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Effects of dietary grape seed oil and linseed oil on growth, muscle fatty acid composition and expression of putative Δ5 fatty acyl desaturase in abalone *Haliotis discus hannai* Ino

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ABSTRACT

A 120-day feeding trial was conducted to investigate the effects of dietary increasing levels of grape seed oil (GO) and linseed oil (LO), rich in linoleic acid (LA) and α-linolenic acid (ALA) respectively, on growth, fatty acid composition and expression levels of putative Δ5 fatty acyl desaturases (Fads) in muscle of juvenile abalone (*Haliotis discus hannai* Ino). Seven experimental diets were formulated to contain increasing amounts (0%, 0.875%, 1.75% and 3.5%) of GO or LO as dietary lipids. Tripalmitin (TP), rich in 16:0, was supplemented to reach 3.5% (dry weight) total lipid. These diets were named as 0%GO/LO (TP), 25%GO, 50%GO, 100%GO, 25%LO, 50%LO and 100%LO. With the increase of dietary GO or LO inclusion, growth parameters (such as specific growth rate of weight) first increased and then decreased ($R^2 > 0.72$, $P = 0.000$). The survival rate of abalone was not significantly correlated with dietary GO ($R^2 = 0.04$, $P = 0.815$) or LO inclusion ($R^2 = 0.50$, $P = 0.046$). The contents of muscle n-6 polyunsaturated fatty acids (PUFA), including LA, 20:2n-6, 20:4n-6, 22:2n-6, were significantly positive correlated with dietary GO inclusion ($R^2 > 0.895$, $P = 0.000$). A positive relationship was significantly noted between the values of muscle n-3 PUFA (ALA, 20:3n-3, 20:5n-3 and 22:5n-3) and the dietary LO inclusion ($R^2 > 0.905$, $P = 0.000$). DHA was significantly increased and then decreased with dietary LO increase ($R^2 = 0.937$, $P = 0.000$). The expression levels of putative Δ5 Fads in 50%GO group were significantly higher than those of other GO groups ($P < 0.05$). With the increase of dietary LO, expressions of putative Δ5 Fads first increased and thereafter reached a plateau ($R^2 > 0.929$, $P = 0.000$). These results indicated that biosynthesis of long chain polyunsaturated fatty acid in muscle of abalone could be increased in response to increasing levels of dietary LA or ALA through increased expressions of putative Δ5 Fads. High intakes of dietary LA and/or ALA inhibited the biosynthesis of DHA and compromised the growth performance of abalone.

1. Introduction

Fish oil (FO) is the traditional lipid source in aquaculture industries, including abalone mariculture. However, being heavily reliant on a declining global marine fish stock presents a risk to the aquaculture industries (Peron et al., 2010; Tacon and Metian, 2008). The substitution of FO with alternative oils could be a viable option for normal development of aquaculture industries (Olsen, 2011). Vegetable oil (VO), rich in C18 polyunsaturated fatty acid (PUFA), is a good alternative to FO for relatively low cost and stable production. However, previous studies have shown that exceeded levels of dietary VO could result in accumulation of C18 PUFA and reduction of long chain polyunsaturated fatty acid (LC-PUFA), and therefore decrease the flesh quality of aquatic animals (Bell et al., 2003; Su et al., 2004). For this reason, it is crucial to understand regulatory mechanism of LC-PUFA biosynthesis in abalone under dietary VO inclusion and find practical ways to stimulate such biosynthesis activity.

The biosynthesis of LC-PUFA is a process of catalysis on C18 substrates, linoleic acid (LA) or α-linolenic acid (ALA), through Δ6 fatty acyl desaturase (Fad), elongase and Δ5 Fad. LC-PUFA biosynthesis in fish was well investigated and many studies have found functional Fads and elongases in different fish species (Agaba et al., 2005; González-Rovira et al., 2009; Gregory et al., 2010; Monroig et al., 2010; Ren et al., 2012; Seiliez et al., 2003; Tocher et al., 2006; Zheng et al., 2005, 2009). In addition, Fads in some fishes were reported to be bifunctional, such as Δ5/6 Fad in zebrafish (*Danio rerio*) (Hastings et al., 2001), Δ5/6 and Δ5/4 Fads in white spotted rabbitfish (*Siganus canaliculatus*) (Li et al., 2010). In contrast, there is little information available on LC-PUFA synthesis across a wide range of aquatic invertebrates including abalone. Previously, some studies suggested that abalone might have the ability to convert LA to arachidonic acid (ARA) or ALA to eicosapentaenoic acid (EPA) based on the results of feeding experiments (Bautista-Teruel et al., 2011; Durazo-Beltrán et al., 2003;

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Xu et al., 2004). Mateos et al. (2011, 2012a,b) reported some studies about putitive-Δ6 Fad and elongase (without confirmation of actual enzymatic activities) in Jade Tiger Hybrid abalone (*Haliotis. laevigata* × *H. rubra*). Monroig et al. (2011) found that the common octopus (*Octopus vulgaris*) expresses a Fad-like gene that encodes an enzyme with similar Δ5 enzymatic activities (conversion rate of 39%) towards 20:3n-6 and 20:4n-3, which suggested that Δ5-like Fad in octopus could participate in the production of ARA and EPA from fatty acid substrates. Recently, we successfully cloned two putative Δ5 Fads (Hdhfad1 and Hdhfad2) in abalone (*H. discus hannai* Ino). Functional analysis in yeast (*Saccharomy cescerevisiae*) showed that the rate of Hdhfad1 and Hdhfad2 converting 20:4n-3 to EPA is 31% and 16% respectively while percentages of 20:3n-6 to ARA is similar, about 15% (unpublished data by Li et al.).

Many studies have shown that the increased transcript levels of desaturases and elongases occurred in fishes (González-Rovira et al., 2009; Monroig et al., 2010; Tocher et al., 2006; Zheng et al., 2005, 2009) and shellfishes (Mateos et al., 2011, 2012a,b) when dietary FO was replaced by VO. Ren et al. (2012) found that expressions of Δ6 Fad and elongase regulate the conversions of C18 precursors to LC-PUFA in common carp (*Cyprinus carpio* var. Jian) fed diets full of LA or ALA, but no LC-PUFA. This was different with the report on rat that n-3 LC-PUFA synthesis from ALA is regulated independently of changes in the expressions of synthetic enzymes, including Δ5 Fad, Δ6 Fad and elongases (Tu et al., 2010). As far as we know, there is no information on the regulation of LC-PUFA biosynthesis by dietary increasing levels of LA or ALA (the precursor for biosynthesizing LC-PUFA) in abalone without dietary LC-PUFA interference. And the expressions of putative Δ5 Fads in response to such diets in abalone are also unknown.

This study was conducted to investigate the effects of dietary increasing levels of LA or ALA by adding increasing proportions of grape seed oil (GO, 67.40% LA in total fatty acids) or linseed oil (LO, 70.19% ALA in total fatty acids) in diets on growth, fatty acid composition and expressions of putative Δ5 Fads in muscle of abalone. These data will provide useful information about effects of VO on growth performance, LC-PUFA biosynthesis capability and gene regulations of putative Δ5 Fads in abalone.

