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Growth Performance of Critically Endangered *Hypselobarbus pulchellus* in Biofloc Systems with Various C/N Ratios

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ABSTRACT

Background: Biofloc technology relies on adding a carbon source to maintain optimal carbon to nitrogen ratio for transforming the waste into microbial biomass that aquatic animals can use. In the present study, the growth performance of the critically endangered *Hypselobarbus pulchellus* in biofloc-based seed rearing with different Carbon to Nitrogen ratio was studied.

Methods: Healthy early fry (0.01 g) was randomly stocked into 15 circular FRP tanks (1000 L) at a density of 100 individuals/m^3 and reared for 90 days. The carbon to nitrogen ratios of 5:1(CN5), 10:1(CN10), 15:1(CN15) and 20:1(CN20) were maintained using jaggery without considering carbon content derived from the feed and the control group received no additional source of carbon.

Result: The results indicated that *H. pulchellus* had better growth performance in biofloc systems having higher C/N ratios. The highest weight gain (0.48±0.01 g) and weight gain percentage (3931.15±120.68%) and specific growth rate (4.11±0.03% per day) were found in the CN10 group (P<0.05), followed by CN15 and CN20, while the control group values were lowest. In terms of survival, all treatments had high survival rates ranging from 92% to 94.67%, with no significant variations (P>0.05). The CN10, CN15 and CN20 treatments had significantly higher amylase, protease and lipase activities than control (P<0.05). Moreover, the immunological and antioxidant activity increased with CN ratios. The CN10, CN15 and CN20 groups had significantly lower total ammonia nitrogen, nitrite and nitrate levels than control group (P<0.05). Overall, this study suggests that C/N ratios of 10:1 to 20:1 are optimal for rearing *H. pulchellus* fingerlings from early fry, with 10:1 presenting the best growth results.

Key words: Aquaculture, BFT, Biofloc, C/N ratio, *Hypselobarbus pulchellus*, Sustainability.

INTRODUCTION

Aquaculture is a rapidly growing food producing industry that is meeting global food demand (FAO, 2022). However, as the industry intensifies production, there is a growing demand for sustainable aquaculture practices (Aanesen *et al.*, 2023; Campanati *et al.*, 2021). A major issue in intensive fish farming is the build-up of nitrogen compounds like NH_4 and NO_2 (Abu Bakar *et al.*, 2015) due to ammonia nitrogen from the cultured organism, food remains and faeces and these can negatively impact water quality, which is a major concern in aquaculture (Crab *et al.*, 2007; Piedrahita, 2003). Intensifying production methods can increase the efficiency and output of aquaculture operations, but it must be done in a responsible way to ensure industry sustainability (Engle *et al.*, 2017). Biofloc Technology (BFT) has gained attention in recent times owing to its numerous benefits and it is a promising method for enhancing fish growth performance in aquaculture (Najdegerami *et al.*, 2016; Nisar *et al.*, 2022; Wang *et al.*, 2015). The main principle behind BFT is maintaining the carbon to nitrogen (C/N) ratio to increase the heterotrophic bacteria population through the utilization of nitrogen compounds and their subsequent transformation into microbial biomass that may be utilised as fish food (Avnimelech, 1999). BFT is a bio-secure system that minimizes the need for water exchange and improves water quality through self-generated bioremediation processes (Emerenciano *et al.*, 2011; Panigrahi *et al.*, 2018).

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The nutrients in faeces and leftover feed in the water are converted into microbial communities of bacteria, protozoa, phytoplankton and zooplankton that form bioflocs, which are high-value food for shrimp and fish (Dauda, 2019; Emerenciano *et al.*, 2012; Khanjani *et al.*, 2022; Mohammady *et al.*, 2023). BFT has emerged as a method to prevent the buildup of nitrogen metabolites. By assimilating nutrient waste, it keeps toxic substances in the culture medium at low levels, allowing for reduced water exchange and enhancing water usage efficiency (Ekasari *et al*., 2014). Additionally, these microbial particles serve as valuable sources of nutrients, including proteins, lipids, amino acids and fatty acids. This nutritional contribution lowers the dietary requirements for fish (Emerenciano *et al*., 2013). The effectiveness of BFT in enhancing water quality has been reported in earlier research (Deb *et al*., 2020; Elayaraja *et al*., 2020; Toledo *et al*., 2016). There are a vast number of research that are focussing on exploring species that have the potential to grow well in BFT system.

