Effects of Replacing Squid Oil with Dietary Vegetable Oils on Growth, Fatty Acid Composition and Expression Levels of *Fatty Acyl Delta6 Desaturase* mRNAs in Black Ear Catfish, *Pangasius larnaudii*

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ABSTRACT

A 6-month feeding trial on four experimental diets containing squid oil (SO), palm oil (PO), soybean oil (SBO) and sunflower oil (SFO) in juvenile black ear catfish (*Pangasius larnaudii*) was performed. Fish fed each source of vegetable oils exhibited a higher final weight and ADG (months 4 – 6) than those fed the SO diet ($P < 0.05$). Final weight gain (FWG) and specific growth rate (SGR) of fish fed PO and SBO were greater than those fed with SO diet ($P < 0.05$). The EPA/ARA ratio in muscle of fish fed SO and PO was higher than that of fish fed the SFO diet ($P < 0.05$). In addition, the full-length cDNAs of *fatty acyl delta6 desaturase* (*Plfadsd6*; 1748 bp) were isolated. Its expression levels in fish fed the PO diet were greater than those fed other diets ($P < 0.05$). *PlFadsd6* in fish fed SO was not significantly different from fish fed SBO ($P > 0.05$) but greater than those fed the SFO diet ($P < 0.05$). Results of the present study suggest that the diet containing SO may be more appropriately replaced with PO than SBO and SFO in the grow-out diet for *P. larnaudii*.

Keywords: vegetable oils, *fatty acyl delta6 desaturase* mRNAs, *Pangasius larnaudii*

INTRODUCTION

Source of oil in aquaculture feed relies mainly on fish oil (and squid oil) which contain high n-3 HUFA and essential fatty acids (EFA). The demand for fish oil has consistently increased for farming activities (Barlow, 2000). Nevertheless, the reduction in supply and an increase in prices of fish oil have resulted in efforts to partially or completely substitute with vegetable oils in aquafeeds (Tocher *et al.*, 2001; Bransden *et al.*, 2003; Bahurmiz and Ng, 2007; Izquierdo *et al.*, 2008; Jaya-Ram *et al.*, 2011; Gao *et al.*, 2012; Ren *et al.*, 2012).

Pangasiid catfish have been cultured in the Mekong Delta since the 1960s for local consumption (Van Zalinge *et al.*, 2002). Black ear catfish (*Pangasius larnaudii* Bocourt, 1866) a member of Family Pangasiidae, is one of main species cultured in Mun River, Ubon Ratchathani Province (Chansri *et al.*, 2002). It is an omnivorous species with very broad natural diets (Termvidchakorn and Hortle, 2013). The harvest size is approximately 1–2 kg with a culture period of 1–2 years.

Small-scale fish farmers search for feeds which are alternative to commercial feeds containing fish meals and/or fish/squid oils. Unlike marine fishes, freshwater teleosts can effectively use polyunsaturated fatty acid (PUFA) precursor like linoleic (LA) and α-linolenic (ALA) acids for the synthesis of C20/22 highly unsaturated fatty acid (HUFA) (Sargent *et al*., 2002; Hastings *et al*., 2005). As a result, several vegetable oils have been partly or completely substituted in fish diets across a variety of freshwater species (Bell *et al*, 2002; Bransden *et al*., 2003; Fonseca-Madrigal *et al*., 2005)

Variable compositions of fatty acid are found from different sources of vegetable oils, for example, palm oil (PO) is rich in saturated fatty acid (SFA) while soybean oil (SBO) and sunflower oil (SFO) are rich in LA but linseed oil is rich in ALA (Zambiazi *et al*., 2007). The suitable replacement of fish oils depends on the efficiency of fish species that can convert C18 PUFA to C20/22 HUFA (Sargent *et al*., 2002). The fatty acid profiles of fish liver (reflecting lipid biosynthesis efficiency) and muscle (reflecting the quality of fillets) partially are influenced by the fatty acid composition in diets.

PUFA, particularly LA and ALA, plays an important role as the precursor of HUFA biosynthesis (Hastings *et al*., 2005). The processes require the activities of fatty acyl desaturase (Fads) and very long-chain fatty acyl elongase (Elovl) enzymes. Several studies reported an increase in their transcript levels in freshwater fish (e.g. *Danio rerio* and *Oreochromis niloticus* and *Cyprinus carpio*) fed diets containing vegetable oils (Bell *et al*., 2001; Tocher *et al*., 2002b; Zheng *et al*., 2005; Ren *et al*., 2012). Effects of various vegetable oils in feed diets on the expression of either enzyme in cultured freshwater fish should be studied.

In this study, the possibility to replace squid oil with different vegetable oils (PO, SBO and SFO) in the grow-out diets of *P. larnaudii* was investigated. Effects of different formulated diets on the expression levels of *fatty acyl delta6* desaturase (*PlFadsd6* also called *Fads2*, and *Fadsd6-like*) mRNA were examined using quantitative real-time PCR. In addition, lipid compositions in muscle of the experimental fish were also examined.

MATERIALS AND METHODS

Experimental animal, diet formulation and feeding trial

SO, PO, SBO, and SFO were purchased from a local market in Ubon Ratchathani. Four diets with the same basal composition (40% protein and gross energy 420.50 Kcal $\cdot$100 g^{-1}), but formulated with different sources of oils. SO, PO, SBO, and SFO were subsequently produced. The ingredients were identified by proximate analysis (AOAC, 1997; Table 1). Fingerlings of *P. larnaudii* were obtained from Yasothon Inland Fisheries Research and Development Center, Department of Fisheries, Thailand. Twenty individuals (average body weight = 20.66 ± 3.44 g for overall specimens) were randomly placed into each cage (1x1x1.5 m). Fish were acclimated at the ambient conditions (28 ± 2ºC and natural light) for 2 weeks and fed with a commercial diet. A 6 month experiment was carried out in triplicate. Experimental fish were fed twice daily until satiation with four different formulated diets and the body weight was monthly measured. At the end of the experimental period, muscle and liver were dissected out (*N* = 9, 9, 9 and 9 for SO, PO, SBO, and SFO diets, respectively) and the expression level of *PlFadsd6* mRNA was examined using quantitative real-time PCR. Fatty acid profiles were analyzed by gas chromatography (AOAC, 1997). During the experiment, water quality (dissolved oxygen, pH, water temperature, and unionized ammonia) was monitored every two weeks.

