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Effects of n3-HUFA enriched *Daphnia magna* on growth, survival, stress resistance, and fatty acid composition of larvae of Persian sturgeon (*Acipenser persicus*)

A.M. Abedian Kennari[1*] ; M.R. Oveisipour[1] and R.M. Nazari[2]

aabedian@modares.ac.ir

1- Department of Fisheries, Faculty of Natural Resources and Marine Sciences, Tarbiat Modares University, P.O. Box: 46414-356 Noor, Iran.

2- Shahid Rajaee Sturgeon Hatchery Center, P.O. Box: 338 Sari, Iran

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Abstract: Effects of *Daphnia magna* enriched with cod liver oil (CLO) as a source of highly unsaturated fatty acid (HUFA) on growth, survival, stress resistance, and fatty acid composition of the Persian sturgeon larvae were evaluated. *Daphnia* enriched for three different time periods (3, 6, and 9 hours) and non-enriched *Daphnia* were fed to the Persian sturgeon larvae (average weight 61.6±0.4mg) during 14 days. The highest n3-HUFA content was found in *Daphnia* enriched for 9 hours (0.69mg g^{-1} DW) and the highest n3-HUFA content of the larvae was also found in those larvae fed with *Daphnia* enriched for 9 hours (2.39mg/g^{-1}DW). A significant growth difference between larvae fed with enriched and non-enriched *Daphnia* was observed ($P<0.05$), while survival rate did not significantly differ among the treatments ($P>0.05$). Furthermore, the highest pH stress resistance was found in those larvae fed with *Daphnia* enriched for 9 and 6 hours ($P<0.05$). A salinity stress test did not show significant differences among the treatments.

Keywords: Live food, *Daphnia*, Persian sturgeon, Enrichment, n3-HUFA, Growth performance, Fatty Acids Composition

* **Corresponding author**

Introduction

Rearing of larval fish is the most critical stage in the production cycle for many species. The primary problem in rearing relates to the transitional period from endogenous to exogenous food resources and thus to adequate feed supply (Leger *et al.*, 1986; Abi-Ayad & Kestemont, 1994). A readily available diet which has a high nutritional quality and is easily accepted and digested by the larval fish is essential to success (Kim *et al.*, 1996). Dietary lipids play an important role in fish nutrition for provision of both essential fatty acids (EFA) and energy. Dietary lipids also assist in the absorption of fat-soluble nutrients (Sargent *et al.*, 1999).

Live prey organisms, especially zooplankton, are generally used as initial larval food for certain species of fish (Leger *et al.*, 1986). Being naturally low in EPA, live foods commonly used for first feeding of larvae, such as *Rotifer* and *Artemia*, have to be enriched with lipids rich in EFA prior to feeding (Copeman *et al.*, 2002).

It has been suggested that white sturgeon may require both n3 and n6 fatty acids based on growth and the 20:3n9/20:4n6 and 20:3n9/22:6n3 ratio in liver phospholipids (Hung & Deng, 2002). *Daphnia* sp., however poor in EFA, is one of the most important starter live food in sturgeon larviculture in Iran. Therefore, *Daphnia* EFA- enrichment as a live food is needed to meet the requirements of sturgeon for EFA.

Some methods have significantly enhanced EFA level in *Daphnia* sp. (Sundbom & Vrede, 1997; von Elert, 2002; Ravet *et al.*, 2003), but no study exists to test the effects of EFA-enriched *Daphnia* on the performance of Persian sturgeon larvae. This study presents a specific approach to enrich *Daphnia* with cod liver oil. The study also aims at evaluating the role of HUFA in the first feeding of the Persian sturgeon larvae and their effects on the growth performance and body composition of the larvae.

Material and methods

Sturgeon larvae (12 days post hatch) were obtained from the Shahid Rajaee Sturgeon Hatchery Center in Sari, Iran. The larvae were fed with *Artemia* nauplii

(Instar 1) for two days before collection. The larvae with initial wet weight of 61.6±0.4mg and total length of 22.3±0.3mm were randomly distributed in groups of 150 individuals per tank into 12 rectangular elliptic fiberglass tanks of 15 L each (10 larvae/L). Each tank was supplied with water via 1 inch PVC pipe at a flow rate of 3 L min^{-1}. Water was continuously aerated (compressed air) to keep oxygen levels close to 5.8±0.3mg L^{-1} (n=56). The tank outlet and inlet was protected by a 250μm net screen. Water quality was checked periodically; pH was about 7.8±0.02 (n=28), temperature was 19±1°C (n=56) and photoperiod was 12 L: 12 D.

