

## **Effects of feeding *Artemia franciscana* enriched with HUFA, vitamins C and E on growth performance, survival and stress resistance of cuttlefish (*Sepia pharaonis*) (Ehrenberg, 1831)**

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Received: July 2016

Accepted: June 2017

### **Abstract**

This study aimed to evaluate the effects of using *Artemia franciscana* enriched with cod liver oil and vitamins C and E on the growth, survival and stress resistance of juvenile *Sepia pharaonis* (Pharaoh cuttlefish). Twenty-five days after hatching, the larvae of *S. pharaonis* were transferred into culture tanks with an average length of  $15.97 \pm 0.15$  mm and average body weight of  $3.53 \pm 0.03$  g, in eleven treatments (with 3 replicates). The treatments included: highly unsaturated fatty acids (HUFA) +5, 10 and 15% vitamin E-enriched *Artemia* (E1, E2 and E3 groups, respectively), HUFA+5, 10 and 15% vitamin C-enriched *Artemia* (C1, C2 and C3 groups, respectively), HUFA+2.5% (w/w) vitamins C- and E-enriched *Artemia* (CE1 group), HUFA+5% (w/w) vitamins C- and E-enriched *Artemia* (CE2 group), HUFA+7.5% (w/w) vitamins C- and E-enriched *Artemia* (CE3 group), HUFA without vitamins (HUFA group) and non-enriched *Artemia* (control). For each replicate, 20 larvae were introduced into tanks of 20 L, and 10 *Artemia* L<sup>-1</sup> day<sup>-1</sup> were used to feed the larvae. At the end of the experiment, to investigate stress resistance, three groups of larvae were submitted to one hour stress at three levels of salinity (5, 15 and 25 ppt), and temperature (10, 25 and 32 °C). The results showed that cuttlefish fed with CE1, CE2, CE3 and larvae fed with HUFA without vitamins were significantly different ( $p < 0.05$ ) with regards to growth performance and survival as compared with the control. Feeding with vitamin C and unsaturated fatty acid elevated the survival and growth factors of *S. pharaonis* larvae. Also, the survival of larvae against induced stress increased ( $p < 0.05$ ).

**Keywords:** *Artemia franciscana*, Growth performance, Stress resistance, *Sepia pharaonis*, Survival, Vitamins C and E.

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## Introduction

The Pharaoh cuttlefish, *Sepia pharaonis*, is a broadly distributed species found in waters from eastern Africa to Japan (Anderson *et al.*, 2007). *S. pharaonis* is a dominant cephalopod species and primary fishing activity occurs during the spawning season, when adults migrate from deep to shallow waters in the littoral zone (Ghazvineh *et al.*, 2012).

Juvenile cuttlefish are predatory; their diets consist mainly of live prey. Many attempts have been made to rear juvenile cuttlefish with alternative diets, but the young animals in these trials have tended to be fragile and have low growth rates (Choe, 1966). Therefore, this study reviewed feeding the cuttlefish with enriched *Artemia franciscana*. One of the most important nutritional factors for marine fish larvae is the dietary content of highly unsaturated fatty acids (HUFA) with 20 or more carbon atoms (Watanabe, 1982). Therefore, HUFA must be included in live prey and weaning diets to satisfy the requirements for growth, organ and tissue development and functioning, stress resistance and survival (Izquierdo *et al.*, 2001). The n-3 HUFA requirements have been extensively studied in fish larvae such as *Sepia officinalis* (Koueta *et al.*, 2002), *Fenneropenaes indicus* (Citarasu *et al.*, 1998) and *Argyrosamus regium* (El Kertaouie *et al.*, 2017). However, these fatty acids, particularly DHA, are very prone to oxidation and are more exposed in formulated diets for marine fish larvae (Izquierdo *et al.*, 2013). Moreover, at a physiological level,

oxidative risk is particularly high in the fast-growing larvae, due to the high metabolic rate, oxygen consumption and water content in the larval tissues (Betancor *et al.*, 2012). Thus, dietary inclusion of adequate levels of antioxidant nutrients is required to avoid *in vivo* lipid peroxidation and the determination of excessive HUFA requirements.

