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Enrichment of *Artemia* nauplii with Essential Fatty Acids and Vitamin C: Effect on Rainbow Trout (*Oncorhynchus mykiss*) Larvae Performance

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Abstract. The effect of essential fatty acids (EFA) and vitamin C-enriched *Artemia* nauplii on growth, survival, and resistance to temperature (high) stress in rainbow trout larvae reared in tanks were investigated. The larvae (average weight 120.43mg±13.5) were fed 6 times daily starting at the onset of exogenous feeding for 1 week. Triplicate groups of fish were offered one of four treatments (1) commercial starter food for rainbow trout ، (2) newly hatched *Artemia* nauplii (unenriched)، (3) highly unsaturated fatty acid (HUFA) + vitamin C-enriched *Artemia* nauplii and (4) combination of 10 % HUFA+ vitamin C enriched nauplii and commercial starter food. After 1 week, all groups of fish were switched to the commercial diet for an additional period of 3 weeks. Statistical analysis of growth after first week and at the end of the experiment, showed that growth of larvae in various treatments were significantly different ($P<0.05$). After 4 weeks, the larvae in treatment 3 with the average weight of 657.50 ± 57.93 mg had the highest body weight ($P<0.05$).The highest percentage of survival (96%) was observed in treatment 3 ($P<0.05$). Proximate compositions of trout larvae in after one week feeding with experimental diets showed that the protein in the larvae of treatments 3 and 4 was significantly different compared to other treatments ($P<0.05$). The best result of resistance to temperature (up 24°C) was observed in larvae reared on treatment 3 with 91.34 ± 1.52 percent ($P<0.05$).

Keywords: essential fatty acid, vitamin C, Oncorhynchus mykiss, stress resistance, growth and survival

1. Introduction

Successful rearing of larval fish is the most critical stage in the production cycle for many species. *Oncorhynchus mykiss* has a promising market potential in Europe, East and South Asia. It is also an important aquaculture species in Iran. The high nutritional quality of its flesh encourages investigations on the aquaculture potential of this excellent food fish (Sedgwick, 1990). The use of Artemia nauplii is well established due to its many advantages: year- round availability as on- the shelf cysts; good nutritional value for some fish; and relatively easy improvement through simple enrichment techniques (Leger et al., 1987). Nutritional deficiencies have been another concern when using brine shrimp. Some stocks of *Artemia* nauplii have shown a deficiency in eicosapantaenoic acid (EPA; 20:5n-3) and doccosahexanoic acid (DHA; 22:6n-3) (Takeuchi and Watanabe 1982). The essential fatty acids (EFA) for fish are broadly recognized to comprise polyunsaturated fatty acids (PUFA) with carbon chain lengths of 18 and HUFA with carbon chain lengths of 20 and 22, of both the n-3 and n-6 series. On the other hand, ascorbic acid also is an important micronutrient in fish diet. It is needed in the synthesis of collagen necessary in the formation of connective tissues and bone matrix (Dabrowski et al., 1994).The effects of supplemental ascorbic acid in enriched live food for *Clarias garipinus* larvae of start feeding (Merchie et al., 1997), labrox and *Clarias gariemus* (Merchie et al., 1995b), *Chanos chanos* (Gapasin et al.,1998), *Penaeus monodon* (Merchie et al 1998), *Penaeus vannamei*

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(Wouters et al.,1999) and *Bidyanus bidyanus* (Smith et al.,2004) has been investigated. The aim of this study was to test the effect of EFA and ascorbate supplementation in enhancing rainbow trout larval growth, survival and resistance to temperature stress (up 24 °C).

