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Preliminary Evaluation of Freshwater Pufferfish Supplementation on Growth Performance and Hematology Parameters in the Nile Tilapia (*Oreochromis niloticus*)

Khaled Youssef AbouelFadl[1], Mahmoud M. S. Farrag[2], Manar T. E. Tolba[1], Ragaa A. Ahmed[3]

[1] Faculty of Fish and Fisheries Technology, Aswan University, Egypt
[2]Department of Zoology, Faculty of Science, Al-Azahr University (Assuit Branch), Assuit, Egypt
[3]Aquaculture department, Faculty of Fish and Fisheries Technology, Aswan University, Egypt

[*]**Corresponding Author: ragaaahmed@aswu.edu.eg**

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ABSTRACT

The pufferfish, a freshwater *Tetraodon lineatus*, is often neglected in commercial fishing because of its venomous qualities and, yet, is highly nutritional. This particular study aimed to test the pufferfish muscle powder as an alternative dietary protein source in the feed of the Nile tilapia (*Oreochromis niloticus*), focusing on changes in growth performance and blood-related parameters. For this experiment, 1050 healthy Nile tilapia were taken, divided into five groups, and each group received different diets containing varying amounts of pufferfish powder (0, 25, 50, 75, and 100%) as a substitute to commercial fishmeal. The experiment was set to run for 90 days and during this period, multiple growth parameters like weight gain, specific growth rate (SGR), feed conversion ratio (FCR), feed efficacy (FE), and hematological parameters were tracked. The findings indicated that pufferfish powder inclusion levels above a certain threshold positively impacted growth performance, with the best results in final weight, SGR, FCR, and FE obtained from the 100% replacement diet. Furthermore, all fish health parameters monitored were within normal limits, suggesting the treatments did not have negative effects on fish health. Based on the results, it can be concluded that the muscle powder of freshwater pufferfish is an economically and ecologically attractive alternative protein source for the feed of the Nile tilapia, as it is feasible and sustainable. Additional research, however, is needed to evaluate the safety of the diet for humans.

INTRODUCTION

Dietary protein is a costly component in fish diets **(Halver & Hardy, 2002)**. Aquaculture represents the important sustainable option for enhancing fishery resources and rejuvenating ecosystems **(Okechi, 2004)**. The growing demand, high costs, inconsistent fish meal supply, and aquaculture expansion highlight the need for alternative protein sources **(Phumee *et al.*, 2011)**. Numerous studies have investigated substituting fish meal with other protein sources, such as plant-based proteins, for tilapia diets **(Nguyen & Davis, 2009)**. Rising costs and demand for conventional protein sources drive fish farmers in developing countries to explore alternative solutions **(Panigrahi *et al.,* 2014)**.

Discard is a significant problem in global fisheries with an estimated 7.3 million tons of discard generated annually worldwide **(Kelleher, 2005; Mukhopadhyay *et al.,* 2020)**. Discarded seafood, which is non-marketable, is released back into the sea (dead or alive) for various reasons (such as not meeting legal or market size, deformities, or visible parasites) **(Tsagarakis *et al.,* 2017)**. These discards cause the ecological and environmental pollution and lead to the loss of valuable components such as proteins, lipids, and minerals. However, this waste contains substantial nutrients and is widely used to produce low-value by-products such as fertilizers, animal feed ingredients, and fish meal or oil **(Özyurt *et al.,* 2019)**.

The inland fisheries of Lake Nasser and the River Nile are essential for fish production in Egypt, housing key species like tilapia and *Lates niloticus*, as well as by-catch species such as the pufferfish *Tetraodon lineatus*, which is illegal to fish or trade **(Farrag *et al.,* 2021)**. Consequently, the pufferfish population has surged, damaging fishing tools and catch. While many studies have explored various species in these waters **(Shalloof & El-Far, 2017)**, few have focused on *T. lineatus*, including its toxicological aspects **(Farrag *et al*., 2021)**. *T. lineatus* once made up 11.1% of Lake Nasser's annual fish production (2730 MT) (all discarded) **(El-Far *et al.,* 2020)**. The increased size and abundance of pufferfish particularly marine species have led to significant damage to fishing equipment, encouraging illegal species sales **(Farrag *et al.,* 2016)**. This behavior is also present in freshwater species. Pufferfish are well known for containing tetrodotoxin (TTX), a potent toxin in their liver, gall bladder, intestine, gonads, eggs, and skin. The TTX levels in pufferfish vary by species and season **(Barbier *et al.,* 2003; Farrag 2022)**. Toxicity tests on freshwater pufferfish from Lake Nasser revealed that most muscle samples were predominantly non-toxic (87.10%) **(Farrag *et al.,* 2021)**.

