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Replacement of fishmeal with Quinoa Husk (*Chenopodium quinoa*) for mitigating multiple stresses in *Pangasianodon Hypophthalmus*

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The fishmeal is boon for aquaculture production in this recent pollution and climate change era. However, the demand of fishmeal is enhancing in many folds which needs to find alternative to fishmeal in cheap price. The present investigation addresses these issues with quinoa husk (QH). An experiment was performed to evaluate replacement of fishmeal by QH in different proportionate at 0, 15, 20, 25, 30 and 35%. The study was designed with 12 treatments as control, stressors group (concurrent exposed to ammonia, arsenic and high temperature stress, NH_3+As+T), group fed with QH at 15, 20, 25, 30 and 35% without and with stressors (NH_3+As+T) in *Pangasianodon hypophthalmus* for 105 days. The optimization of QH dose for growth performance such as food conversion ratio, growth rate, protein efficiency ratio and specific growth rate with respect to protein percentage and obtained 26%. The oxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione-s-transferase (GST) and glutathione peroxidase (GPx) in gill, kidney and liver tissues were significantly lowered by replacement of fishmeal by QH at 25% in fish reared under arsenic and ammonia toxicity and high temperature stress (NH_3+As+T). The neurotransmitter enzyme (AChE) in brain tissue was noticeably enhanced by QH at 25%. The aspartate amino transferase (AST) and alanine amino transferase (ALT) as well as malate dehydrogenase (MDH) and lactate dehydrogenase (LDH) in gill and liver were significantly reduced by QH at 25% in fish reread under multiple stresses (NH_3+As+T). The nitro blue tetrazolium (NBT), blood glucose, albumin, globulin, total protein, A:G ratio, myeloperoxidase (MPO) and total immunoglobulin (Ig) were noticeably improved by supplementation of QH at 25–30% in fish reared under NH_3+As+T. The amylase, protease and lipase were significant improved with replacement of fishmeal by QH at 25%. The histo-pathological alterations were marked in liver and gill tissues, whereas these tissues were protected by QH at 25% in fish reared under control and stress condition (NH_3+As+T). The present study revealed that replacement of fishmeal at 25% by QH could be a better replacement for improvement in anti-oxidative status, acetylcholine esterase and growth performance in fish reread under NH_3+As+T stress.

Keywords Quinoa husk, Anti-oxidative status, Metabolic enzymes, Histopathology, Stress

Aquaculture is one of the world's fastest-growing food sectors, with total production projected to reach 202 million tons by 2030[1]. Over 70% of the aquaculture sector relies on feed-based systems, which require high levels of animal protein. Traditionally, the sector relied on fishmeal and fish oil generated from wild-caught forage fish, which is unsustainable, with an extra 38 million tons required by 2025[2]. This has promoted scientists to explore alternative sources of fishmeal that do not compromise aquaculture production. Plant-based protein ingredients have emerged as a promising solution to fill this gap[3]. Among these alternatives, quinoa (*Chenopodium quinoa*) stands out as an excellent candidate for providing a cost-effective and complete protein source for aquatic organisms. Our previous research on quinoa husk demonstrated its potential to replace fishmeal while supporting better growth performance in fish[4]. Notably, quinoa was designated the "International Year of Quinoa" in 2013 due to its high amounts of unsaturated fatty acids and large protein content[5]. Quinoa

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is highly nutritious, containing protein (11–19%), fat (5–9%), and complex carbohydrates such as starch and dietary fiber (46–77%)[6]. It is rich in vitamins and minerals, including magnesium, iron, potassium, phosphorus, and zinc, as well as vitamins B complex such as niacin (B_3), folate (B_9), and riboflavin (B_2)[7]. Additionally, it is a good source of antioxidants, including phenolic and flavonoid compounds, which can help protect against oxidative damage[8]. Quinoa is gluten-free, rich in essential amino acids, and has a low glycemic index[9]. It is also considered a functional food, known for its antioxidant, anti-inflammatory, and antimicrobial properties[10,11].

Moreover, in the current scenario, unexpected temperature variations, climate change, excessive rainfall, drought conditions, water pollution, and various other abiotic factors are increasingly prominent[12,13]. These conditions are significantly impacting ecosystems and aquatic organisms. Moreover, rising temperatures are contributing to escalating levels of contamination, particularly arsenic contamination, which is prevalent in many Asian countries, including India, Bangladesh, China, and Nepal. In Bangladesh alone, nearly 200 million people are at high risk, with 43,000 fatalities annually due to arsenic contamination[14,15]. The International Agency for Research on Cancer (IARC) classifies arsenic as a Group I carcinogen[16]. Arsenic is widely used in various industries, including metal alloy manufacturing, pharmaceuticals, agriculture, veterinary medicine, wood preservatives, glassware production, and microelectronics[17,18]. The toxicity of contaminants is exacerbated when it, combined with other factors, such as ammonia (NH_3) and high temperatures. The combination of arsenic and ammonia, particularly under high-temperature conditions, significantly increases toxicity[19]. Ammonia is particularly harmful to aquatic organisms, as even low concentrations in fish ponds can lead to mass mortality. It is predominantly derived from high-protein meals, fish waste, and aquatic creature metabolism[20,21]. The toxicity of NH_3 results in reduced growth performance, weakened immunity, neurotoxicity, oxidative stress, tissue erosion, and increased mortality in fish[5]. As poikilothermic animals, fish have body temperatures that match the surrounding water, making them especially vulnerable to changes in water temperature, which can affect their physiology, metabolism, and behavior[22]. Additionally, the synthesis and release of stress hormones in fish are likely influenced by temperature and other stress factors[23,24].

Interestingly, the current study looked at the effects of replacing fishmeal with QH on growth performance, antioxidative status, cellular and metabolic stress, and tissue histopathology in fish reared under ammonia and arsenic toxicity, as well as high-temperature stress. Growth performance, antioxidative status, cellular metabolic stress, and tissue histopathology are reliable indicators for assessing the efficacy of nutrients against stressors[25–27]. Stressors have a significant impact on oxidative stress enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST) and glutathione peroxidase (GPx), which are responsible for the excessive synthesis of reactive oxygen species (ROS) in cells.

Pangasianodon hypophthalmus is a suitable fish species for feed ingredient evaluation and culture in abiotic and biotically stressed environments[25–27]. This species is known for its high demand, sturdiness, adaptability, high growth rate, and medicinal value. It is highly suitable for intensive aquaculture, with global production reaching 2,520.4 thousand tonnes in 2020[1]. The present investigation focused on two main objectives: To standardize the dosage of QH for replacing fishmeal in fish reared under multiple stresses (NH_3+As+T) and to determine the efficacy of QH in alleviating numerous stresses in fish.

Materials and methods

Animal care and maintainace

The healthy *P. hypophthalmus* were collected from the National Institute of Abiotic Stress Management's farm pond. The fish weighed 7.33 g and measured 4.5 cm in length. The fish were acclimatized for 15 days before commencing the experiment in the rectangular plastic tanks (capacity: 100 L). The experiment was designed with 12 treatments as (1) Control (Ctr), (2) Stressors group (concurrent exposed to ammonia and arsenic toxicity as well as high temperature stress, NH_3+As+T), (3) Group fed with QH at 15%, (4) Group fed with QH at 20%, (5) Group fed with QH at 25%, (6) Group fed with QH at 30% and (7) Group fed with QH at 35% and 8–12 treatments were fed with 15, 20, 25, 30 and 35% with stressors (NH_3+As+T). The details of the treatments are summarised in Table 1. The each treatment was used in triplicate with 15 fish in each tank. The total 540 fish were used for this experiment. The feeding was performed daily at 8:30 AM and 5:00 PM. APHA[28] method was used

S. No	Details of the treatments	Notation
1	Control	Ctr
2	Fed with control diet and concurrent exposure to ammonia, arsenic and high temperature	NH_3+As+T
3	Fed with quinoa husk (QH) at 15% kg^{-1} diet	QH at 15% kg^{-1} diet
4	Fed with quinoa husk (QH) at 20% kg^{-1} diet	QH at 20% kg^{-1} diet
5	Fed with quinoa husk (QH) at 25% kg^{-1} diet	QH at 25% kg^{-1} diet
6	Fed with quinoa husk (QH) at 30% kg^{-1} diet	QH at 30% kg^{-1} diet
7	Fed with quinoa husk (QH) at 35% kg^{-1} diet	QH at 35% kg^{-1} diet
8	Fed with quinoa husk (QH) at 15% kg^{-1} diet and concurrent exposure to ammonia, arsenic and high temperature	QH at 15% kg^{-1} diet+NH_3+As+T
9	Fed with quinoa husk (QH) at 20% kg^{-1} diet and concurrent exposure to ammonia, arsenic and high temperature	QH at 20% kg^{-1} diet+NH_3+As+T
10	Fed with quinoa husk (QH) at 25% kg^{-1} diet and concurrent exposure to ammonia, arsenic and high temperature	QH at 25% kg^{-1} diet+NH_3+As+T
11	Fed with quinoa husk (QH) at 30% kg^{-1} diet and concurrent exposure to ammonia, arsenic and high temperature	QH at 30% kg^{-1} diet+NH_3+As+T
12	Fed with quinoa husk (QH) at 35% kg^{-1} diet and concurrent exposure to ammonia, arsenic and high temperature	QH at 35% kg^{-1} diet+NH_3+As+T