2. Materials and methods

2.1. Experimental diets

Seven experimental diets were formulated to contain increasing amounts (0%, 0.875%, 1.75% and 3.5%) of GO or LO, and tripalmitin (TP) was supplemented to make each diet contain 3.5% (dry weight) total lipid. Diet containing 3.5% TP, a triacylglycerol of palmitic acid (16:0), served as control diet as TP is usually used as filler in balanced diets to study fatty acid metabolism (Durazo-Beltrán et al., 2003). These diets were named as 0%GO/LO (TP), 25%GO, 50%GO, 100%GO, 25%LO, 50%LO and 100%LO according to the ratio of GO/LO to TP in diets. The nutrient composition of the diets was analyzed following the usual procedures, Association of Official Analytical Chemists (AOAC, 1995). Crude protein was determined by measuring nitrogen (N × 6.25); crude lipid was measured by ether extraction using Soxhlet method; ash was analyzed, and carbohydrates were

Table 1
Formulation and proximate compositions of the experimental diets.

	Diets [a]						
Ingredients (g/kg)	TP	25%GO	50%GO	100%GO	25%LO	50%LO	100%LO
Casein	250	250	250	250	250	250	250
Gelatin	60	60	60	60	60	60	60
Dextrin	335	335	335	335	335	335	335
CM-cellulose	50	50	50	50	50	50	50
Sodium alginate	200	200	200	200	200	200	200
Choline chloride	5	5	5	5	5	5	5
Vitamin mix[b]	20	20	20	20	20	20	20
Mineral mix [c]	45	45	45	45	45	45	45
TP	35	26.25	17.5	0	26.25	17.5	0
GO	0	8.75	17.5	35	0	0	0
LO	0	0	0	0	8.75	17.5	35
Proximate analysis [d]							
Moisture (mg g^{-1})	538.25	539.95	538.21	537.78	538.22	541.85	539.44
Crude protein (mg g^{-1})	145.65	142.94	145.14	145.91	146.58	144.68	145.58
Crude lipid (mg g^{-1})	15.44	15.53	15.61	15.61	15.38	15.39	15.41
Ash (mg g^{-1})	54.31	55.04	54.51	54.12	55.30	55.19	55.08
Carbohydrate (mg g^{-1})	246.35	246.54	246.53	246.59	244.52	242.88	244.50
Gross energy (kcal kg^{-1}) [e]	1706.96	1697.69	1707.17	1710.49	1702.82	1688.75	1699.01
Energy from lipid (kcal kg^{-1}) [f]	138.96	139.77	140.49	140.49	138.42	138.51	138.69
Lipid (en%) [g]	8.14	8.23	8.23	8.21	8.13	8.20	8.16
LA (en%) [h]	0.08	1.30	3.24	5.54	0.30	0.66	1.22
ALA (en%) [i]	0.04	0.08	0.12	0.13	1.10	2.67	5.73

[a] TP, Tripalmitin; GO, Grape seed oil; LO, Linseed oil.
[b] Vitamin mix (kg^{-1}): Thiamin HCl, 120.0 mg; Riboflavin, 100.0 mg; folic acid, 30.0 mg; Pyridoxine HCl, 40 mg; Niacin, 800 mg; Ca pantothenate, 200 mg; Inositol, 4000; Biotin, 12 mg; Vitamin B12, 0.18 mg; Ascorbic acid, 4000 mg; Vitamin E, 450 mg; Menadione, 80 mg; Retinal acetate, 100,000 IU; Cholecalciferol, 2000 IU.
[c] Mineral mix (kg^{-1}): NaCl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 6.0 g; $NaH_2PO_4 \cdot 2H_2O$, 10.0 g; KH_2PO_4, 12.8 g; $Ca(H_2PO_4)_2 \cdot H_2O$, 8.0 g; $ZnSO_4 \cdot 7H_2O$, 141.2 mg; $CoCl_2 \cdot 6H_2O$, 0.4 mg; KIO_3, 1.2 mg; $CuSO_4 \cdot 5H_2O$, 12.4 mg; $Na_2SeO_3 \cdot 5H_2O$, 0.4 mg; Fecitrate, 1.0 g.
[d] Means of three analyses.
[e] Gross energy (kcal kg^{-1}) was calculated based on the standard physiological fuel values for protein, lipid and carbohydrate of 4, 9 and 4 kcal/g, respectively.
[f] Energy from lipid (kcal kg^{-1}) was calculated by crude lipid (g kg^{-1}) * 9 kcal/g.
[g] Lipid (en%): the percent of energy (kcal kg^{-1}) from lipid in gross energy (kcal kg^{-1}).
[h] LA (en%) = (% LA in total fatty acids) * lipid en%] * 100.
[i] ALA (en%) = (% ALA in total fatty acids) * lipid en%] * 100.

eventually Nitrogen free extract (NFE) according to the AOAC. Gross energy (kcal kg^{-1}) was calculated based on the standard physiological fuel values for protein, lipid and carbohydrate of 4, 9 and 4 kcal/g, respectively. Lipid (en%) means the percent of energy from lipid in gross energy (energy from lipid/gross energy * 100). LA (en%) means the percent of energy provided from LA in gross energy and was calculated by [(% LA in total fatty acids) * lipid en%] * 100. ALA (en%) was calculated by [(% ALA in total fatty acids) * lipid en%] * 100 (Gibson et al., 2013; Tu et al., 2010, 2013). The ingredients and proximate compositions of the experimental diets were listed in Table 1.

2.2. Experimental procedure

Juvenile abalones (*H. discus hannai* Ino) were obtained from Aoshanwei fishery Co. Qingdao, China and became acclimatized to laboratory condition for one week in recirculation water system prior to the feeding experiment. At the initiation of the experiment, the abalones were fasted for 24 h and weighed. Abalones of similar sizes (initial weight: 0.38 ± 0.01 g; initial shell length: 15.06 ± 0.21 mm) were randomly distributed into 21 tanks (35 L) and each tank was stocked with 50 abalones. There were three replicates for each of the seven dietary treatments and all abalones were raised under 24 h dark photoperiod for 120 days. Abalones were hand-fed with experimental feeds at a rate of 2%–5% equal to wet body weight at 6:00 pm once daily. Faces and excess feeds were removed in the next morning for good water quality. To clean alga possibly eaten by abalone, aquariums and corrugated boards were cleaned three times a week. During the 120-day feeding experiment, temperature was registered between 16 °C to 18 °C, salinity 31 to 35 ppt, and pH 7 to 8. Meanwhile, dissolved oxygen was measured at about 7 mg/L.

2.3. Analyses and measurement

2.3.1. Sample collection

At the end of the feeding experiment, abalones were starved for 2 days prior to sample collection. They were dried with gauze and then individually weighed, length measured and dissected. Muscle tissue was used to analyze the fatty acid composition and gene expressions of putative Δ5 Fads. Samples for determining the fatty acid composition were stored at −20 °C. Other samples for gene expression analysis were immediately collected in 1.5 ml RNase-free centrifuge tubes, frozen in liquid nitrogen and then stored in −80 °C refrigerator.

