtained blood samples were transferred to ViroMed Laboratory, located in Iranian Science and Technology Park in Guilan Province, Iran. The laboratory conducted analyses including red blood cells (RBCs), white blood cells (WBCs) and differential leukocytes counts, according to Kazemi *et al*. (2010). Mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) were calculated using equation 2 (Kazemi *et al*. 2010). Equation 2: Standard relations for calculating MCV, MCH and MCHC values (Kazemi *et al*. 2010).

$$\mathrm{MCV} = \mathbf{Hct}\ (\%) \times \mathbf{10} / \mathbf{RBC}\ (\mathbf{10^6\ /\ mm^3})$$

$$\mathrm{MCH} = \mathbf{Hb\ (gr/dL)} \times \mathbf{10} / \mathbf{RBC}\ (\mathbf{10^6\ /\ mm^3})$$

$$\mathrm{MCHC} = \mathbf{Hb\ (gr\ /\ 100\ ml)} / \mathbf{Hct}\ (\%)$$

Biochemical parameters

The quantification of total protein in blood plasma was conducted using the Biuret method and the Auto Analyzer machine (Biotecnica, model 1500 BT) at a wavelength of 540 nm, as described by Zargari *et al*. (2023). Additionally, measurements of triglyceride, cholesterol, and glucose levels were performed using commercial kits from Pars Azmoun (Karaj, Alborz Province, Iran) and a spectrophotometer (USA, Unico UV-Vis 2100). These analyses were carried out at a wavelength of 546 nm, in accordance with the methodology detailed in the study by Zargari *et al*. (2023).

Immunological indicators

The quantification of lysozyme levels involved turbidity measurements using a spectrophotometer at a wavelength of 670 nm (Ellis, 1977). Immunoglobulin M (IgM) levels were assessed through the Immunoturbidimetric method utilizing a spectrophotometer (model VIS-2100 manufactured by Unico, USA) at a wavelength of 340 nm, following the methodology detailed by Khoshbavar Rostami *et al*. (2006). Total immunoglobulin (Total Ig) was determined using the Biuret method (Siwicki *et al*. 1994), employing equation 3. The activity levels of C3 and C4 complements were measured through the turbidity method, utilizing a commercial kit from Pars Azmoun (Tehran, Iran).

Equation 2:

$$\text{Total Ig (mg mL}^{-1}\text{)} = \text{Total protein - Total protein after mix with Polyethylene glycol}$$

Tissue accumulation of elements

To determine the tissue cumulation of Mn, calcium (Ca), phosphorus (P), sodium (Na), and iron (Fe) in blood, muscle, liver, and cartilage tissues, a 1-gram sample of each target tissue is meticulously weighed. Subsequently, the samples were subjected to the digestion process. Then the resulting digested samples, along with a container equipped with ice packs, were sent to Aria Shimi Laboratory (Karaj, Alborz Province, Iran) for analysis. In the Laboratory, the tissue aggregation of in each sample was quantified using inductively coupled plasma atomic emission spectroscopy (ICP-OES), as outlined by Şaşi *et al*. (2018). This analytical technique provides precise measurements, ensuring an accurate assessment of the elemental composition within the examined tissues.

Statistical analysis

The research was conducted employing a completely random statistical design. The normal distribution of data in groups and replicates for all growth and safety variables was assessed using the Shapiro-Wilk test. Subsequent to

confirming the normal distribution, statistical comparisons between groups in the treatments were carried out using One-Way ANOVA test. The homogeneity of variance was performed, and Duncan's test was subsequently applied to compare the groups. All statistical analyses were performed utilizing SPSS Version 23 software, while Excel 2010 software was employed for figure plotting. The presentation of data was in the form of mean ± standard deviation (SE).

RESULTS

Growth indices

The results indicate that there was no significant difference in growth indices between the experimental and control treatments ($p > 0.05$). However, the treatments Mn_0 and Mn_3 exhibited the highest average weight and growth indices (final biomass, SGR, BWI, CF, PBWI, and GR), while the lowest values for these indices were observed in Mn_4 and Mn_5. Notably, Mn_3 demonstrated the best FCR level at the conclusion of the rearing time (Table 1).

Table 1. Growth indices and survival rate calculated based on standard relationships in beluga, *H. huso* fed with different levels of manganese at the end of the experiment.