Daphnia magna of the mean length size 1.6±0.15mm (n=30) were collected from an earthen pond in Shaid Rajaee Hatchery Center (Sari, Iran). Enrichment solution was prepared with cod liver-oil (Seven Seas), polysorbate (Tween 80, Merck) and freshwater according to Ako *et al.* (1994). In this method, first, 5mL polysorbate was added to 50mL freshwater and mixed carefully, then 50mL cod liver oil was added to the solution and mixed; 0.3-0.5mL of the final solution as an enrichment solution was used for 1 L of the incubator. *D. magna* at a density of 10,000 ind. L^{-1} were enriched in an incubator at a temperature of about 20°C according to methods described by Von Elert (2002) and three different enrichment exposure times were employed (3, 6, 9 hours). The solutions were prepared daily in order to maintain the quality at comparable level throughout the experiment.

Four different *Daphnia* enrichment treatments were tested (analysis was performed in triplicate): (I) non-enriched *Daphnia*, (II) Enriched *Daphnia* with cod liver oil (CLO) in 3 hours, (III) Enriched *Daphnia* with CLO in 6 hours, and (IV) Enriched *Daphnia* with CLO in 9 hours. Larvae were fed *Daphnia ad libitum* (Kolkovski *et al.*, 2000) 4 times per day.

The survival rate in each treatment was calculated, based on counting the number of dead larvae. The wet weight of larvae was measured by randomly sampling of 10 larvae in each replicate on 3rd, 5th, 12th and 14th days. Growth indexes of fish calculated based on following equations (Lee *et al.*, 2003).

$$\text{Weight gain (\%)} = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}} \times 100 \quad (1)$$

$$\text{Specific Growth Rate (SGR)} = \frac{\ln(\text{final weight}) - \ln(\text{initial weight})}{\text{days}} \times 100 \quad (2)$$

After 14 days of larval rearing, the resistance of larvae against extreme pH values was determined at pH levels of 4.5 and 11, respectively. First, 10L larval rearing water was poured into a 60L aquarium and 12 small baskets were placed inside the aquarium. The designated pH of the water was adjusted with 0.1 N HCl and 1 N NaOH. Then, 30 larvae were taken randomly from each replicate and transferred to the small baskets inside the aquarium. The larval survival was calculated by counting of the dead larvae after 30 minutes pH stress test. Artificial sea water (25g L^{-1}) was made by adding marine salt into the water in order to evaluate larvae resistance against salinity stress (Kolkovski *et al.*, 2000). From each tank, 30 larvae were collected and transferred into the small baskets inside the aquarium. The larval survival was computed by counting of the dead larvae after 15 minutes salinity stress test.

Enough samples of *Daphnia* and 10 larvae from each replicate were collected and kept in a freezer before analyzing. The extraction of lipid from *Daphnia* and fish larvae was done through saponification with 2g NaOH in 100ml methanol according to Folch *et al.*, 1957. Fatty acid methyl esters were prepared by transesterification with borontrifluoride (BF_3) in methanol (Metcalfe & Schmitz, 1961). Fatty acids methyl esters were measured in a Unicam 4600 gas chromatograph (USA) with flame ionization detector. The column was BPX 70 with a capillary column of 30m length and ID of 0.25mm. The carrier gas was helium at a flow rate of 30 ml s^{-1}. Detector and injector temperature were 250 and 240°C, respectively. The thermal gradient was 170°C for 5 min, then increased by 3°C min^{-1} to 200°C and held this temperature for 20 min. The fatty acids were quantified by comparing areas of their peaks with a peak of an internal standard, (C18:0). The peaks of fatty acids were carried out by connecting the GC with

personal computer and utilizing Millenium software. Peak identification was performed by means of standard sample.