2.3.2. Analysis of fatty acid composition

The fatty acid profiles were analyzed using the procedures described by Metcalfe et al. (1966) with some modification. About 100 mg freeze-dried samples were added into a 10 ml volumetric screwed tube with cover. Then 3 ml KOH-methanol (1 N) was added and heated on 80 °C water bath for 20 min. After that, 3 ml HCL-methanol (2 N) was added and the mixture was heated on 80 °C water bath for another 20 min. Previous tests were conducted to make sure that all fatty acids can be esterified following the procedures above (Ai et al., 2008; Xu et al., 2004). After cooling to room temperature, 1 ml hexane was added into the mixture above, shaken vigorously for 1 min, and then allowed to separate into two layers. The top phase was removed carefully into a clean test tube for fatty acid methyl esters (FAME) identification.

FAME was separated and quantified using HP6890 gas chromatograph (Agilent) equipped with a fused silica capillary column (007-CW) and a flame ionization detector. The column temperature was programmed to rise from 150 °C up to 200 °C at a rate of 15 °C min^{-1}, then from 200 °C to 250 °C at a rate of 2 °C min^{-1}. Injector and detector temperature was 250 °C, respectively. Individual FAME was identified by comparison of retention time with fatty acid standards (Cayman and Sigma).

2.3.3. RNA extraction, cDNA synthesis and quantitative real-time PCR (qRT-PCR) analysis for putative Δ5 Fads

Muscle tissue of abalone fed with different diets was respectively grinded in liquid nitrogen. Total RNA was extracted using Trizol reagent (Invitrogen, USA) then electrophoresed on 1.2% agarose gel to test the quality and integrity followed by concentration determination with NanoDrop®ND-1000 (Nano-Drop Technologies, Wilmington, DE, USA). 1 μg total RNA was subjected to PrimeScript®RT reagent Kit with gDNA Eraser (TaKaRa, Japan) in 10 μl volume for reverse transcription and DNA erasability. The expression levels of putative Δ5 Fads in muscle of abalone fed with different diets were studied by qRT-PCR method: $2^{-\Delta\Delta CT}$ (Livak and Schmittgen, 2001). TP fed abalone served as control group. The qRT-PCR primer pairs were designed to guarantee the primer amplification efficiencies of target gene and reference gene (ribosomal protein S9, RPS9) approximately equal. Meanwhile expression levels of reference gene (RPS9) were proved to be unresponsive to different diets. *Hdh*Fad1 (F1: 5′-CCATTTACTCCACCACATT-3′ and R1: 5′-AACAGTTCCTCCACCCTA-3′, GQ470626) and *Hdh*Fad2 qualified primers (F2: 5′-CTGTTCAAGCCCAACG-3′ and R2: 5′-TTAAAGACGG AAATGTGAC-3′, GQ466197) were designed to evaluate mRNA expressions of putative Δ5 Fads. RPS9 primers (F: 5′-GTCGGCTCGTGCGTAT-3′ and R: 5′-GGATGTTCACCACCTGTTT-3′, EU247757) were used to normalize the template amounts of putative Δ5 Fads. The real-time PCR was carried out in a quantitative thermal cycler (Mastercycler *ep* realplex, Eppendorf, German) in a final volume of 25 μl containing 2 × SYBR Green Real-time PCR Master Mix (TaKaRa, Japan), primer pairs and cDNA. The program was 95 °C for 30s followed by 35 cycles of 95 °C for 5 s, 58 °C for 15 s and 72 °C for 20s. Melting curve (1.85 °C increments/min from 58 °C to 95 °C) was performed after the amplification phase for confirmation. Each sample was run in triplicate, and reactions without templates were used as negative control.

2.4. Calculations and statistical methods: statistical analysis

The survival of abalone was expressed by survival rate (SR, %), which was calculated by final abalone number/initial abalone number × 100.

The growth parameters of abalone was calculated as follows:

$$\text{Weight gain}(g) = W_f - W_i;$$

$$\text{Weight gain rate } (WGR, \%) = \left(W_f - W_i\right)/W_i \times 100;$$

Table 2
Fatty acid compositions (percentage of total fatty acids) of experimental diets.

	Diets						
Fatty acids [a]	TP	25%GO	50%GO	100%GO	25%LO	50%LO	100%LO
14:0	2.12	1.74	1.62	1.55	1.85	1.58	1.70
16:0	91.35	73.01	45.28	9.26	75.37	51.09	4.37
18:0	2.38	2.84	2.93	3.21	2.16	1.79	0.99
∑ SFA [b]	95.85	77.59	49.83	14.02	79.38	54.46	7.06
16:1n-7	0.31	0.38	0.37	0.42	0.38	0.56	0.90
18:1n-9	1.29	4.20	7.59	14.72	2.00	3.57	5.53
18:1n-7	0.56	0.49	0.96	1.77	0.68	0.73	1.05
∑ MUFA [c]	2.16	5.07	8.91	16.90	3.06	4.86	7.48
18:2n-6	1.02	15.83	39.38	67.40	3.71	8.01	15.00
18:3n-3	0.53	0.99	1.46	1.55	13.54	32.52	70.19
∑ PUFA [d]	1.55	16.82	40.84	68.95	17.25	40.53	85.19

[a] Some fatty acids, of which the contents are minor, trace amount in all diets such as 14:1, 20:0, 20:1n − 9, 22:0, 22:1n − 11, were not listed in the table. Values are means of three analyses.
[b] SFA: saturated fatty acid.
[c] MUFA: monounsaturated fatty acid.
[d] PUFA: polyunsaturated fatty acid.

Table 3
Growth response and regression analyses of dietary GO inclusion (X) against growth performance (Y) in abalone.

	Diets				ANOVA [1]		Regression analysis		
Parameters	0%GO (TP)	25%GO	50%GO	100%GO	P.S.M.E [2]	*P*	Equation	R^2	*P*
Initial weight (g)	0.39	0.38	0.38	0.37	0.00	0.519			
Final weight (g)	0.89^{b}	1.04^{a}	0.98^{ab}	0.88^{b}	0.02	0.003	$Y = -0.0633X^2 + 0.3077X + 0.6548$	0.74	0.002
Weight gain (g)	0.50^{b}	0.66^{a}	0.60^{a}	0.51^{b}	0.03	0.001	$Y = -0.0618X^2 + 0.3043X + 0.2715$	0.75	0.002
WGR (%) [3]	130.95^{c}	173.65^{a}	155.99^{ab}	136.81^{bc}	5.51	0.001	$Y = -15.472X^2 + 77.352X + 72.011$	0.72	0.003
SGR_W (% day^{-1}) [4]	0.69^{c}	0.84^{a}	0.78^{ab}	0.72^{bc}	0.02	0.001	$Y = -0.0518X^2 + 0.2602X + 0.4974$	0.72	0.003
Initial shell length (mm)	15.19	15.08	15.08	14.91	0.05	0.349			
Final shell length (mm)	18.24^{b}	19.35^{a}	19.03^{a}	18.17^{b}	0.16	0.001	$Y = -0.4931X^2 + 2.4149X + 16.361$	0.84	0.000
Shell increase (mm)	3.04^{b}	4.27^{a}	3.95^{a}	3.26^{b}	0.15	0.000	$Y = -0.4793X^2 + 2.4298X + 1.1549$	0.89	0.000
SIR (%) [5]	20.06^{b}	28.33^{a}	26.23^{a}	21.89^{b}	1.02	0.000	$Y = -3.155X^2 + 16.112X + 7.5092$	0.88	0.000
DGR_{SL} (μm day^{-1})[6]	25.40^{b}	35.59^{a}	32.97^{a}	27.19^{b}	1.28	0.000	$Y = -3.9942X^2 + 20.248X + 9.6238$	0.89	0.000
Survival rate (%)	86.67	84.67	84.67	86.00	1.23	0.943	$Y = 0.8333X^2 - 4.3667X + 90.167$	0.04	0.815

