



Impact of Graded Levels of Dietary Ferrous and Ferric Citrate Supplementation on Expression of Iron Regulatory Genes in *Clarias magur*

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ABSTRACT

Background: The 60-day experimental study investigated the expression of iron regulatory genes, namely ferritin, transferrin and hepcidin, in *Clarias magur* fingerlings fed diets supplemented with two dietary iron sources. The experimental study comprised two groups in which *C. magur* fingerlings received diets supplemented with ferrous or ferric citrate at doses of 0, 15, 30, and 45 mg Fe/kg above the minimum dietary iron requirement of 30 mg Fe/kg diet, respectively.

Methods: Ferritin, transferrin, hepcidin expression levels in fish intestine, liver, muscle and gill tissues were determined using quantitative-real time PCR (qRT-PCR). Regardless of the dietary iron source, all tissues exhibited expression of the three genes under investigation, however, the degree of expression varied amongst tissues.

Result: Liver showed highest ferritin mRNA expression among all tissues under both iron sources. Maximum expression (6.77-fold) was observed at 45 mg/kg ferrous citrate. Muscle showed modest changes while gill exhibited the lowest ferritin expression. In intestine, under ferrous citrate supplementation, transferrin expression increased with increasing iron level. Maximum expression (4.21±0.14) was recorded at 45 mg/kg iron supplementation. Liver transferrin expression increased with increasing ferrous citrate. Highest expression was observed at T₃ (3.31±0.07). In intestine, the highest hepcidin expression occurred at T₁ (5.62±0.01). Ferric citrate showed comparatively lower expression in all treatments. Liver showed the highest hepcidin expression among all tissues. Expression increased significantly with increasing iron supplementation. Maximum expression (7.99±0.06) was observed in fish fed 45 mg/kg ferrous citrate (T₃).

Key words: Ferric citrate, Ferritin, Ferrous citrate, Gene expression, Hepcidin, Transferrin.

INTRODUCTION

Iron is an essential micronutrient that plays a crucial role in various physiological processes and immune functions in fish including oxygen transport, drug metabolism, steroid synthesis, DNA synthesis, ATP production, electron transport and cellular respiration (Lim *et al.*, 2001; Ganz and Nemeth, 2015; Sangeetha and Rajan, 2021). In aquaculture, dietary supplementation of various inorganic and organic forms of iron has been reported to significantly enhance growth performance, health status and survival rates of cultured fish species (Yu *et al.*, 2024). However, excessive iron accumulation can promote the generation of reactive oxygen species (ROS), resulting in oxidative stress and cellular damage (Sevcikova *et al.*, 2011; Bresgen and Eckl, 2015).

To maintain iron homeostasis, fish possess tightly regulated iron-metabolism proteins that control iron uptake, transport, storage and export (Arosio *et al.*, 2009; Ganz and Nemeth, 2012). These proteins also contribute to host defense by limiting iron availability to invading pathogens, thereby enhancing immune responses during infection. (Shike *et al.*, 2004; Das *et al.*, 2015). Key components of this regulatory network include ferritin, the major intracellular iron-storage protein; transferrin, responsible for iron transport; ferroportin, which mediates cellular iron export; and hepcidin, a central regulator of systemic iron

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homeostasis that controls iron egress from cells (Torti and Torti, 2002; Nemeth *et al.*, 2004; Bao *et al.*, 2005; Shi and

Camus, 2006; Fraenkel *et al.*, 2009; Neves *et al.*, 2009; Gkouvatso *et al.*, 2012; Yin *et al.*, 2018).

The general recommendation for dietary iron content in fish feeds to improve growth and feed utilization ranges from 30-150 mg/kg of dietary feed (NRC, 2011). In channel catfish (*Ictalurus punctatus*) improved growth and feed efficiency was obtained at 30 g/kg of iron supplementation (Lim *et al.*, 1996). According to Gatlin and Wilson (1986), normal growth and feed efficiency were observed for channel catfish fed 10 mg or more of supplemental iron/kg; however, they observed that 20 mg supplemental iron/kg was required to maintain optimum hematological values. Based on these data, the dietary iron requirement of fingerling channel catfish was determined to be not more than 30 mg Fe/kg diet. Viani *et al.* (2025) suggested that 60 mg/kg dose of Fe₃O₄-NPs significantly improves growth, feed conversion and physiological safety in *Clarias batrachus*.