	Treatments					
	Mn_0	**Mn_1**	**Mn_2**	**Mn_3**	**Mn_4**	**Mn_5**
Initial weight (g)	267 ±3.01^a	267 ±3.75^a	266.50 ±1.04^a	266.83 ±0.44^a	266.67 ±5.55^a	265.67 ±4.53^a
Initial length (cm)	39 ±0.28^a	37.85 ±0.75^a	38.53 ±0.75^a	38.68 ±0.45^a	37.33 ±0.59^a	38 ±0.32^a
Final weight (g)	742.5 ±11.57^a	719 ±28.04^a	703.67 ±8.25^a	737.33 ±6.72^a	678.33 ±20.48^a	693.5 ±10.69^a
Final length (cm)	52.02 ±0.23^a	51.42 ±0.57^a	51.77 ±0.43^a	52.13 ±0.30^a	50.29 ±0.29^a	51.62 ±0.11^a
BWI (g)	475.5 ±13.32^a	452 ±24.68^a	437.17 ±7.51^a	470.5 ±7.02^a	411.67 ±19.97^a	427.83 ±6.80^a
PBWI (%)	125.49 ±5.09^a	119.68 ±4.60^a	119.01 ±0.52^a	122.49±0.99^a	107.68 ±7.16^a	115.34 ±3.33^a
CF	0.53 ^{a}	0.53 ±0.01^a	0.51 ±0.01^a	0.52 ±0.01^a	0.51 ±0.01^a	0.50 ±0.01^a
SGR (%/day)	1.70 ±0.01^a	1.65 ±0.04^a	1.62 ±0.02^a	1.69 ±0.02^a	1.56 ±0.06^a	1.60 ±0.01^a
FCR	1.41 ± 0.01^a	1.46 ± 0.04^a	1.58 ± 0.09^a	1.38 ± 0.08^a	1.45 ± 0.04^a	1.45 ± 0.04^a
Initial biomass (g)	2670 ±30.14^a	2670 ±37.35^a	2665 ±10.41^a	2668.33 ±4.41^a	2666.67 ±55.48^a	2656.67 ±45.31^a
Final biomass (g)	7425 ±117.51^a	7190 ±280.42^a	7036.67 ±82.48^a	7373.33 ±67.23^a	6783.33 ±204.78^a	6935 ±106.93^a
SR	100	100	100	100	100	100

Regarding haematological parameters, the number of RBCs and the amount of haemoglobin (Hb) exhibited a significant difference among the treatments ($p < 0.05$). Conversely, there was no significant difference in haematocrit (Hct) rate (%) between the treatments ($p > 0.05$). In addition, the levels of MCV, MCH and MCHC showed no significant differences among the treatments ($p > 0.05$). Notably, a significant difference in the number of WBCs was observed among different treatments ($p < 0.05$), with the highest and lowest levels recorded in Mn_5 and Mn_2 respectively (Table 2).

Table 2. Haematology studies of beluga, *H. huso* fed with different levels of manganese at the end of the experiment.

	Treatments					
	Mn_0	**Mn_1**	**Mn_2**	**Mn_3**	**Mn_4**	**Mn_5**
WBC	6500 ± 200^c	6900 ± 200^c	5500 ± 100^d	8250 ± 50^b	9050 ± 450^b	10550 ± 250^a
RBC	803500 ± 8500^{ab}	770500 ± 5500^{bc}	831500 ± 18500^a	820000 ± 11000^a	816500 ± 18500^a	748500 ± 8500^c
Hb (g/dL)	6.34 ± 0.09^{ab}	6 ± 0.02^{ab}	6.49 ± 0.17^a	6.37 ± 0.08^{ab}	6.41 ± 0.13^{ab}	5.89 ± 0.06^b
Hct (%)	28.5 ± 0.50^a	27 ± 0.01^a	29 ± 1^a	28.5 ± 0.5^a	28.5 ± 0.5^a	26.5 ± 0.5^a
MCV	345.5 ± 2.5^a	350.5 ± 2.5^a	348.5 ± 4.5^a	347 ± 1^a	348.5 ± 1.5^a	353.5 ± 2.5^a
MCH	78.85 ± 0.25^a	77.85 ± 0.35^a	77.95 ± 0.25^a	77.65 ± 0.05^a	78.45 ± 0.25^a	78.55 ± 0.05^a
MCHC	22.2 ± 0.10^a	22.25 ± 0.05^a	22.35 ± 0.25^a	22.35 ± 0.15^a	22.45 ± 0.05^a	22.25 ± 0.15^a
Lymphocyte (%)	86.5 ± 0.50^a	86.5 ± 1^{ab}	85.5 ± 1.5^{ab}	82.5 ± 0.5^{abc}	79.5 ± 1.5^c	80.5 ± 0.5^c
Neutrophil (%)	9.5 ± 0.50^b	10 ± 2^b	8.5 ± 0.5^b	12.5 ± 0.5^{ab}	15 ± 1^a	14.5 ± 0.5^a
Monocyte (%)	4 ± 1^a	3.5 ± 0.5^a	6 ± 1^a	5 ± 1^a	5 ± 1^a	5 ± 1^a
Eosinophil (%)	0^b	1^a	0^b	0^b	1^a	0^b

As shown in Table 3, there was no significant difference between the control and other treatments in the levels of total protein and blood glucose in young beluga ($p > 0.05$). However, blood cholesterol and triglyceride levels exhibited a significant difference between Mn_0 and Mn_1 compared to Mn_4 and Mn_5 ($p < 0.05$). The highest cholesterol level was observed in Mn_2, while the lowest triglyceride level was recorded in Mn_0 (Table 3).

