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Effects of dietary soybean meal levels on reproduction parameters, the growth and gonad, gut, hepatopancreas histology of female African Cichlids *Pseudotropheus socolofi*

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ABSTRACT

This study was conducted to determine the effect of different soybean meal dietary supplementation levels on the growth, reproductive parameters, gonad, intestine and hepatopancreas histology of *Pseudotropheus socolofi*. Experimental groups were hand-fed to satiety with diets supplemented with soybean meal at levels of 3, 16, 27, 35, and 44% twice daily for 90 days. The results of this study showed that specific growth rate, weight gain, and survival rate were not affected by dietary soy proportions ($P > 0.05$). The worst FCR and final weight were found in those fed the diet containing 44% soy ($P < 0.05$). In female reproductive parameters, there was no significant difference between the groups in fertilization rate, egg production, hatching rate, egg diameter, gonadosomatic index and broodstock ovulation percentage ($P > 0.05$). However, due to pathological examinations in female individuals, a significant decrease was observed in the number of mature oocytes in the ovaries and goblet cells in the intestines with increasing soy levels in the diet ($P < 0.05$). As a result, using soybean meal up to 35% did not negatively affect growth. However, adding 44% soybean meal to diets caused histopathologically serious inflammatory reactions and decreased growth.

Keywords: *Pseudotropheus socolofi*, soybean meal, reproduction, histology, growth

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Diyetteki soya küspesi oranlarının Dişi Afrika Çiklitleri *Pseudotropheus socolofi'*nin üreme, büyüme parametreleri, ve gonad, bağırsak, hepatopankreas histolojisi üzerine etkileri

Öz: Bu çalışma, *Pseudotropheus socolofi*'nin büyüme, üreme parametreleri, gonad, bağırsak ve hepatopankreas histolojisi üzerine farklı düzeylerde soya küspesi diyet takviyesinin etkisini belirlemek amacıyla yapıldı. Deney grupları, 90 gün boyunca günde iki kez %3, 16, 27, 35 ve 44 seviyelerinde soya küspesi ile desteklenen diyetlerle doyana kadar elle beslendi. Bu çalışmanın sonuçları spesifik büyüme hızının, kilo alımının ve hayatta kalma oranının diyetteki soya oranlarından etkilenmediğini gösterdi ($P> 0,05$). En kötü FCR ve son ağırlık, %44 soya içeren diyetle beslenenlerde bulundu ($P < 0,05$). Dişi üreme parametrelerinde ise döllenme oranı, yumurta verimi, kuluçka oranı, yumurta çapı, gonadosomatik indeks ve anaç yumurtlama yüzdesinde gruplar arasında anlamlı bir fark bulunmamıştır ($P > 0,05$). Ancak dişi bireylerde yapılan patolojik incelemeler sonucunda diyetteki soya düzeylerinin artmasıyla birlikte yumurtalıklardaki olgun oosit sayısında ve bağırsaklardaki goblet hücrelerinde önemli bir azalma gözlendi ($P< 0,05$). Sonuç olarak soya küspesinin %35'e kadar kullanılması büyümeyi olumsuz etkilememiştir. Ancak diyetlere %44 oranında soya fasulyesi küspesinin eklenmesi histopatolojik olarak ciddi inflamatuar reaksiyonlara ve büyümede azalmaya neden olmuştur.

Anahtar kelimeler: *Pseudotropheus socolofi*, soya küspesi, üreme, histoloji, büyüme