The Indian walking catfish, *Clarias magur* (Hamilton, 1822), is one of the most important freshwater aquaculture species in India owing to its high market demand and culture potential (Jayasankar *et al.*, 2018; Chakraborty *et al.*, 2020). It is rich in easily digestible high-quality protein, essential omega-3 fatty acids and abundant iron. These properties contribute to cardiovascular health and help prevent iron-deficiency anemia and protein-energy malnutrition (Bolaji *et al.*, 2022). In fish, the minimum dietary iron requirement has been estimated to be approximately 30 mg kg⁻¹ diet (NRC, 2011). However, information regarding the physiological and molecular responses of *Clarias magur* to supplemental iron sources remains limited. Therefore, present study focuses on evaluating the tissue-specific expression profiles of key iron-regulatory genes (Ferritin, transferrin and hepcidin) in response to graded dietary supplementation of ferric and ferrous citrate in order to have a comprehensive understanding of how organic iron sources influence iron metabolism, storage and regulation at the mechanistic level. Such knowledge can contribute to the development of nutritionally optimized diets that maximize growth performance while maintaining iron homeostasis.

MATERIALS AND METHODS

Experimental fish and laboratory conditions

Clarias magur weighing approximately 18.0±0.5 g were obtained from Kolkata, West Bengal, India and acclimatized in tanks containing 500 L freshwater with continuous aeration for 10 days at the wet laboratory of College of Fisheries, Kishanganj, Bihar where the experimental trial was conducted during 2024-2025.

Experimental design, diet and feeding schedule

In a completely randomized design, fish (n=120) were divided equally among the two experimental groups and fed with basal feed supplemented with Ferrous (Fe²⁺) and Ferric (Fe³⁺) citrate, respectively. Each experimental group was further divided into four groups (control and three

treatments, T₁, T₂ and T₃) with three replicates consisting of 10 fish each. Control contains 30 mg/kg iron citrate which is the basic dietary iron requirement of fish with no further supplementation. Fish in the three treatment groups received diet containing the minimum dietary iron required by fish (30 mg/kg feed) supplemented with graded dietary concentrations of 15 mg/kg (T₁), 30 mg/kg (T₂) and 45 mg/kg (T₃) of ferric or ferrous citrate, respectively in both the experimental groups. Fish were fed for 60 days and 30-40% water exchange was done on alternate days. During this period, fish were fed with formulated feed consisting of 35% crude protein at the rate of 3% of their body weight (Table 1).

Quantification of mRNA expression of ferritin, transferrin and hepcidin gene

Tissue collection

At the end of the experiment (60th day), fish (n=2) from the triplicate treatment and control tanks of each dietary group were randomly selected and anesthetized using clove oil (50 µl/L) before sacrifice. The fish were dissected for liver, intestine, gill and muscle tissues. These tissues were collected for gene expression study and preserved immediately in RNAlater™ solution (Qiagen, India) at -80°C until RNA extraction.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from equal portions of each tissue collected using Trizol™ reagent (Invitrogen, USA) as per manufacturer's instructions. Briefly, 100 mg of RNA later™ stored tissue was homogenized with 1 ml of Trizol reagent, followed by centrifugation for 5 min at room temperature. To the aqueous phase transferred to a fresh tube, 200 µl of chloroform (0.2 vol/ml Trizol™ reagent) was added, vortexed for 1 min and incubated for 10 min at room temperature. The mixture was centrifuged at 12,000 rpm for 15 min at 4°C and the upper aqueous phase transferred to a fresh 1.5 ml tube. RNA was precipitated out using 500 µl of isopropanol (0.5 vol/ml Trizol™ reagent) followed by centrifugation at 12000 rpm for 15 min at 4°C. The RNA isolated was washed with 70% ethanol, air-dried and re-suspended in 35 µl of nuclease-free water and stored at -80°C. The RNA concentration and purity was estimated based on absorbance at OD260/280 nm using a Nanodrop 2000 (Thermo Fisher Scientific, USA). Total RNA extracted was purified for residual genomic DNA contamination using DNase I (Fermentas International Inc., Canada). Total RNA obtained was reverse transcribed to synthesize cDNA using Revert Aid cDNA synthesis kit (Thermo Scientific) as per the manufacturer's protocol. cDNA was synthesized using a thermal cycler (Bio-Rad laboratories, Inc) with the following cycling conditions: initial denaturation at 94°C for 4 min followed by 30 cycles of 94°C for 30s, 55°C for 30 s, annealing at 72°C for 30 s and a final extension for 7 min at 72°C. The cDNA was stored at -20°C until use.

Primer design for RT-qPCR

The primers for RT-qPCR were designed using Gene runner software (version 3.05) based on available gene sequences in NCBI GenBank. The primer sequences for the gene ferritin, transferrin and hepcidin, their product size and NCBI accession numbers are provided in Table 2. β -actin was used as a reference gene and served as an internal standard gene (Buwono *et al.*, 2015) Primer synthesis was outsourced to Bioserve Biotechnologies, Hyderabad and Bioinnovations, Maharashtra, India.