Studies have demonstrated that exposure to sub-lethal doses of TTX can lead to alterations in haematological indices such as red blood cell (RBC) count, hemoglobin concentration, and hematocrit levels **(Wibowo *et al.,* 2017; Santos *et al.,* 2020)**. These changes can affect fish's overall health and immune response, potentially leading to compromised growth performance and increased disease susceptibility. The present study aimed to determine the benefits of discarded fish, especially freshwater puffer fish, as a substitute in fish diet for the Nile tilapia, monitoring the growth and haematological parameters to ensure the benefits of protein supplementation in various percentages.

MATERIALS AND METHODS

This study was carried out in an agricultural greenhouse in the Faculty of Fish and Fisheries Technology at Aswan University, Egypt. A total of 1050 healthy live Nile tilapia (*Oreochromis Niloticus*) with an average initial body weight (3g) was obtained from the Central Laboratory for Aquaculture Research – Aswan, Egypt. The fish were kept in a rectangular tank, ensuring the optimum water quality, and the system was thermostatically controlled at 23 ± 2°C. The study was conducted in fifteen rectangular tanks (500 L each) filled with 400L of de-chlorinated water. Fishes were acclimatized for two weeks before the start of the experiment. They were divided into five equal groups, including control in three replicates each (70 fish/replicate). Fish were weighed at the beginning of the experiment and then biweekly for 90-day experimental period.

Diets were formulated from commercial ingredients, the study procured various feed materials, including a commercial fish meal, soya bean, wheat flour, wheat bran, yellow corn, fish oil, gelatin, ascorbic acid, vitamin, and mineral mix.

Fresh pufferfish discards were collected from the commercial landing site of Lake Nasser. The fish were cleaned, the skin, viscera, and hard bones were removed. The samples of muscles were oven-dried at 60ºC for 24 hours; then, they were ground in a grinder to turn into powder. The powder was packed and maintained in a fridge (3-4ºC) The fish were fed five treatments (five experimental diets). For these five experiments, five fish diets were prepared and supplemented with the pufferfish muscles (poweder) in different percentages. The percentage of the main protein source of the pufferfish was utilized according to **Farrag *et al.* (2021)** with average muscles **(**16.91%) in different seasons.

This involved partial or total replacement of commercially used fish meal with discarded freshwater pufferfish/ Fahka as follows. The first diet only consisted of commercial fish meal (0% Fahka Powder (FP). The second diet replaced 25% of the commercial fish meal with Fahka Powder (FP). For the third diet, 50% of the commercial fish meal was substituted for Fahka Powder (FP). In the fourth diet, 75% of the commercial fish meal was replaced with Fahka Powder (FP). The commercial fish meal was completely replaced with FP for each of the five alternative main protein sources for the fifth diet. They were mechanically mixed with commercial fish meal and Puffer fish, and water was added until the desired texture was achieved. Pellets were formed using a pelleting machine and air-dried to minimize moisture. The formulation of the present research diets is presented in Table (1), including the commercial fish meal weight, discarded fish, and other common ingredients.

Fish feeding was carried out six days/week, three times daily; all fish were fed with 5% of their body weight at 9:00 AM, 12:00 PM, and 3:00 PM, six days a week.

Table 1. The composition of the experimental diets

	C	T1 (25%)	T2 (50%)	T3 (75%)	T4 (100%)
Wheat flour	23.00	23.00	23.00	23.00	23.00
Wheat bran	19.40	19.40	19.40	19.40	19.40
Fish meal	9.00	6.75	4.50	2.25	0.00
protein source	0.00	2.25	4.50	6.75	9.00
Soybean meal	25.00	25.00	25.00	25.00	25.00
Yellow corn	15.00	15.00	15.00	15.00	15.00
Fish oil	5.00	5.00	5.00	5.00	5.00
CMC	3.00	3.00	3.00	3.00	3.00
Vit. & Min.Mix	0.40	0.40	0.40	0.40	0.40
Ascorbic acid	0.20	0.20	0.20	0.20	0.20
TOTAL	100.00	100.00	100.00	100.00	100.00
CP%	24.9	25.3	25.8	26.2	26.6

All growth parameters were estimated every 15 days. The following equations were used to determine every growth parameter.

· Weight gain (WG, g) = FW – IW.

· Specific growth rate (SGR %) = log FW – log IW/ t * 100.

· Feed conversation ratio (FCR) = FI / WG (g).

· Condition factor (K) = W (g) * 100 / L^3 (cm).

· Survival rate (SR) = Number of live fishes / Total fish initial number * 100.