The *Hypselobarbus pulchellus* (Day, 1870) is a critically endangered fish native to the peninsular rivers of India and is commonly known as 'Haragi meenu' (Rema Devi and Ali, 2020). It has potential as a species for aquaculture in India (Hemaprasanth *et al*., 2016) and breeding technology has been developed for it at the ICAR-CIFA Regional Research Centre in Bengaluru (ICAR-CIFA, 2019). Seed production of this endangered species deserves importance for alleviating its endangered status. This will result in higher availability of fish to the dependent fisher folk. To produce a substantial quantity of healthy fingerlings in a controlled environment, it is necessary to examine the performance of *H. pulchellus* in biofloc seed production systems, as these systems have shown effectiveness for seed rearing (Hosain *et al*., 2021; Panigrahi *et al*., 2020; Samocha *et al*., 2007). Therefore, exploring the potential benefits that biofloc technology could offer for seed production is essential to promote the development of diversified aquaculture. Optimizing C/N ratio with an appropriate carbon source is essential for floc formation (Avnimelech, 1999). Finding the optimal C/N ratio is crucial for efficient nutrient removal in biofloc-based seed production of *H. pulchellus*, which can contribute to sustainable aquaculture practices. However, no information is available on the various C/N ratios effects on growth, digestive enzymes activity, water quality, antioxidant and immune response of *H. pulchellus* seed during biofloc-based seed production. Therefore, this research was conducted to determine the various C/N ratio effects.

MATERIALS AND METHODS

Experimental condition

In the current study, *H. pulchellus'* growth performance was examined under different C:N ratios during early fry to fingerling rearing (Fig 1). The experimental setup included four C/N ratio levels: 5:1 (CN5), 10:1 (CN10), 15:1 (CN15) and 20:1 (CN20) and a control group, each with three replications. No carbon source was added to the control group. *H. pulchellus* early fry were produced in peninsular carp hatchery using the brood-stock maintained at this centre. The early fry having an average length of 1.36 cm and weight of 0.01 g were stocked randomly in 15 circular fiberglass 1000 L tank at the rate of 100 individuals per m^3. Powdered feed containing 35 per cent protein was used to feed the fish twice a day. Jaggery was used as the carbon source in the study. The amount of jaggery required was calculated using the method of Avnimelech (1999), without considering the Carbon content derived from the diet (Miao *et al*., 2020; Panigrahi *et al*., 2018).

Estimation of growth parameters

Scale and digital weighing balance were used to measure the fish length and weight. Subsequently, fish growth parameters were estimated using the following formulas:

$$\text{Weight gain (\%)} = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}} \times 100$$

Specific growth rate (SGR) (% day^{-1}) =

$$\frac{\text{Ln (Final weight) - Ln (Initial weight)}}{\text{Experimental days}} \times 100$$

$$\text{Survival rate (\%)} = \frac{\text{Final count}}{\text{Initial count}} \times 100$$

Enzyme activity analysis

To analyse the digestive enzyme in the intestine tissue, immune parameter in the kidney tissue and antioxidant enzymes in the gills tissue, tissue samples were obtained aseptically through dissection on ice cold conditions. A 5% homogenate of each tissue was made by utilizing a chilled solution of sucrose (0.25 M). Following homogenization, it was then centrifuged at 10,000 rpm for 10 minutes and the supernatant was then stored at -20°C. The amylase activity was determined using 3,5-dinitrosalicylic acid colorimetric method (Rick and Stegbauer, 1974). Protease activity was assessed by incubating samples with casein substrate at 25°C (Drapeau, 1976). Amylase and protease activities were expressed in nanomoles of maltose and tyrosine release per minute, respectively. Lipase activity was evaluated by measuring the fatty acids produced due to enzymatic hydrolysis of triglycerides olive oil emulsion (Cherry and Crandall, 1932) and represented as Units/mg protein. Myeloperoxidase (MPO) activity was measured using the technique developed by Quade and Roth (1997) with 3',3'5',5'- tetramethylbenzidine hydrochloride. Lysozyme activity in kidney tissue was assessed using a turbidimetric assay method outlined by Ellis (1990) using *Micrococcus lysodeikticus*. Superoxide dismutase (SOD) activity was determined by following the Misra and Fridovich (1972) technique, based on the inhibition of 50% of epinephrine's autooxidation. Catalase (CAT) activity was evaluated by initiating the reaction with hydrogen peroxide (H_2O_2) solution (Takahara *et al*., 1960).

