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The effect of Spirulina on reproductive parameters, body composition, immune indices and digestive enzyme in dwarf gourami (*Trichogaster lalius*)

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Abstract

Rearing of ornamental fishes allocated high levels of annual income from the aquaculture industry. Spirulina is a blue-green algae, spiral shape with a diameter of 12 microns and rich source of protein, vitamins, amino acids and essential fatty acids, minerals and antioxidant pigments. Using different levels of spirulina was evaluated on immune indicators, digestive enzymes and reproductive parameters (fecundity and sexual maturation) of dwarf gourami. One hundred and forty five dwarf gourami with an average weight of 3.6±0.23g were randomly distributed in twenty 12-liter aquarium. Four diets were prepared consisting of spirulina at levels of 3%, 7% and 10% substituted by fish meal and with a control diet. Parameters related to body biochemical, reproductive, digestive enzymes and immune index were measured. Increasing spirulina up to 7% enhance the ovarian weight (p<0.05). Hatching percentage and fecundity working has shown a significant increase with spirulina increased (p<0.05). Lipase, amylase and protease has significantly increased with increasing spirulina (p<0.05). Lysozyme and serum bactericidal have significantly increased with the increase of spirulina (p<0.05). Based on results 3% of spirulina due to improved reproductive factors and 10% to enhance immune indices and digestive enzymes in dwarf gourami considered as the best levels of substitution with fishmeal.

Keywords: Spirulina, Dwarf gourami, Reproductive, Immune, Digestive enzymes

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Introduction

Production of ornamental fishes is one of the parts which have high levels of income in the aquaculture industry. So far, more than 90 percent of freshwater ornamentals fish cultured artificially while this feature is only available in a handful of salt water species (30-25 species) were reported. Generally, brood stock management and rearing larvae, as two critical issues are considered for reproduction and rearing in the aquaculture industry. However, low fecundity brood stocks and larvae high mortality of ornamental fishes during the larval culture still are the most important challenges of this industry. To alleviate these problems, previous studies emphasized the effects of diets on the stimulation of brood stock maturation, increase fecundity, egg quality and improved developments of larvae (Degani, 1990; Oliviotto *et al*., 2006; Güroy *et al*., 2012). Fish meal due to high levels of protein and essential amino acids are considered as one of the main constituents the aquatic foods (Gatlin *et al*., 2007). In recent years, much effort has done for partial or complete substituting of fish meal with variety of plant sources available, powder of Spirulina (*Spirulina platensis*) as a source of plant, capable of producing dense and value of proper food can play a promising trends in future years (Lu and Takeuchi, 2004; Choonawala, 2007). Spirulina is a blue-green algae, spiral shape with a diameter of 12 microns and a rich source of protein, vitamins, amino acids and essential fatty, mineral and antioxidant pigments (Diraman *et al*., 2009). Positive results in many aquatic species of Spirulina were reported over the determinants of growth, increased food intake, pigmentation, increase immunity and improve reproductive parameters (Regunthan and Wesley, 2007; Güroy *et al*., 2012.). However, very limited data there are on the use of Spirulina on the characteristics of ornamental fish reproduction (Güroy *et al*., 2012; Lu and Takeuchi, 2004). Dwarf gourami (*Trichogaster lalius*), because of the beauty and reasonable price, is popular among aquarists. In terms of reproductive performance, the fecundity in dwarf gourami is between 300-800 eggs per fish that these differences dependent on the size of brood stocks and type of diets (Goldestein, 1971; Degani, 1990). Also, brood stock fed with commercial diets caused that at larval rearing, survival rates varied between 35-20 percent. It seems to increase growth indices and reproductive performance, improved diets brood stocks and larvae feed are considered as two main causes (Güroy *et al*., 2012.). Reproduction (accelerated maturation, higher rates of fecundity and the percentage of hatching) and immunity (enzyme lysozyme and serum bactericidal) is focused by optimizing of brood stocks through the influence of diet on Dwarf Gourami. For these reasons, using the different levels of spirulina powder substituted with part of fish meal were studied on growth indices, colorimetric, immune, digestive enzymes and reproductive in Dwarf Gourami.

