J. Ocean Univ. China (Oceanic and Coastal Sea Research)
https://doi.org/10.1007/s11802-020-4470-
ISSN 1672-5182, 2020 19 (6): 1-11
http://www.ouc.edu.cn/xbywb/
E-mail:xbywb@ouc.edu.cn

Fatty Acid Composition and Digestive Enzyme Activities of Rainbow Trout in Response to Dietary Docosahexaenoic Acid (DHA) and Eicosapentaenoic Acid (EPA) During Salinity Acclimation

HUANG Ming[1)], ZHOU Yangen[1), *], GE Jian[1)], AGUSTSSON Thorleifur[2), 3)], LI Li[1)], GAO Qinfeng[1), 4)], and DONG Shuanglin[1), 4)]

1) *Key Laboratory of Mariculture (Ocean University of China)*, *Ministry of Education*, *Qingdao* 266100, *China*
2) *NORCE Norwegian Research Center*, *Mekjarvik* 12, 4072, *Randaberg*, *Norway*
3) *RORUM Brynjolfsgata* 5, 107, *Reykjavík*, *Iceland*
4) *Function Laboratory for Marine Fisheries Science and Food Production Processes*, *Qingdao National Laboratory for Marine Science and Technology*, *Qingdao* 266235, *China*

(Received January 24, 2020; revised May 8, 2020; accepted September 1, 2020)

Abstract This physiological study aimed to evaluate the effects of dietary docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) on the fatty acid composition and digestive enzyme activities of rainbow trout (*Oncorhynchus mykiss*) during salinity acclimation. Rainbow trout with an average initial weight of 90.61 g ± 9.25 g were fed diets with the quantities of DHA and EPA equaling to 0.54%, 0.95%, 1.40% and 1.79% (abbreviated as DE-0.54, DE-0.95, DE-1.40, and DE-1.79, respectively) for eight weeks, after which the gastric and intestinal fatty acids composition were analyzed. Subsequently, the fish underwent salinity acclimation. On days 1, 4, 7, and 14 after the freshwater was replaced by seawater and at the end of the 8-week period, gastric and intestinal digestive enzyme activities were determined. The results showed that the gastric and intestinal DHA and EPA contents of the fish were positively correlated to their dietary DHA and EPA levels. Low dietary DHA and EPA levels inhibited the protease activity of rainbow trout. Fish in the DE-0.54 group increased the lipase activity to enhance the utilization of lipids for essential fatty acids demand. Hence, rainbow trout in the DE-0.54 group failed to maintain suitable activities of digestive enzymes after salinity acclimation. Therefore, a diet with minimum 0.95% DHA and EPA levels is necessary for rainbow trout during salinity acclimation.

Key words digestive enzymes; docosahexaenoic acid; eicosapentaenoic acid; rainbow trout

1 Introduction

Rainbow trout (*Oncorhynchus mykiss*), which is classified as salmonids, is one of the world's most important aquaculture species and is farmed in many countries (Esmaeili *et al*., 2017). Due to the increasing demand for rainbow trout, Chinese researchers are establishing mariculture system using deep-sea cages far offshore in the Yellow Sea Cold Water Mass of China (Evans, 2018; Dong, 2019). The 'mountain-sea transfer' culturing method is used, in which juvenile fish are hatched and cultivated in freshwater in mountainous areas before being transferred to seawater cages for growth until harvest.

The parr-smolt transformation process of salmonids (smoltification) requires up to 50% of the total available energy (Moser *et al*., 1994; Boeuf *et al*., 2001). Fishes take energy mainly from the proteins and lipids and the demand for essential fatty acids varies among different species (Glencross, 2009; NRC, 2011). It has been reported that the demand for polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid (DHA, C22:6n3) and eicosapentaenoic acid (EPA, C20:5n3), in salmonids increase during smoltification (Ackman *et al*., 1986; Tocher *et al*., 2000). Furthermore, previous studies have revealed that adequate accumulation of DHA and EPA is a prerequisite for salmonids before being transferred to the sea (Sheridan *et al.*, 1985; Li *et al.*, 1992; Bell *et al.*, 1997).

It has been suggested that digestive enzyme activities can be regulated by dietary composition (Fountoulaki *et al*., 2005; Santigosa *et al*., 2008; Castro *et al*., 2013; Sivaramakrishnan *et al*., 2017; Fuentes-Quesada *et al*., 2018). Xie *et al.* (2018) reported that the activities of protease, lipase and α-amylase in the digestive tract of rabbitfish (*Siganus canaliculatus*) changed depending on the levels of dietary proteins, lipids, and carbohydrates, respectively. The digestive ability of fish changes in response to the

* Corresponding author. Tel: 0086-532-82031590
E-mail: zhouyg@ouc.edu.cn

replacement of fish oil with vegetable oil in the diet, and this replacement causes a decrease in the dietary DHA and EPA levels. You *et al.* (2019) reported that dietary soybean oil significantly decreases the amylase, trypsin, and lipase activities in the intestine of golden pompano (*Trachinotus ovatus*). However, Bowyer *et al.* (2012) found that dietary coconut oil decreases trypsin and lipase activities but had no effect on amylase activity in yellowtail kingfish (*Seriola lalandi*).

The exposure of fish to a hypersaline environment accelerates the drinking rate and thereby changes the ion content and pH in the gastrointestinal tract of fish, influencing the activities of digestive enzyme (Noda *et al*., 1981; Squires *et al*., 1986a; Usher *et al*., 1988; Moutou *et al*., 2004). The effects of salinity on digestive enzyme activities have been widely reported in many species, such as turbot (*Scophthalmus maximus*) (Chen *et al*., 2006), Caspian kutum (*Rutilus frisii kutum*, Kamensky, 1901) (Gheisvandi *et al*., 2015), Japanese flounder (*Paralichthys olivaceus*) (Bolasina *et al*., 2007), and fat snook (*Centropomus parallelus*) (Tsuzuki *et al*., 2007).

