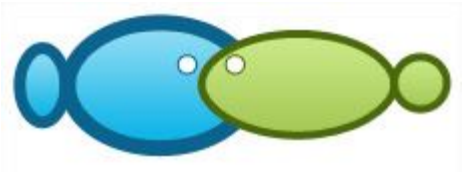

The effect of substituting soybean meal with duckweed (*Wolffia arrhiza*) in the diet of Siamese fighting fish (*Betta splendens* Regan, 1910)

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Abstract. The objective of this research was to investigate the effect of substituting soybean meal (SBM) with *Wolffia arrhiza* (WA) in the diet of Siamese fighting fish (*Betta splendens*) on growth performance, skin color expression, carotenoid accumulation, and intestinal morphology. The experiment was conducted as a completely randomized design with four treatments and three replicates. The experimental diets consisted of: control diet (W0), SBM substitution with 5% WA (W5), 10% WA (W10), and 15% WA (W15). Two-month male fish were obtained from a reliable commercial farm and acclimated to the rearing environment. After a 30-day rearing period, the results showed that the survival rate, growth performance, and feed utilization were not significantly different between treatment groups ($p > 0.05$). The color expression indicated that the fish fed with the control diet showed the highest L* and a* ($p \leq 0.05$). Furthermore, the b* of the fish fed W15 was higher than in all treatment groups ($p \leq 0.05$) followed by the fish fed W10, W5, and W0, respectively. Carotenoid accumulation in each organ increased in all treatment groups. The fish fed with W15 showed the highest carotenoid accumulation in skin and scales including the caudal fin ($p \leq 0.05$). The midgut histological showed that there was a significant increment in intestinal diameter with the dietary substitution of WA 5% (W5) ($p \leq 0.05$). The morphology analysis of intestinal fold height was not significantly different among treatment groups ($p > 0.05$). However, evident differences were detected in intestinal enterocyte height which was significantly decreased in the control group ($p \leq 0.05$). A significant difference in microvillus height were also detected ($p \leq 0.05$).

Key Words: *Betta splendens*, carotenoid accumulation, duckweed, growth performance, protein alternative.

Introduction. *Wolffia arrhiza* (WA) or duckweed is a small aquatic plant which has a round shape, is approximately 1.00 mm in length, and is generally found throughout Thailand. Two species of *Wolffia*, *Wolffia arrhiza* (L.) and *Wolffia globosa* (L.) were discovered in the upper northeast and east of Thailand (Rodroil et al 2012; Ruekaewma et al 2015). WA provides high moisture content of about 95.0% and its frond contains an average protein in dry matter (DM) yield ranging from 18.9 to 36.5% (Castanares 1990; Bhanthumnavin & McGarry 1971; Bergmann et al 2000; Rusoff et al 1980). WA consists of 2.81 to 4.63% fat, 11.6 to 13.54% fiber, 17.6 to 18.19% ash, 0.16 to 1.37% calcium, 0.41 to 0.88% phosphorus, and 3,605 to 4,740 kcal Kg^{-1} of gross energy (Chareontesprasit & Jiwyam 2001; Sirirustananun 2018). Moreover, Jairakphan (1999) reported that WA has complete amino acids and fatty acid. The above information indicates that it is possible to use WA as protein source in animal feed. From previous studies, WA has been a successful substitution for fish meal and soybean meal (SBM) in animal feed including fish feed with reduced feed costs (Haustein et al 1990; Chareontesprasit & Jiwyam 2001; Tavares et al 2008; Ariyaratne 2010; Chantiratikul et al 2010a; Chantiratikul et al 2010b; Suppadit et al 2012; Gogoi et al 2017). Moreover, WA has the potential to be used as human food (Bhanthumnavin & McGarry 1971; Suppadit et al 2008; Ruekaewma et al 2015; Appenroth et al 2017) and utilized in the

treatment of wastewater (Hillman & Culley 1978; Edwards et al 1992). In terms of production, WA also has the advantage of doubling its biomass in 16 hr. to 4 days under optimal nutrition, sunlight availability, water and temperature and it is also easy to harvest (Fujita et al 1999; Suppadit et al 2012). The cultivation of WA has been commercialized in Thailand and Bangladesh (Chantiratikul et al 2010a). In addition to WA containing high nutrients, it has also been found to have beta-carotene of approximately 120-627 mg kg^{-1} DM where the beta-carotene content is higher than that of SBM diet (Suppadit et al 2012). Moreover, Gogoi et al (2017) also reported that the carotenoids content of WA was estimated at 559.76 µg g^{-1} and they play an important role in the regulation of skin and muscle color in fishes (Ezhil et al 2008), making it an interesting source of natural pigment for animals. For all these reasons, the idea of studying the substitution of SBM with WA in the diet of ornamental fish has emerged.