Vitamin E is recognized as the major hydrophobic chain-breaking antioxidant that prevents the propagation of free radical reactions in membranes and lipoproteins (Izquierdo and Betancor, 2015). The specific location of vitamin E as a structural component of cell membranes confers on this vitamin a particular role in the control of peroxidation of HUFA (Izquierdo and Betancor, 2015). Early nutritional studies have shown that vitamin E is essential for marine fish larvae (Gonzalez *et al.*, 1995). Moreover, dietary vitamin E must be increased when dietary HUFA are high as found in gilthead seabream (Izquierdo *et al.*, 2013) or European seabass (*Dicentrarchus labrax* L.) (Betancor *et al.*, 2011). Thus, elevation of dietary PUFA causes a reduction in vitamin E content in the liver of Atlantic salmon (Waagbø *et al.*, 1993) or African catfish (*Clarias gariepinus*) (Lim *et al.*, 2001). Nevertheless, vitamin E requirements depend on the interactions of this vitamin with other nutrients (Hamre, 2011). The high vitamin E requirement of fish larvae is associated with the high HUFA needs during the larval stages (Atalah *et al.*, 2012). For instance, increase in dietary vitamin E

supplementation in high-DHA feed protected this fatty acid from oxidation and reduced the occurrence of chondroid bone anomalies (Izquierdo *et al.*, 2013). Symptoms of vitamin E deficiency in fish larvae include accumulation of lipid oxidation products, muscle dystrophy and reduced growth and survival (Izquierdo and Betancor, 2015).

Unless it is regenerated, after neutralizing free radicals, vitamin E must be replenished through the diet or from reserves elsewhere (Burton and Traber, 1990). Thus, the vitamin E radicals produced can probably be regenerated to vitamin E by vitamin C in the interface between water and lipids (Packer *et al.*, 1979). Ascorbic acid seems to play a significant role in  $\alpha$ -tocopherol metabolism, reducing  $\alpha$ -tocopheroxyl radicals and regenerating them to  $\alpha$ -tocopherol (Niki *et al.*, 1985). Consequently, optimum dietary vitamin E levels may also be determined by the levels of vitamin C (Sealey and Gatlin, 2002). For instance, elevation of dietary vitamin C from 1800 to 3600 mg kg<sup>-1</sup> during weaning of European seabass increases tissue content of  $\alpha$ -tocopherol and reduces the occurrence of muscular dystrophy and tissue TBARs, denoting its sparing effect over dietary vitamin E (Betancor *et al.*, 2012).

At present, there is no information on the requirements of HUFA, vitamin E or C for cuttlefish juveniles (*S. pharaonis*), and therefore, this study was conducted to determine the importance of these nutrients with enriched *Artemia* on the growth,

survival and stress resistance of cuttlefish.

### Materials and methods

*A. franciscana* cysts were decapsulated using Treece (2000) for hatching. Newly hatched nauplii were separated and aerated for 5 h until most of the nauplii molted into the second larval stage (Instar II) and were then transferred to the tanks with 300 L volume at a density of 7-8 nauplii L<sup>-1</sup>.

*Artemia* were not fed during the first 24 h in order to allow yolk resorption. The nauplii were fed with a rice bran suspension (D'Agostino, 1980; Intriago and Jones, 1993) from day 2 to 4 (3 days) of the trial. This suspension was prepared with 3 g of rice bran micronized with a screen (100  $\mu$ m) and suspended in 1 L of seawater. Following this, the diet formula was homogenized using a kitchen blender and filtered (30  $\mu$ m) before being cold stored (Dobbeleir *et al.*, 1980). From day 5, the organisms were fed with the microalgae *Tetraselmis suecica* at 200,000 cells ml<sup>-1</sup> (Ahmadi *et al.*, 1990; De Roeck-Holtzhauer *et al.*, 1993; Odile *et al.*, 1994) until the end of the trial on day 15 (11 days). The microalgae were cultivated in Guillard f2 medium (Guillard, 1975). Rice bran particles and algal cells were counted using a hematocytometer.

At this stage, *A. franciscana* 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  $\alpha$ -tocopherol acetate (Sigma, USA)

were used as lipid, vitamins C and E sources. Eleven different *Artemia*-enrichment treatments were set up as shown in Table 1.

The enrichment emulsion was prepared according to the method described by Larger *et al.* (1987). Vitamins were added as percentage of fish oil to the emulsion. The enrichment solution was given (2 ml per liter) in two portions at 12-h intervals. After 24 h incubation during the enrichment, the *Artemia* were washed with salt water (28 ppt) to discard non-absorbed lipids and were then kept aerated at 4°C until they were served to fish (Noshirvani *et al.*, 2006). Each day, a new batch of enriched *Artemia* was used. At the end of the enrichment period, the vitamin E and C content of enriched, non-enriched *Artemia* and cuttlefish larvae were analyzed by reverse phase high-performance liquid chromatography (HPLC, Waters 600) according to Trenzado *et al.* (2007). The fatty acid composition of both *Artemia* diets and tested animals (*S. pharaonis*) were analyzed and estimated following the method of Desvilettes *et al.* (1994) using gas chromatography. The results were expressed as area percent of fatty acid methyl esters (FAME).