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Materials and methods

Spirulina algae cultivation and preparation

Algae cultivation (*S. platensis*) was conducted in 20-liter containers and temperature 34-35 °C, with a 24-hour exposure of lighting and aeration. Guillard (f/2) was used as medium. Spirulina centrifuged with Sigma 8K in 5.000 rpm for 5 minutes. Algae biomass dried out in 40 °C for 24 hours. (Lavens and Sorgeloos, 1996).

Preparation of dwarf gourami

One hundred and forty five dwarf gourami with an average weight of 3.6±0.23g were purchased. Randomly, they were distributed in 12 aquarium 20-liter and were fed for 7 days with commercial food contained 4% fat and 30% protein. During rearing, physicochemical parameters were set in the optimum range (pH 7-7.2, Temperature 23-24 °C and gentle aeration). Feeding was performed on a daily basis for 3 times and satiation (Oliviotto *et al.*, 2006). Fish were cultured for 60 days and period light was 12:12 (light: dark), also all aquariums filtered by central filtration.

Preparation of diets

Four diets were prepared consisting of spirulina powder substituted at 3, 7 and 10 percent in diet with fish meal and a control diet (only fishmeal). The ingredients used for the preparation of diets as followed (Table 1).

Table 1: The combination of dietary ingredients used in study.

Diet composition	Spirulina replacement levels in the diet (%)			
	Control	**3**	**7**	**10**
Fish meal	45.5	45.5	38.5	35.5
Spirulina	0	3	7	10
Soy-meal	30	30	30	30
Wheat	12	12	12	12
Starch	5	5	5	5
Vitamin premix[1]	0.5	0.5	0.5	0.5
Mineral premix[2]	0.5	0.5	0.5	0.5
Canola oil	6.5	6.5	6.5	6.5
The biochemical composition of diets (%)				
Dry matter	91.16	91.26	91.64	91.73
Protein	41.50	41.68	41.92	41.62
Ether extraction	12.8	12.6	12.34	12.13
Ash	4.9	5.1	5.1	5.2
Digestible Energy (Kcal g^{-1})	3.98	3.96	3.93	3.90

[1]In terms of Kg:
vitamin A, 10,000 IU; vitamin D3, 2,000 IU; vitamin E, 100 mg; vitamin K, 20 mg; vitamin B_1, 400 mg; vitamin B_2, 40 mg. vitamin B_6 20 mg; vitamin B_{12}, 0.04 mg; biotin, 0.2 mg; choline chloride, 1200 mg; folic acid, 10 mg; inositol, 200 mg; niacin, 200 mg; pantothenic calcium, 100mg.
[2]In terms of Kg:
$MgS0_4$. $2H_20$, 127.5; KCl, 50.0; NaCl, 60; $CaHPO_4$. $2H_2O$, 727.8; $FeSO_4$. $7H_2O$, 25.0; $ZnSO_4$. $7H_2O$, 5.5; $CuSO_4$. $5H_2O$, 0.785; $MnSO_4$. $4H_2O$, 2.54; $CoSO_4$. $4H_2O$, 0.478; Ca $(IO_3)_2$. $6H_2O$, 0.295; $CrCl_3$. $6H_2O$, 0.128.

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The reproductive parameters

To determine reproductive parameters were obtained some index include absolute fecundity, relative fecundity and working fecundity, ovarian weight, gonadosomatic Index, the average time required for maturation, the average diameter of eggs, larvae yolk sac diameter, total length of larvae and percentage of hatching. In order to determine the reproductive fecundity, 5 females from each treatment were randomly separated and the eggs obtained were counted by the loop. Ovarian weight done with incision abdominal and isolated ovarian and weighing it. Then as much as 0.1 g of eggs were separated and counted with loops (in 5 replicate).

The biochemical composition of body

Chemical body analysis such as moisture, ash, crude protein and crude fat was conducted based on AOAC (1990).

Calculation of digestive enzyme and immune indicators

For enzyme analysis, the intestine was washed with cold deionized water to remove as much mucus as possible and were then homogenized in cold sodium phosphate buffer (0.1 M, at pH 7.0, and 4 ºC) by a ratio of 1:9 (m/v) (Liu *et al*., 2008).

