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Effect of Enriched *Artemia urmiana* on Growth, Survival and Composition of Larval Persian Sturgeon

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Abstract

Recently, the nutritional requirements of marine finfish larvae have received considerable attention, and studies have shown that docosahexaenoic acid (DHA) affects on the growth and survival of marine finfish larvae. We investigated the effects of different *Artemia* diets containing variable amounts of DHA on the growth and survival of larval *Acipenser persicus*.

Four different commercial *Artemia* enrichment formulations were used: ICES30/4, Sturgeon Ovary Oil (SOO), Cod Liver Oil (CLO) and Linseed Oil (LO). The resultant *Artemia* contained a different 45 L concentration of DHA (0.00-5.99 mg/g DW) and eicosapentaenoic acid EPA (0.69-4.97 mg/g DW). Seventy-five aquaria were used with three replicates per treatment.

Larvae were fed with *Artemia* from 3 to 20 days after active feeding at 250 prey L^{-1}. At the end of the experiment, total length and wet weight of fish larvae showed significant differences among treatments (P<0.05), but no dry weight (P>0.05). However, larvae reared on LO were of significantly higher dry weight than larvae reared with ICES30/4 and SOO. Survival in fish larvae fed with SOO *Artemia* enriched (93.3±1.6%) was significantly higher than ICES30/4 and LO (P<0.05), but not CLO (P>0.05). Protein/ lipid ratio in larvae enriched with CLO showed significant differences with other treatments (P>0.05). DHA/EPA ratio in the larvae fed with ICES30/4 (1.11±0.00) was the highest among the treatments. This study resulted that the requirement of the Persian sturgeon larvae to dietary DHA and EPA; is high also, our results showed that there is a positive effect of *Artemia* DHA proportions on growth and survival rates.

Keywords: *Acipenser persicus*, HUFA, enrichments, *Artemia urmiana*.

Introduction

Lipids and amino acids are major sources of metabolic energy during the embryonic and pre-feeding larval stages in fish. At hatch, yolk-sac larvae have high levels of these energy sources, but they are dramatically reduced during the endogenous feeding stage (Evans *et al*., 2000). Thus, start-feeding larvae require a live feed that provides sufficient levels of these energy sources. Studies have shown that essential fatty acids (EFA), such as docosahexaenoic acid (DHA, 22:6n-3), eicosapentaenoic acid (EPA, 20: 5n - 3), and arachidonic acid (ARA, 20: 4n - 6) are also important in larval fish nutrition (Takeuchi, 1997; McEvoy *et al*., 1998; Estevez *et al*., 1999; Sargent *et al*., 1999). These fatty acids, as components of phospholipids (PL), are critical structural and physiological components of the cell membranes of most tissues. However, the live feeds commonly used for the first-feeding larval stages, such as rotifers and *Artemia*, are naturally poor in these fatty acids, so enrichment of live foods with lipids rich in EFA is necessary to achieve better growth and survival through metamorphosis (Rainuzzo *et al*., 1997). Recently, absolute and relative levels of DHA, EPA, and ARA in the diets of marine fish larvae have received considerable attention (Sargent *et al*., 1999; Harel *et al*., 2002; Bell and Sargent, 2003). DHA, which has a competitive relationship with EPA, is particularly important for normal neural development and function, including that of retina and brain (Sargent *et al*., 1999). Studies have shown that the DHA requirement in the diet differs among fish species, especially in cold-water fish species such as yellowtail flounder (*Limanda ferruginea*) and Atlantic halibut (*Hippoglossus hippoglossus*), which require high levels of dietary DHA (McEvoy *et al*., 1998; Copeman *et al*., 2002). However, Planas and Cunha (1999) reported that the requirement of turbot larvae (*Scophthalmus maximus*) to DHA in their diet for better growth and survival is lower. Other specific benefits of feeding DHA-enriched diets to fish larvae include successful metamorphosis, reduced pigmentation problems, enhanced vision capabilities, improved neural development and stress resistance (Watanabe, 1993).