Table 3. Biochemical parameters of beluga, *H. huso* fed with different levels of manganese (Mn) at the end of the experiment.

	Treatment					
	Mn_0	**Mn_1**	**Mn_2**	**Mn_3**	**Mn_4**	**Mn_5**
Total protein	2.39 ± 0.15^a	2.39 ± 0.04^a	2.49 ± 0.08^a	2.09 ± 0.04^a	2.07 ± 0.12^a	2.12 ± 0.1^a
Glucose (mg dL^{-1})	58.95 ± 4.75^a	63.85 ± 4.55^a	70.25 ± 3.95^a	59.55 ± 2.05^a	59.45 ± 4.25^a	58.85 ± 2.35^a
Cholesterol (mg dL^{-1})	137.50 ± 14.5^{ab}	145 ± 9^a	155 ± 7.5^a	110.5 ± 4.5^{bc}	92.5 ± 5.5^c	99 ± 3^c
Triglyceride	340 ± 29^a	329.5 ± 22.5^a	318 ± 4^{ab}	297 ± 6^{ab}	263 ± 22^b	245 ± 20^b

According to Table 4, Mn_2 exhibited the highest activity levels of lysozyme, IgM, total Ig, as well as C_3 and C_4 complements, whereas their lowest levels in Mn_5.

Table 4. Immunological indicators of Beluga, *H. huso* fed with different levels of Manganese at the end of the experiment.

	Treatment					
	Mn_0	**Mn_1**	**Mn_2**	**Mn_3**	**Mn_4**	**Mn_5**
Lysozyme (U mL^{-1})	36.95 ±0.45^a	36.75 ±2.15^a	39.95 ±0.25^a	33.2 ±0.3^b	31.95 ±0.45^b	31.2 ±0.6^b
IgM (mg dL^{-1})	43.45 ±1.25^a	47.45 ±0.85^a	50.2 ±3^a	44 ±1.6^a	42.1 ±2.4^a	41.85 ±0.35^a
Total Ig (g dL^{-1})	15.35 ±1.63^a	15.85 ±1.34^{ab}	17.15 ±0.49^{ab}	15.3 ±0.57^{ab}	14.25 ±0.78^b	13.9 ±0.42^b
C3 complement (mg dL^{-1})	26.5 ±3.55^a	27.25 ±4.95^a	31.65 ±2.45^a	30 ±2.7^a	27.95 ±3.55^a	25.5 ±1.9^a
C4 complement (mg dL^{-1})	8.15 ±0.35^a	10 ±1.7^a	10.95 ±0.45^a	9.35 ±1.15^a	8.75 ±0.25^a	7.7 ±1^a

According to Table 5, the Ca and P in the experimental treatments with the addition of Mn supplement did not show any significant differences ($p > 0.05$). Similarly, Na and Fe did not exhibit any significant differences between different diets ($p > 0.05$). However, there was a significant difference in the amount of dietary Mn between the control and the other treatments ($p < 0.05$).

Table 5. Dietary element (Mn, Ca, P, Fe and Na) levels in different diets of Beluga, *H. huso*.

	Treatment					
	Mn_0	**Mn_1**	**Mn_2**	**Mn_3**	**Mn_4**	**Mn_5**
Mn (mg kg^{-1})	5 ± 0.46^f	7.33 ± 0.21^e	13.60 ± 0.12^d	20 ± 0.06^c	25.2 ± 0.12^b	27.04 ± 0.04^a
Ca (g kg^{-1})	5 ± 0.29^a	6.28 ± 0.29^a	9 ± 0.32^a	7.89 ± 0.29^a	6.12 ± 0.12^a	3.67 ± 0.34^a
P (g kg^{-1})	1.45 ± 0.9^a	1.67 ± 0.02^a	2 ± 0.02^a	1.75 ± 0.06^a	1.62 ± 0.07^a	1.33 ± 0.05^a
Fe (mg kg^{-1})	260 ± 0.58^a	258.39 ± 0.36^a	255.1 ± 0.32^a	254.25 ± 0.55^a	251.46 ± 0.29^a	249.7 ± 0.49^a
Na (mg kg^{-1})	23.5 ± 0.29^a	21.63 ± 0.22^a	22.52 ± 0.16^a	23.3 ± 0.17^a	22.94 ± 0.03^a	23.15 ± 0.08^a

The findings indicate that the tissue aggregation of Ca, P, and Fe in the blood, muscle, cartilage, and liver tissues remains unaffected by varying concentrations of Mn in the diet. No significant differences ($p > 0.05$) were observed in the accumulation of these elements in different tissues (Figs. 1-A, B, C and Table 6).