Interactions between osmoregulation and digestion have been reported in fish (Psochiou *et al*., 2007). Digestive enzyme activities are indicators of digestive processes and nutritional conditions that can be easily and reliably analyzed (Barman *et al*., 2005; Bolasina *et al*., 2006; Rungruangsak-Torrissen *et al*., 2006; Nikolopoulou *et al*., 2011; Sutthinon *et al*., 2015; Liu *et al*., 2017). However, previous studies have mainly focused either on the effects of dietary components or salinity acclimation on the digestive enzyme activities, while few studies have considered these two factors together at the same time. The objective of this study was to explore the effects of dietary DHA and EPA levels on the gastric and intestinal digestive enzyme activities in rainbow trout during salinity acclimation.

2 Materials and Methods

2.1 Experimental Diets

The basal diet was formulated to be both isoproteic (45% proteins) and isolipidic (16% lipids) using fish meal, soybean meal, chicken meal, and corn gluten meal as the primary protein sources and fish oil and soybean oil as the lipid sources. With the adjustment of the proportions of fish oil and soybean oil, four different practical diets with DHA + EPA levels equal to 0.54%, 0.95%, 1.40%, or 1.79% were designed (Table 1). The fatty acid composition of the experimental diets is presented in Table 2.

2.2 Experimental Design and Sample Collection

The experiment was conducted at the Key Laboratory of Mariculture, Ocean University of China (Qingdao, Shandong, China). Juvenile rainbow trouts were obtained from the Linqu Cold Water Fish Farm (Linqu, Shandong, China). Prior to the experiment, stomachs and intestines of three fish were collected as initial samples, after which fish were acclimated by being stocked in circular tanks (volume, 180 L; height, 0.60 m; upper diameter, 0.65 m; lower diameter, 0.60 m) for two weeks and fed experimental diets (mixed by the four aforementioned diets). After the acclimation period, fish with an average weight of (90.61 ± 9.25) g were randomly assigned to four groups, and the fish in the various groups were fed diets with DHA and EPA levels equaling to 0.54%, 0.95%, 1.40% and 1.79%

Table 1 Composition of the four diets (45% protein and 16% lipid) formulated with0.54%, 0.95%, 1.40%, and 1.79% of total dietary DHA and EPA

Component		DE-0.54	DE-0.95	DE-1.40	DE-1.79
Ingredient (%, as-is)	Fish meal	30.00	30.00	30.00	30.00
	Chicken meal	12.00	12.00	12.00	12.00
	Soybean meal	17.80	17.80	17.80	17.80
	Corn gluten meal	16.40	16.40	16.40	16.40
	Corn starch	11.83	11.83	11.83	11.83
	Choline chloride	0.50	0.50	0.50	0.50
	Vitamin premix	1.00	1.00	1.00	1.00
	Mineral premix	1.00	1.00	1.00	1.00
	Soybean lecithin	1.00	1.00	1.00	1.00
	Fish oil	1.17	2.90	4.62	6.33
	Soybean oil	7.30	5.57	3.85	2.14
Proximate composition (%, as-is)	Ash	9.85	9.66	9.31	10.09
	Crude protein	47.89	47.51	48.13	46.53
	Lipid	15.69	15.50	15.34	15.31
	DHA + EPA	0.54	0.95	1.40	1.79

Notes: Fish meal, crude protein 63.83%, crude lipid 10.0%; Chicken meal, crude protein 59.51%, crude lipid 15.47%; soybean meal, crude protein 42.17%, crude lipid 4.42%; corn starch, crude protein 67.06%, crude lipid 3.37%. Ingredients were obtained from Great Seven Bio-Tech (Qingdao, Shandong, China). Vitamin premix (mg (kg diet)$^{-1}$): 5 mg vitamin D; 10 mg vitamin K; 10 mg vitamin B12; 20 mg vitamin B6; 20 mg folic acid; 25 mg vitamin B1; 32 mg vitamin A; 45 mg vitamin B2; 60 mg pantothenic acid; 60 mg biotin; 200 mg niacin acid; 240 mg α-tocopherol; 800 mg inositol; 2000 mg ascorbic acid. Mineral premix (mg (kg diet)$^{-1}$): 10 mg $CuSO_4 \cdot 5H_2O$; 25 mg Na_2SeO_3 (1%); 50 mg $ZnSO_4 \cdot H_2O$; 50 mg $CoC_{12} \cdot 6H_2O$ (1%); 60 mg $MnSO_4 \cdot H_2O$; 80 mg $FeSO_4 \cdot H_2O$; 180 mg $Ca(IO_3)_2$; 1200 mg $MgSO_4 \cdot 7H_2O$; 8345 mg zeolite. DHA, docosahexaenoic acid, C22:6n3. EPA, eicosapentaenoic acid, C20:5n3.

Table 2 Fatty acid composition of the experimental diets (% of total fatty acids)