Quantitative real-time PCR (RT-qPCR) for mRNA expression of ferritin, transferrin and hepcidin gene in different tissues of *Clarias magur* fingerlings

After reverse transcription, target cDNA was amplified and relative quantification of ferritin, transferrin and hepcidin

gene in different tissues of *Clarias magur* fingerlings was done in a quantitative real-time PCR using SYBR green qPCR master mix without ROX (Thermo scientific) in aria MX real-time PCR system (Agilent Technology LDA). The reaction took place in a volume of 10 μ l containing 5 μ l of 2 $\times$ maxima™ SYBR green qPCR master mix (Thermo Scientific), 1 μ l of (5 pmol) each gene-specific primer, 1 μ l cDNA template and remaining nuclease-free water prepared for quantification. The quantified value of mRNA was expressed in terms of CT (threshold cycle) value. The PCR program included 10-minute activation and initial denaturation step at 95°C for 5 min, followed by 45 cycles for amplification with each denaturation cycle at 95°C for 15s, annealing at 53°C for 30s and extension at 72°C for 30s. At the end, the specificity of reaction was confirmed using melt curve analysis. A comparative CT method was

Table 1: Feed composition and proximate composition of the experimental diets.

Feed Ingredients (g/100 g)	Experimental treatments			
	Control	T ₁	T ₂	T ₃
Casein ^a	31.5	31.5	31.5	31.5
Gelatin ^a	10	10	10	10
Dextrin ^a	10	10	10	10
Starch ^a	25	25	25	25
Cellulose ^a	11.28	11.28	11.27	11.27
Cod liver oil ^b	3	3	3	3
Soya oil ^b	3	3	3	3
Vitamin mix ^c	2	2	2	2
Mineral mix ^c	2	2	2	2
Carboxymethyl cellulose ^a	2	2	2	2
Butylated hydroxy toluene ^a	0.02	0.02	0.02	0.02
Choline Chloride ^a	0.05	0.05	0.05	0.05
Vitamin C ^a	0.1	0.1	0.1	0.1
Betaine ^a	0.05	0.05	0.05	0.05
Fe ²⁺ /Fe ³⁺ Citrate*	0.0030	0.0045	0.0060	0.0075
Proximate composition of feed				
Dry matter	92.67±1.45	93.21±1.09	93.5±1.73	93.66±1.06
Crude protein (CP %)	35.13±0.41	35.24±0.27	35.26±0.32	35.22±0.09
Ether extract (EE %)	6.09±0.11	6.15±0.12	6.21±0.07	6.18±0.21
Crude fiber (CF %)	6.11±0.22	6.13±0.31	6.34±0.20	6.29±0.29
Nitrogen free extract (NFE)	43.99±1.24	44.33±1.43	44.01±1.08	43.89±0.96
Total ash (TA %)	8.08±0.19	8.15±0.24	8.19±0.37	8.22±0.16
Digestible energy (DE Kcal/100 g)	360.91±2.30	358.75±2.43	361.58±1.64	362.86±1.16

*Supplemented dietary iron (Fe²⁺ or Fe³⁺ Citrate) source; Data are expressed as mean±SE; (n = 3).

Minimum dietary iron requirement is 30 mg/kg feed; therefore, control contains 30 mg/kg iron citrate which no further supplementation. Experimental treatments (T₁-T₃) included: T₁ (Supplemented with 15 mg/kg); T₂ (Supplemented with 30 mg/kg); T₃ (Supplemented with 45 mg/kg) above the minimum dietary requirement of Fe²⁺/Fe³⁺ citrate respectively to the basal diet.

Ingredients procured from ^a Himedia Pvt., India, ^b local retail shop; ^c Sigma-Aldrich, India.

^aMineral premix (g/100 g mixture): NaCl, 4.33; MgSO₄·7 H₂O, 13.63; NaH₂PO₄·2H₂O, 8.67; KH₂PO₄, 23.86; Ca(H₂PO₄)₂·H₂O, 13.51; Ca-Lactate, 32.53; AlCl₃, 0.015; KI, 0.015; CuCl₂, 0.010; MnSO₄, 0.080; CoCl₂, 0.100; cellulose used as carrier.

^aVitamin premix (mg/100 g mixture): Thiamin-HCl, 5; riboflavin, 20; pyridoxine-HCl, 5; choline chloride, 500; nicotinic acid, 75; calcium pantothenate, 50; inositol, 200; folic acid, 1.5; cyanocobalamin, 0.01; Vitamin K (menadione), 4; α -tocopherol, 40; retinyl acetate, 400 IU; Vitamin D (Cholecalciferol) 2000 IU, cellulose used as carrier.

NFE = 100- (% Crude protein + % Ether extract + % Crude fiber + % ash).

DE (Kcal/100 g) = (% EE $\times$ 9) + (% CP $\times$ 4) + (% NFE $\times$ 4), Halver (1976).

used to estimate the relative expression of mRNA. All PCR reactions were run in duplicate and normalized by the value by $2^{-\Delta\Delta CT}$ method. The fold change in gene expression was normalized to β -actin and the expression level of the target gene was calculated by the $2^{-\Delta\Delta CT}$ CT-method (Livak and Schmittgen, 2001).

