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Effect of organic and in-organic chromium supplementation on growth performance and genotoxicity of *Labeo rohita*

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ABSTRACT

In aquaculture industry, the aim of feed formulation is to provide the fish with feasible diet to enhance their body. Carbohydrates supplemented with chromium compounds (organic and in-organic) are major energy currencies for biological machinery of fish. Here, this article presents a description that emphasizes the effect of chromium picolinate (organic) and chromium chloride hexahydrate (in-organic) on growth execution and genotoxicity of *Labeorohita*. Seven groups (each with a replica) with 30 *Labeorohita* fingerlings were formed: a control group, three groups were supplemented with chromium chloride Hexahydrate (0.3, 0.5 and 0.6 mg kg^{-1}) and three groups were supplemented with Cr-Pic, 0.3, 0.5 and 0.6 mg kg^{-1}) respectively. The experimental group T4 fortified with (Cr-Pic) along with carbohydrates by dose of 0.3 mg/kg demonstrated significant results ($P < 0.05$). Superior growth for *Labeo rohita* was observed as compared to control and other experimental groups. Minimum growth trend was observed in group T5 (CP-0.5 mg/kg), T6 (CP-0.6 mg/kg) and T7 (control) respectively. Comet assay results indicated the dose and Cr related (organic or in-organic) genetic damage in fish erythrocytes. Hence, maximum comet parameters (Tail length, Tail DNA and Olive Tail Moment) were observed in (in-organic Cr) by 0.3 mg/kg concentration. This study suggested the toxicity corresponding to in-organic Cr but organic Cr could be used as growth promoter if so. Overall results demonstrated supplementation of organic chromium compounds by 0.3 mg/kg should be reconsidered for growth. This drive of research address the fish farmers to utilize the feed supplemented with organic Cr compounds which is most appropriate to provide sustainable yield as part of increment in growth performance and beneficial health effects for consumers on indices to reduce the toxicity risks.

1. Introduction

Fish are the main component of marine habitat. They are complexly linked to other organisms through food web and other mechanisms. Fish and human societies have had special bonding with one another for thousands of years. This infrastructural relationship mainly based on food sources. Fish is supplying nutrients, proteins and minerals which are not probably offered by any other food stuff. It is involved in provision of protein to whole of the world (Lynch et al., 2016). In nations such as Bangladesh and Ghana, fish accounts more than 50% of the animal protein diet. According to an expert prediction fish and the products derived from fisheries are among the best sources of micronutrients. Aquaculture supplied 148 million tons of fish, of which 128 million tones is being used as food source. Sustainable fish industries defend a particular significance in developing countries. Taking in account the cost effectiveness of particular diet, food content may have striking effects on feed behavior and fish nutrition. The trial to improve the feeding strategies is to improve the fisheries welfare (Silva et al., 2016). The current aquaculture practice was administered to search for feed ingredients that can be used to formulate significant fish feed and to enhance fish production (see Fig. 1)

Carbohydrates are now being evaluating as an alternative protein source to fish meal in aqua feeds. It is possible to replace the fish meal up to 80% with plant protein concentrates mixed with organic form of Cr without compromising the growth performance of fish (Ngugi