Table 1. Details of experimental design and treatments.

to check the water quality periodically which was well within the recommended level of this species[29]. The QH (QH) was obtained from ICAR-NIASM with School of Drought Stress Management (SDSM). Sodium arsenite ($NaAsO_2$) and $(NH_4)_2SO_4$ were added as sources of arsenic and ammonia toxicity (NH_3) respectively on every alternate day with exchange of two-thirds of the water. The dose was used for ammonium sulfate (1/10th of LC_{50} 2.0 mg L^{-1} of $(NH_4)_2SO_4$)[27] and arsenic (1/10th of LC_{50} 2.68 mg L^{-1} of arsenic)[30], as well as a high temperature of 34 °C, to induce the stress in this experiment. The aerator was used to provide oxygenation in throughout the experiment. The experimental diets (Iso-nitrogenous and iso-caloric) of QH were prepared. After harvesting of the quinoa crop, the trashing was performed to separate the seed coat and outer layer of the quinoa. The husk parts of quinoa were used for this experiment. The feed ingredients (Fishmeal, soybean meal, groundnut meal, what flour, cellulose) including quinoa was moist heat up to 80–100 °C for 30–40 min. The details of the ingredients were mentioned in the Table 2. Moreover, the proximate analysis of diets was also conducted as AOAC[31] and determined the gross energy based on Halver method[32] (Table 2).

Sample preparation for enzymatic analysis

After the experiment, the fish were dissected aseptically, and different tissues were collected. Before dissection, the fish were euthanized using 100 µg L^{-1} of clove oil. Tissues included the liver, gills, kidneys, intestines, and brain were collected. Then, using a tissue homogenizer (Omni Tissue Master Homogenizer, Kennesaw, GA) with a 0.25 M cooled sucrose solution and 1 mM EDTA, the dissected tissues were homogenized. After centrifuging the homogenized tissues at 6000 xg for 20 min at 4 °C, the supernatants were kept cold, at -80 °C. Using the Lowry protein assay[33], the protein content of the liver, gills, kidneys, intestine, and brain was ascertained.

Antioxidant enzyme activities

The Catalase assay (EC 1.11.1.6) was conducted utilizing Takahara et al.[34]. In summary, the reaction mixture was composed of 2.45 mL of 50 mM phosphate buffer (pH 7), 50 µL of tissue homogenate, and 1.0 mL of newly made H_2O_2 substrate solution. Following thorough mixing, the absorbance drop was tracked for three minutes at 240 nm. Superoxide Dismutase (SOD) assay (EC 1.15.1.1) was performed in accordance with Misra and Fridovich's[35]. 50 µL of tissue homogenate, 1.5 mL of phosphate buffer, and 0.5 mL of newly made epinephrine were all included in the reaction mixture. A UV spectrophotometer was used to detect the absorbance at 480 nm over a three-minute period following full mixing. Using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate, the Glutathione S-transferase (GST) assay (EC 2.5.1.18) was ascertained spectrophotometrically in accordance with the protocol outlined by Habig et al.[36]. The foundation of this technique is the adduct that forms between CDNB and glutathione (GSH), more precisely S-2,4-dinitrophenyl glutathione. The rise in absorbance at 340 nm

Ingredients	Q-0	Q-15	Q-20	Q-25	Q-30	Q-35
Fish Meal	30	25.5	24	22.5	21	19.5
Groundnut meal	10	10	10	10	10	10
Soyabean	10	10	10	10	10	10
Wheatflour	18	18	18	18	18	18
Quinoa husk	0	12.3	16.4	20.5	24.55	28.64
Veg oil	3	3	3	3	3	3
Cod liver oil	2	2	2	2	2	2
Vit-mineral mixture	2	2	2	2	2	2
Cellulose	23	15.2	12.6	10	7.45	4.86
Lecithin	2	2	2	2	2	2
Total	100	100	100	100	100	100
Proximate composition of the diets						
Crude protein (CP)	34.45 ± 0.27	35.20 ± 0.06	34.82 ± 0.02	35.21 ± 0.01	35.19 ± 0.05	34.97 ± 0.15
Ether extract (EE)	9.13 ± 0.06	9.24 ± 0.04	9.23 ± 0.04	9.27 ± 0.05	9.43 ± 0.03	9.59 ± 0.11
Total carbohydrate (TC)	39.80 ± 0.37	37.43 ± 0.38	35.90 ± 1.33	37.44 ± 0.06	37.87 ± 0.13	38.02 ± 0.17
Organic matter (OM)	90.75 ± 0.04	90.19 ± 0.49	89.61 ± 0.74	90.19 ± 0.24	90.72 ± 0.08	90.69 ± 0.03
Dry matter (DM)	60.20 ± 0.11	62.57 ± 0.18	64.10 ± 1.33	62.56 ± 0.08	62.13 ± 0.18	61.98 ± 0.27
Digestible energy (DE)	360.93 ± 1.07	355.19 ± 1.71	357.48 ± 2.74	355.47 ± 0.62	358.22 ± 2.74	359.11 ± 1.94
Saponin (gram/100 gram)	0.75	0.74	0.65	0.71	0.63	0.94

Table 2. Ingredient composition and proximate analysis of experimental diets (% dry matter) of quinoa husk (QH), diets fed to *Pangasianodon hypophthalmus* during the experimental period of 105 days. Data expressed as mean ± SE, $n = 3$. Digestible energy (DE) (Kcal/100 g) = (% CP × 4) + (% EE × 9) + (TC × 4). *Manual prepared Vitamin mineral mixture; Composition of vitamin mineral mix (quantity/250 g starch powder): vitamin A 55,00,00 IU; vitamin D3 11,00,00 IU; vitamin B1:20 mg; vitamin E 75 mg; vitamin K 1,00 mg; vitamin B12 0.6 mcg; calcium pantothenate 2,50 mg; nicotinamide 1000 mg; pyridoxine: 100 mg; Zn 500 mg; I 1,00 mg; Fe 750 mg; Cu 200 mg; Co 45 mg; Ca 50 g; P 30 g; Se: 2 ppm. [a]Procured from local market, [b]Himedia Ltd, Himedia Ltd, [c]SD Fine Chemicals Ltd., India.

is measured in comparison to a blank. Glutathione Peroxidase (GPx) assay (EC 1.11.1.9) was carried out using Paglia and Valentine's[37] methodology.

Neurotransmitter enzyme activities

A modified Hestrin method by Augustinsson[38] was used to test the activity of acetylcholinesterase (EC 3.1.1.7). In a nutshell, the assay mixture included 0.2 mL of the sample, 1 mL of cholinergic buffer, and 1 mL of phosphate buffer. For thirty minutes, the mixture was incubated at 37 °C. After the solution was incubated, alkaline hydroxylamine, HCl, and ferric chloride were added. The absorbance at 540 nm was used to measure the reaction that resulted.

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and malate dehydrogenase (MDH)

Using Wootton's[39] approach, the activities of ALT (E.C.2.6.1.2) and AST (E.C.2.6.1.1) were ascertained. In short, 1 N sodium hydroxide, dipotassium hydrogen phosphate, potassium dihydrogen phosphate, and 2,4-dinitrophenylhydrazine (DNPH) were made. DL-alanine and α-ketoglutarate were the substrates for the ALT assay, whilst DL-aspartic acid and α-ketoglutarate were utilized in the AST assay. The substrate and tissue homogenate were combined, and the mixture was then incubated for an hour at 37 °C. After the incubation period, DNPH was added, and the absorbance at 540 nm was calculated. Lastly, the solution was mixed with 0.4 N NaOH. Using the technique developed by Wroblewski and Ladue[40], LDH activity was measured. To put it briefly, 0.1 M sodium dihydrogen phosphate and disodium hydrogen phosphate were added to a phosphate buffer to create a reaction mixture. Substrates such as sodium pyruvate and fresh NADH were then introduced. The sample or enzyme extract was combined with the reaction mixture, allowed to sit for 20 min, and then the absorbance at 320 nm was determined. Similarly, Ochoa's approach[41] was employed to determine MDH activity. With the exception of using oxaloacetate as the substrate rather than sodium pyruvate, the reaction mixture was the same as for LDH.

