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Research Article

Keywords: copper, Artemia enrichment, hybrid grouper, Chlorella-mediated bioencapsulation, Keap1/Nrf2

Posted Date: November 10th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-10942127/v1

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Additional Declarations: No competing interests reported.

Chlorella-mediated methionine-copper bioencapsulation in *Artemia* nauplii modulates the Keap1/Nrf2 antioxidant axis, innate immunity, and appetite regulation in hybrid grouper larvae

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Abstract

Copper (Cu) serves as an essential trace element and obligate cofactor of Cu/Zn superoxide dismutase and ceruloplasmin; however, its nutritional delivery to marine fish larvae via live prey has not been fully investigated. This study assessed the dose-response effects of trophic Cu transfer on hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) larvae over a 15-day period, employing a three-tier bioencapsulation system where *Chlorella* was primarily enriched with methionine-copper (Met-Cu; 0, 5, 10, 15, and 20 mg/L) and delivered via *Artemia* nauplii. The Cu content in enriched *Artemia* ranged from 173.92 to 606.68 mg/kg dry weight (DW), resulting in dose-dependent increases in larval whole-body Cu (140.9–178.71 mg/kg DW). Larvae fed with 15 mg/L Met-Cu enrichment (Cu15) exhibited peak survival (71.67 ± 2.08%), final body weight (250.37 ± 43.58 mg), and specific growth rate (14.17 ± 2.97 %/d). This optimal dose significantly enhanced digestive capacity (pepsin, trypsin, lipase, and amylase) and antioxidant enzyme activities (GSH-Px, CAT, and SOD), while concurrently minimizing malondialdehyde (MDA) accumulation. Gene expression analysis suggested activation of the Keap1/Nrf2 pathway in the Cu15 treatment, leading to the upregulation of downstream antioxidant genes (*CAT*, *GPX*, *GR*, *LZM*). A balanced innate immune response was achieved, exemplified by upregulation of *IL-1β*, *IL-8*, and *IL-10*, alongside suppression of *TNF-α* and *TGF-β*. Additionally, optimal Cu enrichment influenced appetite regulation by downregulating anorexigenic signals (*CGRP*, *NUCB2*) and upregulating the orexigenic receptor *NPY8R*. Conversely, 20 mg/L Met-Cu induced signs of Cu toxicity, as indicated by reduced Nrf2 expression, decreased SOD activity, and increased TNF-α levels. In conclusion, 15 mg/L Met-Cu represents the optimal *Artemia* enrichment concentration for hybrid grouper larviculture. This study offers new mechanistic insights into the effects of live-prey Cu biofortification on antioxidant signaling, innate immune homeostasis, and neuroendocrine appetite control.

Keywords: copper; *Artemia* enrichment; hybrid grouper; *Chlorella*-mediated bioencapsulation; *Keap1*/*Nrf2*

1. Introduction

Known commercially as the pearl dragon grouper, the hybrid grouper (*Epinephelus fuscoguttatus* ♀ × *E. lanceolatus* ♂) has emerged as one of the most significant marine finfish species cultivated in southern China and Southeast Asia, attributed to its rapid growth, high flesh quality, and stress tolerance (Ch'ng and Senoo 2008; Mohd Faudzi et al. 2022). However,

seed production is hindered by high larval mortality, with survival rates in the first three weeks post-hatch often falling below 30% (Mohd Faudzi et al. 2022). During this critical period, larvae cannot process formulated diets due to an immature digestive system, relying entirely on live prey, thereby transmitting any nutritional deficiencies present in the live feed directly to the larvae. Such deficiencies likely contribute more significantly to larval loss than is commonly recognized.

Artemia nauplii serve as the standard live feed in marine hatcheries worldwide. However, their inherent nutrient profile is inadequate in long-chain highly unsaturated fatty acids (LC-HUFAs), vitamins, and trace minerals (Hamre et al. 2013; Watanabe et al. 1997). The practice of enriching *Artemia* through supplemental nutrients before they reach the larvae—termed bioencapsulation—has become routine for lipids and vitamins (Sorgeloos et al. 2001; Ramena et al. 2025). Conversely, trace mineral enrichment has received significantly less attention. One of the few studies addressing this, by Sun et al. (2013), demonstrated that copper-enriched *Artemia* enhanced growth, antioxidant enzyme activity, and stress tolerance in Chinese mitten crab (*Eriocheir sinensis*) larvae. The response of marine fish larvae to similar enrichment remains unexplored, and the fundamental question of how *Artemia*'s native Cu content (typically 150–180 mg/kg DW) aligns with actual larval requirements has not been addressed.

Cu is an essential nutrient in fish physiology, functioning as a metallic cofactor for Cu/Zn superoxide dismutase (Cu-Zn SOD), the primary enzymatic defense against reactive oxygen species (ROS), and is also vital for ceruloplasmin, cytochrome c oxidase, and lysyl oxidase (Prohaska 2008). A deficiency in Cu results in decreased Cu-Zn SOD activity, reduced antioxidant capacity, and adverse effects on growth and feed efficiency (Ogino and Yang 1980; Gatlin and Wilson 1986). In groupers specifically, Lin et al. (2008) found that Cu-deficient diets decreased hepatic SOD activity in juvenile *Epinephelus malabaricus*. Furthermore, Tang et al. (2013) reported that adequate dietary Cu improved digestive enzyme activity (trypsin, lipase, amylase) and hepatopancreatic antioxidant status in grass carp. However, excess Cu can trigger Fenton chemistry and hydroxyl radical generation, leading to lipid peroxidation, cellular apoptosis, and inflammatory dysregulation (Wang et al. 2015).

Dietary Cu requirements have been established for several juvenile teleosts consuming formulated diets, specifically approximately 3 mg/kg in rainbow trout (Ogino and Yang 1980), 4 mg/kg in channel catfish (Gatlin and Wilson 1986), and 4.37 mg/kg in *E. malabaricus* (Lin et al. 2008). However, these values do not directly apply to early larvae, as they rely on live feed and possess intestines that are not yet fully absorptive. To our knowledge, few studies

have quantified Cu nutritional requirements or systematically examined its dose-response dynamics during the early larval stage of marine fish.

