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Effects of n-3 PUFA Levels in Live Foods on Albinism, Growth, Survival, and Salinity Tolerance of Flounder (*Paralichthys olivaceus*) Larvae in Large-Scale Artificial Rearing

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Abstract

The effects of feeding enriched rotifers and *Artemia* nauplii on albinism in flounder (*Paralichthys olivaceus*) larvae raised in a large-scale artificial system were investigated. Larvae were first fed an S-type rotifer (*Brachionus angularis*) enriched with *Nannochloropsis oculata* for 11-12 days, which raised the n-3 polyunsaturated fatty acids (PUFA) content in the rotifer from 5.36% to 17.63% of the total fatty acids. Next, the larvae were fed one of three strains of *Artemia* enriched with microcapsule (50DE), vitamin A (9000 IU/l), and vitamin D (2000 IU/l) for 35 days, which raised the n-3 PUFA contents in the *Artemia* to 38.62%, 36.53%, and 33.86% of the total fatty acids, respectively. Among the larvae fed the enriched feeds, no more than 3‰ were albino, much fewer than in the control groups fed non-enriched foods (p<0.01). In addition, n-3 PUFA contents in the muscles, growth and survival rates, and salinity tolerance were greater in founder fed the enriched foods. *Artemia* nauplii from Qixiangcuo (Tibet, China), enriched to 38.62% of total fatty acids, was superior in preventing albinism than the strains from Pikou or Yingkou (Liaoning, China).

Introduction

Shellfish and shrimp culture in north China has suffered a host of diseases (Huang et al., 1998), causing farmers to seek alternative species to culture. Aquaculture production of flounder has greatly increased over the past decades, reaching about 5000 tons annually. Flounder is now one of the most important large-scale industrial marine fish cultures in north China.

One of the most common problems in the

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production of larvae and juvenile flounder is albinism and ambicoloration that influence economic value, growth, and survival. Many factors influence the rate of albinism including water temperature and flow rate (Sugiyama et al., 1985), lighting (Seikai, 1991), aeration (Fukusho et al., 1986), UV irradiation (Matsumoto and Ishii, 1986), stocking density (Takahashi, 1994), salinity of the seawater (Wang, 1997), and substrate (Alicia and Seikai., 2001). Seikai (1985) suggested that stage D (total length 7.4-8.6 mm, 16-19 days after hatching, i.e., at the onset of metamorphosis) is the crucial stage for induction of albinism. Moreover, the key factor is not timing but the duration of dietary supplementation (Dickey-Collas, 1993).

Nutritional studies indicate that dietary amino acids, polyunsaturated fatty acids (PUFA), vitamins A, D, E, and K, and others are responsible for the rate of albinism (Kim and Lee, 2004). Kim and Lall (2000) analyzed the amino acid composition of whole body tissue of flounder and reduced the rate of albinism by improving the nutritional components. n-3 PUFA is an essential factor for normal growth and survival in flounders (Furuita et al., 1998) and high levels of n-3 PUFA, especially eicosapentaenoic acid (EPA) and docosahexenoic acid (DHA), play a crucial role in reducing albinism (Furuita et al., 2002). Kim and Lee (2004) found that the necessary n-3 PUFA content for the development of flounder is 0.8-1.0% of the diet, but the precise requirement is unknown.

The rate of albinism of flounder also could be influenced by *Artemia* strain. Seikai (1985) demonstrated that flounder fed San Francisco *Artemia* had a lower rate of albinism than those fed Tianjin's (21.1-26.1% vs 81.3-84.0%).

Therefore, enrichment of live foods seems to be an important approach to reducing albinism in flounder raised in large-scale artificial systems. The present study investigated the effects of n-3 PUFA enrichment of rotifer and three *Artemia* strains on albinism, survival, growth, and salinity tolerance of larvae and juvenile flounder raised in large-scale systems in north China.