2. Materials and Methods

Artemia cysts (Urmia Lake, Iran) were hatched following standard procedures (Sorgeloos et al., 1993; Lavens and Sorgeloos., 1996). Newly hatched *Artemia* (Instar I) nauplii (200,000 nauplii / l) were divided in batches in 5 liter plexiglass tanks. Enrichment protocol followed the method of Treece, (2000). 0.5 ml of the enrichment suspension (assuming a density of 200 *Artemia* per ml) was added per liter of the incubation water at the onset of the enrichment period. Another 0.5 ml/l of the enrichment diet was added 12 hours before harvesting and nauplii were harvested after 24 hours. Newly hatched *Artemia* nauplii served as the control (Leger et al., 1987; Treece, 2000). Samples of unenriched and enriched *Artemia* were also collected regularly and stored at -20ºC. These samples were later used for the analysis of fatty acid methyl esters. Vitamin C was not analyzed in the present study. The four treatments (in a completely randomized design with 3 replicates per treatment) were: (1) larvae fed commercial starter food ≠2 or ≠3 (Bioproducts, Inc., France, 58% protein, 15%lipid and 11 %ash) ;(2) larvae fed newly hatched *Artemia* nauplii;(3) larvae fed HUFA+vitamin C enriched *Artemia* nauplii and (4) larvae fed combination of 10% HUFA+vitamin C enriched nauplii and commercial starter food. Treatment 1 served as control. The fish larvae in all treatments were fed 6 times per day (4, 8,12,16,20 and 24 hours). The daily ration was adjusted according to larvae weight gain after 7 and 14 days of rearing. 1200 uniformly sized yolk- sac larvae (120.63mg±13.50 SD) were randomly divided into 12 groups (four treatments, three replicates) of 100 individuals. Water quality was maintained within optimum range: temperature (9.3 ±1.36 °C), dissolved oxygen (7.8-8.6 mg/l), pH (8-8.2), total ammonia (0.5±0.03 mg/l), residual chlorine (0.05±0.03 mg/l) and the photoperiod was set at 12L: 12D cycle (light period from 8-20 hours) and light intensity was kept at 40 lux at the tank surface. Samples were oven- dried at 60 °C for 24 h then stored at -20 °C. These dried samples were later analyzed for fatty acid methyl esters (Lepage and Roy, 1986), using flame ionization, (DANI-1600 models, Italy). Proximate composition; moisture, crude protein, crude lipid and ash were also measured in duplicate using standard methods (AOAC, 1990). Moisture by drying samples at 105 °C overnight, protein by measuring kjeldahl nitrogen, lipid was analyzed by ether extraction using a soxhlet system, and ash by heating for 5h at 550 °C in a muffle furnace. Twenty nine day- old trout larvae were subjected to temperature stress test following the method described by Ako et al., 1994; and Kanazawa,1995) .The test involved immersing fish 10 fish larvae/replicate in 24 °C for a period of one hour. The mortality was recorded at every 5 min intervals. Results were expressed as% total body dry weight At the end of the experiment the number of surviving fish was recorded and used for calculating mortality. Diet effects on total length, survival, weight, SGR and temperature stress were analyzed using two- way analysis of variance (ANOVA) (SPSS version 9).

3. Results

Table 1: Certain fatty acids (mg FA/g DW) of newly Artemia nauplii (A), Artemia enriched with HUFA +vitamin C (B)

Fatty acid	A	B
14:0	1.30±0.10	1.30±0.20
16:0	15.79± 0.39	15.50±1.95
18:0	3.99±0.78	4.53±0.07
18:1n-9	18.35±0.47	16.67±0.43
18:2n-6	1o.12±1.95	11.33±0.54
18:3n-3	30.48±1.83	36.43±0.37
20:5n-3	2.80±0.43	7.72±0.32
22:6n-3	tr	1.27±0.25
SFA	21.41±0.84	22.59±0.30
USFA	62.51±0.57	67.83±0.37
PUFA	27.18±1.84	43.42±3.5
HUFA	2.80±0.43	8.99±0.28

Data are mean±SD (n=3), SFA=saturated fatty acid, USFA= unsaturated fatty acid, HUFA= highly unsaturated fatty acid and PUFA=poly unsaturated acid, tr=trace

Table 2: Whole- body fatty acid composition (mg FA/g DW) of 29-day old trout larvae fed of different diets