Respiratory burst activity, serum protein and A: G ratio

The Secombes method, modified by Stasiack and Baumann[42], was used to perform the respiratory burst activity assay. Using a protein estimation kit and the BCA method, total plasma protein was measured colorimetrically. The bromocresol green binding method, as reported by Doumas et al.[43], was utilized to test albumin. By deducting the albumin values from the total plasma protein, the amounts of globulin were calculated. The albumin levels were divided by the globulin values to get the albumin/globulin ratio (A/G ratio).

Blood glucose

An estimation of blood glucose level was performed using the Nelson[44] and Somoyogi[45] methods. After filtering the blood and deproteinizing it with zinc sulfate and barium hydroxide, the supernatant was used to measure the amount of glucose. At 540 nm, the absorbance was measured in relation to the blank.

Myeloperoxidase content and total immunoglobulin level

Myeloperoxidase was quantified using the Quade and Roth[46] method with some adjustments[47], and Anderson and Siwicki's[48] method was also used to quantify the total immunoglobulin level.

Digestive enzymes

Protease activity

Protease activity was established by applying Drapeau's[49] approach. In short, 1% casein was added to a 0.05 M Tris phosphate buffer, and the mixture was incubated for five minutes at 37 °C. Following the addition of the tissue enzyme homogenate to the reaction mixture, 10% TCA was added to stop the reaction. After filtering the mixture, the absorbance at 280 nm was calculated.

Amylase activity

The technique outlined by Rick and Stegbauer[50] was used to measure the activity of amylase. A phosphate buffer including sodium dihydrogen phosphate and disodium hydrogen phosphate was made, to put it briefly. The substrate in this case was starch solution. The tissue homogenate, starch solution, and phosphate buffer reaction mixture were incubated at 37 °C. Following the incubation period, 5 min of boiling water heating were applied to the solution before adding dinitro salicylic acid (DNS). Following a dilution with distilled water, the mixture's ultimate absorbance was measured at 540 nm.

Lipase

The activity of intestinal lipase (EC.3.1.1.3) was assayed using the method described by Cherry and Crandell Jr[51]. Two test tubes were labeled as the test (T) and control (C). Into each tube, 3 ml of distilled water and 1 ml of homogenate were added. The control tube (C) was placed in boiling water at 100 °C for 5 min to inactivate the lipase and then cooled. Subsequently, 0.5 ml of phosphate buffer solution (pH 7) and 2 ml of olive oil emulsion were added to both tubes, which were shaken thoroughly and incubated at 37 °C for 24 h. After incubation, 3 ml of 95% alcohol and 2 drops of phenolphthalein solution were added to each tube. The contents of each tube were then titrated with 0.05 N NaOH until a permanent pink color appeared.

Histopathology

The gill and liver tissues of the fish were fixed in neutral buffered formalin for 48 h. The tissues were then washed under running tap water and dehydrated in ascending grades of alcohol. Following dehydration, they were

cleared twice in xylene for 30 min each and embedded in paraffin for 45 min. Paraffin-embedded tissue blocks were prepared, and 5 µm thick sections were cut using a rotary microtome and collected on glass slides. The sections were dewaxed in xylene, rehydrated in a series of alcohol dilutions, and washed in tap water for 1 min. They were stained with hematoxylin for 12 min, rinsed in tap water, dipped in 1% acid alcohol, and washed again in tap water. The sections were then dehydrated through 50%, 70%, and 90% alcohol for 2 min each, stained with eosin for 4 min, and briefly dipped in absolute alcohol for 1 min. Finally, the stained sections were cleared in xylene for 5 min and mounted with DPX (Distrene di-n-butylphthalate-polystyrene-xylene). The prepared slides were examined under a microscope (Leica Microsystems Ltd., DM 2000, Heerbrugg, Switzerland) following the method described by Robert[52].

Statistics

Software called the Statistical Package for the Social Sciences (SPSS) version 16 was used to examine the data. The Shapiro-Wilk and Levene's tests were used to determine the homogeneity of variance and normality, respectively. The current study used one-way ANOVA (Analysis of Variance) and Duncan's multiple range tests. The data analysis significance level was established at $p < 0.05$.

Results

Optimization of replacement of fishmeal by QH for growth performance

In the present investigation, *Pangasianodon hypophthalmus* reared for 105 days under low doses of arsenic, ammonia, and high temperature stress (34 °C) were fed QH at levels of 15%, 20%, 25%, 30%, and 35% in place of fishmeal. To ascertain the ideal replacement level of fishmeal, growth performance measures including growth rate, feed conversion ratio (FCR), protein efficiency ratio (PER), and specific growth rate (SGR) were assessed. The results indicated that the highest growth performance was achieved with fishmeal replacement by QH at levels of 26.08%, 26.02%, 25.53%, and 25.70%. The detailed results are presented in Fig. 1A-D. Our previous study also reported on growth performance in *P. hypophthalmus* reared under multiple stressors (NH_3+As +T).

Effect of QH on oxidative stress in *P. Hypophthalmus*

Tables 3 and 4 show the oxidative stress parameters, which include CAT, SOD, GPx and GST that were measured in the liver, gills, and kidney of *P. hypophthalmus* reared under multiple stressors (NH_3+As+T). In comparison to control and QH-fed groups, catalase (CAT) activity was significantly increased in the liver ($p = 0.0014$), gill ($p = 0.023$), and kidney ($p = 0.0019$) during concurrent exposure to ammonia and arsenic toxicity, as well as high-temperature stress (34 °C). With 25% QH replacement, CAT activity in these tissues was considerably lower than in the control and all other groups, both with and without stressors (NH_3+As+T). Under stress, glutathione S-transferase (GST) activity was significantly higher in the kidney ($p = 0.0025$), liver ($p = 0.018$)

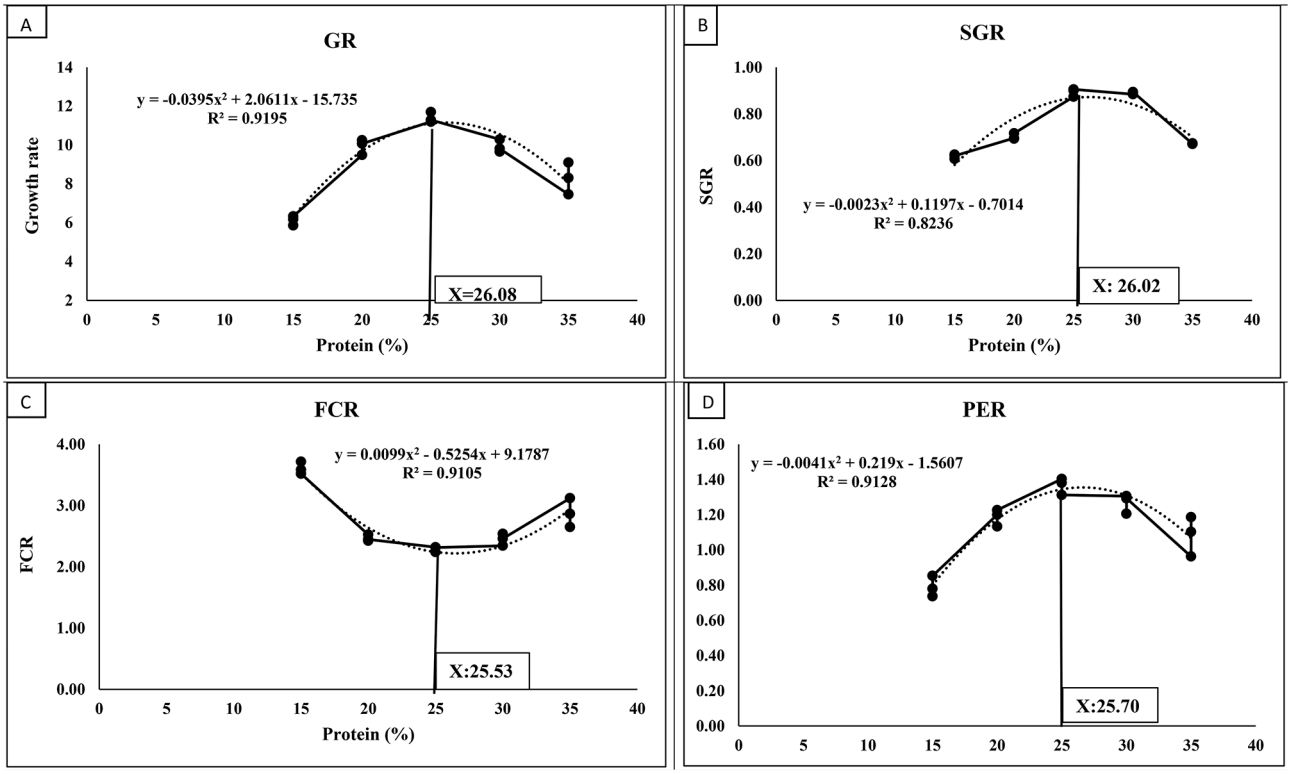

Fig. 1. (**A**–**D**) Optimization of replacement of fishmeal by quinoa husk (QH) for growth rate, SGR, FCR and PER of the fish reared under control and multiple stressors (As+NH_3+T).