Pharaoh cuttlefish egg clusters, attached to cuttle traps and rocks, were collected from the Persian Gulf near the shore of Bandar-e-Lengeh city in the south of Iran. The eggs were immediately transferred to plastic containers filled with sea water (salinity 35%, temperature 28°C) and transported to the laboratory of the Persian Gulf Mollusk Research Station

in Bandar-e-Lengeh. The egg masses were placed in an incubation tank containing 1000 L filtered sea water and were acclimated gradually to the temperature and salinity of the water. Aeration was provided through airstones from an air blower. The water temperature was kept at 27.5±0.5 °C, salinity was 37-38 ppt, a gentle and constant aeration was done, and a diurnal light/dark cycle was maintained at 12:12 h. After hatching, on the 25<sup>th</sup> day, they were randomly distributed in eleven groups into experimental tanks (20 L) with three replicates at a density of 1 cuttlefish juvenile per liter (Nabhitabhata *et al.*, 2005), with an initial mean body weight of 3.53±0.03 mg. Again, water temperature was kept at 27.5±0.5°C, salinity was 37-38 ppt, pH was 7.6, and a gentle and constant aeration was provided during the feeding periods. Ten (10) *Artemia* L<sup>-1</sup> in a day were transferred to tanks to feed the juveniles (Anil *et al.*, 2005) during the experimental period, for 15 days.

Growth performance parameters were calculated according to the following formulae:

Weight gain= $W_2 - W_1$ ;

Percent weight gain= $[(W_2 - W_1) / W_1] \times 100$ ;

Length gain= $L_2 - L_1$ ; and

%SGR= $((\ln W_2 - \ln W_1) / (T_2 - T_1)) \times 100$ ;

where  $W_2$  and  $W_1$  represent the final and initial weight, respectively,  $L_2$  and  $L_1$  represent the final and initial length, respectively, and  $T_2$  and  $T_1$  represents the final and initial time (i.e., duration) of the experimental period.

CF was calculated as:

$$CF=(W/L^3)\times 100;$$

where W is weight and L is length.

In addition, survival rate was calculated at the end of the experiment as:

$$\text{Survival}=(Nf/N0)\times 100;$$

where N0=initial number of fish, and Nf =final number of fish.

**Table 1: Eleven different *Artemia* enrichment treatments for *Sepia pharaonis* juvenile.**

Treatment	(C)Vitamin	(E)Vitamin	
E1	-	5	+
E2	-	10	+
E3	-	15	+
C1	5	-	+
C2	10	-	+
C3	15	-	+
CE1	2.5	2.5	+
CE2	5	5	+
CE3	7.5	7.5	+
HUFA	-	-	+
control	-	-	-

#### Stress tests

At the end of the experiment, 3 cuttlefish from each rearing tank were removed carefully and directly transferred from salt water to water at salinities of 5, 15 and 25 ppt, individually. Osmotic shocks lasted for 1h. Moreover, the larvae were also suddenly exposed to thermal stress (10, 25 and 35°C for 1 h). After exposure to stress, mortality was recorded.

#### Statistical analysis

**Table 2: Average fatty acid content of the HUFA source *Artemia* before and after enrichment, control and HUFA treatments (in mg day<sup>-1</sup> g<sup>-1</sup> of cod liver oil, *Artemia* or cuttlefish juvenile).**

Fatty acids	Cod liver oil	<i>Artemia</i> before enrichment	<i>Artemia</i> after enrichment	Control treatment	HUFA treatment
18:1 (n-9)	16.11	17.28	18.651	19.796	18.816
20:1 (n-9)	7.34	4.34	4.999	1.078	1.372
16:1 (n-7)	0.3	0.168	0.421	0.3038	0.4312

Data were analyzed using SPSS statistical software (Version 16). Data were checked for normality (Kolmogorov-Smirnov test) and homogeneity of variances (Bartlett's test) prior to their comparison. All the data were expressed as mean±SD ( $n=3$ ), by means of one-way ANOVA, and the mean comparison was performed using Duncan's test at a reliability level of 5%.

#### Results

The fatty acid composition of cod liver oil and *Artemia* before and after enrichment is presented in Table 2. The eicosapentaenoic acid (EPA, 20:5n-3) content in non-enriched *Artemia* was about 2.42 mg g<sup>-1</sup> DW and Docosahexaenoic acid (DHA, 22:6 n-3) was 0 mg g<sup>-1</sup> DW. After enrichment with cod liver oil, the EPA content increased to 7.88 mg g<sup>-1</sup> DW and the DHA content increased to 0.991 mg g<sup>-1</sup> DW. The DHA/EPA ratio increased to 0.125 after the enrichment. This ratio was zero (0) before the enrichment.