The Siamese fighting fish (*Betta splendens* Regan, 1910) is a freshwater fish that is distributed in all regions of Thailand and it has continued to gain popularity. The size and color expression of fish are important factors in determining the purchase price. Nowadays, synthetic and natural pigments are widely used in ornamental fish diet (Boonyaratpalin & Unprasert 1989; Daniel & Kumuthakalavalli 1991; Gomes et al 2002; James et al 2006; Gupta et al 2007; Ezhil et al 2008; Bagre et al 2012; Gogoi et al 2017; Ninwichian et al 2020). Feeding carotenoids can improve the skin color and market value of ornamental fish (Gupta et al 2007). WA are a viable candidate as an ingredient in fish diet, for both high nutritional value and color enhancement. Therefore, the objective of this study was to investigate the effect of substituting SBM with WA in the diet of Siamese fighting fish on growth performance, skin color expression, carotenoid accumulation, and intestinal morphology.

Material and Method

Experimental design. Two-month old male Siamese fighting fish were obtained from a reliable commercial farm and acclimatized to the rearing environment for a week prior to feeding them the experimental diets. One-liter clear plastic bottle were used and fish were randomly distributed, 1 fish to each bottle. The experiment was carried out using completely randomized design with 4 treatments and 3 replicates (25 fish for each replication). The four treatments consisted of four different amounts of WA replacing the SBM at 0% (W0), 5% (W5), 10% (W10), and 15% (W15) (Table 1).

Table 1

Formulation (% dry weight) and proximate analysis of experimental diets

Ingredients (%)	*Experimental diets*			
	W0	*W5*	*W10*	*W15*
Fish meal	26	26	26	26
Soybean meal	40	35	30	25
Wolffia arrhiza	0	5	10	15
Cassava starch	10	10	10	10
Wheat flour	5	5	5	5
Corn meal	10	10	10	10
Soybean oil	4	4	4	4
Di-calcium phosphate	1	1	1	1
Vitamin and mineral premix[a]	1	1	1	1
Vitamin C	2	2	2	2
Butylated hydroxytoluene (BHT)	1	1	1	1
Proximate analyses				
Crude protein (%)	35.28	34.36	33.43	32.51
Crude fat (%)	7.00	7.06	7.15	7.23
Crude fiber (%)	4.33	4.60	4.88	5.12
Ash (%)	9.04	10.12	10.57	10.94
Moisture (%)	9.64	9.45	9.79	9.68
Gross energy (kcal Kg^{-1})	3076	3088	3101	3115
Total carotenoid (µg g^{-1})	180.14	260.09	290.88	350.81

[a] Vitamin premix provided per kg of diet: V_A 12,000,000 IU; V_D 2,000,000 IU; V_E 6000 IU; V_K 2000 mg; V_{B1} 800 mg; V_{B2} 2500 mg; V_{B6} 800 mg; V_{B12} 10 mg. Mineral provided per kg of diet: Mn 18 mg; Mg 200 mg; Co 0.1 mg; I 0.25 mg; Fe 140 mg; Cu 2.5 mg; Zn 65 mg; Se 0.2 mg.

The fish were fed to satiation twice a day (09.00 A.M. and 3 P.M.) for 30 days. The experiment was conducted at facilities of Department of Fisheries, Faculty of Agriculture and Technology, Rajamangala University of Technology Isan, Surin campus from September to December 2020.

Experimental diet. WA were collected from the reservoir of Rajamangala University of Technology Isan, Surin campus, Thailand. The fresh WA were washed, cleaned, and dehydrated by hot air oven at 65°C until the moisture content less than 10% and then finely ground into meshes. The dried WA samples were evaluated for proximate analysis using the method of AOAC (2005) and the results are shown in Table 2. Dried WA were stored in airtight bags until use. The feed ingredients were ground and mixed according to the experimental design. The experimental diets were pelleted by using extruder machine and dried in a hot air oven at 65°C for 12 hr. The diets were ground into a size between 2-3 mm and they were analyzed for nutrients composition using proximate analysis (AOAC 2005) with the results presented in Table 2.