[1] ANOVA: one-way analysis of variance. Different superscript letters in each row show significant differences among dietary treatments by Tukey's test ($P < 0.05$).
[2] P.S.M.E: pooled standard error of mean.
[3] WGR: weight gain rate.
[4] SGR_W: specific growth rate of weight.
[5] SIR: shell increase rate.
[6] DGR_{SL}: daily growth rate of shell length.

$$\textit{Specific growth rate of weight}\left(SGRw, \% \, day^{-1}\right) = \left(LnW_f - LnW_i\right) \times 100/t;$$

$$\textit{Shell length increase}\,(mm) = L_f - L_i;$$

$$\textit{Shell length increase rate}\,(SIR, \%) = \left(L_f - L_i\right)/Li \times 100;$$

$$\textit{Daily growth rate of shell length}\left(DGR_{SL}, \mu m \, day^{-1}\right) = \left(L_f - L_i\right)/t \times 1000;$$

Where, W_f and W_i are final and initial mean weight (g), respectively; L_f and L_i are final and initial mean shell length (mm), respectively; t was duration of experimental days (120).

The statistical analysis was performed using SPSS 13.0 for Windows. All values are presented as means with S.E.M. (standard error of the mean) and *P*-value. All data was subjected to one-way analysis of variance (ANOVA) followed by Tukey's test. The level of significance was chosen at $P < 0.05$. Regression analysis was used to access the potential relation of the growth, main fatty acid composition and gene expression levels of putative Δ5 Fads by dietary GO or LO inclusion. Curve selection was based on highest regression coefficient (R^2) and lowest *P* value of all curves available.

3. Results

3.1. Fatty acid content in experimental diets

The dominant fatty acid in TP diet was 16:0 (91.35%). Inclusion of graded amounts of GO or LO resulted in increasing percentages of LA (from 1.02% to 67.40%) in GO diets (including 0%, 25%, 50% and 100% GO diets) and ALA (from 0.53% to 70.19%) in LO diets (including 0%, 25%, 50% and 100% LO diets), respectively (Table 2). LC-PUFA was not detected in all experimental diets.

3.2. Growth performance and survival of abalone

The growth performance and survival of abalone fed diets with increasing GO inclusion were presented in Table 3. The SR ranged from 86.00 to 86.67 in abalone fed GO diets, but no significant difference was found among GO dietary treatments ($P > 0.05$). Compared with the abalone fed 0%GO diet (control), the growth of abalone fed 25%GO and 50%GO diets were significantly improved in terms of weight gain, shell length increase, WGR, SGR_W, SIR and DGR_{SL} ($P < 0.05$). However, no significant difference was observed in values of growth parameters between 100%GO and 0%GO groups ($P > 0.05$). Regression analysis showed that the all growth parameters of abalone were first increased and then decreased with the increase of dietary GO

Table 4
Growth response and regression analyses of dietary LO inclusion (X) against growth performance (Y) in abalone.

	Diets				ANOVA [1]		Regression analysis		
Parameters	0%LO (TP)	25%LO	50%LO	100%LO	P.S.M.E [2]	*P*	Equation	R^2	*P*
Initial weight (g)	0.39	0.38	0.38	0.38	0.00	0.861			
Final weight (g)	0.89^{b}	1.15^{a}	1.08^{a}	0.73^{c}	0.05	0.000	$Y = -0.1554X^2 + 0.7236X + 0.3237$	0.94	0.000
Weight gain (g)	0.50^{b}	0.77^{a}	0.72^{a}	0.35^{c}	0.05	0.000	$Y = -0.1586X^2 + 0.7414X - 0.0788$	0.95	0.000
WGR (%) [3]	130.95^{b}	199.81^{a}	192.63^{a}	90.80^{c}	13.84	0.000	$Y = -42.673X^2 + 200.6X - 27.91$	0.96	0.000
SGR_W (% day^{-1}) [4]	0.69^{b}	0.91^{a}	0.90^{a}	0.54^{c}	0.05	0.000	$Y = -0.1436X^2 + 0.6688X + 0.1661$	0.96	0.000
Initial shell length (mm)	15.19	15.07	15.01	15.07	0.05	0.692			
Final shell length (mm)	18.24^{b}	20.11^{a}	20.01^{a}	17.57^{b}	0.34	0.000	$Y = -1.0782X^2 + 5.1817X + 14.117$	0.95	0.000
Shell increase (mm)	3.04^{b}	5.04^{a}	5.00^{a}	2.51^{c}	0.35	0.000	$Y = -1.1217X^2 + 5.4426X - 1.2938$	0.98	0.000
SIR (%) [5]	20.06^{b}	33.47^{a}	33.30^{a}	16.65^{c}	2.31	0.000	$Y = -7.5151X^2 + 36.537X - 9.1076$	0.99	0.000
DGR_{SL} (μm day^{-1})[6]	25.4^{b}	42.03^{a}	41.66^{a}	20.91^{c}	2.88	0.000	$Y = -9.3474X^2 + 45.355X - 10.781$	0.98	0.000
Survival rate (%)	86.67	90.67	92.00	82.67	1.51	0.097	$Y = -3.3333X^2 + 15.6X + 74$	0.50	0.046

[1] ANOVA: one-way analysis of variance. Different superscript letters in each row show significant differences among dietary treatments by Tukey's test ($P < 0.05$).
[2] P.S.M.E: pooled standard error of mean.
[3] WGR: weight gain rate.
[4] SGR_W: specific growth rate of weight.
[5] SIR: shell increase rate.
[6] DGR_{SL}: daily growth rate of shell length.

Table 5
Lipid and fatty acid compositions in muscle of abalone fed dies with graded increase of GO.