Methionine-chelated copper (Met-Cu) was selected as the enrichment source due to its enhanced bioavailability and lower ionic toxicity compared to inorganic salts such as CuSO4, a trend demonstrated in gibel carp (*Carassius auratus gibelio*) by Shao et al. (2010). To further mitigate the risk of direct Cu^{2+} toxicity to the larvae and enhance absorption, a *Chlorella*-mediated step was incorporated between the Met-Cu source and *Artemia*. In this three-tier approach (Met-Cu → *Chlorella* → *Artemia* → larvae), microalgae initially uptake ionic Cu, transforming it into organically bound copper prior to transfer up the food chain. This strategy mimics the natural movement of minerals within an ecological food web and provides hatcheries with a safe and efficient delivery method. A comparable approach—enriching *Artemia* with selenium via yeast—was validated by Cao et al. (2023) in largemouth bass (*Micropterus salmoides*) larvae, resulting in improved survival, growth, and antioxidant status, thereby supporting the feasibility of trace mineral biofortification through live-feed vectors.

In this study, a *Chlorella*-mediated Met-Cu *Artemia* enrichment protocol was developed using five concentrations (0, 5, 10, 15, and 20 mg/L) and tested for its dose-response effects on hybrid grouper larvae over a 15-day trial. This study systematically assessed: (1) Cu transfer efficiency through the trophic chain; (2) larval growth performance and thermal stress resistance; (3) digestive enzyme activities; (4) antioxidant enzyme activities and lipid peroxidation; and (5) transcriptional regulation of antioxidant, innate immune, and appetite-related genes. Ultimately, the study aims to determine the optimal Met-Cu enrichment concentration for hybrid grouper larviculture and establish a mechanistic framework linking live-feed copper biofortification to digestive, antioxidant, and immune homeostasis in marine fish larvae.

2. Materials and methods

2.1 Experimental larvae and rearing conditions

Fertilized eggs of hybrid grouper (*E. fuscoguttatus* ♀ × *E. lanceolatus* ♂) were sourced from Qionghai Deyifeng Aquaculture Base (Hainan, China), with all larvae derived from a single spawning batch. Following an 18-day acclimation period in a green-water system, healthy larvae (initial body weight 37.76 ± 9.06 mg) were selected and stocked at 100 individuals per tank. Larvae were reared in 60-L glass aquaria under controlled conditions: temperature 27 ± 2°C, salinity 30 ± 1 ppt, dissolved oxygen > 7.0 mg/L, and a photoperiod of

12L:12D. Fifty percent of the tank water was replaced daily.

2.2 *Artemia* nauplii enrichment protocol

Artemia cysts were hatched in filtered seawater at 28°C for 24 hours. Instar II nauplii were collected via phototaxis, rinsed three times, and used immediately. The enrichment medium was prepared by adding a concentrated *Chlorella* suspension (3.5×10^{10} cells/mL; 5 mL/L) and methionine-copper (Met-Cu; ≥ 98% purity; Cu ≥ 21%; Shanxi Rankang Biotechnology, China) to filtered seawater at nominal concentrations of 0, 5, 10, 15, and 20 mg/L. After one hour of continuous aeration to facilitate microalgal Cu uptake, freshly hatched *Artemia* nauplii were introduced at a density of 5,000 nauplii/mL and incubated for 8 hours at 28°C. Enriched nauplii were collected on a 75-μm mesh sieve, rinsed three times with clean seawater, and then fed immediately to the larvae. This enrichment procedure was conducted freshly before each of the four daily feedings. Subsamples of enriched nauplii were pooled per replicate, freeze-dried, and stored at -80°C for Cu analysis.

Methionine-copper was selected as the Cu source for two main reasons: (i) organic Cu chelates demonstrate enhanced intestinal bioavailability compared to inorganic Cu sulfate in teleost fish (Shao et al. 2010), and (ii) the amino acid chelate scaffold reduces the fraction of bioavailable free Cu^{2+} ions in the enrichment medium, minimizing acute toxicity to *Artemia* nauplii during the 8-hour enrichment period.

2.3 Experimental design and feeding management

Five dietary groups were established based on the *Chlorella*-mediated Met-Cu enrichment concentrations: Control (0 mg/L), Cu5 (5 mg/L), Cu10 (10 mg/L), Cu15 (15 mg/L), and Cu20 (20 mg/L). Each group consisted of three replicate tanks (n = 3), with each tank containing 100 larvae at 18 days post-hatch. Larvae were fed enriched *Artemia* nauplii four times daily (06:00, 12:00, 18:00, and 24:00) over a 15-day trial.

2.4 Sample collection and growth parameters

At the end of the trial, all surviving larvae in each tank were counted. Following a 24-hour fasting period, ten individuals per replicate were weighed (0.01 mg precision) and measured for total body length (mm) using a vernier caliper. The remaining larvae were snap-frozen in liquid nitrogen and stored at −80°C for biochemical analyses. Additionally, five larvae per replicate were transferred into RNAstore Stabilizer (Servicebio, Wuhan, China) for RNA preservation. Growth indices were calculated as follows:

Survival rate (SR, %) = (FN/IN) × 100;

Specific growth rate (SGR, %/d) = [ln(FBW) - ln(IBW)] / days × 100.

Where: FN = final number of surviving fish at the end of culture; IN = initial number of larvae at the start of the trial; FBW = final body weight of larvae; IBW = initial body weight of larvae.

2.5 Acute thermal stress challenge

Ten larvae per replicate were randomly selected and transferred to 2-L aerated containers maintained at 35.5 ± 0.2°C. The time to 50% mortality (LT_{50}) and 100% mortality (LT_{100}) were recorded in minutes, with loss of equilibrium followed by cessation of opercular movement serving as the endpoint criterion.

2.6 Copper content determination in *Artemia* and larval whole body

Samples (0.2–3 g) were digested with HNO_3:$HClO_4$ (5:1, v/v) on a graphite digestion block at temperatures of 120°C (0.5–1 h), 180°C (2–4 h), and finally 200–220°C until the solution reached a colorless state. Following cooling, the digests were diluted to 100 mL using ultrapure water, with a blank digestion conducted simultaneously. Copper concentrations were determined via atomic absorption spectrophotometry and expressed on a dry weight (DW) basis.

2.7 Digestive enzyme assays and antioxidant indices

Frozen larval samples were homogenized in 0.9% saline (1:9 w/v) and centrifuged at 2,500 × g for 10 minutes at 4°C. The activities of pepsin (EC 3.4.23.1), trypsin (TPS; EC 3.4.21.4), lipase (LPS; EC 3.1.1.3), and amylase (AMS; EC 3.2.1.1) were measured in the supernatant using commercial colorimetric kits (Grices Biotech, Suzhou, China). Antioxidant indices, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA), were assayed using kits from the same supplier. Total protein concentration was quantified via the Bradford method, and all activities are expressed per mg protein.