To analyze statistical results, one-way ANOVA was applied and the mean comparison was done through LSD test at reliability level of 5%. All variances were checked for normality and homogeneity. Data analysis was done in SPSS software (release 12.0).

Results

Result on the fatty acid profile of non-enriched and enriched *Daphnia* for 3, 6 and 9 hours are shown in Table 1. Eicosapentaenoic acid (EPA, 22:5n3) content in non-enriched *Daphnia* was about 0.06mg/g^{-1}DW and docosahexanoic acid (DHA, 22:6n3) was not detected. After enrichment with cod liver-oil for 3, 6 and 9 hours, EPA content increased to about 0.13, 0.43 and 0.69mg/g^{-1}DW of *Daphnia*, respectively; however, DHA content was only detected in *Daphnia* enriched for 3 and 6 hours. The highest n3-HUFA concentration observed for 9 hours enriched *Daphnia* (0.69mg/g^{-1}DW).

Table 2 shows results on the fatty acid composition in sturgeon larvae fed the enriched and non-enriched *Daphnia* diets. Content of n3-HUFA in larvae fed the enriched *Daphnia* was improved accordingly with increments of the enrichment times. This means that larvae with the highest n3-HUFA (2.39mg/g^{-1}DW) were those that were fed with the *Daphnia* enriched diet exposed at 9 hours.

Wet weight of the sturgeon after the 1^{st}, 3^{rd}, 5^{th}, 12^{th} and 14^{th} day of rearing are shown in Figure 1. Growth of those larvae fed the 9 hours enriched *Daphnia* was better than in all the other treatments. The lowest growth was observed in larvae fed with non-enriched *Daphnia*. Table 3 shows significant differences between growth of the fish larvae fed with non-enriched and enriched *Daphnia* ($P< 0.05$), but no significant differences are observed among the enriched treatments ($P>0.05$). The highest weight gain, SGR and final total length were observed in the larvae that fed with 9 hours enriched *Daphnia*. Survival of fish fed enriched *Daphnia* was not significantly different from that fed non enriched *Daphnia* ($P> 0.05$).

The results of low and high pH stress are shown in Figure 2. The highest resistance was observed in those larvae fed with enriched *Daphnia* in 9 hours. Although significant differences between enriched and non-enriched treatments were evident ($P<0.05$), the difference between 6-hours and 9 hours enrichment was not significant ($P>0.05$). In addition, results of high pH condition showed significant differences among the treatments ($P<0.05$). The highest and lowest survivals were observed in 9-hours enriched and non enriched *Daphnia*, respectively. Results of salinity stress showed that there were not any significant differences among the treatments; survival was very low in all groups, as it is shown in Figure 2.

Table 1: Average fatty acid content in *Daphnia magna* before and after enrichment in three different times during 3, 6 and 9 hours (in mg per dry gram of *Daphnia*)*

Fatty acids	*Daphnia* enrichment			
	Non-enriched	CLO-3	CLO-6	CLO-9
14:0	0.39	0.52	2.00	1.32
16:0	1.14	1.75	3.66	4.49
16:1(n-7)	0.36	1.23	1.96	2.12
18:0	0.35	0.46	1.08	1.16
18:1(n9)	1.40	2.26	3.71	5.64
18:2(n6)	0.40	0.42	0.60	1.18
18:3(n3)	n.d.	n.d.	n.d.	n.d.
EPA 20:5(n3)	0.06	0.13	0.43	0.69
DHA 22:6(n3)	n.d.	0.07	0.13	n.d.
∑SFA	1.88	2.73	6.74	6.97
∑UFA	2.19	4.10	6.82	9.62
∑PUFA	0.46	0.61	1.15	1.86
∑n3-HUFA	0.06	0.19	0.55	0.69

*** Values are expressed the means from two replicate.**
∑SFA= total saturated fatty acid
∑UFA= total unsaturated fatty acid
∑PUFA= total polyunsaturated fatty acid
∑n3-HUFA= total n3 highly unsaturated fatty acid
n.d. = not detected.