Persian Sturgeon (*Acipenser persicus*) is the first rehabilitated species in the Caspian Sea (Iranian Fisheries Organization, data manager, 2003). In order to develop this species, a consistent production of juvenile fish must be achieved. Understanding the nutritional requirements of early larval sturgeon, especially of EFA such as DHA and EPA, is important for successful mass production. This study

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investigated the effect of DHA level and DHA/EPA ratio in four *Artemia* enriched diets on growth and survival of Persian Sturgeon larvae.

Materials and Methods

Getting Enriched *Artemia* after 4 Differ Feeding with Oils

Four different commercial enrichment formulations were used: ICES30/4 (an emulsion with 30% HUFA), Sturgeon ovary oil (SOO), Cod liver oil (CLO) and Linseed oil (LO) (Table 1).

Artemia were enriched with ICES30/4, SOO, CLO, and LO in 12 L vessels at a density of 300 *Artemia nauplii* L^{-1}. Each batch of artemia was enriched by 0.08 g of enrichment during 24 h. Enrichment diets were divided into two portions and added 0-12 h. Water temperature and salinity for enrichment were 22°C and 32 ppt, respectively. One-gram samples of 24 h enriched artemia were taken from each enrichment vessel for lipid analysis.

Feeding with Enriched *Artemia* of Sturgeon Larvae

The sleeping stage larvae of the Persian sturgeon from Shahid Beheshti propagation complex were moved to Artemia research center in Urmia University in plastic bag with 1/3 water and 2/3 Oxygen and transferred in two tanks (1000 L) with suitable water flow for 4 days. Water temperature was maintained at 20°C. After adaptation and start to active feeding, 20250 larvae (250 larvae L^{-1}) were transferred to each of the seventy-five 45 L rectangular glass aquaria (three replicates per treatment) that were randomly placed in a thermo-regulated water bath. This was considered day 1 of the experiment.

The water temperature in the experimental tanks was maintained at 20°C and monitored twice daily. A flow through water system was provided at an initial flow of 150 ml min^{-1}. Dissolved oxygen was monitored weekly. Light was provided around the clock at 1000 lux. Larvae were fed four times a day with Artemia enriched (one treatment with three replications as the control group were fed with Artemia non-enriched). Each experimental tank was aerated, which ensured a homogenous distribution of prey within the tank.

Five larvae (15 per treatment) were randomly sampled for morphometric measurements from each experimental tank on 3, 8, 13, and 20 days. The length of fish larvae was measured with 0.001 scales and then wrapped in a 1.0 cm^2 pre-weighed aluminum foil dried. At the end of 20th day, survival rate was determined by counting all larvae from each tank.

Lipid Analyzing from Larvae

Fish larval samples were placed directly in chloroform and stored under nitrogen 20°C, until extraction.

Lipids were extracted in chloroform/methanol according to Parrish (1999), using a modified Folch procedure (Folch *et al*., 1957). Lipid classes were determined using thin layer chromatography with flame ionization detection (TLC/FID) as described by Parrish (1987). Extracts were spotted on silica gel-coated Chromarods, and a three-stage development system was used to separate lipid classes.

The first separation consisted of a 25 min and a 20 min development in 99:1:0.05 hexane/diethyl ether/formic acid. The second separation consisted of a 40 min development in 80:20:1 hexane/diethyl ether/formic acid. The last separation consisted of two 15 min developments in 100% acetone followed by two 10 min developments in 5:4:1 chloroform/ methanol/water. After each separation, the rods were scanned, and three chromatograms were combined using T-Data Scan software (RSS, Bemis, Tennessee, USA). The signal detected in millivolts was quantified using lipid standards (Sigma).

Fatty acid methyl esters (FAME) were prepared by transesterification with 10% boron triflorate (BF3) in methanol at 85°C for 1.5 h (Morrison and Smith, 1964; Budge, 1999).