2.8 RNA extraction and quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using the Eastep Super Total RNA Extraction Kit (Promega Biotech, Beijing), incorporating on-column DNase I digestion to remove genomic DNA contamination. RNA purity (A260/A280 1.90–2.10) was assessed using a NanoPhotometer. First-strand cDNA was synthesized from 1000 ng RNA using HiScript III All-in-one RT SuperMix Perfect for qPCR with gDNA Eraser (Vazyme Biotech Co., Ltd., Nanjing, China).

qPCR was conducted on a LightCycler 96 Instrument (F. Hoffmann-La Roche Ltd) using ChamQ SYBR qPCR Master Mix (Vazyme Bio) with the following thermal cycling conditions: 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. Primer sequences were designed based on previously published sequences (Sun et al., 2021; Liu et al., 2024) and are listed in **Table 1**. Target genes related to antioxidant signaling (*CAT*, *GPX*, *GR*, *LZM*, *Keap1*, and *Nrf2*), innate immune response (*IL-1β*, *IL-8*, I*L-10*, *TNF-α*, and *TGF-β*), and appetite regulation (*CGRP*, *NPY8R*, and *NUCB2*) were evaluated, with *β-actin* serving as the endogenous reference gene. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001), normalizing the expression levels in the Control group to 1.0.

Table 1. Sequences of primer used in quantitative real-time PCR.

Gene name	Forward primer	Reverse primer
IL-8	AGTCATTGTCATCTCCATTGCG	AAACTTCTTGGCCTGTCCTTTT
IL-1β	CCTCATCATCGCCACACAGA	TGCCTCACAACCGAACACAT
TNF-α	GCAGCGATGGTGACGAGAAGG	TCCTCCTGTGCCGTGCTCTG
IL-10	TTCGACGAGCTCAAGAGTGAG	TGCCGTTTAGAAGCCAGATACA
TGF-β	AACATCCCGCTACCTCGCTT	TCCGCTCATCCTCATTCCT
Nrf2	ACTGATCTGCCGTTCTCTTTC	TTTCTGACGGTGGTTGTAGTC
Keap1	CCAGAAGGAATGTGTGGCTAAA	TGGTTGGTCATCGGGTTGTA
CAT	GCGTTTGGTTACTTTGAGGTGA	GAGAAGCGGACAGCAATAGGT
GPX	TACCCTACCAGTCCTCCAACC	AACAACACCCGACACCCA
GR	CTTTCACTCCGATGTATCACGC	GCTTTGGTAGCACCCATTTTG
LZM	ATGGGACAGTGAGGAACACC	GTTGCGGATGCGTCCAATA
CGRP	CCCATGTCTAAGCGCTGCAC	GTCGGTGCGGGGATAGGTTT
NPY8R	GGGCTTCCTCAGCCCTCATT	TCAGTCGGCTCCCTCTGGAC
NUCB2	TCCATCTCTGCTGGCGTCTG	AAGGCCAGTGTCCCCGTTTT
β-actin	TACGAGCTGCCTGACGGACA	GGCTGTGATCTCCTTCTGC

Abbreviations: *IL-8*, Interleukin-8; *IL-1β*, Interleukin-1 beta; *TNF-α*, Tumor necrosis factor alpha; *IL-10*, Interleukin-10; *TGF-β*, Transforming growth factor beta; *Nrf2*, Nuclear factor erythroid 2–related factor 2; *Keap1*, Kelch-like ECH-associated protein 1; *CAT*, Catalase; *GPX,* Glutathione peroxidase; *GR*, Glutathione reductase; LZM, Lysozyme; *CGRP*, Calcitonin gene-related peptide; *NPY8R*, Neuropeptide Y receptor type 8; *NUCB2*, Nucleobindin-2; *β-actin*, Beta-actin.

2.9 Statistical analysis

All data are presented as mean ± SD (n = 3). Statistical differences among dietary groups were assessed using one-way analysis of variance (ANOVA) followed by Tukey's HSD post-hoc test, with the significance level set at $p < 0.05$. Statistical analyses were performed using SPSS version 26.0, and graphical representations were created using GraphPad Prism version 10.1.2.

3. Results

3.1 Copper accumulation in *Artemia* Nauplii and larval whole body

Copper content in enriched *Artemia* nauplii increased progressively in a dose-dependent manner relative to the *Chlorella*-mediated Met-Cu enrichment concentration, with each group showing statistically significant differences from the others ($p < 0.05$; **Table 2**). Correspondingly, the whole-body Cu content of larvae exhibited a dose-dependent increase, with the Cu15 and Cu20 groups accumulating significantly higher tissue Cu levels compared to the lower enrichment groups ($p < 0.05$). These results confirm an efficient and dose-dependent transfer of copper from the enrichment medium through *Artemia* to hybrid grouper larvae.

Table 2. Copper content in *Artemia* and larvae whole body following Met-Cu fortification (Dry Weight).

Parameters	Groups				
	Control	Cu-5	Cu-10	Cu-15	Cu-20
Cu in *Artemia* (mg/kg)	173.92±1.31^{e}	311.99±1.63^{d}	400.42±4.44^{c}	488.98±9.28^{b}	606.68±5.06^{a}
Cu in larvae (mg/kg)	140.9±3.44^{d}	149.3±3.58^{c}	153.4±1.79^{c}	167.09±0.85^{b}	178.71±0.44^{a}

Note: Control, Cu-5, Cu-10, Cu-15, and Cu-20 represent five different concentrations of Met–Cu accumulated in *Chlorella*: 0, 5, 10, 15, and 20 mg/L, respectively. Different letters indicate significant differences within the same row ($p < 0.05$).

3.2 Growth performance and thermal stress resistance

Following the 15-day feeding trial, SR, FBW, and SGR all exhibited significant dose-dependent responses to Met-Cu enrichment ($p < 0.05$; **Table 3**). The Cu15 group achieved the highest values across all three primary growth indices, significantly surpassing both the unenriched Control and all other treatment groups ($p < 0.05$). Final body length (FBL) was notably greater in Cu10 and Cu15 compared to all other groups ($p < 0.05$). Conversely, the Cu20 group displayed significantly reduced growth performance relative to the Cu15 group, indicating that excessive copper supplementation negatively impacted larval development.