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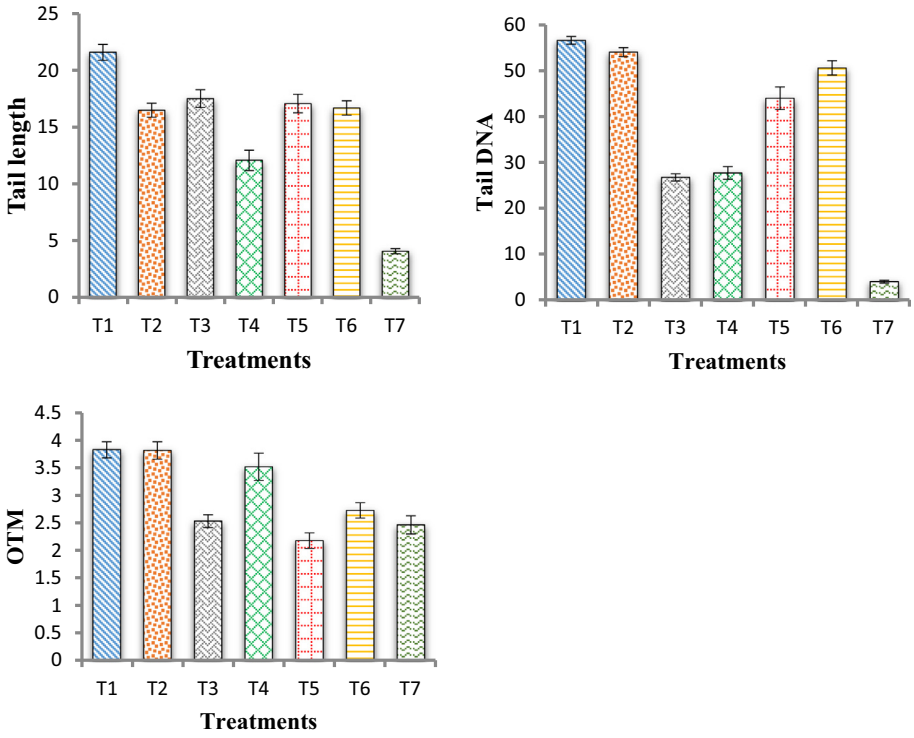

Fig. 1. (Graphical Demonstration of six treated groups, that are Cr-Pic by 0.3 mg/kg, 0.5 mg/kg, 0.6 mg/kg, $CrCl_2$ Hexahydrate by 0.3 mg/kg, 0.5 mg/kg, 0.6 mg/kg and a control group corresponding to Tail length, Tail DNA and Olive Tail moment (OTM) respectively). There could be many reasons of DNA damage in an animal cell including radiations or ultraviolet rays, but the DNA alterations caused due to chemical exposure was an indirect reason. It was perceived during trail that in aquatic organisms, the blood is at first pace of attack of toxicity. Hexavalent chromium taken through diet is more toxic than trivalent form. In result of toxicity, Cr got infused in blood and ultimately in membranes of blood cells, nucleus got deformed, electrophoresed DNA fragments protrude out from cell because eyrythrocytes are more sensitive to toxic substance after gill epithelium. Comet assay proved as unmatched technique to check the genotoxicity. Whereas by comparing both forms of Cr compounds, organic form of Cr (Cr-Pic) demonstrated improved growth, feed efficiency ratio, by mixing with carbohydrates while in-organic form of Cr was also there to demonstrate genotoxicity. Comet assay showed that Cr induced the DNA damage when treated with erythrocytes of *Labeorohita* for 90 days. Results stated that maximumfrequency of Tail DNA and Tail length were shown by in-organic chromium (chromium chloride hexahydrate 0.3 mg/kg) while minimum trend towards the comet parameters was shown by organic chromium compounds by concentration of 0.5 mg/kg and 0.6 mg/kg respectively. Presence of longer tail, there were noteworthy DNA damages.

et al., 2017). Gross production of major cereal grains e.g. wheat, rice and corn come to be 2.5 billion tons in year of 2013. The fisheries growth potential can be increased by paying more attention to fish feed quality and by addressing the issues to eradicate the harmful effects of fish feed (Mohsin et al., 2015). Various feeding trials were conducted to assess the effects of carbohydrates on growth performance and other physiological processes. Because carbohydrates are involved with augmentation in plasma triglycerides and cortisol level as in work piece of and it is estimating through this study that this efficiency of carbohydrates can be enhanced by providing carbohydrates with a right union to organic Cr.