Table 1. Fatty acid composition (%) of different enrichment oils

Sample	ICES30/4	SOO	CLO	LO
C14:0	4.12	2.85	6.29	0.75
C16:0	20.05	21.65	16.66	22.36
C16:1n7	4.77	6.87	8.47	0.60
C17:0	1.26	0.92	0.46	0.00
C17:1n7	0.27	1.21	0.78	0.00
C18:0	6.26	1.83	3.10	2.58
C18:1n9	13.47	27.24	16.02	18.35
C18:1n7	2.70	4.21	3.93	1.14
C18:2n6	4.03	0.94	2.69	51.84
C18:3n3	0.68	0.65	1.04	0.27
C20:3n3	1.51	0.00	0.64	0.12
C20:5n3 (EPA)	6.29	7.55	11.39	0.03
C22:6n3 (DHA)				

A Varian model 3400 Gas Chromatograph (GC) equipped with a Varian 8100 Auto-Sampler was used for fatty acid analysis (Varian, California, and USA). An Omegawax 320 column, 30 m long, 0.32 mm i.d., 0.25 μm film thickness (Supelco, Bellefonte, Pennsylvania, USA) was used for separations. Hydrogen was used as the carrier gas, and the flow rate was set at 2 ml min^{-1}. The column temperature profile was as follows: 65°C for 0.5 min, hold at 195°C for 15 min after ramping at 40°C min^{-1}, and hold at 220°C for 0.75 min after ramping at 2°C min^{-1}. The injector temperature was increased from 150°C to 250°C at 200°C min^{-1}. Peaks were detected by flame ionization with the detector held at 260°C. Fatty acid peaks were integrated using Varian Star Chromatography Software (version 4.02), and identification was made with reference to known standards (PUFA 1, 3 and 37 Component FAME Mix, Supelco Canada, Ontario, Canada).

Statistical Method

All data were tested for normality to satisfy the assumptions of ANOVA. Two-way ANOVA were used to determine the statistical significance of treatment on dry weight and standard length of sturgeon larvae. One-way ANOVA with the Duncan multiple comparison test was used to compare differences in survival of larvae and lipid class, and fatty acid composition of *Artemia* and larvae between treatments. Differences were considered significant at the P<0.05 level.

Results

The total protein content of enriched *Artemia* in all treatments was significantly higher (P<0.037) than in the initial nauplii (control), and in ICES30/4, it was significantly higher than in the SOO, CLO and LO treatments (Table 2). The total lipid content of *Artemia* increased in all treatments after 24 h enrichment, but it was significantly higher (P<0.011) in the ICES30/4(20.87%) and LO (19.45%) treatments than in the SOO and CLO treatments. The protein/lipid ratio of the enriched *Artemia* in all groups was lower than in the initial *Artemia*. However, CLO and SOO showed a significantly higher ratio than ICES30/4 and LO.

All treatments resulted in higher levels of EPA than DHA except for ICES30/4, and the highest levels of EPA and DHA (P<0.033) were 4.97 and 5.99 respectively in ICES30/4 treatment. *Artemia* from ICES30/4, SOO and CLO had significantly higher DHA/EPA ratios than *Artemia* from LO and Control (P<0.021).

Initial and final length and weight of sturgeon larvae during first three days are demonstrated in Table 3. The effects of enrichment with ICES30/4 and SOO on length of sturgeon larvae were significant (P<0.034). From the 8th day to the end of the experiment, larvae reared on ICES30/4 and SOO were significantly longer than larvae reared on other treatments (Table 4, 5 and 6).

Enrichment of live food had no significant effect on the dry weight, but had significant effect on the wet weight of larval sturgeon. ICES30/4 performed the highest WW in sturgeon larvae in the 8th day, but there are no significant difference between ICEA30/4 and SOO during the 8th till 20th days (Table 4, 5 and 6).

ICES30/4 and SOO enrichment had a significant effect on the survival rate of larval sturgeon. At the end of experimental period (day 20), the larvae fed with *Artemia* enriched with SOO and CLO oils had higher survival rate than ICES30/4 and LO (Table 6). Cochran's test for variance outliers (Kanji, 1994) was used to determine outliers in the data, and a significant critical value (P<0.05) was found for the SOO and CLO survival data. When the data were analyzed after removing this outlier, larval survival in the SOO and CLO treatments was significantly higher (P<0.019), while no significant difference was found

Table 2. Average total Protein, lipid (% DW), DHA and EPA Fatty acid composition (mg/g DW) and DHA: EPA ratios in of all enriched *Artemia* and control