Where, IW is the initial fish weight (g); FW is the final fish weight (g); WG is the fish weight gain (g); FI is feed intake (g); t is the total days' number of experiments; W is the fish weight (g), and L is the fish length (cm) **(Tacon *et al.*, 2008)**.

For hematological parameters peripheral blood samples withdraw from caudal puncture according to **Ezzat *et al.* (1974)**. No anesthetic will be applied to fish as it may affect blood parameters and hemolysis tissues **(Hoffman, 1977)**. Blood samples of fish were freshly collected in glass tubes using anticoagulant (EDTA) and were stored in polystyrene cool until used for hematological analysis.

The whole blood was used for the estimation of hemoglobin concentration (Hb), hematocrit value (Hct), and red blood cell count (RBCs) by using an automated technical analyzer (Celltac α MEK- 6400J/K). Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cell count (WBCs) were calculated according to **Dacie and Lewis (2002)**. MCHC (g/dl) = Hb / Hct * 100, MCH (pg) = Hb / RBCs * 10, MCV (mm3) = Hct/RBCs * 10 **(Osman *et al.*, 2018)**.

Data were presented as mean±SD (Standard Deviation). The results were subjected to a one-way analysis of variance (ANOVA) to test the effect of treatment inclusion on fish performance. Data were analyzed using **SPSS (1997)** program, Version 16. Differences between means were compared using **Duncan's (1955)** multiple range test at $P< 0.01$ level.

RESULTS

Growth performance analysis

Data in Table (2) show that, the initial weight was significantly lower in treatments T2, T3, and T4 compared to the control group, with values of 3.27, 3.27, and 3.27, respectively, indicating a consistent starting point for the experiment.

In terms of final weight, treatments T3 and T4 demonstrated significantly higher results than the control and T1 groups, with T4 achieving the highest final weight of 14.73. This trend was mirrored in weight gain, where T4 showed a significant increase of 11.47, indicating superior growth performance. Growth in weight also followed a similar pattern, with T4 showing the highest growth of 351.99, significantly outperforming all other treatments. The specific growth rate (SGR) was the highest in T4 (1.88), reflecting the enhanced growth efficiency in this treatment. Total feed intake was significantly higher in T4 (16.13), suggesting increased consumption, contributing to the observed growth. Correspondingly, the feed conversion ratio (FCR) was significantly lower (better) in T4 (1.41). Feed efficiency (FE) was also at its highest level in T4 (0.71), corroborating the improved FCR and growth metrics. Importantly, the survival rate was consistently 100% across all treatments, indicating no adverse effects on fish health under the different experimental conditions. The condition factor (K) showed no significant differences across all treatments.

Table 2. Growth performance parameters of *Oreochromis niloticus* with different levels of Fahka powder

Parameters	C	T1 (25%)	T2 (50%)	T3 (75%)	T4 (100%)
Initial weight (g)	3.7±0.26^{b}	3.4±0.26^{ab}	3.27±0.21^{a}	3.27±0.9^{a}	3.27±0.67^{a}
Final weight (g)	10.27±0.51^{a}	10.3±1.02^{a}	11.53±0.70^{ab}	11.87±1.03^{b}	14.73±0.75^{c}
weight gain (g/fish)	6.57±0.55^{a}	6.9±1.04^{ab}	8.27±0.87^{bc}	8.6±0.82^{c}	11.47±0.74^{d}
Growth in weight (g)	178.33±22.5^{a}	204.2±38.39^{ab}	254.67±40.1^{bc}	262.92±8.3^{c}	351.99±32.19^{d}
SGR (% /day)	1.28±011^{a}	1.38±0.16a^{b}	1.58±0.15^{bc}	1.61±0.029^{c}	1.88±0.89^{d}
Total feed intake	12.16±0.53^{a}	11.76±1.14^{a}	12.95±0.42^{ab}	13.77±0.84^{b}	16.13±0.76^{c}
FCR	1.86±0.2^{c}	1.72±19^{bc}	1.58±0.12^{ab}	1.6±0.054^{ab}	1.41±0.04^{a}
Feed efficiency (FE)	0.54±0.059^{a}	0.59±0.064^{ab}	0.64±0.049^{ab}	0.62±0.021^{bc}	0.71±0.02^{c}
K	1.18±0.19^{a}	1.20±0.074^{a}	1.15±0.29^{a}	1.08±0.093^{a}	1.3±0.21^{a}
Survival rate (%)	100%^{a}	100%^{a}	100%^{a}	100%^{a}	100%^{a}

Means in the same row with different superscript letters are significantly different (P<0.05).