The homogenate was centrifuged at 4 ºC at 10000 g for 30 minutes. The soluble protein content in the enzyme extract was measured by Lowry method (Lowry *et al*., 1951). α-Amylase was determined by starch-hydrolysis method according to Robyt and Whelan (1968). The enzymatic reaction mixture consisted of 2% (w/v) starch solution (0.125 ml), 0.1 M citrate–phosphate buffer at pH 7.5 (0.125 ml) and a digestive extract (0.05 ml). The reaction mixture was incubated for 1 hour at 37 ºC. Absorbance was determined at 600 nm. Maltose was used as a standard and the activity unit of a-amylase was defined as the quantity of enzyme that produced 1mmol of maltose ml^{-1} min^{-1}. Lipase activity was determined by the evaluation of the degradation of triacylglycerols, diacylglycerols, and monoacylglycerols to free fatty acids following the method of Metin and Akpinar (2000). For the emulsion, a 1% solution of polyvinyl alcohol (PVA) in distilled water was used. Then 5 ml of 0.1 N HCl were added, heating to 75–85 ºC for 1 hour, followed by cooling, filtering, and adjusting pH to 8.0 with 0.1 N NaOH. To an aliquot of the above solution, virgin olive oil was added to a substrate concentration of 0.1 M. The mixture was emulsified for 5 minutes. The reaction mixture composed of a PVA solution-emulsified substrate (1 ml), McIlvaine buffer at pH 8 (0.5 ml), and digestive extract (0.5 ml). The McIl-vaine's buffer was prepared from 0.1 M citric acid and 0.2 M bisodium phosphate. The reaction mixture was incubated for 4 hours at 37° which 3 ml of a 1:1 ethanol–acetone solution was added to stop the reaction and break the emulsion. A few drops of 1% phenolphthalein in ethanol were added to the reaction mixture and titrated with 0.01 M NaOH. For the blank tubes, the same procedure was followed but with boiled enzyme. One unit of lipase

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activity was defined as the hydrolysis of 1.0 micro-equivalent of fatty acids from triacylglycerols in 1 hour at pH 7.7 and 37 °C. Total proteolytic activity was measured using the casein hydrolysis method by Walter (1984). The assay was conducted using a wide range of pH values. The buffers used were 0.1 M KCl–HCl (pH 1.5), 0.2 M glycine–HCl (pH 3.0), 0.1 M citrate–0.2 M phosphate (pHs 4.0 and 7.0), 0.1 M Tris–HCl (pHs 8.5 and 9.0) and 0.1 M glycine–NaOH (pH 10.0), at 25 °C. Enzyme reaction mixtures consisted of 1% (w/v). Casein in water (0.25 ml), buffer (0.25 ml) and enzyme sample (0.1 ml) were incubated for 1 hour at 37 ºC. The reaction was stopped by adding 0.6 ml of 8% w/v trichloroacetic acid. After holding for 1 hour at 28 ºC, samples were centrifuged at 1800g for 10 minutes and the absorbance of the supernatant recorded at 280 nm. Tyrosine was used as standard and one unit of enzyme activity was defined as the amount of enzyme needed to catalyze the formation of 1 mg of tyrosine per 1 minute.

Statistical analysis

The results were analysed using a standard one-way analysis of variance (ANOVA) using SPSS (version 22). Kolmogo-rov–Smirnov and Bartlettl's tests were applied to check the normality and homogeneity of variances. To compare data obtained from treatments was used of Tukey test at 5% significance level. Excel 2013 was used for diagramming.

Results

Spirulina composition

Analysis showed that spirulina powder contains 61.1% protein, 5.3% fat, 19.5% carbohydrates and 9.8% ash and 4.2 percent moisture.

The effects of spirulina replacement in diets on body biochemical composition of dwarf gourami

Moisture of male body showed no significant difference ($p>0.05$), while in females the lowest moisture has been observed at 3% and the most was in 10% and control that there was no significant difference ($p>0.05$). Male and female protein increased with rising spirulina ($p<0.05$). Male and female fat decreased with rising spirulina ($p<0.05$). Although, there was no significant differences between male and female ash ($p>0.05$), by rising spirulina, in terms of numerical can be observed increase (Table 2).