	Control	ICES30/4	SOO	CLO	LO
Protein	55.12^{a}	59.90^{c}	58.43^{b}	58.47^{b}	58.17^{b}
Lipid	16.79^{a}	20.87^{d}	18.86^{b}	18.72^{b}	19.45^{c}
Protein/Lipid	3.28^{b}	2.87^{a}	3.09^{b}	3.12^{b}	2.99^{a}
C22:6n3 (DHA)	0.00^{a}	5.99(0.06)^{c}	0.69(0.05)^{b}	0.70(0.09)^{b}	0.00^{a}
C20:5n3 (EPA)	0.82(0.11)^{b}	4.97(0.26)^{e}	1.71(0.12)^{c}	2.55(0.06)^{d}	0.69(0.05)^{a}
DHA/EPA	0.00^{a}	1.20^{d}	0.40^{c}	0.29^{b}	0.00^{a}

Letters in front of the row numbers demonstrated the significant differences

Table 3. Initial and final Length (mm) and Weight (mg) of sturgeon larvae, during first three days

	TL	DW	WW
Initial length	19	4.8	33
Final length	21.2	6.5	46.8

Table 4. Total Length (mm), Wet and Dry Weight (mg) of sturgeon larvae in day 8

Treatments	TL	DW	WW
Control	23.7(0.3) [a]	9.8(0.4) [a]	81.7(2.5) [b]
ICES30/4	25.1(0.5) [c]	9.8(2.1) [a]	87.3(1.00) [c]
SOO	24(0.7) [b]	9.4(1.6) [a]	80.1(7.4) [b]
CLO	23.7(0.5) [a]	9.7(1.5) [a]	75.1(8.9) [a]
LO	24.3(0.7) [b]	9.2(1.2) [a]	80.3(3.8) [b]

Letters in front of the row numbers demonstrated the significant differences

Table 5. Total Length (mm), Wet and Dry Weight (mg) of sturgeon larvae in day 13

	TL	DW	WW
Control	32. (0.7) [b]	18.7(0.5) [a]	162.2(2.4) [a]
ICES30/4	32.2(1.2) [b]	19.0(3.7) [a]	174.8(16.2) [b]
SOO	32.3(0.8) [b]	18.4(2.4) [a]	171.3(2.1) [b]
CLO	32.5(0.3) [b]	18.2(0.9) [a]	163.9(5.4) [a]
LO	31.7(0.9) [a]	8.8(0.4) [a]	158.3(3.4) [a]

Letters in front of the row numbers demonstrated the significant differences

Table 6. Total Length (mm), Wet and Dry Weight (mg) and Survival rate (%) of sturgeon larvae in day 20

	TL	DW	WW	Survival
Control	40.3(2.1)[a]	29.3(4.3)[a]	297.3(31.4)[a]	87.9(2.3)[a]
ICES30/4	41.3(2.1)[b]	30.5(6.9)[a]	307.3(53.9)[b]	88.0(0.4)[a]
SOO	41.6(1.0)[b]	30.9(4.2)[a]	309.9(25.9)[b]	93.3(1.6)[b]
CLO	39.9(0.9)[a]	28.0(1.9)[a]	292.1(10.8)[a]	91.7(0.8)[b]
LO	40.2(1.1)[a]	31.5(4.6)[a]	302.4(28.1)[a]	87.2(0.2)[a]

Letters in front of the row numbers demonstrated the significant differences

among the other three treatments.

The total protein content of enriched sturgeon larvae in all treatments was significantly lower ($P<0.047$) than in the control, but in ICES30/4 (69.62%) and LO(68.93%) was significantly higher than in the SOO and CLO treatments (Table 7). The total lipid content of sturgeon larvae increased only in SOO treatment (22.14%) compared to control group. The protein/lipid ratio of the enriched sturgeon larvae in CLO was the highest (5.84) while between controls, ICES30/4 and LO treatments there were not significant differences. SOO treatment had the lowest protein/lipid ratio (3.06).

There were no difference between controls, CLO and LO treatments in ARA ($P>0.067$), but their amount of this fatty acid was higher than ICES30/4 and SOO treatments (Table 7).

ICES30/4 was the only oil, which can improve the DHA amount (2.75±0.08) in larvae compares to the control, other treatments resulted in lower levels of EPA than the control.

Although there were significant differences between groups regarding to EPA ($P<0.043$) none of the treatments can improve levels of EPA in sturgeon larvae compared with the control (2.97±0.12).