Materials and Methods

Larvae and treatments. Flounder eggs were provided by the Yellow Sea Fisheries Research Institute. Larvae were randomly divided into 36 groups of 300 each (12 treatments in triplicate) and reared in 1-m^3 concrete ponds (Table 1). Larvae were fed enriched or non-enriched rotifer (*Brachionus angularis*, S-type) from the third day after hatching and until day 15 (Fig. 1). During the following three days, larvae were fed rotifers plus one of three strains of *Artemia* nauplii obtained from Qixiangcuo (Q) in Tibet, China; Pikou (P) in Liaoning, China; or Yingkou (Y), in Liaoning, China, enriched or non-enriched. For the final 33 days, larvae were fed enriched or non-enriched *Artemia* nauplii. The density of the rotifers and *Artemia* was measured daily. Water temperature was maintained at 19.0±0.2°C, supplied at a rate of 300-1500 ml/min. Illumination was 400-500 lux at the water surface, the photoperiod was 13 h light:11 h dark, and aeration was 80-160 ml/min. The rate of albinism, growth, survival, and salinity tolerance were analyzed. Salinity tolerance was based on Dhert et al. (1992).

Table 1. Treatment groups were fed rotifer from day 3 to 18 and *Artemia* from day 16 to 50.

Group	*Rotifer*	*Artemia**
A	non-enriched	non-enriched Q
B	non-enriched	non-enriched P
C	non-enriched	non-enriched Y
D	non-enriched	enriched Q
E	non-enriched	enriched P
F	non-enriched	enriched Y
G	enriched	non-enriched Q
H	enriched	non-enriched P
I	enriched	non-enriched Y
J	enriched	enriched Q
K	enriched	enriched P
L	enriched	enriched Y

* Q = Qixiangcuo, P = Pikou, Y = Yingkou

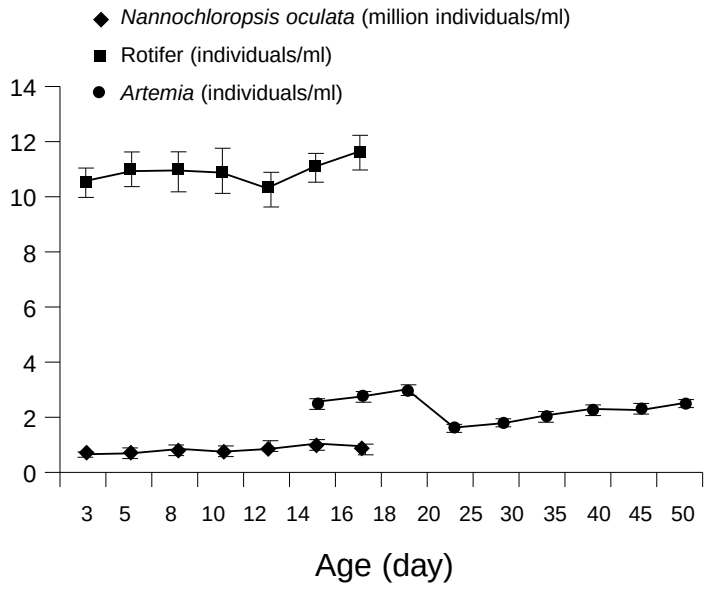

Fig. 1. Density of *Nannochloropsis oculata* in the rotifer growing ponds and of rotifers and *Artemia* in the flounder growing ponds.

Enrichment of live feed. Rotifers were reared at 300-500 individuals/ml. Non-enriched rotifers were fed yeast. Enriched rotifers were fed *Nannochloropsis oculata* stocked at 0.7-0.9 million individuals/ml for 12 h in the rotifer rearing water (25-28°C with vigorous aeration) which raised the n-3 PUFA content in the rotifers from 5.36% to 17.63% of the total fatty acids (Table 2).

Newly hatched *Artemia* nauplii were enriched with microcapsules (50DE, Shengsuo, Shandong, China) for 8-10 h at a water temperature of 25°C, then with vitamin A (9000 IU/l) and vitamin D (2000 IU/l) for 12-15 h, which enhanced the n-3 PUFA content in Q, P, and Y to 38.62%, 36.53%, and 33.86% of total fatty acids, respectively (Table 3).