Table 6. Dietary element (Mn, Ca, P, Fe and Na) levels in the blood, muscle, cartilage, and liver tissues of Beluga, *H. huso*.

		Treatments					
		Mn_0	**Mn_1**	**Mn_2**	**Mn_3**	**Mn_4**	**Mn_5**
Cartilage	**Mn (mg kg^{-1})**	8 ± 0.23^f	9.88 ± 0.58^e	11.7 ± 0.04^d	13 ± 0.58^c	14.95 ± 0.59^b	17.6 ± 0.55^a
	Ca (g kg^{-1})	160 ± 1.16^a	163.2 ± 0.46^a	165.1 ± 0.69^a	163 ± 0.87^a	161.03 ± 0.59^a	159.8 ± 0.55^a
	P (g kg^{-1})	110.03 ± 0.97^a	112 ± 0.84^a	115.36 ± 0.69^a	113.25 ± 0.64^a	110.09 ± 0.58^a	105 ± 0.58^a
	Fe (mg kg^{-1})	32.61 ± 0.64^a	35 ± 0.28^a	37.17 ± 0.08^a	36.2 ± 0.46^a	34.05 ± 0.12^a	31.95 ± 0.23^a
	Na (mg kg^{-1})	13.7 ± 0.06^a	14.2 ± 0.12^a	13.27 ± 0.42^a	13.99 ± 0.57^a	13.19 ± 0.53^a	14 ± 0.14^a
Blood	**Mn (mg kg^{-1})**	3.96 ± 0.29^f	5.01 ± 0.14^e	6.97 ± 0.01^d	9.05 ± 0.12^c	12.3 ± 0.4^b	16 ± 0.26^a
	Ca (g kg^{-1})	10 ± 0.29^a	11.54 ± 0.31^a	12.5 ± 0.29^a	12 ± 0.2^a	11.23 ± 0.46^a	9.5 ± 0.29^a
	P (g kg^{-1})	2 ± 0.03^a	2.21 ± 0.09^a	2.6 ± 0.11^a	2.4 ± 0.06^a	2.18 ± 0.12^a	2.13 ± 0.08^a
	Fe (mg kg^{-1})	60 ± 0.87^a	63.6 ± 0.14^a	67.23 ± 0.59^a	65.91 ± 0.51^a	63.61 ± 0.34^a	59 ± 0.29^a
	Na (mg kg^{-1})	138.2 ± 0.46^a	139.4 ± 0.32^a	138.7 ± 0.54^a	140.2 ± 0.55^a	140.4 ± 0.35^a	139.8 ± 0.03^a
Muscle	**Mn (mg kg^{-1})**	1.98 ± 0.02^f	3.65 ± 0.09^e	6.25 ± 0.26^d	8.2 ± 0.26^c	11 ± 0.17^b	14.5 ± 0.12^a
	Ca (g kg^{-1})	4 ± 0.17^a	4.7 ± 0.06^a	6 ± 0.12^a	4.8 ± 0.12^a	4 ± 0.26^a	3.5 ± 0.18^a
	P (g kg^{-1})	5 ± 0.06^a	5.3 ± 0.12^a	5.49 ± 0.23^a	5.4 ± 0.12^a	5 ± 0.07^a	4.5 ± 0.04^a
	Fe (mg kg^{-1})	10 ± 0.14^a	11.72 ± 0.33^a	11.5 ± 0.4^a	11.1 ± 0.58^a	9 ± 0.06^a	6.54 ± 0.11^a
	Na (mg kg^{-1})	6.6 ± 0.06^a	7.2 ± 0.12^a	6.5 ± 0.17^a	6.7 ± 0.17^a	7.01 ± 0.08^a	7.5 ± 0.29^a
Liver	**Mn (mg kg^{-1})**	1.3 ± 0.08^d	1.5 ± 0.09^d	1.6 ± 0.07^{cd}	1.9 ± 0.06^c	2.5 ± 0.17^b	3.1 ± 0.09^a
	Ca (g kg^{-1})	0.55 ± 0.06^a	0.68 ± 0.04^a	0.85 ± 0.06^a	0.71 ± 0.04^a	0.65 ± 0.06^a	0.59 ± 0.03^a
	P (g kg^{-1})	0.75 ± 0.03^a	0.8 ± 0.04^a	0.92 ± 0.04^a	0.83 ± 0.04^a	0.76 ± 0.58^a	0.69 ± 0.01^a
	Fe (mg kg^{-1})	52.01 ± 0.51^a	55.30 ± 0.17^a	59.63 ± 0.22^a	55 ± 1.16^a	53.4 ± 0.16^a	49.63 ± 0.33^a
	Na (mg kg^{-1})	0.71 ± 0.03^a	0.63 ± 0.01^a	0.57 ± 0.03^a	0.69 ± 0.04^a	0.75 ± 0.06^a	0.62 ± 0.02^a