Fatty acid	Treatment 1	Treatment 2	Treatment 3	Treatment 4
14:0	3.49±0.17[a]	0.69±0.03[c]	0.85±0.094[c]	3.12±0.06[b]
16:0	23.86±0.25[b]	22.68±0.15[c]	19±0.19[d]	28.44±0.11[a]
18:0	1.8±0.16[d]	6.23±0.25[a]	5.82±0.11[b]	4.22±0.18[c]
18:1n-9	14.99±0.08[c]	14.67±0.08[d]	16.61±0.14[a]	15.48±0.19[b]
18:2n-6	18.07±0.11[b]	13.95±0.21[c]	12.23±0.12[d]	19.55±0.26[a]
18:3n-3	1.36±0.069[d]	3.26±0.20[b]	5.31±0.08[a]	2.6±0.15[c]
20:5n-3	-	-	2.98±0.032	-
22:6n-3	-	-	0.36±0.10	-
SFA	19.14±0.11[b]	17.84±0.06[d]	21.01±0.032[a]	18.84±0.20[c]
USFA	39.15±0.19[b]	34.10±0.19[d]	37.91±0.032[c]	41.15±0.12[a]
PUFA	1.29±0[d]	3.30±0.21[b]	8.14±0.26[a]	2.91±0.16[c]
HUFA	-	-	3.44±0.23	-

Values in each rows with different superscripts are significantly different (P<0.05)

Table 3: Average weight and total length [a], percent weight gain, of fish fed various dietary treatments .Values are mean ± standard deviation (n=3)

Treatment	Time (day)	Average weight (mg)	Average total length (mm	%weight gain
1	8	180.1±7.6[d]	21.07±1.1[b]	8.63±1.50[d]
2	8	202.76±8.3[c]	27.72±0.4[b]	11.82±1.1[c]
3	8	219.4±20.5[a]	28.44±0.9[a]	13.63±1.8[a]
4	8	210.7±19.1[b]	28.16±0.8[a]	12.73±1.5[b]
1	21	362±22.2[b]	33.44±0.7[c]	11.53±0.4[c]
2	21	358.86±26[b]	34.02±0.7[b]	11.37±0.6[c]
3	21	428.13±28.2[a]	35.42±0.81[a]	14.48±0.9[a]
4	21	417.33±19[a]	35.07±0.5[a]	14.08±0.9[a]
1	29	568.3±20.7[c]	38.7±0.4[c]	15.47±0.2[c]
2	29	560.63±27.3[c]	38.75±0.6[c]	15.19±0.5[c]
3	29	657.5±57.9[a]	40.74±1.1[a]	18.39±1.7[a]
4	29	596.5±39.2[b]	40.01±0.7[b]	16.37±1[b]

Within columns values with different superscripts are significantly different (P<0.05)
[a] Initial weights and lengths of trout larvae 120.63(mg) ±13.50 SD and 23.26(mm)± 0.90 SD respectively

Table 4: Specific growth rate (SGR), food conversion ratio, body weight increase (BWI) per day and coefficient of variation for SGR of fish fed various dietary treatments. Values are mean ± standard deviation (n=3)

Treatment	Time (day)	SGR%/day	%BWI	CV of SGR %	Feed conversion ratio
1	8	5.95±1.52[b]	52.57±17.1[b]	26.98±6[c]	0.7±0.05[c]
2	8	7.59±1.3[a]	70.9±16.8[a]	18.10±2.8[b]	0.48±0[b]
3	8	8.12±0.5[a]	76.72±6.3[a]	6.30±0.4[a]	0.42±0.01[a]
4	8	7.83±0.5[a]	73.23±6.6[a]	7.18±0.5[a]	0.46±0[ab]
1	21	25.30±0.4[b]	205.79±27.7[b]	7.77±0.5[d]	0.69±0.04[a]
2	21	5.24±0.32[b]	201.41±21.3[b]	6.12±0.34[b]	0.69±0.01[a]
3	21	5.90±0.11[a]	245.47±8.3[a]	1.91±0.03[a]	0.74±0.03[a]
4	21	5.88±0.18[a]	244.08±13.5[a]	1.82±0.03[a]	0.77±0.03[b]
1	29	5.40±0.[3bc]	381.37±51.5[b]	6.50±0.4[c]	0.62±0.03[a]
2	29	5.34±0.3[c]	372.42±45.2[b]	5.82±0.3[b]	0.79±0.01[c]
3	29	5.74±0.11[b]	429.82±18[a]	1.91±0.03[a]	0.74±0.02[c]
4	29	5.48±0.1[a]	391.12±14.9[b]	1.82±0.03[a]	0.69±0.02[b]

Within columns values with different superscripts are significantly different (P<0.05)

