Journal of the Hellenic Veterinary Medical Society

Vol 77, No 2 (2026)

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doi: 10.12681/jhvms.41060

To cite this article:

Ameli, Z., Eynizadeh, Z., Ghasemi, M., Hosseini, A., Azizi, V., & Mortazavi Moghadam, F. (2026). Nitrite acts as an endocrine disruptor by downregulating hepcidin gene expression: Evidence from common carp (Cyprinus carpio). *Journal of the Hellenic Veterinary Medical Society*, *77*(2), 10387–10393. https://doi.org/10.12681/jhvms.41060 (Original work published May 15, 2026)

J HELLENIC VET MED SOC 2026, 77 (2): 10387-10393
ΠΕΚΕ 2026, 77 (2): 10387-10393

Research article
Ερευνητικό άρθρο

Nitrite acts as an endocrine disruptor by downregulating *hepcidin* gene expression: Evidence from common carp (*Cyprinus carpio*)

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ABSTRACT: Nitrite, a common nitrogen-based compound, can enter aquatic environments through agricultural runoff, industrial discharge, and wastewater, where it poses a threat to fish health and ecosystem balance. One potential mechanism of toxicity involves disruption of iron metabolism and immune function, mediated by key regulatory genes such as *hamp1* (hepcidin). In this study, we used common carp (*Cyprinus carpio*) to investigate how high concentrations of nitrite affect oxidative stress levels and the expression of the *hamp1* gene. A total of 120 fish (15 ± 2 g) were randomly assigned to four treatment groups, each with three replicate aquaria containing 10 fish. Fish were exposed for 96 hours to nitrite concentrations of 0 (control), 80, 160, and 320 mg·L^{-1}. Following exposure, blood samples were collected for plasma analysis, and liver and kidney tissues were harvested for gene expression assays. Nitrite exposure significantly increased plasma total oxidant status (TOS) at all treatment levels ($P < 0.05$, $P < 0.05$, and $P < 0.01$, respectively) and significantly reduced total antioxidant capacity (TAC) at 160 and 320 mg·L^{-1} ($P < 0.01$). In both liver and kidney tissues, *hamp1* expression was significantly downregulated across all nitrite concentrations ($P < 0.05$). These findings suggest that nitrite exposure induces oxidative stress and may impair iron regulation and immune function in fish by suppressing *hamp1* gene expression.

Keyword: *Nitrite toxicity; Oxidative stress; hamp1; Methemoglobinemia; Iron regulation*

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Date of submission: **11-4-2025**
Date of acceptance: **15-10-2025**