Fig. 1-D and Table 4 illustrate that the Mn accretion in all tissues is dependent on the concentration of Mn in the diet, exhibiting a significant increase ($p < 0.05$). The highest tissue accumulation was observed in Mn_5, while the lowest in Mn_0. As demonstrated in Fig. 1-E and Table 6, Na levels in all tissues were not influenced by dietary Mn supplementation ($p > 0.05$). The consistent Na levels among tissues suggest that Mn concentration in the diet did not impact the accumulation of Na in the examined tissues.

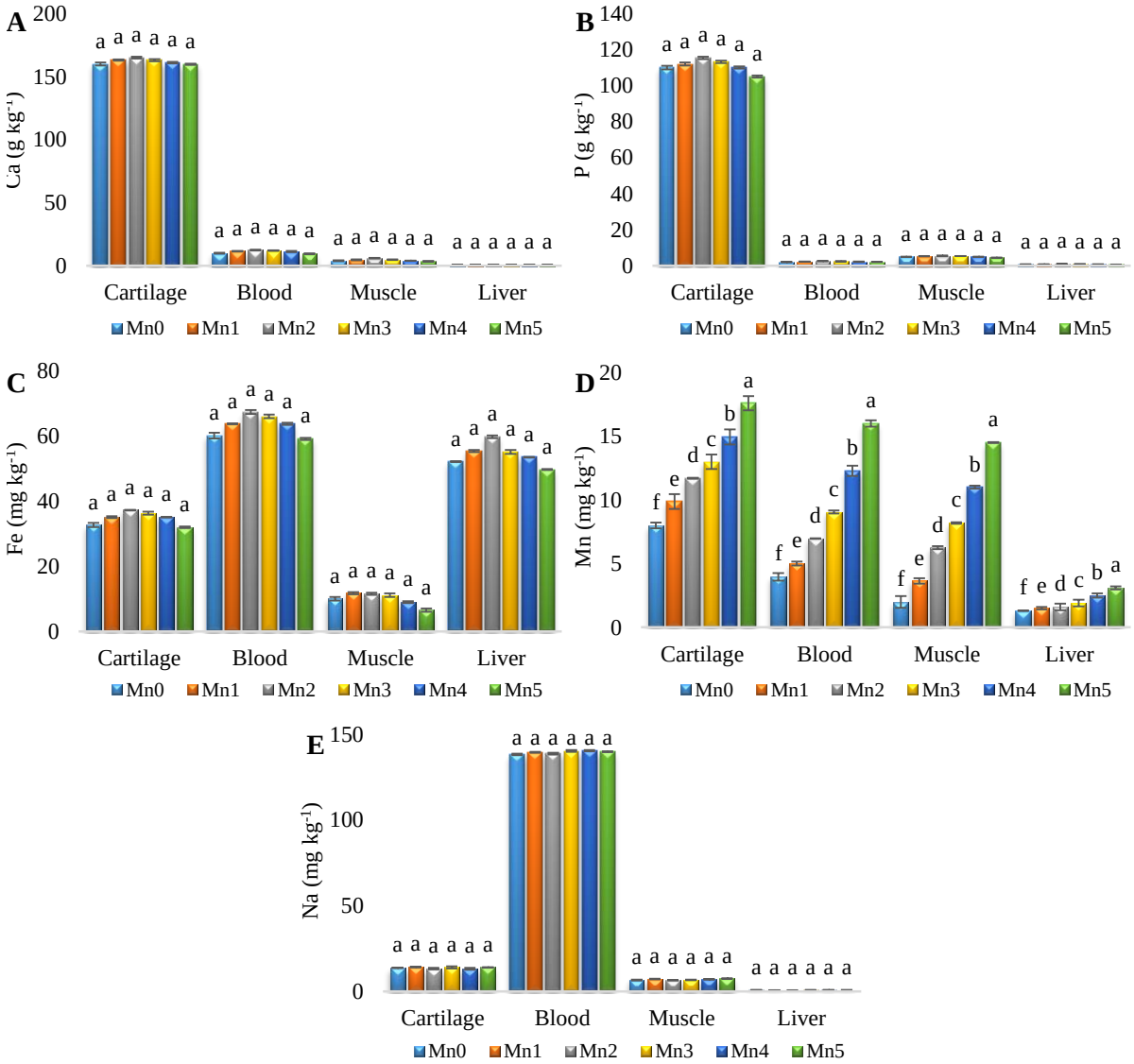

Fig 1. Comparison of the average levels of different elements in the cartilage, blood, muscle and liver tissues in Beluga (*H. huso*) fed with different levels of Mn at the end of the experiment; A: Calcium (Ca); B: Phosphorus (P); C: Iron (Fe); D: Manganese (Mn); E: Sodium (Na). Data are mean ± SE.

DISCUSSION

Despite the absence of significant differences in final weight and growth indices among treatments in the present study, Mn_0 and Mn_3 exhibited the highest growth indices and optimal values. The observed increase in the CF in fish fed the control diet and low levels of Mn may be attributed to the enhanced fat deposition in the body, as suggested by Musharraf & Khan (2021). Additionally, Mn_3 displayed the lowest FCR at the end of the experiment, while an increase in the concentration of dietary Mn in treatments 4 and 5 corresponded to an elevation in FCR values. The outcomes of the current study are in accordance with findings from research conducted on various fish species, including *Pelteobagrus fulvidraco* (Tan *et al*. 2012), *C. auratus* (Pan *et al*. 2008), *Ctenopharyngodon idella* (Liang *et al*. 2015), *H. fossilis* (Zafar & Khan 2019), and *L. rohita* (Musharraf & Khan 2021). Similarly, studies by Ye *et al*. (2008) on *E. coioides* and Maage *et al*. (2000) on *S. salar* did not observe a significant difference in growth indices with the addition of Mn supplements to fish diets. Noteworthy, however, Pan *et al*.