Fatty acids		DE-0.54	DE-0.95	DE-1.40	DE-1.79
Saturated fatty acids	C10:0	0.33	0.34	0.32	0.33
	C12:0	0.40	0.43	0.40	0.44
	C14:0	1.89	2.66	3.39	4.18
	C15:0	0.19	0.29	0.36	0.45
	C16:0	16.96	18.04	19.16	20.15
	C17:0	0.59	0.81	1.02	1.25
	C18:0	5.05	5.10	5.14	5.12
	C20:0	1.18	1.20	1.19	1.22
	C21:0	ND	ND	0.14	0.17
	C22:0	0.82	0.79	0.74	0.72
	C23:0	0.17	0.21	0.23	0.28
	C24:0	0.40	0.42	0.29	ND
	ΣSFA	27.97	30.30	32.37	34.30
Monounsaturated fatty acids	C14:1	0.11	0.14	0.15	0.18
	C15:1	ND	ND	0.09	0.11
	C16:1	2.09	2.99	3.92	4.83
	C17:1	0.15	0.23	0.26	0.34
	C18:1n9	25.94	24.82	23.88	22.54
	C20:1	0.86	1.40	1.96	2.53
	C22:1n9	0.62	1.31	2.03	2.77
	C24:1	0.21	0.25	0.30	0.34
	ΣMUFA	29.98	31.13	32.60	33.65
Polyunsaturated fatty acids	C18:2n6c	33.55	27.79	21.65	16.21
	C18:3n3	3.80	3.26	2.66	2.06
	C18:3n6	0.16	0.19	0.23	0.28
	C20:2	0.29	0.29	0.28	0.33
	C20:3n3	0.38	0.49	0.55	0.66
	C20:4n6	ND	ND	0.15	0.27
	C20:5n3	1.56	2.79	4.04	5.30
	C22:2	0.43	0.41	0.39	0.54
	C22:6n3	1.88	3.37	5.07	6.37
	ΣPUFA	42.05	38.57	35.02	32.04
	Σn-3	7.62	9.91	12.32	14.40
	Σn-6	33.71	27.97	22.03	16.77
	n3/n6	0.23	0.35	0.56	0.86
	DHA+EPA	3.45	6.16	9.11	11.68
	DHA/EPA	1.21	1.21	1.26	1.20

Notes: Values are the means of three replicates. n-3, omega-3 series polyunsaturated fatty acids. n-6, omega-6 series polyunsaturated fatty acids. DHA, docosahexaenoic acid, C22:6n3. EPA, eicosapentaenoic acid, C20:5n3. ND, not detected.

(DE-0.54, DE-0.95, DE-1.40 and DE-1.79 groups, respectively) of the total diets, respectively. Three replicates were included in each group, and each tank contained 12 fish. The experimental diets were offered twice daily at 8:30 a.m. and 4:00 p.m. for an 8-week period, and a 12-h light: 12-h dark photoperiod was maintained during this period. Approximately 70% of the water was changed daily at 12:00 a.m. During this period, the following water quality parameters (mean±standard deviation) were maintained: temperature, (16±1.2)℃; pH, 7.89±0.09; dissolved oxygen (DO), (8.32±0.34) mg L^{-1}; ammonia nitrogen, (0.04±0.01) mg L^{-1}; and nitrite nitrogen, (0.02±0.01) mg L^{-1}. Every two weeks, the experimental fish were starved for one day before being anesthetized by a moderate level of methanesulfonate (MS-222) and then weighed.

At the end of the 8-week period, three fish per treatment were anesthetized using MS-222 (70 mg L^{-1}), and their stomachs and proximal intestines were dissected out, after which the gastric and intestinal contents were squeezed out by sterile tweezers. Sampled stomachs and proximal intestines were kept at −80℃ for fatty acid and digestive enzyme activities determination.

Salinity acclimation was performed in all tanks by mixing brackish water (salinity 14 g L^{-1}) with natural seawater. Salinity was increased to final 30 g L^{-1} at a rate of 4 g L^{-1} per day, after which it was kept constant for an additional period of 14 days. A hand-held salinometer (Dedu DT-Y100, Changzhou, China) was used to measure the salinity. On days 1, 4, 7, and 14 after salinity acclimation, stomachs and proximal intestines of three fish per treatment were collected following the method discussed above. All sampling was performed at 24 h post-feeding. The stomachs were used for determination of the gastric amylase, pepsin and lipase activities, and the intestines were used for determination of the intestinal amylase, trypsin and lipase activities.

2.3 Measurements of Indicators and Analytical Methods

Fatty acids were extracted according to the method described by Bligh *et al.* (1959).They were esterified into methyl esters using methyl esterification reagent (hydrochloric acid/methanol, 1:5) and analyzed by gas chromatography (Shimadzu GC-2010 plus, Kyoto, Japan). The related parameters and instruments used in this experiment were described by Liu *et al*. (2018).

All digestive enzyme activities were measured using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. The stomachs and intestines were weighed and homogenized together with cold saline (0.75% NaCl, pH 7.0). The homogenates were immediately centrifuged at $800 \times g$ for 10 min at 4℃, after which the supernatants were collected and used for determination of enzyme activities after appropriate dilution.

2.4 Statistical Analysis

All statistics were performed using SAS 9.4 (SAS Institute Inc., Cary, NC). Data were analyzed using a one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls (SNK) multiple comparisons test to identify significant differences among different treatment groups. Additionally, interactions between dietary DHA and EPA levels and culture time on digestive enzyme activities were evaluated by the two-way ANOVA among treatment means. All statistical tests for significance were set at $P<0.05$. All figures were constructed by GraphPad Prism 7 (GraphPad Software, San Diego, CA, www.graphpad.com).

3 Results

3.1 Growth

The survivals of rainbow trout in all treatment groups were 100%. No significant differences of final mean weight, final biomass, weight gain percentage and feed conversion ratio (*FCR*) of fish were observed among different treatments (Table 3).

3.2 Fatty Acid Composition

The gastric and intestinal DHA and EPA contents of rainbow trout were positively correlated to their dietary DHA and EPA levels (Tables 4 and 5). At the end of 8-week period, the gastric DHA and EPA contents in the DE-1.40 and DE-1.79 groups were significantly higher than in DE-0.54 and DE-0.95 groups. The intestinal DHA content was the lowest in the DE-0.54 group and the highest in the DE-1.79 group. The intestinal EPA in the DE-1.40 and DE-1.79 groups were significantly higher than in DE-0.54 and DE-0.95 groups. Moreover, the linoleic (C18:2n6) contents in the DE-1.40 and DE-1.79 groups were significantly lower than in DE-0.54 and DE-0.95 groups according to the dietary content of soybean oil.