Animal care and all experimental procedures were approved by the Animal Experiment Committee, Ubon Ratchathani University (Approval no.21/2552).

Table 1. Formulation (g·100 g^{-1} diet), proximate analysis (% dry matter basis) and fatty acid composition (% of total fatty acids by weight) of experimental diets

Component	Diet			
	SO	PO	SBO	SFO
Ingredients				
Fish meal	35.0	35.0	35.0	35.0
Soybean meal	44.0	44.0	44.0	44.0
Rice bran	7.0	7.0	7.0	7.0
Cassava	5.0	5.0	5.0	5.0
Rice mill	3.0	3.0	3.0	3.0
Squid oil	4.0	0	0	0
Palm oil	0	4.0	0	0
Soybean oil	0	0	4.0	0
Sunflower oil	0	0	0	4.0
Vitamin premix	2.0	2.0	2.0	2.0
Total ingredients	100	100	100	100
Proximate composition				
Protein (%)	40.34	40.52	40.61	40.32
Fat (%)	7.87	7.76	7.93	7.93
Fiber (%)	2.65	3.30	2.91	2.79
Moisture (%)	2.92	2.78	2.67	3.74
Ash (%)	13.27	13.17	13.01	13.52
Fatty acid composition				
Saturated fatty acids	22.30	42.00	13.80	10.30
Monounsaturated fatty acids	37.60	12.10	23.10	14.70
Polyunsaturated fatty acids	32.20	9.41	64.80	70.99
Linoleic acid	1.20	9.20	57.20	70.67
α-Linolenic acid	10.40	0.21	7.50	0.32

Preparation of total RNA and first-strand cDNA

Total RNA was extracted from a piece of liver (approximately 50 mg) of each fish using Trizol following the protocol recommended by the manufacturer (Invitrogen). The concentration of extracted total RNA was spectrophotometrically measured (Sambrook and Russell, 2001). One and a half microgram of DNase I-treated total RNA was reversed-transcribed using an ImProm-II™ Reverse Transcription System (Promega).

Degenerate primer design and PCR

Protein sequences of fatty acyl desaturase (Fads) from zebrafish, *Danio rerio* (accession no. AAG25710), Atlantic salmon, *Salmo salar* (AAL82631), rainbow trout, *Oncorhynchus mykiss* (AAK26745) and gilthead seabream, *Sparus aurata* (AAL17639) were retrieved from GenBank and multiple sequence alignments were performed using Clustal W (Thompson *et al*., 1994). Degenerate primers for amplification of the partial ORFs of *PlFadsd6* (primers Fad-Degen-F/R, Table 2) were designed. PCR was carried out composing of predenaturation at 94°C for 3 min followed by 30 cycles of 94°C for 30 s, 55°C for 45 s and 72°C for 90 s. The final extension was carried out at 72°C for 7 min. The resulting products were size-fractionated through agarose gels. The amplification fragment was eluted from the gel, cloned into pGEM-T Easy

Rapid amplification of cDNA end-polymerase chain reaction (RACE-PCR) of *PlFadsd6*

The full-length cDNAs of *PlFadsd6* were isolated using 5'- and 3'-RACE-PCR using

Table 2. Nucleotide sequences of primers used for isolation, characterization and expression analysis of *Plfadsd6*

Application	Primer name	Primer sequence
Plfadsd6		
Degenerate RT-PCR	Fads-deg-F	5'-AGCAYGACTWCGGYCAYCTGTC-3'
	Fads-deg-R:	5'-GTSACCCAHACAAACCAGTG-3'
5'RACE	Fads-5'RACE	5'-GACCACGTTGGGCTTAGCATGGTGCTG-3'
3'RACE	Fads-3'RACE	5'-CACAACGGGATTGGGTGGATTTCGCC-3'
RT-PCR	Fads-RT-F	5'-CATGACTTCGGTCATCTGTCAG-3'
	Fads-RT-R	5'-GCTGATGGTTATAGGGCATGTTC3'
Quantitative real-time PCR	Fads-qRT-F	5'-CACTTTCAGCACCATGCTAAGCC-3'
	Fads-qRT-R	5'-GCTGATGGTTATAGGGCATGTTC3'
β-actin		
RT-PCR	Actin-RT-F	5'-AGAGAGAAATTGTCCGTGACATC-3'
	Actin-RT-R	5'-CTCCGATCCAGACAGAGTATTTG-3'
Quantitative real-time PCR	Actin-qRT-F	5'-GGTACCACCATGTACCCTGGCAT-3'
	Actin-qRT-R	5'-CTCCGATCCAGACAGAGTATTTG-3'

a SMART™ RACE cDNA Amplification Kit (Clonetech). RACE-PCR was carried out in a 50 µl reaction mixture containing 1 µl of the template cDNA, 1x *Taq* buffer, 1.0 mM MgCl$_2$, 0.2 mM each dNTP, 0.2 mM each primer (see RACE primers in Table 2), 1 unit *Taq* DNA polymerase. PCR was carried out with the thermal cycling of 94ºC for 3 min, followed by 35 cycles of 94ºC for 30 s, 62ºC for 30 s, 72ºC for 1.5 min. The final extension was carried out at 72ºC for 10 min. RACE-PCR products were electrophoresed in a 1.2% agarose gel. Each gel-eluted product was ligated with pGEM-T Easy vector, transformed into *E. coli* JM 109 and sequenced for both directions. Nucleotide sequences of RACE-PCR fragments and that from degenerate PCR were assembled and searched against data in GenBank using Blast*N* and Blast*X* (Altschul *et al.*, 1990; http://ncbi.nlm.nih.gov). The p*I* value and molecular weight of the deduced proteins were examined using ProtParam (http://www.expasy.org/tools/protparam.html). The functional domains in the deduced proteins were predicted using SMART (http://smart.embl-heidelberg.de).