Table 3. Growth performance of hybrid grouper larvae fed copper-enriched *Artemia* at different copper concentrations

Parameters	Groups				
	Control	Cu-5	Cu-10	Cu-15	Cu-20
SR (%)	45.33±4.04^{c}	65.33±3.51^{a}	70.67±1.53^{a}	71.67±2.08^{a}	46.33±3.21^{c}
FBW (mg)	159.97±27.15^{c}	191.74±25.26^{bc}	219.29±31.15^{ab}	250.37±43.58^{a}	192.53±47.9^{b}
FBL (mm)	22.11±1.63^{b}	22.91±2.35^{b}	25.99±1.09^{a}	26.39±1.69^{a}	22.71±2.91^{b}
SGR(%/d)	8.15±2.12^{bc}	10.26±1.67^{b}	12.1±2.38^{ab}	14.17±2.97^{a}	10.32±2.77^{b}

Note: SR, Survival rate; FBW, Final body weight; FBL, Final body length; SGR, Specific growth rate. Different superscript letters within the same row indicate significant differences ($p < 0.05$).

During the 35.5°C acute thermal stress challenge, the LT_{50} exhibited a numerical trend of Cu15 > Cu5 > Cu10 > Cu20 > Control, although differences among groups did not reach

240 statistical significance ($p > 0.05$; **Table 4**). The LT_{100} was significantly longer in the Cu10 and
241 Cu15 groups compared to Control ($p < 0.05$), with no significant difference observed between
242 these two groups.
243

Table 4. Lethal Time Under 35.5°C stress of hybrid grouper larvae.

Items	Groups				
	Control	Cu-5	Cu-10	Cu-15	Cu-20
LT_{50} (min)	4.82±0.32^{a}	5.89±0.75^{a}	5.8±0.49^{a}	6.15±0.52^{a}	5.35±0.72^{a}
LT_{100} (min)	7.42±0.69^{b}	8.93±0.80^{b}	10.82±1.20^{a}	10.28±2.30^{a}	9.1±0.25^{b}

Note: LT50, Median lethal time; LT100, Lethal time for 100% mortality. Different superscript letters within the same row indicate significant differences ($p < 0.05$).

3.3 Digestive enzyme activities

Pepsin activity was significantly greater in all Met-Cu-enriched groups compared to the Control ($p < 0.05$), yet no significant differences were found among the various enrichment levels ($p > 0.05$; **Figure 1**). In contrast, for trypsin (TPS), lipase (LPS), and amylase (AMS), the Cu15 enrichment elicited the highest activities across all groups ($p < 0.05$). Notably, TPS and AMS activities in the Cu20 group declined to levels significantly below those of the unenriched Control ($p < 0.05$), indicating a toxicity-induced impairment of pancreatic secretory function at the highest Cu concentration. Overall, *Artemia* nauplii enriched at 15 mg/L Met-Cu significantly enhanced larval digestive capabilities, except for pepsin, which appeared to reach saturation at lower enrichment thresholds.

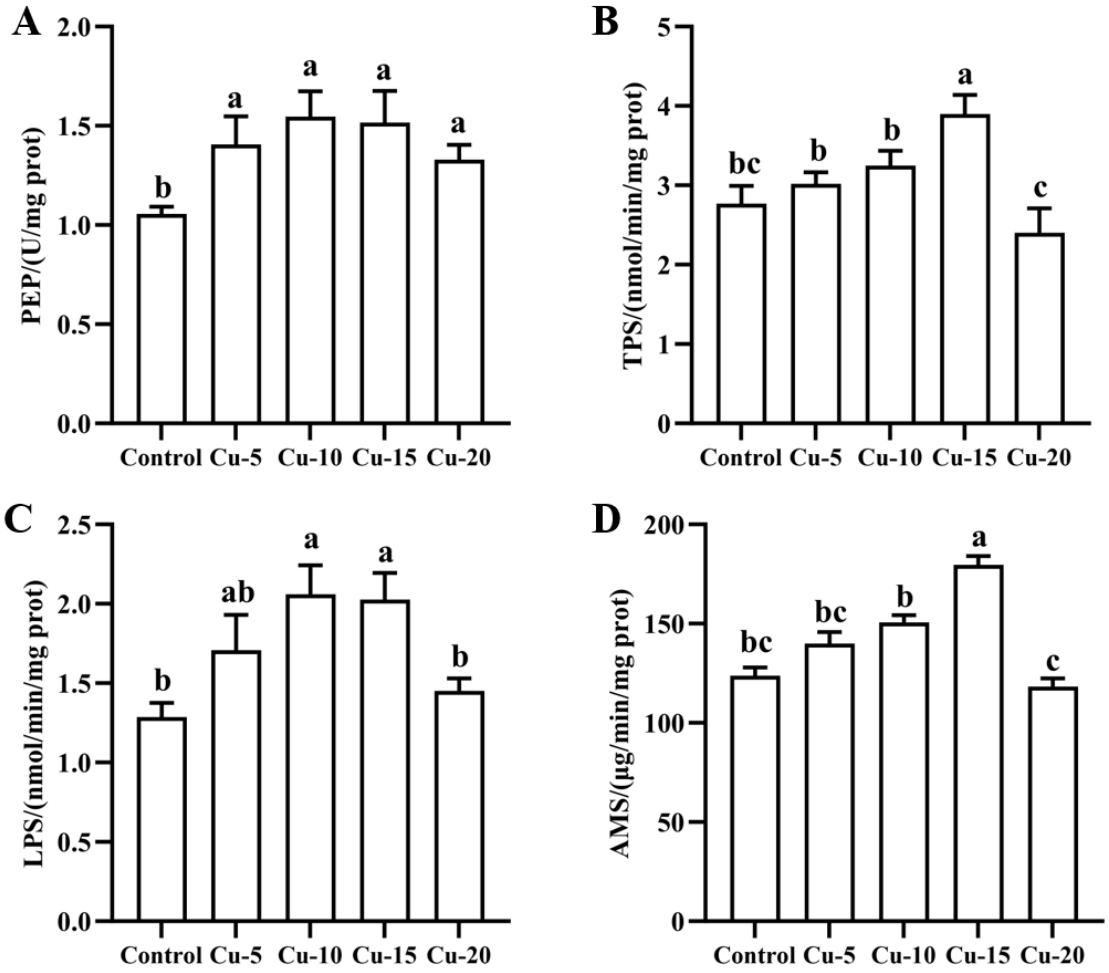


Figure 1. Digestive enzyme activities of hybrid grouper larvae fed copper-enriched *Artemia* at different copper concentrations. Control, Cu-5, Cu-10, Cu-15, and Cu-20 represent five different concentrations of Met–Cu accumulated in *Chlorella*: 0, 5, 10, 15, and 20 mg/L, respectively. (A) PEP, Pepsin; (B) TPS, Trypsin; (C) LPS, Lipase; (D) AMS, α-Amylase. Different letters above bars indicate significant differences ($p < 0.05$).