The ratio of DHA/EPA was the highest in ICES30/4 (1.11), and had significant difference with other treatments and control which they have no difference between them.

Discussion

While evaluating effect of DHA levels and DHA:EPA dietary ratio on growth and survival rate of Persian sturgeon, primarily cold water fish; the traits of early ontogenesis in this species, as anadromous freshwater species and its thermal capacity should be considered by the authors. A relationship between DHA levels and DHA/EPA ratios of *Artemia*, and the growth and survival of sturgeon larvae was found in the present study. Noori *et al.* (2002) found that *Acipenser persicus* larvae were fed with *Artemia* nauplii enriched with essential fatty acid supplemented with vitamin C, had significantly better growth and resistance to salinity stress compared with control group. Takeuchi *et al.* (1994) investigated the effect of DHA levels of rotifers on the growth, survival rate, and abnormalities of larval cod (*Gadus macrocephalus*), and suggested that the appropriate level of DHA that should be contained in the rotifers was around 1% DW. In their study, any amount higher than 1% DHA resulted in a high percentage of abnormal fish, together with high mortality. However, in our study, larval sturgeon fed with SOO containing 1.44±0.24 DW of DHA had higher growth and survival than larvae fed with ICES30/4 and 2.75±0.08 DW of DHA. Although Sargent *et al.* (1999) suggested that species-specific requirements for DHA exist among marine finfish larvae but several other

Table 7. Average total Protein, lipid (% DW), ARA, DHA and EPA Fatty acid composition (mg/g DW) and DHA: EPA ratios in of all enriched sturgeon larvae and control

	Control	ICES30/4	SOO	CLO	LO
Protein	69.93 [c]	69.62 [c]	67.90 [a]	68.00 [a]	68.93 [b]
Lipid	17.37 [b]	16.65 [b]	22.14 [c]	11.63 [a]	15.40 [b]
Protein/Lipid	4.02 [b]	4.18 [b]	3.06 [a]	5.84 [c]	4.47 [b]
C20:4n6 (ARA)	0.47(0.07) [b]	0.98(0.11) [a]	0.98(0.12) [a]	1.53(0.20) [b]	1.35(0.10) [b]
C22:6n3 (DHA)	2.30(0.17) [b]	2.75(0.08) [c]	1.44(0.24) [a]	2.28(0.18) [b]	1.48(0.10) [a]
C20:5n3 (EPA)	2.97(0.12) [c]	2.46(0.09) [b]	1.87(0.21) [a]	2.90(0.13) [b]	2 .17(0.15) [b]
DHA/EPA	0.77 [a]	1.11 [b]	0.77 [a]	0.78 [a]	0.68 [a]

Letters in front of the row numbers demonstrated the significant differences

studies suggested that much higher levels of DHA (or n-3 highly unsaturated fatty acids e HUFA) could reduce larval survival (Planas and Cunha, 1999). Izquierdo *et al.* (1992) showed that, in larval Japanese flounder (*Paralichthys olivaceus*), lower (or higher) DHA content (1.5%) of *Artemia* did not affect survival, but larvae were significantly larger when fed with *Artemia* containing a higher percentage of DHA (up to 3.5%). However, Salhi *et al.* (1994), in their study with gilthead sea bream (*Sparus aurata*), showed that larvae fed with a lower DHA micro diet (>0.5%) had a significantly lower survival rate than larvae fed with a higher DHA micro diet (1.2-1.3%).

They suggested that the growth of larvae was affected by a combination of DHA content and total dietary lipid. In our study, however, the SOO (1.44±0.24 DW of DHA) treatment gave a significantly higher survival than the ICES30/4 (2.75±0.08 DW of DHA) but total dietary lipid was higher in SOO than ICES30/4.