Water quality

The data on water quality was collected every 15 days. Dissolved oxygen, pH and temperature were measured by using an oxygen meter (PDO-519, Lutron) and pH-temperature meter (PH-222, Lutron). Alkalinity, nitrite, nitrate and total ammonia nitrogen (TAN) were analysed as per standard methods (APHA, 1995). The biofloc volume from each tank was measured using the Imhoff cone. Total heterotrophic bacterial (THB) counts of experimental tank water were analysed using tryptone soya agar at the end of the experiment. The plates were then incubated for forty-eight hours and the bacterial colonies were represented as CFU/ml.

Statistical analysis

The data were analysed using a one-way ANOVA test using SPSS (version 23 for Windows) software. The Duncan's multiple range test was used in order to identify the significance of the treatment means.

RESULTS AND DISCUSSION

Growth and survival of *H. pulchellus*

The results indicated that *H. pulchellus* had better growth performance in biofloc systems with higher CN ratios (Table 1). Significant variations were found among the treatments in weight gain and SGR ($P<0.05$). The highest weight gain (0.48±0.01g) and weight gain percentage (3931.15±120.68%) were found in the CN10 group ($P<0.05$), followed by CN15 (0.42±0.02 g; 3441.8±125.88%) and CN20 (0.41±0.01 g; 3322.13±113.1%), whereas the control group had the lowest values (0.33±0.02 g; 2667.76±169.66%). Furthermore, the CN10 treatment had the highest specific growth rate (4.11±0.03% per day) ($P<0.05$). However, all treatments had higher survival rates ranging from 92% to 94.67%, without any significant variations between the groups ($P>0.05$). Bioflocs are natural food sources that are continuously available and can provide additional nutrients to fish and shrimp (Avnimelech, 1999; Emerenciano *et al.*, 2012; Mohammady *et al.*, 2023). BFT not only improves feed efficiency and offers high-quality protein sources but also contains abundant growth promoters and bioactive compounds. Optimal C/N ratios have been found to substantially enhance the growth performance of fish (Panigrahi *et al.*, 2018; Wang *et al.*, 2015). The CN

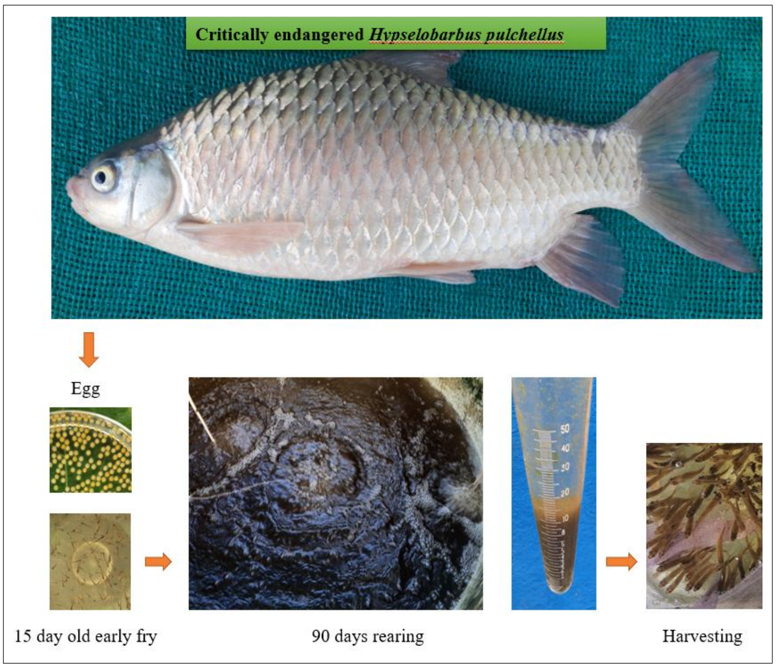

Fig 1: Schematic representation of critically endangered *Hypselobarbus pulchellus* seed rearing using biofloc systems with varying carbon-to-nitrogen ratios.

Table 1: Growth parameters of *H. pulchellus* under different biofloc systems with various C/N ratios (Mean±SE).