Chemical analysis. Fifty days after hatching, the fatty acid compositions of the *N. oculata*, rotifer, *Artemia*, and muscle of normal and albino juvenile flounders were analyzed. Lipid was extracted from the *Artemia* with a mixture of chloroform and methanol (2:1, v/v). Lipids were saponified with potassium hydrate and fatty acid methyl esters (FAME) were prepared by trans-esterification with borontrifluoride in methanol (Metcalfe and Schmitz, 1961). The FAME were analyzed on a gas chromatograph (Alilent-6890, US) equipped with a PEG-20M column (50 m x 0.25 mm).

Statistical analysis. Data were subjected to one-way analysis of variance (ANOVA) using software SPSS (version 10.0). Significant differences (p<0.05) between means were determined by Duncan's multiple range test.

Results

Fatty acid composition of flounder. The ∑n-6 PUFA content was higher in groups fed enriched feeds than in groups fed non-enriched feeds (groups J-L>D-I>A-C; Table 4). Within each group, the ∑n-6 PUFA was higher in albino fish than in normal fish while the ∑n-3 PUFA content was higher in normal fish than albino. The highest ∑n-3 PUFA content was in normally pigmented fish fed enriched rotifer

Table 2. Fatty acid composition (% weight) of yeast, rotifers fed yeast, *Nannochloropsis oculata*, and enriched rotifers fed *N. oculata*.

Fatty acid	*Yeast*	*Rotifers fed yeast*	*Nannochloropsis oculata*	*Rotifers fed N. oculata*
14:0	0.31±0.01	1.91±0.25	5.27±0.05	3.25±0.15
15:0	trace	0.56±0.18	0.14±0.03	0.61±0.16
16:0	13.20±0.04	10.28±0.05	29.44±1.28	15.56±0.14
17:0	-	0.14±0.03	0.21±0.04	0.15±0.02
18:0	7.84±0.16	8.65±0.70	1.39±0.46	7.04±0.35
20:0	trace	0.38±0.04	0.97±0.20	0.48±0.08
14:1n-9	0.84±0.17	3.51±0.07	0.45±0.09	0.98±0.13
16:1n-9	29.87±1.25	23.13±0.46	25.08±0.94	24.71±1.67
17:1n-9	-	trace	0.38±0.06	trace
18:1n-9	39.51±3.15	34.17±0.38	13.16±0.97	17.24±0.58
18:1n-6	-	trace	trace	trace
22:1n-6	-	trace	trace	trace
18:2n-6	trace	8.24±0.34	3.51±0.37	9.04±0.64
20:4n-6	-	-	-	-
22:5n-6	-	-	-	-
18:3n-3	3.91±0.61	4.38±0.39	0.67±0.04	3.25±0.41
20:3n-3	trace	0.95±0.23	2.92±0.27	1.27±0.04
18:4n-3	-	-	-	-
20:5n-3	trace	trace	15.42±1.64	13.11±1.72
22:6n-3	-	-	trace	trace
∑n-3 PUFA	3.91	5.36	19.01	17.63
∑n-6 PUFA	-	8.24	3.51	9.04
∑n-3/∑n-6	-	0.65	5.42	1.95

Results are means±SE.

and *Artemia* (group J) while the lowest was in albino fish fed non-enriched rotifer and *Artemia* (group A). There were no significant differences in EPA contents but normal flounder had a higher DHA level than albino, and differences were greater in fish fed enriched rotifer and enriched *Artemia* (groups J-L).

Albinism, survival, and salinity tolerance. Albinism decreased and survival and salinity tolerance increased from group A to group L (Fig. 2). Albinism in groups J-L fed enriched rotifers and enriched *Artemia* was significantly lower than in the other groups (p<0.01). Group J, fed Qixiangcuo *Artemia*, had the lowest rate (0.12%). Survival increased from group A to group L but there were no significant differences among groups G to L. Salinity tolerance increased from A to L and was significantly higher in groups G-L than in groups A-F.

Growth. Growth rates in groups G-L were significantly higher than in groups A-F, except

Table 3. Fatty acid compositions (% weight) of non-enriched and enriched *Artemia* from three different sources.