Table 3 Growth performance of rainbow trout fed four experimental diets (initial weight of 90.61 g ± 9.25 g, 8 weeks)

Diet	Final mean weight (g)	Final biomass (g)	Percent weight gain (%)	Survival (%)	FCR
DE-0.54	142.58	1711.00	56.02	100	1.78
DE-0.95	131.45	1577.40	44.77	100	1.81
DE-1.40	137.30	1647.60	51.22	100	1.78
DE-1.79	132.78	1593.30	47.91	100	1.80
PSE	5.6880	68.2624	5.9371	0.0000	0.1071
P-value	0.5365	0.5358	0.6008	1.0000	0.9931

Notes: The initial weight was presented as mean ± standard deviation. Values are the means of three replicates. Means with different superscripts indicate significant differences ($P < 0.05$) based on one-way ANOVA and the Student-Newman-Keuls (SNK) test. PSE, pooled standard error. FCR, feed conversion rate. Final biomass was measured by weighting 12 fish from each tank, *FCR* = (Dry feed offered) / (Wet weight gain). Percent weight gain = 100 × (Final body weight − Initial body weight) / (Initial body weight).

Table 4 Fatty acid composition of the stomach fat of rainbow trout fed the experimental diets for 8 weeks (% of total fatty acids)

Fatty acid		Initial	DE-0.54	DE-0.95	DE-1.40	DE-1.79	PSE	*P*-value
Saturated fatty acids	C14:0	2.58	1.99^{b}	2.26^{ab}	2.60^{a}	2.46^{ab}	0.1191	0.0329
	C15:0	0.34	0.24	0.30	0.40	0.35	0.0639	0.3698
	C16:0	17.06	17.2^{ab}	16.08^{b}	17.64^{a}	16.9^{ab}	0.3060	0.0375
	C17:0	0.67	0.78^{b}	0.75^{b}	0.91^{a}	0.75^{b}	0.0320	0.0246
	C18:0	6.47	7.22	6.36	6.45	6.23	0.2229	0.0523
	C20:0	0.43	0.59	0.48	0.53	0.46	0.0354	0.1143
	C22:0	0.36	0.47	0.36	0.36	0.32	0.0366	0.0996
	C23:0	0.23	0.20	0.18	0.28	0.29	0.0707	0.6528
	ΣSFA	28.13	28.70^{a}	26.77^{b}	29.15^{a}	27.76^{ab}	0.4311	0.0189
Monounsaturated fatty acids	C16:1	4.64	3.62	4.47	3.83	4.16	0.3080	0.2949
	C17:1	0.36	0.57	0.41	0.32	0.33	0.5594	0.0427
	C18:1n9	23.26	22.75	24.13	23.14	23.58	0.3785	0.1375
	C20:1	0.16	ND	0.07	0.26	0.26	0.0455	0.0416

(*to be continued*)

(*continued*)

Fatty acid		Initial	DE-0.54	DE-0.95	DE-1.40	DE-1.79	PSE	*P*-value
Monounsaturated fatty acids	C22:1n9	0.30	ND	0.29	0.27	0.27	0.0127	0.7266
	C24:1	0.93	1.27	0.94	0.74	0.92	0.1200	0.0751
	ΣMUFA	29.65	28.21^{b}	30.30^{a}	28.57^{ab}	29.52^{ab}	0.4541	0.0434
Polyunsaturated fatty acids	C18:2n6	21.30	21.47^{a}	20.47^{a}	18.30^{b}	18.37^{b}	0.4191	0.0015
	C18:3n3	3.13	2.66	2.79	3.01	2.83	0.1197	0.2967
	C18:3n6	0.67	0.77	0.62	0.68	0.63	0.0806	0.5855
	C20:2	1.66	1.22^{b}	1.70^{a}	1.63^{a}	1.71^{a}	0.0596	0.0012
	C20:3n3	1.53	1.23	1.25	1.36	1.32	0.0490	0.2651
	C20:3n6	0.72	1.28^{a}	0.85^{b}	0.87^{b}	0.68^{b}	0.0673	0.0014
	C20:4n6	1.50	3.34^{a}	2.17^{b}	1.65^{b}	1.49^{b}	0.2571	0.0036
	C20:5n3	2.06	1.61^{b}	1.74^{b}	2.23^{a}	2.35^{a}	0.0825	0.0005
	C22:2	0.73	0.70^{b}	0.74^{b}	0.93^{a}	0.91^{a}	0.0475	0.0173
	C22:6n3	8.93	8.81^{c}	10.61^{b}	$11.60a^{b}$	12.41^{a}	0.3341	0.0003
	ΣPUFA	42.21	43.09	42.93	42.27	42.72	0.4635	0.6460
	Σn-3	15.65	14.31^{c}	16.39^{b}	18.20^{a}	18.92^{a}	0.3299	<0.0001
	Σn-6	24.18	26.86^{a}	24.10^{b}	21.51^{c}	21.17^{c}	0.5494	0.0003
	DHA+EPA	10.98	10.42^{c}	12.36^{b}	13.83^{a}	14.76^{a}	0.3079	<0.0001

Notes: The values represent the means from three replicates. Means with different superscripts indicate significant differences ($P < 0.05$) based on one-way ANOVA and the Student-Newman-Keuls (SNK) test. PSE, pooled standard error. SFA: saturated fatty acid. MUFA, monounsaturated fatty acid. PUFA: polyunsaturated fatty acid. n-3, omega-3 series polyunsaturated fatty acid. n-6, omega-6 series polyunsaturated fatty acid. DHA: docosahexaenoic acid, C22:6n3. EPA, eicosapentaenoic acid, C20:5n3. ND, not detected.