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Introduction

Soybean meal (SBM) is used as plant-derived protein because of its abundance, relatively low cost, high protein levels and amino acid profiles in fish feeds (DiMaggio et al. 2016). SBM contains high levels of phenolic compounds, known as estrogenic compounds called isoflavonoid phytoestrogens (Bagheri et al. 2013). Therefore, the inclusion of SBM in fish feeds may introduce endocrine-disrupting compounds in the form of phytoestrogens (DiMaggio et al. 2016). SBMs phytoestrogens with estrogenic activity may affect reproductive development and sex differentiation in fish (El-Sayed et al. 2012).The pure forms of phytoestrogens such as genistein and daidzein in SBM were added to fish diets and their effects on growth and sex reversal were investigated by various researchers (Pelissero et al. 1991; Kaushik et al. 1995; Ko et al. 1999; Oca et al. 2005; Hernandez et al. 2007; El-Sayed et al. 2012; Bagheri et al. 2013; Brown et al. 2014; Jourdehi et al. 2014; Chakraborty et al. 2015; Ahmed et al. 2015; DiMaggio et al. 2016; Dong and Qiuyan 2016; Nezafatian et al. 2017). However, there are few studies about the effects of using high levels of SBM in fish feed on the reproductive systems of fish. Bagheri et al. (2013) reported that average egg number, sperm quality, fertilization and hatching decreased in *Carassius auratus*-fed diets added to high SBM. Pelissero et al. (1991) declared that the SBM (30%) based diet raised the plasma vitellogenin level of *Acipenser baeri*. In particular, no study has been detected on fish that reproduce more than one generation yearly. The objective of this study was to evaluate the effect of different levels SBM based diets on the growth, reproduction parameters, gonad, gut and hepatopancreas histology of *Pseudotropheus socolofi*.

Materials and Methods

The experiment conditions and design

In the present study, *Pseudotropheus socolofi* species was preferred, which gives offspring every two months, regarding the reliability of the hypothesis. *Pseudotropheus socolofi* broodstocks were obtained from the Faculty of Eğirdir Fisheries at the Isparta University of Applied Sciences. Each treatment was replicated three times. A total of 75 females with a mean weight of 5.43 g and 15 males with an average weight of 8.52 g ± were randomly distributed (5♀:1♂) in 15 aquariums (30 x 40 x 100 cm).

The experiment fish were fed diets containing SBM additions with three replicates for each treatment group for 90 days. The experimental groups were fed by hand ad libitum twice daily at 8:30 and 20:30. 100-watt thermostat heaters were used to keep the temperature in the aquariums at approximately 27 ± 1 °C. In addition, suitable shelters have been placed where fish can be easily stored. The aquariums cleaned 2 days a week, and the residual feed and feces were siphoned out. The dissolved oxygen ratio ranged from 6.15 to 6.35 mg L^{-1}. Fish were fed for 90 days under a natural lighting environment. Experimental fish were weighed at the beginning of the experiments and 90th day (at the end of the experiments). All fish were weighed individually a day before and at weighing days of fasting. Feed consumption was recorded daily.

Experimental Diets

Experimental diets were isonitrogenic (46% crude protein) and isoenergetic (9%). Five diets were prepared by adding different SBM levels (3%, 16%, 27%, 35% and 44%). The doses were determined based on the highest level of soybean in commercial feeds. The composition of experiment diets is shown in Table 1. Feed ingredients were obtained from a local fish feed producer. All ingredients were ground into small particles (0.5 mm) in a mill. Dietary ingredients were mixed in a mixer. Room temperature water was added to obtain a 30% moisture level. Diets were passed through a mincer with a 2 mm sieve. The pellets were fan-dried and stored frozen at –20 °C until used. YSI Pro Plus multi-measurement set and DAIHAN Wiseven model oven were used to prepare the diets.

Reproductive Performance

The females were observed as daily for spawning activity, and eggs were gently removed from the buccal cavity of females after ovulation. Photos of eggs were taken with a smartphone and the eggs were counted over photos. Fertilized eggs were separated based on the different coloring of unfertilized eggs (Ikhwanuddin et al. 2015). The diameters of eggs were measured with a micrometer using a microscope. The eggs were hatched in a special incubator (Biolife Turbojet Star X6) used for the first time in literature.

Histopathological examination

Histopathological examination was performed on 5 fish from each group. A complete necropsy was performed on each fish and visceral organ samples were collected during the necropsy. Tissue samples were fixed in 10% neutral formalin and processed by automatic tissue processing equipment (Leica ASP300S; Leica Microsystem, Nussloch, Germany). The samples were embedded in paraffin, and 5 μm sections were taken by a Leica RM 2155 rotary microtome (Leica Microsystem, Nussloch,

Germany). Then, sections were stained with hematoxylin and eosin (H&E) for histopathological examination, and periodic acid-Schiff (PAS) staining was used for histochemical analysis. After the coverslip, all slides were microscopically examined under a light microscope.