	Initial abalone	Diets				ANOVA [1]	
		0%GO(TP)	25%GO	50%GO	100%GO	P.S.E.M [2]	*P*
Lipid (%)	6.88	6.58	6.56	6.63	6.61	0.04	0.930
Fatty acid (the percentage in total fatty acids) [3]							
14:0	3.86	3.60^{a}	3.17^{ab}	3.74^{a}	2.77^{b}	0.13	0.012
16:0	22.61	30.45^{a}	25.13^{b}	21.46^{c}	18.08^{d}	1.41	0.000
18:0	7.25	7.54^{c}	7.74^{bc}	8.64^{a}	8.50^{ab}	0.16	0.005
20:0	5.67	6.49	5.63	5.65	6.22	0.15	0.062
22:00	5.68	5.84^{a}	5.66^{ab}	4.66^{c}	5.18^{bc}	0.15	0.002
∑ SFA [4]	45.07	53.92^{a}	47.34^{b}	44.15^{c}	40.75^{d}	1.49	0.000
16:1n-7	8.33	7.59^{b}	8.46^{ab}	9.18^{ab}	10.15^{a}	0.36	0.042
18:1n-9	6.26	10.16^{a}	7.71^{b}	5.71^{c}	4.80^{c}	0.63	0.000
18:1n-7	5.72	5.41^{a}	4.30^{b}	3.83^{b}	3.84^{b}	0.21	0.00
∑ MUFA [5]	20.32	23.16^{a}	20.46^{ab}	18.72^{b}	18.79^{b}	0.62	0.008
18:2n-6	7.38	3.36^{d}	6.82^{c}	9.12^{b}	10.69^{a}	0.83	0.000
18:3n-3	3.51	2.72	2.30	2.51	2.14	0.10	0.156
20:2n-6	1.70	1.25^{d}	2.35^{c}	2.85^{b}	3.45^{a}	0.25	0.000
20:3n-3	0.60	Nd[6]	0.33^{c}	0.39^{b}	0.43^{a}	0.05	0.000
20:4n-6	5.49	4.59^{c}	5.83^{bc}	7.03^{ab}	7.93^{a}	0.40	0.000
20:5n-3	6.61	4.14^{b}	5.46^{ab}	5.45^{ab}	5.72^{a}	0.23	0.033
22:2n-6	1.79	1.17^{c}	1.67^{bc}	2.17^{ab}	2.40^{a}	0.16	0.001
22:5n-3	5.75	4.55^{b}	5.02^{ab}	5.52^{ab}	6.02^{a}	0.21	0.042
22:6n-3	0.90	0.39^{bc}	0.54^{a}	0.52^{ab}	0.29^{c}	0.03	0.001
∑ n-6 PUFA [7]	16.36	10.38^{d}	16.67^{c}	21.17^{b}	24.47^{a}	1.61	0.000
∑ n-3 PUFA	17.37	11.79	13.64	14.39	14.60	0.46	0.106

[1] ANOVA: one-way analysis of variance.
[2] P.S.M.E: pooled standard error of mean.
[3] Some fatty acids, of which the contents are trace amount or not detected, were not listed in the table. Means in the same row sharing a same superscript letter are not significantly different determined by Tukey's test.
[4] SFA: saturated fatty acid.
[5] MUFA: monounsaturated fatty acid.
[6] Nd: not detectable.
[7] PUFA: polyunsaturated fatty acid.

($R^2 > 0.72$, $P < 0.05$). The SR of abalone was not significantly correlated with dietary GO contents ($R^2 = 0.04$, $P = 0.815$) (Table 3).

The growth response and survival of abalone fed diets supplemented with graded LO level were shown in Table 4. No significant difference was observed in SR of abalone fed various LO diets ($P > 0.05$). Compared with those of 0%LO group, abalone fed 25% and 50% LO diets showed significantly higher values of weight gain, shell length increase, WGR, SGR_W, SIR and DGR_{SL} ($P < 0.05$). However, the values of those significantly declined when abalones were fed 100% LO diet. A significant relationship was noted between the values of these growth parameters of abalone and the dietary LO inclusion ($R^2 > 0.94$, $P = 0.000$) (Table 4).

3.3. Lipid and fatty acid analysis in muscle of cultured abalone

Lipid and fatty acid profiles of abalone fed diets with increasing level of GO or LO were shown in Tables 5 and 6, respectively. There is no significant difference in crude lipid level of muscle in abalone fed diets containing increasing amount of GO or LO ($P > 0.05$). Fatty acid compositions of abalone were significantly affected by experimental diets (GO and LO diets) in composition with that of initial abalone. Abalone fed TP diet accumulated the highest level of SFA, especially 16:0, and monounsaturated fatty acid (MUFA). Total SFA and MUFA in muscle of abalone were progressively decreased with the increase of dietary GO or LO inclusion ($R^2 > 0.681$) (Tables 5 and 6). Total n-6 PUFA in GO groups (Table 5) was significantly increased with the increasing amounts of GO in diets ($R^2 = 0.981$, $P = 0.000$) (Fig. 1E). A significant positive relationship ($R^2 = 0.977$, $P = 0.000$) was observed between muscle total n-3 PUFA in LO groups and dietary LO level (Fig. 2F).

The relationship between dietary increasing LA (en%) and values of n-6 series fatty acids in muscle of GO groups were regression analyzed in Fig. 1. The levels of muscle LA, 20:2n-6, ARA, 22:2n-6 and total n-6 PUFA were progressively increased with the increase of dietary LA (0.08–5.54 en%) in GO diets ($R^2 > 0.895$, $P = 0.000$). There are slight amounts of ALA in all GO diets (from 0.04–0.13 en%), and this also translated into moderate augments of 20:3n-3, EPA, docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA) in muscle tissue (Table 5). In addition, DHA in muscle of 100%GO group was significantly lower than those of 25%GO and 50%GO groups. Values of ALA, 20:3n-3, EPA, DPA or DHA in muscle of abalone fed GO diets were lower than those of initial abalone.

The regression analyses of dietary increasing ALA (en%) on values of ALA, 20:3n-3, EPA, DPA, DHA and total n-3 PUFA in muscle of LO groups were presented in Fig. 2. The proportions of muscle ALA, 20:3n-3, EPA and DPA were progressively increased with the graded increase of dietary ALA (0.04–5.73 en%) in LO diets ($R^2 > 0.905$, $P = 0.000$). DHA was initially increased and then decreased with the increase of dietary ALA ($R^2 = 0.937$, $P = 0.000$). Low levels of LA in all LO diets, ranging from 0.08% to 1.22%, had no significant effect on values of muscle LA and 20:2n-6 ($P > 0.05$), but significantly increased ARA and 22:2n-6 in muscle tissue (Table 6).

3.4. Expressions of putative Δ5 Fads in muscle of abalone

The expression levels of putative Δ5 Fads in muscle of abalone fed various diets were shown in Fig. 3. All values are expressed as relative expressions of putative Δ5 Fads normalized against the expression levels of RPS9. Compared to those of abalone fed control diet (TP diet), expression levels of Hdhfad1 and Hdhfad2 were increased to 163.12 ± 0.09% and 248.67 ± 0.27% in abalone fed with 50%GO diet ($P < 0.05$). There was no significant difference in abalone fed with 25%GO or 100%GO diet in comparison with those of control group

Table 6
Lipid and fatty acid compositions in muscle.