(2008) reported contrasting results in *C. auratus*, where they observed a significant elevation in growth rate and FCR by the upraise of Mn concentration in the diet. These discrepancies highlight the species-specific responses to Mn supplementation, emphasizing the importance of considering individual fish species in dietary interventions. Asaikkutti *et al*. (2016), in their investigation about the impact of manganese oxide nanoparticles (Mn_2O_3 NPs) as a nutritional supplement on *Macrobrachium rosenbergii*, reported a significant increase in length, final weight, food consumption, specific growth rate, and survival with the addition of Mn_2O_3 NPs up to a concentration of 18 mg kg^{-1} in diet. Similarly, Liang *et al*. (2015) conducted a study on *C. idella* and observed linear increases in the indices of SGR, BWI, and food efficiency in fish fed with $MnSO_4$ up to a concentration of 18.7 mg kg^{-1} of diet. These indices remained constant at higher concentrations. In contrast, Ye *et al*. (2009) found the lowest FCR in their study on *E. coioides*, with the treatment containing 15 mg kg^{-1} of diet. Additionally, Welker *et al*. (2018) observed optimal FCR levels in *O. mykiss* treated with diet containing 5 mg of $MnSO_4$ per kg of diet. These studies collectively demonstrate the varied effects of Mn supplementation on different species of aquatic organisms and emphasize the importance of dosage considerations in optimizing growth and food efficiency. In the study conducted by Sotoudeh *et al*. (2018) on *O. mykiss*, a direct relationship was reported between the average final weight and the addition of Mn supplements in the diet. Similar findings were reported by Satoh *et al*. (1991) on *O. mykiss*, Pan *et al*. (2008) on *C. auratus*, Tan *et al*. (2012) on *P. fulvidraco*, and Liang *et al*. (2015) on *C. idella*, indicating that a reduction in Mn intake through the diet leads to the decreased growth performance in fish. To draw more accurate and valid conclusions about determining the mineral requirements of living organisms, it is crucial to not only measure growth indices, but also assess the accumulation of elements in body tissues. Additionally, examining the mineral composition of the whole body, biochemical parameters, and immune system activity under the influence of mineral food supplements should be considered (Lin *et al*. 2008; Antony Jesus Prabhu *et al*. 2016). In the present study, the number of RBCs and Hb exhibited significant differences between treatments, while Hct percentage did not show a significant difference. Mn_2 showed the highest levels, while Mn_5 the lowest. Subsequently, MCV, MCH, and MCHC blood indices did not show any significant differences between the treatments. Mohammadi & Rajabi (2016) observed an elevation in the number of RBCs, Hb levels, and Hct rate (%) in *O. mykiss* fingerlings by adding Mn_2O_3 NPs to the diet. Given that any alteration in the levels of MCV, MCH, and MCHC can serve as indicators of dysfunction in hematopoietic organs such as the spleen and liver (Munker *et al*. 2000), the lack of alterations in these indicators suggests the absence of its negative effects on these tissues. This indicates the favourable conditions of haematopoiesis in fish treated with oral Mn supplements, particularly in the Mn_3 group. The number of RBCs, Hct (%), and Hb level reflect a high oxygen demand to meet the increased oxygen requirements for higher metabolism (Satheeshkumar *et al*. 2012). Consequently, it can be inferred that a diet enriched with Mn may lead to an elevation in the number of RBCs, Hb levels, and subsequently, Hct (Kori-Siakpere *et al*. 2005). Baruthio *et al*. (1988) proposed that RBCs possess the ability to store Mn. Therefore, alterations in the quantity of RBCs can serve as a suitable indicator to measure the effectiveness of oral Mn supplementation. In the context of the current research, the levels of total protein and glucose in the blood plasma of young *H. huso* showed no significant differences between the control and other treatments. However, the maximum levels were observed in Mn_2. On the other hand, cholesterol and triglyceride values exhibited significant differences. Mn_2 demonstrated the maximum cholesterol levels, while Mn_0 the maximum triglyceride levels. Cholesterol levels are considered crucial indicators of fish health (Gul *et al*. 2011). It has been established that the composition of the diet and feeding practices can influence the biochemical indices of fish blood plasma, including cholesterol and triglyceride levels (Mambrini & Kaushik 1995). Sotoudeh *et al*. (2018) reported no significant differences in the protein, glucose, triglycerides, and cholesterol levels among different treatments. Given the limited number of studies and data on the impact of Mn on the biochemical indices in fish blood, further supplementary studies should be conducted in this regard. In this study, aside from the lysozyme and Total Ig levels, no significant differences were observed in other immune factors among the treatments. Mn_2 exhibited the highest levels of lysozyme enzyme and Total Ig levels, while Mn_5 the lowest. Sotoudeh *et al*. (2019) also reported the positive effects of Mn on lysozyme by adding 15 mg $MnSO_4$ to the diet of *O. mykiss*. In their study, the levels of C3 and C4 complements in fish fed with diets containing $MnSO_4$ were significantly higher compared to the control group. These findings suggest that Mn supplementation may positively influence certain immune parameters in fish. In fishes, the innate immune system is recognized as a crucial defence mechanism against pathogenic agents. Strengthening this system holds great significance for farmed fishes, particularly in aquaculture settings where higher stocking densities make fish more