The effects of spirulina replacement in diets on reproductive performance and body size of larvae proceeds dwarf gourami

Although ovarian weight increased with 7% of spirulina, so it was observed the highest weight of ovarian in 3% of spirulina ($p<0.05$). In 10% of spirulina, loss of ovarian weight was observed, that is why ovarian weight has been reduced by use of spirulina ($p<0.05$). There was significant decrease in the gonad somatic index by increasing spirulina from 3% up to 10% ($p<0.05$), but 3% of spirulina compared to the control was significant increase and differences ($p<0.05$). The average time

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of maturation at the level of 3% spirulina had significant decrease compared to control (p>0.05). Generally, absolute, relative and working fecundity have gained by increasing spirulina (p<0.05). The average egg diameter decreased with increasing spirulina (p<0.05) but egg diameter in 3% of spirulina increased compared to the control and showed significant differences (p<0.05). The yolk sac diameter in 3% of spirulina increased (p<0.05) and after that up to 10% spirulina that was reduced (p>0.05).

Table 2: The biochemical composition of dwarf gourami fed with different levels of spirulina for 8 weeks.

Body composition	Spirulina replacement in the diets (%)			
	Control	3	7	10
Moisture of male	65.70±0.14^{a}	66.05±0.50^{a}	66.10±0.99^{a}	66.15±0.50^{a}
Moisture of female	65.80±0.14^{ab}	65.45±0.21^{a}	66.20±0.14^{b}	65.95±0.35^{ab}
Crude protein of male	63.20±0.50^{a}	62.95±0.02^{a}	65.04±0.42^{b}	66.09±0.89^{b}
Crude protein of female	63.38±0.41^{a}	63.13±1.5^{a}	65.68±1.90^{a}	70.05±0.31^{b}
Fat of male	29.04±1.70^{b}	28.64±1.70^{b}	27.14±0.20^{ab}	24.52±0.41^{a}
Fat of female	27.32±0.26^{b}	26.85±0.39^{b}	25.78±0.03^{b}	19.58±2.16^{a}
Ash of male	7.87±2.35^{a}	8.42±1.72^{a}	7.83±0.62^{a}	9.39±0.48^{a}
Ash of female	9.31±0.16^{a}	10.04±1.92^{a}	8.55±1.87^{a}	10.37±1.57^{a}

Different letters indicate significant differences at the level of 5 percent.

Table 3: Reproductive parameters and size of larvae dwarf gourami fed wit diets containing different levels of spirulina for 8 weeks.

Parameters	Replacement level of spirulina in diets (%)			
	Control	3	7	10
Ovarian weight (g)	0.38±0.05^{a}	0.81±0.02^{d}	0.69±0.05^{c}	0.58±0.07^{b}
Gonadosomatic Index (GSI)	8.33±1.28^{a}	16.90±0.21^{c}	15.64±1.22^{c}	12.42±1.37^{b}
Average time of maturation (Day)	64.80±2.86^{d}	52.60±1.67^{a}	59.40±2.07^{c}	56.20±1.48^{b}
Absolute fecundity	1022.00±38.5^{a}	1288.00±46.65^{c}	1138.60±24.05^{b}	1043.60±36.36^{a}
Relative fecundity	217/20.9.01^{a}	263.20±6.38^{c}	248.80±11.23^{b}	239.80±11.35^{b}
Working fecundity	361.40±9.48^{a}	587.00±20.46^{b}	717.20±9.26^{d}	695.60±6.54^{c}
Average egg diameter (µm)	742.00±10.44^{c}	786.20±4.76^{d}	679.20±3.03^{b}	647.20±8.29^{a}
Average diameter of the yolk sac larvae (µm)	651.40±5.03^{c}	673.40±2.41^{d}	620.00±1.58^{b}	603.80±3.96^{a}
Hatching (%)	52.31±1.35^{a}	88.72±2.79^{d}	63.79±1.69^{b}	69.32±0.84^{c}
Length of larval (mm)	3.38±0.57^{d}	3.23±0.06^{c}	3.01±0.11^{b}	1.96±0.10^{a}

Different letters indicate significant differences at the level of 5 percent.