Chromium since naturally occurring element found in rocks, animals, plants, and soil. Mostly found in its insoluble trivalent form Cr (III). If Cr is in reduced form it is insoluble, while as it get oxidized, get dissolve in aquatic medium (Jaishankar et al., 2014). Chromium salts such as Chromium picolinate and Chromium chloride hexahydrate had play a considerable role as vital micronutrients for better growth of human and animals. Various forms of chromium compounds have been used as fed additives in fish diet due to their prevalence role in carbohydrate, fats and protein metabolism. Interaction of carbohydrates with other elements is directly correlated to fish health. Cr has been investigated as cytotoxic element causing genotoxicity in fish liver and blood (Stankiviciute et al., 2017). Cr exposure has been shown to cause DNA strand breakage and formation of Cr-DNA complex including Cr-DNA adducts and DNA-Cr-DNA crosslinks. Role of Cr in glucose, fats and protein metabolism in animals and humans is true but noticeable toxicological features are also there. High dose of Cr (VI) can cause stomach tumor. The toxicity of Cr (VI) form can be reduced to Cr (III) though it cannot be transported inside the cell. Cr (VI) may interact with cell integrity and various functions (Aslam & Yousafzai, 2017). There is vast investigation on significance of Cr as chromium picolinate because of it high nutritional value (Vincent & Stallings, 2007). Hexavalent and trivalent form of are most commonly found. Hexavalent is considered more toxic because plasma membrane easily permits to pass out through it. Once enters inside the cell, forms major complexes with macromolecules including DNA (Bakshi & Panigrahi, 2018). Both forms of Cr organic and inorganic are significant for humans as well as for animals. Cr has pronounced effect on metabolic rates if ingested through diet. It improves the growth rate and efficiency ratios (Pires et al., 2015). Chromium compounds brought about DNA damage in indigenous fish fauna (Kousar & Javed, 2015). Cr is well known carcinogenic element. A concentration determined rise in tail formation of blood cells is present. Hexavalent Cr has genotoxic potential through various modes of action and it can be perceive through comet assay. It is unclear yet that either what mechanisms are followed by Cr compounds to cause toxicity (Fang et al., 2014).

2. Experimental methods-general considerations

2.1. Experimental fish

Labeo rohita has auspicious market potential in Asia as well as other regions in world. Many steps have been taken including betterment of nutritive value to improve the health quality of major carps.

Labeo rohita fingerlings were bought from fish seed hatchery, Satyana road, Faisalabad, Pakistan. Prior to start the experiment, fish were acclimatized for 10 days feeding on control diet. Fish was fed with advised diet at 4% of live wet weight.

2.2. Feed ingredients and diet preparation protocol

The basic diet ingredients were fish meal, soybean meal, yellow corn, wheat bran and maize gluten were purchased from local market and ground to make powder for making fish feed. Every single dry fixing was measured watchfully and blended completely for 30 min, an adequate amount of water was added to make mixture, and this mass was spread in plate and placed in oven at (60 °C) to dry it. This material was grounded to make pellets of 2 mm with hand pelletizer. These pellets were steamed in autoclave for 5 min. Each pellet was dried at 60 °C in oven. Six diets were prepared by adding increasing amount of Chromium picolinate (CP1, CP2 and CP3) and Chromium chloride hexahydrate (CCH1, CCH2 and CCH3), whereas the control diet was maintained without Cr supplementation. Feed was quantified and prepared by using Winfeed 2.6 linear formulation method (Winfeed U.K.) Ltd., Cambridge, U.K).

2.3. Experimental design

After acclimatization fish was disseminated to 14 aquaria each had just about 29 L water limit. Every aquarium had thirty fingerlings of almost equal size dispersed arbitrarily. The water quality parameters i.e. pH, dissolved oxygen, and temperature was checked daily as per standard strategies (APHA 1985). The fingerlings were given the food to satiation twice day by day (morning and evening) at 4% of live body weight, and daily feed intake was observed.

2.4. Study of growth parameters

During trial, on the basis of fortnight from all treatments morphometric characteristics of sampled fish were recorded which includes body weight and body length. Using 30 cm ruler total body length of fish was measured from tip of the mouth to the most anterior tip of tail fin. Body weight was measured using analytical balance. After recording data fish fingerlings were again transferred into their respective aquaria. The ideal relationship between feed ratio and two parameters commonly used to determine the nutritional value of a diet that are specific growth rate and feed conversion ratio.

By following calculations fish fingerling's growth was determined.

I. **Increase in wet body weight (g) by following formula**

Average body weight of 1st observed fortnight – Average body weight of next observed fortnight.

II. **Increase in net total body length (cm)**

Average body length of 1st observed fortnight – Average body length of next observed fortnight.