Table 2

Mean value of proximate analysis on dry weight basis of duckweed

Proximate analysis	*Means ±SD*
Crude protein (%)	29.62±7.33
Crude fat (%)	2.65±1.02
Crude fiber (%)	12.54±2.08
Ash (%)	18.00±1.44
Calcium (%)	1.52±0.60
Phosphorus (%)	0.78±0.48
Gross energy (Kcal Kg^{-1})	3475±200.32
Carotenoid content (µg g^{-1})	542.85±69.04

Data collection. The survival rate, growth performance and feed utilization following: total length, specific growth rate (SGR), average daily gain (ADG), and feed conversion ratio (FCR) were recorded before and after the 30-day rearing period and calculations were done according to the method of Ariyaratne (2010):

$$\text{ADG (g fish}^{-1}\text{ day}^{-1}\text{)} = \frac{\text{Final weight of fish - initial weight of fish}}{\text{Rearing period}}$$

$$\text{SGR (\% day}^{-1}\text{)} = \frac{\text{Ln(final weight) - Ln(initial weight)}}{\text{Experiment duration}} \times 100$$

$$\text{FCR} = \frac{\text{Dry weight of given feed (g)}}{\text{Weight gain (g)}} \times 100$$

$$\text{Survival rate (\%)} = \frac{\text{No. of harvested fish}}{\text{No. of stocked fish}} \times 100$$

Twelve fish of each replicate were collected to analyze the color expression by using the Color Reader Model, and it was calibrated to white and black standard sites to measure values for lightness (L*), redness (a*) and yellowness (b*).

Carotenoid content in the experimental diet was analyzed following the method of Gogoi et al (2017). The diets were weighted as much as 0.5 g by using precision digital balance (FX-2000i, A&D Company Limited) and homogenized by using tissue homogenizer with 10 mL dimethyl sulfoxide (DMSO) solvent. Then, the samples were centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was separated and 0.5 mL of it mixed with 4.5 mL of the respective solvent. The solution mixture was analyzed for chlorophyll-*a*, chlorophyll-*b*, and carotenoids content by a UV/vis spectrophotometer.

The total carotenoid in each organ of the fish were analyzed including the skin and scales as well as the caudal fin. One g of samples was weighed and placed in a 10-mL screw-capped clear glass vial. Then, 2.5 g of anhydrous sodium sulphate was added and the sample was gently mashed with a glass rod against the side of the vial until it reasonably mixed well with sodium sulphate. To this sample, 5 mL of chloroform was

added and the vial was sealed and placed at 0°C overnight. When the chloroform formed a clear 1-2 cm layer above the caked residue, the optical density was read at 380, 410, 440, 450, 460, 475, and 500 nm in spectrophotometer then 0.3-mL aliquots of chloroform were taken and diluted with absolute ethanol in a column of 3 mL. A blank prepared in similar manner was used for comparison. The wavelength at which maximum absorption was obtained was used for the calculation. The total carotenoids content was calculated as µg g^{-1} following Gogoi et al (2017).

For the intestinal morphology, approximately 0.5 cm length segments of midgut samples were fixed with 10% formalin solution and dehydrated in ethanol, embedded in paraffin, cut by microtome at 4 µm sections, and stained with hematoxylin and eosin (H&E) according to standard histology procedures. Tissue slides were digitally photographed with a light microscope (Nikon Eclipse Ci) equipped with a CCD camera and NIS-elements D software. Intestinal diameter (ID), fold height (FH), enterocyte height (EH), and microvillus height (MH) were measured according the method of Peng et al (2013).

Statistical analysis. Mean values and standard deviations (S.D.) were calculated. The significance of difference among treatments was tested using One-way analysis of variance (ANOVA) and Duncan's multiple range test (DMRT) at 0.05 of significance level. Statistical analysis was performed with IBM SPSS statistics version 20.

Results. The growth performance of the fighting fish fed with experimental diets for 30 days are shown in Table 3. The results showed that the final weight, final length, SGR, ADG were not significantly different between treatment groups ($p > 0.05$). Feed utilization in terms of FCR were also not significantly different between treatment groups ($p > 0.05$). A 100% survival rate was observed in all treatments groups.