3.4 Antioxidant enzyme activities

GSH-Px and CAT activities were significantly highest in the Cu15 group compared to all other groups ($p < 0.05$; **Figure 2**). Similarly, SOD activity peaked in the Cu15 group; however, SOD activity in the Cu20 group was significantly lower than that of the Control ($p < 0.05$), indicating oxidative inactivation of the SOD enzyme at supraphysiological Cu concentrations. MDA content, a key biomarker of lipid peroxidation, was significantly lowest in the Cu10 and Cu15 groups but displayed a partial rebound in the Cu20 group. These findings illustrate that 15 mg/L Met-Cu enrichment maximally enhances the antioxidant defense system, while 20 mg/L results in a deterioration of antioxidant capacity.

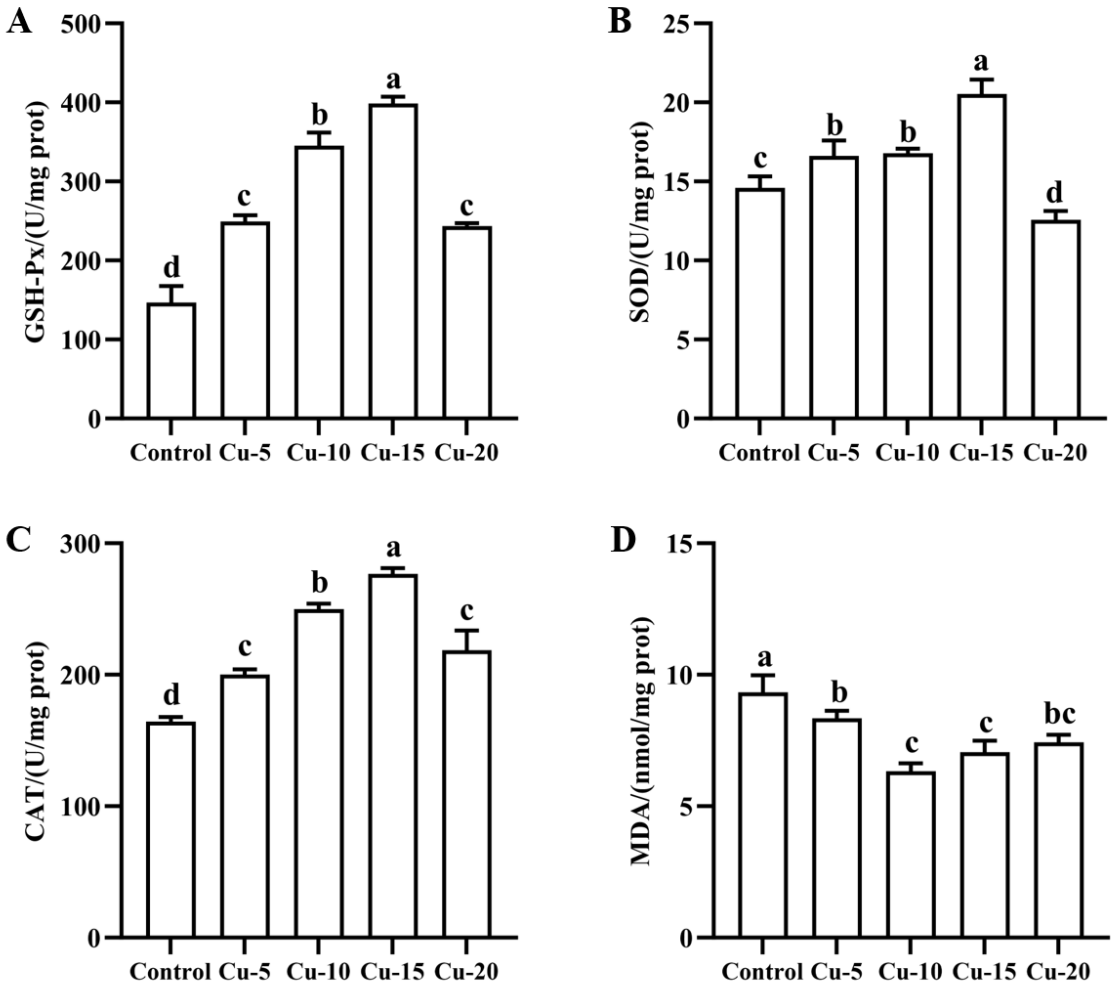


Figure 2. Antioxidant enzyme activities of hybrid grouper larvae fed copper-enriched Artemia at different copper concentrations. Control, Cu-5, Cu-10, Cu-15, and Cu-20 represent five different concentrations of Met–Cu accumulated in Chlorella: 0, 5, 10, 15, and 20 mg/L, respectively. (A) GSH-Px, Glutathione peroxidase; (B) CAT, Catalase; (C) SOD, Superoxide dismutase; (D) MDA, Malondialdehyde. Different letters above bars indicate significant differences ($p < 0.05$).

3.5 Antioxidant-related gene expression

Transcripts for *CAT* and *LZM* were significantly upregulated in the Cu10 and Cu15 groups compared to all other treatments ($p < 0.05$; **Figure 3**). Expression of *GPX* peaked in the Cu10 group but was paradoxically suppressed below control levels in the Cu5 and Cu20 groups ($p < 0.05$). *GR* mRNA expression was significantly elevated specifically in the Cu10 group. Regarding the Keap1/Nrf2 pathway, *Keap1* expression peaked at Cu10 before declining, while *Nrf2* mRNA levels were significantly highest in Cu10 and Cu15, followed by a sharp decline in Cu20 to below the Control level ($p < 0.05$). These transcriptional profiles indicate that the antioxidant defense system was optimally activated under Cu10 and Cu15 treatments. The concurrently suppressed *Nrf2* expression and diminished enzymatic output observed in Cu20 further confirm that supraphysiological copper levels compromise both the transcriptional activation and functional execution of the *Keap1*/*Nrf2* pathway.