Also, the fatty acid composition of the control treatment (cuttlefish juvenile fed with non-enriched *Artemia*) and HUFA treatment (cuttlefish juvenile fed *Artemia* enriched with HUFA without vitamin) is shown in Table 2.

Table 2 continued:

<b>20:4 (n-6)</b>	6.37	0.012	0.423	0.9898	0.98
<b>18:3 (n-3)</b>	1.14	2.98	2.861	0.0882	0.1176
<b>18:4 (n-3)</b>	0.28	0.0456	0.0189	0	0.0896
<b>20:3 (n-3)</b>	0.77	0	0	0.1862	0.196
<b>EPA 20:5 (n-3)</b>	6.84	2.42	7.88	13.034	14.21
<b>22:5 (n-3)</b>	1.08	0.0856	0.009	0	0.098
<b>DHA 22:6 (n-3)</b>	5.98	0	0.991	2.0678	3.43
<b>14:0</b>	7.19	5.31	5.0123	3.038	3.52
<b>16:0</b>	9.09	15.65	13.89	21.658	19.796
<b>18:0</b>	2.48	4.24	4.321	6.762	5.782
<b>DHA/EPA</b>	0.87	0	0.125	0.1586	0.2413

Eicosapentaenoic acid (EPA, 20:5n-3) content in the control was about 13.034 mg g<sup>-1</sup> DW and Docosahexaenoic acid (DHA, 22:6 n-3) was 2.067 mg g<sup>-1</sup> DW. After the cuttlefish juvenile was fed *Artemia* enriched with HUFA, the EPA content increased to 14.21 mg g<sup>-1</sup> DW

and DHA content increased to 3.43mg.g<sup>-1</sup> DW. The DHA/EPA ratio increased to 0.2413 in HUFA treatment. This ratio was 0.1586 in the control.

The results of determining growth factors are summarized in Table 3.

Table 3: Response of cuttlefish juvenile to various test diets at the end of the experiment.

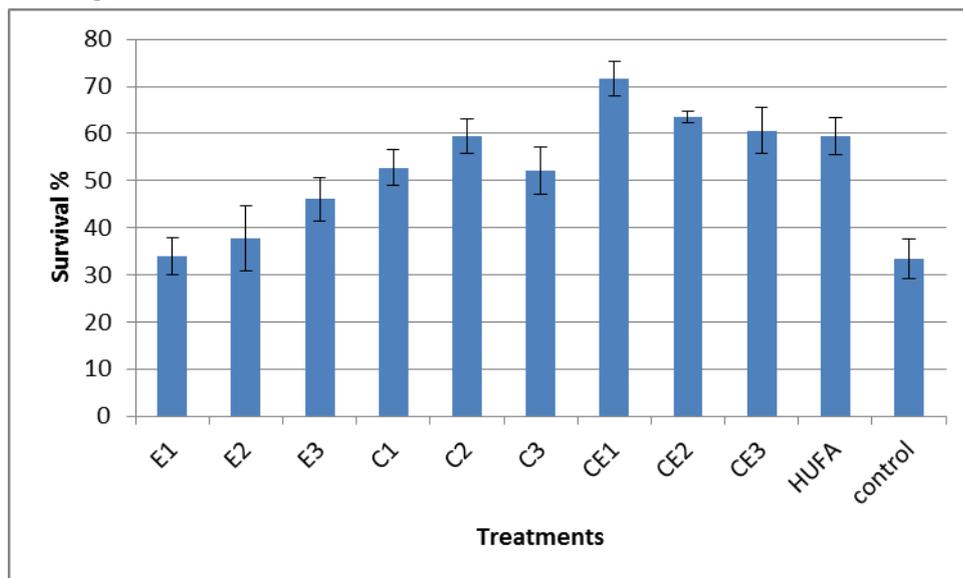
Treatment	WG (g)	WG%	Length gain (mm)	SGR	CF
E1	0.21 ± 0.03 <sup>ab</sup>	6.16 ± 1.03 <sup>ab</sup>	1.5 ± 0.13 <sup>ab</sup>	0.77 ± 0.01 <sup>a</sup>	0.082 ± 0.001 <sup>b</sup>
E2	0.25 ± 0.03 <sup>ab</sup>	7.14 ± 1.09 <sup>ab</sup>	1.35 ± 0.13 <sup>a</sup>	0.98 ± 0.02 <sup>a</sup>	0.085 ± 0.002 <sup>bc</sup>
E3	0.23 ± 0.02 <sup>ab</sup>	6.67 ± 0.64 <sup>ab</sup>	1.06 ± 0.16 <sup>a</sup>	0.66 ± 0.02 <sup>a</sup>	0.089 ± 0.002 <sup>c</sup>
C1	0.42 ± 0.02 <sup>c</sup>	11.92 ± 0.71 <sup>c</sup>	2.57 ± 0.14 <sup>c</sup>	1.24 ± 0.02 <sup>a</sup>	0.071 ± 0.001 <sup>a</sup>
C2	0.36 ± 0.04 <sup>bc</sup>	10.35 ± 1.24 <sup>bc</sup>	2.25 ± 0.12 <sup>c</sup>	0.89 ± 0.02 <sup>a</sup>	0.074 ± 0.001 <sup>a</sup>
C3	0.31 ± 0.05 <sup>abc</sup>	9.03 ± 1.62 <sup>abc</sup>	2.13 ± 0.18 <sup>bc</sup>	1.07 ± 0.01 <sup>a</sup>	0.075 ± 0.001 <sup>a</sup>
CE1	0.44 ± 0.01 <sup>c</sup>	12.68 ± 0.48 <sup>c</sup>	2.75 ± 0.14 <sup>c</sup>	1.19 ± 0.02 <sup>a</sup>	0.07 ± 0.001 <sup>a</sup>
CE2	0.42 ± 0.02 <sup>c</sup>	12.02 ± 0.71 <sup>c</sup>	2.66 ± 0.11 <sup>c</sup>	1.05 ± 0.02 <sup>a</sup>	0.07 <sup>a</sup>
CE3	0.43 ± 0.04 <sup>c</sup>	12.18 ± 1.14 <sup>c</sup>	2.68 ± 0.11 <sup>c</sup>	1.18 ± 0.02 <sup>a</sup>	0.07 ± 0.001 <sup>a</sup>
HUFA	0.44 ± 0.06 <sup>c</sup>	12.49 ± 0.77 <sup>c</sup>	2.75 ± 0.27 <sup>c</sup>	1.14 ± 0.01 <sup>a</sup>	0.07 ± 0.002 <sup>bc</sup>
Control	0.18 ± 0.02 <sup>a</sup>	5.35 ± 1.73 <sup>a</sup>	1.32 ± 0.17 <sup>a</sup>	0.76 ± 0.01 <sup>a</sup>	0.084 ± 0.002 <sup>a</sup>