In the morphometric analysis of the small intestines, the villi length was measured in each fish at 40x with an Olympus CX41 light microscope. Five different villi were measured in each fish for statistical analysis. Morphometric evaluation was performed using the Database Manual Cell Sens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan). The mucous cell counting was conducted in the anterior part of the intestine and the same region of the digestive tract for each fish. The mean number of mucous cells per 100 000 μm^2 of epithelial area in sections was compared and statistically evaluated. Histopathological and histochemical changes were graded in a blinded manner by a specialized pathologist from another university (Luna 1968).

Table 1. Formulation and proximate composition of experimental diets

Ingredients (%)	3%	16%	27%	35%	44%
Fish meal	30	30	30	30	30
Casein	22.5	16.6	11.34	6.85	3
Soybean meal	3	16	27	36	44
Corn starch	17.5	12.89	8.91	4.44	1.44
Corn gluten	5	5	5	5	5
Wheat meal	5.5	5.12	4.77	4.7	4.4
Sunflower meal	7.2	5	3.4	3.01	2.01
Fish oil	6.3	6.39	6.58	7	7.15
Vit[1]+Min[2]	2	2	2	2	2
Pellet binder	1	1	1	1	1
Crude protein (%)	46.24	46.28	46.18	46.29	46.254
Crude fiber (%)	2.44	2.32	2.28	2.48	2.48
Crude matter (%)	76.44	80.46	84.02	88.06	89.34
Crude lipid (%)	9.08	9.18	9.22	9.83	9.99
Crude ash (%)	12.49	11.67	10.80	11.31	11.03
GE:	3761.45	3761.91	3760.47	3761.61	3760.4

Vitamin premix.[1]; per kg, 4,000,000 IU vitamin A, 480,000 IU vitamin D3, 40,000 mg vitamin E, 2400 mg vitamin K3, 4,000 mg vitamin B1, 6,000 mg vitamin B2, 40,000 mg niacin, 10,000 mg calcium D-pantothenate, 4,000 mg vitamin B6, 10 mg vitamin B12, 100 mg D-biotin, 1,200 mg folic acid, 40,000 mg vitamin C and 60,000 mg inositol. Mineral premix.[2]; per kg 23,750 mg Mn, 75,000 mg Zn, 2,000 mg Co, 2,750 mg I, 100 mg Se, 200,000 mg Mg.

NFE: Nitrogen Free Extract = 100-(% Moisture + % Crude protein + % Crude lipid + % ash + % Crude fiber) (Yeşilayar et al. 2020).

GE: Gross energy = (% crude protein×23.6) + (% crude lipids×39.5) + (% carbohydrates×17.3) (Koshio et al. 1993).

Statistical Analysis

The significance of differences among results of the intestinal data was analyzed by one-way analysis of variance (ANOVA). SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) was used to analyze the data.

The variables were assessed by the Bonferroni test, and ANOVA tests were used to compare groups. In comparing ovulation and egg quality, growth performance and gonodasomatic index data in among the groups were used ANOVA test (variance analysis). $P < 0.05$ was considered statistically significant.

Results

Growth Performance

The growth parameters of *P.socolofi* (female 93%; male 7%) fed diets added with different rates of SBM were given in Table 2. No statistical differences were found in terms of specific growth rate (SGR), weight gain (WG) and survival rate among groups ($P > 0.05$). Each other killings when many males in the aquarium

However, among groups, there were statistically significant differences in FCR and final weight ($P < 0.05$). Fish fed with a diet comprising 44 % SBM exhibited the highest feed conversion ratio (FCR) and the lowest final weight compared to other diets as statistical ($P < 0.05$).