Treatments	Superoxide dismutase			Catalase			Glutathione-S-Transferase		
	Liver	Gill	Kidney	Liver	Gill	Kidney	Liver	Gill	Kidney
Control	54.57 ± 1.93	44.11 ± 1.32	43.81 ± 1.31	20.91 ± 0.95^{bc}	34.23 ± 2.12^{d}	29.91 ± 0.88^{c}	0.35 ± 0.02^{b}	0.49 ± 0.03^{b}	0.42 ± 0.02^{c}
As+NH_3+T	56.50 ± 2.52	44.70 ± 2.05	44.56 ± 1.95	35.81 ± 1.40^{e}	52.93 ± 1.34^{f}	42.17 ± 1.4^{d}	0.55 ± 0.05^{e}	0.66 ± 0.04^{e}	0.60 ± 0.03^{f}
QH-15	58.29 ± 1.91	46.84 ± 0.96	45.19 ± 0.83	22.20 ± 0.80^{c}	52.10 ± 5.28^{f}	30.18 ± 2.57^{c}	0.37 ± 0.03^{b}	0.52 ± 0.03^{bc}	0.38 ± 0.02^{bc}
QH-20	54.42 ± 2.36	46.84 ± 1.18	42.97 ± 0.87	21.29 ± 1.24^{c}	49.60 ± 2.39^{e}	28.80 ± 2.59^{c}	0.36 ± 0.02^{b}	0.51 ± 0.03^{bc}	0.40 ± 0.05^{c}
QH-25	54.00 ± 0.57	45.17 ± 0.91	43.59 ± 0.59	13.58 ± 0.42^{a}	23.15 ± 1.65^{a}	20.92 ± 1.18^{a}	0.24 ± 0.01^{a}	0.29 ± 0.02^{a}	0.22 ± 0.03^{a}
QH-30	56.46 ± 2.12	44.39 ± 2.10	44.21 ± 2.03	20.30 ± 1.92^{bc}	31.16 ± 1.89^{c}	31.79 ± 2.4^{cd}	0.36 ± 0.02^{b}	0.62 ± 0.07^{d}	0.49 ± 0.02^{d}
QH-35	55.73 ± 1.39	44.10 ± 1.29	43.33 ± 1.23	28.11 ± 1.38^{d}	31.22 ± 2.12^{c}	30.01 ± 2.13^{c}	0.35 ± 0.03^{b}	0.53 ± 0.03^{c}	0.40 ± 0.02^{c}
QH-15+As+NH_3+T	57.04 ± 0.48	42.80 ± 1.31	43.38 ± 1.01	18.53 ± 1.41^{b}	31.72 ± 1.93^{c}	32.95 ± 1.4^{d}	0.36 ± 0.03^{b}	0.45 ± 0.04^{b}	0.50 ± 0.05^{d}
QH-20+As+NH_3+T	55.14 ± 3.28	44.64 ± 1.19	44.19 ± 1.1	25.42 ± 0.52^{cd}	33.18 ± 2.39^{d}	31.20 ± 1.78^{cd}	0.41 ± 0.02^{d}	0.49 ± 0.02^{b}	0.54 ± 0.01^{e}
QH-25+As+NH_3+T	55.38 ± 0.45	46.89 ± 1.18	46.63 ± 1.22	14.97 ± 1.15^{a}	24.75 ± 2.71^{a}	23.77 ± 2.34^{b}	0.22 ± 0.01^{a}	0.28 ± 0.01^{a}	0.35 ± 0.03^{b}
QH-30+As+NH_3+T	54.79 ± 1.53	44.58 ± 1.09	44.52 ± 1.16	22.80 ± 2.18^{c}	29.22 ± 2.15^{bc}	31.90 ± 1.55^{cd}	0.38 ± 0.02^{cd}	0.48 ± 0.05^{b}	0.55 ± 0.04^{e}
QH-35+As+NH_3+T	57.04 ± 0.69	44.10 ± 1.17	43.82 ± 1.10	24.29 ± 0.70^{cd}	28.78 ± 3.02^{b}	29.98 ± 1.52^{c}	0.39 ± 0.02^{cd}	0.52 ± 0.05^{bc}	0.48 ± 0.03^{d}
P-Value	0.077	0.092	0.085	0.0014	0.023	0.0019	0.018	0.0011	0.0025

Table 3. Effect of quinoa husk (QH) diets in superoxide dismutase (SOD), catalase (CAT), glutathione-S-Transferase (GST) and glutathione peroxidase (GPx) in liver, gill and kidney tissues of fish reared under control and multiple stresses (NH_3+As+T). Values in the same row with different superscript (a, b, c, d, e, f) differ significantly. CAT, SOD, and GST and GPx: Units/mg protein; Data expressed as Mean ± SE ($n = 6$).

and gill ($p = 0.0011$) than in the control and QH-fed groups. In comparison to the control and other groups, GST activity was dramatically reduced with 25% QH replacement, both with and without stresses. With 15%, 20%, 30%, and 35% QH replacement without stresses, the liver's GST activity was comparable to the control group. Under stress, the liver, kidney and gills showed considerably higher glutathione peroxidase (GPx) activity in comparison to the control and QH-fed groups. With 25% QH replacement, GPx activity was considerably decreased both in the presence and absence of stresses. Superoxide dismutase (SOD) activity, did not differ significantly among the group.

Effect of QH on Neurotransmitter enzymes in *P. Hypophthalmus*

Fish fed either a control diet or QH diets for 105 days were used to measure the activity of the neurotransmitter enzyme acetylcholinesterase (AChE) in the brain tissues of *P. hypophthalmus* under concurrent exposure to low dose ammonia and arsenic toxicity, as well as high temperature. Table 4 presents the findings. In brain tissue, exposure to low ammonia concentrations, arsenic toxicity, and high temperature (34 °C) dramatically reduced ($p = 0.0031$) AChE activity. Conversely, whether or not NH_3+As+T was a stressor, 25% QH replaced fishmeal and significantly increased AChE activity.

Effect of QH on alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes in *P. Hypophthalmus*

Table 5 presents the findings of ALT and AST activities in the gill and liver tissues of *P. hypophthalmus* that were reared for 105 days while being given either a control diet or diets containing QHs and concurrently exposed to low doses of ammonia and arsenic toxicity, high temperature stress. In comparison to the control and other groups, ALT activity in the gill ($p = 0.0018$) and liver ($p = 0.019$) tissues was considerably lower in the groups given 25% QH without stressors and 15% and 25% QH with stressors. On the other hand, groups fed the control diet and 35% QH diet and exposed to NH_3+As+T had significantly higher levels of ALT activity in the liver. When groups were fed a diet consisting 35% QH and the control diet, 15% and 20% QH, and were exposed to low doses of arsenic and ammonia toxicity as well as high temperature stress, the gill tissue showed a substantial increase in ALT activity ($p = 0.0018$). Interestingly, compared to the control and other groups, 25% QH diet dramatically decreased ALT activity in the gill tissue, with and without stresses. Similarly, when exposed concurrently to low doses of arsenic and ammonia toxicity, together with high temperature stress, AST activity in the liver ($p = 0.022$) and gill ($p = 0.016$) tissues was considerably enhanced in comparison to the control and other groups. In comparison to the control and other groups, the 25% QH diet dramatically decreased the AST activity in the liver and gill tissues, both with and without stresses.