susceptible to various opportunistic bacteria (Dixon & Stet 2001). Enhancing the innate immune response is essential for maintaining the health and well-being of farmed fish populations and minimizing the impact of infectious agents in aquaculture environments. In the present study, the levels of IgM, Total Ig, and complements C_3 and C_4 in different treatments suggest that the consumption of an oral supplement containing Mn by beluga does not result in a significant alteration in the levels of Total Ig, as well as C_3 and C_4. However lysozyme, recognized for its potent antimicrobial properties in the body fluids of animals, including fish, plays a crucial role in the humoral immune system. Lysozyme stands out as one of the most important factors in the non-specific immunity of fish (Magnadottir 2006). Strengthening the innate immune system is particularly vital for farmed fish, given their vulnerability to various stresses and opportunistic pathogens in aquaculture conditions (Dixon & Stet 2001). Therefore, utilizing food supplements holds the potential to enhance the immune system performance of fish (Dawood 2021; Ali *et al*. 2022). In the present study, the tissue Mn accretion was influenced by the concentration of dietary Mn supplements, exhibiting a significant increase in blood, cartilage, muscle, and liver tissues. According to this study, the accumulation of Ca, P, and Fe did not exhibit significant differences among treatments. However, their levels in the aforementioned tissues displayed a rise up to Mn_2, followed by a drop until Mn_5. Albeit, these alterations were not significant. Furthermore, the Na level in all tissues remained unaffected by dietary Mn supplementation. In line with the present study, Tan *et al*. (2012) observed in their examination on the whole-body composition of *P. fulvidraco* that an elevation in Mn concentration resulted in a decline in the Ca, P, Na, and Fe levels. Similarly, Ye *et al*. (2009) did not find a significant difference in the effects of Mn on the Ca, Fe, P, and Na concentrations in the bones and whole body of young *E. coioides*. They reported that increasing the dietary Mn concentration only led to an upraise in the Mn level in various tissues and bones, and elevating the Mn concentration to 1000 mg kg^{-1}, reduced the amount of Fe in the whole body. In a study by Liang *et al*. (2015) on *C. idella*, oral Mn supplementation significantly affected the total body and bone mineral content. The tissue accumulation of Mn, Ca, and P in the whole body and bone tissues increased in fish fed with 18.7 g kg^{-1}, but higher Mn concentrations led to a drop in Ca and P levels. In contrast to the current study, the effect of increasing Mn concentration on Fe level was not significant. Pan *et al*. (2008) found in *C. auratus* that increasing the concentration of $MnSO_4$ from 7.21 to 22.17 mg kg^{-1} diet led to an increase in the amount of Mn in skeletal tissue. Liu *et al*. (2016) reported an increase in Mn in the whole body, liver, and vertebral column in young hybrid grouper with an increased concentration of $MnSO_4$ in the diet. Additionally, Musharraf & (2021) reported a significant increase in the concentration of Mn in the vertebral column and the whole body at a concentration of 1.1 mg Mn per kg of diet by adding a Mn supplement to the diet of *L. rohita*. At higher concentrations of dietary Mn, the tissue accumulation of this element in the vertebral column and the whole body remained constant. They reported that the amount of Fe in the whole body