INTRODUCTION

Water pollution is a growing environmental concern that threatens both human health and aquatic ecosystems. Among nitrogen-based contaminants, nitrite is particularly known to disrupt physiological and biochemical processes in aquatic organisms when present at elevated levels. Although nitrite is a natural intermediate in the nitrogen cycle, its accumulation, especially in aquaculture systems, typically results from imbalances in bacterial nitrification, primarily involving *Nitrosomonas* and *Nitrobacter* species. In such environments, nitrite concentrations can exceed 1 mM, far above the typical <1 µM found in natural waters (Kroupova *et al.*, 2005;Madison and Wang, 2006;Wuertz *et al.*, 2013). Nitrite is absorbed by gill chloride cells in freshwater fish through competition with chloride uptake and by the intestinal epithelium in marine fish. Once in the bloodstream, nitrite is taken up by erythrocytes, where it reacts with hemoglobin (Hb) by oxidizing the ferrous iron (Fe^{2+}) in its heme group to the ferric state (Fe^{3+}), thereby converting functional Hb into methemoglobin (MetHb), also known as ferrihemoglobin, a form that cannot bind or transport oxygen (Kiese, 1974;Jensen, 2003;Deane and Woo, 2007;Tomasso, 2012). The formation of MetHb reduces the blood's total oxygen-carrying capacity, leading to functional anemia. Additionally, the blood develops a brownish discoloration, characterized by a distinct optical absorption peak at approximately 635 nm (Svobodova *et al.*, 2005). Moreover, in a study by (Jensen *et al.* and Jensen *et al.*, 1987) nitrite exposure significantly increased the concentrations of Hb and nucleoside triphosphates in red blood cells, suggesting cellular shrinkage. Although arterial PO_2 (partial pressure of oxygen) may rise, oxygen delivery remains impaired due to the formation of MetHb, which cannot bind oxygen. This leads to reduced O_2 saturation and diminished oxygen affinity in the remaining functional Hb. Iron homeostasis in vertebrates is primarily regulated by hepcidin, a 25-amino-acid peptide hormone secreted by hepatocytes. In a classical endocrine feedback loop, low iron levels suppress hepcidin production, promoting iron absorption and mobilization, whereas high iron levels stimulate its expression to limit further uptake (Nemeth and Ganz, 2022). Beyond its role in iron regulation, hepcidin also serves as a potent antimicrobial peptide. Structurally, it is a disulfide-stabilized, hairpin-shaped β-sheet that is one of the most widely conserved antimicrobial peptides across vertebrates. Unlike many other antimicrobial peptides, hepcidin is highly conserved from fish to humans. It was first identified in humans, where its gene comprises three exons and two introns, and is predominantly expressed in the liver—a pattern preserved across all vertebrates (Shi and Camus, 2006;Hilton and Lambert, 2008;Valero *et al.*, 2013). Notably, the hepcidin gene shows remarkable sequence conservation between humans and fish, reflecting over 400 million years of evolutionary divergence (Merryweather-Clarke *et al.*, 2003). The main objective of the present study was to evaluate the regulation of *hamp1* (hepcidin) gene expression under high nitrite exposure using common carp (*Cyprinus carpio*) as a model organism.

MATERIALS AND METHODS

Experimental Animals

A total of 126 healthy juvenile Common carp (*C. carpio*), with an average weight of 15 ± 2 g, collected from Shahid Ansari Reconstruction and Proliferation Center of bony fish stocks, Rasht, Iran, immediately transferred alive to the laboratory. The fish were acclimated for 15 days in freshwater for 15 days in fresh water at 22-25 °C in aerated aquaria (pH 7.5, dissolved oxygen 6.5 ± 0.5 mg/L, Salinity 34 ± 0.5 ppt, total ammonia < 0.05 mg/L, and nitrite < 0.001 mM) at a density of 10 fish per 50 L. Photoperiod was maintained at a constant 12 h light/12 h dark cycle. Also, aquariums were cleaned daily by siphoning the bottom to remove wastes. They were fed daily with commercial fish feed during adaptation time. All experiments were performed in accordance with the guidelines of Iran national committee for ethics in biomedical research (Approval number: IR.SBU.REC.1401.139) (Mardani *et al.*, 2019).

Determination of Lethal Concentration of Nitrite

In accordance with OECD Guideline for Testing of Chemicals 203 (Fish, Acute Toxicity Test) (OECD, 2000), for the actual experiment, the minimum number of fish needed for this experiment, were employed. Fishes were randomly distributed into 6 groups with 7 individuals per aquaria which were tested in triplicate (18 aquaria in total). The 96 hours' nitrite exposer challenge was performed with desired doses in each treatment set as Table 1; and the number of daily deaths of each aquarium was recorded. All procedures followed internationally accepted standards for the ethical treatment of aquatic animals.

Table 1. NO_2 concentration for each treatment of 96-h LC_{50} dose determination assay.

Treatment name	Concentration of NO_2 added to water (mg. L^{-1})
Treatment 1 (negative control)	0
Treatment 2	80
Treatment 3	160
Treatment 4	320
Treatment 5	480
Treatment 6	540

In order to make a stock solution, $NaNO_2$ (Merck Co., Germany) dissolved in 5 L distilled water. The stock solution added daily to desired aquaria due to adjusting to the required value. The feeding was stopped to sustain water quality. During the test period, levels of nitrite were assayed every day, according to the Colorimetric determination of Griess method (Bryan and Grisham, 2007).