Phylogenetic analysis

The deduced amino acid sequence of *PlFadsd6* (accession no. AGR45585) was phylogenetically compared with Fadsd6, Fadsd5, Fads2 or Fads2-like of *Haplochromis burtoni* (XP_005941747), *Maylandia zebra* (XP_004553069), *Oreochromis niloticus* (XP_005470691 and AGV 52807), *Pundamilia nyererei* (XP_005730157), *Fundulus heteroclitus* (XP_012720846 and XP_012720374), *Solea senegalensis* (accession no. AEQ92868), *Oryzias lapites* (XP_004069637), *Poecilia formosa* (XP_007556957), *Lates calcarifer* (ACY25091), *Scatophagus argus* (AHA62794), *Epinephelus coioides* (ACJ26848), *Sparus aurata* (ADD50000), *Dicentrarchus labrax* (accession no. ACD10793), *Anguilla japonica* (AHY22375), *Salmo salar* (NP_001165752, XP_014025862 and NP_001117014), *Oncoryhnchus masou* (ABU 87822), *Onchorhynchus mykiss* (AFM77867), *Danio rerio* (NP_571720), *Cyprinus carpio* (AIA 19310), *Astyanax mexicanus* (XP_007235183), *Tachysurus fulvi* (AJQ20793), *Clarias macrocephalus* (AGR45589), *Pangasianodon hypophthalmus* (AFN21428), *Xenopus* (*Silurana*) t*ropicalis* (NP_001120262), *Rattus norvegicus* (AEX15918), *Mus musculus* (NP_062673), *Bos taurus* (NP_001076913) and *Homo sapiens* (NP_004256). Multiple alignments were carried out with Clustal W (Thompson *et al.*, 1994). A bootstrapped neighbor-joining tree bootstrapped 1000 times (Saitou and Nei, 1987) was constructed using MEGA 6.0 (Tamura *et al.*, 2013).

RT-PCR and tissue distribution analysis

The expression of *PlFadsd6* (primers Fads-RT-F/R) in various tissues of *P. larnaudii* (4-month-old females) was analyzed by RT.PCR. *β-actin* (primers actin-F/R, Table 2) amplified from the same template was included as the positive control. The thermal profiles were 94ºC for 3 min followed by 30 cycles of denaturation at 94ºC for 30 s, annealing at 55 ºC for 30 s and extension at 72ºC for 1.5 min. The final extension was carried out at 72°C for 10 min. The amplicon was electrophoretically analyzed on a 1.5% agarose gel and visualized with a UV transilluminator after ethidium bromide staining (Sambrook and Russell, 2001).

Quantitative real-time PCR

Total RNA was transcribed as above. Standard curves representing 10^2-10^8 copies of recombinant plasmids of *PlFadsd6* (primers Fads-qPCR-F/R; Table 2) and the internal control, *β-actin* (actin-F/R) were constructed. *PlFadsd6* and *β-actin* mRNAs in liver of *P. larnaudii* were separately amplified in a 10 μl reaction volume containing 100 ng of first strand cDNA template, 0.4 μl of each primer and 5 μl of green master mix. The thermal profile for quantitative real-time PCR was 95ºC for 5 min followed by 40 cycles of 95ºC for 30 s, 56ºC for 30 s and 72ºC for 30 s. Quantitative real-time PCR of each specimen was carried out in triplicate.

Statistical analysis

The body weight and fatty acid profiles in muscle are presented as mean ± standard error of the mean (or standard deviation). Differences in growth parameters between different groups of samples were analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test (R-package). Results were considered significant if *P*<0.05.

For gene expression analysis, the quantification of *PlFadsd6* and *β-actin* in each sample well was evaluated by reference to the relevant standard curve. The relative expression levels (copy number of *PlFadsd6* and that of *β-actin*) among treatments were statistically tested using ANOVA and Duncan's new multiple range test ($P < 0.05$).

RESULTS

Growth performance of *Pangasius larnaudii* fed with different sources of vegetable oil-supplemented diets

The body weight of *P. larnaudii* fed different sources of dietary oils was measured monthly (Table 3). The average body weight (ABW) and average daily gain (ADG) between treatments were not significantly different during the first three months of the feeding trial ($P < 0.05$). After 6 months of feeding, the final weight (FW) and ADG at months 4-6 of the experimental animals fed PO, SBO and SFO diets (320.24 ± 13.74, 322.52 ± 8.49 and 270.53 ± 7.09 g and 1.96 ± 0.71, 1.97 ± 0.44 and 1.47 ± 0.20 g·day^{-1}, respectively) were significantly greater than those of the black ear catfish fed SO (194.42 ± 5.67 g and 0.79 ± 0.06 g·day^{-1}; $P < 0.05$). However, final weight gain (FWG) and specific growth rate (SGR) of *P. larnaudii* fed PO (305.64 ± 13.66 g and 1.65 ± 0.02%) and SBO (307.92 ± 8.53 g and 1.68 ± 0.01%) but not SFO (255.57 ± 7.13 g and 1.57 ± 0.01%) diets were significantly greater than those of fish fed the SO diet (179.75 ± 5.63 g and 1.40 ± 0.01%; $P < 0.05$).

Isolation of *PlFadsd6* transcript

The amplification products of 478 bp in length were obtained using degenerate primers for *Fads*. Nucleotide sequences of this gene segments significantly matched *Fadsd6* of *Pangasius hypophthalmus* (GenBank accession no. AFN21428.1; *E-value* = 6e-92). The 5´- and 3´ RACE-PCR of this gene was further carried out and generated 772 bp and 792 bp fragments.

Table 3. Average body weight (± SEM) and growth parameters of *P. larnaudii* fed with different supplementary diets

Parameter	Average body weight ± SEM (g)			
	SO	PO	SBO	SFO
Initial weight (g)	14.67 ± 0.08^{ns}	15.20 ± 0.08^{ns}	14.60 ± 0.04^{ns}	14.97 ± 0.06^{ns}
Final weight (g)	194.42 ± 5.67^{a}	320.24 ± 13.74^{b}	322.52 ± 8.49^{b}	270.53 ± 7.09^{b}
WG (month 1) (g)	25.55 ± 1.17^{ns}	27.17 ± 1.27^{ns}	26.92 ± 2.52^{ns}	26.76 ± 0.51^{ns}
WG (month 2) (g)	11.01 ± 0.57^{ns}	28.53 ± 3.62^{ns}	25.35 ± 1.57^{ns}	16.54 ± 2.39^{ns}
WG (month 3) (g)	70.84 ± 1.99^{ns}	69.14 ± 7.34^{ns}	74.42 ± 4.27^{ns}	77.48 ± 6.58^{ns}
WG (month 4) (g)	26.75 ± 0.53^{ns}	3.75 ± 4.41^{ns}	41.88 ± 9.02^{ns}	30.11 ± 5.24^{ns}
WG (month 5) (g)	39.17 ± 3.07^{ns}	49.68 ± 5.71^{ns}	54.13 ± 10.27^{ns}	48.28 ± 4.26^{ns}
WG (month 6) (g)	6.42 ± 7.87^{a}	126.76 ± 13.76^{b}	85.22 ± 7.42^{ab}	56.40 ± 4.22^{ab}
FWG (g)	179.75 ± 5.63^{a}	305.64 ± 13.66^{b}	307.92 ± 8.53^{b}	255.57 ± 7.13^{ab}
ADG (mo 1-3; $g \cdot day^{-1}$)	1.17 ± 0.01^{ns}	1.36 ± 0.05^{ns}	1.38 ± 0.05^{ns}	1.31 ± 0.25^{ns}
ADG (mo 4-6; $g \cdot day^{-1}$)	0.79 ± 0.06^{a}	1.96 ± 0.71^{b}	1.97 ± 0.44^{b}	1.47 ± 0.20^{b}
SGR (%)	1.40 ± 0.01^{a}	1.65 ± 0.02^{b}	1.68 ± 0.01^{b}	1.57 ± 0.01^{ab}