Effect of QH on lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) enzymes in *P. Hypophthalmus*

The gill and liver tissues of fish reared under several stressors (NH_3+As+T) were used in the present experiment to determined LDH and MDH activities. The results are displayed in Table 6. When low doses of arsenic and ammonia toxicity were concurrently exposed to, LDH activities in the gill ($p = 0.0017$) and liver ($p = 0.0024$) tissues were considerably higher than in the control and other groups. When compared to the control and other groups, a 25% QH diet dramatically decreased LDH activities in both the liver and gill tissues, both with and without stresses. Additionally, a 25% QH diet significantly decreased MDH activities in the gill ($p = 0.0004$) ad liver ($p = 0.0009$) tissues when compared to the control and other groups, with and without stresses. But when given the control diet, MDH activities in the gill and liver tissues were markedly increased under concurrent

	Glutathione peroxidase			AChE
Treatments	**Liver**	**Gill**	**Kidney**	**Brain**
Control	2.02 ± 0.14^{c}	2.18 ± 0.13^{b}	2.14 ± 0.05^{c}	0.64 ± 0.02^{c}
As+NH_3+T	3.59 ± 0.38^{f}	3.29 ± 0.38^{f}	2.69 ± 0.26^{f}	0.35 ± 0.03^{a}
QH-15	1.82 ± 0.16^{b}	2.40 ± 0.17^{e}	2.10 ± 0.22^{c}	0.47 ± 0.05^{bc}
QH-20	3.05 ± 0.21^{e}	2.47 ± 0.25^{e}	2.42 ± 0.30^{e}	0.48 ± 0.04^{bc}
QH-25	0.94 ± 0.09^{a}	1.18 ± 0.13^{a}	1.20 ± 0.09^{a}	0.69 ± 0.06^{d}
QH-30	2.21 ± 0.35^{d}	2.42 ± 0.11^{d}	2.16 ± 0.24^{d}	0.49 ± 0.04^{bc}
QH-35	1.79 ± 0.26^{b}	2.54 ± 0.19^{d}	2.27 ± 0.21^{d}	0.43 ± 0.01^{b}
QH-15+As+NH_3+T	1.98 ± 0.11^{bc}	2.05 ± 0.12^{b}	1.89 ± 0.18^{b}	0.44 ± 0.03^{b}
QH-20+As+NH_3+T	2.07 ± 0.41^{c}	2.22 ± 0.51^{cd}	2.35 ± 0.43^{d}	0.67 ± 0.05^{d}
QH-25+As+NH_3+T	1.03 ± 0.04^{ab}	1.09 ± 0.06^{a}	1.22 ± 0.22^{a}	0.68 ± 0.03^{d}
QH-30+As+NH_3+T	1.98 ± 0.15^{bc}	2.18 ± 0.14^{b}	2.15 ± 0.30^{c}	0.66 ± 0.04^{cd}
QH-35+As+NH_3+T	2.13 ± 0.17^{c}	2.02 ± 0.09^{b}	2.02 ± 0.44^{c}	0.41 ± 0.03^{b}
P-Value	0.012	0.0023	0.0015	0.0031

Table 4. Effect of quinoa husk (QH) diets in glutathione peroxidase (GPx) in liver, gill and kidney tissues and acetylcholine esterase in brain of fish reared under control and multiple stresses (NH_3+As+T). Values in the same row with different superscript (a, b, c, d, e, f) differ significantly. GPx: Units/mg protein; AChE: nmole/min/mg protein; Data expressed as Mean ± SE ($n = 6$).

Treatments	Alanine Aminotransferase		Aspartate aminotransferase	
	Liver	Gill	Liver	Gill
Control	3.73 ± 0.38^{c}	3.05 ± 0.35^{b}	3.06 ± 0.38^{c}	5.64 ± 0.41^{d}
As+NH_3+T	10.20 ± 0.57^{g}	7.28 ± 0.73^{f}	7.13 ± 0.83^{g}	10.09 ± 0.52^{f}
QH-15	3.15 ± 0.24^{b}	4.10 ± 0.32^{c}	5.36 ± 0.40^{e}	6.58 ± 0.78^{e}
QH-20	4.14 ± 0.35^{d}	4.63 ± 0.54^{d}	3.06 ± 0.46^{c}	3.80 ± 0.35^{b}
QH-25	1.93 ± 0.24^{ab}	1.77 ± 0.29^{a}	1.63 ± 0.10^{a}	2.58 ± 0.31^{a}
QH-30	5.04 ± 0.77^{ef}	3.59 ± 0.57^{bc}	6.47 ± 0.92^{f}	5.31 ± 0.79^{d}
QH-35	4.82 ± 0.56^{e}	5.45 ± 0.49^{e}	6.95 ± 0.53^{f}	6.67 ± 0.66^{e}
QH-15+As+NH_3+T	1.98 ± 0.35^{ab}	5.56 ± 0.36^{e}	5.23 ± 0.68^{e}	5.38 ± 0.78^{d}
QH-20+As+NH_3+T	3.93 ± 0.41^{c}	5.65 ± 0.86^{e}	3.08 ± 0.44^{c}	4.11 ± 0.58^{c}
QH-25+As+NH_3+T	1.42 ± 0.08^{a}	1.71 ± 0.23^{a}	1.90 ± 0.18^{b}	2.55 ± 0.13^{a}
QH-30+As+NH_3+T	3.97 ± 0.63^{c}	4.21 ± 0.73^{cd}	3.50 ± 0.53^{cd}	6.42 ± 1.0^{e}
QH-35+As+NH_3+T	5.63 ± 0.49^{f}	8.30 ± 1.88^{g}	4.45 ± 0.51^{d}	6.54 ± 0.50^{e}
P-Value	0.019	0.0018	0.022	0.016

Table 5. Effect of quinoa husk (QH) diets in alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in liver and gill tissues of fish reared under control and multiple stresses (NH_3+As+T). Values in the same row with different superscript (a, b, c, d, e, f) differ significantly. ALT: nmole of sodium pyruvate formed/mg protein/min at 37 °C, AST: nmole Oxaloacetate released/min/mg protein at 37 °C; Data expressed as Mean ± SE ($n = 6$).

Treatments	Lactate dehydrogenase (LDH)		Malate dehydrogenase (MDH)	
	Liver	Gill	Liver	Gill
Control	2.29 ± 0.30c	3.52 ± 0.18^{e}	1.38 ± 0.05^{c}	2.40 ± 0.11^{e}
As+NH_3+T	4.48 ± 0.23d	6.92 ± 0.41^{f}	3.05 ± 0.20^{f}	4.01 ± 0.23^{f}
QH-15	2.35 ± 0.15c	3.46 ± 0.26^{e}	1.11 ± 0.09^{b}	1.53 ± 0.22^{b}
QH-20	2.01 ± 0.19b	3.47 ± 0.20^{e}	1.35 ± 0.07^{c}	1.74 ± 0.13^{c}
QH-25	1.17 ± 0.08a	1.94 ± 0.12^{ab}	0.80 ± 0.02^{a}	0.81 ± 0.14^{a}
QH-30	2.25 ± 0.17c	2.83 ± 0.19^{d}	1.38 ± 0.07^{c}	1.79 ± 0.09^{c}
QH-35	2.19 ± 0.08b	2.81 ± 0.47^{d}	1.37 ± 0.03^{c}	1.76 ± 0.07^{c}
QH-15+As+NH_3+T	2.34 ± 0.35c	2.59 ± 0.42^{c}	1.40 ± 0.02^{cd}	1.83 ± 0.05^{c}
QH-20+As+NH_3+T	2.34 ± 0.22c	2.01 ± 0.30^{b}	1.54 ± 0.06^{d}	1.33 ± 0.08^{b}
QH-25+As+NH_3+T	1.29 ± 0.19a	1.62 ± 0.25^{a}	0.73 ± 0.04^{a}	0.79 ± 0.09^{a}
QH-30+As+NH_3+T	2.08 ± 0.27b	3.82 ± 0.24^{e}	1.66 ± 0.05^{e}	2.07 ± 0.14^{d}
QH-35+As+NH_3+T	2.25 ± 0.16b	3.41 ± 0.25^{e}	1.63 ± 0.04^{e}	2.16 ± 0.09^{d}
P-Value	0.0024	0.0017	0.0009	0.0004

Table 6. Effect of quinoa husk (QH) diets in lactate dehydrogenase (LDH) and malate dehydrogenase (MDH) in liver and gill tissues of fish reared under control and multiple stresses (NH_3+As+T). Values in the same column with different superscript differ significantly ($p < 0.01$). Data expressed as Mean ± SE ($n = 6$). LDH and MDH: units/min/mg protein at 37 °C,

exposure to NH_3+As+T. Increases were also seen when QH diets were given at 30% and 35%, in contrast to the control and other groups.