Nitrite exposure and sampling

For the experimental challenge, a total of 120 fish were initially used. These were randomly assigned to 12 aquaria, with 10 fish per aquarium. The experiment included four treatment groups (0, 80, 160, and 320 mg·L^{-1} nitrite), each conducted in triplicate. During acclimation, 6 fish were lost and excluded from the study.

Based on preliminary tests, the 96 h LC_{50} dose of nitrite was determined to be 323.59 mg·L^{-1}. Therefore, sublethal concentrations (0, 80, 160, and 320 mg·L^{-1}) were selected for the 96-h exposure experiment. To maintain water quality, feeding was suspended during this period.

At the end of 96 hours, 5 fish were randomly sampled from each aquarium (i.e., 15 fish per treatment group, 60 fish in total). Fish were anesthetized using *Syzygium aromaticum* solution, and blood was collected from the caudal vein using heparinized syringes. Plasma was separated via centrifugation (20 min at 4°C, 4000 rpm), and liver and kidney tissues were excised and stored at −85 °C for hamp1 gene expression analysis.

Biochemical analysis

Plasma total antioxidant capacity (TAC) was assessed using a commercial TAC assay kit (Kiazist Total Antioxidant Capacity Kit, Kiazist Chemistry Laboratories, Iran; Cat. No.: KTAC96), and total oxidant status (TOS) was measured using a TOS detection kit (Kiazist Total Oxidant Status Kit, Cat. No.: KTOS-96). Assays were performed according to the manufacturer's instructions. Absorbance was read using a BioTek® 800 TS absorbance microplate reader. TAC was determined via the Ferric-Reducing Antioxidant Power (FRAP) method, in which ferric tripyridyltriazine is reduced to the ferrous form, producing a blue color measurable at 450 nm. Based on a standard curve, TAC values were expressed as Trolox equivalents (nmol/mL).

TOS levels were determined by measuring absorbance at 545 nm using the same BioTek® microplate reader and were expressed in nmol/mL.

RNA extraction and cDNA preparation

Total RNA was extracted from liver and kidney tissues using the AnaCell Super RNA Extraction Kit. The NanoDrop Microvolume Spectrophotometer (Thermo Fisher Scientific) was used to quantify total RNA concentrations. AnaCell cDNA synthesis kit was used to synthesize cDNA by reverse transcription with M-MLV reverse transcriptase (Promega) and Oligo (dT). The synthesized cDNA concentration was measured by Nano Drop Microvolume Spectrophotometer (Thermo Fisher Scientific), the quality was assessed by 1% agarose electrophoresis and then prepared for the next step.

Real-time PCR

The Real-time PCR analysis of *hamp1* gene expression was performed with Mic Real-time PCR cycler (Bio Molecular Systems) using SYBR Green Real- time MasterMix (Tiangen). The expression of the *hamp1* gene was also normalized against β-actin (*Cyprinus carpio* actin, cytoplasmic 1), which served as the housekeeping gene. The primers used in this study are shown in Table 2. The *C. carpio hamp1* primers were designed by Oligo7 software. The amplification scheme was: incubated for 5 min at 95 °C, followed by 35 cycles of 20 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C. Cycle threshold (Ct) assays were conducted on transcript levels, and relative expression of target genes was calculated using equation $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Statistical analysis

Following the 96-h nitrite challenge, statistical analysis was carried out using GraphPad Prism 9.5.1(GraphPad, 2023). Data are presented as mean ± standard deviation (SD) to reflect variability among

Table 2. Primer sequence used in this study.

Gene name	Template strand sequence (5'→ 3')	Accession no.
hamp1	Forward: AAGTTGACACGTGTGGCTCT Reverse: TCAGTCTGCTGTGTGAAGGG	XM_019085197.2
actb1	Forward: AGACATCAGGGTGTCATGGTTGGT Reverse: CTCAAACATGATCTGTGTCAT	XM_042721308.1

individuals within each treatment group. The normality of the data was confirmed using the Shapiro-Wilk test. Differences between groups were evaluated using one-way ANOVA followed by Tukey's post-hoc test. Although the Duncan test offers higher sensitivity, we retained the Tukey method due to its robustness and widespread application in toxicological studies. The significance level for all tests was set at $P < 0.05$.