Table 2. Growth performance of *P. socolofi* fed different SBM levels for 90 days (mean ± SE)

	3%	16%	27%	35%	44%	df	F	P
Initial weight (g)	5.99 ± 0.06	6.00 ± 0.02	5.90 ± 0.11	5.95 ± 0.03	5.92 ± 0.11	4	0.34	0.85
Final weight (g)	7.95 ± 0.25[a]	7.52 ± 0.30[ab]	7.33 ± 0.21[ab]	7.23 ± 0.11[ab]	7.12 ± 0.28[b]	4	1.83	0.02
WG	1.96 ± 0.30	1.52 ± 0.30	1.33 ± 0.12	1.38 ± 0.14	1.20 ± 0.36	4	1.27	0.35
FCR	3.69 ± 0.60[a]	4.80 ± 1.42[a]	5.26 ± 0.39[a]	4.92 ± 0.58[a]	7.48 ± 2.13[b]	4	5.95	0.01
SGR	0.31 ± 0.04	0.25 ± 0.04	0.23 ± 0.02	0.23 ± 0.02	0.20 ± 0.06	4	1.09	0.41
Survival rate (%)	94.44 ± 5.56	88.89 ± 5.56	100 ± 0.00	83.33 ± 16.67	88.89 ± 5.56	4	0.54	0.71

Significant differences between treatments are indicated with different letter ($P < 0.05$).
Growth parameters were calculated using the following formulas
Weight gain (WG) g = (final body weight (g) - initial body weight (g))
Feed conversion ratio (FCR) = (total feed intake (g)) / (final body weight (g) - initial body weight (g))
Specific growth rate (SGR) % = [(ln final body weight - ln initial body weight)/experiment days] x 100
Survival (%) = 100*(final number fish−initial number fish) / initial number fish.

Table 3. Reproductive performance parameters of *Pseudotropheus socolofi* fed with experimental diets (mean ± SE)

	3 %	**16 %**	**27 %**	**35 %**	**44 %**	**df**	**F**	**P**
Fertility rate (%)	92.87 ± 1.41	94.68 ± 1.19	93.73 ± 1.46	95.31 ± 1.32	92.16 ± 1.57	4	0.65	0.60
Fecundity rate (%)	4.68 ± 0.20	4 ± 0.22	4.43 ± 0.26	4.37 ± 0.26	4.31 ± 0.30	4	1.17	0.33
Hatching rate (%)	89.24 ± 2.67	87.00 ± 3.09	91.50 ± 2.10	80.00 ± 5.00	88.71 ± 3.52	4	0.94	0.45
Egg diameter (mm)	3.01 ± 0.02	2.71 ± 0.18	2.90 ± 0.10	2.72 ± 0.19	2.83 ± 0.10	4	1.17	0.33
GSI	1.99 ± 0.40	1.82 ± 0.76	1.87 ± 0.53	0.79 ± 0.10	0.85 ± 0.35	4	1.16	0.39
Laying broodstock (%)	243.33 ± 54.87	235 ± 111.69	206.67 ± 35.28	196.67 ± 27.28	216.66 ± 20.28	4	0.11	0.98

Fertility rate (%) = (No. of fertilized eggs/No. of total eggs) *100
Fecundity rate (%) = No. of eggs/body weight of female(gr).
Hatching rate (%) = (No. of hatched eggs/No. of fertilized eggs) *100
Gonado somotic index (GSI) = (weight of gonad (g)/weight of fish) *100
Percentage of laying broodstock (%) = (Total number of laying broodstock / Number of female) *100
Significant differences between treatments are indicated with different letter in the same row ($P < 0.05$).

Reproductive performance

The reproductive performance data of *P. socolofi*-fed diets added with different SBM levels are given in Table 3. According to the end of experiment results, no statistical difference was detected between the groups regarding fertilization rate, egg productivity, egg opening rate, egg diameter, gonodosomatic index and percentage of laying eggs ($P > 0.05$).

Histopathological examination of the female

No lesions were observed in any group at the gross examinations. Microscopical analysis revealed that guts were markedly affected by the increased ratio of the SBM. There was no inflammatory reaction, but numerous goblet cells were noticed in the anterior section of the gut in the 3% group. Meanwhile, there was a gradual decrease in goblet cells related to increased SBM additions. Severe inflammatory reaction was also observed in the 44% SBM added group (Figure 1). Statistical analysis results of villi length and goblet cell numbers are shown in Table 4. Our findings indicated that excessive SBM addition to a fish diet can cause harmful effects. No marked lesion in other organs in any group (Figure 2) except ovaries.