Copeman *et al.* (2002) found that yellowtail flounder fed with high DHA/EPA ratio (8:1) had a higher growth and survival than those fed with low DHA/EPA ratio (1.9:1). However, there was no significant difference in the growth of Japanese flounder and turbot larvae when they were fed with different dietary ratios of DHA and EPA (Estevez *et al.*, 1999; Furuita *et al.*, 1999). Harel *et al.* (2002) investigated the effect of commercial enrichment materials on early development of three larval fish. They reported no significant difference in growth between striped bass (*Morone saxatilis*) and gilthead sea bream larvae fed with *Artemia* enriched with Algamac 2000 or PL-Cr (DHA-rich phospholipids extract of *Crypthecodinium sp*.). However, the growth of halibut larvae fed with *Artemia* enriched with DHA Selco was lower than the growth of larvae fed with PL-Cr. Our studies also showed that sturgeon larvae fed with low DHA/EPA diets (SOO) showed better growth and survival than those fed with high DHA/EPA diets (ICES30/4). On the other hand, sturgeon larvae fed LO, which has almost equivalent level of DHA had compared with SOO, had a lower survival than those fed with SOO treatments. All these studies, including our own, suggest the existence of species-specific requirements for the DHA/EPA ratio for growth and survival of marine finfish larvae.

The lipid composition of eggs/yolk was suggested as an indicator for determining the nutritional requirements of first-feeding larvae. Typically, a dietary DHA/EPA ratio of 2:1 is found in marine species, and has been suggested as adequate for larval feeding (Tocher and Sargent, 1984; Sargent *et al.*, 1999). However, in our experiment, growth and survival of larval sturgeon improved with increasing ratio. However, increase in the DHA/EPA ratio was significantly higher in the ICES30/4 treatment, which yielded not better growth than the other two treatments. From our results, it seems that larval sturgeon requires a lower DHA: EPA ratio than some other marine finfish species. Copeman *et al.* (2002) suggested that larval yellowtail flounder require higher dietary DHA levels for better growth. Thus, our results indicate that the presence of high DHA and lower EPA levels in the diet may not be important for better growth of sturgeon larvae.

Watanabe (1993), suggested that the DHA content of Atlantic cod larvae could be reduced rapidly during larval development after hatching. In our study, the DHA levels of Persian Sturgeon larvae in CLO treatment did not change compared with the initial value. Meanwhile, the DHA levels of larval sturgeon fed with SOO and LO were lower than the initial value. This suggests that the DHA levels of sturgeon larvae should be kept close to the initial levels for better larval growth, and that this can be accomplished by feeding diets with a relatively high DHA level and high DHA/EPA ratio. Copeman *et al.* (2002) found that supplementing diets with high EPA levels was not effective for the growth of yellowtail flounder. Similarly, in our experiment, EPA levels of larvae enriched with SOO was very low (1.87±0.21) in all treatments and had effect on the survival of sturgeon larvae.

Recently, studies have indicated that arachidonic acid (ARA) levels in marine fish larvae may be important for stress tolerance, pigmentation, growth, and survival (Bell and Sargent, 2003). In particular, the competitive interactions between EPA and ARA are important in the formation of eicosanoids (Harel and Place, 2003). In our study, ARA levels in larvae were higher in all treatments than in their respective diets. Thus, larval cod appear to have the ability to selectively incorporate dietary ARA into their body tissues.

Similarly, Copeman *et al*. (2002) found that yellowtail flounder larvae have the ability to increase the dietary ARA levels in the body tissue inspite of lower dietary ARA levels (as low as 2.2% of total fatty acid). Zheng *et al*. (1996), reported that prey enriched with higher ARA provided no improvement in survival for Pacific cod larvae. Similarly, in our studies, ARA levels in *Artemia* did not affect growth and survival of Persian Sturgeon. However, studies have shown that dietary ARA levels are important for improved growth and survival in gilthead sea bream (Bessonart *et al*., 1999; Koven *et al*., 2001). Sargent *et al*. (1999) suggested that both the concentration and ratio, not only between DHA and EPA, but also between EPA and ARA, is important in larval marine fish nutrition. Thus, it appears that the ARA levels in diet have a species-dependent effect on sturgeon fish.

Chemical composition of the enrichment diets used in our experiment differed not only in essential fatty acids, but also in phospholipids, proteins, and micronutrients. Although our results showed that DHA, EPA, and DHA: EPA ratio had significant effects on the growth, survival, and composition of larval sturgeon, differences in other nutrients could have also affected our results. Unfortunately, it is difficult to control all the nutrients in studies involving commercial enrichments and live prey, and could only be achieved using formulated inert diets.

In conclusion, high dietary lipid appears effective in improving the nutritional value of *Artemia* for the improvement of growth and survival of sturgeon larvae.

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