The quantified result was analyzed by the following formulae:

$$\Delta ct = \text{Target gene ct value} - \text{reference gene ct value.}$$

$$\Delta\Delta \text{ ct value} = \Delta ct \text{ value of treatment group} - \Delta ct \text{ value of control group}$$

Statistical analysis

For each tissue, the gene transcript values were calculated for triplicate samples and expressed as mean $\pm$ standard error. The statistical significance of all parameters was determined using one-way ANOVA followed by Duncan's new multiple range test using the statistical package with SPSS 22.0 for Windows. A significant difference was considered at a p value <0.05 .

RESULTS AND DISCUSSION

Table 3, Fig 1-2; Table 4, Fig 3-4; Table 5, Fig 5-6 presents the expression of ferritin, transferrin and hepcidin gene, respectively in different tissues of *C. magur* fed diets supplemented with varying levels of ferrous or ferric citrate.

Ferritin

Liver showed the highest ferritin mRNA expression among all tissues under both iron sources. Expression increased significantly with increasing ferrous citrate supplementation. Maximum expression (6.77-fold) was observed in the liver at 45 mg/kg ferrous citrate (T_3). Ferric citrate produced considerably lower expression than ferrous citrate.

Intestine showed moderate ferritin expression. Under ferrous citrate, expression peaked at T_2 (3.44) and then declined. Under ferric citrate, expression increased progressively from T_1 to T_3 , suggesting active regulation of iron absorption and storage.

Muscle exhibited relatively low expression. Under Ferrous citrate expression resulted in gradual increase with dose while with ferric citrate expression peaked at T_2 and decreased at T_3 .

Gill exhibited the lowest ferritin expression overall. Expression increased significantly at the highest iron supplementation level.

Transferrin

In intestine, under Ferrous citrate supplementation, transferrin expression increased significantly with increasing iron level. Maximum expression (4.21 ± 0.14) was recorded at T_3 . Under ferric citrate, expression peaked at T_2 (3.98 ± 0.03) and declined at T_3 . The intestine responds

Table 2: Primers used for expression study in real-time PCR.

Gene	Primer sequence (5'-3')	Product size	NCBI accession no.	Purpose
Ferritin_Fwd	F: 5'-CGGTGTGGAAGCTCTCGAAT-3'	147 bp	KC994611.1	quantitative PCR
Ferritin_Rev	R: 5'-GACTTCACCTGCTCGTCCAA-3'			
Hepcidine_F	F: 5'-GTGCCTTTCTCTGAGGTCCG-3'	127 bp	XM_053487903.1	quantitative PCR
Hepcidine_R	R: 5'-GACGTTTGCCTGAACAGC-3'			
Transferrin_F	F: 5'-ATTACGGCACAGAAGCGACA-3'	160 bp	JN993348.1	quantitative PCR
Transferrin_R	R: 5'-TGTCCTGCTCGATGAGAGT-3'			
β -actin_F	F: 5'-TTGAGACCTTCAACACCTCAGCCA-3'	148 bp	EU 184877	Internal control
β -actin_R	R: 5'-ACCATCACCGGAGTCCATCAACAAT-3'			

Table 3: Relative mRNA expression of ferritin gene in different tissues of *Clarias magur* fed with experimental diets supplemented with ferrous and ferric citrate.

Tissue	Fish diet containing ferrous citrate (Fe ⁺²)			p-value	Fish diet containing ferric citrate (Fe ⁺³)			p-value
	Ferritin expression values (Mean±S.D)				Ferritin expression values (Mean±S.D)			
	T ₁	T ₂	T ₃		T ₁	T ₂	T ₃	
Intestine	2.47 ^{ab} ±0.09	3.44 ^a ±0.53	2.21 ^b ±0.03	0.014	1.67 ^c ±0.02	2.64 ^b ±0.03	2.88 ^a ±0.06	0.000
Liver	5.24 ^c ±0.11	5.92 ^b ±0.03	6.77 ^a ±0.12	0.000	2.59 ^c ±0.02	3.07 ^a ±0.01	2.69 ^b ±0.04	0.000
Muscle	1.69 ^c ±0.01	1.85 ^b ±0.01	2.03 ^a ±0.02	0.000	1.62 ^b ±0.00	1.90 ^a ±0.01	1.50 ^c ±0.00	0.000
Gill	1.00 ^b ±0.06	0.97 ^b ±0.02	1.58 ^a ±0.02	0.001	0.47 ^d ±0.01	0.90 ^c ±0.00	1.09 ^a ±0.01	0.000

Values are expressed as mean $\pm$ SE (n=6). Mean values in the same row with different superscript differ significantly, whereas those with the same letter are similar ($p\leq0.05$). Treatments- T_1 ; T_2 and T_3 represent supplementation of 15, 30 and 45 mg/kg of ferrous and ferric citrate respectively.

actively to dietary iron supplementation, reflecting its role in iron absorption and transport.