Parameters	Control	CN5	CN10	CN15	CN20
Initial length (cm)	1.36±0[a]	1.36±0[a]	1.36±0[a]	1.36±0[a]	1.36±0[a]
Initial weight (g)	0.0122±0[a]	0.0122±0[a]	0.0122±0[a]	0.0122±0[a]	0.0122±0[a]
Final length (cm)	3.63±0.03[c]	3.73±0.01[b]	3.9±0.04[a]	3.76±0.03[b]	3.74±0.01[b]
Final weight (g)	0.34±0.02[c]	0.41±0.02[b]	0.49±0.01[a]	0.43±0.02[b]	0.42±0.01[b]
Weight gain (g)	0.33±0.02[c]	0.39±0.02[b]	0.48±0.01[a]	0.42±0.02[b]	0.41±0.01[b]
Weight gain per cent	2667.76±169.66[c]	3227.05±159.01[b]	3931.15±120.68[a]	3441.8±125.88[b]	3322.13±113.1[b]
Specific growth rate (%/day)	3.68±0.07[c]	3.89±0.05[b]	4.11±0.03[a]	3.96±0.04[b]	3.93±0.04[b]
Survival per cent	92±1.53[a]	92.67±1.76[a]	94±1.53[a]	93.67±1.86[a]	94.67±1.67[a]

Values in the same row with different letters are significantly different (P<.05).

Table 2: Water quality parameters of different C/N ratio treatments (Mean±SE).

Parameters	Control	CN5	CN10	CN15	CN20
Dissolved oxygen (mg L^{-1})	8.1±0.23^{a}	7.83±0.3^{ab}	7.67±0.25^{ab}	7.54±0.24^{ab}	7.26±0.19^{b}
Temperature (°C)	21.24±0.29^{a}	21.38±0.19^{a}	21.44±0.23^{a}	21.53±0.26^{a}	21.55±0.25^{a}
PH	8.12±0.07^{a}	8.05±0.09^{ab}	7.93±0.1^{ab}	7.86±0.06^{ab}	7.78±0.11^{b}
Alkalinity (mg L^{-1} $CaCO_3$)	264.29±5.77^{a}	258.1±5.85^{a}	257.62 ±6.62^{a}	254.29±7.33^{a}	249.05±4.69^{a}
TAN concentration (mg L^{-1})	0.61±0.07^{a}	0.48±0.01^{ab}	0.35±0.03^{bc}	0.3±0.05^{c}	0.25±0.02^{c}
Nitrite concentration (mg L^{-l})	0.56±0.03^{a}	0.51±0.02^{a}	0.4±0.02^{b}	0.36±0.04^{b}	0.32±0.03^{b}
Nitrate concentration (mg L^{-l})	2.98±0.29^{a}	2.66±0.27^{a}	1.78±0.2^{b}	1.69±0.16^{bc}	1.01±0.22^{c}
THB (CFU/mL)	2.00±0.15^{d}	3.33±0.23^{d}	5.33±0.34^{c}	9.00±0.78^{b}	11.66±0.94^{a}
Floc volume (mL/L)	3.83±0.44^{a}	12.67±0.93^{b}	18.67±1.3^{c}	19.83±1.17^{c}	21.5±1.89^{c}

Values in the same row with different letters are significantly different (P<.05).

treatments had better *H. pulchellus* growth performance than the control in our study. Specifically, the CN10 had the highest weight gain percentage and SGR than the other groups. The ratio of 10:1 C/N in the CN10 group had a positive impact on fish growth performance and can be considered balanced. Conversely, the control group had the lowest growth performance parameters, indicating that the presence of biofloc and an appropriate ratio of C/N can have a positive influence on *H. pulchellus* growth. Previous studies had reported that maintaining a C/N ratio of 10:1 by adding external carbohydrate sources in the BFT system enhanced growth performance in fishes like *Oreochromis niloticus* (Aboseif *et al.*, 2022) and *Heteropneustes fossilis* (Zafar *et al.*, 2021). In the present investigation, we found that CN treatments had higher survival rates than the control group, which suggests that microbial floc can enhance fingerling growth and survival. Further, our findings indicate that maintaining the CN ratio to 10:1 in biofloc systems can benefit *H. pulchellus* growth performance during early fry to fingerling rearing.

