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Effects of Feeding Enriched Artemia Fransiscana with HUFA, Vitamin C and E on Growth Performance, Survival and Stress Resistance of Yellowfin Seabream Larvae

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Abstract

Effects of feeding highly unsaturated fatty acid (HUFA), vitamins E and C enriched *Artemia fransiscana* nauplii on growth, survival and stress resistance of yellowfin seabream, *A. latus* were investigated. Larvae at first exogenous feeding with 53 ± 5 mg body weight were fed with HUFA+5%, 10% and 15% vitamin C-enriched *Artemiai* (C1, C2 and C3 groups, respectively), HUFA+5% and 10% vitamin E-enriched *Artemia* (E1 and E2 groups, respectively), HUFA+2.5% (W/W) vitamin C and E enriched *Artemia* (CE2 group), HUFA+5% (W/W) vitamin C and E-enriched *Artemia* (CE1 group), HUFA without vitamin (HUFA group) and non-enriched *Artemia* (control) in 3 replicates. Enriched *Artemia* feeding for all treatments was initialized on day 17 and finished on day 23 post hatch. After the period of enrichment, the larvae were fed with Coppens diet from day 23 to 36 and then, the sampling was done. Larvae resistance to osmotic and temperature stresses was performed with submersion in fresh water (0.5-1 ppt) and cold water (15°C) for 1h, respectively. Larvae growth factors except for DGR were not significantly different between the groups ($p>0.05$). Fish fed non-enriched *Artemia* had significantly higher DGR (0.63 ± 0.04) ($p<0.05$). Mortality rate was significantly different between control and other groups from day 17 ($p<0.05$). Stress tolerance, cortisol and total protein were not significantly different among the groups. Glucose was only significantly different between C3 and CE2. Results indicate that enrichment of *Artemia fransiscana* with HUFA and vitamins E and C increases survival in yellowfin seabream larvae at its first feeding.

Keywords: *A. latus, Artemia fransiscana,* Enrichment, Growth, Survival, Stress resistance

Introduction

In commercial culture of marine species, rearing of fish larvae is the most critical stage. A high nutritional quality diet which is easily accepted and digested is essential for better growth and development of the larvae. Lipids play an important role in provision of both essential fatty acids (EFA) and energy for larval fish [1]. Start-feeding larvae require a live feed that provides sufficient levels of this energy source because lipids are dramatically reduced during the endogenous feeding stage [2].

The use of *Artemia* nauplii as live feed for crustacean and fish larvae is widespread in marine and freshwater aquaculture. However, live foods that are commonly used for the first feeding of marine larvae, such as *Artemia*, are naturally poor in EFAs [3]. Therefore, enrichment of live food with lipids rich in EFA is necessary to achieve better growth and survival through metamorphosis [3,4]. These enrichments provide levels of phospholipids containing highly unsaturated fatty acids (HUFAs), especially Eicosapentaenoic acid (EPA, 20:5n-3) and Docosahexaenoic acid (DHA, 22:6 n-3) [5]. Studies have shown that EPAs and DHAs are also critical structural and physiological components of the cell membranes of most tissues [6].

Vitamin C (ascorbic acid) is an essential vitamin for normal growth and physiological function of fish. Fish larvae are particularly sensitive to vitamin C deficiency [7]. Larvae rapid growth rate suggests that they have higher vitamin requirements than both juveniles and adults. The addition of vitamin C to larval diets improves survival, growth performance, skeletal development, stress resistance, as well as immune response [8]. Vitamin E (a-tocopherol) acts as a lipid soluble antioxidant and can be supplemented in *Artemia* enrichment [9]. Among natural antioxidants, α-tocopherol is a potent biological antioxidant that can protect biomembranes and lipid components containing unsaturated fatty acids against attack from oxygen free radicals [10]. Vitamin E is an indispensable nutrient required in maintaining flesh quality, immunity, normal resistance of red blood corpuscles to haemolysis, and permeability of capillaries and heart muscle [11].

Yellowfin seabream *A. latus* is widely distributed in shallow coastal waters, lagoons and estuaries of the Indo–West Pacific as well as in the Persian Gulf. Adults spawn in coastal waters and the larvae move to estuaries. *A. latus* is emerging as a candidate aquaculture species in coastal waters of southern Iran. In order to develop this species for aquaculture at a commercial scale, a consistent production of juvenile fish must be achieved. Understanding the nutritional requirements of early larval yellowfin seabream, especially of EFA such as DHA and EPA, and vitamins C and E is important for successful mass production. This study aims at evaluating the role of dietary EFAs, vitamins C and E enriched *Artemia fransiscana* in the first feeding of *A. latus* larvae and their effects on growth, survival and stress resistance of the larvae.