Effect of QH on blood glucose, nitroblue tetrazolium, total protein, albumin, globulin, A:G ratio, total immunoglobulin and myeloperoxidase in *P. Hypophthalmus*

Table 7 shows the blood glucose, NBT, total protein (TP), albumin, globulin, AG ratio, immunoglobulin (Ig), and myeloperoxidase (MPO) results for *P. hypophthalmus* that were reared under high temperature stress and concurrently exposed to low doses of ammonia and arsenic toxicity. The fish were also fed either a control diet or diets made of QHs for 105 days. In comparison to the control and other groups, blood glucose levels were considerably higher ($p = 0.0011$) when subjects simultaneously experienced high temperature stress, low doses of ammonia and arsenic toxicity. On the other hand, QH diets at 15%, 25%, and 30% significantly lowered blood glucose levels in comparison to the control and other groups with and without stresses. Under stressful condition, NBT ($p = 0.0008$) and total protein (TP) ($p = 0.0016$) levels were significantly lower. In contrast to the control and other groups, these values were significantly higher with a 30% QH diet, followed by 25% and 20%

Treatments	BG	NBT	TP	Albumin	Globulin	A: G ratio	Ig	MPO
Control	83.23 ± 1.42^{b}	0.42 ± 0.02^{c}	1.11 ± 0.02^{e}	0.27 ± 0.01d	0.83 ± 0.02	0.33 ± 0.01^{d}	0.87 ± 0.03^{f}	0.31 ± 0.01^{c}
As+NH_3+T	126.67 ± 1.54^{e}	0.26 ± 0.01^{a}	0.51 ± 0.01^{a}	0.21 ± 0.04c	0.30 ± 0.04	0.74 ± 0.18^{e}	0.23 ± 0.01^{a}	0.12 ± 0.05^{a}
QH-15	93.69 ± 0.66^{c}	0.37 ± 0.03^{bc}	0.87 ± 0.02^{d}	0.16 ± 0.02ab	0.71 ± 0.03	0.24 ± 0.04^{c}	0.63 ± 0.03^{c}	0.28 ± 0.03^{c}
QH-20	93.84 ± 0.39^{c}	0.46 ± 0.02^{d}	1.13 ± 0.01^{e}	0.15 ± 0.01ab	0.98 ± 0.01	0.16 ± 0.02^{ab}	0.94 ± 0.05^{g}	0.29 ± 0.01^{c}
QH-25	82.93 ± 1.23^{b}	0.45 ± 0.03^{d}	1.15 ± 0.81^{e}	0.17 ± 0.01ab	0.97 ± 0.04	0.17 ± 0.02^{ab}	0.90 ± 0.06^{g}	0.31 ± 0.02^{c}
QH-30	81.36 ± 0.68^{ab}	0.44 ± 0.01^{cd}	1.04 ± 0.27^{e}	0.12 ± 0.02a	0.93 ± 0.03	0.13 ± 0.01^{a}	0.93 ± 0.03^{g}	0.31 ± 0.01^{c}
QH-35	106.82 ± 0.46^{d}	0.31 ± 0.01^{b}	0.86 ± 0.05^{d}	0.19 ± 0.01b	0.67 ± 0.02	0.29 ± 0.02^{c}	0.70 ± 0.02^{de}	0.25 ± 0.01^{b}
QH-15+As+NH_3+T	78.23 ± 0.53^{a}	0.32 ± 0.02^{b}	0.74 ± 0.03^{c}	0.15 ± 0.01ab	0.59 ± 0.05	0.25 ± 0.01^{c}	0.54 ± 0.01^{c}	0.27 ± 0.02^{bc}
QH-20+As+NH_3+T	82.12 ± 2.06^{b}	0.45 ± 0.04^{cd}	0.83 ± 0.04^{d}	0.13 ± 0.01a	0.70 ± 0.02	0.19 ± 0.01^{b}	0.62 ± 0.02^{d}	0.30 ± 0.01^{c}
QH-25+As+NH_3+T	80.61 ± 1.04^{ab}	0.45 ± 0.03^{cd}	1.13 ± 0.11^{e}	0.24 ± 0.02c	0.89 ± 0.05	0.29 ± 0.01^{c}	0.78 ± 0.04^{e}	0.33 ± 0.02^{c}
QH-30+As+NH_3+T	82.07 ± 2.55^{b}	0.53 ± 0.04^{e}	1.00 ± 0.06^{e}	0.19 ± 0.01b	0.81 ± 0.03	0.23 ± 0.02^{c}	0.78 ± 0.03^{e}	0.32 ± 0.01^{c}
QH-35+As+NH_3+T	93.64 ± 1.01^{c}	0.32 ± 0.02^{b}	0.60 ± 0.02^{b}	0.16 ± 0.02ab	0.44 ± 0.02	0.35 ± 0.01^{d}	0.40 ± 0.03^{b}	0.23 ± 0.01^{b}
P-Value	0.0011	0.0008	0.0016	0.015	0.01	0.022	0.0002	0.014

Table 7. Effect of quinoa husk (QH) diets on blood glucose (BG), NBT, total protein (TP), albumin, globulin, A:G ratio, ig and MPO in fish reared under control and multiple stresses (NH_3+As+T). Values in the same row with different superscript (a, b, c, d, e) differ significantly. Total protein, albumin, globulin: g dL^{-1}Blood glucose: mgdL^{-1}; Data expressed as Mean ± SE ($n = 3$).

QH diets. The control group had considerably greater albumin levels ($p = 0.015$) than the other groups, with all other groups exhibiting lower albumin levels. Under stressful conditions, globulin levels were considerably lower ($p = 0.01$) (NH_3+As+T). As contrast to the control and other groups, globulin levels were considerably higher with QH diets at 20%, 25%, and 30% without stressors and at 25% and 30% with stressors. In comparison to the control and other groups, the AG ratio was significantly lower with QH diets at 20, 25, and 30% with and without stressors, but it was significantly higher ($p = 0.022$) during stress condition. In comparison to the control and QH groups, immunoglobulin (Ig) levels were significantly lower ($p = 0.002$) when low dosages of arsenic, ammonia toxicity, and high temperature stress were experienced simultaneously. In comparison to the control and other groups, the fish that were fed diets containing 20, 25, and 30% QH had the greatest levels of Ig. In contrast, myeloperoxidase (MPO) activity was significantly higher in the control group and in groups fed QH diets at 20, 25, and 30%, with and without stressors. MPO activity was significantly reduced ($p = 0.014$) under NH_3+As+T stress conditions.

Effect of QH on digestive enzymes in *P. Hypophthalmus*

Protease, lipase, and amylase were among the digestive enzymes measured in the intestines of fish reared in various stressful conditions. In comparison to the control and other groups, fish fed 25% diet of QH without stressors had significantly higher protease activity ($p = 0.031$). In contrast, fish subjected to high temperatures, low concentrations of ammonia and arsenic, and other conditions showed a significant decrease in protease activity when compared to the control and other groups. When compared to the control and other groups, fish given a 25% QH diet with stressors exhibited considerably ($p = 0.012$) higher lipase activity. These fish also showed enhanced activity in the absence of stressors. Similarly, a 25% QH diet free of stressors resulted in significantly ($p = 0.042$) increased amylase activity, with the control group showing the lowest amylase activity (Fig. 2A–C).

Effect of QH on histopathology of *P. Hypophthalmus*

The histopathology of liver and gill was observed in liver and gill tissues of *P. hypophthalmus* fed with QH diet and reared under arsenic, ammonia and high temperature stress (Figs. 3 and 4). Histopathology of gill tissue was shown normal primary and secondary lamella. Whereas, secondary lamella shorten and curved, primary lamella altered and karyokinesis shown in the group concurrently exposed to As+NH_3+T. Moreover, QH diet at 25% showed normal primary and secondary lamellae. The group fed with QH fed at 25% with NH_3+As+T showed secondary lamella shorten; swelling of secondary lamella and secondary lamella clubbed (Fig. 3A–D). Similarly, the histopathology in liver tissues were showed normal hepatic cell with nucleus in control group. The group concurrently exposed to NH_3+As+T group, lipid vacuoles, loss of nucleolus and pyknotic nuclei. The group fed with 25% QH showed normal hepatic cell. Whereas, the group fed with QH at 25% with NH_3+As+T showed altered nucleus with nucleolus and pyknotic nuclei (Fig. 4A–D).

Discussion

The present study investigates the utilization of QH, a waste planting material, as a replacement for fishmeal in the preparation of low-cost fish feed. Fishmeal was replaced with QH at 15, 20, 25, 30, and 35% to evaluate its effectiveness against multiple stressors, including concurrent exposure to ammonia, and arsenic toxicity as well as, and high temperature stress (34 °C), in *P. hypophthalmus*. QH contains an anti-nutritional factor in the form of saponin. We determined the saponin content in QH to be 2.06 g/100 g. After heating the QH, the saponin content varied from 0.63 to 0.94 g/100 g. The results showed that heating method reduced saponin content, aligning with traditional practices to control anti-nutritional factors in feed ingredients[53].