was unchanged up to the concentration of 11.1 mg Mn in the diet, and by the dietary increase in Mn, the Fe level in the whole body dropped significantly. At the Mn concentration of 11.1 mg kg^{-1}, the Fe level in the liver tissue increased, and by an elevation in the concentration of oral Mn, the Fe level in the liver tissue declined. These results may be attributed to competitive inhibition between Mn and Fe for similar binding sites during the adsorption process, as there is a known negative interaction between Fe and Mn (Andersen *et al*. 1996). Additionally, Mn influences Fe absorption, causing the intestine to have difficulty distinguishing between Mn and Fe (Hallberg *et al*. 1991). SandstroEm (2001) reported that high Mn consumption can interfere with Fe absorption. In a study on *Solea senegalensis* post-larvae, Viegas *et al*. (2021) found that the increased Mn levels in the diet from 45 to 90 mg, upraised the Mn level in the whole body, with no alterations in the Fe, Na, Ca, and P levels. Research on *E. coioides* (Nie *et al*. 2015) and *Scophthalmus maximus* (Ma *et al*. 2015) suggests that to a certain extent, the level of Mn in the diet can influence the stabilization process of other elements in different body tissues. An upraise in dietary Mn may lead to a decreased intestinal absorption, elevated liver metabolism, and biliary excretion, causing disturbances in the element stabilization process (Aschner & Aschner 2005). Unlike terrestrial vertebrates, fish can acquire the minerals they need from both the water environment and their diet (Liang *et al*. 2015). Regarding Ca and P, consistent with the present study, a negative correlation was observed between high levels of dietary Mn and total body P in *S. salar* (Maage *et al*. 2000). Similar results have been reported regarding the accumulation of Ca and P in the whole body and vertebral column in *E. coioides* (Ye *et al*. 2009), *P. fulvidraco* (Tan *et al*. 2012), *C. idella* (Liang *et al*. 2015), *H. fossilis* (Zafar & Khan 2019), and *L. rohita* (Musharraf & Khan 2021). The decrease in Ca and P levels in the whole body, cartilage tissues, liver, and blood may be attributed to competitive inhibition between Mn and Ca (Liang *et al*. 2015) or between Mn and P (Ye *et al*. 2009) during intestinal absorption. Lall

et al. (2002) concluded that different fish species tend to maintain a constant ratio of Ca to P in the range of 0.7 to 1.6. In the present study, this ratio in cartilage was between 1.4 and 1.5. Some animals, including fish, can maintain a constant level of tissue Mn through strong homeostatic regulation, involving both endogenous excretion and intestinal absorption (Malecki *et al*. 1996; Zhang *et al*. 2016). Therefore, fish can increase Mn absorption from the diet and reduce its excretion to compensate for Mn deficiency.

CONCLUSION

Based on the results of various investigated indices and the accumulation of elements in different tissues of farmed beluga fish, it can be concluded that the optimal amount of $MnSO_4$ supplement needed for young farmed beluga falls within the range of 10-15 mg kg^{-1} of diet. The observed disparity between the obtained level for beluga and the reported requirements for other species by different researchers could be attributed to variations in experimental methodologies, the base diet, the form of oral Mn supplementation, the concentration of Mn in the water environment, and the specific characteristics of the species under study.

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