Table 5 Fatty acid composition of the intestine fat in rainbow trout fed the experimental diets for 8 weeks (% of total fatty acids)

Fatty acid		Initial	DE-0.54	DE-0.95	DE-1.40	DE-1.79	PSE	*P*-value
Saturated fatty acids	C14:0	2.69	1.90^{b}	2.16^{b}	2.54^{a}	2.65^{a}	0.1029	0.0031
	C15:0	0.31	0.27^{b}	0.29^{b}	0.40^{a}	0.35^{ab}	0.0251	0.0241
	C16:0	16.45	15.97^{bc}	15.47^{c}	16.78^{ab}	17.37^{a}	0.3248	0.0139
	C17:0	0.68	0.68^{b}	0.75^{ab}	0.92^{a}	0.87^{ab}	0.0520	0.0376
	C18:0	5.87	6.58	6.29	7.36	6.61	0.3691	0.2831
	C20:0	0.34	0.45	0.45	0.56	0.47	0.0398	0.2493
	C22:0	0.26	0.33	0.30	0.39	0.28	0.0243	0.0541
	C23:0	0.35	0.24	0.24	0.34	0.29	0.0225	0.0374
	ΣSFA	26.94	26.42^{b}	25.95^{b}	29.28^{a}	28.89^{a}	0.4814	0.0023
Monounsaturated fatty acids	C16:1	4.82	4.17^{a}	4.05^{a}	3.62^{b}	4.33^{a}	0.0984	0.0054
	C17:1	0.23	0.25^{b}	0.25^{b}	0.40^{a}	0.27^{b}	0.0312	0.0274
	C18:1n9	27.37	27.83^{a}	26.78^{b}	21.33^{c}	21.92^{c}	0.2813	<0.0001
	C20:1	0.20	0.21^{b}	0.22^{b}	0.30^{a}	0.24^{ab}	0.0185	0.0388
	C22:1n9	0.26	0.27	0.25	0.24	0.29	0.0143	0.1702
	C24:1	0.56	0.66	0.62	0.86	0.68	0.0742	0.1764
	ΣMUFA	33.44	33.39^{a}	32.19^{b}	26.76^{d}	27.73^{c}	0.2967	<0.0001
Polyunsaturated fatty acids	C18:2n6	19.93	20.33^{a}	20.64^{a}	18.84^{b}	17.89^{b}	0.3664	0.0023
	C18:3n3	2.62	2.54^{c}	2.82^{b}	2.95^{ab}	3.04^{a}	0.0510	0.0006
	C18:3n6	0.31	0.69	0.56	0.68	0.51	0.0588	0.1421
	C20:2	1.74	1.56^{b}	1.60^{b}	1.65^{b}	1.99^{a}	0.0890	0.0328
	C20:3n3	1.49	1.64	1.44	1.48	1.47	0.1148	0.6439
	C20:3n6	0.62	1.09^{ab}	0.94^{ab}	1.256^{a}	0.72^{b}	0.1093	0.0438
	C20:4n6	1.08	1.56	1.61	1.47	1.42	0.0581	0.1855
	C20:5n3	1.76	1.42^{c}	1.45^{c}	2.02^{a}	1.87^{b}	0.0444	<0.0001
	C22:2	0.70	0.71^{b}	0.72^{b}	0.87^{ab}	1.07^{a}	0.0634	0.0113
	C22:6n3	9.36	8.66^{d}	10.08^{c}	12.74^{b}	13.38^{a}	0.1574	<0.0001
	ΣPUFA	39.62	40.19^{c}	41.86^{b}	43.96^{a}	43.38^{a}	0.2927	<0.0001
	Σn-3	15.24	14.25^{c}	15.80^{b}	19.18^{a}	19.77^{a}	0.2019	<0.0001
	Σn-6	21.94	23.68^{a}	23.74^{a}	22.26^{b}	20.55^{c}	0.3552	0.0007
	DHA+EPA	11.12	10.08^{c}	11.53^{b}	14.75^{a}	15.25^{a}	0.1785	<0.0001

Notes: The values represent the means from three replicates. Means with different superscripts indicate significant differences ($P < 0.05$) based on one-way ANOVA and the Student-Newman-Keuls (SNK) test. PSE, pooled standard error. SFA: saturated fatty acid. MUFA, monounsaturated fatty acid. PUFA, polyunsaturated fatty acid. n-3, omega-3 series polyunsaturated fatty acid. n-6, omega-6 series polyunsaturated fatty acid. DHA, docosahexaenoic acid, C22:6n3. EPA, eicosapentaenoic acid, C20:5n3.

3.3 Gastric Amylase, Pepsin and Lipase Activities in Rainbow Trout

No significant difference of gastric amylase activity in rainbow trout was observed with different dietary DHA and EPA levels before salinity acclimation (Fig.1A). Gastric amylase activity in rainbow trout in the DE-1.40 and DE-1.79 groups continually increased following salinity increase, reaching maximum values on day 7, and subsequently recovered to freshwater values on day 14. Moreover, gastric amylase activity in the DE-0.54 group was significantly lower compared to the other three groups on day 4 after salinity acclimation.

No significant difference of gastric pepsin activity in rainbow trout was observed with different dietary DHA and EPA levels before salinity acclimation (Fig.1B). Gastric pepsin activity of rainbow trout in the DE-0.54 group peaked on day 1 after salinity acclimation, and then tended to decrease, reaching a minimum value on day 7. The activity increased again on day 14, but failed to return to the freshwater value. Salinity stress increased the gastric pepsin activity of rainbow trout in the DE-1.79 group before fully recovering on day 14.

Gastric lipase activity of rainbow trout in the DE-1.79 group was significantly lower compared to other groups before salinity acclimation (Fig.1C). Gastric lipase activity of rainbow trout in the DE-0.54 group decreased to the lowest value on day 7 and increased to the highest value on day 14. In contrast, gastric lipase activity in the DE-0.95 and DE-1.79 groups decreased to their freshwater values following an increasing trend, peaking on days 4 and 7, respectively. The gastric lipase activity of rainbow trout in the DE-0.54 group was significantly lower than that in the other groups on day 7, and the gastric lipase activity in the DE-0.95 group was significantly lower than that in the DE-0.54 and DE-1.40 groups on day 14.