RESULTS

Effect of 96-hour nitrite exposure on oxidative stress

A comparison of the mean changes in plasma **total oxidant status (TOS)** among different treatment groups is shown in **Figure 1a**. The nitrite challenge significantly affected TOS levels ($F(3,16) = 7.09$, $P = 0.003$). Specifically, exposure to nitrite at **80, 160, and 320 mg L^{-1}** led to significantly higher plasma TOS compared to the control group (0 mg L^{-1}), with P values of 0.048, 0.022, and 0.003, respectively.

As shown in **Figure 1b**, nitrite exposure also significantly influenced plasma **total antioxidant capacity (TAC)** ($F(3,16) = 7.67$, $P = 0.002$). The TAC levels were significantly decreased in fish exposed to **80, 160, and 320 mg L^{-1}** of nitrite compared to controls, with corresponding P values of 0.005, 0.002, and 0.005, respectively.

Effect of 96-hour nitrite exposure on *hamp1* gene expression in liver and kidney tissue

In the **liver**, mean *hamp1* gene expression significantly decreased across treatment groups ($F(3,16) = 17.19$, $P < 0.001$; Figure 2a). Fish exposed to **80, 160, and 320 mg L^{-1}** nitrite showed significantly reduced *hamp1* expression compared to the control group, with all P values < 0.001.

In the **kidney**, *hamp1* expression was also affected by nitrite ($F(3,16) = 4.66$, $P = 0.016$; Figure 2b). A significant downregulation was observed only in the group treated with **320 mg L^{-1}** ($P = 0.018$), while the reductions at **80 and 160 mg L^{-1}** were **not statistically significant**, with P values of 0.26 and 0.09, respectively.

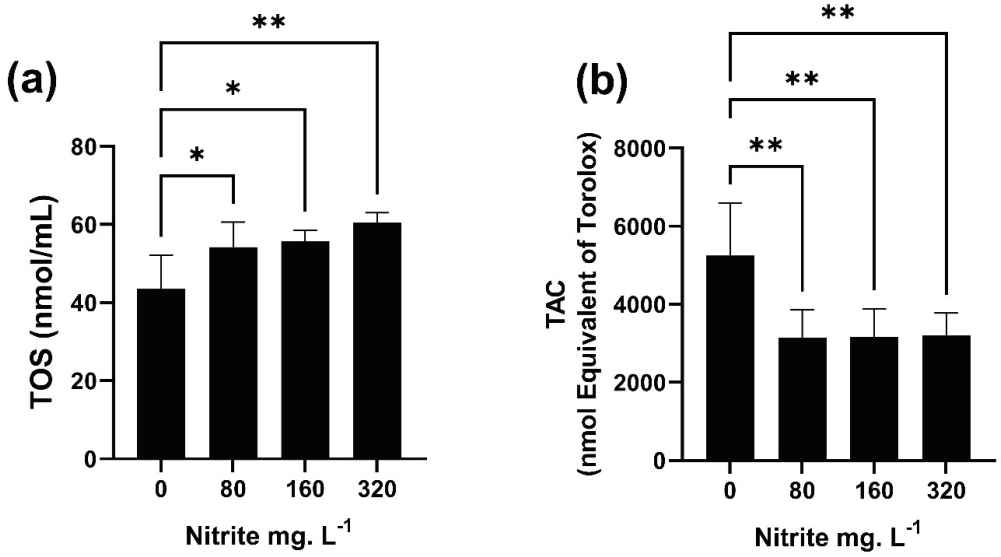

Figure 1. Effect of 96-h nitrite exposure on plasma level of TOS (a) and TAC (b) in common carp. Data are shown as mean ± SD of five animal per group. *$P < 0.05$, **$P < 0.01$.