Table 3

Growth performance and feed utilization of *Betta splendens* fed with experimental diets for 30 days

Performance	*Experimental diets*				*P-value*
	W0	*W5*	*W10*	*W15*	
Initial weight (g $fish^{-1}$)	0.90±0.05	0.86±0.04	0.88±0.05	0.91±0.04	0.500
Initial length (cm $fish^{-1}$)	4.13±0.31	4.07±0.23	4.14±0.24	3.96±0.20	0.174
Final weight (g $fish^{-1}$)	1.53±0.03	1.51±0.03	1.47±0.03	1.51±0.02	0.129
Final length (cm $fish^{-1}$)	4.38±0.27	4.41±0.25	4.40±0.22	4.27±0.21	0.340
SGR (% day^{-1})	1.78±0.15	1.89±0.10	1.71±0.20	1.68±0.14	0.392
ADG (g day^{-1})	0.02±0.00	0.02±0.00	0.020±0.00	0.02±0.00	0.248
FCR	3.54±0.04	3.49±0.10	3.47±0.11	3.55±0.03	0.500
Survival rate (%)	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	

Data represented as mean±SD.

The results of color measurement on skin of the fish is shown in Table 4. The initial color measurement in terms of lightness (L*), redness (a*), and yellowness (b*) were not significantly different between treatment groups ($p > 0.05$).

Table 4

Color measurement on skin of *Betta splendens* fed with experimental diets for 30 days

Parameters	*Experimental diets*				*P-value*
	W0	*W5*	*W10*	*W15*	
		0-day rearing period			
Lightness (L*)	26.44±1.46	27.00±0.63	26.83±0.88	26.76±1.05	0.519
Redness (a*)	4.98±0.65	5.16±0.67	5.22±0.80	5.06±0.90	0.834
Yellowness (b*)	4.19±0.71	3.86±0.57	4.16±0.70	3.82±0.70	0.296
		30-day rearing period			
Lightness (L*)	$27.29±1.28^{b}$	$25.26±1.28^{a}$	$25.61±1.50^{a}$	$26.02±0.87^{a}$	< 0.001
Redness (a*)	$4.91±0.84^{b}$	$4.37±0.48^{a}$	$4.16±0.75^{a}$	$4.27±0.73^{a}$	0.028
Yellowness (b*)	$2.89±1.13^{a}$	$4.98±1.31^{b}$	$4.98±1.16^{b}$	$5.67±0.94^{c}$	< 0.001

Data represented as mean±SD. Different superscripts in the same row indicate significant differences at $p \leq 0.05$.

After 30-day rearing period, the fish fed with control diet showed the highest L* and a* ($p \leq 0.05$) while the L* and a* of the fish fed with test diet (W5, W10, and W15) were not significantly different between treatment groups ($p > 0.05$). In addition, the b* of fish fed W15 was higher than in all treatment groups ($p \leq 0.05$) followed by the fish fed W10, W5, and W0, respectively.

Carotenoid accumulation (µg g^{-1}) in each organ of the fish fed with different experimental diets is shown in Table 5. The results showed that the total carotenoid in skin and scales including the caudal fin of fish at the start of the trial was not significantly different between treatment groups ($p > 0.05$). After the 30-day rearing period, the carotenoid accumulation in each organ increased in all treatment groups. The fish fed with W15 showed the highest carotenoid accumulation in skin and scales including the caudal fin ($p \leq 0.05$) followed by the fish fed with W10, W5, and control diet, respectively. The results showed the highest carotenoid values in skin and scales followed by the caudal fin in all treatment groups.

Table 5

Carotenoid accumulation (µg g^{-1}) in each organ of *Betta splendens* fed with different experimental diets for 30 days

Organ	*Experimental diets*				*P-value*
	W0	*W5*	*W10*	*W15*	
			0-day rearing period		
Skin and scales	12.42±0.18	12.47±0.17	12.44±0.21	12.45±0.19	0.367
Caudal fin	10.02±0.05	10.03±0.04	10.03±0.06	10.01±0.04	0.420
			30-day rearing period		
Skin and scales	19.17±0.15^{a}	32.19±0.31^{b}	38.82±0.59^{c}	40.40±0.31^{d}	< 0.001
Caudal fin	17.48±0.33^{a}	29.04±0.23^{b}	33.85±0.42^{c}	36.51±0.46^{d}	< 0.001

Data represented as mean±SD. Different superscripts in the same row indicate significant differences at $p \leq 0.05$.

The results of midgut histological evaluation of fighting fish fed with experimental diets for 30 days are shown in Figure 1 and detailed in Table 6. There was a significant increment of intestinal diameter in the dietary substitution of SBM with WA 5 % (W5) ($p \leq 0.05$). The morphology analysis of intestinal fold height was not significantly different among treatment groups ($p > 0.05$). The fold height values were between 993.72-1102 µm. However, evident differences were detected in intestinal enterocyte height which was significantly decreased in the control group ($p \leq 0.05$). A significant difference in microvillus height were also detected ($p \leq 0.05$). The fish fed with W15 showed the highest microvillus height followed by the ones fed with W10, W5, and W0, respectively.