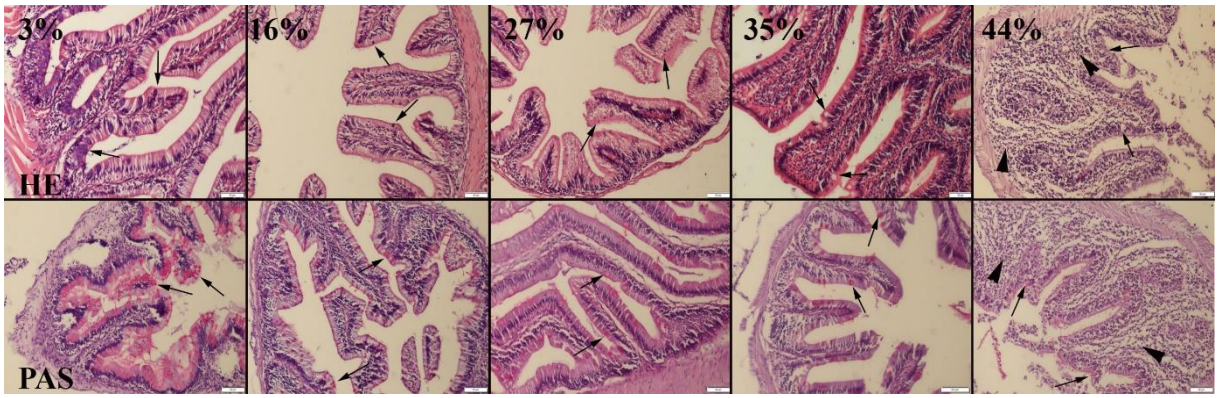

Figure 1. Representative histopathological figures between the group. Decreased number of goblet cells in 16%, 27%, 35% and 44% soy bean added groups compared to 3% group, severe inflammatory reaction 44% added group in gut (arrow head), Bars=50 µm.

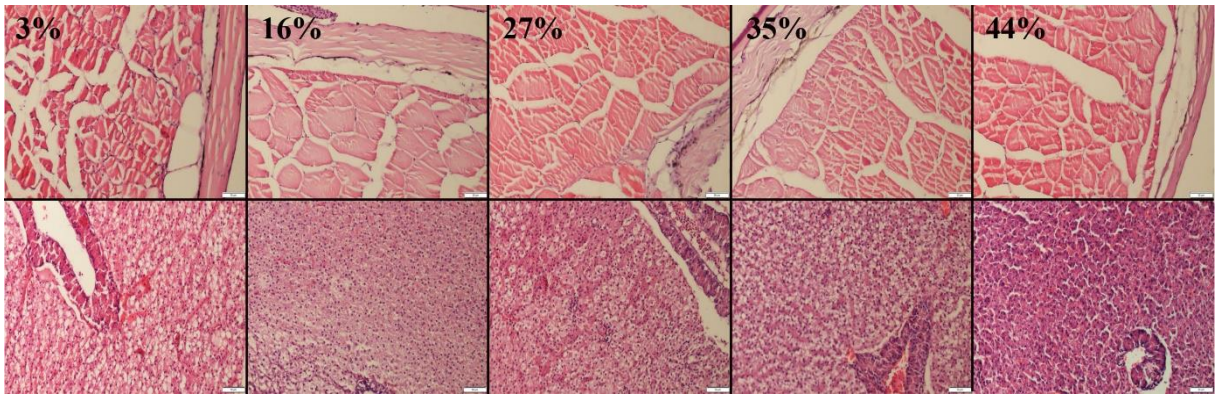

Figure 2. Histopathological appearance of muscle (upper row) and hepatopancreas (bottom row) between the group, HE, Bars=50 µm.

Table 4. Statistical analysis results of the intestinal data

Groups	Goblet cell numbers (mean ± SD)	Villi length (µm) (mean ± SD)
3%	53.80 ± 3.42^{a}	517.60 ± 10.73^{a}
16%	49.60 ± 1.67^{b}	517.40 ± 4.21^{a}
27%	34.40 ± 1.94^{c}	521.00 ± 6.78^{a}
35%	27.20 ± 1.30^{d}	521.40 ± 8.41^{a}
44%	16.40 ± 3.36^{e}	525.20 ± 11.54^{a}
P value	< 0.001	> 0.05

*: One-way ANOVA Bonferroni test

**: The differences between the means of groups carrying different letters in the same column are statistically significant ($P < 0.001$).