Water quality

The temperatures and alkalinity did not significantly vary between treatments (Table 2). However, the pH and dissolved oxygen (DO) were significantly reduced in the CN20 ratio group than control. In addition, TAN, NO_2 and NO_3 levels were significantly lower in CN10, CN15 and CN20 groups than in control. Further, the addition of carbohydrates in the CN treatment increased the total count of heterotrophic bacteria (THB) than the control group. The CN20 group had a significantly higher (P<0.05) THB count (11.66±0.94 × 10^5 CFU/ml), followed by CN15 (9.00±0.78 × 10^5 CFU/ml) and CN10 (5.33±0.34 × 10^5 CFU/ml), compared to the CN5 and control group (3.33±0.23 × 10^5 CFU/ml and 2.00±0.15 × 10^5 CFU/ml, respectively). However, no statistically significant variation (P>0.05) found between the control and CN5 treatment. Over the culture period, the biofloc volume in all of the CN treatment groups increased than the control and no significant variations were found among the CN10, CN15 and CN20 groups at 90 days (P<0.05). One of the significant challenges in intensive aquaculture systems is the accumulation of harmful nitrogen metabolites in the water. However, pollutants like nitrogen and phosphorus can reach high concentrations in cultured water, posing a crucial obstacle to the development of aquaculture. Numerous studies have demonstrated that BFT has the ability to efficiently transform nitrogen, phosphorus and other pollutants in cultured water. This capability not only enhances water quality but also mitigates the environmental impact, reducing the adverse effects on the aquatic ecosystem (Avnimelech, 1999). Water quality management is an essential aspect of aquaculture, as it plays a critical role in the success of the endeavour. In our study, we observed that increasing CN ratio in treatments led to a decrease in TAN, NO_2 and NO_3 than the control group. This decrease may have been caused by nutrient cycling or microbial assimilation. The proliferation of bacteria is stimulated by the addition of carbohydrates through microbial protein synthesis by nitrogen absorption (Avnimelech, 1999). According to Ebeling *et al.* (2006), heterotrophic bacteria produce microbial biomass weighing 8.07 g and use 4.71 g of oxygen for every gram of total ammonia nitrogen (TAN) that is taken up by them. In the current research, CN treatments had higher levels of THB than the control group. This indicates nitrogen compounds were assimilated by heterotrophic bacteria in CN treatments. Further, the reduction of nitrogen compounds levels was notable when the ratio of C/N was at 10:1, with no substantial increase in CN15 and CN20 groups. In the current study, the biofloc volume in CN treatments increased compared to the control group over the culture period. The floc volume increased along with the CN ratio from CN5-20. This increase in floc volume may have led to the reduction in pH, dissolved oxygen and alkalinity.

Digestive enzymes antioxidant and immune response

The digestive enzymes (lipase, amylase and protease), immunological (lysozyme activity and MPO), as well as antioxidant enzymes (SOD and catalase) parameters of *H. pulchellus* under different BFT systems with various C/N ratios are presented in Table 3. The fingerlings raised in CN10, CN15 and CN20 BFT treatments had significantly higher activity of amylase, protease and lipase than the control (P<0.05). However, no significant variations were

Table 3: The digestive enzymes, immunological and antioxidant activity of *H. pulchellus* reared in biofloc systems with varying C/N ratios (Mean±SE).

Parameters	Control	CN5	CN10	CN15	CN20
Amylase (U/mg protein)	349.17±10.08[b]	363.38±8.39[b]	465.48±13.44[a]	433.92±12.53[a]	441.55±7.65[a]
Protease (U/mg protein)	247.71±11.44[c]	302.7±6.99[b]	366.27±12.69[a]	368.62±10.64[a]	374.27±15.13[a]
Lipase (U/mg protein)	392.72±9.07[c]	399.16±9.22[c]	551.45±15.92[a]	500.98±11.57[b]	467.19±16.18[b]
Lysozyme activity (U ml^{-1} min^{-1})	58.02±2.19[c]	70.1±2.11[b]	134.34±5.41[a]	122.27±3.36[a]	125.78±4.75[a]
MPO (OD at 450)	0.1±0.01[c]	0.28±0.04[c]	1.28±0.03[a]	0.73±0.14[b]	0.55±0.04[b]
SOD (U/mg protein)	5.01±0.17[d]	6.54±0.11[c]	9.47±0.49[a]	8.12±0.52[b]	8.75±0.35[ab]
Catalase (U/mg protein)	2.25±0.12[c]	3.85±0.09[b]	5.09±0.44[a]	4.94±0.37[a]	4.59±0.19[ab]

Values in the same row with different letters are significantly different (P<.05).