Materials and Methods

Artemia enrichment

Artemia fransiscana cysts were decapsulated using chlorine prior to hatching [12]. Cyst hatching was carried out in a 40-l conical tank

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containing salt water (30 ppt) at 28°C with heavy aeration. Newly hatched nauplii were separated and aerated for 5 h until most of the nauplii molted into the second larval stage (Instar II) and then started filter feeding on particles effectively. At this stage, *A. fransiscana* nauplii were washed with salt water and stocked into the enrichment tanks (2 l) at 150 nauplii per milliliter. Cod liver oil (EPA 6.84% and DHA 5.98%), ascorbyl-6-palmitate (Serva, USA) and a-tocopherol acetate (Sigma, USA) were used as lipid, vitamins C, and E sources. Nine different *Artemia* enrichment treatments were set up as is shown in table 1. Enrichment emulsion was prepared according to the method described by Noshirvani et al. [13]. Vitamins were added as percent of fish oil to the emulsion. The enrichment solution was given (0.5 ml per liter) in two portions at 12-h intervals. After 24 h incubation during enrichment, the nauplii were washed with salt water (28 ppt) to discard non-absorbed lipids and were then kept aerated at 5°C until they were served to fish [13]. Each day, a new batch of enriched *Artemia* nauplii was used. At the end of enrichment period, vitamin E and C content of enriched, non-enriched *Artemia* and fish larvae were analyzed by reverse-phase high-performance liquid chromatography (HPLC, Waters 600) according to Trenzado et al. [14]. Fatty acid composition of both *Artemia* nauplii diets and tested animals (*A. latus*) were analyzed and estimated following the method of Desvilettes et al. [15], using Gas Chromatography. The results are expressed as area percent fatty acid methyl esters (FAME).

Fish and Experimental design

Yellowfin seabream larvae at the stage of first feeding, on day 2 post hatch, with initial mean body weight of 53 ± 0.08 mg were obtained from the Emam Khomaini Marin Fish station, Emam Khomaini harbour, Southern Iran. The larvae were acclimatized with laboratory conditions for two weeks. Then they were randomly distributed into nine groups in experimental tanks (40 l), with three replicates at a density of 330 fish per tank. Feeding rate was adjusted to the water volume in each treatment for 6 times a day. All treatments fed rotifer for the first 5 days of their feeding and then fed non-enriched *Artemia* for the later 11 days. Enriched *Artemia* feeding was initialized on day 17 and finished on day 23 post hatch. Fish larvae in the control group were fed with non-enriched *Artemia*. After the period of enrichment, the larvae were fed with Coppens diet (Netherlands), from the day of 23 to 36 post hatch. Uneaten food and dead larvae were removed daily from the bottom and mortality was also recorded. Water quality was checked daily during the trial. Temperature was 28.83°C, Salinity was 54.67 ppt, pH was 7.91 and dissolved oxygen was 4.84 ppm during the feeding periods. Diurnal light/dark cycle was at 16:8 h.

Fish performance

Five fish larvae from each individual tank were randomly selected

Treatment	Vitamin C Amount (%)	Vitamin E Amount (%)	
HUFA	-	-	+ Cod liver oil
C1	5	-	+ Cod liver oil
C2	10	-	+ Cod liver oil
C3	15	-	+ Cod liver oil
E1	-	5	+ Cod liver oil
E2	-	10	+ Cod liver oil
CE1	2.5	2.5	+ Cod liver oil
CE2	5	5	+ Cod liver oil
control	-	-	-

Table 1: Nine different *Artemia* enrichment treatments for *A. latus* larvae.