\*Mean ± SD of three replicates. Means in the same column with different superscripts are significantly different ( $p < 0.05$ ). 1- Weight gain, 2- Percent of weight gain, 3- length gain, 4- Specific growth rate, 5- Condition factor; E1, E2, E3: Larvae fed with HUFA + 5, 10 and 15% (w/w) vitamin E-enriched *Artemia*; C1, C2, C3: Larvae fed with HUFA + 5, 10 and 15% (w/w) vitamin C-enriched *Artemia*; CE1, CE2, CE3: Larvae fed with HUFA + 2.5, 5 and 7.5% (w/w) vitamins C and E enriched *Artemia*, respectively; HUFA: Larvae fed with HUFA without vitamin; Control: Larvae fed with non-enriched *Artemia*.

When compared with the control treatment, cuttlefish fed with HUFA + 2.5, 5 and 7.5 % (w/w) vitamin C- and E-enriched *Artemia*, respectively (CE1, CE2, CE3) and larvae fed with HUFA and without vitamin (HUFA) had significant differences with regard to growth performance. Cuttlefish fed with HUFA + 2.5% (w/w) vitamin C- and E-enriched *Artemia* (CE1) showed better growth performance ( $p < 0.05$ ), but no significant difference was observed between the other treatments, CE1 and CE2 that were fed *Artemia* enriched with a mixture of cod liver oil and vitamins, and HUFA treatment ( $p > 0.05$ ). Although the growth index of SGR was higher in C1 treatment than

the control and other groups, it was not significantly different ( $p > 0.05$ ).