Hematological analysis

Data in Table (3) show the hematological parameters assessed in this study across different treatment groups (C, 25, 50, 75, and 100%) exhibiting both consistency and variation. Hemoglobin (HB) levels remain stable, with values ranging from 11.65 to 11.75, indicating no significant differences among the groups. Similarly, red blood cells (RBCs) counts show minor fluctuations within a narrow range of 1.70 to 1.77, suggesting consistent erythropoiesis across treatments. Hematocrit (HCT) values exhibit slight variability, with the highest value of 27.25% observed in the 75% group and the lowest of 24.75% in the 25% group. Mean corpuscular volume (MCV) shows slight variability, ranging from 139.5 to 152, reflecting minor changes in erythrocyte size. Mean corpuscular hemoglobin (MCH) values range from 66.62 to 71.9, and mean corpuscular hemoglobin concentration (MCHC) values range from 46.55 to 50.4, both indicating no significant treatment effects on hemoglobin content and concentration within erythrocytes.

White blood cells (WBCs) counts vary slightly across groups, with values from 59 to 60.65, suggesting stable leukocyte production. Lymphocytes (Lym) counts remain consistent, ranging from 54.25 to 55.5, while monocytes (MO) show slight variations with values from 15.5 to 19.25, indicating a potential but non-significant treatment effect. Neutrophils (Net) counts range from 24.25 to 28.5, showing moderate variability, while eosinophils (Eso) remain constant, ranging from 1.5 to 1.75. Platelet (PLT) counts vary slightly from 129.75 to 159.25.

Table 3. Hematological parameters of *Oreochromis niloticus* with different levels of Fahka powder

Parameter	C	T1 (25%)	T2 (50%)	T3 (75%)	T4 (100%)
HB (g/dL)	11.75±0.89^{a}	11.72±0.79^{a}	11.67±0.65^{a}	11.65±0.95^{a}	11.69±1.11^{a}
RBCs(106 cell/mL)	1.7±0.37^{a}	1.75±0.52^{a}	1.77±0.26^{a}	1.77±0.44^{a}	1.75±0.5^{a}
HCT (%)	25.75±5.20^{a}	24.75±5.12^{a}	25.25±3.09^{a}	27.25±3.09^{a}	25.5±6.02^{a}
MCV	139.5±19.43^{a}	148.5±15.71^{a}	145.75±8.09^{a}	148.75±16.87^{a}	152±10.55^{a}
MCH	70.85±11.04^{a}	70.12±13.86^{a}	66.62±7.91^{a}	71.9±9.03^{a}	70.25±11.44^{a}
MCHC	50.4±3.80^{a}	47.25±7.36^{a}	46.55±3.91^{a}	48.15±5^{a}	46.9±5.95^{a}
WBCs(103cell/mL)	60.65±5.28^{a}	59.75±4.11^{a}	59±8.08 ^{a}	60.25±6.80^{a}	60.6±12.75^{a}
Lym%	54.25±4.34^{a}	55.25±8.53^{a}	55.5±5.25^{a}	54.75±4.64^{a}	54.75±8.05^{a}
MO%	15.5±2.08^{a}	15.75±3.09^{a}	16.5±3.41^{a}	18.5±3.10^{a}	19.25±1.70^{a}
Net%	28.5±2.64^{a}	25.25±6.84^{a}	26.5±2.64^{a}	25.25±2.21^{a}	24.25±5.73^{a}
Eso%	1.75±0.5^{a}	1.5±0.57^{a}	1.5±0.57^{a}	1.5±0.57^{a}	1.75±0.95^{a}
PLAT(103 cell/mL)	159.25±13.22^{a}	129.75±18.83^{a}	130.75±17.91^{a}	130.5±27.40^{a}	132±25.15^{a}

Means in the same row with different superscript letters are significantly different (P<0.05).

WBC's= White blood cells, RBC's= Red blood cells, HB= Hemoglobin, Lym = Lymphocytes, MO= Monocytes, Net = Neutrophils, Eso = Eosinophils.

DISCUSSION

In recent years, research has increasingly focused on the effects of using fish as feed beyond the aquaculture industry. This has brought attention to the impacts of aquaculture on solving the problem of poverty and nutrition, while some studies indicate that discards are influential **(Catchpole *et al.,* 2006)**, others suggest they have limited effects **(Lejeune *et al.,* 2022)**.

Among the discard fish species found in large quantities is the freshwater pufferfish, which has garnered significant attention from researchers, particularly in studies related to nutritional values and toxicity **(Farrag *et al.,* 2022)**. Due to its toxicity, there is considerable controversy about the use of the pufferfish as human food, despite the fact that its high protein content has potential in the fish food industry.