Expression remained relatively low in muscle. Ferric citrate produced a sharp increase at T_2 followed by a marked

decline at T_3 . Ferrous citrate treatments showed only minor variation. From this it can be concluded that muscle tissue is not a primary site of iron transport regulation and therefore exhibited comparatively lower transferrin expression.

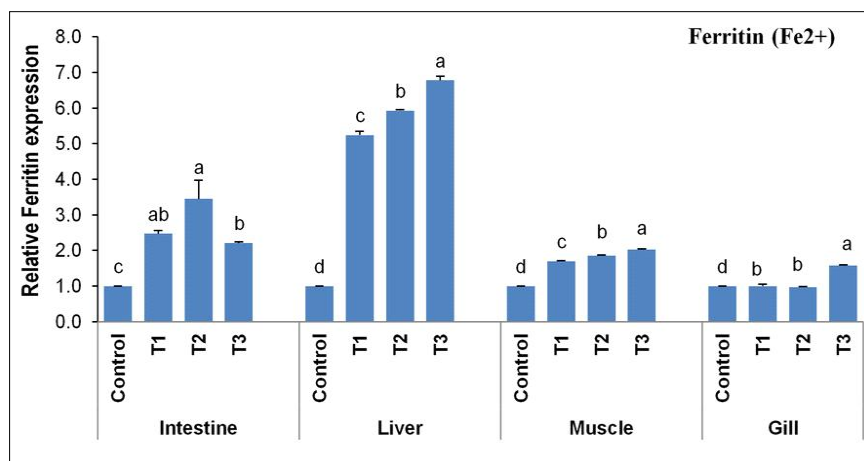


Fig 1: Relative mRNA expression of ferritin gene in different tissues of *Clarias magur* fed with diets supplemented with ferrous citrate.

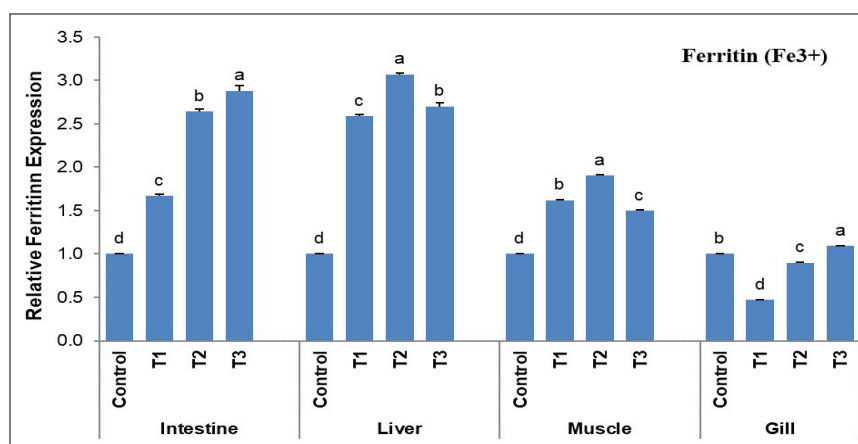


Fig 2: Relative mRNA expression of ferritin gene in different tissues of *Clarias magur* fed with diets supplemented with ferric citrate.

Table 4: Relative mRNA expression of transferrin gene in different tissues of *Clarias magur* fed with experimental diets supplemented with ferrous and ferric citrate.

Tissue	Fish diet containing ferrous citrate (Fe ²⁺)				Fish diet containing ferric citrate (Fe ³⁺)			
	Transferrin expression values (Mean±S.D)			p-value	Transferrin expression values (Mean±S.D)			p-value
	T ₁	T ₂	T ₃		T ₁	T ₂	T ₃	
Intestine	2.26 ^c ±0.28	3.15 ^b ±0.03	4.21 ^a ±0.14	0.001	2.51 ^c ±0.03	3.98 ^a ±0.03	2.88 ^b ±0.01	0.000
Muscle	1.84 ^a ±0.01	1.13 ^c ±0.05	1.55 ^b ±0.04	0.000	2.15 ^b ±0.04	3.44 ^a ±0.02	1.02 ^c ±0.06	0.000
Liver	2.02 ^b ±0.05	2.15 ^b ±0.05	3.31 ^a ±0.07	0.000	1.20 ^c ±0.07	1.42 ^b ±0.01	1.69 ^a ±0.06	0.002
Gill	2.24 ^b ±0.03	3.17 ^a ±0.05	3.05 ^a ±0.14	0.000	1.36 ^c ±0.00	2.30 ^b ±0.01	3.08 ^a ±0.05	0.000

Values are expressed as mean±SE (n=6). Mean values in the same row with different superscript differ significantly, whereas those with the same letter are similar ($p \leq 0.05$). Treatments- T_1 ; T_2 and T_3 represent supplementation of 15, 30 and 45 mg/kg of ferrous and ferric citrate respectively.