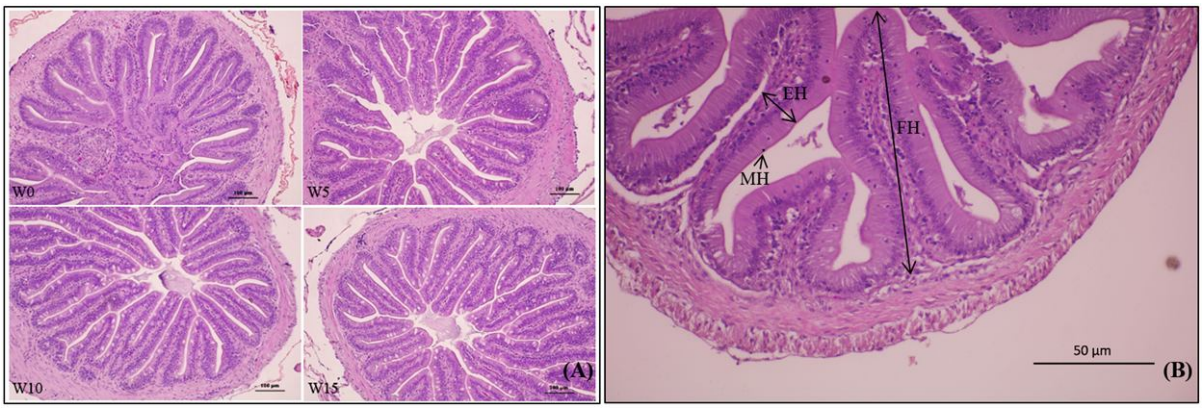

Figure 1. Midgut section photomicrographs of *Betta splendens* fed with experimental diets were displayed in a lower magnification of objective lens of microscope (magnification ×10) (Figure 1A). Fold height (FH), enterocyte height (EH), and microvillus height (MH) were analyzed in a higher magnification of objective lens of microscope (magnification ×40) (Figure 1B).

Table 6

Intestinal morphology analysis of *Betta splendens* fed with different experimental diets

Experimental diets	*Intestinal micromorphology (µm)*			
	Intestinal diameter (ID)	*Fold height (FH)*	*Enterocyte height (EH)*	*Microvillus height (MH)*
W0	4026.5±44.83^{a}	993.72±88.01^{a}	31.93±0.58^{a}	2.80±0.26^{a}
W5	4253.1±49.66^{b}	1063.00±94.75^{a}	33.27±0.89^{b}	3.34±0.31^{b}
W10	4067.6±17.08^{a}	1102.93±88.27^{a}	34.45±1.11^{b}	3.54±0.23^{b}
W15	4036.0±61.22^{a}	1019.59±49.42^{a}	34.56±0.72^{b}	3.98±0.48^{c}
P-value	< 0.001	0.297	0.030	< 0.001

Data represented as mean±SD. Different superscripts in the same row indicate significant differences at $p \leq 0.05$

Discussion. No statistically significant differences were observed on growth performance and survival rate of the fish. From the results, it was observed that using WA instead of SBM did not negatively affect the growth performance of *B. splendens* as similarly to the utilization of colorants from other pigment sources in aquaculture feeds such as, sweet potato, *Phaffia rhodozyma*, *Paracoccus* sp., *Haematococcus pluvialis* and astaxanthin diet (Seyedi et al 2013; Ninwichian et al 2020; Kattakdad et al 2021). However, Sirirustananun (2018) studied the effect of WA on growth performance of Nile tilapia (*Oreochromis niloticus*) fingerlings and their results showed that growth performances of fish fed with considerable WA tended to be lower than that of the fish fed with commercial diet. It was indicated that the utilization pattern of WA by direct feeding may be unfavorable for fish consumption because WA had limited water stability. Conversely, the study of Ariyaratne (2010) showed that Nile tilapia fish fed with WA had specific growth rate lower than the fish fed with commercial diet. The utilization of WA in aquatic animal fee needs to be maintained in a suitable level for each species. From the results, the utilization of WA at 15% in the diet of *B. splendens* was suggested. According to the research of Chareontesprasit & Jiwyam (2001), the results revealed that the highest total production occurred in the fish fed with formulated ration containing 15% *Wolffia* meal which could be successfully used in place of soybean meal but the amount being used should not exceed 15%.