WG = weight gain; FWG = final weight gain; ADG = average daily gain; SGR = specific growth rate; mo = month

After sequence assembly, the full-length cDNA of *PlFadsd6* was 1748 bp in length containing an ORF of 1338 bp corresponding to 445 amino acids with 5' and 3' UTRs of 88 and 276 bp, respectively. The poly A additional signal (AATAAA) was located between nucleotides 1700-1705 of the characterized transcript (GenBank accession no. KC994461; Figure 1). The deduced *PlFadsd6* protein contained a cytochrome b5 (Cyt-b5) domain and a heme-binding motif (HPGG) located at amino acid positions 22 – 96 (*E-value* = 1.7e-22) and 54-57 of the deduced *PlFadsd6* protein. A transmembrane domain was predicted at positions 125-144. A FA desaturase domain was observed at positions 157-419 (*E-value* = 1.1e-36). Three histidine-rich regions (HDFGH, HFQHH, and QIEHH) were found at positions 181-185, 218-222, and 383-387 of the deduced protein, respectively. The deduced amino acid sequence of *PlFadsd6* showed the greatest similarity to *delta-6 desaturase* of *Pangasius hypophthalmus* (identity = 95%; *E-value* = 0.0).

Phylogenetic analysis of *PlFadsd6*

Large sequence differences between *Fads* of fish, *Xenopus* and mammalian species were observed.

In fish, *Fadsd6* from all species except that of *Salmo salar* and *Fadsd5* was allocated into different phylogenetic clusters (Figure 2). *Fads* from catfishes clustered together and *PlFadsd6* showed a close phylogenetic relationship with that of *P. hypophthalmus*. A bootstrapped neighbor-joining tree further indicated that the newly isolated *Fads* in *P. larnaudii* should be recognized as *Fadsd6*.

Tissue expression analysis of *PlFadsd6* and its expression in liver following the feeding trial

Tissues expression analysis indicated that *PlFadsd6* was abundantly expressed in brain and liver. No expression of *PlFadsd6* was found in gill, gut, spleen, pituitary gland, and muscle. Based on a conventional RT-PCR in this study, *PlFadsd6* mRNA were not found in pituitary gland and muscle (Figure 3A).

The expression level of *PlFadsd6* in liver of catfish fed the PO diet was greater than those fed other diets (*P* < 0.05). The expression level of *PlFadsd6* in those fed SO was not significantly different from that in fish fed SBO (*P* > 0.05) but significantly greater than those fed SFO diets (*P* < 0.05; Figure 3B)