Liver transferrin expression increased significantly with increasing ferrous citrate supplementation. Highest expression under ferrous citrate was observed at T₃ (3.31±0.07). Ferric citrate resulted in consistently lower expression values. The liver plays a central role in iron

metabolism and transferrin synthesis. Higher expression under ferrous citrate suggests better iron utilization.

In gill, transferrin expression increased significantly with increasing iron supplementation. Under ferrous citrate, maximum expression occurred at T₂ whereas, under ferric

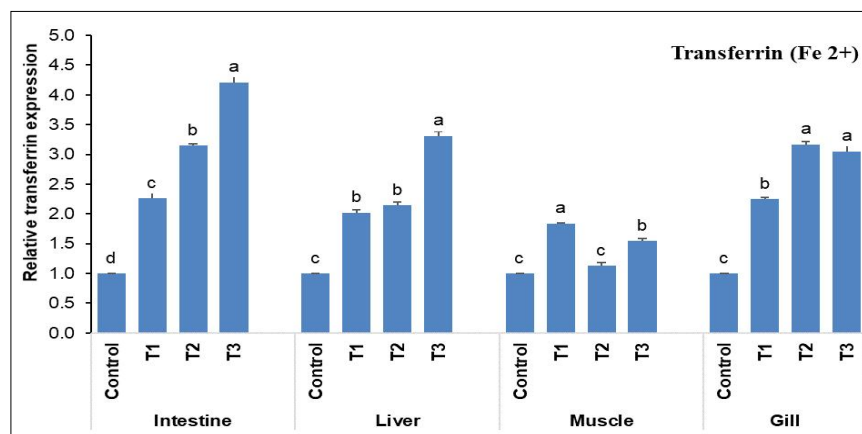


Fig 3: Relative mRNA expression of transferrin gene in different tissues of *Clarias magur* fed with diets supplemented with ferrous citrate.

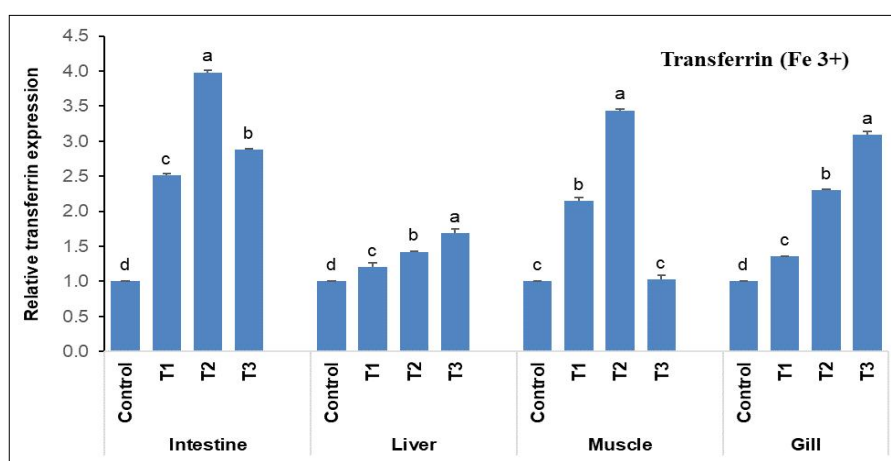


Fig 4: Relative mRNA expression of transferrin gene in different tissues of *Clarias magur* fed with diets supplemented with ferric citrate.

Table 5: Relative mRNA expression of hepcidin gene in different tissues of *Clarias magur* fed with experimental diets supplemented with ferrous and ferric citrate.

Tissue	Fish diet containing ferrous citrate (Fe ⁺²)			<i>p-value</i>	Fish diet containing ferric citrate (Fe ⁺³)			<i>p-value</i>
	Hepcidin expression values (Mean±S.D)				Hepcidin expression values (Mean±S.D)			
	T ₁	T ₂	T ₃		T ₁	T ₂	T ₃	
Intestine	5.62 ^a ±0.01	2.98 ^b ±0.02	3.18 ^b ±0.16	0.000	1.69 ^c ±0.08	2.51 ^a ±0.03	1.92 ^b ±0.03	0.000
Muscle	1.98 ^a ±0.00	1.59 ^b ±0.01	1.47 ^b ±0.02	0.000	1.41 ^b ±0.00	1.60 ^a ±0.01	2.07 ^a ±0.03	0.000
Liver	4.38 ^c ±0.18	5.17 ^b ±0.14	7.99 ^a ±0.06	0.000	1.38 ^c ±0.02	2.70 ^b ±0.06	5.41 ^a ±0.02	0.000
Gill	3.12 ^a ±0.00	2.58 ^b ±0.07	1.73 ^c ±0.03	0.000	0.70 ^c ±0.01	1.75 ^a ±0.03	0.69 ^c ±0.00	0.000