3.4 Intestinal Amylase, Trypsin and Lipase Activities in Rainbow Trout

No significant difference of intestinal amylase activity in rainbow trout was observed with different dietary DHA and EPA levels prior to salinity acclimation (Fig.2A). Intestinal amylase activity of rainbow trout was inhibited by salinity stress on day 4. The activities in DE-0.54 and DE-0.95 groups turned to freshwater values on day 14, whereas the activity in DE-1.40 and DE-1.79 groups recovered to high values on day 7, before decreasing significantly again on day 14. The intestinal amylase activities in rainbow trout were significantly lower on days 4 and 14, but significantly higher on day 7 in the DE-1.40 and DE-1.79 groups compared to DE-0.54 and DE-0.95 groups.

No significant difference of intestinal trypsin activity in rainbow trout was observed with different dietary DHA and EPA levels before salinity acclimation (Fig.2B). The intestinal trypsin activity of rainbow trout in all groups decreased immediately on day 1 after salinity acclimation, followed by continuous increase before reaching the freshwater values on day 14. At the end of the experiment, intestinal trypsin activity of rainbow trout in the DE-1.40 and DE-1.79 groups were significantly higher compared to the DE-0.54 and DE-0.95 groups.

No significant difference of intestinal lipase activity in

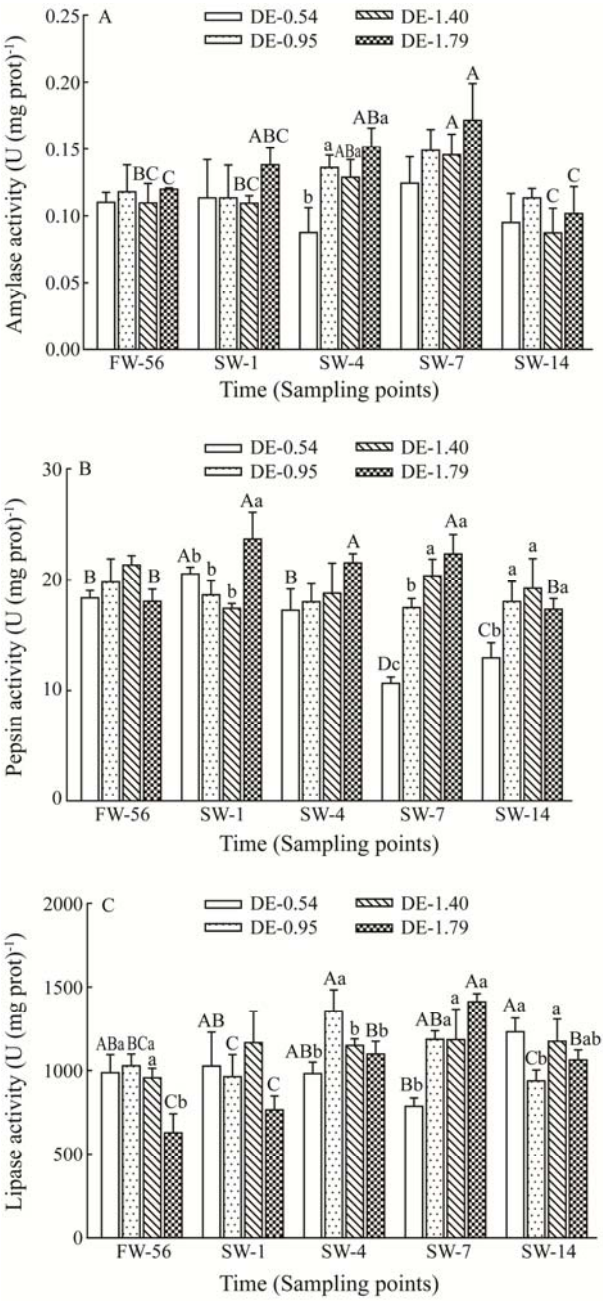

Fig.1 Variations in the gastric amylase activity (A), pepsin activity (B) and lipase activity (C) in rainbow troutfed different levels of dietary DHA and EPA after salinity acclimation. The values represent the means of three replicates. The different lowercase letters indicate significant differences ($P < 0.05$) among different dietary treatments at the same time point, and the different capital letters indicate significant differences ($P < 0.05$) at different time points in the same dietary treatment based on two-way ANOVA and the Student-Newman-Keuls (SNK) test. FW-56, before salinity acclimation; SW-1, one day after salinity reached 30 g L^{-1}; SW-4, four days after salinity reached 30 g L^{-1}; SW-7, seven days after salinity reached 30 g L^{-1}; SW-14, fourteen days after salinity reached 30 g L^{-1}.