Hatching rate with increasing of spirulina decreased but totally was more than control and there was significant difference (p<0.05). The larvae length have shown a significant increase with rising of spirulina (p<0.05) (Table 3).

Digestive enzymes and immune indicators

Lipase activity has significantly increased with increasing spirulina (p<0.05), but in 7% observed overlapping with 3% and 10% (p>0.05). Amylase activity showed a significant

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increase with rising in spirulina (p<0.05). Although protease activity among control and 3% and also between treatments 7% and 10% there was not significant differences (p<0.05), overall, there was significant difference by increasing up 3% (p<0.05) (Table 4). Enzyme lysozyme has shown a significant increase compared to control when spirulina raised (p<0.05), but there were no significant differences between 7% and 10% (p>0.05). Serum bactericidal hydrophila has been increased significantly until spirulina raised (p<0.05) (Table 5).

Table 4: Digestive enzyme of dwarf gourami fed with diets containing different levels of spirulina for 8 weeks.

Parameters	Replacement level of spirulina in diets (%)			
	Control	3	7	10
Lipase activity (µg per mg soluble protein)	4.11±0.04[a]	4.31±2.12[b]	4.42±0.02[bc]	4.58±0.05[c]
Amylase activity (µg per mg soluble protein)	3.78±0.05[a]	3.96±0.03[b]	4.12±0.04[c]	4.26±0.04[d]
Protease activity (µg per mg soluble protein)	247.62±0.05[a]	252.33±2.00[a]	261.83±2.28[b]	264.37±1.81[b]

Different letters indicate significant differences at the level of 5 percent.

Table 5: Immune index of dwarf gourami fed with diets containing different levels of spirulina for 8 weeks.

Parameters	Replacement level of spirulina in diets (%)			
	Control	3	7	10
Enzyme lysozyme (µg per mg)	76.06±0.17[a]	80.41±1.49[b]	91.81±1.14[c]	92.94±1.22[c]
Serum bactericidal hydrophila (Number of bacterial cell, CFU) ($\times 10^2$)	16.20±4.25[a]	1.865±0.71[b]	1.935±2.12[bc]	1.950±2.83[c]

Different letters indicate significant differences at the level of 5 percent.

Discussion

In terms of biochemical composition, moisture content of male and male and female ash in Dwarf Gourami were not affected by spirulina. Males' protein in control and 3% and for females in control, 3% and 7% were no significant differences. Without taking into account the different levels of replacement, the results of this study's Is consistent with findings Nandeesha *et al*. (1998) on common carp, El-Sayed (1994) on silver sea bream and Chou and Shiau (1996) on tilapia (*Oreochromis* sp.). With the increasing levels of algae replacement, body fat reduced and observed at 10% the lowest amount of fat (24.52% for male and 19.58% for female) that are significantly more effective than the other treatments. These results are similar to Kim *et al*. (2013) reveled effects replacement of fish meal with spirulina in *Oplegnathus fasciatus* in levels of 5%, 10% and 15% that fat was significantly reduced in 15%. In general, the use of plant

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resources in the diets reduces the amount of body fat. Unlike the results of this study, Mustafa *et al*. (1994) on sea bream and Puwastien *et al*. (1999) on tilapia observed significant difference in body fat feeding algal sources. Polyphenol compounds are other antioxidant compounds in plants (Balasundram *et al*., 2006). Kim *et al*. (2013) known the reason for reduced the body fat fish, increasing concentrations of polyphenols in diet with increasing levels of spirulina that diet was positively correlated with antioxidant capacity.

The results show that reproductive success in fish was affected by factors such as: brood stock, nutrient and diets, sex ratio, storage density, size and age (Izquierdo *et al*., 2001; Chong *et al*., 2004). In this study, process of reproduction in dwarf gourami was affected by dietary spirulina; such that all fish fed with diets contain spirulina compared to control, reached earlier maturity and spawning. The results of the present study, reached to sexual maturity in the period between 52-59 days after storage that is comparable to Seifi Berenjestanaki *et al*. (2014) in terms of rate of sexual maturation.