Values are expressed as mean±SE (n=6). Mean values in the same row with different superscript differ significantly, whereas those with the same letter are similar (p<0.05). Treatments- T₁; T₂ and T₃ represent supplementation of 15, 30 and 45 mg/kg of ferrous and ferric citrate respectively.

citrate, expression increased steadily and reached its highest value at T_3 . Gill tissue participates in ion and mineral regulation, which may explain the increased transferrin expression with higher iron availability.

Hepcidin

In intestine, the highest expression occurred at T_1 (5.62 ± 0.01), under ferrous citrate supplementation followed by a decline at higher levels. Ferric citrate showed comparatively lower expression in all treatments. Ferrous citrate induced approximately 2-3 times higher hepcidin expression than ferric citrate. Intestine exhibited a strong response to ferrous iron, indicating activation of iron homeostasis mechanisms.

Muscle showed relatively low expression levels of hepcidin compared with other tissues. Under ferrous citrate, expression decreased as iron level increased. Under ferric citrate, expression increased and reached its maximum at

T_3 . Muscle plays a limited role in systemic iron regulation and therefore exhibited lower hepcidin expression.

Liver showed the highest hepcidin expression among all tissues. Expression increased significantly with increasing iron supplementation. Maximum expression (7.99 ± 0.06) was observed in fish fed 45 mg/kg ferrous citrate (T_3). Ferric citrate also increased expression but remained lower than ferrous citrate. Since the liver is the major site of hepcidin synthesis, elevated expression reflects enhanced regulation of iron metabolism in response to increased dietary iron.

In gill under ferrous citrate, hepcidin expression decreased with increasing iron levels. Ferric citrate induced lower expression overall, peaking at T_2 . Gill showed moderate responsiveness to dietary iron.

In this study, Ferrous citrate induced higher ferritin gene expression than ferric citrate in most tissues, indicating better bioavailability and utilization of iron. The liver was

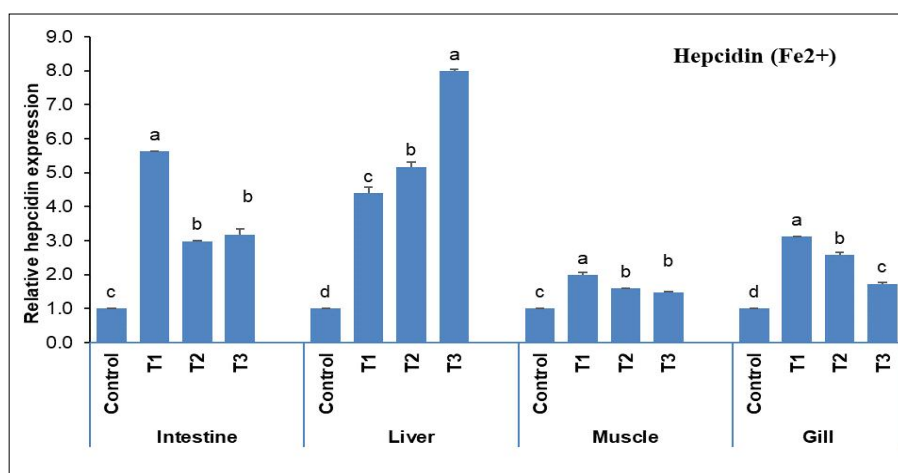


Fig 5: Relative mRNA expression of hepcidin gene in different tissues of *Clarias magur* fed with diets supplemented with ferrous citrate.

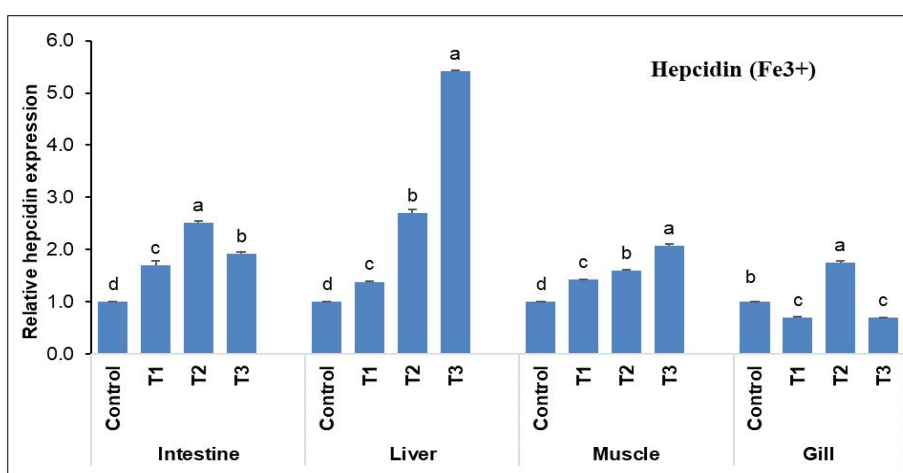


Fig 6: Relative mRNA expression of hepcidin gene in different tissues of *Clarias magur* fed with diets supplemented with ferric citrate.