4. Discussion

Several studies have demonstrated the positive effect of enriched live food on the growth performance of various species. HUFA- enriched *Artemia* nauplii fed to *fenneropenaeus indicus* (Taiebi, 2001), *Sepia oficinalis* (Koueta et al., 2002), and *Chanos chanos* (Gapasin et al.,1998) exhibited better growth and survival .Gilthead sea bream larvae also grow better if fed rotifers enriched with highly unsaturated n-3

HUFA (Mourente et al., 1993). Similar to the finding of Tamaru et al.,1993 , in the present study significant differences were found in the growth of 8- day – old trout larvae fed different diets, larvae fed Artemia enriched with HUFA+vitamin C (treatment 3) exhibited significantly higher growth than larvae fed with commercial food (treatment1) after 29 day of culture (Table3 and 4). On the other hand, survival of 29 day-old trout fed various diets were significantly different (Table 6) supporting the results of Ako et al., (1994); Gapasin et al.,(1998) and Taebi, (2001). In the first week, *the Oncorhynchus mykiss* larvae fed unenriched *Artemia* nauplii, obtained higher growth rate compared to those fed commercial diet (treatment1) (table3). In our study the proximate composition of *Artemia* nauplii was 61.7% protein; 11.44% lipid; and 6.78% ash on a dry weight basis (DW). It may be explained that the higher dietary protein level can meet the requirements of body protein synthesis in early stages and then support fast growth of larvae (Watanabe et al.,1987b). The proteolytic enzymes in *Artemia* may play a significant role in contributing to the digestion process, in addition to digestion brought about by the proteolytic enzymes of the fry itself (Bengeston et al., 1991). Milkfish larvae given *Artemia* enriched with HUFA+ vitamin C showed better growth and higher survival after a stress test (Gapasin et al., 1998). Ako et al., 1994; Gapasin et al., 1998 observed no or few mortalities among fish fed *Artemia* enriched with menhaden oil (high DHA: EPA ratio) compared to high mortalities among fish fed unenriched *Artemia*. Red sea bream (*Pagrus major*) and marble sole *(Euryglossa orientalis*) larvae given diets containing DHA and lecithin tolerated temperature and salinity changes, low oxygen and air exposure better than the larvae given DHA and lecithin- free diets (Kanazawa, 1995).Furuita et al, (1995 a, b) reported that yellowtail larvae and red sea bream juvenile fed *Artemia* enriched with DHA exhibited higher survival in stress test than those fed *Artemia* enriched with EPA. In the present study, trout larvae fed *Artemia* enriched with HUFA+ vitamin C(treatment3) showed better growth and increased resistance to temperatures than those given unenriched diet (treatment 2)and commercial diet (treatment1) this result is similar to Chanos chanos (Gapasin et al., 1998)Sepia officicinalis (Kouetaet al., 2002). Takeuchi and Watanabe (1987); Takon (1990) reported that a dietary deficiency of the essential fatty acids (EFA) is manifested as poor growth, increased water content of the muscle, high liver lipid content and poor feed efficiency that were similar to our study (Table 7). In the current investigation, the larvae fed by HUFA+ vitamin C for 1 week exhibited more resistant to temperature stress (Table5, compared to treatment 1. The lowest mortality value consistently occurring in fish treated with HUFA+ vitamin C (treatment 3) may indicate that ascorbic acid supplementation enhanced resistance to stress. When subjected to salinity stress test, 20 day old *Clarias gariepinus* larvae fed ascorbate- supplemented diet exhibited significantly low mortality than those larvae fed an ascorbate free diet (Merchie et al., 1995). Although it is possible that HUFA alone may have improved growth performance in trout (as reported in *chonos chonos* by Gapasin et al., 1998), the synergistic effect of vitamin C cannot be neglected. Better growth was observed among tilapia finger lings (Anadu et al., 1990) and plaice (Rosenlund et al., 1990) fed diets supplemented with ascorbic acid.

The preceding studies attest the importance of EFA and or vitamin C in fish growth and development. Trout fed with EFA+vitamin C exhibited significantly ($P<0.05$) higher growth than those given unenriched live food after 29 days of culture. When subjected to temperature stress, mortality of the EFA+vitamin C-treated fish was significantly lower ($P<0.05$) among the treatment groups. Optimum requirements of these nutrients in trout, however, are not yet known.

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