Fatty acids	Cod liver oil	Artemia nauplii before enrichment	Artemia nauplii after enrichment
18:1 (n-9)	16.11	20.98	21.39
20:1 (n-9)	7.34	0.13	0.08
16:1 (n-7)	0.3	14.8	13.08
18:1 (n-7)	3.05	11.74	9.38
18:3 (n-6)	2.26	0.61	2.39
20:3 (n-6)	0.45	1.77	0.27
20:4 (n-6)	6.37	0.41	0.04
22:5 (n-6)	0.27	0.12	0.26
14:1 (n-5)	17.28	0.19	0.18
18:3 (n-3)	1.14	4.31	3.03
18:4 (n-3)	0.28	0.06	0.08
20:3 (n-3)	0.77	0.24	4.1
EPA 20:5 (n-3)	6.84	4.54	2.04
22:5 (n-3)	1.08	0.15	1.35
DHA 22:6 (n-3)	5.98	0.18	2.32
14:0	7.19	1.85	3.41
16:0	9.09		19.61
18:0	2.48		4.2
22:0	0.24		1.02
DHA/EPA	0.87		1.14

Table 2: Average fatty acid content of HUFA's source and artemia nauplii before and after enrichment (in mg/day/g of cod liver oil or artemia).

and dried with filter paper and then weighed by 0.001g electronic balance. Growth parameters were calculated as following [16]:

Weight gain (WG) = W_2-W_1; where W_1 and W_2 are the initial and final weight (g)

Specific growth rate (SGR)=ln W_2-W_1/t×100; where W_1 and W_2 are the initial and final weight (g), respectively, and t is the number of days in the feeding period.

Daily growth index (DGI)=(Wt–W0)×100/t; where Wt and W0 were final and initial fish weights, respectively.

Condition factor (CF)=W/L^3×100, where W is final weight (g), L is total length (cm).

Stress tests

At day 36, 30 fish from each rearing tank were removed carefully and directly transferred from salt water to fresh water (0.5-1 ppt). Osmotic shocks lasted for 1 hour. Moreover, the larvae were also suddenly exposed to thermal stress (15°C for 1 hour). To regulate the temperature during the thermal stress, the tanks were equipped with ice and the water temperature was maintained at 15°C. After stress period, fish larvae were sampled from each treatment for biochemical analysis and frozen at -80°C.

Tissue extract and biochemical analysis

To evaluate the osmotic stress level in larvae, concentration of cortisol, total protein and glucose were determined. whole frozen larvae was defrosted, weighted and put into the mortar and added some liquid Nitrogen, then pressed with pestle samples. The samples were homogenated with electronic homogenizer at 4500×g, for 1.5 min and diluted with ratio of 1:9 deionized distilled water. Tissue suspensions were centrifuged at 10000×g for 15 min at 0°C and frozen at -80°C. Cortisol content was determined with Radio immune assay (RIA).

The quantitative determination of glucose was carried out using commercially available diagnostic Experimental Protocol kits, Pars Azmoon, Iran (1 500 0178), at 546 nm and 37°C by the glucose oxidase

Growth parameter	Treatments								
	C1	**C2**	**C3**	**E1**	**E2**	**CE1**	**CE2**	**HUFA**	**Control**
WG [1]	0.20 ± 0.01	0.25 ± 0.07	0.23 ± 0.01	0.22 ± 0.04	0.24 ± 0.04	022 ± 0.06	0.20 ± 0.04	0.22 ± 0.03	0.28 ± 0.06
SGR [2]	6.61 ± 0.76[a]	6.4 ± 0.72[a]	7.16 ± 1.04[a]	6.73 ± 0.7[a]	6.93 ± 0.95[a]	6 ± 0.6[a]	7.16 ± 1.1[a]	6.63 ± 1.18[a]	7.23 ± 0.68[a]
DGI [3]	0.55 ± 0.11	0.76 ± 0.09	0.69 ± 0.15	0.65 ± 0.09	0.72 ± 0.14	0.65 ± 0.14	0.56 ± 0.12	0.65 ± 0.13	0.65 ± 0.38
CF [4]	1.61 ± 0.29	1.75 ± 0.22	1.71 ± 0.19	1.55 ± 0.22	1.7 ± 0.21	1.66 ± 0.21	1.57 ± 0.17	1.6 ± 0.29	1.69 ± 0.16

*Mean ± SD of three replicates. Means in the same row with different superscripts are significantly different (P<0.05). 1-Weight gain 2-Specific growth rate, 3-Daily growth index, 4-Condition factor; C1, C2, C3: larvae fed with HUFA + 5%, 10% and 15%(w/w) vitamin C-enriched artemia nauplii; E1, E2: larvae fed with HUFA + 5% and 10% (w/w) vitamin E-enriched *Artemia* nauplii; CE1, CE2: larvae fed with HUFA + 2.5% and 5% (W/W) vitamin C and E enriched artemia nauplii respectively; HUFA: larvae fed with HUFA without vitamin; Control: larvae fed with non-enriched artemia.