found between CN5 and the control group in amylase and lipase activity. Ju *et al.* (2008) reported that bioflocs could supply adequate cellular nutrition, in addition to a variety of bioactive substances and may even contain certain yet-to-be-discovered constituents. Further, bioflocs include a variety of live microorganisms that may interfere with the resident gut microflora (Huang *et al.*, 2020; Wang *et al.*, 2015). Research has indicated that BFT can enhance the activity of digestive enzymes in aquatic animals (Liu *et al.*, 2018; Long *et al.*, 2015). Fish treated with CN ratios higher than 10:1 had enhanced digestive enzyme activity in their intestines tissue compared to the control in the current study. This might be because of BFT with floc developed using different C/N ratios can alter the balanced microbial flora in the intestinal tract when ingested. This alteration can induce the production of digestive enzymes or influence the existing enzymes' activity. BFT system contains unicellular algae, plankton, bacteria and other microorganisms. Consequently, consuming flocs developed with varying C/N ratios might have significantly increased the activity of digestive enzymes in the intestine. The change in the activity of digestive enzymes might be attributed to the consumption of flocs developed using different C/N ratios. When ingested, these flocs can disrupt the balanced microbial flora in the intestinal tract. This disruption can either induce the production of digestive enzymes or influence the activity of existing ones. In the BFT system, there are unicellular algae, plankton, bacteria and various other microorganisms. Therefore, consuming flocs developed with varying C/N ratios could have led to a substantial increase in digestive enzyme activity in the intestine. This increase in digestive enzyme activity might have led to enhanced digestive activity and contributed to the growth of *H. pulchellus*.

Furthermore, the activities of catalase and SOD in the gills were higher significantly in the CN10, CN15 and CN20 groups compared to the control. The first line of defence against harmful reactive oxygen species (ROS) in the body involves the secretion of antioxidant enzymes by host cells (dos Reis Goes *et al.*, 2019). The enhanced antioxidant activity (SOD and CAT) in the gill tissue of CN10, CN15 and CN20 groups in our study indicate that the BFT system can reduce lipid peroxidation levels. This positive effect significantly contributed to the overall health of the fish and improving their survival rates. Moreover, biofloc contains a diverse natural microorganisms and bioactive growth factors, which play a crucial role in enhancing the stress response (Liu *et al.*, 2017). It indicates that BFT can have a positive effect on the health of *H. pulchellus* by reducing lipid peroxidation levels and effectively combating oxygen free radicals.

Similarly, the immunological parameters (lysozyme activity and MPO) increased with carbon source concentration, with CN10 showing the highest values. However, no significant variations among the CN10, CN15 and CN20 treatments in terms of lysozyme activity and between CN5 and the control group in MPO activity (P>0.05). This phenomenon can be attributed to the presence of numerous probiotics and bioactive substances in BFT. When consumed by fish, BFT leads to an increase in immune enzyme activity, benefiting the host through secondary metabolites (Defoirdt *et al.*, 2007). Additionally, biofloc may contains various microorganisms whose cellular components or metabolites act as immune stimulants, enhancing the innate immune system. Lysozyme is an enzyme that hydrolyses bacterial cell walls and is involved in the immune response by recognizing and destroying foreign bodies (Saurabh and Sahoo, 2008). Lysozyme and myeloperoxidase (MPO) activities were enhanced in the CN treatment group in the current study, with the highest values observed in the CN10 group than in control. It can be attributed to the presence of probiotics in the bioflocs (Khanjani *et al.*, 2022). The elevated levels of Lysozyme and myeloperoxidase (MPO) activities in the groups with different C/N ratios indicate an enhancement in immune factors. As a result of these enhanced immune responses, the fish are better protected against potential pathogen infections and environmental stressors.

CONCLUSION

The current study presents the first information regarding the performance of critically endangered *H. pulchellus* in biofloc systems with various C/N ratios. Our findings indicate that a higher ratio of C/N from 10:1 to 20:1 in biofloc systems effectively reduces total ammonia nitrogen, nitrite and nitrate levels. Furthermore, the higher C/N ratio had a positive effect on digestive enzymes, immune system function and antioxidant activity, indicating that the C/N ratio of 10:1 to 20.1 are optimal for rearing *H. pulchellus* fingerlings from early fry, with 10:1 presenting the best growth results. Further research exploring the microbial diversity in different C/N ratios could provide a better understanding of its effect on the critically endangered *H. pulchellus* performance in biofloc systems.

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Ethics

All the necessary ethical considerations were taken into account during the experiment based on guidelines.

Conflict of interest

The authors declare that they have no conflict of interest.

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