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                                                          A    1
CATGGGACACACACTTATCACGGAGGACTGAGCACAGTTAAATGCACATCACGACTACGT   61
GCAGAGAAGGAACACCTTAACACAGAGATGGGCGGCGGAGGACACCGGGGCGAGCAGCTG  121
                       M  G  G  G  G  H  R  G  E  Q  L   11
GGGTCAGGAGAGAGCGGATCATGCACACAGTACACCTGGGAAGAAGTGCAGAAGCACAAT  181
 G  S  G  E  S  G  S  C  T  Q  Y  T  W  E  E  V  Q  K  H  N   31
CACAGGGGTGATCAATGGCTGGTGATCGAGAGAAAGGTGTACAAGGTGACTGAGTGGATG  241
 H  R  G  D  Q  W  L  V  I  E  R  K  V  Y  K  V  T  E  W  M   51
AAAAGACACCCTGGAGGACGCAGAGTTCTAGCGCACTATGCTGGAGAGGACGCAACGGAT  301
 K  R  H  P  G  G  R  R  V  L  A  H  Y  A  G  E  D  A  T  D   71
GCATTTATGGCCTTCCATCCGGATCAGCGTTTCGTGCGGAAGTTCATGAAGCCACTGCTG  361
 A  F  M  A  F  H  P  D  Q  R  F  V  R  K  F  M  K  P  L  L   91
CTGGGAGAGTTGGCGCCTTCGGAGCCCAGTCAAGACCATGGCAAAAACGCTGCTTTAATG  421
 L  G  E  L  A  P  S  E  P  S  Q  D  H  G  K  N  A  A  L  M  111
GAAGATTTCCGGGCCTTGCGTAAGCAGTTGGAGGCACAGGGCTTGTTCCGCACCAGTCCG  481
 E  D  F  R  A  L  R  K  Q  L  E  A  Q  G  L  F  R  T  S  P  131
CTGTTTTTTATCCTATACCTTGGACACATTCTGCTTTTGGAGGCCCTGTCTTTAGCACTC  541
 L  F  F  I  L  Y  L  G  H  I  L  L  L  E  A  L  S  L  A  L  151
CTTTCGACATTTGGCAATGGCTGGATTATGACAATTCTAGTATCTGTCATACTGGCCACT  601
 L  S  T  F  G  N  G  W  I  M  T  I  L  V  S  V  I  L  A  T  171
GCACAGGCTCAGGCTGGCTGGCTTCAGCATGACTTTGGCCACCTATCAGTTTTTAAAAAT  661
 A  Q  A  Q  A  G  W  L  Q  H  D  F  G  H  L  S  V  F  K  N  191
TCTACCTGGGATCACCTAGTACACAAATTTGTCATTGGACACCTGAAGGGGGCTTCTGCA  721
 S  T  W  D  H  L  V  H  K  F  V  I  G  H  L  K  G  A  S  A  211
AATTGGTGGAACCATCGCCACTTTCAGCACCATGCTAAGCCCAACGTGGTCAAGAAGGAC  781
 N  W  W  N  H  R  H  F  Q  H  H  A  K  P  N  V  V  K  K  D  231
CCTGACATTAACATGCTGAACATACTGGTCTGTGGGAAACACCCTGCCTGTTGAGTACGGG  841
 P  D  I  N  M  L  N  I  L  V  L  G  N  T  L  P  V  E  Y  G  251
ACTAAAAAGTTGAAGAACATGCCCTATAACCATCAGCACAAGTACTTCTTTTTGATTGGC  901
 T  K  K  L  K  N  M  P  Y  N  H  Q  H  K  Y  F  F  L  I  G  271
CCTCCCCTGCTCATACCACTGTACTTTAACATGCACATTTTGCAAACTATGTATTCACAA  961
 P  P  L  L  I  P  L  Y  F  N  M  H  I  L  Q  T  M  Y  S  Q  291
CGGGATTGGGTGGATTTCGCCTGGTATCTGTCCTTCTATGCACGTTACCTCTCGTGCTAT 1021
 R  D  W  V  D  F  A  W  Y  L  S  F  Y  A  R  Y  L  S  C  Y  311
GTTCCCTATTATGGAATTCTGGGTTCTTTGGTGCTTCTGACTTTTGTAAGGTTTCTAGAG 1081
 V  P  Y  Y  G  I  L  G  S  L  V  L  L  T  F  V  R  F  L  E  331
AGTCACTGGTTTGTGTGGGTGACCCAAATGAACCACATTCCCATGGACATTGACTATGAC 1141
 S  H  W  F  V  W  V  T  Q  M  N  H  I  P  M  D  I  D  Y  D  351
AAAAATGATGACTGGCTCAGCATGCAGTTGAAAGCTACTTGTAATATTGAGCAGTCGCCA 1201