Table 3: Response of seabream larvae to various test diets after 36 days of feeding*.

Parameters	Treatments								
	C1	**C2**	**C3**	**E1**	**E2**	**CE1**	**CE2**	**HUFA**	**Control**
Thermal stress	0.33 ± 0.5[a]	1 ± 1.73[a]	0	0.66 ± 1.15[a]	1 ± 1.73[a]	2.33 ± 2.08[a]	0.66 ± 1.15[a]	0.66 ± 0.57[a]	1 ± 1.73[a]
Salinity stress	0	0	0	0	0	0	0	0	0

Mean ± SD of three replicates. Means in the same row with different superscripts are significantly different (P<0.05). C1, C2, C3: larvae fed with HUFA + 5%, 10% and 15%(w/w) vitamin C-enriched artemia nauplii; E1, E2: larvae fed with HUFA + 5% and 10% (w/w) vitamin E-enriched *Artemia* nauplii; CE1, CE2: larvae fed with HUFA + 2.5% and 5% (W/W) vitamin C and E enriched artemia nauplii respectively; HUFA: larvae fed with HUFA without vitamin; Control: larvae fed with non-enriched artemia.

Table 4: Mortality rate of seabream larvae after thermal and salinity shock.

method according to Trinder [17]. The limit of detection (LOD) of the procedure was 5 mg/dl. Total protein was estimated following the method of Lowry et al. [18].

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using the statistical software SPSS, version 11.0. When ANOVA identified differences among groups, multiple comparisons among means were made with Duncan's new multiple-range tests. Data are presented as treatment mean ± SD. The values of P<0.05 were considered significantly different.

Results

Fatty acid composition of cod liver oil and *Artemia* nauplii before and after enrichment is presented in table 2. Eicosapentaenoic acid (EPA, 20:5n-3) content in non enriched *Artemia* was about 4.54 mg g^{-1} DW and Docosahexaenoic acid (DHA, 22:6 n-3) was 0.18 mg g^{-1} DW. After enrichment with cod liver oil, EPA content decreased to 2.04 mg g-1 DW and DHA content increased to 2.32 mg g^{-1} DW. The decrease of EPA after enrichment could be attributed to long storage of lipids before starting the enrichment process. DHA/EPA ratio increased to 1.14 after the enrichment. This ratio was 0.03 before the enrichment.

The results of determining growth factors are summarized in table 3. Seabream larvae growth factors except for daily growth rate were not significantly different between the groups (p>0.05).

Mortality rate of fish from day 1 to day 17 (before enrichment period) was not significantly different between the treatments (Figure 1). Mortality rate of fish fed with enriched Artemia from day 17 to end of the experiment (after enrichment period) was significantly lower than the control group (p<0.05) (Figure 2).

The supplementation of HUFA, vitamins C and E to the *Artemia* enrichment did not improve significantly larvae tolerance to thermal stress (p>0.05). Fish in the C3 group showed higher resistance to stress (Table 4).

There was no significant difference in cortisol and total protein under stress conditions between the groups (p> 0.05). (Figure 3 and 4). About the glucose, the only significant difference was observed between C3 and CE2 but the other groups were not significantly different (p>0.05) (Figure 5).

Discussion

In this research, effect of dietary enriched *Artemia* with unsaturated fatty acids (cod liver oil), vitamin C and vitamin E on survival, growth and stress resistance of yellowfin seabream was evaluated. The supplementation of dietary HUFAs, α-tocopherol acetate and ascorbyl-6-palmitat caused significant effect on seabream (*A. latus*) larvae survival, when they fed enriched *Artemia* from day 17 to the end of the enrichment period. Many marine fish larvae are believed to require HUFAs, especially EPA and DHA for better survival during larval period. Copeman et al. showed that use of EPA and DHA increased survival of Flounder (*Limanda ferruginea*) larvae [3]. On the other hand, freshwater catfish (*Clarias gariepinus*) fed *Artemia* nauplii enriched with unsaturated fatty acids plus 10% and 20% ascorbyl-6-palmitat, was not significantly different in survival at the end of day 15 [19]. Moreover, unsaturated fatty acids, vitamin C and E enriched *Artemia* nauplii did not have significant effect on walleye (*Stizostedion vitreum*) larvae survival [20].