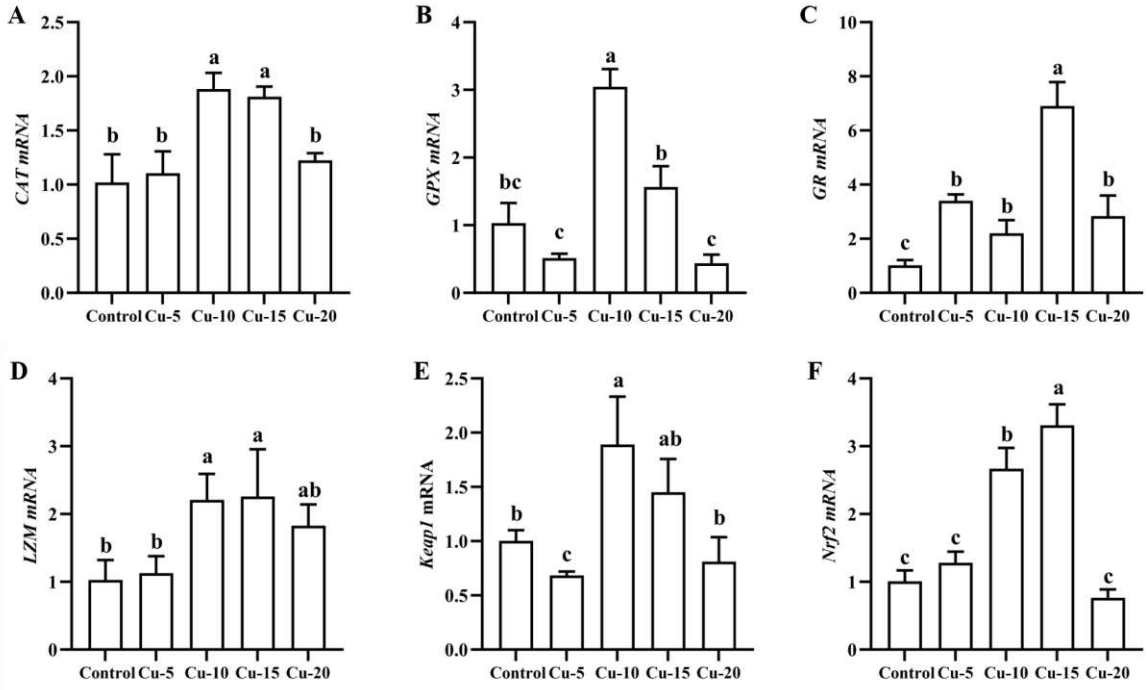


Figure 3. Expression of antioxidant-related genes in hybrid grouper larvae fed copper-enriched Artemia at different copper concentrations. Control, Cu-5, Cu-10, Cu-15, and Cu-20 represent five different concentrations of Met–Cu accumulated in Chlorella: 0, 5, 10, 15, and 20 mg/L, respectively (Control group set to 1.0). (A) CAT, Catalase; (B) GPX, Glutathione Peroxidase; (C) GR, Glutathione Reductase; (D) LZM, Lysozyme; (E) Keap1, Kelch-like ECH-associated protein 1; (F) Nrf2, Nuclear factor erythroid 2-related factor 2. Different letters above bars indicate significant differences ($p < 0.05$).

3.6 Innate immune gene expression

The Cu15 enrichment resulted in the highest expression of both pro-inflammatory (*IL-1β*, *IL-8*) and anti-inflammatory (*IL-10*) cytokine genes among all groups ($p < 0.05$; **Figure 4**), while simultaneously exhibiting the lowest expression levels of *TNF-α* ($p < 0.05$). In contrast, the Cu20 group showed the highest TNF-α expression across all groups, with *IL-1β*, *IL-8*, and *IL-10* levels returning to near-control levels. *TGF-β* mRNA expression peaked in the Cu5 group and reached its lowest point in the Cu15 group. The simultaneous upregulation of both pro- and anti-inflammatory genes and the suppression of *TNF-α* in the Cu15 group suggest that optimal copper enrichment fosters a balanced innate immune response. Conversely, excess copper at Cu20 appears to induce significant inflammatory dysregulation.

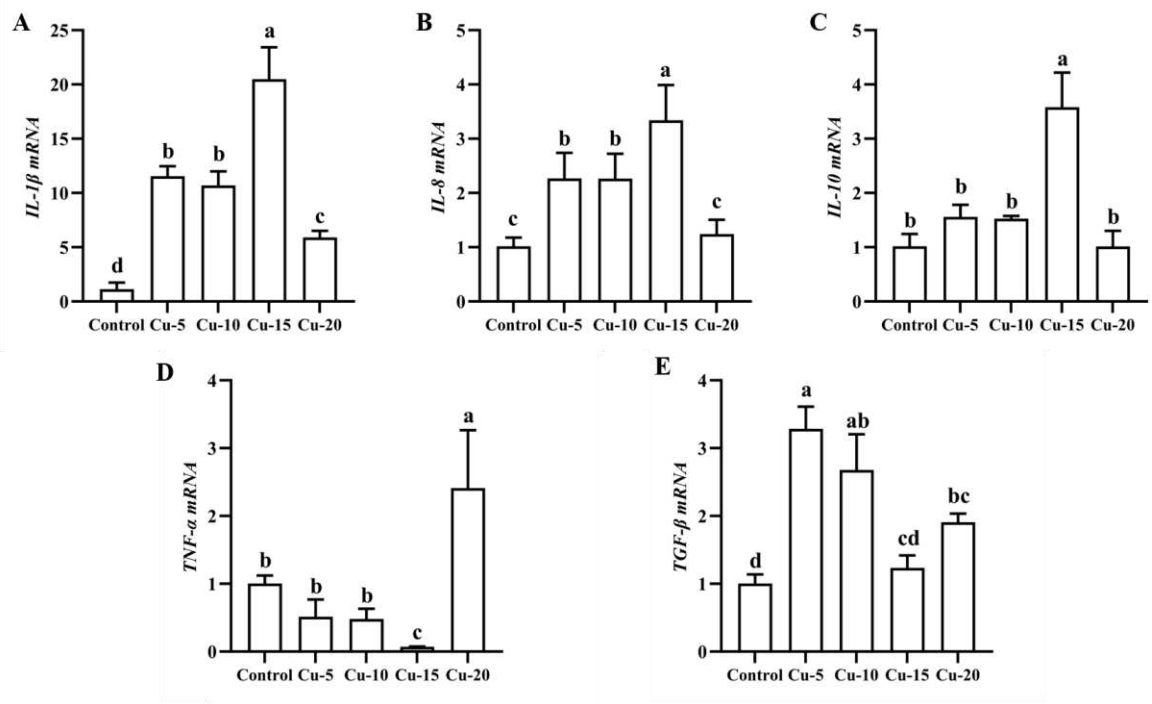


Figure 4. Expression of innate immune genes in hybrid grouper larvae fed copper-enriched *Artemia* at different copper concentrations. Control, Cu-5, Cu-10, Cu-15, and Cu-20 represent five different concentrations of Met–Cu accumulated in *Chlorella*: 0, 5, 10, 15, and 20 mg/L, respectively (Control group normalized to 1.0). (A) IL-1β, Interleukin-1 beta; (B) IL-8, Interleukin-8; (C) IL-10, Interleukin-10; (D) TNF-α, Tumor Necrosis Factor-alpha; (E) TGF-β, Transforming Growth Factor-beta. Different letters above the bars indicate significant differences ($p < 0.05$).