	Initial abalone	Diets				ANOVA [1]	
		0%LO(TP)	25%LO	50%LO	100%LO	P.S.E.M [2]	*P*
Lipid (%)	6.88	6.58	6.61	6.56	6.69	0.04	0.779
Fatty acid (the percentage in total fatty acids) [3]							
14:0	3.86	3.60	3.76	3.42	3.30	0.09	0.330
16:0	22.61	30.45^{a}	27.5^{b}	20.24^{c}	17.22^{d}	1.62	0.000
18:0	7.25	7.54	7.87	7.93	8.46	0.16	0.247
20:0	5.67	6.49	5.80	5.60	5.71	0.14	0.057
22:00	5.68	5.84	6.44	6.09	6.07	0.12	0.377
∑ SFA [4]	45.07	53.92^{a}	51.37^{a}	43.28^{b}	40.76^{b}	1.67	0.000
16:1n-7	8.33	7.59^{b}	7.53^{b}	10.73^{a}	11.63^{a}	0.58	0.000
18:1n-9	6.26	10.16^{a}	8.01^{b}	6.31^{c}	4.74^{d}	0.61	0.000
18:1n-7	5.72	5.41^{a}	3.21^{b}	2.8^{b}	3.23^{b}	0.32	0.000
∑ MUFA [5]	20.32	23.16^{a}	18.75^{b}	19.85^{b}	19.6^{b}	0.55	0.001
18:2n-6	7.38	3.36	3.45	3.24	3.05	0.10	0.587
18:3n-3	3.51	2.72^{d}	4.61^{c}	7.42^{b}	8.74^{a}	0.72	0.000
20:2n-6	1.70	1.25	1.27	1.68	1.68	0.08	0.017
20:3n-3	0.60	Nd [6]	0.43^{c}	0.88^{b}	1.41^{a}	0.16	0.000
20:4n-6	5.49	4.59^{b}	5.25^{ab}	6.73^{a}	6.17^{ab}	0.32	0.046
20:5n-3	6.61	4.14^{d}	5.57^{c}	6.31^{b}	7.05^{a}	0.33	0.000
22:2n-6	1.79	1.17^{b}	1.13^{b}	1.62^{a}	1.6^{a}	0.08	0.006
22:5n-3	5.75	4.55^{c}	6.23^{b}	6.40^{ab}	7.60^{a}	0.35	0.001
22:6n-3	0.90	0.39^{d}	0.85^{b}	1.07^{a}	0.52^{c}	0.08	0.000
∑ n-6 PUFA [7]	16.36	10.38^{b}	11.10^{ab}	13.26^{a}	12.5^{ab}	0.43	0.043
∑ n-3 PUFA	17.37	11.79^{d}	17.69^{c}	22.08^{b}	25.32^{a}	1.53	0.000

[1] ANOVA: one-way analysis of variance.
[2] P.S.M.E: pooled standard error of mean.
[3] Some fatty acids, of which the contents are trace amount or not detected, were not listed in the table. Means in the same row sharing a same superscript letter are not significantly different determined by Tukey's test.
[4] SFA: saturated fatty acid.
[5] MUFA: monounsaturated fatty acid.
[6] Nd: not detectable.
[7] PUFA: polyunsaturated fatty acid.

($P > 0.05$) (Fig. 3A). Expression levels of *Hdhfad1* and *Hdhfad2* in abalone fed with 50%LO diet were about 2.34 and 2.50 times as those of control group. And the levels were about 2.09 and 2.46 times as those of TP group when abalone was fed with 100%LO diet ($P < 0.05$). There is no significant difference between 25%LO group and control group in expressions of putative Δ5 Fads (Fig. 3B). A significant positive relationship ($R^2 > 0.929$, P = 0.000) was noted between putative Δ5 Fads expression levels and dietary LO inclusion.

4. Discussion

Previous studies have suggested that LA, ALA, ARA and EPA are essential fatty acid (EFA) for *H. discus hannai* Ino. And diets containing EFA contributed to the faster growth of abalone (Bautista-Teruel et al., 2011; Durazo-Beltrán et al., 2003; Mai et al., 1996; Xu et al., 2004). Limited growth of abalone in all experimental groups, even though for 120-days period, may be related to the absence of dietary ARA and EPA. Similarly, lower growth rate was also observed in abalone fed diets containing lower levels of n-3 LC-PUFA (Mateos et al., 2013). The present study confirmed the contributing effects of EFA (LA and ALA) on growth of abalone in comparison with the group fed diet without EFA supplementation. Furthermore, it was also found that extremely high levels of LA or/and ALA might vanish the stimulating effect of EFA or even reduce the growth of abalone. Similar findings have also been observed in other studies that excessive EFA, especially n-3 LC-PUFA, might impair the growth of fishes (Furuita et al., 2002; Kim and Lee, 2004). Dietary fatty acids are known to be energy provision for organisms through β-oxidation (Mishra and Samantaray, 2004). Moreover, PUFA (such as LA and ALA) is not preferred in this process than SFA and MUFA (Lim et al., 2001). Therefore, high levels of LA and ALA in 100%GO and 100%LO diets may have consequences on the protein sparing effect, using protein as energy instead of PUFA, and eventually on growth performance of abalone.

LC-PUFA in muscle of abalone fed GO or LO diets, despite the absence of LC-PUFA in these diets, confirmed that abalone has the capacity for converting dietary C18 precursors to LC-PUFA and retaining LC-PUFA in tissue (Durazo-Beltrán et al., 2003; Mai et al., 1996; Xu et al., 2004). Moreover, increasing levels of dietary LA in GO diets or ALA in LO diets progressively elevated the levels of total n-6 PUFA ($R^2 > 0.895$, $P = 0.000$) or total n-3 PUFA ($R^2 > 0.905$, $P = 0.000$) respectively in muscle tissue.

In the absence of other n-6 PUFA, dietary LA is the sole contributor to tissue ARA, linked biochemically via two desaturases and an elongase. Theoretically, increasing dietary LA, without the competition of other C18 fatty acids, should contribute to the increase of ARA level in tissue. And this relationship has been suggested in rodent (Gibson et al., 2013; Mohrhauer and Holman, 1963; Renaud et al., 1995) and fish (Ren et al., 2012), and also established in our study (Table 3, Fig. 1C). However, other papers on human concluded that increasing dietary LA has no effect on increase of plasma phospholipid ARA level, or even may have an inverse relationship (Adam et al., 2003, 2008; Liou and Innis, 2009; Liou et al., 2007; Rett and Whelan, 2011; Thijssen and Mensink, 2005). The possible reason for no response of tissue ARA content by dietary increasing LA in those studies was considered to be related to fractional conversion from LA to ARA and limited catalysis of Δ6 Fad on LA (Demmelmair et al., 1999; Rett and Whelan, 2011; Vermunt et al., 2001). Present study has suggested that abalone has certain conversion of dietary LA to muscle ARA. In addition, such biosynthetic process was regulated on mRNA levels of Δ6 Fad, Δ5 Fad and elongase in abalone (Fig. 3) (Mateos et al., 2011, 2012a,b). This probably suggested the differences in the affinity of