According to the results of this study, although growth index of SGR was higher in control group than the others but it was not significantly different (p>0.05). Daily growth ratio was increased significantly in control group (p<0.05).

The nutritional value of high levels of HUFA and vitamins C and E have been confirmed by enhanced growth in many marine fish larvae such as turbot (*Scophthalmus maximus*) [21], Red seabream (*Pagrus major*) [22], gilthead seabream (*Sparus aurata*) [23], yellowtail flounder (*Limanda ferruginea*) [3].

In agreement with this research results, Rainuzzo et al. in turbot and Dickey-Collas and Geffen in plaice (*Pleuronectes platessa*) did not find a relationship between (n-3) HUFA levels in live food fed to fish larvae and their growth [24,25]. Kolkovski et al. found no significant difference of growth between walley larvae fed newly hatched *Artemia* nauplii and larvae fed nauplii enriched with cod liver oil and fatty acids emulsion with high ratio of EPA/DHA plus vitamin E [20]. Oral administration of unsaturated fatty acids and vitamin E had no significant effect on growth of rainbow trout [26,27]. Merchie et al. showed that the larvae

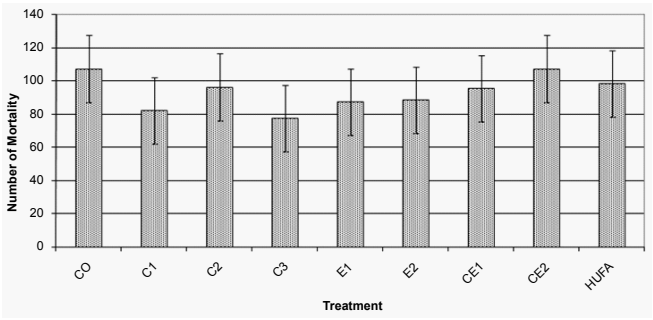

Figure 1: Mortality of seabream larvae in different treatments from day 1 to day 17 (p>0.05). (Values are mean ± S.D., mean values bearing same superscript are not statistically significant, P>0.05).

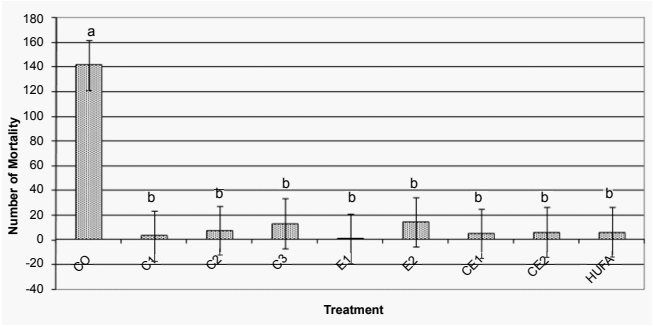

Figure 2: Mortality of seabream larvae in different treatments from day 17 to end of the experiment. (Values are mean ± S.D., mean values bearing same superscript are not statistically significant, P>0.05).

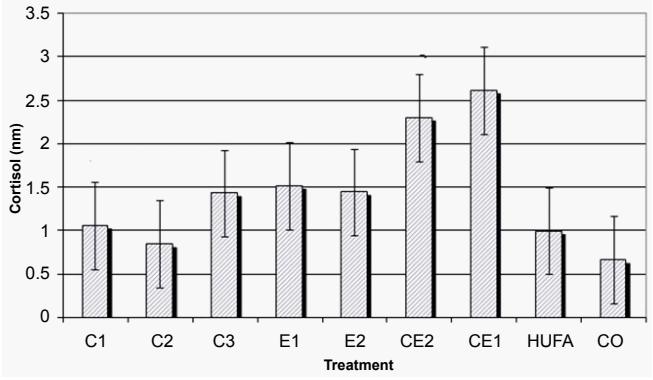

Figure 3: Cortisol observed in seabream larvae after salinity shock.

of freshwater prawn fed *Artemia* nauplii enriched with unsaturated fatty acids and 10 and 20 percent vitamin C were not significantly different in growth and survival after 25 days Also showed that European bass larvae (*Dicentrachus labrax*) fed with enriched unsaturated fatty acids *Artemia* nauplii and 0, 10 and 20 percent vitamin C were not significantly different on physiological condition after 20 and 35 days [28]. Moreover, Merchie et al. concluded that turbot larvae fed enriched nauplii with 5 and 10 percent vitamin C were not significantly different in growth [8]. 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.