3.7 Appetite-related gene expression

The anorexigenic genes *CGRP* and *NUCB2* were significantly downregulated in all Met-Cu-enriched groups compared to the Control ($p < 0.05$; **Figure 5**), with *CGRP* expression reaching its lowest point in the Cu15 group before rebounding to control-equivalent levels in Cu20. The orexigenic receptor gene *NPY8R* was significantly upregulated across the Cu5 through Cu15 groups, peaking at Cu10, but returned to control-equivalent levels in Cu20 ($p < 0.05$). This pattern indicates a coordinated transcriptional shift in groups Cu5 to Cu15, characterized by the simultaneous suppression of satiety signals and enhancement of appetite-stimulating receptors. This neuroendocrine configuration aligns with improved growth performance. In the Cu20 group, however, this pro-appetite gene profile was entirely dismantled, as all three appetite-related genes reverted to or fell below control levels.

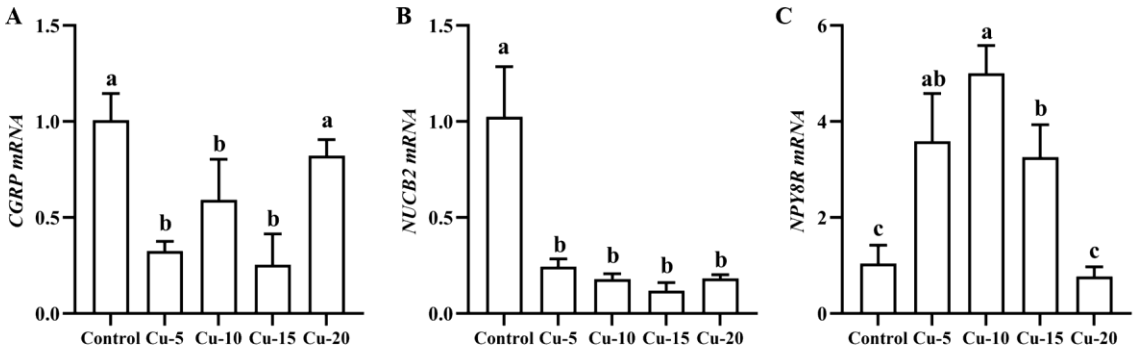


Figure 5. Expression of appetite-related genes in hybrid grouper larvae fed copper-enriched *Artemia* at different copper concentrations. Control, Cu-5, Cu-10, Cu-15, and Cu-20 represent five different concentrations of Met–Cu accumulated in *Chlorella*: 0, 5, 10, 15, and 20 mg/L, respectively (Control group normalized to 1.0). (A) CGRP, Calcitonin Gene-Related Peptide; (B) NUCB2, Nucleobindin-2; (C) NPY8R, Neuropeptide Y Receptor Type 8. Different letters above the bars indicate significant differences ($p < 0.05$).

4. Discussion

Cu accumulated in enriched *Artemia* and larval tissues in a dose-dependent manner, confirming that *Chlorella*-mediated bioencapsulation serves as a promising and potentially scalable method for live-feed copper biofortification. Direct exposure to waterborne Cu^{2+} is acutely toxic to aquatic organisms at comparable ionic concentrations (Clearwater et al. 2002). The microalgal intermediate matrix circumvents this issue by converting ionic Cu into an organically complexed form, thereby enhancing bioavailability while reducing toxicity risks to both *Artemia* vectors and larvae (Shao et al. 2010). The enhanced growth observed in the Cu10 and Cu15 groups reflects two fundamental roles of Cu: it acts as the catalytic core of cytochrome c oxidase in aerobic energy metabolism and supports extracellular matrix remodeling through lysyl oxidase, both of which are critical for rapid larval growth (Prohaska 2008). Similar dose-dependent growth responses to dietary Cu have been documented in juvenile grouper (*Epinephelus malabaricus*) (Lin et al. 2008), grass carp (Tang et al. 2013), and *Pangasianodon hypophthalmus* (Kumar et al. 2024), confirming the conserved essentiality of Cu across teleost species. Enhanced activities of TPS, LPS, and AMS at Cu15, along with the saturation response of pepsin, align with previous findings indicating that optimal Cu levels stimulate pancreatic exocrine secretion and brush-border enzyme function (Tang et al. 2013; Peng et al. 2023). Conversely, at Cu20, activity of TPS and AMS declined below control levels, mirroring Cu toxicity-associated pancreatic dysfunction observed in Atlantic salmon (Berntssen et al. 1999), where oxidative damage to acinar cell membranes disrupts zymogen secretion without necessarily impairing gastric proteolysis.

GSH-Px, CAT, and SOD activities increased progressively up to Cu15. This pattern is consistent with the established Keap1–Nrf2 regulatory mechanism, in which oxidative or

electrophilic stress can modify critical cysteine residues of Keap1, weaken Keap1-mediated Nrf2 repression, and promote Nrf2-dependent transcription of antioxidant response element (ARE)-regulated genes such as GPX, CAT, and GR (Itoh et al. 1999; Kobayashi et al. 2009; Suzuki and Yamamoto 2015). The Cu10 group's upregulation of *Keap1*, in conjunction with these downstream genes, further supports this phenomenon, as Nrf2 induces *Keap1* transcription in a negative feedback loop to restore redox balance (Kobayashi et al. 2009). Dietary Cu-dependent activation of the Nrf2 pathway has also been reported in largemouth bass (Xu et al. 2024), indicating that this regulatory axis functions efficiently across teleost lineages. At Cu20, however, both *Nrf2* mRNA levels and downstream antioxidant enzyme activity decreased sharply, a result that the basic Keap1 cysteine-sensing model does not fully explain, as heightened oxidative stress would typically promote further Nrf2 activation rather than inhibition (Ngo et al. 2022). A more plausible explanation posits that supraphysiological Cu levels directly impair Nrf2 transcriptional activity once toxicity surpasses a specific threshold, exacerbating rather than mitigating the collapse of antioxidant defenses. The decline of SOD activity below control at Cu20 supports this notion: the Cu/Zn-SOD holoenzyme appears to be inactivated, aligning with reports of concurrent declines in SOD activity and *Nrf2*/*Keap1* transcription following severe Cu exposure in other teleosts (Jiang et al. 2016). The accompanying rise in MDA confirms that antioxidant protection has largely failed at Cu20, reflecting observations in *Epinephelus coioides* subjected to excessive Cu exposure (Wang et al. 2015).