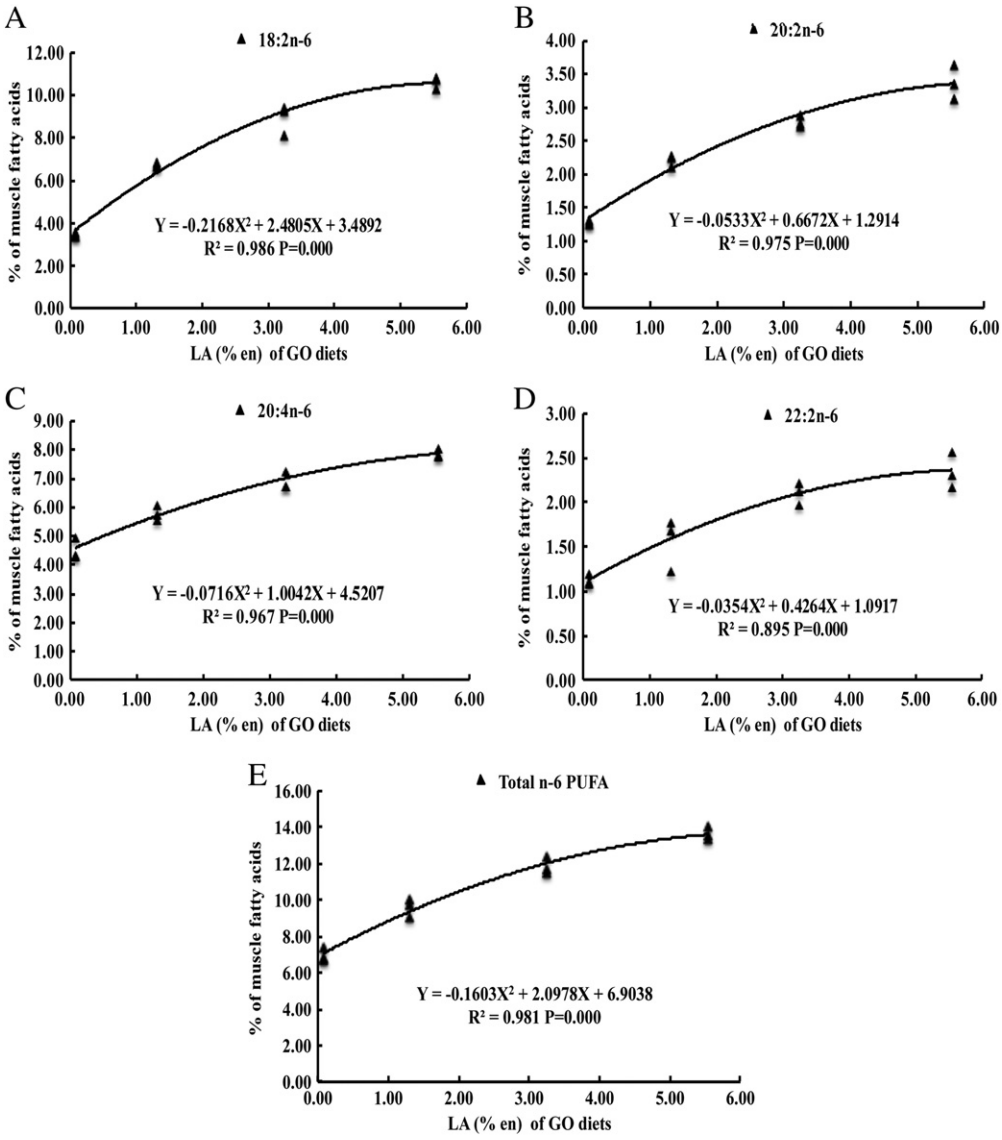

Fig. 1. Effects of increasing levels of dietary LA (18:2n-6) on n-6 PUFA in muscle of abalone fed GO diets. The relationship between n-6 PUFA (A: 18:2n-6, B: 20:2n-6, C: 20:4n-6, D: 22:2n-6, E: Total n-6 PUFA) in muscle of abalone against dietary LA (%en) was analyzed by second-degree polynomial regression.

desaturases or elongases on substrates in different species. And more researches are needed to explain this question.

In present study, dietary increasing ALA in LO diets resulted in augments of total n-3 series fatty acids (especially for n-3 LC-PUFA) in muscle of abalone in a curvilinear manner ($R^2 > 0.905$, $P = 0.000$), which is consistent with the study on chicken tissue (Kartikasari et al., 2012). However, it was failed to demonstrate an increase of n-3 LC-PUFA in tissue of barramundi (*Lates calcarifer*) under increasing proportions of dietary ALA (Tu et al., 2013). Such difference in n-3 LC-PUFA biosynthesis was attributed to, at least to some extent, the regulation of mRNA expression levels of crucial enzymes by dietary ALA. In present result, compared with those of control group, putative Δ5 Fads expressions were significantly up regulated in 50%LO and 100%LO groups, which accordingly have higher muscle n-3 LC-PUFA levels than those of other groups (Table 6). However in study of barramundi, the expression levels of two genes (Δ6 Fad and elongase) were not different between fish fed differing ALA levels (Tu et al., 2013). The other possible reason for such difference may be related to the substrate level, which has been suggested in other studies (Goyens et al., 2006; Tu et al., 2010). Coincidentally, the ALA levels in study of barramundi varied from 0.1% to 3.2% energy (Tu et al., 2013), which were significantly lower than those of ALA diets for chicken study ranging from 1% to 8% energy (Kartikasari et al., 2012), and LO diets in our study from 0.04% to 5.73% energy.

The value of DHA was gradually increased in muscle of abalone when dietary ALA was less than about 3.00 en%. Further increase of dietary ALA in LO diets resulted in the decline of DHA in muscle tissue ($R^2 = 0.937$, $P = 0.000$). Similar finding was reported on rat that DHA synthesis from ALA is inhibited by diets extremely high in C18 PUFA (Gibson et al., 2013). The biosynthesis of DHA from ALA involved two uses of Δ6 Fad, with the first converting ALA to 18:4n-3 and the second converting 24:5n-3 to DHA. And this pathway has been suggested in many species, such as rat and trout (Buzzi et al., 1997; D'andrea et al., 2002; Sprecher, 2000). Recently, another pathway for DHA synthesis was reported in some vertebrate species that involves direct Δ4 desaturation of DPA (Li et al., 2010; Morais et al.,