	Qixiangcuo		*Pikou*		*Yingkou*	
Fatty acid	*Unenriched*	*Enriched*	*Unenriched*	*Enriched*	*Unenriched*	*Enriched*
14:0	0.60±0.02^{b}	0.62±0.02^{b}	0.33±0.06^{a}	0.38±0.11^{a}	0.29±0.01^{a}	0.28±0.04^{a}
15:0	0.22±0.01^{a}	0.25±0.01^{b}	0.21±0.01^{a}	0.25±0.01^{b}	0.19±0.03^{a}	0.36±0.07^{b}
16:0	10.98±1.02^{b}	7.21±0.36^{a}	11.16±1.17^{b}	7.89±0.64^{a}	10.65±0.25^{b}	7.37±0.39^{a}
17:0	0.68±0.14	0.65±0.10	0.44±0.22	0.49±0.09	0.67±0.11	0.61±0.05
18:0	0.80±0.04^{a}	0.73±0.09^{a}	2.65±0.28^{b}	3.08±0.37^{b}	2.31±0.16^{b}	3.31±0.21^{b}
20:0	0.61±0.15^{b}	0.55±0.06^{b}	0.22±0.05^{a}	0.24±0.01^{a}	0.65±0.02^{b}	0.75±0.23^{b}
14:1n-9	0.31±0.10	0.32±0.08	0.28±0.12	0.25±0.14	0.23±0.21	0.26±0.16
16:1n-9	24.54±1.65^{b}	18.13±0.42^{a}	23.16±2.12^{ab}	17.98±1.54^{a}	23.96±0.64^{b}	18.98±0.68^{a}
17:1n-9	2.90±0.15^{b}	1.50±0.82^{a}	3.56±0.69^{b}	2.12±0.46^{ab}	1.65±0.05^{a}	1.74±0.61^{a}
18:1n-9	2.98±0.14^{b}	1.66±0.34^{a}	2.53±0.34^{ab}	1.26±0.92^{a}	2.67±0.87^{ab}	1.54±0.71^{a}
18:1n-6	25.68±1.61^{b}	19.35±1.23^{a}	27.32±2.35^{b}	21.16±2.58^{a}	26.18±0.67^{b}	20.65±0.67^{a}
22:1n-6	-	-	0.53±0.01^{b}	0.30±0.08^{a}	0.49±0.05^{b}	0.27±0.12^{a}
18:2n-6	1.71±0.17^{a}	1.68±0.06^{a}	2.99±0.25^{b}	2.86±0.44^{b}	2.72±0.31^{b}	2.69±0.49^{b}
20:4n-6	1.12±0.33	1.19±0.96	1.25±1.02	1.32±0.39	1.41±0.68	1.51±0.58
22:5n-6	0.52±0.03	0.54±0.04	0.53±0.06	0.56±0.04	0.55±0.08	0.57±0.02
18:3n-3	8.21±0.78^{b}	7.20±0.16^{b}	7.51±0.97^{b}	6.20±1.39^{b}	4.31±0.11^{a}	3.92±0.37^{a}
20:3n-3	0.15±0.08	0.18±0.16	0.13±1.05	0.10±0.04	0.17±0.07	0.18±0.10
18:4n-3	2.01±0.68^{bc}	2.21±0.24^{c}	1.56±0.33^{b}	1.36±0.09^{a}	1.56±0.02^{b}	1.35±0.08^{a}
20:5n-3	12.62±0.37	19.88±1.32	13.54±1.48	20.71±1.54	12.01±0.64	19.21±2.41
22:6n-3	trace[5]	9.15±0.36	trace	8.96±0.25	trace	9.02±0.02
Σn-3 PUFA	25.99	38.62	22.74	36.53	18.05	33.86
Σn-6 PUFA	3.35	3.41	4.77	4.74	4.68	4.77
DHA/EPA	-	0.46	-	0.43	-	0.47
Σn-3/Σn-6	7.76	11.33	4.77	7.71	3.86	7.10

Values within a row with different superscripts are significantly different (p<0.05).

Table 4. Fatty acid compositions (% weight) of normal and albino flounder in each treatment.