the primary site of iron storage, showing the highest ferritin transcription, followed by the intestine. Increasing dietary iron generally increased ferritin expression, particularly in the liver and gill. The highest ferritin expression was observed in the liver at the highest ferrous citrate level (45 mg/kg), indicating enhanced iron storage capacity in response to increased iron availability. The results suggest that ferrous citrate is more effective than ferric citrate in enhancing iron storage mechanisms in *magur* fingerlings.

Transferrin expression levels revealed discrepancies among the tissues tested. Such discrepancies have been previously reported and been attributed to iron sources and concentrations in fish diets (Buyinza *et al.*, 2024). Ferrous citrate generally induced higher transferrin gene expression than ferric citrate, particularly in the intestine, liver and gill. The highest transferrin expression was observed in intestine of fish fed 45 mg/kg ferrous citrate (4.21 ± 0.14), reflecting its crucial role in dietary iron absorption and transport. Elevated transferrin expression in liver and gill further suggests enhanced iron mobilization and distribution within the body. The generally higher transferrin expression observed in fish fed ferrous citrate compared to ferric citrate indicates greater bioavailability of ferrous iron, which may facilitate more efficient iron transport and utilization in *magur* fingerlings.

Hepcidin, another key regulator protein of iron homeostasis is primarily expressed in liver with bacterial challenge and iron overload (Ganz, 2003; Nemeth and Ganz, 2006). It is an iron regulating hormone and can be used as a biomarker in determining the serum iron bioavailability (Rajamanickam *et al.*, 2020, 2021). Here, hepcidin expression exhibited a distinct tissue-specific pattern, with liver showing the highest transcriptional activity among all tissues in both the diets. As the principal regulator of systemic iron homeostasis, hepcidin expression increased markedly with increasing dietary iron levels, particularly in fish fed ferrous citrate. The highest hepatic hepcidin expression observed at 45 mg/kg ferrous citrate suggests activation of regulatory mechanisms to prevent excessive iron accumulation. Increased hepcidin expression under high iron conditions has been widely reported and is considered a feedback response to maintain iron balance by reducing intestinal iron absorption and iron release from storage tissues. This is in accordance to prior studies, where higher transcript of hepcidin was detected in fish liver due to iron overload (Nemeth and Ganz, 2006; Wang *et al.*, 2009; Shen *et al.*, 2019). The genes encoding the hepcidin protein is also regulated by anemia, hypoxia and inflammation thereby limiting iron availability (Shike *et al.*, 2004; Das *et al.*, 2015).

Overall, the three gene expression profiles collectively indicate that dietary iron supplementation modulates iron metabolism in *Clarias magur* through coordinated regulation of iron storage (ferritin), transport (transferrin) and homeostasis (hepcidin). The liver emerged as the

principal organ involved in iron regulation, while the intestine played a major role in iron absorption and transport. These findings highlight the effectiveness of ferrous citrate as a dietary iron source and provide molecular evidence for its role in enhancing iron metabolism and homeostasis in *Clarias magur* fingerlings.

CONCLUSION

Dietary iron supplementation significantly influenced the expression of ferritin, transferrin and hepcidin genes in different tissues of *magur* fingerlings. The liver showed the highest expression of ferritin and hepcidin, while the intestine exhibited the highest transferrin expression, highlighting their major roles in iron storage, regulation and transport. Ferrous citrate consistently induced greater gene expression than ferric citrate, indicating higher bioavailability and more efficient utilization of ferrous iron. Among the tested levels, 45 mg Fe/kg diet as ferrous citrate produced the strongest transcriptional response, suggesting improved iron metabolism and homeostasis. Overall, ferrous citrate is a more effective dietary iron source for *Clarias magur* fingerlings.

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Disclaimers

The views and conclusions expressed in this article are solely those of the authors and do not necessarily represent the views of their affiliated institutions. The authors are responsible for the accuracy and completeness of the information provided, but do not accept any liability for any direct or indirect losses resulting from the use of this content.

Informed consent

All animal procedures for experiments were approved by the Committee of Experimental Animal care and handling techniques were approved by the University of Animal Care Committee.

Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this article. No funding or sponsorship influenced the design of the study, data collection, analysis, decision to publish, or preparation of the manuscript.

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