The ovarian follicles were microscopically evaluated at five different stages according to the appearance and diameters of the oocyte. At stage I (immature stage), oocyte diameter changed 55 to 60 µm without zona radiating and was irregular. At stage II, the diameter of the oocytes was between 163 to 175 µm throughout the long axis, and they had granular cytoplasm and ellipsoid in shape. At stage III, the oocyte's diameters were changing from 400 to 425 µm, and they had zona radiata with a 2 to 5 µm thickness. They had large cortical vacuoles with wavy margins in this stage. At stage IV, the diameters of the oocytes were between 758 to 825 µm; in addition, they had 8 to 10 µm thick zona radiate and were generally globular in shape. At stage V (maturation stage), oocyst diameter ranged between 843 to 889 µm and with 6 to 7 µm thick zona radiate, the oocytes without nucleus and granules in this stage. SBM caused decreased mature oocytes in ovaries related to ratio and a marked decrease was observed in the 44% added group (Figure 3).

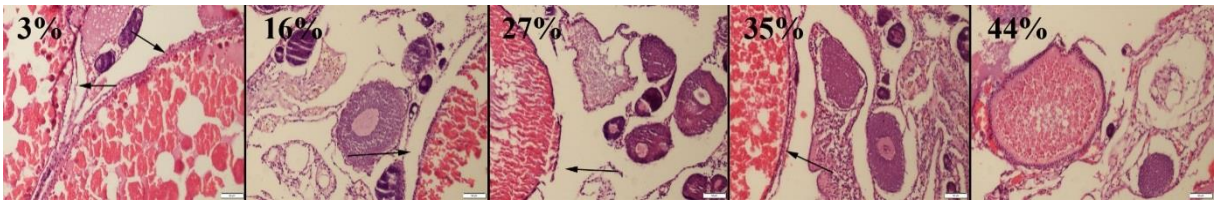

Figure 3. Microscopical appearance of the ovaries between the group, mature oocytes (stage V) (arrows) decreased according to increase of SBM ratio, HE, Bars=50 µm.

Statistical analysis of the percentage of the ovum developmental stages between the groups is shown in Table 5.

Table 5. Statistical analysis of ovum developmental stage percentages between the groups.

Groups	Stage I	Stage II	Stage III	Stage IV	Stage V
3%	9.00 ± 1.22^{a}	13.40 ± 2.51^{a}	21.40 ± 2.79^{ab}	39.20 ± 2.49^{a}	38.00 ± 3.53^{a}
16%	14.60 ± 2.70^{b}	20.80 ± 1.30^{b}	27.00 ± 4.12^{bd}	38.40 ± 8.61^{b}	40.20 ± 1.780^{ab}
27%	18.40 ± 1.51^{b}	25.40 ± 3.13^{c}	43.20 ± 1.92^{c}	24.00 ± 2.54^{c}	43.20 ± 3.76^{b}
35%	26.20 ± 3.89^{c}	41.60 ± 4.03^{d}	24.40 ± 3.64^{d}	18.40 ± 1.51^{d}	8.40 ± 1.14^{c}
44%	41.40 ± 6.10^{d}	42.00 ± 2.34^{d}	17.40 ± 2.07^{a}	10.60 ± 0.89^{e}	5.60 ± 2.60^{c}
P value	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001

* The differences between the means of groups carrying different letters in the same column are statistically significant.
**: Values expressed as mean ± standard deviation.