III. **Condition factor by using following formula**

$$K = W \times 10^5 \div L^3$$

where

W = Average wet body weight (g)

L = Average body length (cm)

IV. **Specific growth rate (SGR) evaluated by using the following formula**

$$SGR = \frac{\ln(\text{Final wet body weight}) - \ln(\text{Initial wet body weight})}{\text{Time duration(Days)}} \times 100$$

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

2.5. Comet assay protocol

Comet assay (single cell gel electrophoresis) is a simple method for measuring de-oxyribonulceic acid strand breaks in eukaryotic cell (Collins AR, 2004). For comet assay application, five fish per aquarium were randomly selected. Genotoxicity of *Labeo rohita* was evaluated by using comet assay according to procedure by Singh et al. (1988).

2.5.1. Blood removal and preparation and layering of slide

After providing the different concentrations of organic and inorganic Cr compounds for 90 days of trial, blood from caudal vain of fish was removed. To preserve the blood sample, Sterilized EDTA tubes were used. For storage purposes, the blood sample was allowed to place in lab's refrigerator Department of Zoology, Government College University Faisalabad, Pakistan. Prior to begin the comet assay, slides were immersed in methanol and allowed to burn at blue flame to avoid the dust and oil particles from slides. LMP agarose gel (0.5%) was used to layer the slides.

2.5.2. Cell lysis

Prepared slides were immersed in chilled lysing buffer solution (contained 18.62 g EDTA, 73.05 g NaCl, 0.60 g Tris-Hcl with 10 pH adjusted NaOH pellets) in glass container employed in dark box. The purpose to immerse in lysing buffer was to disrupt the proteins, RNA content and bonding bridges of blood proteins. While the DNA of blood cells got fixed inside gel on slide and that fixed DNA was then tagged as nucleoid (single cell). Slides were allowed to leave over 1.5 h. Timing may vary for sample.

2.5.3. Electrophoresing & neutralization

Electrophoresing require cold electrophoresing buffer (Contained with 0.18 g EDTA, 6 g of NaOH with dist. water) for 20 min, 300 mA current, and 20 V voltage in horizontal electrophoresing container. Electrophoresing was done to separate the DNA fragments and to detect the DNA damage. During this step, damaged DNA becomes unwind and moved along resulting in formation of comet tail. Length of comet corresponded to damage. This process was carried out in complete darkness. Neutralization was done by using cold neutralizing buffer (24.22 g in dist. water, pH adjusted at 7.5 using HCl). After electrophoresing, neutralizing was done. The pH of solution was maintained at 7.5. Each slide was washed with neutralizing buffer three times after interval of 5 min. This process was also carried out in dim light.

2.5.4. Staining and comet scoring of slides

Staining was done by using Ethidium Bromide by amount of *75ul* per slide. After loading the staining solution, coverslip was placed and slides were allowed to dry for 20 min. For scoring purposes dried slides were perceived under epifluorescent microscope (Nikon DS-Fi2). Magnification was adjusted at 40×. 50 cells were counted for each slide. Comets were appeared in damaged cells having different tail lengths depending on the level of damage. Cells with spherical shape were not counted during scoring. Com-

puter image analysis (Casp software) was used to measure length of head, length of tail, length of comet, head DNA, tail DNA and tail movement. Cells with maximum damage demonstrate highest value for tail DNA and minimum value for head DNA while opposite values was observed in opposite case or minimum damage.

2.5.5. Statistical analysis

After finding the conceivable results, information of growth performance and genotoxicity was subjected to measurable examination and ±SE qualities was computed by utilizing one way ANOVA through SPSS software (Steel, Torrie and Dickey 1997). Results of comet assay were statistically analyzed by using one way ANOVA and TUKEY test (Snedecor & Ochran, 1994).

3. Results

The recorded data of experimental trial showed significantly variant results for growth and comet parameters in *Labeo rohita* fingerlings.

3.1. Growth performance evaluation

Fish were mass weighed every fortnight during experimental trail. The efficacy of growth and length parameters are shown in Table 1. At the end of trial after applying the same weighing procedures, the growth execution parameters were assessed by SGR, Condition factor, total increment in weight and length. Pronounced growth, feed efficiency ratio, by mixing with carbohydrates was shown by T4 (Cr-Pic.0.3 mg/kg) with average gain in weight 3.01 g and length as 5.68 cm with initial weight and length 1.81 g and 4.00 cm, respectively. While the highest value of specific growth rate was shown by treatment-T6 (1.06) which includes carbohydrates along with in-organic form of chromium. Significant variations were seen in Co-efficient of determination graphs drawn between fortnights and treatments (Fig. 1).