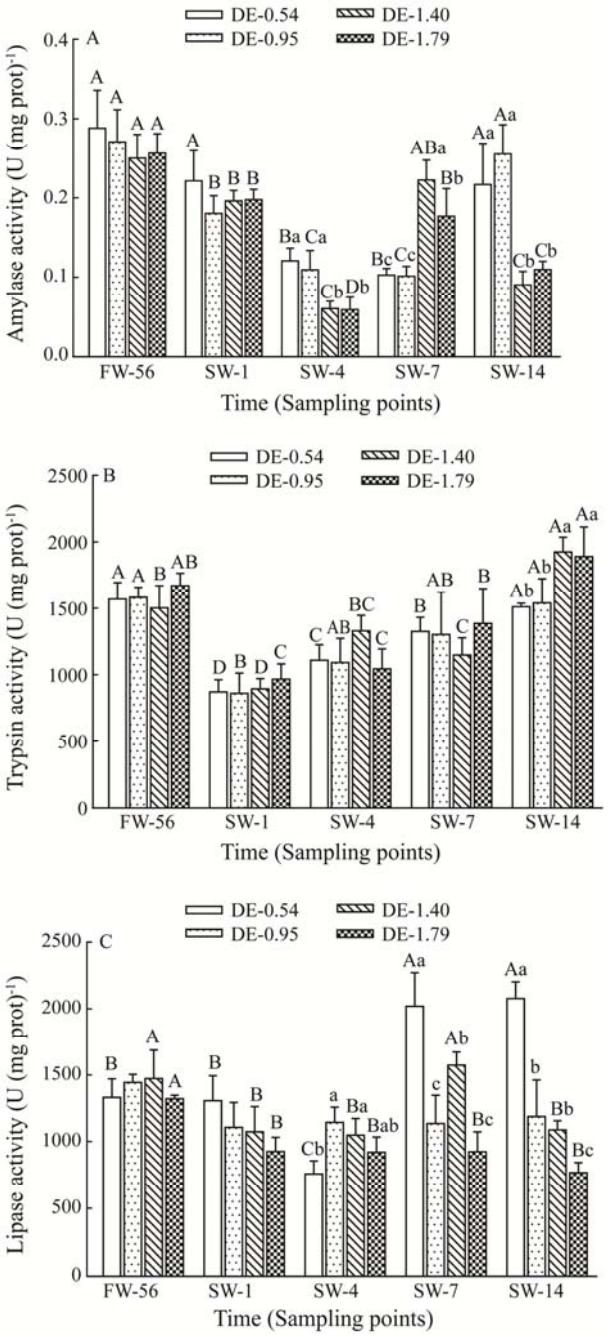

Fig.2 Same as those in Fig.1 but for variations in the intestinal amylase activity (A), trypsin activity (B) and lipase activity (C).

rainbow trout was observed with different dietary DHA and EPA levels during freshwater stage (Fig.2C). Intestinal lipase activity of rainbow trout in the DE-0.54 group decreased to the minimum value on day 4 of salinity acclimation, and then increased significantly on day 7, and the values on day 7 and 14 were significantly higher compared to the freshwater value. Intestinal lipase activity of rainbow trout in the DE-1.40 group decreased significantly after salinity acclimation. Although it recovered to its freshwater value on day 7, it declined again on day 14. Intestinal lipase activity of rainbow trout in the DE-1.79 group decreased significantly on day 1 after salinity acclimation before leveling off. On days 7 and 14, the intestinal lipase activity of rainbow trout in the DE-0.54 group was significantly higher than in other groups.

3.5 Interaction Effect of Culture Time and Dietary DHA and EPA Level on Digestive Enzyme Activities

Two-way ANOVA showed a significant interaction effect of culture time and dietary DHA and EPA on gastric pepsin and lipase activities in rainbow trout. This interaction effect was also found for intestinal amylase, trypsin and lipase activities in rainbow trout (Table 6).

4 Discussion

The present study found that dietary DHA and EPA supplementation (0.54%–1.79%) did not significantly improve the final biomass, survival and FCR of rainbow trout reared in freshwater for 8 weeks, which is consistent with the results of several previous studies on rainbow trout (Guler *et al*., 2011; Thanuthong *et al*., 2011) or Atlantic salmon (*Salmo salar*) (Bell *et al*., 1997; Tocher *et al*., 2000).

DHA and EPA play important roles in the anti-stress response, salinity acclimation, and digestive ability (Cornet *et al*., 2018; Fuentes-Quesada *et al*., 2018). Fish need to obtain these essential fatty acids from food, and food is the main source of deposited DHA in fish tissues (Van Anholt *et al*., 2012). In general, high dietary DHA and EPA levels can improve the DHA and EPA contents in fish tissues (Guler *et al*., 2011; Hixson *et al*., 2014; Wijekoon *et al*., 2014). Similarly, our results showed that the gastric and intestinal DHA and EPA contents were positively correlated to the dietary DHA and EPA levels. In addition, high levels of DHA and EPA in membrane can increase membrane fluidity and change the microenvironment of the membrane, which will further affect the activity of attached enzymes. Thus, it is reasonable to speculate that digestive enzyme activities are influenced by the changes in the gastric and intestinal fatty acid composition in rainbow trout.

Table 6 The *P*-values of two-way ANOVAs for the effects of culture time and dietary DHA and EPA levels on the gastric and intestinal digestive enzyme activities in rainbow trout after salinity acclimation *P*-values 是无单位的

Variable	Stomach			Intestines		
	Amylase (U (mg prot)$^{-1}$)	Pepsin (U (mg prot)$^{-1}$)	Lipase (U (mg prot)$^{-1}$)	Amylase (U (mg prot)$^{-1}$)	Trypsin (U (mg prot)$^{-1}$)	Lipase (U (mg prot)$^{-1}$)
Model	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Time	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
Level	0.0002	<0.0001	0.0026	0.0115	0.1087	<0.0001
Time × Level	0.1741	<0.0001	<0.0001	<0.0001	0.0495	<0.0001

Carbohydrate is hydrolyzed by amylase to glucose absorbed by fish. In our results, the gastric and intestinal amylase activities of rainbow trout have not been significantly influenced by dietary DHA and EPA levels before salinity acclimation, which is consistent with the results of several previous studies. Murashita *et al.* (2008) found that α-amylase activity in the digestive tract was not affected by dietary proteins, lipids or starch in yellowtail. Santigosa *et al.* (2011) reported that amylase activity in the proximal intestine of gilthead sea bream (*Sparus aurata* L.) was not affected when the fish oil in diets was replaced by vegetable oil causing low dietary DHA and EPA level. In addition, our findings indicate that gastric amylase activity was lower and more stable than intestinal amylase activity. A possible reason for this finding is that amylase is synthesized by the hepatopancreas and directly secreted into the intestines. Moreover, because the optimal pH for amylase is 7.0−8.0 (Ugwumba, 1993; Munilla-Morán *et al*., 1996; Fernandez *et al*., 2001), the low pH in the stomach inhibits the activity of amylase.

In this study, short-term exposure to salinity stress inhibited intestinal amylase activity of rainbow trout, and this effect might be due to the high concentration of Cl^- in seawater, which negatively affects amylase activity (Podoler *et al*., 1971). Although the intestinal amylase activity of rainbow trout in the DE-1.40 and DE-1.79 groups could recover to the freshwater values on day 7 after salinity acclimation, the activities significantly decreased again on day 14. This result may indicate that high dietary DHA and EPA inhibits carbohydrate digestion in rainbow trout during long-term mariculture; however, its underlying mechanism still needs further study.