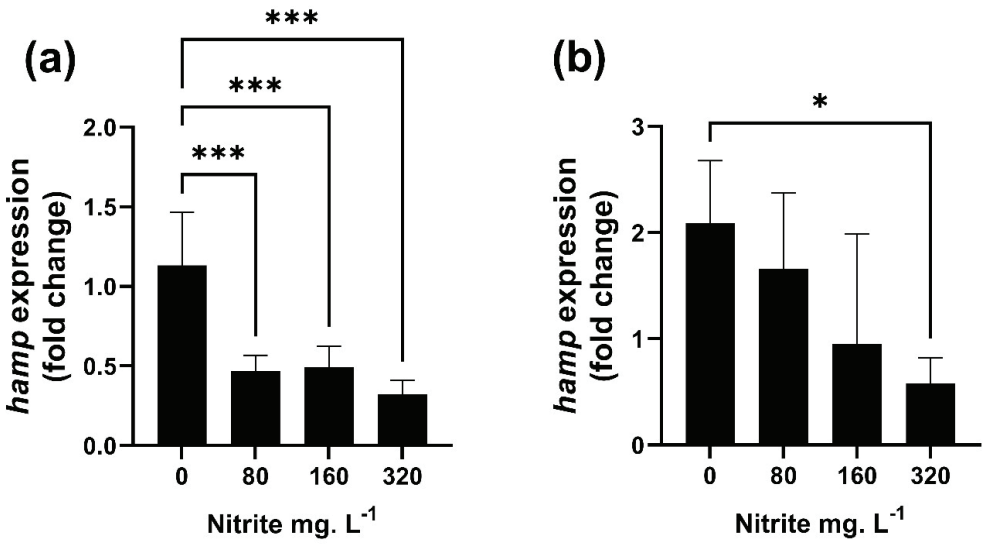

Figure 2. Effect of 96-h nitrite exposure on *hamp1* gene expression in liver (a) and kidney (b) tissue of common carp. Data are shown as mean ± SD of five animal per group. *$P < 0.05$, ***$P < 0.001$.

DISCUSSION

Experimental vertebrates that share a common evolutionary ancestry with humans play a vital role in advancing our understanding of gene function, particularly those involved in human diseases. Comparative genomic analyses between fish species and humans have highlighted the conservation of both coding and non-coding elements, revealing that many teleost fish share key aspects of developmental pathways and physiological mechanisms with mammals, including *Homo sapiens* (Cossins and Crawford, 2005;Roest Crollius and Weissenbach, 2005).

Hepcidin, a key hormone regulating iron homeostasis and functioning as an antimicrobial peptide, exhibits strong sequence conservation between mammals and fish (Shi and Camus, 2006). Most teleost fish, including common carp, possess two hepcidin homologues: *hamp1* and *hamp2* (Neves *et al.*, 2016), Of these, *hamp1* is considered the functional orthologue of the mammalian hepcidin gene and was the focus of this study. In 2006, Shi and Camus hypothesized that the highly conserved evolution of hepcidin enables many amphibians and fish hepcidins to regulate iron metabolism in vivo similarly to their mammalian counterparts—a hypothesis further supported by subsequent research (Gracey *et al.*, 2001;Fraenkel *et al.*, 2005). These evolutionary and functional similarities make *Cyprinus carpio* a suitable model for investigating hepcidin gene regulation under environmental stress.

Endocrine-disrupting compounds (EDCs) are widespread in aquatic ecosystems and can result in harmful alterations to development, physiological homeostasis, and overall health in aquatic vertebrates (Segner, 2009). Nitrite is one such EDC that, when present above safe environmental levels, poses significant toxicological risks. In humans, excessive nitrite exposure has been linked to carcinogenicity and tumor formation (Mueller *et al.*, 2001). According to the WHO Guidelines for Drinking-water Quality (WHO, 2002), one of the primary biological effects of nitrite is methemoglobinemia, resulting from the oxidation of Hb to MetHb, which is incapable of transporting oxygen. Similar mechanisms of toxicity are observed in fish species inhabiting natural water bodies and aquaculture systems. Elevated nitrite levels have been shown to cause physiological damage, economic loss in aquaculture, and accumulation of nitrite in edible fish tissues, raising additional concerns related to food safety and potential mutagenesis (De Flora and Arillo, 1983;Cheng and Chen, 2000).