 K  N  D  D  W  L  S  M  Q  L  K  A  T  C  N  I  E  Q  S  P  371
TTCAACGATTGGTTCAGTGGCCATCTTAACTTTCAGATTGAACACCATCTCTTTCCCATG 1261
 F  N  D  W  F  S  G  H  L  N  F  Q  I  E  H  H  L  F  P  M  391
ATGCCTCGCCATAACTATAGCCGTGCAGCTCCTCATGTACAAAAACTTTGTGAGAAGTAT 1321
 M  P  R  H  N  Y  S  R  A  A  P  H  V  Q  K  L  C  E  K  Y  411
GGTGTTGTGTATCAGGTCAAAGGCCTGTGGGAATCCTGGTGTGACATTGTCACGTCTTTG 1381
 G  V  V  Y  Q  V  K  G  L  W  E  S  W  C  D  I  V  T  S  L  431
AAGAAGTCTGGTGAGCTGTGGCTAGATGCATACCTCCACAAATAATCATCTCTCCTGTTT 1441
 K  K  S  G  E  L  W  L  D  A  Y  L  H  K  *               445
TTCCTTCAGTGCTTGTTCAAAACTATATATACATTGTAACCACAGTTCAACAATATTGAC 1501
CTAGGATTTTAATTTAATATCAGGAATATCCATAACAATTCTTGAATTCACATGTTAATC 1561
CTCATGTTATATCTTGTCCTATATGCTGGAACTAATGTGGTATTTTGGTACTTTATTTGT 1621
AAGTGAATTGGGACCACAGATCTCTCTTGAATGAAAATGTTTTTTTATCTTTCCCATGCT 1681
GTTGGTTACTCCGACACAAATAAAGTCAAATTATAGTGCTGGAAAAAAAAAAAAAAAAAA 1741
AAAAAAA                                                      1748
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Figure 1. The full-length cDNA and deduced amino acids of *PlFadsd6*. The putative start (ATG) and stop (TAA) codons are boldfaced and underlined. A poly A additional signal (positions 1700 - 1705) is boldfaced, italicized and underlined. Three histidine boxes are underlined. The predicted Cyt-b5 (positions 22 - 96) and FA_desaturase (positions 157 - 419) domains are highlighted. The heme binding domain (HPGG; positions 54 - 57) is italicized and underlined. A transmembrane domain (positions 125 - 144) is illustrated in italics and highlighted.
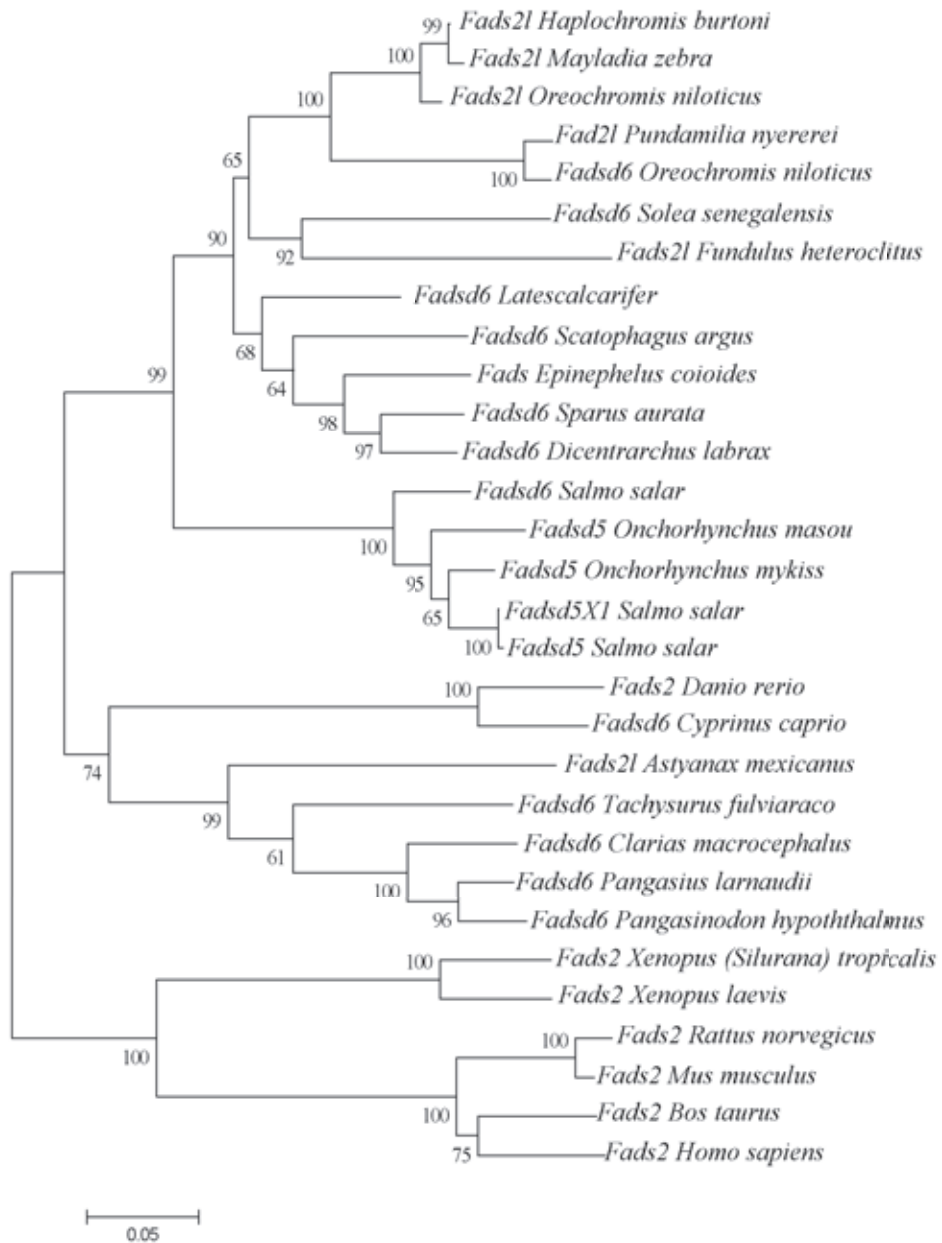
Figure 2. Phylogenetic tree of deduced amino acid sequences of *PlFadsd6* and *Fads* from other species. Values at the node represent the percentage of times that the particular node occurred in 1000 trees generated by bootstrapping the original aligned sequences.
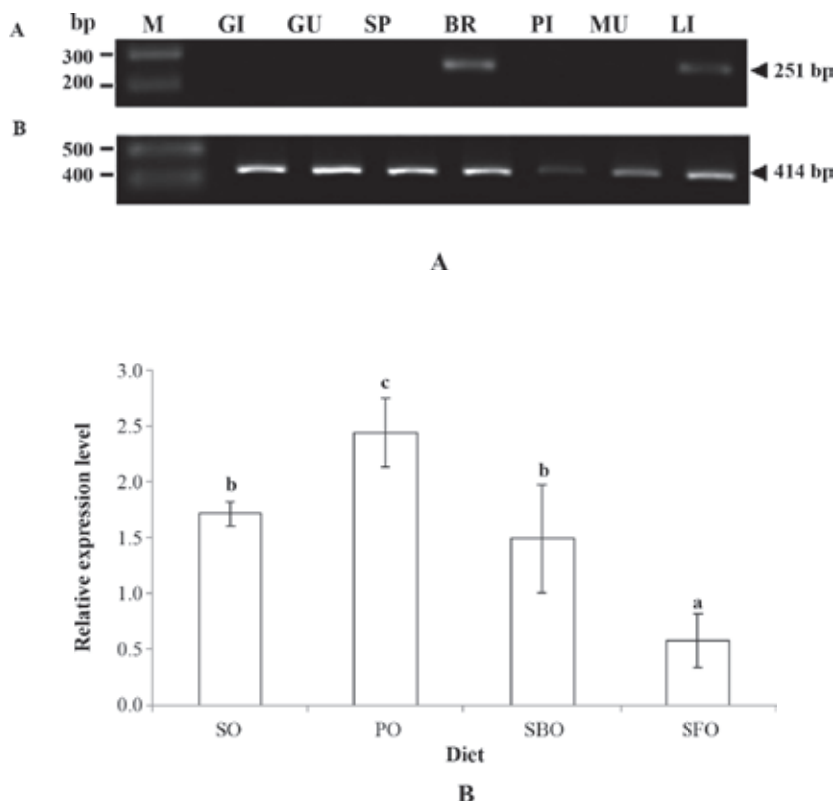
Figure 3. (A) Tissue distribution analysis of *PlFadsd6* (A) in various tissues of *P. larnaudii*. *β-actin* (B) amplified from the same template was included as the reference gene. GI = gills, GU = gut, SP = spleen, BR = brain, PI = pituitary gland, MU = muscle, LI = liver. Lanes M are a 100 bp DNA marker. (B) Histogram showing relative expression levels of *PlFadsd6* in liver of *P. larnaudii* fed various sources of lipids (SO = squid oil; PO = palm oil; SBO = soybean oil; SFO = sunflower oil). The same letters above histograms indicate that the expression levels were not significantly different ($P > 0.05$).