The total carotenoid of the experimental diets was found to increase with an increase in the levels of WA which resulted in different color measurement on skin of the experimental fish after 30 days of the test. The fish fed with WA showed significantly decreased lightness and yellowness while there was a significant increase in the redness resulting from the different amounts of carotenoids in the experimental diets. The redness value generally shows a positive relationship with carotenoids as it increases as the carotenoid content (Gogoi et al 2017). Skrede & Storebakken (1986) reported that there was a close relationship between instrumental color measurements and carotenoid concentration. This was similar to the results in the present study. The results indicated that higher levels of WA also resulted in higher carotenoids accumulation and there were more deposits in the skin and scales than in the caudal fin. This suggests that high carotenoid content can promote an increase in skin pigmentation (Gomes et al 2002). The present study was in conformity with the report of *Botia dario* (Gogoi et al 2017) and laying hens (Chantiratikul et al 2010b) that skin pigmentation of chicken increased with increase in dietary *Wolffia* meal. Thongprajukaew et al (2012) reported that *B. splendens* had the highest carotenoid accumulation in muscle (44-51%) followed by skin, caudal fin, anal fin, dorsal fin, pelvic fins, and pectoral fins, respectively. Carotenoids are synthesized by plants and microorganisms including algae and photosynthetic microorganisms. Aquatic animals cannot synthesize carotenoids, but they are able to absorb carotenoids from their diet and store them in adipose tissue (Parker 1996; Rao & Rao 2007). The accumulation of carotenoids affects the expression of yellow, orange, and red colors in fish skin pigmentation. However, the accumulation of carotenoids in fish changes throughout their life depending on many factors such as, pigment source, concentration, length of carotenoids feeding, dietary ingredients present in the diet,

genetics, age (reproductive age), sex, species digestion, and absorption capacity (Thongprajukaew et al 2012; Ezhil & Narayanan 2013; Kelestemur & Coban 2016). According to Suppadit et al (2012), WA contains approximately 120-627 mg kg^{-1} DM of beta-carotene. Beta-carotene is one of the carotenoids responsible for the orange and red pigmentation of fish and it has also been shown to have other biological and nutritional functions essential for growth and health.

In the present study, intestinal micromorphology in terms of ID, FH, EH, and MH were evaluated. The results indicated that higher levels of WA significantly increased EH and MH. Higher SBM levels in the diet induced negative influence on intestinal micromorphology. According to the research of Van den Ingh et al (1991) and Chen et al (2011), it was observed that the expanded structure of intestine would rapture when fed with high SBM levels, which could be related to anti-nutritional factors such as lectin or saponin in SBM. Carotenoids, such as beta-carotene and astaxanthin can improve and contribute to the gut immune system, thereby preventing and/or delaying the development of dysbiosis, that is a broad spectrum of imbalanced gut microbiota which causes the disease (Lyu et al 2018). It was demonstrated by Perera & Yen (2007) that the bile salt micelles serve as a reservoir for carotenoids and that carotenoids move from the bile salt micelles through the microvilli membrane to deliver their contents to the apical portion of the enterocytes. There is general agreement that the rate of transfer is dependent on the concentration of carotenoids and that the carotenoids are taken up by the mucosal cell. Thus, the utilization of WA in fish diet can increase the concentration of carotenoids, which can improve gut health.

Conclusions. The substitution of SBM with WA in the diet of Siamese fighting fish showed non-significant differences in growth performance, feed utilization, and survival rate but it was positively affected the color expression, carotenoid accumulation, and gut health. Thus, it can be concluded that a diet of WA can substitute SBM as it had positive effects on Siamese fighting fish. The research suggests replacing SBM with WA at 15% as it was the highest level of this experiment. Further studies on the increased level of SBM replacement with WA are needed.

Acknowledgements. The authors would like to express their thanks for the support from the Faculty of Agriculture and Technology, Rajamangala University of Technology Isan Surin Campus, Surin, Thailand.

Conflict of interest. The authors declare that there is no conflict of interest.

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Received: 02 September 2021. Accepted: 19 November 2021. Published online: 24 December 2021.
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How to cite this article:
Kattakdad S., Suratip N., Phungam N., Udduang S., 2021 The effect of substituting soybean meal with duckweed (*Wolffia arrhiza*) in the diet of Siamese fighting fish (*Betta splendens* Regan, 1910). AACL Bioflux 14(6):3654-3663.