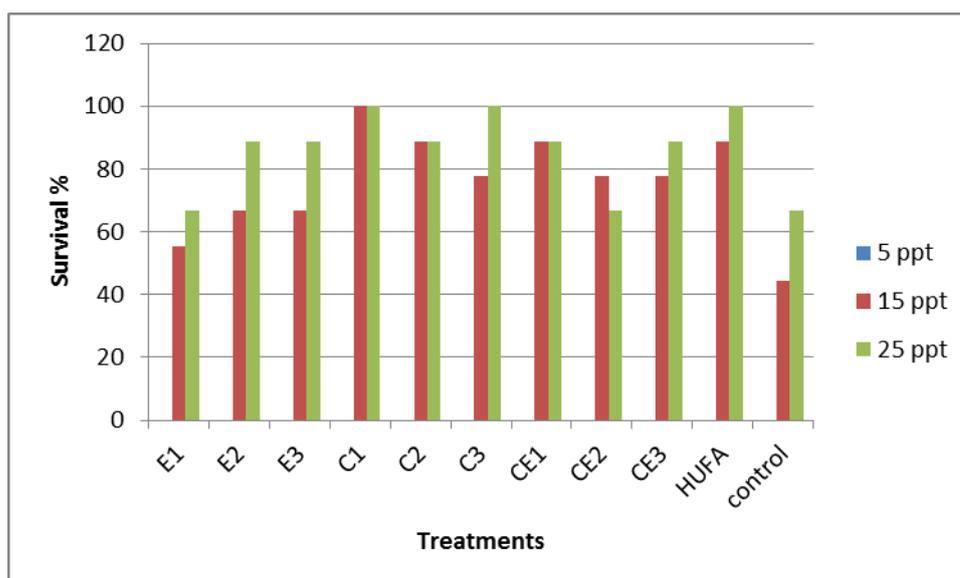
Enriched *Artemia* with cod liver oil and mixture of vitamins C and E increased the survival of cuttlefish that fed on them, and CE1, CE2, CE3 showed better results than the other treatment but these treatments were not significantly different from each other ( $p > 0.05$ ). Cuttlefish in treatment CE1 with 71.7%, showed the highest survival in this experiment which was statistically different ( $p < 0.05$ ) with the control group and treatments E1, E2, and E3. The control group and E1 treatment showed the lowest survival, at 33.9% (Fig. 1).



**Figure 1: Survival percentage of juvenile cuttlefish in different treatments at the end of experiment (data represent the mean ± SD. Bars assigned with different superscripts are significantly different ( $p > 0.05$ )).**

The survival percentage of cuttlefish juveniles in different treatments after salinity and thermal shock at the end of

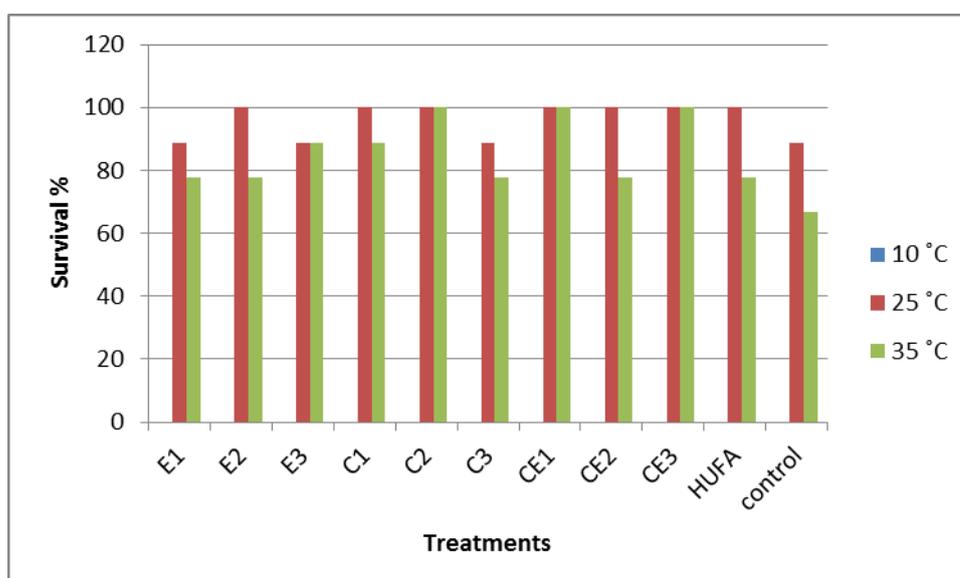
experiment are shown in Figs. 2 and 3, respectively.



**Figure 2:** Survival percentage of juvenile cuttlefish in different treatments after salinity shock at the end of experiment (data represent the mean $\pm$ SD. Bars with different superscripts are significantly different ( $p>0.05$ )).

To investigate the effect of vitamins E and C and cod liver oil on survival, at the end of the experiment, thermal shock at 10, 25 and 35°C, and salinity 5, 15 and 25 ppt were used. Cuttlefish juvenile fed the *Artemia*-enriched HUFA with 5, 10 and 15% vitamin E,

5, 10 and 15% of ascorbyl palmitate and a mixture of 2.5, 5 and 7.5% of vitamins C and E showed no significant difference with salinity and temperature stress. All cuttlefish died at 5 ppt and 10°C treatment.



**Figure 3:** Survival percentage of juvenile cuttlefish in different treatments exposed to thermal shock at the end of the experiment (data represent the mean $\pm$ SD. Bars with different superscripts are significantly different ( $p>0.05$ )).