Ovary weight between treatments was significantly difference and all treatments fed by spirulina had more ovarian weight than the control (3% was most ovarian weight, 0.81 g). Gonadosomatic index were significant differences between treatments and the highest amount at 3% (16.90) were observed that with 7% showed no significant difference. According to Amornsakun *et al*. (2004) GSI is 8%-10% in freshwater fish and noted that GSI in *Pectoralis Trichopoda* is 10.9%. It also amounts for the red sword fish (*XiPhophorus helleri*) fed by 8% spirulina was 123% and there was significant difference with control (9.36%) and also goad weigh was 4 times more than control (James *et al*., 2006).

In this study, fish fed with Spirulina In terms of absolute, relative and working fecundity had significant differences with control (except 10% for absolute fecundity) and the best results were obtained at the level of 3% for the Absolute and relative fecundity and to 7% for working fecundity. Similar results in this study were observed in James *et al*. (2006) that swordtail fish fed with 8% of Spirulina. However, have been mentioned higher fecundity is about 4,000 eggs in gourami families in some cases (Richte, 1988). Fecundity rate in three spotted gourami fish was reported in range of 8021 to 9104 egg by Reyes-Bustamente and Ortega-Salas (2002). Previous studies have reported that essential fatty acids, alpha-tocopherol, ascorbic acid and carotenoids in the diets were effective to stimulate sexual maturity, reproduction, high quality eggs and larvae survival rates (Scabini *et al*., 2011).

Also, Güroy *et al*. (2012) reported that fed with 2.5% spirulina on cichlid yellow tail (*Pseudotro pheusacei*) had the highest eggs produced and hatching percentage and there was a significant difference compared to other treatments. As well as was not observed significant differences in fecundity, egg

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size and spawning intervals on tilapia fish (*O. niloticus*) fed with spirulina compared to the control (Lu and Takeuchi, 2004).

Hatching percentage in treatments which were used spirulina with reduced spirulina from 10% to 3% has significantly increased, but in general the percentage of hatching was higher than control. Also, in 3% spirulina, the average diameter of the egg and yolk sac diameter was higher than all treatments and higher amounts of spirulina made reduced these two factors. The reason it can be probably due to decreased fat Dwarf Gourami, because dropped body fat by increasing spirulina. In general, there is no exact cause for the results of increasing reproductive parameters resulting from the use of spirulina, nevertheless, previous studies have pointed to the role of pigments, precursors of vitamin A and fatty acids (Watanabe and Vassallo-Agius, 2003).

Use of spirulina in the diet of Dwarf Gourami showed increasing of activity enzyme lysozyme, so that by increasing the Spirulina up to 10 percent was significantly increased that matched with Ragap *et al*. (2012) based on Increase lysozyme activity within several weeks in effect continuous use. Spirulina help to build lysozyme enzyme in the body of fish so that by increasing spirulina and period of using in the diets, production of enzyme lysozyme increased, according to the results obtained in this study, similar results there was by Ibrahim *et al*. (2013). Spirulina can be considered as a prebiotic, so that by increasing spirulina in feed increased indicators related to non-specific immune and as a prebiotic caused enhances activity of serum and bactericidal activity in different fish species (Ye *et al*., 2011; Ebrahimi *et al*., 2012; Akrami *et al*., 2013).

By increasing spirulina in diets of Dwarf Gourami was increased digestive enzyme so that amylase in each treatment showed a significant increase with others which is represents an increase of carbohydrates in the diet. Protease in the digestive tract was significant increase also, that could be due to high levels of vegetarian's protein in the diet contain spirulina, because Dwarf Gourami is a omnivorous fish and consumed better of food with nature of plant. Increasing lipase in the digestive tract up to 7% spirulina showed significant differences and further did not significantly increased in lipase that could be because ability to lower in fat digestion omnivorous fish.

According to the results obtained 3% spirulina due to improved reproductive factors and 10% to enhanced immune indexes and digestive enzymes in dwarf gourami considered as the best levels of substitution with fishmeal.

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