Table 2: Average fatty acid content in the Persian sturgeon larvae at the end of the experiment (in mg per dry gram)*

Fatty acids	*Daphnia* enrichment			
	Non-enriched	CLO-3	CLO-6	CLO-9
14:0	0.13	0.53	0.28	0.38
16:0	2.71	4.35	4.83	4.33
16:1(n-7)	0.52	1.28	1.48	1.25
18:0	1.56	1.21	1.80	1.84
18:1(n9)	3.62	5.39	6.53	6.53
18:2(n6)	0.41	0.81	1.08	1.00
18:3(n3)	n.d.	n.d.	n.d.	n.d.
EPA 20:5(n3)	0.65	0.74	0.84	1.30
DHA 22:6(n3)	0.27	0.47	0.62	1.09
∑SFA	4.40	6.09	6.91	6.55
∑UFA	6.17	8.69	10.55	11.16
∑PUFA	1.33	2.02	2.54	3.39
∑n3-HUFA	0.92	1.21	1.46	2.39

***Values expressed are the means from two replicates.**
n.d. = not detected.

Table 3: Average growth and survival of the Persian sturgeon larvae fed with *Daphnia* enriched at various levels of time*

	Daphnia enrichment times			
Growth parameter	non	3 hours	6 hours	9 hours
Initial total length (mm)	22.8± 0.1	22.2±0.2	22.05±0.5	22.2±0.4
Final total length (mm)	31.76±0.38 [a]	34.18±0.34 [b]	36.13±0.16 [c]	36.76±0.31 [c]
Weight gain (g)	144.1±2.4 [a]	162.1±5.5 [b]	169.1 ±2.4 [b]	172.4 ±1.2 [b]
Body weight increase(%)	233.9±3.8 [a]	263.1±9 [b]	274.5 ±3.8 [b]	279.9 ±1.9 [b]
SGR	9.27±0.08 [a]	9.91±0.18 [b]	10.15±0.07 [b]	10.26±0.03 [b]
Survival Rate (%)	99.75±0.31	99.82±0.53	99.78±0.48	99.88±0.75

***Mean±S.D. of three replicates. Numbers within the same row with different superscripts are significantly different (P<0.05).**

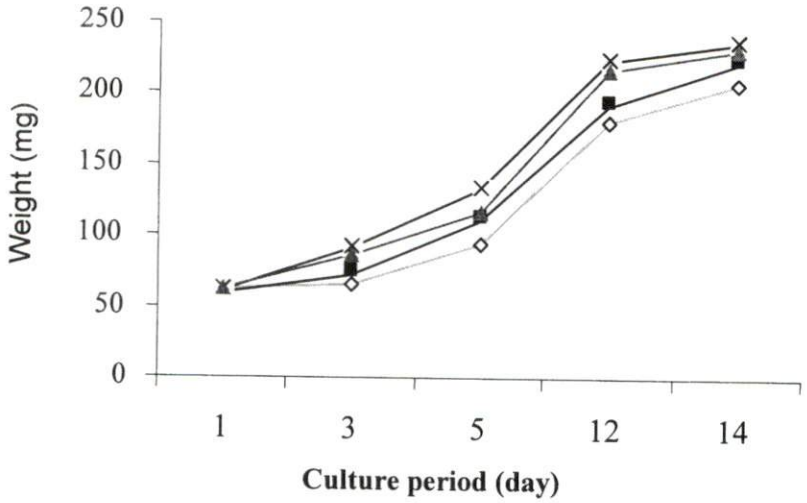

Figure 1: Average weight of the Persian sturgeon larvae during 14 days, fed non enriched *Daphnia* (◊) and 3 (■), 6 (▲) and 9 (×) hours enriched *Daphnia*. Each data point represents mean weight of three replicates (n=30)