## Discussion

In this research, the effect of dietary enriched *Artemia* with unsaturated fatty acids (cod liver oil), vitamins C and E on survival, growth and stress resistance of pharaoh cuttlefish (*S. pharaonis*) was evaluated. In general, the growth of animals varies with several factors: food availability, environmental factors, the social hierarchy of the population, nutritional qualities of the food, etc. In this study, it was suggested that, the supplementation of dietary HUFAs,  $\alpha$ -tocopherol acetate and ascorbyl-6-palmitate had significant effect on the growth and survival of pharaoh cuttlefish larvae, when they were fed enriched *Artemia* during the experimental period.

Many marine fish larvae are believed to require HUFAs, especially EPA and DHA for better survival during larval period. Growth enhancement as a result of unsaturated fatty acids and vitamin E and C administration has been reported in several previous studies on a variety of fish and aquatic species fed these diets. Koueta *et al.* (2002) showed that the use of EPA and DHA increased the growth and survival of juvenile *S. officinalis*. These data indicated the importance of *n*-3 HUFA such as docosahexaenoic acid (DHA: 22:6*n*-3) and eicosapentaenoic acid (EPA: 20:5*n*-3) in cephalopod juvenile nutrition.

A research study was conducted on the importance of highly unsaturated fatty acids and the antioxidant vitamins E and C on meagre larvae (*Argyrosomus regius*). Increase in dietary HUFA up to 3% significantly

improved larval growth, lipid absorption and deposition. In addition, among fish fed 3% HUFA, increase in vitamins E and C significantly improved body weight, as well as total lipid, 22:6*n*-3 and *n*-3 fatty acid contents in the larvae (El Kertaoui *et al.*, 2015). On the other hand, seabream larvae fed the *Artemia* nauplii enriched with 5, 10 and 15% ascorbyl palmitate, 5 and 10% vitamin E and a mixture of 2.5 and 5% vitamins C and E, showed no significant difference in growth among the groups but increased the survival rate in yellowfin seabream larvae at its first feeding (Adloo *et al.*, 2012). Also, in freshwater catfish (*Clarias gariepinus*), a diet containing unsaturated fatty acids plus 10% and 20% ascorbyl-6 palmitate, showed no significant difference on catfish survival at the end of day 15 (Fermin and Bolivar, 1991).

According to the results of the current study, better growth performance was observed in larvae fed with HUFA+2.5% (w/w) vitamin C- and E-enriched artemia, respectively (CE1), and larvae fed with HUFA without vitamin (HUFA) showed significant difference when compared with the control group. As previously mentioned, in marine fish larvae, HUFA are an important source of metabolic energy, structural components in the phospholipids of cellular membranes and precursors of bioactive molecules, being required for larval growth and development (Izquierdo and Koven, 2011). The results of the present study revealed that the enriched *Artemia* served as suitable

food for pharaoh cuttlefish, although, the growth index of SGR was higher in C1 treatment than the control, and in other groups, it was not significantly different ( $p>0.05$ ).

Also, the positive results observed in cuttlefish juveniles fed vitamins E and C and HUFA, agree well with previous studies that demonstrated that these vitamins protected HUFAs from oxidation, increased their incorporation into larval tissues and promoted larval growth (Hamre, 2011; Atalah *et al.*, 2012; Betancor *et al.*, 2012; Izquierdo *et al.*, 2013). Since vitamin E is the major hydrophobic antioxidant, the increase in dietary HUFA would accelerate the autocatalytic peroxidation of vitamin E, thereby increasing the requirement for this vitamin (Watanabe, 1982; Sargent *et al.*, 1997; Izquierdo *et al.*, 2001). For instance, elevation of dietary HUFA levels in diets for seabream could increase dietary vitamin E and promote incorporation of HUFA in fish membranes and promote larval growth (Atalah *et al.*, 2012). Moreover, vitamin C not only protects tissues from oxidative stress by neutralizing the reactive oxygen species (ROS), but also plays an important role by indirectly protecting HUFA from oxidation, since it is essential to regenerate  $\alpha$ -tocopheroxyl radicals to  $\alpha$ -tocopherol. Despite the fact that information on the synergistic effects of vitamins E and C on marine larvae is very scarce, an antioxidant synergism was demonstrated in seabass larvae fed high 5% DHA (Betancor *et al.*, 2012) as well as in juveniles of several species such

as rainbow trout (*Oncorhynchus mykiss*) (Frischknecht *et al.*, 1994), Atlantic salmon (Hamre *et al.*, 1997), yellow perch (*Perca flavescens*) (Lee and Dabrowski, 2003), golden shiner (*Notemigonus crysoleucas*) (Chen *et al.*, 2004), channel catfish (*Ictalurus punctatus*) (Yildirim-Aksoy *et al.*, 2008), hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (Sealey and Gatlin, 2002) and red seabream (Gao *et al.*, 2013).