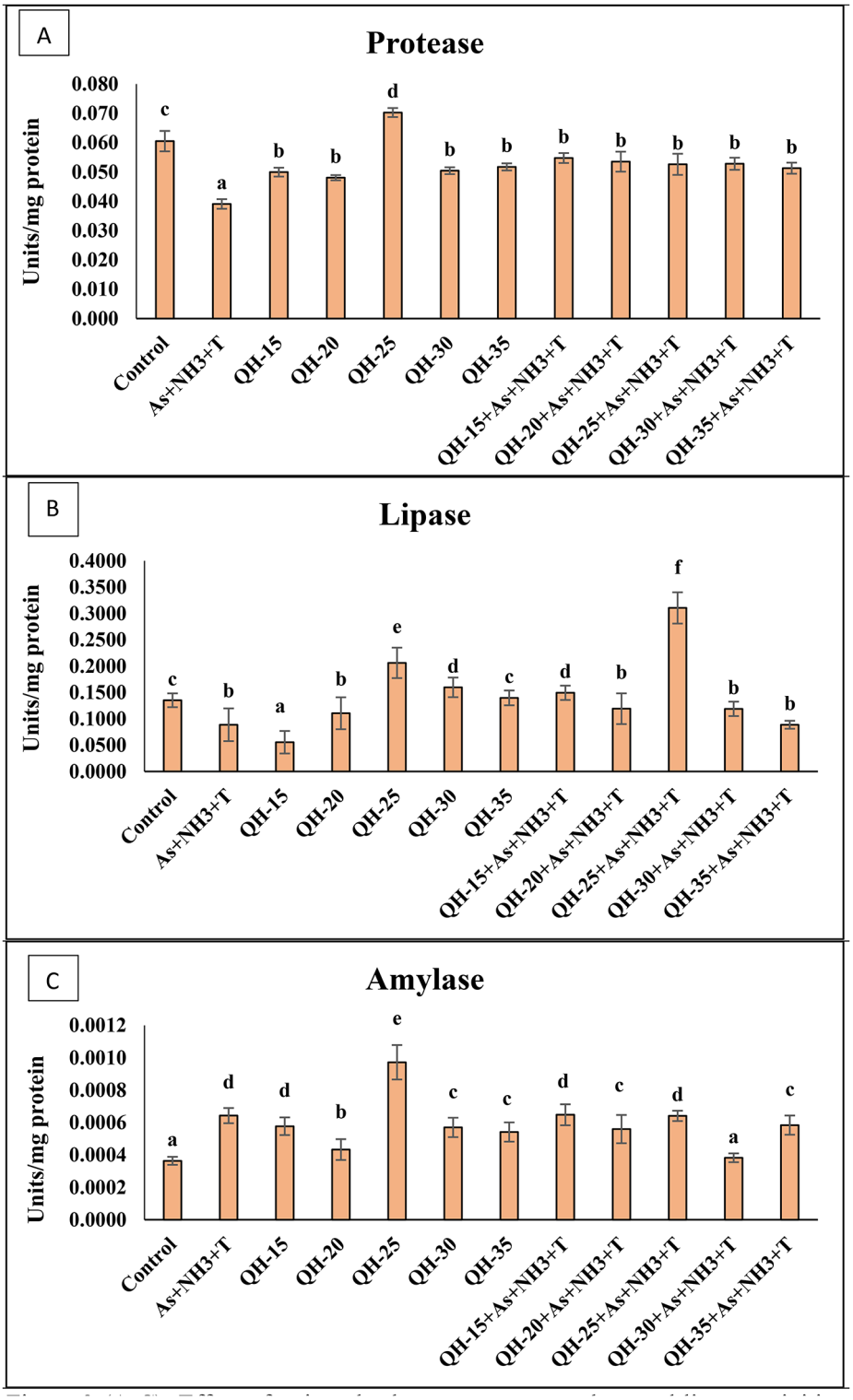

Fig. 2. (**A**–**C**) Effect of quinoa husk (QH) on protease, amylase and lipase activities in fish intestine. Within endpoints and groups, bars with different superscripts differ significantly (a–f). Data expressed as Mean ± SE ($n = 3$).

Our previous study on QH, which involved gene regulation and growth performance indicators under multiple stresses, was reported for *P. hypophthalmus*[4]. The dose of the QH was optimized in this study with respect to improved growth performance, including weight increase %, protein efficiency ratio, feed conversion ratio, and specific growth rate. Under multiple stress condition, (NH_3+As+T), 25.5–26.0% QH showed the best results.

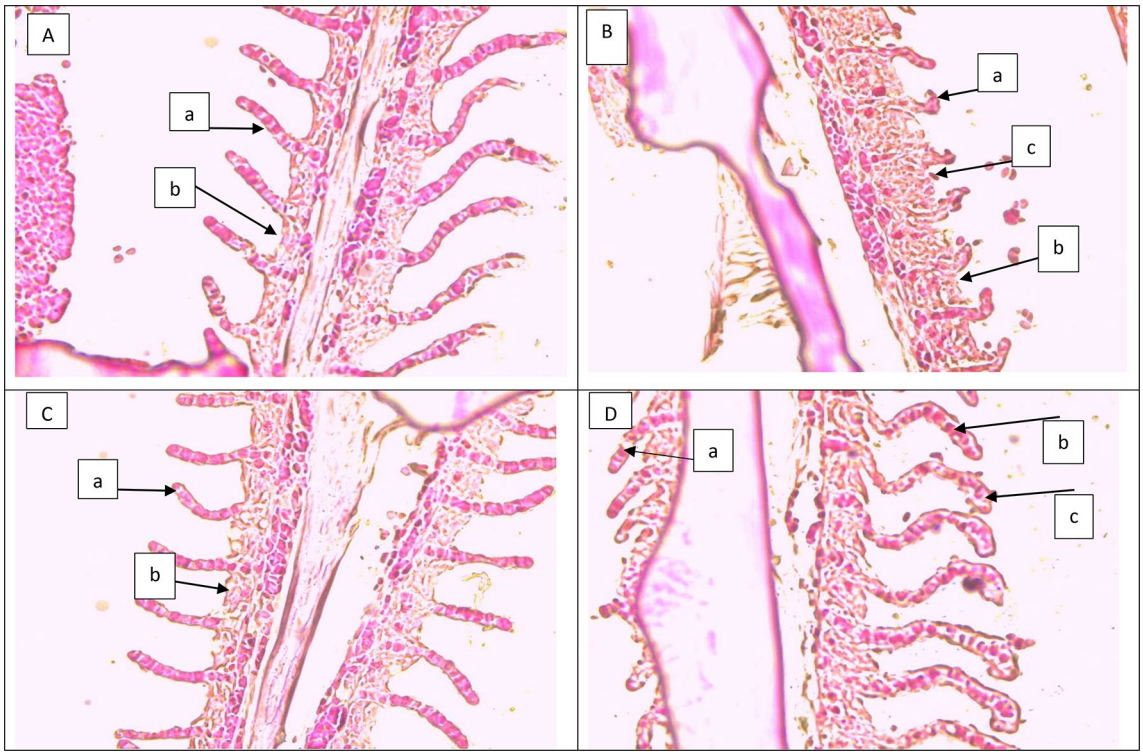

Fig. 3. (**A**–**D**): Histopathology of gill tissue (A (Control treatment): a: normal primary b: secondary lamellae; B (NH_3+As+T group): a: secondary lamella shorten and curved; b; Primary lamella altered c. karyokinesis; C (QH group at 25%): normal primary b: secondary lamellae; D (QH group at 25% with NH_3 + As+T group): a; Secondary lamella shorten; b. Swelling of secondary lamella ; c: Secondary lamella clubbed).

The kidney, liver, and gills showed significantly increased levels of oxidative stress enzymes, such as catalase (CAT), glutathione peroxidase (GPx), and glutathione S-transferase (GST), when simultaneously exposed to low concentrations of ammonia and arsenic, along with high-temperature stress. These findings suggest that high-temperature stress, combined with low doses of ammonia and arsenic, enhances the generation of reactive oxygen species (ROS) and alters peroxide conversion pathways[4]. Arsenic further contributes to its toxic mechanism by binding to thiol groups in cellular proteins and non-proteins and activating NADPH oxidase (Nox)[54]. Additionally, arsenic and ammonia penetrate the fish through the gills, affecting the liver and kidney tissues and exacerbating oxidative stress[4,55]. Notably, replacing fishmeal with 25% QH significantly reduced oxidative stress, as evidenced by decreased enzymatic activities of GST, CAT, and GPx in the liver, kidney, and gill tissues. The ability of QH to alleviate oxidative stress may be attributed to its role in regulating antioxidative enzymes, offering protection against multiple stressors[56,57]. Quinoa is well-known for its potent antioxidant properties, attributed to its high levels of total polyphenols, flavonoids, and other bioactive compounds[58]. Thus, incorporating 25% QH effectively mitigates the combined toxicity of low doses of arsenic and ammonia, as well as high-temperature stress.