Fatty acid composition in muscle

Fatty acid compositions in muscle of *P. larnaudii* fed vegetable oil-containing diets were examined and different fatty acid compositions were compared to those fed the SO diet. The black ear catfish fed the PO-containing diet showed the greatest amounts of palmitic acid (16:0), stearic acid (18:0) and oleic acid (18:1cis-9) than those with other treatments ($P < 0.001$). Palmitoleic acid (C16:1) was not found in those fed SBO and SFO diets while ALA (C20:3n-3) was not found in muscle of fish fed the latter diet. The level of LA (18:2 n-6) in the black ear catfish fed the SBO diet was greater than in those fed SO, PO and SFO diets ($P < 0.01$). The EPA (20:5 n-3) levels in *P. larnaudii* fed SFO were significantly lower than those fed other diets ($P < 0.01$). The EPA/ARA ratio and DHA + EPA in those fed SO and PO diets were significantly greater than those fed the SFO diet ($P < 0.01$) while the DHA/EPA ratio was in the opposite direction (Table 4).

Table 4. Fatty acid composition (percent total fatty acids by weight ± SD) of muscle of *P. larnaudii* fed different experimental diets. SO = squid oil; PO = palm oil; SBO = soybean oil; SFO = sunflower oil

Fatty acid	SO	PO	SBO	SFO
Myristic acid (C14:0)	0.11 ± 0.04^{ns}	0.13 ± 0.03^{ns}	0.08 ± 0.02^{ns}	0.06 ± 0.01^{ns}
Palmitic acid (C16:0)***	1.33 ± 0.11^{b}	1.84 ± 0.06^{c}	1.22 ± 0.05^{b}	0.76 ± 0.03^{a}
Stearic acid (C18:0)*	0.25 ± 0.06^{ab}	0.33 ± 0.02^{b}	0.27 ± 0.02^{ab}	0.17 ± 0.08^{a}
Palmitoleic acid (C16:1)***	0.21 ± 0.05^{b}	0.14 ± 0.04^{b}	0^{a}	0^{a}
Oleic acid (C18:1 cis-9)***	1.40 ± 0.17^{b}	1.81 ± 0.04^{c}	1.23 ± 0.12^{b}	0.79 ± 0.04^{a}
Linoleic acid (C18:2n6)**	0.35 ± 0.02^{a}	0.48 ± 0.02^{b}	0.67 ± 0.03^{c}	0.43 ± 0.15^{ab}
α-Linolenic acid (C18:3n3)***	0.07 ± 0.01^{b}	0.07 ± 0.01^{b}	0.06 ± 0.01^{b}	0^{a}
Docosahexaenoic acid (C22:6n3)	0.12 ± 0.03^{ns}	0.17 ± 0.04^{ns}	0.15 ± 0.03^{ns}	0.09 ± 0.05^{ns}
Arachidonic acid (C20:4n6)	0.04 ± 0.01^{ns}	0.04 ± 0.01^{ns}	0.05 ± 0.02^{ns}	0.03 ± 0.01^{ns}
Eicosapentanoic acid (C20:5n3)**	0.04 ± 0.01^{b}	0.04 ± 0.01^{b}	0.03 ± 0.01^{b}	0.01 ± 0.00^{a}
ALA/LA	0.20 ± 0.04^{a}	0.15 ± 0.02^{b}	0.08 ± 0.02^{c}	0^{d}
DHA + EPA*	0.16 ± 0.01^{ab}	0.21 ± 0.02^{b}	0.18 ± 0.02^{b}	0.10 ± 0.01^{a}
DHA/EPA*	3.00 ± 0.02^{a}	4.25 ± 0.02^{a}	5.00 ± 0.01^{ab}	9.00 ± 0.02^{b}
EPA/ARA*	1.00 ± 0.00^{b}	1.00 ± 0.00^{b}	0.60 ± 0.01^{ab}	0.33 ± 0.00^{a}

*, **, *** = significance at $P < 0.05$, 0.01 and 0.001, respectively; NS = not significant at $P < 0.05$

DISCUSSION

Better growth performance of *P. larnaudii* fed with diets containing dietary vegetable oils than those fed with SO diet

Low profitability of catfish industry demands that diet formulation should be improved to reduce feed costs and increase productivity. In this study, a 6-month feeding trial was carried out for evaluation of the total replacement of added SO in the grow-out diet of *P. larnaudii* with PO, SBO or SFO from fingerlings (about 15 g) to marketable size (>300 g). The environmental conditions (dissolved oxygen 4.00 - 6.40 ppm, pH 6.36 - 8.88, water temperature 29.60 - 31.70°C, and unionized ammonia 0.001 - 0.005 ppm) during the feed trial were in the range of standard for aquaculture (Boyd, 1998). The survival rate of experimental animals was 98-100 % over a 6 month experimental period.

HUFAs are essential fatty acids for normal growth and survival of teleosts (Tocher *et al.*, 2003a and 2003b). Freshwater teleosts have a capacity to bioconvert LA and ALA into HUFAs (Zheng *et al.*, 2004; Hastings *et al.*, 2005;

Tanomman *et al.*, 2013). However, the efficiency for using LA is species-dependent and varied widely. In Nile tilapia (*Oreochromis niloticus*) and tilapia galilae (*Sarotherodon galilaeus*), a replacement of fish oil with vegetable oil for a 17 week feeding trial was carried out. Growth and FCR of both species fed the SBO-containing diet was better than those with fish oil ($P < 0.05$; Goda *et al.*, 2007).

Pongjanyakul *et al.* (2008) substituted the different ratio of soybean meal in the diet (0, 25, 50, 75, 100 %) in 3 month feeding trial of *P. larnaudii* (23% proteins) and its specific growth rate fed the soybean meal diet was lower than the diet containing fish meal. Accordingly, fish meal which contains the essential fatty acids of fish was included in all experimental diets in our study but more cost-effective diets including 4% PO, SBO and SFO (US\$1.0 – 1.5·kg^{-1}) was produced to fully replace the SO diet (US\$1.8·kg^{-1}; Thai Feed Mill Association, 2009).

Asdari *et al.* (2011b) determined the replacement effects of the diet containing fish oil (FO) with SO, crude palm oil (CPO) and linseed oil (LO) on growth performance of *P. hypophthalmus*

in the 12-week feeding trial. Growth performance of fish fed all vegetable oil-based diets was better than those fed the FO diet. Muscle and liver fatty acid composition for all treatments generally reflected the composition in the diet. Similar results were found in *P. nasutus* juveniles where growth performance of fish fed the CPO diet was significantly higher than fish fed the FO diet ($P <$ 0.05; Asdari *et al.*, 2011a). Piedecausa *et al.* (2007) reported that the n-3/n-6 ratio in tissues of fish fed vegetable oils is greatly reduced. Results from previous studies in *P. nasutus* (Asdari *et al.*, 2011a) and *P. hypophthalmus* (Asdari *et al.*, 2011b) was in agreement with the present study on *P. larnaudii* which suggested that excessive amounts of n-3 fatty acids reduce the overall growth performance of *Pangasius* fish.

During larval development, DHA is required for visual response in gilthead sea bream (Benitez-Santana *et al.*, 2007) and schooling behavior in Pacific treadfin (*Polydactylus sexfilis*) (Masuda *et al.*, 2001). In this study, there was no significance in the growth rate of *P. larnaudii* at the first 3 months. Nevertheless, late feed responses were observed during months 4-6 of the experiment where the black ear catfish fed vegetable oil-based diets exhibited a better growth performance than those fed the SO diet. However, fatty acid composition in muscle revealed a lower amount of EPA (C20:2, *n*-6) in the feeding SFO diet than other formulated diets. The EPA/ARA ratio in SO and PO was greater than that of SBO and SFO. Based on the muscle lipid composition, the quality of fish from PO and SO diets was not different while those from SBO and SFO diets may be inferior due to the lack of palmitoleic acid (C16:1) with a moderate level of EPA/ARA in the former and the lack of both C16:1 and ALA (C18:3n-3) and a low level of EPA/ARA in the latter. The elevated HUFA in muscle implied that the dietary vegetable oils (i.e. PO and SBO) can be modulated desaturation and elongation activities in *P. larnaudii* as well as in other freshwater fishes (Tocher *et al.*, 2002b, 2003a, 2003b). Nevertheless, the preference of these enzymes to precursor of the HUFA pathway may be different (Zheng *et al.*, 2009). In cobia, the desaturase was more active for LNA, while the elongase preferred both LA and LNA substrates (Zheng *et al.*, 2009).