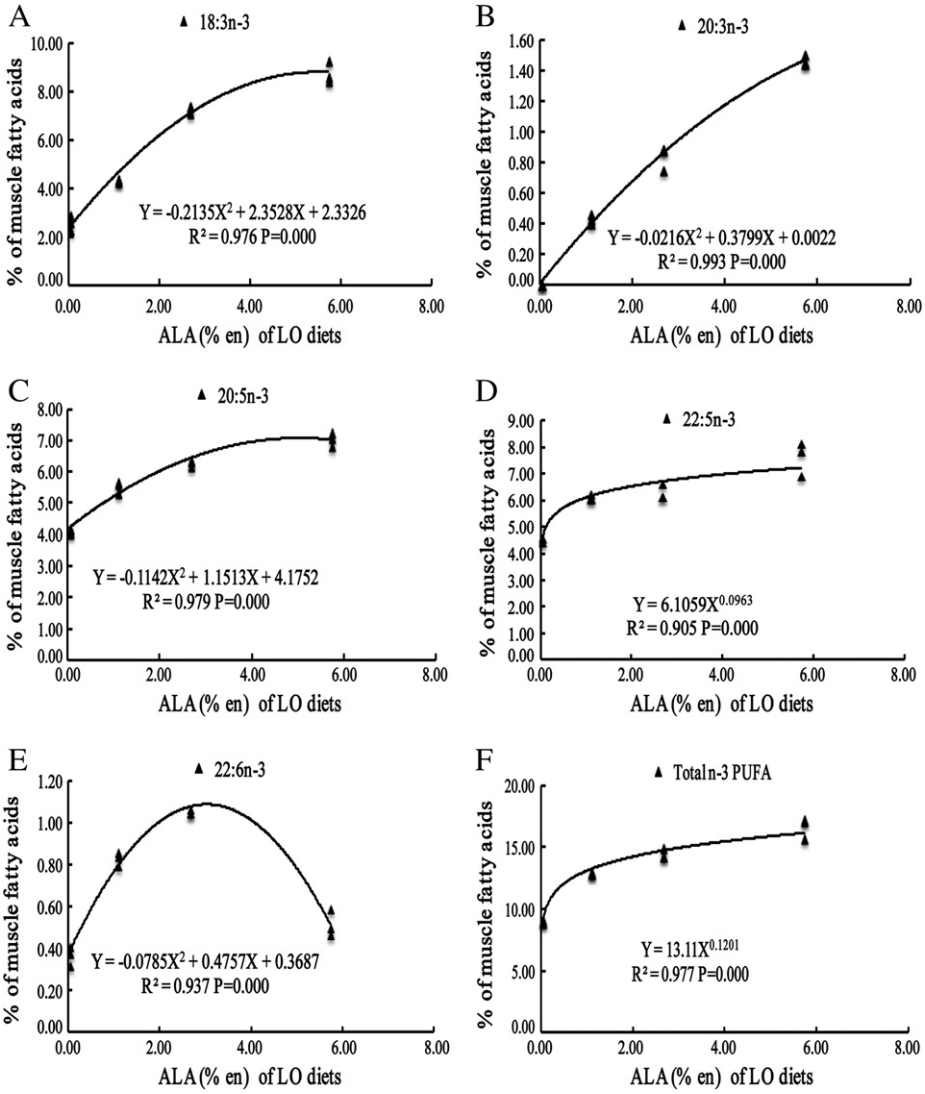

Fig. 2. Effects of increasing levels of dietary ALA (18:3n-3) on n-3 PUFA in muscle of abalone fed LO diets. The relationship between n-3 PUFA (A: 18:3n-3, B: 20:3n-3, C: 20:5n-3, D: 22:5n-3, E: 22:6n-3, F: Total n-3 PUFA) in muscle of abalone against ALA (%en) in LO diets was analyzed by second-degree polynomial or power regression.

2012). However, there is no information on which pathway does abalone exactly use to biosynthesize DHA *in vivo*. With the successfully cloning of Δ6 Fad in abalone (Mateos et al., 2011, 2012a,b), we speculated that the competition between ALA and 24:5n-3 for the Δ6 Fad in the conversion of 24:5n-3 to DHA resulted in the lower accumulation of DHA in abalone at high ALA level in 100%LO diet. This interpretation is supported by the findings that Δ6 desaturase has a higher substrate affinity for C18 PUFA than C24 substrates (Geiger et al., 1993). Furthermore, 100%LO diet also contained higher LA content than other LO diets in present study, which also aggravated the competition between C18 and C24 fatty acid for Δ6 Fad. As expected, DHA has low levels in any groups fed with GO diets, which are extremely rich in LA. This finding is consistent with previous studies that DHA biosynthesis and accumulation is suppressed by dietary LA (Gibson et al., 2013; Liou and Innis, 2009; Sprecher et al., 1995, 1999).

In conclusion, results of the present study demonstrated that the biosynthesis of some LC-PUFA in muscle of abalone could be increased in response to increasing levels of dietary LA or ALA, and this could be accomplished through the promoting effect of LA or ALA on the expressions of putative Δ5 Fads. High intakes of dietary LA or/and ALA resulted in reduced growth performance of abalone and inhibited the biosynthesis of DHA. The biosynthesis of LC-PUFA is very complex and it is affected by dietary fatty acid composition, key enzymes crucial for this biosynthetic process, regulation of some crucial transcription factors, and the feedback of tissue fatty acid profiles. Further studies are warranted to find practical ways to increase such biosynthesis activity from C18 PUFA by means of elevating the activities of crucial enzymes (Δ6, Δ5 desaturases and elongases) or stimulating the regulation of the transcription factors, such as sterol regulatory element-binding protein and peroxisome proliferator-activated receptor, involved in LC-PUFA metabolism.

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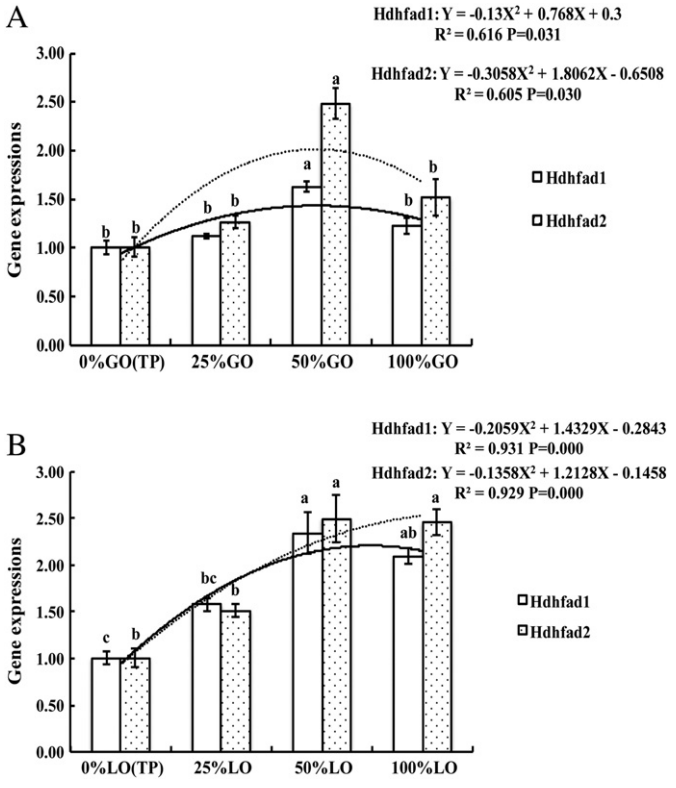

Fig. 3. Effects of dietary increasing amounts of GO (A) or LO (B) on expression levels of putative Δ5 Fads (Hdhfad1 and Hdhfad2) in muscle of abalone, *H. discus hannai* Ino. Values are means ± S.M.E. (n = 3). Different letters above the columns indicate significant difference by Tukey's test ($P < 0.05$). The relationship between muscle expression levels of putative Δ5 Fads (Hdhfad1 and Hdhfad2) against dietary GO or LO inclusion was analyzed by second-degree polynomial regression.

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