3.2. Chromium analysis in composed diet fed

Different doses of Cr were exploited in six experimental diets (three with organic Cr and remaining with in-organic Cr compounds). A significant difference ($P > 0.05$) between organic and in-organic Cr was perceived. Briefly, striking effects were seen on SGR, weight and length parameter by exploiting organic Cr (Cr-Pic) by the dose of 0.3 mg/kg in consideration to other experimental groups with correspondent doses. Organic form of chromium has played the role well in improved growth, feed efficiency ratio, specific growth rates, and length to weight affinity by mixing with carbohydrates.

3.3. Comet assay

Along with all growth investigations, comet formation relied too much on Cr form (organic or in-organic) and their advised doses. Varying concentrations of Cr showed effects on blood parameters of *Labeo rohita* fish subjected for 90 days of trial (Singh et al., 1988). Different comet parameters *viz*, Tail length, Tail DNA and OTM attributed by both forms of Cr showed significant behavior ($P > 0.05$) and shown in Table 2.

3.4. Discussion

The supplementation of chromium compounds for the advancement of (growth factors *viz,* SGR, increment in length and weight and their interaction) have been well reported in scientific community.

3.4.1. Significant growth tools (weight, length and condition factor)

Cr-Pic intensified the body growth and carbohydrate's metabolism profile. Carbohydrates admixed to Cr provide the same results as that of high potency protein diet. Findings were similar to work of Liu et al. (2010). Appropriate Cr concentration is necessarily important to improve the growth it was 0.3 mg/kg (with organic chromium compound). Cr concentration more than a proper dose can reduce the body weight of fish as stated by Ahmad (2012). Present study was also a need of time to compare the both form of Cr used in this trial. Since two forms of Cr has been used during feeding trial. Organic form of Cr (Cr picolinate) demonstrated improved growth, feed efficiency ratio, by mixing with carbohydrates similar to findings of Pires et al. (2015). In this study, T6 (Cr-Pic. 0.6 mg/kg) group showed highest value of specific growth rate which includes carbohydrates along with in-organic form of chromium. This is because of reason that organic forms of Cr were absorbed more efficiently than in-organic forms as in investigations of (de Oliveira et al., 2014). Significant variations were seen in Co-efficient of determination graphs drawn between fortnights and treatments provided. Besides the positive effect of Cr on fat, glucose and protein metabolism, distinguished toxicological features were also there (Aslam & Yousafzai, 2017). Obtained results were deviating from Mehrim (2014) conclusions. Contradictory to the positive effect of Cr supplementation on growth (Seluck et al., 2010) demonstrated that Cr picolinate supplementation have no significant impact on growth and metabolism of fish. Since it is extensively known that toxicity of in-organic complex compounds

Table 2
Comparison of means of various comet parameters analyzed in *Labeo rohita* blood under different treatments of organic and in-organic chromium.

Treatments	Comet parameters		
	Tail DNA	Tail length	OTM
T1 (CCH1, 0.3 mg/kg)	56.57 ± 0.839	21.58 ± 0.696	3.83 ± 0.144
T2(CCH2, 0.5 mg/kg)	54.00 ± 0.987	16.50 ± 0.612	3.81 ± 0.157
T3(CCH3, 0.6 mg/kg)	26.78 ± 0.806	17.52 ± 0.783	2.53 ± 0.115
T4 (CP1, 0.3 mg/kg)	27.74 ± 1.380	12.10 ± 0.890	3.52 ± 0.248
T5(CP2, 0.5 mg/kg)	43.98 ± 2.461	17.08 ± 0.819	2.18 ± 0.142
T6(CP3, 0.6 mg/kg)	50.56 ± 1.566	16.70 ± 0.629	2.73 ± 0.139
T7 (control)	4.12 ± 0.306	4.12 ± 0.221	2.46 ± 0.165

Table 1
Growth performance in *Labeorohita* fingerlings cultured under different treatments of organic (Cr-Pic.) and in-organic ($CrCl_2$ hexahydrate) chromium compounds.