The neurotransmitter enzyme acetylcholinesterase (AChE) in brain tissue was significantly inhibited by low doses of ammonia and arsenic, as well as high-temperature stress. However, dietary inclusion of 25% QH enhanced AChE activity compared to all other groups. Ammonia and arsenic likely inhibit AChE activity by impairing the acetylcholine hydrolysis system, which is essential for maintaining nerve impulses and facilitating transmission across synaptic junctions to other cholinergic neurons. This inhibition may also disrupt the function of ion channels, particularly those regulating sodium ions[59]. The sensitivity of brain tissues to arsenic and ammonia makes their inhibition of AChE activity particularly evident[27]. The observed enhancement of AChE activity with a 25% QH diet may be due to the antioxidant properties of quinoa, which is rich in phenolic and flavonoid compounds[60].

Moreover, activities of ALT, AST, LDH, and MDH in liver and gill tissues were significantly elevated in groups exposed concurrently to low doses of ammonia and arsenic toxicity, as well as high temperature stress. It's crucial to remember that these enzymes ALT, AST, LDH, and MDH are sensitive biomarkers that are frequently employed in ecotoxicological research and that can serve as early warning indicators of potentially dangerous changes in fish[61]. The elevated levels of ALT, AST, LDH, and MDH indicate damage to target organs such as the liver and gill tissues due to ammonia, arsenic, and high temperature stress[62,63]. Further, a 25% QH diet reduced the activities of ALT, AST, LDH, and MDH in liver and gill tissues. Interestingly, quinoa supplementation decreased ALT and AST activities, suggesting benefits for liver homeostasis[64]. A study by Cao et al.[65] also found that quinoa reduced ALT and AST levels in rats. This effect may be attributed to the optimal levels of minerals, proteins, essential amino acids, dietary fibers, and bioactive compounds present in quinoa.

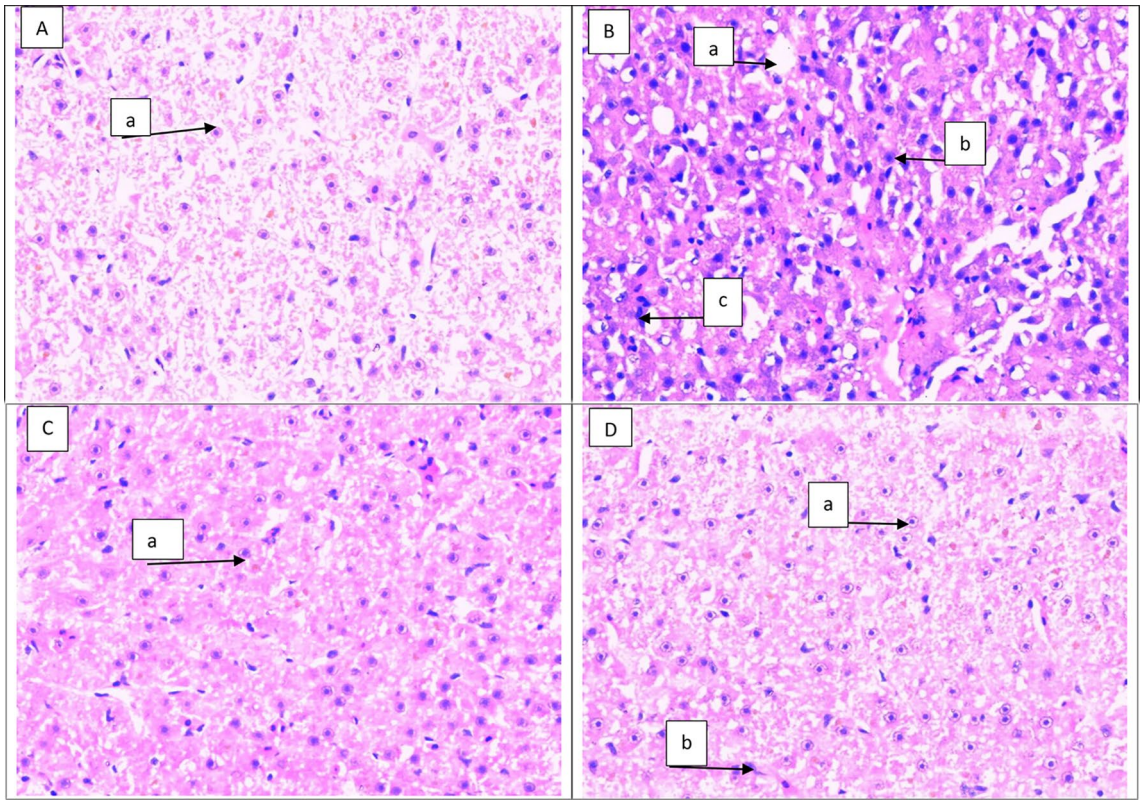

Fig. 4. (**A**–**D**) Histopathology of liver tissue (A (Control treatment): a: normal hepatic cell with nucleus; B (NH_3+As+T group): a: lipid vacuoles; b: loss of nucleolus; c: pyknotic nuclei; C (QH group at 25%): normal hepatic cell; D (QH group at 25% with NH_3+As+T group): a; nucleus with nucleolus; b: pyknotic nuclei).

Blood glucose (BG) levels were significantly reduced in *P. hypophthalmus* reared under concurrent exposure to ammonia, arsenic, and high temperature stress when fed with 25% QH diet. This reduction may be attributed to quinoa's low glycemic index, high fiber content, and the low insulin levels[66]. Other immunological parameters, such as total protein (TP), NBT, globulin, albumin, the albumin-to-globulin (AG) ratio, immunoglobulins (Ig), and myeloperoxidase (MPO) activity, were significantly affected by exposure to low doses of ammonia and arsenic toxicity combined with high temperature stress. However, 25% QH diet improved these immunological attributes in fish subjected to the same stressors. NBT (nitroblue tetrazolium) is a marker of fish health, reflecting the phagocytic activity of leucocytes and the production of intracellular superoxide radicals[67]. Globulins, including α1, α2, β, and γ types, with gamma globulin being a key immunological protein, play important roles in immune response[68,69]. Albumin regulates free hormones, fat metabolism, and the transport of metals, bilirubin, hormones, drugs, and vitamins[70]. These improvements in immunological parameters may be due to the bioactive compounds present in quinoa, particularly polyphenols, flavonoids, essential amino acids, essential fatty acids, and vitamins. This is the first report demonstrating that QH can enhance immunological attributes in fish exposed to multiple stressors. Additionally, MPO, a potent oxidant and hemeprotein, plays a role in the respiratory burst, utilizing H_2O_2 to produce hypochlorous acid[71].

Moreover, digestive enzymes such as protease, amylase, and lipase were significantly enhanced in fish fed 25% QH diet, whether reared under control conditions or exposed to low doses of ammonia and arsenic toxicity, as well as high temperature stress. The improvement in digestive enzyme activity is directly related to the growth performance of the fish. Enhanced nutrient digestion and absorption, along with better resistance to opportunistic indigenous bacteria, are indicators of improved digestion and growth performance[72].

Histopathological examination of the liver and gill tissues was conducted to assess the effects of concurrent exposure to low doses of ammonia and arsenic toxicity, as well as high temperature stress, and to evaluate the protective role of QH against these stressors. The exposure to these stressors resulted in hepatic cell damage, including pyknotic nuclei, loss of nucleolus, shrunken nuclei, and the presence of lipid vacuoles in liver tissues. In gill tissues, the primary and secondary lamellae were damaged and exhibited shrinkage. These findings are consistent with results from our previous study, which also reported similar histopathological changes due to exposure to the same stressors[73,74].

Conclusions

The current study found that replacing fishmeal with QH greatly enhanced the performance of fish reared under both control and multiple stress condition (NH_3+As+T). It was discovered that QH replacement at the level between 26% and 26.5% was the most effective way to improve growth performance as indicated by feed

conversion ratio (FCR), growth rate, specific growth rate (SGR) and protein efficiency ratio (PER). Furthermore, a diet containing 25% QH enhanced the antioxidant status of fish reared under control and stressed condition. In fish subjected to both control and stress conditions, 25% QH also decreased cellular metabolic stress and histopathological changes. These findings unequivocally show that fish performance can be improved by using QH in place of fishmeal.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions

Neeraj Kumar: Conceived and designed the experiments; sampling of the site, performed the experiments; analysed the data; contributed reagents/materials/analysis tools; wrote the paper. Paritosh Kumar: Support in sampling and perform analysis. Aliza Pradhan: Support in sampling, perform analysis and provide the Quinoa husk. Dilip Kumar Singh: Analysis and data validation. Tarkeshwar Kumar: Data validation and editing. Jagadish Rane: Project formulation and monitoring and guiding. Kotha Sammi Reddy: Supervision, monitoring and guiding.

Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

The Aquaculture Central Wet Laboratory facilities and experimental protocols were approved under the Committee for the Purpose of Control and Supervision of Experiments on Animals (CCSEA) under the number 2190/GO/RReBi/SL/2022/CCSEA. This study also strictly adhered to the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines. All methods were performed in accordance with the relevant guidelines and regulations.

Additional information

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