About the effect of vitamins on larval growth, Sau et al. and Paul et al. demonstrated that dietary vitamin E increased growth of rohu (*Labeo rohita*) and marigal (*Cirrhinus mrigala*) [29,30]. Also, ascorbic acid supplementation diet resulted significant positive effect on growth of African catfish (*Clarias gareiepinus*) and larvae fed diet with 20 percent AP, had 30% higher weight than the control group. On the contrary, supplementation of dietary a-tocopherol in the *Artemia* enrichment did not have a significant effect on walleye (*Stizostedion vitreum*) larvae growth which is in consistent with the results of this study [20].

In this research, seabream larvae fed the *Artemia* nauplii enriched with 5, 10 and 15 Percent of Ascorbyl palmitat, 5 and 10% vitamin E and mixed of 2.5 and 5% of vitamins C and E showed no significant difference against salinity and temperature stress. Unlike the results of this study, resistance of African catfish (*Clarias gareiepinus*) larvae fed *Artemia* enriched with 20% of the AP was higher than the larvae fed *Artemia* enriched with 0 and 10% AP, after exposure to 25 ppt salinity stress for 1 hour [28]. Also, 25 day hatched milkfish larvae fed Rotifer and *Artemia* enriched with HUFAs and vitamin C had lower mortality against salinity stress, compared with the control group [31].

In some fishes, cortisol is the principal corticosteroid [32]. Elevated cortisol levels cause gluconeogenesis and glucogenolysis in the liver [33]. The resulting hyperglycaemia helps to satisfy the increased energy demand during stress, allowing the organism to react to stressors [34]. According to the results of this study, however, there was no significant difference between the groups in cortisol level of yellowfin seabream under stress conditions (p> 0.05).

Total protein is measurable humoral component of the non-specific

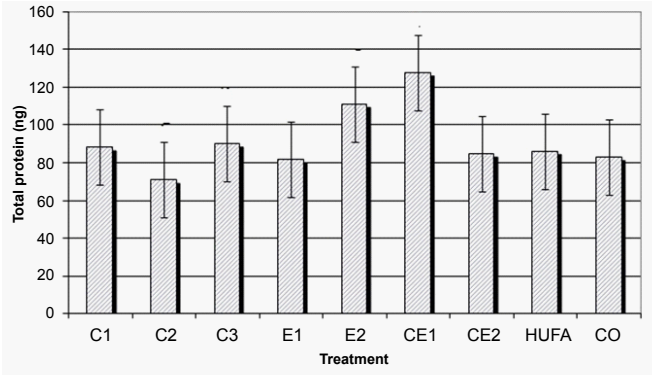

Figure 4: Total protein observed in seabream larvae after salinity shock.

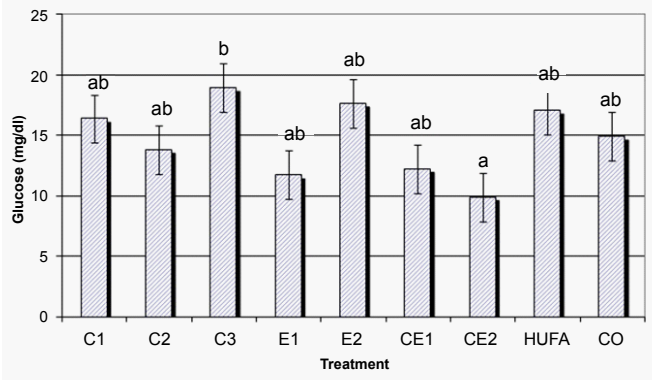

Figure 5: Glucose observed in seabream larvae after salinity shock (Values are mean ± S.D., mean values bearing same superscript are not statistically significant, P>0.05).

defense mechanism [35]. The total protein was higher in the fish fed enriched *Artemia* nauplii with 2.5% vitamin C and E (CE1 group) than the other treatments after salinity stress but it was not significant ($p>0.05$). Very low levels of serum total protein have significance in relation to infectious disease, kidney damage, nutritional imbalance and stressful condition in fish [36].

Conclusion

The results of the present study indicate that use of enriched *Artemia fransiscana* nauplii with HUFAs can increase survival of yellowfin seabream (*A. latus*) in larval stage. Also, the results show that feeding yellowfin seabream with HUFA+15% vitamin C enriched *Artemia fransiscana* nauplii before some stresses can help to prevent negative effects of stress.

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