Discussion

Effect on growth of soybean meal

In this study, the effects on the growth, reproduction parameters of supplementation of different levels of SBM (contains phytoestrogens) to diets were researched. Therefore, diet keeps fishmeal levels constant to avoid affecting reproduction parameters. Generally, there are studies on using SBM as a protein source instead of fish meal in the literature. In the present study, the growth and FCR were adversely affected by increasing SBM levels (especially 44%) in diets. However, a 35% SBM level may be used without adverse effects on the growth and reproduction performance of *Pseudotropheus socolofi*. The antinutrients contained in SBM may adversely affect the growth. Although not directly effective on growth, *Pseudotropheus socolofi*. is not a species fast growing for that is ornamental fish. The number of females in the trial groups was higher than that of males (5♀:1♂); females may have used for gonad development most of the nutrients obtained from

feed. Namely, the growth may be negatively affected by gonadal development. Similarly, Kaushik et al. (1995) indicated that replacing fishmeal with SBM (up to 50%) reduced the growth rate of the rainbow trout. Hernandez et al. (2007) indicated that high levels of SBM (60%) in their diets induced low final weight in *Diplodus puntazzo*. Gu et al. (2016) indicated that SBM caused dose-dependent decreases in growth performance and nutrient utilization of turbot *Scophthalmus maximus*. Krogdahl et al. (2003) reported that growth in Atlantic salmon decreased depending on the raising of the SBM ratio. Li et al. (2012) reported that high SBM levels (45-60%) negatively affected juvenile Japanese seabass growth. In the current study, pure phytoestrogen additions were not made to the diets. Nevertheless, researchers have reported that adding soy phytoestrogens such as genistein and daidzein to fish diets adversely affects growth. Ko et al. (1999) reported that the weight gain decreased in females of yellow perch, *Perca flavescens* fed with genistein diet. DiMaggio et al. (2016) indicated that the administration of diets of high-level genistein reduced the growth and survival of *Paralichthys lethostigma* fry. Dong and Qiuyan (2016) observed negative growth *Oreochromis niloticus* juvenile fed diet supplemented with genistein.

According to histopathological examinations, no remarkable lesions were identified in the muscle and hepatopancreas of all groups. However, a significant reduction of goblet cell numbers was determined in the gut with increasing SBM levels. A severe inflammatory reaction was also observed in the added 44% SBM group. Krogdahl et al. (2003) and Urán et al. (2009) reported that the inclusion of as low as 5–10% SBM level in the diet of Atlantic salmon could produce detrimental inflammatory effects in the intestinal tissues. Gu et al. (2016) indicated that enteritis developed in the distal intestine, and the severity of the inflammation increased depending on the dose of SBM (26–54%) in turbot. Zhang et al. (2018) pointed out that increasing the FM replacement level to 75% in Japanese seabass diets reduced villus height, but not at the 50% level. Similarly, villus height did not change even at the 44% SBM level in this study. This result pointed out that the addition of 44% SBM does not pose a digestive issue.

Effect of soybean meal on reproductive performance

In the present study, the fertilization rate, egg efficiency, egg opening rate, egg diameter, gonadosomatic index and spawning percentage paremeters of *Pseudotropheus socolofi* broodstock fed with diets supplemented with different SBM levels were no significant difference in among groups. However, SBM caused decreasing mature oocytes with increasing ratio and a marked decrease was observed in the 44% added group. There are very few studies investigating the effects of SBM used in fish diets on reproduction; Bagheri et al. (2013) reported that a long-term (5 months) feeding *Carassius auratus* with SBM (35, 65 and 100%) caused a reduction in maturation, fertilization, hatching rates average eggs number with increasing SBM inclusion. Unlike our study, they found a decrease in fertilization and hatching rates. This result may depend on the application of a longer feeding time than our study. Ko et al. (1999) indicated that estrogenic effects on reproductive function were not observed in *Perca flavescens* fed diet supplemented with genistein. Oca et al. (2005) indicated that the supplementation of genistein and daidzein to the diets of Oreochromis niloticus was no effect towards the masculinization of gonads in females. Brown et al. (2014) reported that *β*-sitosterol or genistein did not have significant effects on steroids or gonads of Betta splendens females.

In conclusion, 44% SBM addition to diets of *Pseudotropheus socolofi* negatively affected growth. In pathological examinations, negative effects were observed in gonads while the high SBM levels no affected reproductive parameters of females. The results of this study showed that long-term and high-dose SBM can cause harmful effects in fish, so it should take into account this subject when using SBM.

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