Parameters	Experimental diets						
	T1	T2	T3	T4	T5	T6	Control
Initial weight (g)	1.27	1.93	1.73	1.81	2.17	1.25	2.14
Final weight (g)	3.30	3.34	2.91	3.67	2.93	3.12	3.33
Weight gain (g)	0.26	0.20	0.16	0.27	0.10	0.26	0.17
Initial length (Cm)	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Final length (Cm)	6.22	6.02	5.75	6.44	6.27	6.17	6.17
Increase in length (Cm)	0.31	0.29	0.24	0.35	0.17	0.31	0.27
SGR	1.01	0.60	0.57	0.78	0.33	1.06	0.49

is appear to be more in freshwater fish species. That's why in present study, *Labeo rohita* was selected on an index to measure toxicity effects of organic and in-organic form of Cr. Since fish fingerlings were used during trial because effect of complex compounds can be assessed more effectively in younger active forms in preference to larger ones. Results achieved have shown resemblance to work piece of (El-Shafei, 2016) as well.

Differences among results obtained by different researches for same kind of work (even with same compounds and experimental design) are because of many reasons. Water systems in aquaria, rearing conditions, experimental diet timings are key points for different results. *Labeo rohita* fingerlings fed with 0.3 mg/kg concentration of organic chromium compound showed better survival rate. As that the results of Asad et al. (2017a,b). Likewise, in work of Giri et al. (2014) supporting results were obtained in which fish fed with (0.8 mg/kg) concentration of chromium Picolinate. Organic Cr since has significant effect on growth performance. Cr in appropriate concentration fed to fish cause increase in growth while it may get declined due to increased concentration of Cr as declared by Shaheen and Farhat (2015). Performance a feeding trial to assess the better impacts of dietary chromium Picolinate on feed utilization, growth execution in fish were similar to findings of Wang et al. (2014). Along with reporting the significant results of chromium picolinate on growth (Ahmad et al., 2012) found the genotoxic effect of Cr concentrations on fish blood cells. Concentration of Cr-picolinate by 2.0 mg/kg showed increase in WBC's and decline in number of RBC's in blood.

Results of focal study presenting validate relationship between length and weight parameters. There is straight line graph relationship likely to be seen there in research outcomes. Increment in one parameter showed increment in other one. Studies of (Donea et al., 2017) supporting the current work. Hence it was demonstrated through analysis that supplementation of Organic form of chromium has played the role well in improved growth, feed efficiency ratio, specific growth rates, along with these all findings, its varying concentrations could also have effect on blood parameters of *Labeo rohita.*

In-organic form of Cr may have more genotoxic effects on fish gill, liver and blood. Cr exists in various oxidation states ranging from [7–9] including Cr (IV) which is harmful among them at all. The typical reason is its availability in aquatic medium that it readily obtained by body fluids and tissues (Mannan et al., 2018). Research indicated that organic form of Cr might have shown specific effects on bioactivity of insulin, metabolic rates and inflow of blood glucose, because the alterations induced by organic Cr in morphometric parameters of fish shown signs of positive effects (as given in Table 1). Results were also supported by (Hastuti & Subandiyono, 2014). According to Results of experimental trial obtained, in which fish were cultured in six treatment groups each, having equal number of fish. In-organic chromium administrated through diet by dose of 0.3 mg/kg showed maximum genotoxic effects with the mean values 21.58 ± 0.696, 56.57 ± 0.839 and 3.83 ± 0.144 for Ltail, Tail DNA, and OTM, respectively. Minimum genotoxic effects were observed in control diets and organic treatments, respectively. Analysis approved that genotoxicity detected in *Labeo rohita* blood was caused due to in-organic dietary chromium. These results were supported by Mallesh et al. (2015). The measurement of genotoxicity in erythrocytes using comet assay revealed significant effect ($P < 0.05$) by supplementary in-organic chromium doses.

4. Conclusion

Organic form of chromium (Cr-Pic.) in fish feed with carbohydrates is growth enhancer by making the carbohydrates digestible to fish. While in-organic form may be genotoxic. As the in-organic chromium compounds are toxic so their supplementation should be avoided to get an enhanced growth in *Labeo rohita* fish. A specialized research area to work more with some other fish species is needed for fish farmer awareness to utilize organic chromium in combination to carbohydrates which is cheaper source of feed.

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