The diet containing PO as the whole source of lipid supplement apparently modulate higher desaturase activity than LNA-rich (SO) diet. Izquierdo *et al.* (2008) reported that diets in which fish oil was totally replaced by SBO and rapeseed oils induced a six fold increase of the desaturase mRNA level in *S. aurata*. Similarly, Gao *et al.* (2012) showed that diet fully replaced of PO could increase a higher DHA concentration in liver than those fed fish oil in *Lateolabrax japonicus* juveniles.

An altered fatty acid profile of the aquaculture species following the replacement of fish/squid oil-containing diets with VO-containing diets is a major concern for health benefits. In the present study, *P. larnaudii* fed PO and SBO diets did not show significant reduction of DHA, EPA, ARA from those fed the SO diet while a lower EPA was observed following the SFO feeding. It is interesting to further investigate about the grow-out diet (i.e. PO) containing a low amount of short chain PUFA but rich in SFA provided a better growth performance of those fed a SO diet.

Isolation and expression analysis of *PlFadsd6* in *P. larnaudii* fed different fatty acid sources

The full-length cDNAs of *PlFadsd6* were isolated and reported for the first time in *P. larnaudii*. A predicted Cyt-B5 and FA_ desaturase domains were found in the deduced *PlFadsd6* protein. It functions in heme binding in a diverse range of proteins. In addition, the Cyt-b5 domain is found in proteins that are able to bind to steroids (e.g. progesterone receptor) (Gerdes *et al.*, 1998). The information implied the functional involvement in fatty acid desaturation of the characterized genes.

Phylogenetic analysis indicated that it was closely related with those from other catfishes but distantly related with other teleosts. Tissue distribution analysis illustrated that *PlFadsd6* was expressed only in brain and liver. Similar results were previously reported in *S. solar* (Monroig *et al.*, 2010), *Channa striatus* (Aliyu-Paiko *et al.*, 2012), and cobia (Zheng *et al.*, 2009). This is coincident with the function of liver as the main organ

responsible for the biosynthesis of HUFA. The high expression of *PlFadsd6* in *P. larnaudii* brain suggested its important functionsof fatty acid metabolism in brain, which needs to be further clarified.

   PlFadsd6 transcripts were not expressed in pituitary gland and muscle. As a result, fatty acid composition in muscle should reflect the effects of replacing SO with vegetable oil-containing diets. Abundant expression of *PlFadsd6* observed in liver of fish fed PO implied the ability to biosynthesize HUFA from diet substrates of *P. larnaudii*.

   The fatty acid compositions in vegetable oils readily affect the HUFA biosynthesis pathway. Increased hepatocyte desaturation and elongation activities in tilapia and zebrafish were reported with vegetable oil-based diets (Tocher *et al.*, 2002a). Similarly, *PlFadsd6* transcripts in the black ear catfish fed PO were higher than other experimental diets (SO, SBO and SFO; $P < 0.05$).

   Although several studies reported that high LA and ALA contents can up-regulate *Fadsd6* expression (Turchini *et al.*, 2006; Francis *et al.*, 2007; Li *et al.*, 2008), the excess of PUFAs can also inhibit the desaturation of substrates (Bell *et al.*, 1993; Ruyter *et al.*, 2000). Results in this study revealed significant decreases of *PlFadsd6* transcripts in *P. larnaudii* fed SBO (high LA and ALA) and SFO (high LA but low ALA) diets. Nevertheless, up-regulation of these transcripts was observed in the black ear catfish fed the PO diet (moderate level of LA but low level of ALA). The LA/ALA ratios in SO, PO, SBO and SFO were 0.115, 43.81, 7.63 and 220.84, respectively. We speculate that the up-regulation of *PlFadsd6* mRNA may depend on the optimum level of a LA to ALA ratio. The EPA and DHA levels in *P. larnaudii* muscle fed SFO (high LA) were lower than those fed PO (low LA) and SBO (moderate LA). This result was in agreement with the reduction of *PlFadsd6* transcripts in *P. larnaudii* liver. The circumstance suggested the possible inhibition of DHA synthesis in the formulated diet with the excess LA content.

   The nutritional regulation of desaturase activities could vary among the developmental stage and capacity of fish to transform these lipids through desaturation and elongation pathways (Tocher *et al.*, 2006). Ren *et al.* (2012) illustrated a decrease of DHA in muscle of *Cyprinus carpio* fed high LA. This may probably due to the detrimental effect to maintain DHA in muscle tissue. Apparently, the diet that contains PO as lipid supplement source significantly induce *PlFadsd6* expression, but not significantly affect on increasing the level of HUFA in muscle compared with the SO control.

   The primary mechanism responsible for increased HUFA biosynthesis during limited dietary HUFA intake is through up-regulated expression of desaturase mRNAs (Bell *et al.*, 2003). Reduced transcriptional levels of liver *PlFadsd6* mRNA in *P. larnaudii* fed dietary SBO and SFO diets were found. This was not consonant with several fish species on an increase in both enzymatic and transcriptional activities of liver desaturase with increasing levels of dietary LA and LNA (Tocher *et al.*, 2002b; Zheng *et al.*, 2004; Ling *et al.*, 2006). Apparently, the contradictory results on increased *PlFadsd6* mRNA in black ear fish fed dietary PO is relatively unclear and need further studies. Apparently, the expression of these genes is not solely influenced by dietary levels of n-3 fatty acids alone but could also be positively or negatively regulated by both substrates and products of the HUFA pathway (Zheng *et al.*, 2004).

   Considering results from the feeding trial, the growth performance of fish (4-6 months old) fed formulated diets containing vegetable oils was better than fish fed the SO diet. When HUFA profiles in muscle and expression levels of liver *PlFadsd6* mRNA were also taken into the account, the SO was more appropriately replaced with PO than SBO and SFO in the grow-out diet for *P. larnaudii*. However, the replacement effects of diets containing vegetable oils in < 4 month old fish should be confirmed.

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