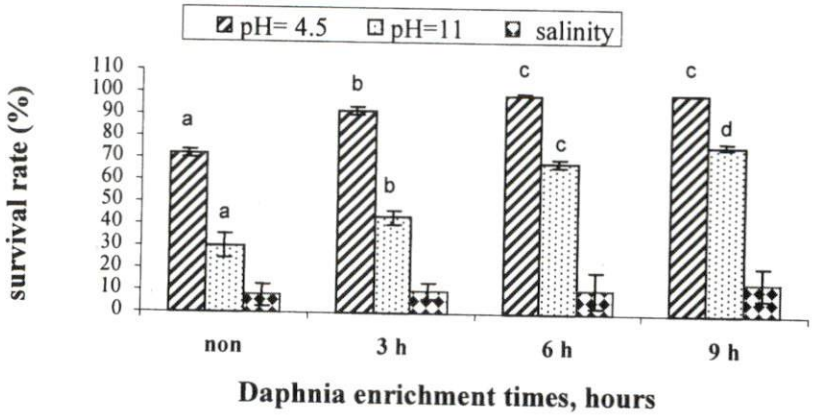

Figure 2: Average survival rate (%) of the Persian sturgeon larvae in pH (30 min exposure) and salinity stress tests (15 min). Each data point represents mean weight and standard deviation of three replicates (n=90)

Discussion

Many different methods have been used for enrichment of live foods (Coutteau & Sorgeloos, 1997; Weers & Gulati, 1997; von Elert, 2002; Ravet *et al.*, 2003). The enrichment method of *Daphina* boosted up considerably its fatty acids content in comparison to similar studies conducted with different oil sources for enrichment of *Artemia* (Kraul *et al.*, 1993; Ako *et al.*, 1994).

The EPA of *Daphnia* was increased with the increment of enrichment times. However, DHA was detected in the 6 hours of enriched *Daphnia* and not detected in 9 hours (Table 1). It maybe related to *Daphnia* ability to convert α-LA and DHA to EPA, which was also supported by Sundbom & Vrede (1997), von Elert (2002), Ravet *et al.* (2003). von Elert (2002) suggested that additional EPA in food of *Daphnia galeata* only increased its EPA content. He also showed that EPA was not converted to other fatty acids but DHA and α-LA could be converted to EPA. This conversion is well-known in *Artemia franciscana* and *Brachionus plicatilis* that have been shown to catabolise DHA selectively during starvation (von Elert, 2002). This reason might be due to fast filtration of *Daphnia* that they use DHA to obviate their starvation in 9 hours enrichment.

It is worth stating that both of EPA and DHA play a critical role in growth and evolution of fish larvae specially sea fishes (Watanabe *et al.*, 1983; Dhert *et al.*, 1990; Sorgeloos *et al.*, 1991; Sorgeloos & Leger, 1992; Watanabe, 1993; Kraul *et al.*, 1993). We also observed that feeding of the Persian sturgeon larvae with HUFA-enriched *Daphnia* resulted in growth improvement compared to non-enriched *Daphina*. Since enriched *Daphnia* provides necessary energy, larvae can pass better through external nutrition and as a result production will increase. Therefore, to achieve higher production, nutrition optimization of the Persian sturgeon larvae especially by using HUFA in daily foods and transferring it to fish body through live food is recommended for sturgeon hatcheries.

Survival rate of sturgeon larvae was not affected by enrichment, which was in agreement with Kolkovski *et al.* (2000) who did not find any relationship between HUFA enrichment and walleye (*Stizostedion vitreum*) larval survival.

The results of this study proved that feeding the Persian sturgeon with live food containing high n3-HUFA content increased larval resistance to pH stress (Fig. 2). The competitive interactions between EPA and AA are important in the formation of eicosanoids. Eicosanoids are a group of biologically active molecules, once known as local hormones, which include prostaglandins, thromboxanes, and leukotrienes (Sargent, 1995). Several studies have demonstrated that prostaglandins (PGs) are involved in the control of osmoregulatory processes and the regulation of the stress-induced hypothalamus–pituitary–interrenal (HPI) axis, which facilitates the release of cortisol, the main corticosteroid in teleost fish (Gupta *et al.*, 1985; Van Praag *et al.*, 1987; Wales, 1988). Relationship between feeding of larvae with HUFA-enriched *Artemia* and enhancing resistance against environmental stress has been reported in other species of fishes (Dhert *et al.*, 1990; Ako *et al.*, 1994). Since the content of all such acids increases due to enrichment, it shows that fatty acid plays the most important role in resistance against stress (Lavens & Sogeloos, 1996).

According to the results of this study, *Daphnia* HUFA level was enhanced by increasing the enrichment time and, therefore, resulting in a better larval development, stress resistance and production enhancement.

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