However, in the present study, there were significant differences in growth and survival between the experimental treatment and control, except E1, E2 and E3 treatments. Similar results were found with turbot (Stéphan *et al.*, 1995) and channel catfish (Bai and Gatlin, 1993) fed diets containing different levels of dietary  $\alpha$ -tocopherol. Stéphan *et al.* (1995) reported that *in-vivo* and *in vitro* oxidation of lipids in turbot larvae muscles was reduced when dietary  $\alpha$ -tocopherol was supplemented in the diet. Supplementation of dietary  $\alpha$ -tocopherol in the *Artemia* enrichment did not have a significant effect on walleye (*Stizostedion vitreum*) larvae growth (Kolkovski *et al.*, 1996), which is consistent with the results of this study. This can be related to the lower dietary HUFA requirements of walleye in the early life stages, as in other freshwater fish (Sargent *et al.*, 1997).

Handling the average fatty acid content of the HUFA source *Artemia* before and after enrichment, control and HUFA treatments (in  $\text{mg day}^{-1} \text{g}^{-1}$  of cod liver oil, *Artemia* or cuttlefish juvenile) showed that these enrichments provide high levels of phospholipids

containing highly unsaturated fatty acids, especially eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) (Léger *et al.*, 1985; Koven *et al.*, 1993). In addition, an increase was seen in the amount of EPA and DHA in the *Artemia* after enrichment, and in cuttlefish with HUFA treatment as compared to the control.

Stress is known to affect many aspects of fish physiology, from immune competence (Rotllant and Trot., 1997) to metabolism (Montero *et al.*, 1999) and growth rate (Procarione *et al.*, 1999). Marine fish larvae are probably subjected to high levels of oxidative stress. Live feed production and enrichment is achieved under highly pro-oxidative conditions, with high levels of n-3 polyunsaturated acids, air or oxygen addition to the culture water, high temperature and bright light. Formulated diets for marine fish larvae also contain high levels of polyunsaturated fatty acids and pro-oxidants, for example, in the form of minerals. The high surface-to-volume ratio of the feed particles also favors oxidation. It is therefore important to supplement marine fish larval diets with vitamin E, but vitamin E at high levels, in the absence of a sufficient amount of vitamin C, has been shown to increase mortality and tissue lipid oxidation in Atlantic salmon and Atlantic halibut juveniles (Hamre *et al.*, 1997). Though, in this research, cuttlefish juveniles fed the *Artemia* enriched with 5, 10 and 15% ascorbyl palmitate, 5, 10 and 15% vitamin E and mixture of 2.5, 5 and 7.5% of vitamins

C and E showed no significant difference with regards to salinity and temperature stress, a significant difference was observed between treatment C1 and the control in 15 ppt salinity. According to the results, seabream larvae fed *Artemia* nauplii enriched with ascorbyl palmitate, and vitamin E showed no significant difference in salinity and temperature stress. It seems that the resulting hyperglycemia helps in satisfying the increasing energy demand during stress, allowing the organism to react to stressors (Gronow, 1974).

Unlike the results of this study, the resistance of African catfish (*C. gareiepinus*) larvae fed *Artemia* enriched with 20% of the ascorbyl palmitate was higher than that of the larvae fed *Artemia* enriched with 0 and 10% ascorbyl palmitate, after exposure to 25 ppt salinity stress for 1 h (Merchie *et al.*, 1995). Also, 25 days hatched milkfish larvae fed Rotifer and *Artemia* enriched with HUFAs and vitamin C, had lower mortality against salinity stress, when compared with the control group (Gapsin *et al.*, 1998).

The effectiveness of HUFA enrichment of *Artemia* on juvenile cuttlefish growth was demonstrated. The effects of HUFA in live food could thus easily be tested in juvenile cuttlefish as suggested by Castro *et al.* (1993). HUFA-enriched *Artemia* may result in the normal growth of juvenile cuttlefish, thus avoiding the nutritional deficiency observed (Choe, 1966; Boletzky, 1989; DeRusha *et al.*, 1989; Castro *et al.*, 1993; Koueta and Boucaud-Camou, 1999). This

hypothesis must therefore be followed up: food enriched with conditioned HUFA could be essential for cephalopod aquaculture. Although it is possible that HUFA alone may improve growth performance, the synergistic effects of vitamins C and E cannot be neglected. Better growth was observed among juvenile cuttlefish fed with HUFA plus vitamin C- and E-enriched *Artemia*.

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