Group	∑n-6 PUFA	∑n-3 PUFA	EPA	DHA	∑n-3/∑n-6	DHA/EPA	
A	normal	4.21±0.24^{a}	33.45±0.83^{c}	6.64±0.47	25.22±1.47^{b}	7.95	3.80
	albino	5.01±0.93^{a}	26.75±0.84^{a}	6.49±0.76	22.54±0.97^{a}	5.34	3.47
B	normal	3.98±0.38^{a}	34.51±0.66^{c}	6.88±0.82	26.01±0.45^{b}	8.67	3.78
	albino	4.72±0.85^{a}	27.05±1.36^{a}	6.74±0.34	22.41±0.84^{a}	5.73	3.32
C	normal	4.02±0.61^{a}	35.09±0.02^{c}	6.77±0.29	25.41±0.41^{b}	8.73	3.75
	albino	4.86±0.67^{a}	26.93±0.84^{a}	6.51±0.81	22.31±0.73^{a}	5.54	3.43
D	normal	6.65±0.22^{b}	38.51±2.14^{d}	6.87±0.85	29.21±1.45^{b}	5.79	4.25
	albino	8.72±0.50^{c}	29.55±1.58^{b}	6.54±0.41	22.65±0.98^{a}	3.39	3.46
E	normal	6.54±0.15^{b}	37.86±0.14^{d}	6.77±0.81	29.32±1.75^{b}	5.79	4.33
	albino	8.69±0.46^{c}	29.62±1.39^{b}	6.87±0.43	21.85±0.93^{a}	3.41	3.18
F	normal	6.57±0.14^{b}	36.51±0.56^{d}	6.65±0.24	29.05±1.09^{b}	5.56	4.37
	albino	8.92±0.14^{c}	30.09±0.99^{b}	6.55±0.71	22.04±0.67^{a}	3.37	3.36
G	normal	6.34±0.05^{b}	38.85±1.28^{d}	6.49±0.80	28.21±0.98^{b}	6.13	4.35
	albino	8.56±0.13^{c}	30.54±1.54^{b}	6.56±0.53	22.44±0.48^{a}	3.57	3.42
H	normal	6.25±0.24^{b}	36.67±0.42^{d}	6.81±0.25	29.07±0.97^{b}	5.87	4.27
	albino	8.91±0.25^{c}	31.37±1.21^{b}	6.58±0.74	22.33±0.85^{a}	3.52	3.39
I	normal	6.86±0.76^{bc}	37.01±1.52^{d}	6.77±0.24	29.34±1.05^{b}	5.40	4.33
	albino	8.54±0.03^{c}	30.87±1.93^{b}	6.67±0.44	22.47±0.99^{a}	3.61	3.37
J	normal	8.01±0.52^{c}	40.21±2.57^{d}	6.88±0.83	32.21±0.12^{b}	5.02	4.68
	albino	9.34±0.86^{c}	31.34±0.65^{b}	6.65±0.72	22.75±0.47^{a}	3.36	3.42
K	normal	7.84±0.35^{bc}	39.37±1.98^{d}	6.79±0.89	31.90±1.45^{b}	5.02	4.70
	albino	9.17±0.96^{c}	32.51±1.08^{b}	6.56±0.97	22.47±0.46^{a}	3.55	3.43
L	normal	7.63±0.82^{bc}	39.06±3.95^{d}	6.94±1.02	31.21±0.15^{b}	5.12	4.50
	albino	9.51±1.07^{c}	30.57±1.20^{b}	6.44±0.94	22.65±0.83^{a}	3.21	3.52^{a}

Values in a column with different superscripts are significantly different (p<0.05).

in group B and there were no significant differences among groups G to L (Fig. 3). The highest growth rate was in group J (0.34±0.05 mm/d) and the lowest in group C (0.18±0.03 mm/d).