The present study investigated the effects of high nitrite concentrations on the regulation of hepcidin, an iron-regulatory hormone, in *Cyprinus carpio* as a model organism. While the role of hepcidin in anemia has been well documented (Pagani *et al.*, 2019;Fathi *et al.*, 2022), this is the first study designed to evaluate *hamp1* gene expression under elevated nitrite exposure. The results clearly demonstrate a significant downregulation of *hamp1* in the

liver across all treatment groups, as well as a significant decrease in kidney tissue at the highest nitrite concentration compared to the control group (Figure 2). These findings support the hypothesis that hepcidin expression in *C. carpio* is influenced by methemoglobinemia. In 2015, Neves and his colleagues reported functional divergence between *hamp1* and *hamp2* in European sea bass (*Dicentrarchus labrax*), including downregulation of *hamp1* during anemia and hypoxic conditions—findings that align with the observed increase in total oxidant status (TOS) and decrease in total antioxidant capacity (TAC) in our study (Neves *et al.*, 2015).

The observed decrease in total antioxidant capacity (TAC) and increase in total oxidant status (TOS) following nitrite exposure indicate a clear shift toward oxidative stress. This redox imbalance aligns with the known biochemical effects of methemoglobinemia, in which nitrite promotes the formation of reactive nitrogen species and disrupts cellular antioxidant defenses. Notably, this stress response is accompanied by significant downregulation of *hamp1* gene expression in both the liver and kidney. Hepcidin (*hamp1*) plays a dual role as a key regulator of iron metabolism and as a component of the innate immune system, with emerging evidence also supporting its involvement in oxidative stress regulation. The suppression of *hamp1* expression under nitrite-induced oxidative stress suggests that redox imbalance may impair iron-regulatory mechanisms, potentially contributing to broader physiological dysfunction. These results support the hypothesis that oxidative stress may modulate *hamp1* transcription through iron-sensing pathways or inflammatory signaling cascades.

While this study specifically investigates nitrite-induced oxidative stress and suppression of *hamp1* expression, it also contributes to a broader understanding of how environmental stressors may impair immune function in fish. Evidence of compromised health has been reported across multiple species, including tumor development in various types of fish species and the presence of viral pathogens in aquaculture ecosystem (Ghasemi *et al.*, 2021;Harshbarger *et al.*, 2021;Ghasemi *et al.*, 2023;Chengula *et al.*, 2024;Ghasemi *et al.*, 2024). These observations underscore the need to monitor immune markers such as *hamp1* to assess fish susceptibility to environmental stressors. Given hepcidin's dual role in iron regulation and innate immunity, the downregulation of *hamp1* observed in this study may reflect a broader immunosuppressive response to chemical or ecological pressures.

Human hepcidin evolved from an ancient antimicrobial peptide, and its structural and transcriptional regulatory mechanisms have been highly conserved throughout vertebrate evolution. Based on the findings of this study, it can be hypothesized that high levels of nitrite accumulation in drinking water or food may suppress hepcidin expression in humans as well—potentially weakening a key component of the innate immune response.

CONCLUSION

In conclusion, this study provides the first evidence that nitrite exposure significantly downregulates *hamp1* gene expression in the liver and kidney tissues of common carp (*Cyprinus carpio*). These findings suggest that nitrite-induced oxidative stress may disrupt iron homeostasis and compromise innate immune regulation through suppression of *hamp1*. Given the conserved function of hepcidin across vertebrates, this research highlights the broader ecological and physiological implications of environmental nitrite contamination. More studies are needed to better understand the specific molecular pathways involved and to assess the long-term consequences of hepcidin suppression on fish health and disease resistance.

Financial support and sponsorship
This study did not receive any external funding.

Conflicts of interest
There are no conflicts of interest.

Data availability statement
All data generated and analyzed during the current study are available with the corresponding author upon reasonable request.

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