Data on innate immune genes reveal a similar distinction between beneficial and toxic Cu exposure. At Cu15, both pro-inflammatory (*IL-1β*, *IL-8*) and anti-inflammatory (*IL-10*) cytokine genes were upregulated, while *TNF-α* and *TGF-β* were at their lowest, suggesting a relatively balanced innate immune response rather than overt inflammation. *IL-1β* and *IL-8* promote neutrophil recruitment and pathogen clearance, while co-elevated *IL-10* prevents excessive tissue damage (Zou and Secombes 2016). The downregulation of TGF-β, a broadly immunosuppressive cytokine in teleosts (Zou and Secombes 2016), suggests that larvae are relaxing some of the usual immune constraints, which may contribute to improved immune readiness under hatchery conditions (Mohd Faudzi et al. 2022). In contrast, the pronounced rise in *TNF-α* at Cu20, coupled with the failure of the coordinated *IL-1β*/*IL-8*/*IL-10* response, indicates ROS-driven, NF-κB-dependent sterile inflammation, rather than directed antimicrobial immunity. Wang et al. (2015) demonstrated this mechanism in juvenile *Epinephelus coioides*, where excess Cu activated intestinal NF-κB, induced *TNF-α* transcription, and triggered apoptosis. The biphasic, concentration-dependent relationship

between dietary Cu and *TNF-α* observed in this study aligns with findings in *Pangasianodon hypophthalmus*, where optimal Cu supplementation mitigated stress-induced *TNF-α* expression (Kumar et al. 2024).

A key novel finding of this study is the demonstration that dietary Cu modulates neuroendocrine appetite regulation in marine fish larvae. Anorexigenic *CGRP* mRNA was suppressed across Cu5–Cu15, reaching its lowest levels at Cu15, which aligns with CGRP's established role as a potent appetite inhibitor in teleosts, where intracerebroventricular CGRP injection reduces food intake in goldfish (Martínez-Alvarez et al. 2009). *NUCB2*, encoding the nesfatin-1 precursor and suppressing food intake through leptin-independent pathways in various teleost species (Gonzalez-Nunez and Rodriguez 2010; Zhang et al. 2018), exhibited uniform suppression across all Cu-supplemented groups regardless of the dosage, functioning more as a threshold on/off switch than a graded response. In contrast, *NPY8R* was upregulated at Cu5–Cu15, enhancing neuropeptide Y signaling, recognized as the most potent appetite-stimulating system in vertebrates (Volkoff 2016; Larsson et al. 2021). Collectively—reduced satiety signals and heightened orexigenic receptor sensitivity—this provides a plausible neuroendocrine explanation for the accelerated growth of Cu15 larvae. At Cu20, this entire profile diminished: *CGRP* and *NPY8R* returned to control levels, suggesting that Cu toxicity disrupts appetite regulation in addition to its metabolic and immune effects, a facet of Cu toxicity previously unreported in early-stage fish larvae. However, the decline at Cu20 may not solely arise from Cu toxicity in the larvae themselves; supraphysiological Cu could also induce lipid peroxidation within the *Artemia* during the 8-hour enrichment period, potentially degrading nutrients before larval ingestion. Future work will profile the nutritional composition of the enriched vectors to differentiate direct larval Cu toxicity from the effects of consuming oxidatively damaged prey.

Integrating growth, enzymatic, antioxidant, immune, and appetite endpoints, 15 mg/L Met-Cu emerges as the optimal *Artemia* enrichment concentration for hybrid grouper larviculture. However, direct quantitative comparisons with dietary Cu requirements established for post-larval juveniles utilizing formulated compound diets (e.g., 4.37 mg Cu/kg in *E. malabaricus* (Lin et al. 2008); approximately 3 mg/kg in rainbow trout (Ogino and Yang 1980); approximately 4 mg/kg in channel catfish (Gatlin and Wilson 1986)) prove challenging due to fundamental discrepancies in the delivery vehicle, larval intestinal maturity, and dynamics between enrichment medium and tissue concentrations (Hamre et al. 2013; Clearwater et al. 2002). The only prior precedent for *Artemia*-mediated Cu enrichment in aquaculture, in Chinese mitten crab (*Eriocheir sinensis*) larvae by Sun et al. (2013), also

identified an intermediate enrichment concentration as optimal, with toxicity emerging at the highest level. This reinforces the conserved dose-response nature of Cu biofortification across diverse aquatic taxa, regardless of the Cu source. Finally, the *Chlorella*-mediated bioencapsulation strategy developed herein—comparable to the selenium-enriched yeast approaches validated in largemouth bass larvae by Cao et al. (2023)—provides a practical, scalable route for trace mineral delivery that mitigates the direct ionic Cu^{2+} exposure to larvae. Future research should evaluate the extension of this methodology to multi-element enrichment protocols to comprehensively address simultaneous trace mineral deficiencies in commercial *Artemia* (Hamre et al. 2013; Watanabe et al. 1997).

5. Conclusion

This study validates a *Chlorella*-mediated bioencapsulation approach for targeted Cu delivery through *Artemia* nauplii in marine larviculture. The optimal enrichment concentration for hybrid grouper (*E. fuscoguttatus* × *E. lanceolatus*) larvae is established at 15 mg/L Met-Cu. At this concentration, growth performance, antioxidant defense, innate immunity, and orexigenic neuroendocrine signaling were generally optimal. However, exceeding this threshold (20 mg/L) was associated with signs of Cu toxicity, characterized by oxidative stress, elevated TNF-α expression, and disruption of appetite-related gene expression. This scalable live-feed protocol addresses a critical bottleneck in early-stage trace mineral nutrition. Future research should assess the applicability of this protocol in other marine taxa and explore potential synergistic effects of co-enriching Cu with essential minerals such as Zn and Se.

Acknowledgments

The research was supported by the earmarked fund for Hainan Modern Agricultural Industrial Technology Research System (HNARS-10-G03). Innovational Fund for Scientific and Technological Personnel of Hainan Province (KJRC2023C40). Project of Collaborative Innovation Center of Marine Science and Technology, Hainan University (XTCX2022HYC23).

CRediT authorship contribution statement

Yujie Zeng: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft. Xi Zhu: Investigation, Data curation. Zheng Li: Investigation, Data curation. Hao Long: Investigation. Wei Ren: Investigation. Yanghua Zeng: Resources. Xin Zhang: Resources. Aiyou Huang: Resources. Zhenyu Xie: Resources. Xiaoni Cai: Conceptualization,

Supervision, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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