Proteases are important components of the digestive enzymes and are responsible for the digestion of dietary proteins (Psochiou *et al*., 2007). In the current study, no significant differences in the gastric pepsin and intestinal trypsin activities were observed among the groups of rainbow trout prior to salinity acclimation. Similarly, Castro *et al.* (2016) found that the dietary replacement of fish oil by vegetable oil had no effect on trypsin activity in European sea bass (*Dicentrarchus labrax*). However, You *et al.* (2019) and Bowyer *et al.* (2012) found decreased trypsin activity in golden pompano and yellowtail kingfish with low dietary DHA and EPA levels, respectively. Santigosa *et al.* (2011) observed increased and decreased alkaline protease activities in the pyloric caeca and proximal intestine, respectively, in gilthead sea bream fed diets including a vegetable oil blend. Therefore, it can be speculated that different effects of dietary DHA and EPA on protease activity might be species- and/or tissue-specific.

Our findings show that gastric pepsin activity in rainbow trout was significantly lower in the DE-0.54 group compared with the other groups on day 14 after seawater acclimation. A possible reason is that fish in this group cannot adapt to the seawater environment (data from this experiment) (Huang *et al*., 2020), and consequently, they need to take in large volumes of alkaline seawater to compensate for the water loss of tissues, resulting in an increase in pH that exceeds the optimal pH range for pepsin in the stomach (Usher *et al*., 1990; Boeuf *et al*., 2001; Gheisvandi *et al*., 2015).

Intestinal trypsin activity was inhibited by short-term salinity stress, which is consistent with the results of several previous studies (Woo *et al*., 1995; Moutou *et al*., 2004; Silva-Brito *et al*., 2019). In our study, the trypsin activity in rainbow trout recovered to the freshwater values on day 14, which indicated that trypsin can regain its normal function after the fish adapt to the hypersaline environment. In addition, our results indicate that the trypsin activities in the DE-1.40 and DE-1.79 groups were significantly higher than those in the DE-0.54 and DE-0.95 groups on day 14 after salinity acclimation. One possible explanation is that rainbow trouts in the DE-1.40 and DE-1.79 groups exhibited higher intestinal DHA and EPA levels, resulting in improved membrane fluidity and material exchange ability (Huster *et al*., 1997; Hishikawa *et al*., 2017; Cornet *et al*., 2018). As a result, redundant ions can be excreted over time after the fish drink large volume of seawater, and the trypsin activity can thus be maintained at a high level because high concentrations of Cl^-, Na^+ and K^+ inhibit the activity of protease (Squires *et al.*, 1986b). Hence, a certain level of dietary DHA and EPA is necessary for the maintenance of high protease activity in rainbow trout after salinity acclimation.

Lipase plays important role in lipid absorption, which can hydrolyze dietary lipids into partial glycerides and free fatty acids (Hamosh *et al*., 1973). In our results, the lowest gastric lipase activity in rainbow trout was found in the DE-1.79 group before salinity acclimation, which is in agreement with the results of Morais *et al.* (2004), who reported lower lipase specific activity in larval sea bass fed fish oil than in larvae fed coconut oil. However, the gastric lipase in the DE-1.79 group could increase to similar value as the other groups after salinity acclimation, indicating that gastric lipase activity of rainbow trout in the DE-1.79 group was only temporarily inhibited and can recover if needed.

The results of this study showed that intestinal lipase activity in rainbow trout was inhibited by short-term salinity stress. Similarly, Liu *et al.* (2017) found that lipase activity in juvenile American shad (*Alosa sapidissima*) decreased after exposure to increased salinity. Our findings showed that on day 14, the highest and lowest intestinal lipase activities were detected in the DE-0.54 and DE-1.79 group, respectively. A possible explanation for this finding is that lipase specificity can change with the unsaturated degree and the chain length of dietary fatty acids (Castro *et al*., 2016). Thus, low lipase activity is detected in fish if the demand for dietary essential fatty acids has been met. An adequate supply of DHA and EPA is an indispensable factor for achieving suitable growth, survival and anti-stress responses in fish (Furuita *et al*., 1998; Hamre *et al*., 2013; Pinto *et al*., 2016; Fuentes-Quesada *et al*., 2018). In the current study, the intestinal lipase activity of rainbow trout in the DE-0.54 group was higher than those in the other groups on days 7 and 14 because the fish in this group need to increase their lipase activity to enhance their utilization of lipids to meet their demands

for DHA and EPA, since rainbow trout needs 1.40% dietary DHA and EPA during salinity acclimation (Huang *et al*., 2020).

5 Conclusions

In this study, the gastric and intestinal DHA and EPA contents of rainbow trout were positively correlated to the dietary DHA and EPA levels. Both dietary DHA and EPA levels together with salinity acclimation play important roles in digestion in rainbow trout. Intestinal amylase, trypsin, and lipase activities of rainbow trout were inhibited by short-term exposure to salinity stress, while the gastric digestive enzyme activities were more stable. In the long term of salinity acclimation, high dietary DHA and EPA levels (DE-1.40 and DE-1.79) improved intestinal trypsin activities of rainbow trout, while rainbow trout in low dietary DHA and EPA group (DE-0.54 group) cannot maintain their activity of gastric pepsin. In addition, fish in the DE-0.54 group kept extremely high intestinal lipase activity. According to the results, rainbow trout fed the DE-0.54 diet cannot maintain an appropriate digestive capacity after entering the seawater. Thus, a diet with a minimum DHA and EPA level equaling to 0.95% is necessary for rainbow trout during salinity acclimation.

Acknowledgements

The authors would like to express their gratitude to everyone who critically reviewed this manuscript and helped with the sample collection. This publication was supported by the National Key Research and Development Program of China (No. 2019YFD0901005), the National Natural Science Foundation of China (Nos. 31702364 and 31572634) and the Primary Research and Development Program of Shandong Province (No. 2018CXGC0101).

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(Edited by Qiu Yantao)