This study focused on the potential using freshwater pufferfish as a supplemented material in fish meal to add value to this species and to utilize it in addressing the protein crisis in fish feed. The present study studied growth performance of the Nile tilapia based on supplemented pufferfish muscles in different regimes, which found that dietary protein sources significantly affect growth performance. Replacing commercial fish meal with 50, 75, and 100% pufferfish protein (FP) diets led to increased final growth. These findings align with other studies, such as that of **Hardy *et al.* (2005)**, who reported that fish fed by-catch meals gained more weight than those fed commercial fish meal diets.

Diets that include fish muscle protein, like by-catch meal, are better because they are easy to digest and contain essential amino acids that come from skeletal muscle, while processing wastes are mostly made up of frames or viscera **(Moon & Gatlin, 1994)**. However, **Babbitt (1990)** noted that some processing by-products, like Alaskan cod fillet waste, contain large quantities of connective tissue, which reduces their usefulness as protein sources due to decreased digestibility and a weaker amino acid profile. In Lake Nasser, this species is a very important discard fish **(El-far *et al.,* 2020; Farrag *et al.,* 2022)**, and also was investigated as nutritional value by **Farrag *et al.* (2022)**.

The feed conversion ratio (FCR) measures the efficiency of converting feed material into body mass. In this study, fish fed a 100% FP diet had the best FCR and the highest K value. This aligns with findings for Atlantic salmon, where diets high in indigestible substances like ash and chitin led to worse FCRs **(Olsen *et al.,* 2006)**. **Olsen *et al.* (2006)** observed similar results when they substituted fish meal with Antarctic krill at various concentrations. Increasing the concentration of puffer fish in the diet improved the K factor, indicating better growth performance without compromising health, as evidenced by normal survival rates across all groups.

Comparative studies, such as that of **Hardy *et al.* (2005)** on the red drum fish and the rainbow trout, support these findings. Blood biochemical indices are crucial for assessing fish health after feeding trials. In this study, blood glucose levels, used as markers of environmental stress, remained within normal ranges, indicating that fish fed with discards maintained normal growth and health status.

Madsen *et al.* (2022) support the belief that discards significantly waste resources and negatively affect the financial sustainability of fisheries, marine biological resources and ecosystems, which in agreement with this study results.

In the present study RBC counts stayed normal at all puffer fish concentrations. These levels are similar to those found for *Prochilodus lineatus* **(Ranzani-Paiva *et al.,* 2000)** and *Schizodon borellii*. However, it is a little lower than those found for Florida red tilapia and *Piaractus mesopotamicus* **(Tavares-Dias *et al.,* 1999)**. The levels of hemoglobin are about the same as those found in *S. borellii* **(Tavares-Dias *et al.,* 1999)** and the hybrid tambacu **(Tavares-Dias *et al.,* 2000)**, but higher than those found in Florida red tilapia **(Tavares-Dias *et al.,* 2000)**. These negligible alterations in RBCs and Hb suggest that the discard diet did not negatively impact *O. niloticus* health.

The levels of hematocrit are lower than those found in *P. lineatus* **(Ranzani-Paiva *et al.,* 2000)** and the hybrid tambacu **(Tavares-Dias *et al.,* 2000b)**. While, MCV values are close to those recorded by **Bittencourt *et al.* (2003)** for the Nile tilapia. However, they are higher than those recorded for *L. macrocephalus* **(Tavares-Dias *et al.,* 1999)** and *P. lineatus* **(Ranzani-Paiva *et al.,* 2000)**. On the other hand, WBC counts are consistent with those found for *O. mossambicus* **(Doggett *et al.,* 1987)** and *Oreochromis hybrid* **(Hrubec *et al.,* 1997)**. All blood parameters indicated no adverse health effects, even at high replacement levels.

Generally, the growth performance showed an increase in growth with the increase of the puffer fish powder concentration showing normal healthy status with no mortality rates , indicating preliminary positive results of the discard freshwater pufferfish in fish meal replacement .

CONCLUSION

This study underscores the potential of utilizing fresh water pufferfish discards as a viable dietary protein source for the Nile tilapia enhancing growth performance. All evaluated blood parameters confirmed that tilapia health remained unaffected by puffer fish supplementation. The findings validate the feasibility of incorporating pufferfish discards into tilapia feed, offering a dual benefit: maximizing the nutritional value of these discards and reducing feeding costs. We recommend conducting further research to confirm the absence of any adverse effects of this type of feed on the health and growth of fish, thereby contributing to scientific knowledge and ensuring the sustainability of aquaculture.

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