Discussion

Environmental (temperature, salinity) and nutritional (DHA, EPA, vitamin A, phospholipids) factors during larvae rearing largely dictate the successful metamorphosis of larvae to juveniles determining, in turn, juvenile quality (Koven, 2003). The n-3 PUFA content in live foods is crucial to the development of flounder, as shown here with three strains of *Artemia* enriched with different levels of n-3 PUFA. The *Artemia* from Qixiangcuo was more effective than from Pikou and Yingkou, indicating that 38.62% of total fatty acids is the appropriate n-3 PUFA content in live foods for flounder, with a ∑n-3/∑n-6 of 11.33 and DHA/EPA ratio of 0.46. Although Furuita et al. (1998) found no clear difference in the effects of EPA and DHA levels on growth and survival

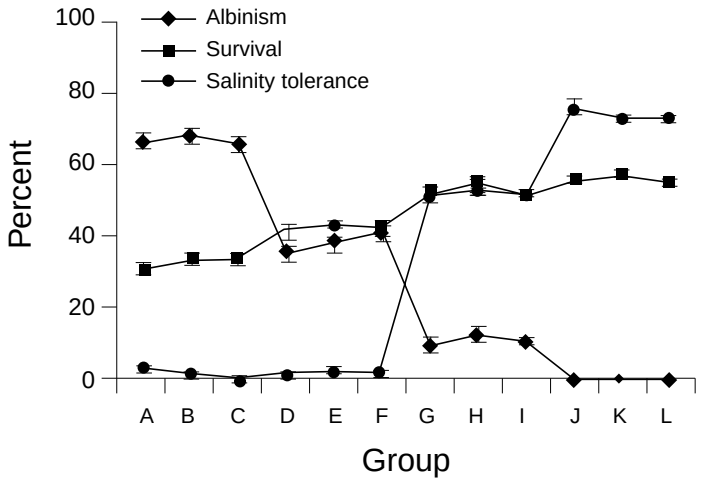

Fig. 2. Albinism, survival, and salinity tolerance of flounder larvae fed enriched or unenriched rotifer and *Artemia*.

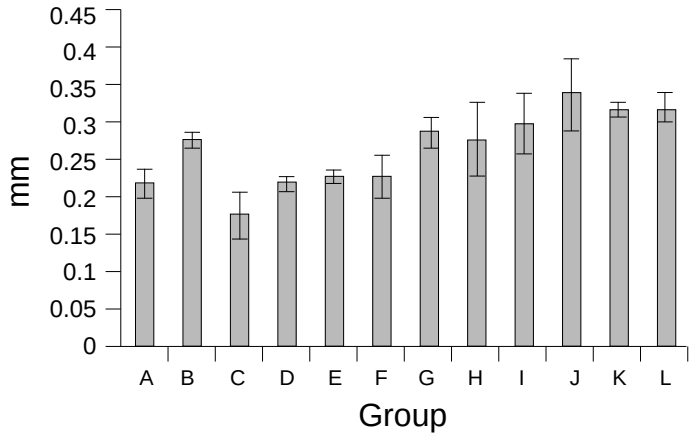

Fig. 3. Growth of flounder larvae (mm/d) fed enriched or unenriched rotifer and *Artemia*.

of flounder larvae, there were higher growth and survival rates in our study in groups with higher ∑n-3/∑n-6 and DHA/EPA ratios. Sargent (1999) found that the DHA/EPA ratio should be 2:1 in the diet of marine fish and that this ratio benefits growth and survival of larvae and juveniles. Although the DHA/EPA ratio was only 0.5 in our study, it prevented albinism and improved growth and survival. As in the present study, Dickey-Collas and Geffen (1992) found no significant difference in the growth and survival of plaice larvae fed non-enriched or enriched *Artemia*.

In comparison, the n-3 PUFA levels were

significantly higher in normal flounder than in albino. The ∑n-3/∑n-6 ratio in normal fish was about 1.5 times higher than in albinos and n-3 PUFA played an important role in preventing albinism. There is a positive relationship between the DHA/EPA ratio in larvae and their pigmentation (Reitan et al., 1994). The DHA/EPA ratio of normal fish was about 1.3 times higher than that of albino. Our results demonstrate that the n-3 PUFA content in live foods can improve the n-3 PUFA content in the fish body from 33.45% to 40.21% and that the ∑n-3/∑n-6 and DHA/EPA ratios should be 1.5 and 1.3, respectively, in large-scale artificial rearing of flounder.

However, it is not clear if albinism that was prevented during the larvae stage may appear at a later stage. Further study is required to learn how to reduce the rate of albinism of flounder larvae by increasing PUFA, vitamins, and other dietary nutritional factors.

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